THE ROLE OF THE ADIPONECTIN-ADIPONECTIN RECEPTOR PATHWAY IN HUMAN CAROTID ATHEROSCLEROTIC PLAQUE INSTABILITY

Karina Gasbarrino

Division of Experimental Medicine
Faculty of Medicine
McGill University
Montreal, Canada

April 2019

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Copyright © Karina Gasbarrino, 2019

ABSTRACT

Stroke is one of the leading causes of mortality and long-term disability in Canada. While men have a higher incidence of stroke than women, women have higher mortality and disability rates. Carotid atherosclerotic plaque instability is a key cause of ischemic strokes. Current guidelines recommend surgical intervention (carotid endarterectomy or carotid artery stenting) for stroke prevention based solely on the degree of carotid artery stenosis. However, stenosis alone is an incomplete determinant of a patient's stroke risk as it does not entirely reflect how unstable a plaque truly is and its likelihood to rupture. Instead it has been increasingly recognized that plaque composition plays a critical role in defining plaque instability. Another major concern in carotid disease management is the lack of sex-specific guidelines, which has led to suboptimal prevention and treatment of strokes particularly in women. Therefore, it is imperative to identify the potential mechanisms involved in carotid plaque instability in women and in men. Adiponectin is the most abundantly secreted adipokine with vasculoprotective and anti-inflammatory properties that interacts with two transmembrane receptors, AdipoR1 and AdipoR2. A series of *ex vivo* and *in vitro* experiments were conducted in the development of this thesis. To achieve this, our large ongoing bio-bank of human carotid plaque specimens and blood samples were used to determine: (1) the contribution of the adiponectin-AdipoR pathway in carotid atherosclerotic plaque instability, (2) the effect of modulating this pathway in the monocyte-macrophage lineage, and (3) sex-specific signatures associated with the adiponectin-AdipoR pathway and plaque instability.

Firstly, our results demonstrated that intraplaque expression of adiponectin plays a more important role in the context of plaque instability than circulating levels of total adiponectin. Moreover, we identified a novel association between decreased adiponectin-mediated signalling through the AdipoR2 pathway and greater plaque instability. This may impair the atheroprotective actions of adiponectin in the plaque and cause adiponectin resistance, thereby contributing to the accumulation of adiponectin observed in more vulnerable lesions.

Secondly, we demonstrated that adiponectin can affect the reverse cholesterol transport pathway, known to be a major protective system against the development of 'unstable' atherosclerotic plaques. We contributed evidence suggesting that adiponectin may significantly improve the efficiency of the rate-limiting step of the cholesterol efflux process and promote

nascent high-density lipoprotein biogenesis from macrophages via activation of both the AdipoR1 and AdipoR2 receptors.

Thirdly, although statins are widely used for the primary and secondary prevention of cardiovascular disease, intensive statin therapy was found to compromise the expression and function of adiponectin and its receptors in the monocyte-macrophage lineage. These findings may partly explain the residual cardiovascular risk in individuals treated with statins, which stresses the need for new therapies aimed specifically at stabilizing atherosclerotic plaques.

Lastly, we demonstrated that men and women who underwent a carotid endarterectomy exhibit clear differences not only at the level of the plaque (i.e., plaque composition) but also at the level of the circulation (i.e., adipokine, lipid, and immune profiles). Specifically, we identified that a decrease in the high molecular-weight to total adiponectin ratio was independently associated with greater plaque instability specifically in women but not in men. Moreover, we have developed a comprehensive method to measure sex hormones using liquid chromatography-tandem mass spectrometry that will be used in the future to identify sex-specific signatures related to plaque instability in men and women.

The results of these studies taken together, have provided evidence for the adiponectin-AdipoR pathway as a: (a) novel mechanism of plaque instability, (b) potential therapeutic target for plaque stabilization and ultimately, stroke prevention, and (c) probable sex-specific marker of plaque instability. Overall, our novel findings might be a stepping stone with the potential to ultimately lead to improvements in the identification, management, and treatment of ‘unstable’ carotid atherosclerosis.

RÉSUMÉ

L'accident vasculaire cérébral est l'une des principales causes de mortalité et d'invalidité au Canada. Alors que les hommes ont une incidence d'accidents vasculaires cérébraux plus élevée que les femmes, les taux de mortalité et d'invalidité des femmes sont plus élevés. L'instabilité de la plaque d'athérome au niveau de la carotide est l'une des principales causes d'accident vasculaire cérébral ischémique. Les recommandations actuelles conseillent une intervention chirurgicale (endartériectomie carotidienne ou endoprothèse carotidienne) pour la prévention des accidents vasculaires cérébraux basées uniquement sur le degré de sténose de l'artère carotide. Cependant, le degré de sténose est à lui seul insuffisant pour prédire le risque d'accident vasculaire cérébral, car il ne reflète pas entièrement l'instabilité d'une plaque ni son risque de rupture. Au lieu de cela, il est de plus en plus reconnu que la composition de la plaque joue un rôle essentiel dans la définition de l'instabilité de la plaque. Une autre préoccupation majeure dans la gestion de cette maladie est l'absence de directives spécifiques au sexe, ce qui a conduit à une prévention et à un traitement sous-optimaux des accidents vasculaires cérébraux, en particulier chez les femmes. Par conséquent, il est impératif d'identifier les mécanismes potentiels impliqués dans l'instabilité de la plaque carotidienne chez les femmes et les hommes. L'adiponectine est l'adipokine la plus abondamment sécrétée, dotée de propriétés antiathérogènes et anti-inflammatoires, interagit avec deux récepteurs transmembranaires : AdipoR1 et AdipoR2. Une série d'expériences *ex vivo* et *in vitro* ont été conduites dans l'élaboration de cette thèse. Pour y parvenir, notre grande banque biologique de spécimens de plaques carotidiennes humaines et d'échantillons de sang ont été utilisées afin de determiner : (1) la contribution de la voie adiponectine-AdipoR dans l'instabilité de la plaque athéromateuse carotidienne, (2) l'effet de la modulation de cette voie dans la lignée des monocytes et des macrophages, et (3) les signatures spécifiques au sexe associées à la voie adiponectine-AdipoR et à l'instabilité de la plaque.

Premièrement, nos résultats ont démontré que le niveau d'expression de l'adiponectine au sein de la plaque joue un rôle plus important que les niveaux circulants d'adiponectine totale dans l'instabilité de la plaque. De plus, nous avons identifié une nouvelle association entre une diminution de la signalisation induite par l'adiponectine par la voie AdipoR2 et une instabilité accrue de la plaque. Cela peut altérer les actions athéroprotectrices de l'adiponectine et provoquer

une résistance à l'adiponectine, contribuant ainsi à l'accumulation d'adiponectine observée dans les lésions plus vulnérables.

Deuxièmement, nos résultats démontrent que l'adiponectine peut affecter la voie de transport inverse du cholestérol, connue pour être un système de protection majeur contre le développement de plaques athéromateuses « instables ». Nous avons apporté des preuves suggérant que l'adiponectine peut considérablement améliorer l'efficacité de l'étape limitante du processus d'efflux de cholestérol et favoriser la biogenèse des lipoprotéines de haute densité à partir des macrophages par l'activation des récepteurs AdipoR1 et AdipoR2.

Troisièmement, bien que les statines soient largement utilisées pour la prévention primaire et secondaire des maladies cardiovasculaires, il a été démontré qu'un traitement intensif aux statines compromettait l'expression et la fonction de l'adiponectine et de ses récepteurs de la lignée des monocytes et des macrophages. Ces résultats pourraient expliquer en partie le risque cardiovasculaire résiduel chez les personnes traitées avec des statines, ce qui souligne la nécessité de nouveaux traitements visant spécifiquement à stabiliser les plaques athéromateuses. Enfin, nous avons démontré que les hommes et les femmes ayant subi une endartériectomie carotidienne présentaient des différences nettes non seulement au niveau de la plaque (sa composition), mais également au niveau de la circulation (profils d'adipokine, de lipides et de cellules immunitaires). Plus précisément, nous avons identifié que la diminution du rapport entre l'adiponectine de haut poids moléculaire et l'adiponectine totale était indépendamment associée à une instabilité accrue de la plaque, en particulier chez les femmes mais pas chez les hommes. De plus, nous avons développé une méthode de mesure des hormones sexuelles à l'aide de la spectrométrie de masse qui sera utilisée à l'avenir pour identifier les signatures spécifiques au sexe liées à l'instabilité de la plaque chez les hommes et les femmes.

Les résultats de ces études ont fourni des preuves sur la voie de l'adiponectine-AdipoR comme étant : (a) un nouveau mécanisme d'instabilité de la plaque, (b) une cible thérapeutique potentielle pour la stabilisation de la plaque et la prévention des accidents vasculaires cérébraux, et (c) un marqueur spécifique au sexe de l'instabilité de la plaque. En somme, nos nouvelles découvertes pourraient éventuellement conduire à des améliorations dans l'identification, la gestion et le traitement de l'athérosclérose carotidienne « instable ».

ACKNOWLEDGEMENTS

As Vincent van Gogh once said, “What would life be if we had no courage to attempt anything?”. Well, six years ago I had the audacity to begin my doctoral journey and as expected it has been a long arduous path of failing experiments, frustrating setbacks and delays, sleepless nights, and moments filled with overwhelmingly unpleasant feelings. However, above all it has been a fulfilling and enriching experience of self-growth and one that I am most proud of. Despite the numerous challenges, I have gained much knowledge and wisdom, despite the countless problems, I have gained multiple skills to solve them, and despite the uneasy path, it has helped me to become the very best version of myself. But most importantly, my doctoral journey was not a lonely process; I have been presented with endless opportunities to meet many brilliant minds along the way and foster new relationships, and what I have achieved today would not have been possible without the help, support, and motivation of these people.

Although no words are certainly enough to express my utmost gratitude, I want to begin by thanking my supervisor and mentor, Dr. Stella Daskalopoulou for her empathy, her heart-warming guidance, her time, her understanding, and her commitment to support me as I evolved into the researcher I am today. She has been my greatest supporter right from the very beginning of my PhD and she has continuously challenged me in various ways to elevate my levels of thinking. Her positive outlook and confidence in my research have inspired me to push my limits time and time again. I am especially grateful for the freedom she has given me to explore various possibilities within my research, which has made this journey all the more exciting and rewarding. But most of all I thank her for every doubt she has reassured, and for always believing in me and in my dreams. She has been my greatest source of inspiration throughout this journey and my reason to want to continue to pursue an academic career in research. As I embark on another chapter in my life, I will hold dear to my heart every memorable experience and every knowledgeable lesson she has taught me.

These dreams certainly would not have seen the light of day if it was not for the incredible team I have had the pleasure to work with on a daily basis. Therefore, importantly, I must give an immense thanks to all current and past members of the lab (research associates/coordinators, lab managers, graduate students, summer bursary students, and volunteers), not only to those who have been directly involved in my thesis work but also to those who have provided me with moral

support and a pleasurable and friendly working atmosphere. I am particularly grateful for the assistance given by Jessica Gorgui and Yessica Haydee-Gomez, who have been instrumental in coordinating the clinical aspect of the project and who have taught me the logistics of dealing with human subject research, Dr. RJ Doonan, who was the senior PhD student at the time I started in the lab and set-up the basis for my thesis work with his novel findings, Dr. Huaien Zheng, who joined the lab several years ago as lab manager and has been there from the beginning of my PhD to the end, providing me with valuable and constructive discussions involving the planning and development of my research work, as well as Dr. Anouar Hafiane, whose help and advice has been instrumental and who has taught me many specialized techniques used in cholesterol efflux and HDL biogenesis. Altogether, their willingness to give their time so generously has been very much appreciated.

My special thanks are extended to all my thesis committee members, my academic advisor, as well as numerous collaborators, who have provided me with valuable knowledge and infinite support and mentorship over the past few years. I am also very appreciative of the funding I have received from the Fonds de la recherche en santé du Québec and the Canadian Institutes of Health Research (doctoral award, travel awards), which have given me endless opportunities to present my research at national and international conferences and to grow my network connections.

I am forever grateful to my parents, my brother, and my friends (as well as my dog), who have patiently stayed by my side throughout the entire journey, offering their sweet words of wisdom and encouragement, their eternal love and support, their shoulders to cry on, and a helping hand whenever I needed it. But most importantly I thank them for being my safe haven. They are the reasons why I am where I am today, why I continue to push myself each day to work hard and achieve my goals. I thank them for always believing in me, even at times when I doubted myself, for being proud of my accomplishments, and for encouraging me to explore my potential.

Last but not least, I want to dedicate this thesis to my grandfather, my guardian angel, who taught me that only those who are courageous enough to take risks and follow their dreams, will accomplish great things in life.

Today I can turn the last page of a fundamental chapter of my life because of the immense support I have received throughout the past years. I will forever be grateful of being blessed with the presence of so many beautiful souls who have helped to positively shape my PhD journey. With this support system by my side I am excited to begin the next big chapter of my life!

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Chapters 3-7 include manuscripts either published, submitted for publication, or in preparation for publication and represent original work as outlined below:

<u>Chapter 3:</u> Circulating chemerin, but not adiponectin, is associated with carotid plaque instability, whereas resistin is related to cerebrovascular symptomatology

- ◊ We are the first to investigate the relationship between circulating adipokine levels (chemerin, resistin, leptin, and adiponectin) and carotid atherosclerotic plaque instability in humans
- ◊ Reported that circulating adiponectin levels are not associated with human carotid atherosclerotic plaque instability

<u>Chapter 4:</u> Decreased adiponectin-mediated signalling through the AdipoR2 pathway is associated with carotid plaque instability

- ◊ Provided evidence that the adiponectin-AdipoR pathway can serve as a novel mechanism of atherosclerotic plaque instability
- ◊ We are the first to show that adiponectin protein (but not gene) expression is present in human carotid atherosclerotic plaques
- ◊ Provided evidence that AdipoR2 (but not AdipoR1) may play a crucial role at the plaque destabilization stage
- ◊ Identified that the local presence of adiponectin at the level of the plaque plays a more important role in the context of plaque instability than circulating levels of adiponectin

<u>Chapter 5:</u> Adiponectin-AdipoR pathway stimulates cholesterol efflux efficiently in human macrophages and modulates HDL-apoA-I biogenesis

- ◊ Provided preliminary evidence that adiponectin can improve the efficiency of the rate-limiting step of the cholesterol efflux process in macrophages and promote nascent HDL biogenesis via activation of both the AdipoR1 and AdipoR2 receptors
- ◊ Provided preliminary evidence that the adiponectin-AdipoR pathway in the monocyte-macrophage lineage may serve as a potential therapeutic target for plaque stabilization

<u>Chapter 6:</u> Intensive statin therapy compromises the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage

- ◊ Identified a novel pleiotropic property of statins in modulating the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage, whereby intensive statin therapy compromised the expression and function of adiponectin and its receptors *in vivo* and *in vitro*

<u>Chapter 7:</u> Sex differences in the adipokine, lipid, and immune profiles of men and women with severe carotid atherosclerosis

- ◊ Provided a comprehensive characterization of sex differences that exist between men and women with severe carotid atherosclerosis who underwent a carotid endarterectomy
- ◊ Identified that a decrease in the HMW:total adiponectin ratio may contribute or act as a potential marker of plaque instability specifically in women but not in men
- ◊ We are the first to report a sexual pattern in the distribution of the adiponectin receptors on circulating monocytes
- ◊ Developed a more sensitive and specific method than existing immunoassays to measure a panel of circulating sex hormones (testosterone, androstenedione, estradiol, and dehydroepiandrosterone) using liquid chromatography tandem mass spectrometry

CONTRIBUTION OF AUTHORS

This manuscript-based thesis is composed of five original manuscripts (Chapters 3-7) and the contributions of each author are stated below:

Chapter 3: Circulating chemerin, but not adiponectin, is associated with carotid plaque instability, whereas resistin is related to cerebrovascular symptomatology

This paper was published in Arteriosclerosis, Thrombosis, and Vascular Biology in 2016;36(8):1670-1678[1].

K Gasbarrino was responsible for refining the protocol of the study, statistical data analysis and interpretation of results, and drafting the manuscript.

C Mantzoros performed the adipokine measurements from collected blood samples and was responsible for critical revision and final approval of the manuscript.

J Gorgui was responsible for patient recruitment, sample collection, critical revision and final approval of the manuscript.

JP Veinot and **C Lai** were responsible for pathological analysis of carotid plaque stability, critical revision and final approval of the manuscript.

SS Daskalopoulou was responsible for conception and design of the study, interpretation of results, critical revision and final approval of the manuscript, and obtaining funding.

Chapter 4: Decreased adiponectin-mediated signalling through the AdipoR2 pathway is associated with carotid plaque instability

This paper was published in Stroke in 2017;48(4):915-924[2].

K Gasbarrino was responsible for the conception and design of the study, participated in patient recruitment and sample collection, performed experimental assays, statistical data analysis and interpretation of results, and drafted the manuscript.

H Zheng participated in the optimization of experimental assays, blood and tissue processing, interpretation of results, and critical revision and final approval of the manuscript.

A Hafiane participated in the optimization of experimental assays and assisted in the design and performance of the *in vitro* study.

JP Veinot and **C Lai** were responsible for pathological analysis of carotid plaque stability, critical

revision and final approval of the manuscript.

SS Daskalopoulou overlooked the proceedings of the experimental work and was responsible for conception and design of the study, interpretation of results, critical revision and final approval of the manuscript, and obtaining funding.

<u>Chapter 5:</u> Adiponectin-AdipoR pathway stimulates cholesterol efflux efficiently in human macrophages and modulates HDL-apoA-I biogenesis

This manuscript represents preliminary analysis (presented at Arteriosclerosis, Thrombosis, and Vascular Scientific Sessions 2016, and abstract published in *Arterioscler Thromb Vasc Biol* **36**,1 Suppl: A28, 2016)[3].

K Gasbarrino was responsible for the conception and design of the study, cholesterol efflux experiments and other experimental assays, statistical data analysis and interpretation of results, and drafting the manuscript.

A Hafiane was responsible for the design of the cholesterol efflux experiments, provided training and assisted in the performance of the cholesterol efflux experiments and other experimental assays (fast protein liquid chromatography, 2D-PAGGE), overlooked the proceedings of the experimental work and participated in statistical data analysis and interpretation of results.

G Cardin was responsible for lentiviral shRNA vector production and transfection experiments (in 293FT cells) and provided all necessary training for me to perform the infection experiments (in THP-1 monocytes).

F Rodier was responsible for conception and design of the shRNA silencing experiments and provided aid in troubleshooting.

SS Daskalopoulou was responsible for conception and design of the study, interpretation of results, and obtaining funding.

<u>Chapter 6:</u> Intensive statin therapy compromises the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage

K Gasbarrino was responsible for the conception and design of the study, participated in patient recruitment for cross-sectional study, sample collection, performed experimental assays, statistical data analysis and interpretation of results, and drafting the manuscript.

A Hafiane participated in the optimization of experimental assays, assisted in the design and performance of *in vitro* experiments (thin layer chromatography), interpretation of results, and critical revision and final approval of the manuscript.

H Zheng performed all peripheral blood monocyte isolations and was responsible for the critical revision and final approval of the manuscript.

SS Daskalopoulou overlooked the proceedings of the experimental work and was responsible for conception and design of the study, patient recruitment and blood collection for the longitudinal study, interpretation of results, critical revision and final approval of the manuscript, and obtaining funding.

<u>Chapter 7:</u> Sex differences in the adipokine, lipid, and immune profiles of men and women with severe carotid atherosclerosis

This manuscript represents preliminary analysis (presented at International Symposium of Atherosclerosis 2018, and abstract published in *Atherosclerosis Suppl.* **32**:13-14, 2018)[4] and will be expanded on (i.e., increase patient sample size and measurement of circulating sex hormones).

K Gasbarrino was responsible for the conception and design of the study, participated in patient recruitment and sample collection, including blood sample and carotid plaque processing, was responsible for experimental assays, assisted with the development and optimization of the mass spectrometry method for analyzing endogenous sex hormones, performed statistical data analysis and interpretation of results, and was responsible for drafting the manuscript.

H Zheng participated in the collection of patient samples, was responsible for peripheral blood monocyte isolation, optimization of experimental assays, and interpretation of results.

Ed Daly was responsible for the development and optimization of the mass spectrometry method for analyzing endogenous sex hormones and undertook all associated troubleshooting.

JP Veinot and **C Lai** were responsible for pathological analysis of carotid plaque stability.

SS Daskalopoulou overlooked the proceedings of the experimental work and was responsible for conception and design of the study, interpretation of results, and obtaining funding.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

CHAPTER 2: GENERAL METHODOLOGY

CHAPTER 4: DECREASED ADIPONECTIN-MEDIATED SIGNALING THROUGH THE ADIPOR2 PATHWAY IS ASSOCIATED WITH CAROTID PLAQUE INSTABILITY

CHAPTER 5: ADIPONECTIN-ADIPOR PATHWAY STIMULATES CHOLESTEROL EFFLUX EFFICIENTLY IN HUMAN MACROPHAGES AND MODULATES HDL-APOA-I BIOGENESIS

CHAPTER 6: INTENSIVE STATIN THERAPY COMPROMISES THE ADIPONECTIN-ADIPOR PATHWAY IN THE HUMAN MONOCYTE-MACROPHAGE LINEAGE

CHAPTER 7: SEX DIFFERENCES IN THE ADIPOKINE, LIPID, AND IMMUNE PROFILES OF MEN AND WOMEN WITH SEVERE CAROTID ATHEROSCLEROSIS

CHAPTER 8: GENERAL DISCUSSION AND CONTRIBUTIONS TO KNOWLEDGE

LIST OF TABLES

CHAPTER 7: SEX DIFFERENCES IN THE ADIPOKINE, LIPID, AND IMMUNE PROFILES OF MEN AND WOMEN WITH SEVERE CAROTID ATHEROSCLEROSIS

LIST OF ABBREVIATIONS

ABCA1 – ATP-binding cassette transporters A1

ABCG1 – ATP-binding cassette transporters G1

ACAS – Asymptomatic Carotid Atherosclerotic Study

ACST – Asymptomatic Carotid Surgery Trial

AdipoR – adiponectin receptors

AF – amaurosis fugax

AHA – American Heart Association

AMPK – 5' adenosine monophosphate-activated protein kinase

apoA-I – apolipoprotein AI

apoB – apolipoprotein B

$apoE^{-/-}$ – apolipoprotein E knock-out

APPL1 – adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1

ARR – absolute risk reduction

AU – arbitrary units

BMI – body mass index

BSA – bovine serum albumin

CAD – coronary artery disease

CAS – carotid artery stenting

CEA – carotid endarterectomy

CI – confidence interval

cIMT – carotid intima-media thickness

CREST – Carotid Revascularization Endarterectomy versus Stenting Trial

CRP – C-reactive protein

CVD – cardiovascular disease

DHEA – dehydroepiandrosterone

ECST – European Carotid Surgery Trial

eNOS – endothelial nitric oxide synthase

E2 – estradiol

ER – estrogen receptor

FBS – fetal bovine serum

FPLC – fast-performance liquid chromatography

FCR – fractional catabolic rate

GPER – G protein-coupled estrogen receptor 1

HDL-C – high-density lipoprotein cholesterol

HMG-CoA – 3-hydroxy-3-methylglutaryl-coenzyme A

HMW – high molecular-weight

HR – hazard ratio

ICAM-1 – intercellular adhesion molecule-1

IL – interleukin

LAA – large artery atherosclerosis

LC-MS/MS – liquid chromatography-tandem mass spectrometry

LDL-C – low-density lipoprotein cholesterol

LMW – low molecular-weight

LXR – liver X receptor

MCP-1 – monocyte chemoattractant protein-1

MI – myocardial infarction

MIP-1α – macrophage inflammatory protein-1 α

MMP – matrix metalloproteinase

MMW – middle molecular-weight

NASCET – North American Symptomatic Carotid Endarterectomy Trial

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NO – nitric oxide

OR – odds ratio

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PMA – phorbol myristyl acetate

PPAR – peroxisome proliferator-activated receptor

PVAT – perivascular adipose tissue

qRT-PCR – quantitative real-time polymerase chain reaction

RCT – reverse cholesterol transport

SD – standard deviation

shRNA – short hairpin RNA

SMC – smooth muscle cell

SR-A – scavenger receptor class A

SR-BI – scavenger receptor class B type I

T2DM – type 2 diabetes mellitus

THP-1 – Tamm–Horsfall protein 1

TIA – transient ischemic attack

TICE – transintestinal cholesterol excretion

TIMP-1 – tissue inhibitor of metalloproteinase-1

TNF-α – tumor necrosis factor alpha

TZD – thiazolidinedione

WAT – white adipose tissue

VCAM-1 – vascular cell adhesion molecule-1

CHAPTER 1: INTRODUCTION

1.1 Preface

Stroke due to carotid atherosclerotic disease is a major health problem for the society at large. Despite advances in prevention and management, it remains a leading cause of mortality and long-term disability worldwide. In Canada alone, every 9 minutes someone suffers a stroke, placing physical, emotional, and financial constraints not only on those affected but also on their family and friends. Furthermore, stroke imposes an immense economic burden on the health-care system.

Unstable atherosclerotic plaques that develop in the internal carotid artery are major etiological factors known to cause strokes. Current clinical practice guidelines recommend carotid endarterectomy (CEA) for stroke prevention in patients with carotid atherosclerotic plaques based solely on the degree of carotid artery stenosis, i.e. typically when stenosis ≥70% in general, and 50-69% in selected patients. However, despite these guidelines, many plaques causing moderate- to high-grade stenoses remain stable and asymptomatic, whereas other plaques causing low-grade stenoses are unstable and increase the risk of disabling and life-threatening strokes. Hence, it is increasingly recognized that stenosis alone is an incomplete feature of stroke risk, as it does not entirely reflect how unstable a plaque truly is. As a result, many people with unstable plaques go undiagnosed or untreated. Another major concern in carotid disease management is the lack of sex-specific guidelines, which have led to suboptimal prevention and/or treatment of strokes particularly in women. Unfortunately, in cardiovascular research, women have been under-represented for many years leading to an under-appreciation of sex differences. However, recently there is increasing awareness that sex differences do exist in atherosclerosis development, plaque morphology and composition, and stroke risk. Thus, it is a research priority to understand the mechanisms that underlie the atherosclerotic process and contribute to plaque instability in men and in women, as it may lead to more accurate patient risk stratification and may pave the way for the identification of novel therapeutic targets aiming to stabilize vulnerable plaques. Over the past few years, we have created a large, prospective bio-bank of human blood and carotid plaque specimens, which we have used herein, to address the current knowledge gaps pertaining to atherosclerotic plaque instability.

Adipose tissue is a source of adipokines, which mediate the crosstalk between adipose tissue, the immune system, and the vascular wall. Adiponectin is the most abundantly secreted adipokine with both anti-inflammatory and vasculoprotective properties. Experimental evidence suggests that by interacting with its receptors, AdipoR1 and AdipoR2 (defined as AdipoR),

adiponectin can exert a protective effect in all stages of atherosclerotic plaque development, particularly through its actions on the macrophage, a major effector cell in plaque instability, and on its precursor in the blood, the monocyte. We hypothesize that dysregulation in these interactions may have numerous pro-atherosclerotic effects on the arterial wall. Thus, herein, we performed a series of studies that sought to unravel in humans 1) the contribution of adiponectin and the adiponectin receptor pathway in carotid atherosclerotic plaque instability and 2) the effect of modulating this pathway in the monocyte-macrophage lineage. Moreover, we aimed to identify sex-specific signatures associated with the adiponectin-AdipoR pathway and plaque instability.

The five studies presented herein (Chapters 3-7) were performed in partial fulfillment of the requirements for my doctoral degree and constitute original work. Below I present a general background of stroke epidemiology, risk factors and types of strokes, the etiology of ischemic strokes, the stages in the development of atherosclerotic plaques, screening and management of carotid artery disease, histological characterization of atherosclerotic plaques, the adipo-vascular axis, and the role of adiponectin and the AdipoR pathway in atherosclerosis development. Furthermore, I present two systematic reviews and meta-analyses that I have performed to comprehensively assess the association between adiponectin and the full spectrum of carotid artery disease, from carotid intima-media thickness (cIMT), to plaque presence, to ischemic stroke risk, to risk of mortality in an ischemic stroke population. I then present methodology common to multiple chapters (Chapter 2), the original studies in Chapters 3-7, followed by a general discussion (Chapter 8).

1.2 Stroke Epidemiology

Stroke along with ischemic heart disease remain the leading causes of mortality and disability world-wide, accounting for a combined 15 million deaths in 2016[5]. Stroke alone represents approximately 40% of these deaths[5]. While 62,000 new strokes occur in Canada each year (one stroke every nine minutes), stroke kills approximately 13,000 Canadians yearly[6]. Furthermore, the Heart & Stroke Foundation's 2017 Stroke Report revealed that over 400,000 Canadians live with long-term disability from stroke and that this number will double in the next 20 years due to the increasingly aging population[7]. The impact of stroke on these survivors can be devastating; in addition to suffering from functional impairments, aphasia, memory loss, depression, and fatigue, stroke survivors remain at a high annual risk of recurrent cerebrovascular events and mortality[7].

Stroke also causes a deep burden on the health care system, costing the Canadian economy an estimated $3.6 billion in direct and indirect costs each year[8].

1.3 Risk Factors of Stroke

Stroke is associated with several risk factors, which are generally categorized as (1) non-modifiable or (2) modifiable. A brief summary of some well-documented risk factors (also referred to as the "traditional" Framingham risk factors) are listed below.

1.3.1 Non-modifiable risk factors

Age

It is well-established that the risk of stroke strongly increases with age. In fact, the incidence of stroke nearly doubles every 10 years after the age of 55[7]. With increasing age, the wall of large arteries becomes increasingly thicker and less elastic, resulting in an increase in pulse wave velocity, which is an important parameter of arterial stiffness[9,10]. Arterial stiffness, a composite indicator of arterial health, is considered to be an independent predictor (over and above traditional risk factors) of several cardiovascular outcomes, including stroke, and is strongly associated with atherosclerosis at various sites of the arterial tree[11-13].

Even though 80% of all strokes occur over the age of 60, stroke in younger adults (aged 20-59) is rising drastically[7]. According to the Framingham Heart Study, the lifetime risk of stroke in middle-aged adults was estimated to be 1 in 6[14]. However, due to the presence of other co-morbidities among older stroke patients, their recovery following a stroke is slower and more challenging than younger stroke patients[7].

Sex

Sex differences in stroke incidence, mortality, and outcomes exist[15]. Although men are known to have a higher incidence of stroke than women among most age groups, after the age of 85 years, significantly more women suffer from strokes than men due at least in part to the longer life expectancy in women[16,17]. Furthermore, it has been reported that women have worse recovery and higher mortality and disability rates than men post-stroke[15,17]. In fact, according to the Heart & Stroke Foundation of Canada, one-third more women die of stroke than men, where women are 35% more likely to die within one year of a stroke compared with men[18]. Much of this excess

mortality in women is believed to be caused by baseline differences between men and women (i.e., age, comorbidities, stroke severity)[15]. However, even after controlling for these factors, women continue to have worse functional outcomes after stroke than men[19,20]. Nonetheless, age remains a powerful risk factor for women when it comes to stroke. During the first 10 years after menopause, the risk of stroke nearly doubles in women, as natural estrogen levels drop and the risk of developing comorbidities increases (i.e., hypertension, hypercholesterolemia)[21].

Family history

Family history of stroke is recognized as an independent risk factor for stroke. A meta-analysis demonstrated that a positive family history of stroke can significantly increase the risk of stroke in an individual by 30% (odds ratio [OR]: 1.3; 95% confidence interval [CI]: 1.2-1.5; P<0.00001)[22]. Interestingly, positive sibling history of stroke has been shown to be more strongly associated with incidence and severity of stroke than a positive maternal or paternal history[23,24]. Family history of stroke has been associated with age; younger stroke patients (aged 60 years or younger) are more likely to have a family history of stroke than older stroke patients[25].

1.3.2 Modifiable risk factors

Blood pressure

There exists an independent and strong relationship between systolic and diastolic blood pressure and stroke risk; an increase in blood pressure is associated with an increase in the risk of stroke and stroke mortality[26-28]. In a meta-analysis of 61 prospective observational studies including patients aged 40-89 years without known cardiovascular disease (CVD) at baseline, each 20 mmHg increase in systolic blood pressure (or equivalently 10 mmHg in diastolic blood pressure) was associated with more than a two-fold increase in the stroke mortality rate[27]. Clinical trials evaluating the efficacy of anti-hypertensive medications have demonstrated that treatment of hypertension is highly effective in the prevention of strokes[26]. In a meta-analysis combining data from 23 clinical trials, anti-hypertensive drug treatment was associated with a 32% reduction in the risk of stroke (95% CI: 0.61-0.76; P=0.004) compared with no drug treatment[29]. Among various anti-hypertensive therapies used as first-line agents, low-dose diuretics were observed to be the most effective in preventing the occurrence of stroke[29], while the combination of

angiotensin-converting-enzyme inhibitor (i.e., perindopril) and long-acting diuretic (i.e., indapamide) was noted to be effective in preventing recurrent strokes[30].

Low-density lipoprotein cholesterol

While high concentrations of low-density lipoprotein cholesterol (LDL-C) were established by the Framingham Heart Study to be a strong risk factor for coronary heart disease[31-33], epidemiological associations between LDL-C and stroke risk were reported to be inconsistent or weak[34-36]. Nevertheless, statin therapy aimed at lowering LDL-C levels, has consistently been shown to be beneficial in reducing stroke risk for both primary and secondary prevention[37-39]. A meta-analysis of randomized trials of statins, including more than 165,000 individuals at high risk for stroke, showed that for each 1 mmol/L decrease in LDL-C there was a 21.1% relative risk reduction for stroke (95% CI: 6.3-33.5; P=0.009)[35]. Furthermore, statin therapy also significantly reduced the risk of recurrent strokes[35]. Greater lipid lowering by more intensive statin therapy (to an LDL-C level <2.1 mmol/L) was reported to achieve significantly greater benefit in reducing stroke risk than moderate statin dosing[37,40]. While statin therapy has demonstrated clear benefits on the risk of stroke, the benefits of other LDL-C lowering interventions and therapies were observed to be small or not statistically significant[41].

Type 2 diabetes mellitus

People with type 2 diabetes mellitus (T2DM) have an increased prevalence of cardiovascular risk factors, such as hypertension, dyslipidemia, and obesity, and an increased vulnerability in developing atherosclerosis[42-45]. Individuals with T2DM are also at an increased risk for stroke; in fact, it has been reported that T2DM more than doubles the risk for stroke in women and in men[46-49]. Furthermore, the duration of diabetes was independently associated with stroke risk, where the risk increased 3% each year and more than tripled with a duration of ≥10 years[49]. Even in individuals without diagnosed T2DM, high fasting plasma glucose levels (≥ 6.1 mmol/L) were associated with a higher risk of age-adjusted fatal, nonfatal, or total stroke events[50]. These associations remained significant even when levels were below the T2DM threshold[50].

Obesity

Obesity is associated with various comorbidities, such as hypertension, dyslipidemia, and T2DM[51,52], as well as an increased risk of stroke[53,54]. The independent and positive relationship between obesity and stroke risk was observed to be graded[55,56]. In women, higher body mass index (BMI ≥27 kg/m^2) was significantly associated with a greater risk of ischemic stroke, with relative risks of 1.75 (95% CI: 1.17-2.59) for BMI of 27-28.9 kg/m^2, 1.90 (95% CI: 1.28-2.82) for BMI of 29-31.9 kg/m^2, and 2.37 (95% CI: 1.60-3.50) for BMI ≥32 kg/m^2, as compared with those with a BMI<21 kg/m^2 (P<0.001).[55] Furthermore, collaborative analyses of 57 prospective studies demonstrated that in the BMI range of 25 to 50 kg/m^2, each 5 kg/m^2 increase in BMI was associated with about 40% higher stroke mortality[56]. However, several studies have reported that abdominal body fat, measured as the waist-to-hip ratio, is a stronger predictor of stroke risk than BMI[57,58]. For example, a study examining the association of BMI and abdominal obesity with stroke incidence in men aged 40-75 years without a history of CVD or stroke, observed that men in the extreme quintiles of waist-to-hip ratio had an age-adjusted relative risk of stroke of 2.33 (95% CI: 1.25-4.37), while the association with BMI was weak and non-significant[58]. Although there is ample evidence that increased weight is associated with an increased incidence of stroke, there is no clear and compelling evidence that weight loss reduces the risk of stroke. While weight loss through bariatric surgery has been associated with a significant reduction in stroke[59], randomized controlled clinical trials assessing the independent effects of weight reduction (controlling for any concomitant risk factors) in overweight or obese individuals on stroke incidence and mortality are urgently needed.

Smoking

Smoking is considered a strong behavioural risk factor for stroke[60,61]. A meta-analysis of 32 studies demonstrated that cigarette smoking is associated with an overall relative risk of stroke of 1.51 (95% CI: 1.45-1.58)[62]. A graded dose-response relationship was noted between the number of cigarettes smoked per day and the relative risk of stroke[60,62,63]. Heavy smokers (>40 cigarettes per day) had an approximate doubling in stroke risk than light smokers (<10 cigarettes per day)[60]. Interestingly, second-hand exposure to tobacco smoke has also been established as a risk factor for stroke and the relative risk associated with this relationship approached similar values as those observed among active smokers[64,65]. Stroke risk is reduced with smoking cessation and this

reduction can occur in a relatively short period. Significant reductions in stroke risk were observed as little as two years after discontinuation of cigarette smoking[60,66].

Smoking likely contributes to increased stroke risk due to rises in heart rate, blood pressure, and arterial wall stiffness, as well as long-term effects related to atherosclerosis development[67,68]. Active smoking was associated with a 50% increase in the progression of atherosclerosis over 3 years, while past smoking was associated with a 25% increase, compared to non-smokers[68].

1.4 Types of Strokes

A stroke occurs when the blood flow to a particular brain region is interrupted or reduced, resulting in damage and death to surrounding brain cells[7]. The severity of the stroke depends on various factors, such as the stroke type, the region of the brain affected by the stroke, the extent of the damaged area, and the duration in the interruption of blood flow to the affected brain region[7]. Strokes can be classified into four types: (1) ischemic stroke, (2) hemorrhagic stroke, (3) transient ischemic attack (TIA), or (4) covert stroke[6,7]. The vast majority of strokes are caused by cerebral ischemia, accounting for 87% of all stroke cases[16]. These strokes occur as a result of reduced blood flow to a particular region of the brain. When loss in blood supply leads to a transient episode of neurologic dysfunction without causing permanent tissue injury, it is referred to as a 'mini stroke' or TIA[69], while an ischemic stroke is defined based on clinical evidence of permanent brain injury. Often times symptoms associated with a TIA (i.e., difficulty speaking, facial drooping, weakness on one side of the body) will resolve within the first hour of their initiation[69]. When blood flow to the ophthalmic arteries is blocked, this results in transient monocular blindness usually lasting seconds to minutes and is specifically referred to as an amaurosis fugax (AF)[70]. A TIA is often a warning sign of future ischemic strokes. Unfortunately, many people who suffer a TIA do not receive proper treatment, and as a result 10-20% of people will have an ischemic stroke within 3 months of their TIA[69].

Other types of strokes occur when a blood vessel in the brain leaks or ruptures, spilling blood into the surrounding tissues (known as hemorrhagic strokes), or when a small blood vessel in the brain becomes permanently blocked (known as covert strokes or 'silent' strokes)[6,7]. Although covert strokes do not result in overt clinical symptoms, they do cause damage to the cells in the affected brain region, particularly in the brain's white matter that controls executive function, rather than specific functions, including movement, speech, and vision[6].

1.5 Etiology of Ischemic Strokes

Ischemic cerebrovascular events are caused by the thrombotic or embolic occlusion of a cerebral artery, and can be classified into the following categories: (1) large artery atherosclerosis (LAA), (2) cardioembolism, (3) small vessel occlusion, (4) stroke of other determined etiology, and (5) stroke of undetermined etiology[71,72].

1.5.1 Large artery atherosclerosis

LAA is one of the most common causes of ischemic cerebrovascular events. Atherosclerotic plaques can cause cerebral ischemia mainly by rupturing and causing a thrombus that can interrupt blood flow locally or that can embolize and lodge in distal arteries. However, in certain cases non-ruptured plaques can also cause strokes by directly reducing cerebral perfusion (generally associated with plaques that exhibit high-grade stenoses) or by surface erosion[71,72]. These plaques mainly form in any major cerebral artery and are common at areas of low shear stress or disturbed laminar flow, particularly at the carotid bifurcation[73]. Other regions frequently affected are the internal carotid artery, the M1 segment of the middle cerebral artery, the first and fourth segments of the vertebral artery, and the first segment of the basilar artery[71]. Furthermore, aortic arch atherosclerotic plaques are another common cause of ischemic strokes[71,74]. However, the carotid artery is the most common site of atherosclerotic plaque formation, causing 15-20% of all ischemic strokes[75]. This thesis will focus specifically on carotid atherosclerotic disease.

1.5.2 Cardioembolism

Atrial fibrillation, the most common type of cardiac arrhythmia affecting about 33.5 million individuals world-wide (representing 0.5% of the world's population)[76,77], is associated with a 4- to 5-fold increased risk of ischemic stroke[26]. In fact, an estimated 10% of all ischemic strokes are a result of atrial fibrillation[26]. Stroke (or TIA) due to atrial fibrillation is believed to be the result of an embolization of intra-cardiac thrombi, which most commonly originate from the left atrial appendage and then travel to the brain[71]. Interestingly, atrial fibrillation has been associated with more severe ischemic strokes than emboli due to carotid atherosclerotic disease[78]. Furthermore, patients with atrial fibrillation who suffer an ischemic stroke also have greater disability and a higher mortality rate[79].

Ischemic cerebrovascular events can also occur in the first weeks following a myocardial infarction (MI), where the majority are believed to be embolic, arising from left ventricular wall mural thrombi[71]. Approximately 1-3% of all MI will lead to an ischemic stroke[80]. Additionally, embolism from diseased native valves or prosthetic valves or thrombus travelling through a patent foramen ovale are other causes of ischemic cerebrovascular events[71].

1.5.3 Small vessel occlusion

Lacunar infarcts are mainly caused by the occlusion of small penetrating arteries (<200 μm) affected by lipohyalinosis or microatheromas and typically occur in the subcortical regions of the brain[71,72].

1.5.4 Stroke of other determined etiology

Rare causes of ischemic stroke can be associated with non-atherosclerotic vasculopathies, hypercoagulable states, or hematologic disorders[72].

1.5.5 Stroke of undetermined etiology

Ischemic strokes of undetermined etiology are not uncommon; in fact, >30% of all ischemic strokes have no identifiable etiology[72,75].

1.6 Stages in the Development of Atherosclerotic Plaques

Atherosclerosis is a progressive and chronic inflammatory disease of the arterial wall that can be converted into an acute clinical event upon plaque rupture and thrombus formation. It is considered a spatially nonrandom process that affects lesion-prone areas of the arterial tree, particularly regions in which laminar flow is disturbed by bends or branch points[81]. Atherosclerotic plaques have complex morphology and composition, characterized by the accumulation of fatty substances, cholesterol, inflammatory cells, smooth muscle cells (SMCs), and cellular waste products that build up over time in the innermost layer of the artery, the intima[82,83].

1.6.1 Endothelial dysfunction and lesion initiation

Atherosclerosis is triggered by an interplay between endothelial dysfunction and the subendothelial retention of apolipoprotein B (apoB)-containing lipoproteins[84]. The healthy

vascular endothelium functions as a selectively permeable barrier between the blood and tissue that plays a crucial role in regulating vascular tone, inflammation, and coagulation. However, the endothelium is exposed to various chemical, mechanical, or immunological insults, which can affect its morphology and integrity. Thus, atherogenesis is perceived to be initiated as a response to injury made to the endothelium, in the form of increased circulating lipoproteins, low shear stress, free radicals from smoking, high blood pressure, or diabetes, or other vascular risk factors[84]. As a result of endothelial dysfunction, the endothelium displays impaired barrier function and exhibits a phenotype that is proinflammatory and proatherogenic. The endothelium increases permeability to LDL, which accumulates, and forms aggregates in the subendothelial matrix of the intima due to interactions with matrix components[85]. Trapped LDL can undergo various modifications. One of the modifications most significant for early lesion formation is lipid oxidation, which triggers an inflammatory response within the intima and promotes foam cell formation[86].

1.6.2 Inflammation

Atherosclerosis is characterized by the recruitment of monocytes and to a lesser degree, lymphocytes, from the circulation to sites of lesion formation[82,83]. Leukocyte recruitment is triggered by the accumulation of oxidized LDL, which stimulates the endothelial cells to produce a number of chemotactic proteins (i.e., monocyte chemoattractant protein-1 [MCP-1]) and growth factors (i.e., macrophage colony-stimulating factor) that interact with receptors on the surface of leukocytes[82,83,87]. Furthermore, endothelial cells increase their expression of cell surface adhesion molecules via activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which mediate the adhesion and entry of leukocytes into the arterial wall. Selectins (e.g., P-selection, E-selectin), expressed by endothelial cells, are responsible for initiating leukocyte adhesion to the endothelium, where leukocytes are described to 'roll' along the inflamed endothelial surface[88]. On the other hand, the firm adhesion of monocytes and lymphocytes to the endothelium and subsequent transendothelial migration of leukocytes are mediated by the interaction of integrins (e.g., CD11/CD18, VLA-4) on leukocytes with immunoglobulin-like adhesion molecules on endothelial cells (e.g., vascular cell adhesion molecule-1 [VCAM-1], intercellular adhesion molecule-1 [ICAM-1])[89]. Once monocytes are recruited into the arterial wall, they accumulate, and differentiate into macrophages[90-92]. Indeed, studies have shown that

resident plaque macrophages arise predominantly from the continuous recruitment of circulating monocytes[91,92]. An animal study using labeled monocytes showed the lesion size to increase in proportion with local monocyte accumulation[90,92]. In the plaque, macrophages are known to orchestrate the inflammatory response by secreting pro-inflammatory and cytotoxic proteins (i.e., interleukin 1 beta [IL-1β] and tumor necrosis factor alpha [TNF-α]), which enhance local inflammation, promoting plaque growth. Macrophages also alter the extracellular matrix of the vessel by releasing proteolytic enzymes, such as matrix metalloproteinases (MMPs), which predispose the plaque to rupture[93-95].

1.6.3 Foam cell formation

Once LDL is modified it can be ingested by macrophages via scavenger receptors, scavenger receptor class A (SR-A) and CD36, to form foam cells[96]. Mice lacking either receptor showed a significant reduction in atherosclerotic lesion development[97,98]. Scavenger receptor expression is upregulated by the presence of cytokines in the plaque, such as TNF-α and interferon-gamma[82]. Environmental factors in the plaque can promote the apoptosis and necrosis of macrophages and foam cells, as well as SMCs[99]. When foam cells die their lipid content, along with other cellular debris, is released into the extracellular space. Defective efferocytosis contributes to advanced lesion progression and the formation of a lipid-rich necrotic core[100,101]. Necrotic cells release damage-associated molecular patterns, which cause an amplification of the inflammatory response in the lesion. A vicious cycle develops where additional macrophages are recruited to the area, transform into foam cells, and die, contributing to further accumulation of cellular and lipid material in the plaque and further growth of the necrotic core[82,83].

Macrophages respond to excess cholesterol accumulation by promoting their own removal of cholesterol via the "reverse cholesterol transport" (RCT) pathway. This pathway is a major protective system against the development of atherosclerotic plaques, as it is responsible for carrying excess cholesterol from the periphery to the liver for excretion via the bile[102,103]. Although the RCT pathway is considered the major cholesterol elimination route, transintestinal cholesterol excretion (TICE) is also considered a significant alternative route to the hepatobiliary pathway[104]. TICE contributes to ~33% of total fecal sterol loss, suggesting a potential anti-atherogenic role for TICE[105]. However, evidence is lacking in this domain, and thus, we will focus particularly on the RCT pathway and macrophage-mediated cholesterol efflux herein.

Cholesterol efflux is the initial step in the RCT pathway by which excess free cholesterol in macrophages is exported and subsequently packaged into high-density lipoprotein (HDL) particles[106,107]. Cholesterol efflux capacity is an important measure of HDL functionality, which has been shown by our group to have strong inverse associations with carotid artery stenosis and advanced plaque morphology[108]. Furthermore, cholesterol efflux capacity is inversely associated with coronary artery disease (CAD), independently of HDL-C levels, and with incident cardiovascular events among apparently healthy individuals[109,110]. ATP-binding cassette transporters A1 (ABCA1), G1 (ABCG1), and scavenger receptor class B type I (SR-BI) are the three most important cellular cholesterol transporters/receptors implicated in cholesterol efflux and are all highly expressed in human and murine macrophages[106]. Studies of macrophage-mediated cholesterol efflux have demonstrated that both apolipoprotein AI (apoA-I) and ABCA1 play pivotal roles in initiating RCT[111-114]. The initial rate-limiting step of the RCT pathway is the formation of nascent HDL particles, which are made up primarily (approximately 65-70%) of apoA-I[111,115]. This step involves the interaction between ABCA1 and monomeric lipid-poor or lipid-free apoA-I, which acts as the primary acceptor of cholesterol. ABCA1 and ABCG1 efflux is sequential, where lipid release via ABCG1 occurs following ABCA1-mediated generation of nascent HDL particles[115]. ABCG1 is responsible for mediating the efflux of cellular cholesterol to lipidated (nascent or mature) HDL particles. Similarly, to ABCG1, SR-BI in peripheral cells can also promote cholesterol efflux to mature HDL particles[116].

1.6.4 Fibrous cap formation and lesion progression

In response to cytokines and growth factors released by immune cells in the plaque, SMCs undergo phenotypic switching from contractile to synthetic SMCs that undergo proliferation and migration[117]. These SMCs have been reported to lose their expression of key SMC markers such as smooth muscle α-actin and smooth muscle myosin heavy chain[117]. SMCs from the medial layer of the artery are stimulated to migrate into the intima, where they proliferate and increase their production of extracellular matrix proteoglycans, collagen, and elastin, giving rise to a fibrous cap that forms over the lipid-rich necrotic core[82,83]. The fibrous cap, composed primarily of vascular SMCs embedded in a matrix that contains type 1 and 3 collagen fibers, provides a 'protective barrier' between platelets in the bloodstream and prothrombotic material in the plaque. While

maintaining the integrity of the plaque, it causes the lesion to grow and expand outwards into the lumen of the artery.

1.6.5 Plaque rupture and thrombosis

Plaques that rupture typically have thin, collagen-poor fibrous caps with few SMCs[118]. An imbalance between collagen synthesis and breakdown can lead to fibrous cap thinning, which renders the cap susceptible to rupture. Immune cells, which infiltrate the fibrous cap of the plaque, are responsible for promoting the cap's degradation. Macrophages secrete proteolytic enzymes, such as MMPs, that cause matrix breakdown and promote plaque instability[94]. T lymphocytes also contribute to plaque instability by stimulating protease production by macrophages and by producing interferon-gamma, which results in impaired collagen synthesis by SMCs[119]. Upon rupture of the fibrous cap, the highly thrombogenic necrotic core becomes exposed to coagulation proteins in the circulation[120]. The thrombogenicity of the necrotic core is likely dependent on the presence of tissue factor, a key protein in the initiation of the coagulation cascade. The major source of tissue factor is the macrophage; upon apoptosis of macrophages, microparticles containing tissue factor are released into the lipid core making it highly thrombogenic[121]. Once the lipid core content comes into contact with the circulation, activation of the clotting cascade along with platelet adhesion, activation, and aggregation triggers the formation of a thrombus[82,83].

1.6.6 The role of sex hormones in atherosclerosis development

It is well recognized that men develop atherosclerosis earlier in life[122]. On the other hand, premenopausal women are more resistant to atherosclerotic disease as its prevalence is lower in these women compared to post-menopausal women or men[122]. It is believed that menopause due to a decrease in estrogen levels, diminishes the 'protection' in women and contributes to an adverse impact on cardiovascular risk variables. Experimental evidence suggests that sex steroid hormones, including estrogens and androgens, have a direct impact on the vascular system, affecting various mechanisms (i.e., vascular reactivity, inflammation, lipoprotein metabolism) that contribute to plaque instability and subsequently stroke risk[123]. These sex hormones have pleiotropic effects on various cell types involved in the atherosclerotic process, particularly the endothelium, SMCs, and macrophages, with estrogens believed to exert more stabilizing and atheroprotective actions than androgens[123]. However, the role of estrogen in atherosclerosis

development is not clearly defined. While experimental and epidemiological studies have shown estrogen to be beneficial, various trials on hormone replacement therapy have yielded contrasting results, demonstrating an excess of thrombotic events with estrogen treatment[124,125]. The timing of hormone initiation relative to menopause, and the dose and duration of hormone exposure play a significant role in the discrepancies observed. According to the timing hypothesis, estrogen is believed to slow the progression of early atherosclerosis if treatment is initiated soon after menopause when the vascular endothelium is relatively healthy[15]. However, if commenced several years after menopause when the vessel wall is already diseased, estrogen may instead exert adverse inflammatory and pro-atherogenic effects[15]. Interestingly, a recent large study demonstrated that total circulating levels of estradiol were associated with the presence of unstable carotid plaques as well as an increased risk of stroke in women, while no such associations were noted with total testosterone levels in either sex[126]. However, a great limitation to this study was that estradiol and testosterone were measured by different methods (immunoassay vs mass spectrometry). Analysis of estrogens in biological samples has been commonly performed using immunoassays for many years. However, this method is suboptimal, as it includes cross-reactivity with similar analytes and has moderate specificity and sensitivity, especially when it comes to detecting estrogen levels in men and in post-menopausal women, where circulating levels are extremely low. Therefore, it has become cumbersome and impractical to study the implication of this hormone in the atherosclerotic process and in other fields due to the lack of a highly sensitive and specific assay to measure estradiol levels.

1.7 Carotid Artery Disease

Carotid artery disease, also known as carotid stenosis, is the progressive narrowing of the carotid arteries, which supply 80% of the blood to the brain. This narrowing is due to the formation of atherosclerotic plaques that form preferentially along the outer wall of the carotid bifurcation and in the proximal internal carotid artery (**Figure 1.1**)[127]. It is believed that the anatomy of the carotid artery influences local hemodynamic forces and plays a role in the site-specific development of atherosclerosis[127-129]. Straight regions of the carotid arterial tree are exposed to steady laminar blood flow and sustained high shear stress, which protect against atherosclerosis[127-129]. On the other hand, disturbed laminar blood flow and low shear stress occur in arterial segments with

geometric irregularities, such as curvatures, branches, and bifurcations, predisposing these areas to plaque formation due to oxidative and inflammatory changes in the vascular endothelium[127-129].

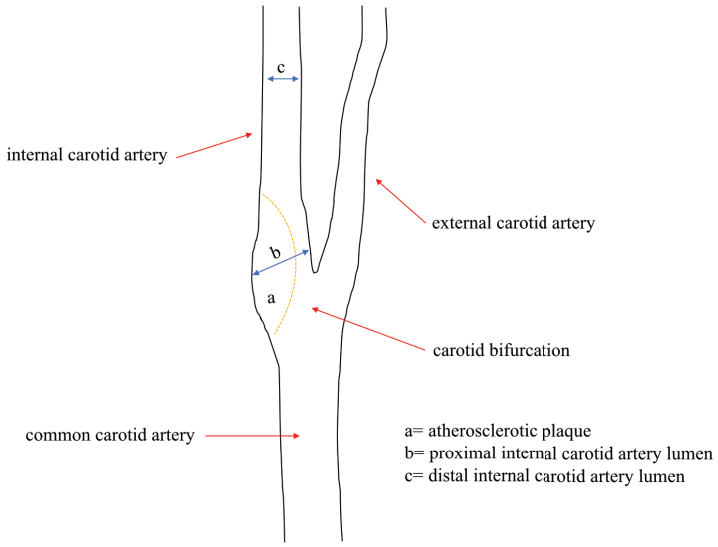


Figure 1.1 Site-specific formation of atherosclerotic plaques in the carotid arteries. *Plaques (a) are most likely to form along the outer wall of the carotid bifurcation (where the common carotid artery divides into the internal and external carotid arteries) or in the proximal internal carotid artery (b) due to disturbed laminar flow and low shear stress that occurs particularly in these regions. Atherosclerosis is less likely to form in the distal internal carotid artery (c) where the shear stress is high and laminar flow is steady.*

1.7.1 Screening for carotid artery disease

Carotid artery disease results in clinical consequences in the form of an AF[130], TIA, or ischemic stroke[131]. In fact, 15-20% of all ischemic strokes are attributable to carotid artery disease[75]. Imaging of the carotid artery is recommended in all symptomatic patients presenting with signs and symptoms of retinal and/or cerebral ischemia[132-134]. A Doppler ultrasound is typically used to screen for carotid artery stenosis in these symptomatic patients[132-134]. Despite some estimated 5-10% of individuals over the age of 65 years living with asymptomatic carotid artery disease[135], screening of the general population for asymptomatic carotid stenosis is not currently indicated, as the magnitude of benefit is believed to be small to none and would unlikely be cost-effective[132-134]. The benefit is limited by the low overall prevalence of disease in asymptomatic individuals

and that revascularization procedures may be associated with greater risks in this population. A Doppler ultrasound may be performed only when there is a high index of suspicion for carotid artery disease due to the presence of a carotid bruit and/or multiple risk factors, such as hypertension, smoking, CAD, and family history of stroke[132-134]. The prevalence of asymptomatic carotid artery stenosis (≥50%) was observed to be >14% in individuals aged >60 years and who had two or more vascular risk factors[136].

High-resolution real-time B-mode ultrasound with Doppler flow imaging is the imaging modality of choice in diagnosing carotid artery disease, as it is non-invasive, reliable, safe, and relatively inexpensive to use[132-134]. A systematic review evaluating the diagnostic value of duplex ultrasonography for the diagnosis of 70-99% carotid artery stenosis reported a pooled sensitivity of 86% (95% CI: 84-89) and a pooled specificity of 87% (95% CI: 84-90), compared to conventional digital subtraction angiography[137]. Furthermore, for recognizing complete artery occlusion, carotid Doppler ultrasound yielded a sensitivity of 96% (95% CI, 94 to 98) and a specificity of 100% (95% CI, 99 to 100)[137]. While its main utility is for evaluation of the severity of carotid artery stenosis, it can also be used to help characterize the morphology of carotid plaques[132-134]. However, other imaging modalities, such as magnetic resonance imaging and computed tomography can characterize the morphology and specific features of the carotid plaque more reliably than Doppler ultrasound[132-134]. Unfortunately, despite their better resolution and their widespread applications, their use remains limited in clinical practice for the diagnosis and monitoring of carotid artery disease, mostly due to limited availability, and high-cost, as well as radiation associated with computed tomography.

1.7.2 Management of patients with carotid artery disease

The ultimate goal of treatment is to reduce the risk of stroke. Cerebrovascular symptomatic status and the degree of carotid artery stenosis are crucial in determining the choice of treatment in patients with carotid artery disease. Therefore, it is important to make a clear distinction between asymptomatic and symptomatic carotid artery disease as non-invasive (medical) and invasive (surgical) management will differ.

Medical management

Optimal medical management is important for the treatment of all patients with carotid artery disease, regardless of the degree of stenosis and whether surgical intervention is planned. It is often considered as the first-line therapy for asymptomatic patients. Treatment includes lifestyle modifications as well as control of traditional vascular risk factors. This comprises anti-hypertensive therapy aimed at reducing blood pressure to a target <140/90 mmHg, lipid-lowering agents such as statins to decrease LDL-C levels to reach a target <2 mmol/L in all patients with carotid artery disease or a more aggressive target <1.8 mmol/L in patients who recently suffered an ischemic stroke or TIA, effective smoking cessation strategies, and a healthy balanced diet[132,134,138]. While T2DM is strongly associated with ischemic stroke risk, the tight control of serum glucose levels in diabetic patients to nearly normoglycemic levels (target glycosylated hemoglobin A_{1C} <7%) was not observed to reduce the risk of stroke[132,134,138]. Nonetheless, diet, exercise, and glucose-lowering drugs are recommended in patients with diabetes and carotid artery disease[132,134,138]. Furthermore, considering that patients with diabetes often have multiple risk factors, it is important to achieve control of these other risk factors to reduce their risk of stroke. Since stroke is an atherothrombotic complication, antiplatelet agents, such as aspirin (75 to 325 mg daily), clopidogrel, and dipyridamole, are highly recommended for secondary prevention of recurrent stroke in patients who had a non-cardioembolic ischemic stroke or TIA[132,134,138]. There is insufficient evidence to demonstrate a similar benefit with antithrombotic therapy in reducing stroke risk in asymptomatic patients with carotid artery disease[132,134,138]. However, in these patients antithrombotic therapy can be used to help reduce overall cardiovascular morbidity. Anticoagulant therapy, on the other hand, is not indicated in symptomatic patients with carotid artery disease, unless there is evidence that the symptoms are cardioembolic in nature[132,134,138].

<u>Statins</u>

Statins remain the first-choice lipid modification therapy for the primary and secondary prevention of CVD[35,37-40]. The major therapeutic action of statins is the reduction in circulating levels of LDL-C as a result of inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity mainly in hepatocytes, which is the rate-limiting step in endogenous cholesterol biosynthesis[139] (**Figure 1.2**). HMG-CoA inhibition leads to a decreased concentration of cholesterol within the cell, which then (1) stimulates the synthesis of LDL receptors on the surface

of the cell membrane, promoting the uptake of LDL-C from the circulation, and (2) decreases the secretion of lipoproteins from the cell into the circulation[139]. There exist various types of statins, which differ in their potency and efficacy in lowering LDL-C levels. Atorvastatin and rosuvastatin are known to be the most effective and potent statins[140].

Beyond cholesterol lowering, statins also possess pleiotropic actions on a multitude of different cell types (**Figure 1.2**), which can lead to the improvement of vascular endothelial function, modulation of immune activation, and reduction of platelet activation/aggregation and thrombus formation[141,142]. Furthermore, statins have direct favorable effects on atherosclerotic plaque burden by promoting plaque regression[143,144]. Various randomized controlled trials demonstrated the effect of statins on plaque regression to be associated with intensive statin therapy[143-145]. In the REVERSAL trial, intensive lipid-lowering treatment with atorvastatin (80 mg) stabilized the progression of coronary atherosclerosis (percentage change in atheroma volume: -0.4%; 95% CI -2.4% to 1.5%; P =0.98) over an 18-month period, while moderate lipid-lowering treatment with pravastatin (40 mg) led to a 2.7% increase in atheroma volume (P =0.001) compared with baseline[145]. In the SATURN trial, treatment with rosuvastatin (40 mg) resulted in a decrease in percent atheroma volume by 1.22% (95% CI -1.52% to -0.90%; P =0.17)[143]. Statins have also been shown to possess anti-inflammatory and immunomodulatory effects which can affect the plaque by 1) inhibiting the adhesion and migration of monocytes to the arterial wall by reducing the expression of various adhesion molecules[146], 2) reducing macrophage accumulation in the plaque[147], and 3) inhibiting the production of MMPs by activated macrophages[147]. Statins can also exert inhibitory effects on vascular SMC migration and proliferation[148]. These non-lipid related effects of statins are believed to be mediated by the concurrent inhibition of protein isoprenylation, a process responsible for a variety of cellular responses downstream of the mevalonate pathway[139] (**Figure 1.2**). While a wide range of patients benefit greatly from statin therapy due to statins' lipid and extra-lipid effects, many statin-treated patients continue to suffer from life-threatening vascular events, due to an issue termed 'residual cholesterol risk' (LDL-C levels remain above target) or 'residual inflammatory risk' (C-reactive protein [CRP] levels remain above target)[149]. Furthermore, various adverse effects have been associated with statin use, such as hepatotoxicity, rhabdomyolysis, intracerebral haemorrhage, nephrotoxicity, cognitive impairment, and new-onset T2DM, which mainly accompany long-term statin treatment and higher doses[139].

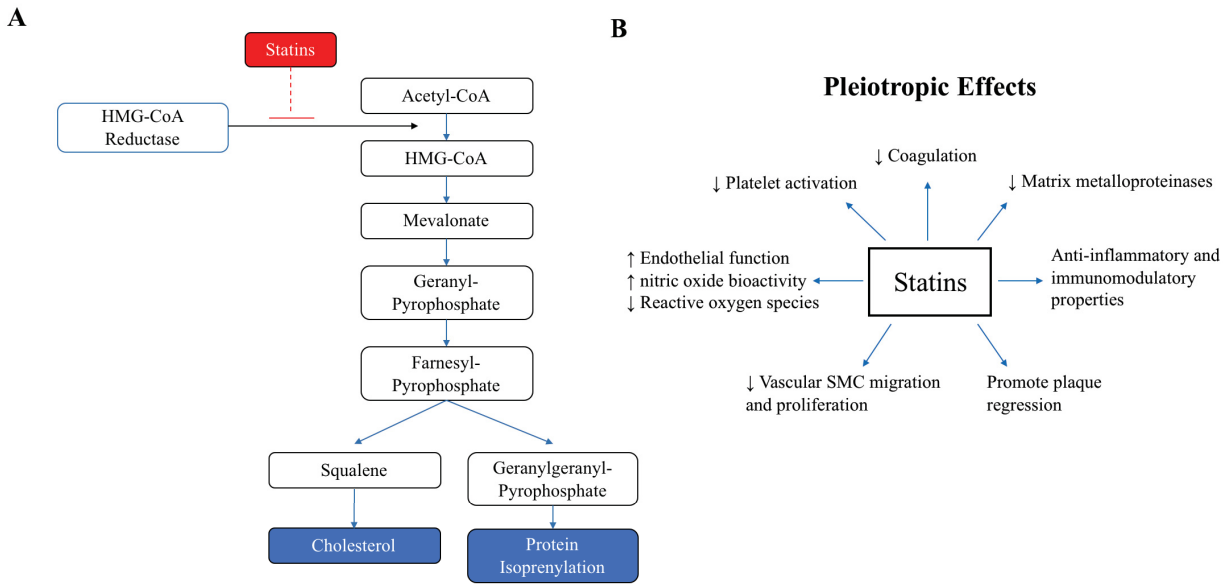


Figure 1.2 Statins' mechanism of action and pleiotropic effects. ***A)*** *Statins' inhibition of the HMG-CoA reductase enzyme leads to inhibition of the mevalonate pathway products. Mevalonate is not only a precursor for cholesterol but also for many isoprenoids, which are important for various cellular functions. Thus, inhibition of this pathway not only affects endogenous cholesterol biosynthesis but can also lead to many pleiotropic effects.* ***B)*** *Statins possess actions beyond lipid-lowering that may be associated with its beneficial effects on atherogenesis and clinical outcomes.*

Surgical management

Surgical management for stroke prevention in individuals with carotid artery disease includes either CEA or carotid artery stenting (CAS). Indications for surgical intervention are based solely on cerebrovascular symptomatic status and the degree of carotid artery stenosis[134,138]. The risk of stroke is known to be higher in patients with a history of carotid artery related ischemic events. Furthermore, the degree of carotid artery stenosis is a strong determinant of stroke risk; studies have demonstrated that the risk of developing cerebral ischemic symptoms increased with increased severity of carotid artery stenosis[150,151]. More stenotic carotid plaques are also more likely to be associated with the presence of unstable histological features such as ulceration, intraplaque hemorrhage, and thrombus formation[152]. Therefore, current guidelines recommend CEA or CAS (plus medical treatment) for stroke prevention in both neurologically symptomatic and asymptomatic patients with carotid plaques, when stenosis is ≥50% or ≥60% respectively[134,138].

Carotid Endarterectomy

CEA is an invasive surgical procedure that involves the removal of carotid plaques from the carotid artery. It is the gold standard treatment for severe symptomatic carotid artery stenosis and recommended in selected asymptomatic patients. Its benefit in reducing the risk of ischemic stroke was demonstrated by large clinical trials, the North American Symptomatic Carotid Endarterectomy Trial (NASCET) and the European Carotid Surgery Trial (ECST) in symptomatic individuals and the Asymptomatic Carotid Atherosclerotic Study (ACAS) and the Asymptomatic Carotid Surgery Trial (ACST) in asymptomatic individuals[150,151,153-155]. The evidence presented in these trials firmly established CEA as the treatment of choice for patients with severe carotid stenosis and thus, CEA has become a widely accepted intervention throughout the surgical community.

Original NASCET findings showed that in symptomatic patients with 70-99% stenosis, CEA was beneficial with a significant 2-year stroke absolute risk reduction of 17±3.5% compared to medical therapy alone[150]. The 'number needed to treat' by surgery was reported to be six patients in order to prevent one adverse event by 24 months[150]. Later findings by the same group also evaluated the benefit of CEA in symptomatic individuals with moderate-grade blockage of 50-69% stenosis; CEA reduced the risk of stroke by 6.5% over a 5-year period compared to those treated medically (P=0.045)[153]. On the other hand, no benefit was observed in patients with <50% stenosis[153]. In fact, in these individuals CEA was associated with increased morbidity compared with medical management. The ECST identified results comparable to those of the NASCET group, despite each trial using different methods of measurement for the degree of stenosis. The NASCET method is known to underestimate the degree of stenosis compared with the ECST method, such that stenoses reported to be 70-99% or 50-99% by the NASCET method were equivalent to 80-99% or 70-99%, respectively, by ECST[156]. On this note, ECST identified a significant 11.6% reduction in major stroke or death at 3 years in symptomatic individuals with >80% stenosis ('number needed to treat' = 9 patients), which was consistent with the 10.1% reduction in major stroke or death at 2 years reported for patients in NASCET with >70 stenosis[154].

The ACST and ACAS also demonstrated that CEA is effective at reducing stroke rates in asymptomatic individuals with ≥60% stenosis (as measured by the NASCET method)[151,155]. A meta-analysis pooling estimates from these trials (plus the Veterans Affairs Cooperative Study) identified the absolute reduction in stroke or death to be 3.5% over 5 years for patients undergoing

CEA compared with those receiving medical therapy alone[157]. However, the strength of these conclusions has been questioned, considering that the medical management used in those trials is now outdated[158]. Therefore, the benefit of CEA in reducing stroke risk is expected to be smaller with the use of contemporary optimal medical management. Furthermore, many argue that the risks associated with CEA may negate the small benefit observed in asymptomatic individuals[157-159]. A 30-day stroke or death rate after CEA of 2.4% was reported, which was 1.9% greater than the stroke or death rate observed in the medical therapy alone group[157]. The ongoing Carotid Revascularization Endarterectomy versus Stenting Trial (CREST)-2 trial comparing CEA or CAS plus current intensive medical management to intensive medical management alone, may provide important data for future recommendations on what may be the best method of preventing strokes in asymptomatic individuals with severe carotid artery stenosis[160].

Carotid Artery Stenting

CAS is a minimally invasive technique that involves placing a self-expanding stent through the carotid lesion to reopen the arterial lumen. Large trials as well as meta-analyses of randomized trials comparing CAS and CEA for prevention of stroke in patients with symptomatic or asymptomatic carotid stenosis found CAS to be inferior to CEA as it was associated with a higher risk of periprocedural stroke[161-165]. In CREST, there were no differences between the two methods of revascularization for the combined outcome of stroke, MI, or death[162]. However, periprocedural stroke was observed to be significantly more frequent with CAS, while the risk of perioperative MI was higher in the CEA group[162]. While CEA remains the preferred method for carotid revascularization, CAS may be superior to CEA in certain patient groups who are considered high-risk surgical candidates due to technical, anatomical, or medical reasons. Therefore, CAS may be offered to patients who have clinically significant cardiac disease, severe pulmonary disease, and who have neck anatomy that is unfavourable for surgery (i.e., contralateral carotid artery occlusion, prior radiation therapy to the neck, or previous ipsilateral carotid artery surgery)[132,134,138].

Current Recommendations

Current Canadian guidelines for surgical management of carotid artery disease are largely shaped by the above-mentioned clinical trials[138]. Symptomatic patients at average or low surgical risk (<6% risk of perioperative stroke or mortality) with ≥50% stenosis (NASCET criteria) are

recommended to undergo CEA. The timing of carotid endarterectomy is essential in the prevention of secondary stroke. Ideally CEA should be performed within 48 hours following the ischemic event or within 14 days of ischemic event onset if patients were not clinically stable for surgery in the first few days[166]. For neurologically symptomatic patients with stenosis <50%, optimal medical therapy is indicated rather than surgical intervention. Furthermore, CEA is also suggested for selected asymptomatic patients with ≥60% stenosis and a low surgical risk (<3% risk of perioperative stroke or death), while patients with stenosis <60% should receive aggressive medical management of risk factors instead. It is important to emphasize that selection of asymptomatic patients for carotid revascularization should include careful consideration of life expectancy, age, sex, and comorbidities[158]. In most patients with carotid stenosis who are candidates for surgical intervention, CEA is preferred to CAS, particularly in patients aged >70 years of age. On the other hand, CAS may be considered for high-risk patients who are not operative candidates for technical, anatomical, or medical reasons, as mentioned above.

1.7.3 Sex differences in carotid artery disease management

Unfortunately, there exists no reliable evidence of benefit of surgical intervention (CEA or CAS) in women with symptomatic or asymptomatic carotid stenosis, as women have been under-represented in carotid stenosis trials, which has led to major uncertainty about the optimal management of carotid artery disease in women. In the majority of these trials, women comprised ≤30% of the total population causing any sex-specific analyses to be under-powered. Thus, there is a dire need for trials with a more representative percentage of women that will properly inform the guidelines for the optimal management of carotid disease, specifically in women. The CREST-2 trial will ideally enrol a more representative percentage of women (>40%) in order to ensure that clinicians have adequate information on the risk and benefit ratio of CEA and CAS for women with asymptomatic stenosis specifically[160].

Overall, previous evidence suggests that women benefit less from surgical intervention than men, particularly those with asymptomatic carotid disease[167,168]. Although women with symptomatic carotid stenosis ≥70% had a similar 5-year absolute risk reduction in stroke as men following CEA (15.1% vs. 17.3%), when stenosis was between 50-69%, CEA was not observed to be beneficial in women (absolute risk reduction [ARR]=3.0%, P=0.94), while it was in men (ARR=10.0%, P=0.02)[167]. In women with asymptomatic carotid stenosis, a Cochrane Systematic

Review reported that the stroke relative risk reduction following CEA was significantly lower compared to that in men; only 4% in women compared to 51% in men (P=0.008)[168]. Differences in plaque morphology and composition may help explain why women benefit less from carotid revascularization than men[122,169]. Evidence not only points toward minimal benefit in women, but also increased perioperative stroke risk. The ACAS trial was the first to suggest that asymptomatic women had an increased perioperative stroke and death rate (3.6%) associated with CEA compared to asymptomatic men (1.7%), although the difference was not significant[155]. Other asymptomatic trials, including ACST, later confirmed these results[151]. Pooled data from ECST and NASCET demonstrated that the periprocedural risk for stroke was also higher in women with symptomatic carotid stenosis who underwent a CEA compared to men (8.7% vs. 6.8%)[166]. It is speculated that women may be at greater operative risk than men because their carotid arteries are 40% smaller than men, making CEA technically more difficult[170].

Timing of surgery for symptomatic carotid stenosis is even more crucial in women than in men, where women were observed to benefit most when CEA was performed within 2 weeks of their last cerebrovascular event[171]. As time increased from most recent event, a decline in benefit was observed in women but not in men[171]. For women with stenosis ≥70%, the ARR in stroke dropped from 41.7% when CEA was conducted within two weeks to only 6.6% when surgery was conducted between two to four weeks[171]. After four weeks, surgery was found to be harmful, with an ARR of -2.2%[171]. In clinical reality, women are not only less likely to undergo CEA than men, but they also receive treatment much later, even in the setting of severe symptomatic stenosis[172]. One study identified that the time from event to CEA was significantly different between men and women, where the median time to CEA was 18 days in men and 35 days in women (P=0.03)[172].
Sex differences are not only observed with surgical intervention but also with medical management. Although women with severe carotid stenosis have been shown to benefit from appropriate medical treatment, they are less likely to receive medical therapy (i.e., statins, anti-hypertensive medication) than men for the primary and secondary prevention of stroke[15]. Furthermore, the rates of administration of thrombolytic therapy (tissue plasminogen activator) to treat patients with acute ischemic stroke were significantly lower in women compared with men[15,173]. This may be due to the atypical presentations seen often times in women presenting with stroke, which leads to delays in the identification of stroke patients.

1.7.4 Knowledge gaps

The current guidelines for carotid disease management in individuals with symptomatic or asymptomatic carotid stenosis are the same for men and women despite the existence of sex disparities in stroke presentation, treatment, and care. Women have been shown to derive less benefit from surgical intervention than men - especially when it is performed outside the 14-day window post-event - and they are also at a higher risk of perioperative complications. As a result, this has led to uncertainty among physicians in deciding which treatment may be appropriate for the management of carotid disease in women. Thus, it is imperative that more carotid trials are performed with greater representation of women in order to put in place sex-specific guidelines for carotid disease management.

All carotid surgical intervention recommendations in patients with symptomatic or asymptomatic carotid disease are based solely on the degree of carotid artery stenosis. Although stenosis is a reasonable predictor of stroke risk, as portrayed by the large carotid stenosis trials, it has also been proven to be an incomplete determinant of a patient's stroke risk (particularly of asymptomatic individuals or women) as it does not entirely reflect how unstable a plaque truly is and its likelihood to rupture. Many plaques causing high-grade stenoses remain stable and asymptomatic, while unstable and potentially dangerous lesions often cause moderate or even low-grade stenoses. In fact, Saam *et al*. evaluated the lesion type according to the American Heart Association (AHA) histological classification in 175 CEA patients and noted that 16.5% of AHA type VI lesions (most advanced and complicated lesion with intraplaque hemorrhage and/or ruptured fibrous cap) occurred in patients with <50% carotid stenosis[174]. This indicates that a large proportion of vulnerable patients may have low-grade carotid stenosis. Furthermore, over 20% of arteries with high-grade stenosis did not have type VI lesions[174]. As a result, the current guidelines for carotid disease management may overlook patients with lower stenosis who have unstable plaques and may misclassify many patients as high-risk when their plaque is stable. The latter group of patients are referred for perhaps unnecessary/premature surgery, imposing unjustified risk and a burden to the health care system.

1.8 Histological Characterization of Atherosclerotic Plaques

Plaque morphology and composition are increasingly recognized as more accurate indicators of plaque instability and better predictors of clinical outcome than the severity of stenosis. Currently,

the gold standard method or the reference method to investigate plaque instability can be performed through histological assessment of the plaque specimen after surgical removal. In this regard, histological classifications of human atherosclerotic lesions have been developed and are used as 'gold standard' scales for the identification of plaque features and the categorization of plaques as stable or unstable: (1) Stary *et al.*'s AHA plaque classification[175,176] and (2) Lovett *et al.*'s semi-quantitative scale of plaque instability[177,178].

1.8.1 American Heart Association plaque classification

The AHA's Committee on Vascular Lesions developed a plaque classification that characterizes lesions from their early development to their more advanced progression (types I to VIII) based on their histological composition and structure (**Table 1.1**)[175,176]. Lesions type I to III are identified as initial, fatty streak, and intermediate lesions, respectively, and develop during the early phases of life. They are always small and clinically silent, while lesions type IV to VIII are considered advanced atherosclerotic lesions, which can be clinically silent or associated with clinical manifestations. The initial (type I) lesion is characterized by a few scattered macrophage foam cells. On the other hand, type II lesions consist primarily of layers of macrophage foam cells as well as lipid-laden SMCs. Type III lesions have similar characteristics as that of type II lesions, but with the addition of isolated pools of extracellular lipids. These lesions typically form the bridge between early and advanced lesions. Type IV, also known as atheroma, is the first lesion considered advanced in the AHA's classification due to severe disruption of intimal structure and the presence of a lipid core. This core of extracellular lipid is more advanced than the small lipid pools observed in the type III lesions. Although considered an advanced lesion, type IV lesions often fail to narrow the vascular lumen. Thus, surgical plaque specimens obtained following a CEA are not typically representative of type IV lesions, but rather of types V-VIII, as these lesions significantly occlude the lumen. Type V, VII, and VIII plaques are characterized by the increased formation of fibrous connective tissue. Lesions that have a lipid core (or multiple lipid cores) and thick layers of fibrous connective tissue are known as type V lesions, or fibroatheromas. Largely calcified fibro-lipid lesions are referred to as type VII (calcific) plaques, while lesions that consist mainly of fibrous connective tissue and little or no lipid core are referred to as type VIII (fibrotic) plaques. Type VII and VIII plaques usually form following lipid regression from Type IV or V lesions. Clinical manifestations and fatal outcomes are often associated with type VI lesions. Type

VI is a complicated or unstable lesion that generally has the underlying morphology of a Type IV or V lesion but is also characterized by the presence of a surface defect (VIa), a hematoma or hemorrhage (VIb), a thrombus (VIc), or a combination of these features (i.e., VIabc).

1.8.2 Semi-quantitative scale of plaque instability

While the AHA classification describes how advanced/complicated a lesion is based on its overall histological composition and structure, semi-quantitative scales were developed to characterize and refine important histological features of the plaque that help determine plaque stability. Lovett *et al*. produced a semi-quantitative scale that classified individual features of the plaque on a 3- or 4-grade scale, including intraplaque haemorrhage, surface thrombus, foam cells, lipid core size, proportion of fibrous tissue, neovascularization, calcification, cap rupture, overall inflammation, and infiltration of the fibrous cap with inflammatory cells (**Table 1.2**)[177]. Inflammation was graded according to the number of macrophages and lymphocytes present. Based on the presence of a combination of these features, the overall instability of the plaque was characterized as definitely stable, probably stable, probably unstable, or definitely unstable (**Table 1.2**)[177].

1.8.3 Features of an unstable plaque

According to the 'traditional concept of plaque vulnerability', unstable plaques are either ruptured or rupture-prone, and generally characterized by a large lipid-rich necrotic core, a thin inflamed fibrous cap, thrombosis, and intraplaque hemorrhage, while stable plaques have a thick fibrous cap, which protects them from rupturing, and small or no lipid core[93]. A large lipid core is the hallmark of a vulnerable plaque as its expansion can 1) erode the fibrous cap of the plaque, 2) increase the thrombogenicity of the plaque material, and 3) provide an environment that is conducive to macrophage activation. Typically, there is a greater abundance of inflammatory cells in unstable plaques than in stable plaques[93]. Macrophages, in particular, are major cellular effectors of the initiation, progression, and complication of atherosclerotic lesions. They are central contributors to plaque instability, as they produce MMPs, which are important in plaque remodeling and erosion of the fibrous cap, causing its rupture[94,95]. In one of the largest studies of symptomatic carotid plaque histology, dense macrophage infiltration into the plaque was associated with a 3.4-fold increased risk of cap rupture[179]. The stability of atherosclerotic lesions may also be influenced by calcification and neovascularization, which are common features of

advanced lesions. Generally, unstable lesions have increased neovascularization, which provide a conduit for entry of inflammatory cells, and less calcification than stable lesions[93].

However, this concept of plaque vulnerability may be challenged in the future due to temporal changes in plaque composition observed over the past decade, which are partly believed to be associated with improvements in risk factor management and secondary prevention strategies[180,181]. Interestingly, plaques obtained from recent CEAs possessed less unstable histological features that are causally associated with symptomatic events (i.e., large lipid core, thrombosis, inflammation) than plaques obtained about a decade ago, despite no reduction in the proportion of CEAs performed on symptomatic subjects[181].

Table 1.1 American Heart Association's histological classification of atherosclerotic lesions

Lesion Type	Description
Type I – Initial lesion	Isolated macrophage foam cells
Type II – Fatty streak	Multiple layers of macrophage foam cells
Type III – Intermediate lesion	Type II changes + small isolated extracellular lipid pools
Type IV – Atheroma	Severe disruption of intimal structure + extracellular lipid core
Type V – Fibroatheroma	Thick layers of fibrous connective tissue + lipid core (or multiple lipid cores)
Type VI – Complicated lesion	Surface defect, hematoma-hemorrhage, thrombus
Type VII – Calcific lesion	Calcification predominates
Type VIII – Fibrotic lesion	Fibrous tissue predominates

*Adapted from *Arterioscler Thromb Vasc Biol. 2000;20:1177-1178*[176]
The evolution of atherosclerotic lesions from type I to type IV is sequential. However, after type IV lesions have developed, the pathway towards further plaque progression may vary. For example, a type IV lesion may develop type VI changes without passing first through a type V stage. Other type IV lesions may develop into type V plaques, which later progress into either type VII or VIII plaques, instead of developing type VI changes. Furthermore, remodeling of Type VI lesions may result in the conversion to type V plaques

Table 1.2 Semi-quantitative scale of plaque instability

Histological Feature	Grade 1	Grade 2	Grade 3	Grade 4
Hemorrhage	No hemorrhage	Small hemorrhage	Large hemorrhage	-
Thrombus	No thrombus	Small thrombus	Large thrombus	-
Lipid core	No lipid core	Small lipid core	Large lipid core (>25% total area)	-
Fibrous tissue	Very little fibrous tissue	~50% fibrous tissue	Predominantly fibrous	-
Foam cells	None	<50 cells	At least 50 cells	-
Neovascularization	None	<10 per section	At least 10 per section	-
Calcification	None	Stippling only	Calcified nodules	-
†Inflammatory cells	None	Occasional cells or one group of >50 cells	2-5 groups of >50 cells	>5 groups of >50 cells or one group >500 cells
†Cap infiltration	None	<10 cells in cap	10-50 cells in cap	>50 cells in cap
Cap rupture	Intact cap	Probably intact (artefactual break in cap created during surgery)	Probably ruptured (site of rupture not clear but thrombus present)	Definitely ruptured
Overall instability	Definitely stable (predominantly fibrous, few inflammatory cells, intact cap)	Probably stable (presence of one feature of instability, such as small hemorrhage or inflamed cap)	Probably unstable (presence of inflammation, thin cap, and large core but no rupture)	Definitely unstable (presence of rupture, thrombus, large hemorrhage, thin inflamed cap)

*Adapted from *Circulation. 2004;110:2190-2197*[177]
† Inflammation graded according to the number of macrophages and lymphocytes present

1.8.4 Sex differences in plaque morphology and composition

There exists compelling evidence demonstrating differences in plaque morphology and composition between men and women. While carotid artery stenosis and carotid plaque area increase with age in both men and women, women have been reported to have greater carotid artery stenosis than men, while men have greater plaque area than women[182]. Histological analysis of carotid plaque specimens collected from patients following a CEA showed that men had a higher prevalence of unstable plaques than women, with high-risk plaque features such as a larger hemorrhage and larger necrotic lipid core[169]. Furthermore, men's plaques were characterized by a greater presence of inflammatory cells, particularly macrophages and foam cells. On the other hand, women had higher rates of stable fibrocalcific plaques than men. As assessed by magnetic resonance carotid plaque imaging, these sex differences in plaque features remained apparent even in plaques with less than 50% stenosis[183].

1.9 The Adipo-Vascular Axis

1.9.1 Adipose tissue – an active endocrine organ

White adipose tissue (WAT) in humans is mainly located beneath the skin (subcutaneous adipose tissue) and around the intra-abdominal organs (visceral adipose tissue). Other common sites of adipose tissue accumulation include the bone marrow, the heart (epicardial adipose tissue), and the adventitia of blood vessels (i.e., periadventitial adipose tissue)[184,185]. In addition to adipocytes, which represent the greatest percentage of cells within the adipose tissue, other cell types are also present, including adipocyte precursor cells, capillary endothelial cells, fibroblasts, and inflammatory cells, that are collectively termed the stromal vascular fraction[184,185].

WAT is the largest site in the body for storing dietary energy mainly in the form of triglycerides, and it releases free fatty acids into the circulation during the fasting state. The continuous storage and hydrolysis of triglycerides (i.e., a process known as lipolysis) is essential to maintaining body weight homeostasis[184]. In addition, adipose tissue plays a role in thermo-insulation and mechanical protection of internal organs. Although WAT was traditionally perceived to act simply as a storage depot for excess energy, recently it has also been recognized to act as a highly dynamic endocrine organ that is involved in the regulation of insulin sensitivity, glucose and lipid metabolism, as well as cardiovascular homeostasis. These functions are mediated by WAT's ability to produce and secrete bioactive substances termed adipokines, which may act

both locally (autocrine/paracrine interactions) and systemically (endocrine)[184,186-188]. The term adipokine is used to describe soluble factors that are mainly but not exclusively produced by adipose tissue.

Adipsin was the first adipokine to be discovered in 1987[189]. However, it was the discovery of leptin in 1994 that redefined WAT as an endocrine organ[190,191]. In the following decades, several hundreds of adipokines have been identified, such as adiponectin, chemerin, resistin, visfatin, and omentin. Adiponectin and leptin are the most abundantly produced adipokines. Leptin has been found to play an important role in the regulation of food intake and energy expenditure[190,191], while adiponectin is an insulin-sensitizer that possesses anti-inflammatory properties[188,192]. In addition to adipokines, WAT, because of its diversity of cells, can also produce cytokines and chemokines (TNF-α, IL-6, and MCP-1), mediators of the coagulation cascade (plasminogen-activator inhibitor type 1), and complement factors[184,193]. Adipokines not only modulate the activity of adipocytes, but they also mediate the cross-talk between WAT and other organs to regulate their metabolism (i.e., the liver, the muscle, the pancreas, and the central nervous system)[184,194]. Since they possess both pro- and anti-inflammatory properties, adipokines play a critical role in integrating systemic metabolism with immune function[195]. Furthermore, WAT expresses numerous receptors that allow it to respond to various hormonal stimuli.

1.9.2 Adipose tissue dysfunction

As a consequence of excess weight, the normal function of adipose tissue is disturbed, i.e., adipose tissue dysfunction, which is characterized by an increase in adipocyte size, infiltration of the adipose tissue by inflammatory cells, and dysregulation in the production and secretion of adipokines[184,196].

Obesity is viewed as a state of chronic, low-grade inflammation, as several studies have pointed out an increase in the number of immune cells infiltrating the adipose tissue of obese individuals and mouse models of obesity[197,198]. Particularly, macrophages play a significant role in augmenting the inflammatory response in adipose tissue. In fact, the accumulation of macrophages in adipose tissue has been shown to rise proportionally with increased BMI, adipocyte hypertrophy, and insulin resistance[197]. Specifically, in obese patients, the macrophage content in WAT has been reported to be ~ 50% of the total number of cells, in comparison to ~5-10% in lean individuals[198]. According to bone marrow transplant studies performed in

macrophage-deficient mice, macrophages that infiltrate the adipose tissue are proposed to derive from the bone marrow[198]. However, the signals that induce macrophage recruitment to the adipose tissue of obese individuals are not well understood. Macrophages are hypothesized to be recruited to the dysfunctional adipose tissue in response to hypertrophic adipocyte necrosis, in order to carry out their scavenger function. This theory has been partly proven in studies where mice were treated with an exogenous drug that induces apoptosis specifically in adipocytes[199]. Furthermore, hypertrophic adipocytes secrete large amounts of the chemo-attractant, MCP-1, which can enhance macrophage infiltration in WAT of obese mice and humans[200,201] . Upon recruitment to the adipose tissue, macrophages form aggregates surrounding the dead adipocytes, termed "crown-like structures"[202]. Since macrophages are secretory cells, their cross-talk with adipocytes contributes significantly to the production of inflammatory factors, such as TNF-α, IL-6, and MCP-1 that act locally as well as systemically[203]. Thus, this vicious cycle of leukocyte recruitment and release of pro-inflammatory adipokine and cytokine synthesis by adipocytes and macrophages contributes not only to local inflammation, but also to a chronic systemic state of low-grade inflammation.

Under conditions of normal metabolic status, adipose tissue is known to produce an array of adipokines/cytokines including adiponectin, leptin, TNF-α, IL-6, resistin, omentin, visfatin, chemerin and many others, resulting in a balance in the secretion of pro- and anti-inflammatory adipokines[195]. However, this balance shifts to favor pro-inflammatory mediators as adipose tissue expands during the development of obesity[195,204] (**Figure 1.3**). In fact, IL-6 and TNF-α are among the pro-inflammatory cytokines that are consistently found to be increased in obesity, both at the local level (i.e., in the WAT) and systemically (i.e., in the bloodstream)[205]. On the other hand, anti-inflammatory mediators, including adiponectin and IL-10 are found to be reduced in obese individuals[204]. Furthermore, microarray studies have been performed in humans and animal models, comparing gene expression profiles of obese versus lean adipose tissue. Most studies noted an alteration in gene profiling with obesity, particularly an up-regulation in inflammatory-related genes[206-208]. Thus, an imbalance in the production of pro- and anti-inflammatory factors associated with obesity is believed to form the link between adipose tissue dysfunction and the development of insulin resistance, CVD, and many other pathological conditions.

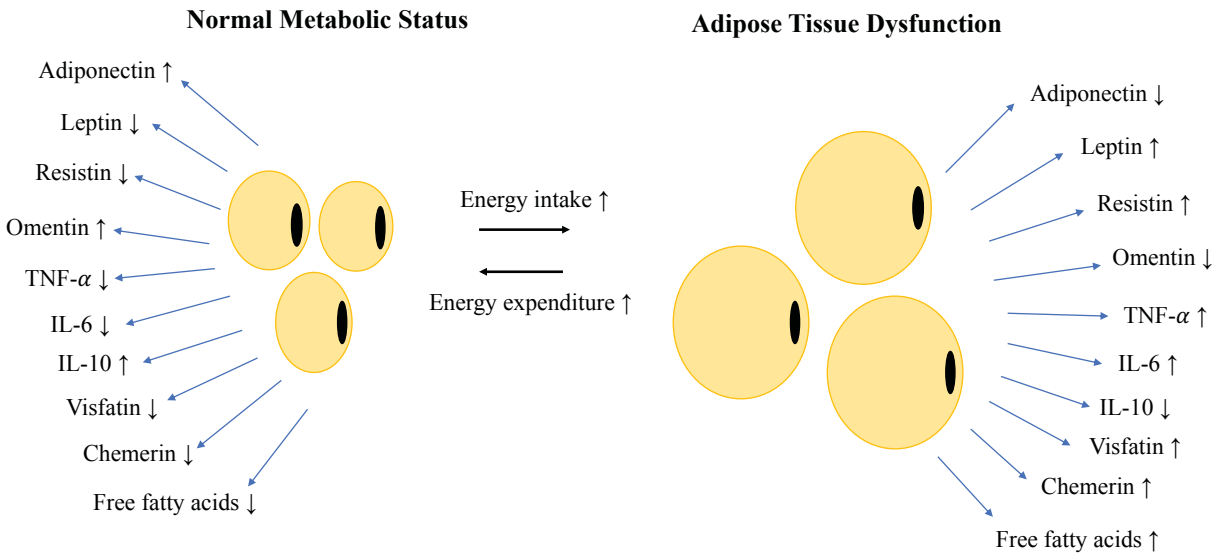


Figure 1.3 The secretory profile of adipose tissue under normal and obese states.

1.9.3 The role of adipose tissue dysfunction in the development of atherosclerosis

Factors secreted by adipose tissue can influence vessel wall homeostasis either by working through the liver or directly at the vessel wall[209,210]. At the liver, adipose-tissue derived factors can influence systemic lipid and lipoprotein metabolism, as well as changes in inflammatory and clotting system components, which can then impact the environment of the vessel wall[209,210]. Furthermore, adipokines can directly affect the function of the major cell types present in the arterial wall, including endothelial cells, SMCs, and macrophages[209,210]. Excess adipose tissue can adversely affect the vasculature by causing a dysregulation in the production of these adipose-tissue derived factors. In fact, obesity is known to accelerate atherosclerosis and has been associated with increased rates of cardiovascular death[211,212].

The regional distribution of body fat is an important determinant of an individual's cardio-metabolic risk. Numerous epidemiological studies have pointed out that individuals with central obesity (i.e., accumulation of fat in visceral depots) are at higher risk for developing T2DM, CVD, and cancer than those with peripheral obesity (i.e., accumulation of fat in subcutaneous depots)[213-215]. Visceral adiposity, rather than subcutaneous adiposity, is believed to contribute to increased comorbidity risk due to **a)** its anatomical site and **b)** its unique adipokine/cytokine gene and secretory profile[216,217]. In comparison to subcutaneous fat, many pro-inflammatory adipokines/cytokines are predominantly secreted by visceral adipose tissue, under obese conditions, whereas adiponectin's (anti-inflammatory adipokine) expression is highly

reduced[218,219]. These factors derived from visceral adipose tissue have favored access to the liver through the portal circulation and can accelerate atherosclerosis development by mechanisms related or not directly related to lipids[220]. For example, increased release of free fatty acids from adipose tissue can increase the synthesis and secretion of LDL, apoB, and triglycerides from the liver, while they can also activate inflammatory processes, induce endothelial cell apoptosis, and impair nitric oxide (NO) production and endothelium-dependent vasodilation[221-224].

In addition to visceral fat, perivascular adipose tissue (PVAT) is also a crucial adipose tissue depot that can play a direct role in atherosclerosis development[209,225]. Most major arteries that are typically affected by atherosclerosis such as the aorta, the coronary arteries, and the carotid arteries are surrounded by PVAT[226]. Originally it was thought that PVAT was simply a structurally supportive tissue for the vasculature, however more recently it has been shown that it can influence vascular homeostasis[226]. Adipokines secreted from PVAT have direct access to the adjacent arterial wall by diffusion. Due to this direct contact, factors derived from PVAT are believed to have more potent effects on the vasculature than factors released from other adipose tissue depots. PVAT also expands with obesity and displays a dysfunctional adipokine profile. In fact, PVAT surrounding atherosclerotic lesions or mechanically-injured arteries displayed pro-inflammatory adipokine profiles and reduced adiponectin expression[227,228]. On the other hand, removal of healthy PVAT enhanced neointimal formation[227]. Thus, under healthy conditions, PVAT has beneficial effects on vessel function. However, under conditions of obesity, in addition to having vasoconstrictive effects, PVAT becomes dysfunctional leading to the release of elevated levels of pro-inflammatory adipokines that can contribute to endothelial dysfunction, atherosclerotic plaque development, and plaque rupture[209,225,226,229].

Many adipokines and cytokines mediate the cross-talk between adipose tissue and the vasculature in the "adipo-vascular axis'. The altered release of these factors by dysfunctional visceral or PVAT can have direct effects on the vessel wall and promote atherosclerotic plaque development. For instance, resistin and leptin levels are increased in obese individuals and are positively associated with coronary atherosclerosis and other cardiovascular complications in humans[230-233]. Resistin can affect the atherosclerotic process by promoting the upregulation of vascular endothelial adhesion molecules, increasing the production of pro-inflammatory cytokines by endothelial cells (i.e., endothelin-1, MCP-1) and macrophages (i.e., TNF-α), and inducing foam cell formation[234-236]. Similarly, leptin can increase the production of MCP-1 and endothelin-1 in

endothelial cells[237,238]. Furthermore, leptin plays a role in neo-intimal formation in response to endothelial damage by promoting the migration, proliferation, and hypertrophy of vascular SMCs[239]. Elevated levels of IL-6 and TNF-α can promote endothelial dysfunction by decreasing the production of endothelial nitric oxide synthase (eNOS) and causing a decrease in the availability of NO[240,241]. With increased plasma concentrations of adipocyte-derived cholesteryl ester transfer protein associated with obesity, the levels of small, dense atherogenic LDL particles also increase[242]. Small, dense LDL particles can easily enter the vascular wall, where they are susceptible to oxidative transformation and can promote endothelial damage and macrophage-to-foam cell transformation[243]. Furthermore, high levels of plasminogen activator inhibitor-1 produced by adipocytes under the influence of TNF-α and free fatty acids, can contribute to atherosclerotic plaque progression by promoting atherothrombosis and inhibiting plasminogen-induced migration of vascular SMCs from the medial layer of the arterial wall to the intima[244]. This promotes the formation of unstable plaques with thin fibrous caps that are prone to rupture[245]. Other pro-inflammatory adipokines that may be associated with the progression of atherosclerotic plaques are chemerin, visfatin, and apelin, which are increased in response to obesity-induced elevation of IL-6 and TNF-α production[246]. On the other hand, some adipokines, like adiponectin, have a protective role in the vasculature and down-regulation of its levels can play a significant contribution to atherosclerosis development, which will be discussed in detail below.

1.10 The Role of Adiponectin and the Adiponectin Receptor Pathway in Atherosclerosis Development

1.10.1 Adiponectin biosynthesis and structural properties

In 1995-1996, both murine and human forms of adiponectin (also termed adipocyte complement-related protein of 30 kDa, AdipoQ, adipose most abundant gene transcript 1, and gelatin-binding protein of 28 kDa) were discovered and isolated by four independent groups[247-250]. Originally, adiponectin was thought to be exclusively synthesized and secreted by adipose tissue and fully differentiated adipocytes. However, it was later determined that various other cells or tissue can also produce adiponectin but to a lower degree than adipose tissue, such as osteoblasts, myocytes, epithelial cells, and placental tissue. Adiponectin is considered the most abundantly secreted adipokine, accounting for about 0.01% of total serum protein, with high levels present in the circulation of healthy individuals (5-30 μg/mL)[251]. However, women have significantly higher

adiponectin levels than men, as testosterone is believed to have direct effects on modulating adiponectin production, complex formation, secretion, and clearance[252].

Human adiponectin is a 244-amino acid polypeptide consisting of four domains: an amino-terminal signal sequence, a hypervariable domain, a collagenous domain comprising 22 Gly-XY repeats, and a carboxy-terminal complement 1q-like globular domain[253]. In the circulation, it exists as oligomeric complexes with different molecular weights (**Figure 1.4**). Its full-length monomer form (which has not been observed in circulation and appears to be confined to the adipocyte) can establish interactions via the collagenous domain to generate three multimeric complexes: a low molecular-weight multimer (LMW, trimer), a middle molecular-weight multimer (MMW, hexamer), and a high molecular-weight multimer (HMW, 12- to 18-mer)[254-256]. A smaller form of adiponectin also exists in the circulation (but in negligible amounts due to a very short half-life); it consists simply of its globular domain, which is generated from the full-length protein by proteolytic cleavage[257]. Leukocyte elastase secreted by activated monocytes and neutrophils may be responsible for the generation of globular adiponectin[258]. These isoforms are suggested to possess different biological activities, but evidence in this domain is still quite limited. However, it is strongly suggested that HMW adiponectin, which makes up 50% of the total adiponectin in the circulation, is the major biologically active isoform[254,256]. Post-translational modifications, such as hydroxylation and subsequent glycosylation, are important for the assembly, secretion, and bioactivity of the HMW isoform[254]. Wang *et al*. demonstrated that mutations in all 4 highly conserved lysine residues within adiponectin's collagenous domain that are crucial for hydroxylation and glycosylation, completely abolish the assembly and secretion of HMW adiponectin[259]. Furthermore, several endoplasmic reticulum-associated proteins also play an important role in the oligomerization and secretion of higher-order adiponectin complexes[260].

Adiponectin is highly expressed by functional adipocytes that are found in lean and healthy individuals. However, its expression is paradoxically lower in obese individuals (**Figure 1.3**)[261]. This is in contrast with other adipokines, whose levels are increased with increased body fat mass. Adipogenic master transcription factor, C/EBPα, and the nuclear hormone receptor, peroxisome proliferator-activated receptor (PPAR)-γ, play an important role in controlling adiponectin transcription[262]. In obese states, increased expression of pro-inflammatory adipocytokines, such as TNF-α and IL-6, can down-regulate the production and secretion of adiponectin by reducing its gene transcription[263]. They can reduce adiponectin mRNA stability, as well as decrease the activity

of C/EBPα and PPAR-γ. The dysregulation of adiponectin production is strongly believed to contribute to the onset of several obesity-related complications, as hypoadiponectinemia (i.e., low levels of circulating adiponectin) has been found to be associated with insulin resistance, T2DM, dyslipidemia, and atherosclerosis[251,264-266].

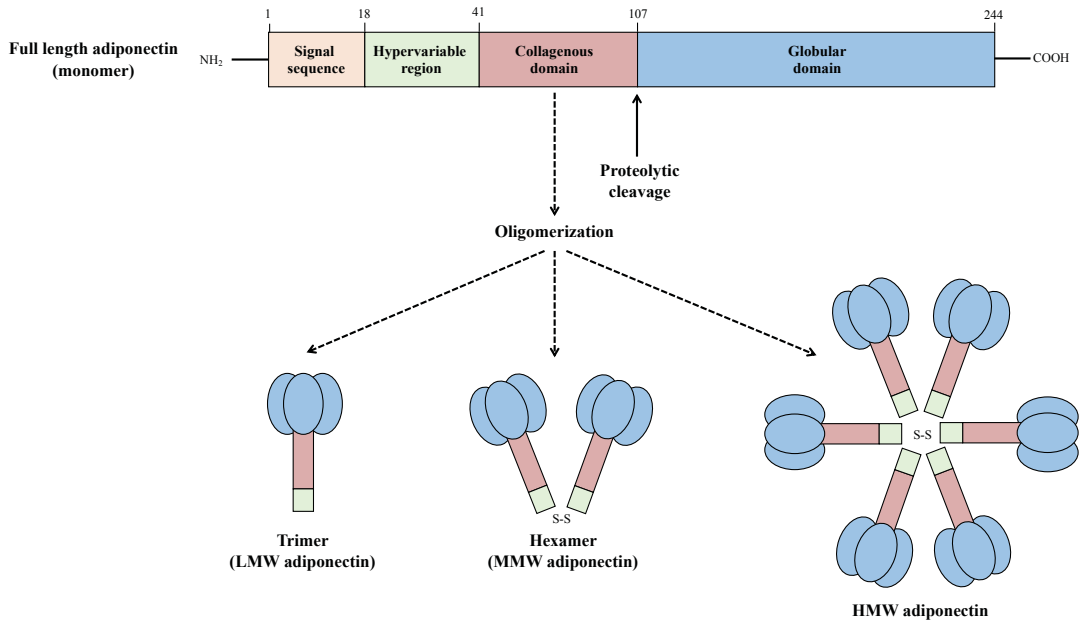


Figure 1.4 Oligomeric isoforms of human adiponectin. *The trimer is the basic building block of oligomeric adiponectin. Disulfide bond formation is crucial for the assembly and stabilization of adiponectin oligomers. Two trimers self-associate via a disulfide-linkage (S-S) mediated by cysteine residue 39 at the hypervariable region to form a hexamer, which further assembles into a bouquet-like HMW multimeric complex that consists of 12 to 18 monomers.*

1.10.2 Adiponectin's pleiotropic functions

Adiponectin acts as a ''pleiotropic cytokine'' linked not only to adipocyte metabolism and homeostasis, but also exhibits a wide range of diverse effects in many different organs and tissues, including WAT, the liver, the skeletal muscle, the heart, the central nervous system, and the vasculature (**Figure 1.5**).

In 2001, three independent groups identified for the first time the important physiological role of adiponectin as an endogenous insulin sensitizer, whereby it modulates glucose and lipid metabolism in insulin-sensitive tissues in both animals and humans[257,267,268]. Fruebis *et al*. first pointed out that acute administration of adiponectin in obese mice significantly decreased free

fatty acid and glucose levels in the blood and increased fatty acid oxidation in skeletal muscle[257]. Berg *et al*. demonstrated effects directly at the level of the liver whereby adiponectin decreased basal glucose levels by inhibiting the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production[268]. Later, clinical studies also supported the idea that lower levels of circulating adiponectin are associated with insulin resistance and a higher incidence of T2DM[264,269,270]. Furthermore, human genetic studies on single nucleotide polymorphisms of the adiponectin gene (located on chromosome 3q27) have pointed out numerous genetic susceptibility loci for T2DM and metabolic syndrome[271-273]. However, in addition to its anti-diabetic effects, adiponectin has also been proven to inhibit endothelial dysfunction and foam cell formation, and suppress atherosclerosis development, as well as modulate lipoprotein remodeling, inflammatory pathways, food intake, and whole-body energy homeostasis[274-276]. Recently, adiponectin has even been suggested to slow the progression of various cancers as it can limit cellular proliferation and induce apoptosis[277].

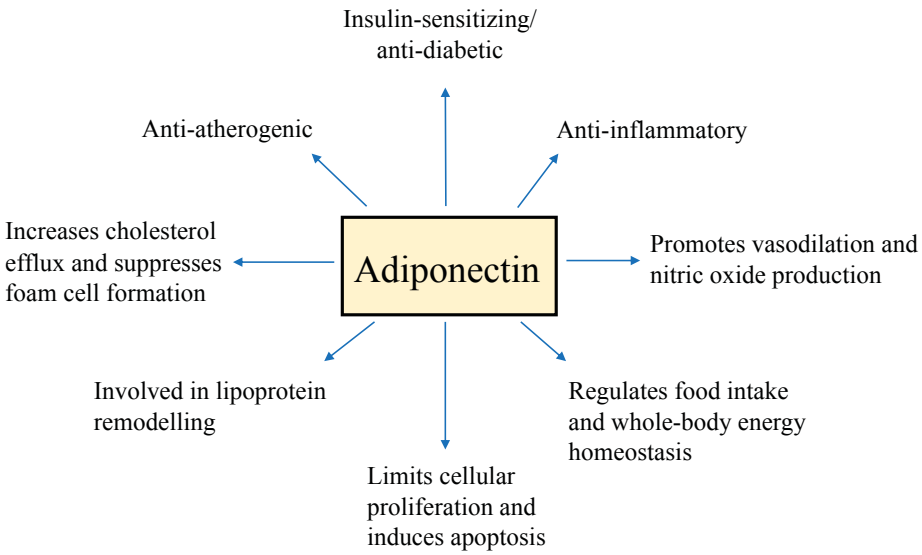


Figure 1.5 Pleiotropic effects of adiponectin. *Adiponectin displays a variety of pleiotropic effects on a multitude of different cell types.*

1.10.3 Adiponectin's vasculoprotective and anti-atherogenic properties

Over the years, adiponectin has attracted much attention because of its anti-inflammatory, vasculoprotective, and anti-atherogenic properties, as it may have the potential to act as a

biomarker and/or therapeutic target for CVD and its complications. Numerous epidemiological studies have identified an association between low circulating levels of adiponectin and CAD, cIMT, and carotid atherosclerosis[265,278-280]. These associations indicate that adiponectin may have a protective role in the prevention of CVD. Low levels of adiponectin were also found to be associated with plaque vulnerability; plasma adiponectin was identified as the strongest predictive factor of the presence of thin-cap fibroatheroma, as assessed by virtual histology intravascular ultrasound in men with stable CAD[281]. Furthermore, evidence suggests that circulating adiponectin is an independent and inverse predictor of cardiovascular events and mortality. Individuals with adiponectin levels in the highest quintile have been shown to have a reduced risk for MI[282]. In the Framingham Offspring Study, elevated plasma adiponectin levels were highly protective of future coronary heart events in men[283]. To comprehensively assess the association between adiponectin levels and the full spectrum of carotid artery disease, from subclinical atherosclerosis (cIMT), to atherosclerotic plaque presence, to ischemic stroke risk, to risk of mortality in an ischemic stroke population, we performed two systematic reviews and meta-analyses, which are presented in sections 1.11 and 1.12 of Chapter 1[284,285].

Animal studies have also demonstrated that adiponectin protects against the development and progression of atherosclerosis. Adenoviral-mediated overexpression of adiponectin in apolipoprotein-E knockout ($apoE^{-/-}$) mice resulted in a reduction in atherosclerotic lesion formation in the aortic sinus by 30% compared with non-treated $apoE^{-/-}$ mice[286]. Interestingly, adenovirus-derived adiponectin accumulated in the fatty streak lesions of the $apoE^{-/-}$ mice, which are predominantly composed of macrophages and foam cells[286], suggesting that adiponectin mediates its beneficial effects directly at the level of the vasculature. Similarly, globular adiponectin transgenic $apoE^{-/-}$ mice had significantly smaller aortic lesions than control $apoE^{-/-}$ mice[287]. On the other hand, adiponectin deficiency in mice led to increased vascular SMC proliferation and enhanced neointimal thickening of arteries in response to vascular injury[288,289]. However, this injury-induced neointimal formation was attenuated upon supplementation of mice with adiponectin[289].

Adiponectin's atheroprotective effects have been established *in vivo* and *in vitro* in all stages of atherosclerotic plaque development, from endothelial dysfunction, plaque initiation and progression, to plaque rupture and thrombosis[290] (**Figure 1.6**). Adiponectin has the ability to attenuate atherogenesis through its direct actions on all major cell types present in the vasculature,

including vascular endothelial cells, macrophages, and SMCs[192]. Adiponectin maintains endothelial function by stimulating the activation of eNOS through 5' adenosine monophosphate-activated protein kinase (AMPK)-dependent phosphorylation of this enzyme and subsequent production of NO in the vascular endothelium[291]. In fact, adiponectin treatment of rat aortic segments protected the endothelium against hyperlipidemic injury, by promoting eNOS activity[292]. Furthermore, adiponectin suppresses the NF-κB inflammatory signalling pathway, decreasing the endothelial inflammatory reaction and reducing TNF-α-induced expression of adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin in endothelial cells[274,293,294]. This attenuates leukocyte attachment to the vascular wall and their migration into atherosclerotic plaques.

Adiponectin can also modulate macrophage function by decreasing its accumulation of cholesterol, thereby suppressing macrophage-to-foam cell transformation[295]. Proposed anti-atherogenic mechanisms by which adiponectin achieves lower intracellular cholesterol levels are by (1) decreasing cholesterol uptake and (2) promoting an increase in cholesterol efflux capacity. Through suppression of macrophage SR-A (but not CD36), adiponectin has the ability to markedly decrease the uptake of oxidized LDL by macrophages[296]. Also, there has been recent interest in adiponectin's ability to enhance ABCA1-mediated cholesterol efflux[295]. Mutations in ABCA1 gene cause Tangier disease and other familial HDL deficiency disorders[297]. Individuals with these diseases are at increased risk for atherosclerotic disease partly due to an over-accumulation of cholesterol in macrophages. Further details concerning adiponectin's effects on cholesterol efflux are presented in section 1.10.5 of Chapter 1. Adiponectin also promotes the polarization of human monocytes into alternative anti-inflammatory M2 macrophages as opposed to the classically activated M1 phenotype, leading to a decrease in M1 markers and a suppression in the release of pro-inflammatory cytokines, such as TNF-α and MCP-1[298,299]. Adiponectin has also been shown in mice to regulate peritoneal macrophage polarization towards a M2 phenotype[299]. An increase in the ratio of M1:M2 macrophages present in the plaque is believed to influence/promote atherogenesis. Lastly, adiponectin can also prevent fibrous cap rupture by inducing the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) by macrophages[300]. In addition to its effects on endothelial cells and macrophages, adiponectin inhibits growth factor-induced SMC proliferation and migration as well as platelet aggregation and thrombus formation[301-304].

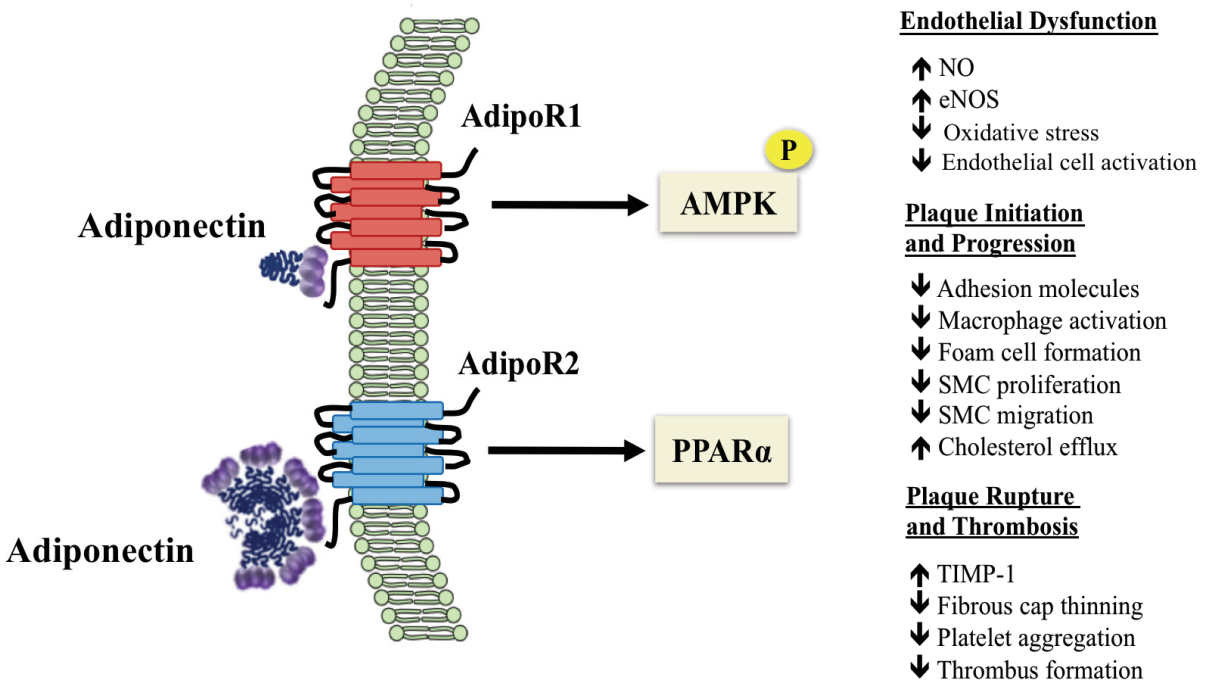


Figure 1.6 Adiponectin's atheroprotective effects in all stages of atherosclerotic plaque development*. Adiponectin's atheroprotective effects are suggested to be mediated via interaction with its transmembrane receptors, AdipoR1 and AdipoR2, leading to the activation of the AMPK and PPAR-α signalling pathways, respectively.*

1.10.4 Adiponectin receptors

Adiponectin exerts its main biological effects via two transmembrane receptors, AdipoR1 and AdipoR2 (AdipoR), which were discovered in 2003 by expression cloning[305]. AdipoR1 and AdipoR2 are encoded by genes situated on chromosomes 1 and 12, respectively, and they display 66.7% homology at the protein level[305]. Despite containing seven transmembrane domains, these receptors are structurally and functionally distinct from the classical G protein-coupled receptors; AdipoR1 and AdipoR2 have an inverted membrane topology with a cytoplasmic N-terminus and an extracellular C-terminal domain[305]. Furthermore, they are not coupled with G-proteins and activate their own unique set of signalling molecules, as detailed below. In fact, determination of the crystal structures of AdipoR1 and AdipoR2 revealed that the AdipoRs represent an entirely novel class of receptors[306]. Scatchard plot analyses demonstrated that AdipoR1 is a high-affinity receptor for globular adiponectin (but it can also bind full-length adiponectin with low-affinity), which mainly leads to activation of AMPK signalling, which is an energy-sensing enzyme[305]. On

the other hand, AdipoR2 has intermediate affinity for both globular adiponectin and its full-length variants (i.e., HMW adiponectin), which mainly leads to stimulation of PPAR-α signalling, which is a key transcription factor in metabolic regulation[305]. Other signalling pathways activated by the interaction between adiponectin and its receptors include IRS1/2, p38 MAPK, Rab5, Akt, and ceramide signaling[307,308]. Recent studies have shown that an adaptor protein called adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1), interacts with the intracellular domains of both receptors, in order to help mediate adiponectin's downstream signalling[309]. T-cadherin has been identified as an additional receptor (or binding protein) for adiponectin that specifically binds the higher-order complexes (hexameric and HMW adiponectin) but not globular adiponectin[310]. It is highly expressed in cardiac myocytes where it plays an important role in mediating the cardioprotective actions of adiponectin[311]. Its structure differs to that of AdipoR1 and AdipoR2 as it is attached to the plasma membrane via a glycosyl phosphatidylinositol anchor[310]. Since it has no intracellular domain it is believed to require the help of other co-receptors, which to date remain unidentified, to mediate its intracellular signalling.

Both AdipoR1 and AdipoR2 are ubiquitously expressed; in mice, AdipoR1 is predominantly expressed in skeletal muscle, while AdipoR2 is most abundantly present in the liver. In humans, however, both AdipoR1 and AdipoR2 are highly expressed in skeletal muscle and the liver. Similar to circulating adiponectin levels, AdipoR expression was significantly decreased particularly in the skeletal muscle, liver, and adipose tissue of mouse models (*ob/ob* or *db/db* mice) of insulin resistance and obesity, as well as of individuals with obesity and T2DM[312-315]. Several gain- and loss-of-function studies have been performed, which have demonstrated that AdipoR1 and AdipoR2 play fundamental roles in glucose and lipid metabolism, inflammation, and oxidative stress[305,313]. Yamauchi *et al*. demonstrated that adenovirus-mediated overexpression of AdipoR1 in *db/db* mice caused a reduction in liver gluconeogenesis via AMPK activation, while overexpression of AdipoR2 enhanced glucose uptake, reduced oxidative stress, as well as decreased the expression of pro-inflammatory cytokines and chemokines, such as TNF-α and MCP-1, via stimulation of the PPAR-α pathway[313]. Both receptors had a significant effect on increasing fatty acid oxidation. On the other hand, systemic disruption of both receptors resulted in abrogation of adiponectin binding and actions, leading to increased glucose production, impaired glucose tolerance, and insulin resistance[313]. Administration of adiponectin to AdipoR1 and AdipoR2 double knockout mice was not sufficient to offset these deleterious effects,

suggesting that these two receptors are responsible for the majority of adiponectin's physiological actions[313].

Adiponectin receptors have been noted by others as well as our group to be expressed in human carotid atherosclerotic plaques as well as in the healthy vascular wall[316,317]. However, their expression was observed to be higher in the lesion area compared to the non-diseased carotid zone. Analysis of AdipoR expression levels in specific cells of the vasculature determined that both AdipoR1 and AdipoR2 are expressed in macrophages, along with smooth muscle cells, and endothelial cells[317]. Moreover, AdipoR are expressed abundantly (93%) in circulating monocytes, as opposed to other circulating cells[318]. Interestingly, AdipoR1 expression decreased upon monocyte differentiation into macrophages, while AdipoR2 expression was not affected during the differentiation process[317]. Nonetheless, AdipoR1 expression remained higher than AdipoR2 expression in monocytes as well as in fully differentiated macrophages[317]. Ultrastructural localization of adiponectin protein by immunoelectron microscopy in the healthy and diseased vasculature of mice was revealed to be similar to that of the AdipoRs, further suggesting that adiponectin mediates its atheroprotective actions via these receptors[319].

While AdipoR1 and AdipoR2 are well known for their involvement in the metabolic action of adiponectin, their role in the vasculature still remains unclear (**Figure 1.6)**. One study determined that both AdipoR1 and AdipoR2 are crucial for mediating adiponectin's actions to suppress lipid accumulation and inhibit macrophage-to-foam cell transformation[320]. On the other hand, these receptors exhibited differential effects in regulating genes that are important for lipid metabolism and inflammation in human macrophages, where AdipoR1 showed greater potency in reducing the levels of pro-inflammatory cytokines, while AdipoR2 had greater potency in suppressing the expression of the scavenger receptor, SR-AI[320]. It was recently noted that AdipoR2 deficiency (but not AdipoR1) led to a reduction in the size of brachiocephalic atherosclerotic plaques in apoE$^{-/-}$ mice; however, these plaques contained a higher degree of macrophages, less collagen content, and no clear fibrous cap, compared with AdipoR2$^{+/+}$apoE$^{-/-}$ mice[321]. This evidence suggests that AdipoR2 may be protective against atherosclerotic plaque instability. Further investigation is clearly needed to determine the contribution of each receptor in mediating adiponectin's actions in the vasculature, in order to highlight their true potential as therapeutic targets for the treatment and/or prevention of atherosclerotic disease.

1.10.5 Adiponectin's effects on cholesterol efflux

Clinical and fundamental evidence suggests that adiponectin may play a critical atheroprotective role in promoting cholesterol efflux via activation of the PPAR-γ/liver X receptor (LXR)-α signalling pathways. AdipoR1 and AdipoR2 are suggested to be implicated in this process, however the data are conflicting and insufficient to establish any firm conclusions.

Clinical evidence

The link between adiponectin and CVD could be partly mediated by adiponectin's effects on lipid metabolism; several studies indicate that the inverse association between adiponectin and CAD is significantly attenuated after adjustments for lipids, particularly HDL-C[282,322]. In fact, a strong correlation between plasma HDL-C and circulating adiponectin concentrations exists in both healthy and diseased populations (i.e., with obesity, metabolic syndrome, T2DM, or CVD), where HDL-C and adiponectin levels are observed to be significantly lower compared to healthy individuals[323,324]. Despite this well-documented association, the mechanisms involved have been poorly investigated.

Several studies demonstrated circulating adiponectin levels to be a robust and independent predictor of cellular cholesterol efflux capacity in humans[295,325-327]. One study showed plasma adiponectin levels to be strongly associated with efflux in healthy adults older than 18 years, independently of age, sex, BMI, glucose, blood pressure, and markers of inflammation and liver function[325]. Moreover, low serum adiponectin levels were also highly correlated with impaired cholesterol efflux capacity in diabetic subjects, and positively associated with low cholesterol efflux to total isolated HDL in subjects with established coronary heart disease[295,326]. Stepwise regression analyses demonstrated that adiponectin accounted for 10.7% of the variance in cholesterol efflux, a value higher than that for apoA-I, which accounted for only 3.9% of the variance[326].

Clinical evidence also demonstrated that adiponectin may play a direct role in HDL catabolism. A strong and negative correlation was observed between adiponectin levels and the HDL-apoA-I fractional catabolic rate (FCR), in various populations, such as in obese subjects, subjects with metabolic syndrome, or in healthy individuals[328-330]. Verges *et al*. determined that plasma adiponectin, on its own, can explain 43% of the variance of HDL-apoA-I FCR[330]. In addition, plasma adiponectin concentrations were also significantly and positively associated with

triglyceride-rich lipoprotein (VLDL-apoB) catabolism, and negatively with triglyceride levels[328,330]. Thus, low circulating adiponectin levels may not only enhance the catabolism of HDL-apoA-I but also reduce the clearance rate of VLDL, apoB-related particles, and triglycerides.

Fundamental evidence

Fundamental evidence also supports the notion that adiponectin plays a key role in promoting HDL/apoA-I cholesterol efflux at various steps throughout the RCT pathway[295,331-341]. Adiponectin has been shown to contribute to this process mainly at the level of the liver and the macrophage.

A co-culture model of adipocytes and hepatocytes revealed a direct involvement of adipose tissue in hepatic cholesterol metabolism, mainly via adiponectin[333]. In HepG2 liver cells, adiponectin treatment (1, 5, 10 μg/ml) increased the synthesis and secretion of apoA-I in a dose-dependent manner and enhanced the cellular expression of ABCA1, while having no effect on ABCG1 and SRB-I expression[336]. This suggests that in the liver adiponectin might increase HDL assembly specifically through ABCA1. Animal studies, using an adiponectin knock-out mouse model, also demonstrated the importance of adiponectin in regulating apoA-I levels in the plasma and ABCA1 expression in the liver[335,338,341].

While the liver is central to the regulation of cholesterol levels in the body, RCT from macrophages is defined as a critical step in protecting against atherosclerotic plaque development. *In vitro* studies indicate that adiponectin treatment might protect against atherosclerosis by significantly enhancing apoA-I-mediated cholesterol efflux from macrophages through an ABCA1-dependent pathway[332,340]. Adiponectin is believed to modulate cellular cholesterol efflux at various doses (1, 5, 10 μg/ml) in murine and human macrophages by positively affecting ABCA1 mRNA and protein expression[332,339,340]. Furthermore, human macrophage-derived foam cells designed to express the adiponectin gene, resulted in a significant reduction in intracellular cholesterol accumulation, an upregulation in ABCA1 and SR-BI protein expression, and increased HDL-mediated cholesterol efflux, compared to control macrophage foam cells not expressing adiponectin[339]. Furthermore, adiponectin knock-out mice were observed to have decreased macrophage ABCA1 expression and apoA-I-mediated cholesterol efflux compared with wild-type mice, and they exhibited apparent aortic atherosclerotic lesions and large lipid deposition in vessel walls[340,341]. While most reports focused on studying ABCA1-mediated cholesterol efflux, there is

little evidence suggesting that adiponectin may also affect cholesterol efflux through the ABCG1 transporter[295].

Potential mechanisms of adiponectin's effects on cholesterol efflux

Various proteins have been suggested to be implicated in adiponectin's effects on ABCA1- and ABCG1-mediated cholesterol efflux, namely PPAR-ɣ and LXR-α[295,332,339,340]. Tian *et al*. was the first to report that activation of the PPAR-ɣ/LXR-α signalling pathways may be necessary for adiponectin's attenuation of lipid accumulation in macrophage-derived foam cells. Activation of LXR-α and PPAR-ɣ by adiponectin, resulted in an increase in ABCA1 expression. Other studies also demonstrated that treatment of macrophages with adiponectin increased LXR-α expression and activation, while LXR-α siRNA completely abolished the promotion effects of adiponectin on ABCA1 expression and cholesterol efflux[295,332,340].

Adiponectin's effects on cholesterol efflux may also be mediated by the same receptors (AdipoR1 and AdipoR2) that transduce its other cellular signals[295,331]. Overexpression and downregulation of AdipoR1 and AdipoR2 in HEK293T human kidney cells provided the first evidence that both receptors are involved in the cholesterol efflux process via upregulation of ABCA1 expression and activity[331]. However, there exists conflicting evidence concerning the importance of each receptor in macrophage lipid metabolism. In macrophages isolated from diabetic patients, adiponectin increased macrophage cholesterol efflux and upregulated ABCG1 and LXR-α expression in the presence of intact AdipoR signalling[295]. However, these effects were blunted in macrophages lacking an AdipoR1 signalling pathway but not AdipoR2[295]. Instead, Li *et al*, found that knockdown of AdipoR2 in human THP-1 macrophages resulted in reduced cellular lipid accumulation, enhanced apoA-I and HDL-mediated cholesterol efflux, and increased expression of PPAR-γ, LXR-α, ABCA1, and ABCG1[334]. Further investigation is needed to elucidate the distinct effects of AdipoR1 and AdipoR2 in macrophage lipid metabolism, including cholesterol efflux capacity, and atherosclerosis development.

1.10.6 Therapeutic modulation of adiponectin and its receptors

Modulation of adiponectin and its receptors is a promising therapeutic strategy for the prevention and/or treatment of cardiometabolic disorders.

Adiponectin

Adiponectin levels are reduced in subjects who suffer from obesity and cardiometabolic disorders. Thus, an important therapeutic approach would be to pharmacologically restore the capacity of adipose tissue to produce and secrete adiponectin, as well as raise circulating adiponectin levels.

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are anti-diabetic therapeutic agents that improve systemic insulin sensitivity and glucose tolerance in obese individuals, T2DM patients, and in animal models of insulin resistance and diabetes[342-344]. Since adiponectin is an insulin-sensitizing adipokine, it is believed that TZDs partly mediate their anti-diabetic properties via upregulation of plasma adiponectin levels[345]. A low-dose treatment of pioglitazone led to an amelioration of insulin resistance in *ob/ob* mice but not in *ob/ob* mice that were adiponectin deficient, suggesting that pioglitazone-mediated reduction in the severity of insulin resistance is partly due to an adiponectin-dependent pathway[346]. A meta-analysis confirmed that administration of TZDs led to an increase in endogenous adiponectin levels in patients with insulin resistance and T2DM[347]. Interestingly, HMW adiponectin is the predominant form of adiponectin upregulated by TZDs[348]. TZDs are agonists of PPAR-γ, a transcription factor that acts as a master regulator of adipocyte differentiation and adipocyte gene transcription. Thus, TZDs are known to raise adiponectin levels by stimulating the transcription of the adiponectin gene via activation of PPAR-γ[349,350]. Combs *et al*. reported circulating levels of adiponectin to be reduced by 5-fold in patients with dominant-negative PPAR-γ mutations, highlighting the importance of PPAR-γ in the regulation of adiponectin synthesis[350]. In addition to improving insulin sensitivity, TZD therapy in association with enhanced adiponectin levels has also been shown to ameliorate the stability of atherosclerotic plaques in patients with T2DM by reducing the necrotic core component of coronary plaques[351].

Other therapeutic agents have also been identified to raise adiponectin levels, such as anti-hypertensive drugs (i.e., angiotensin II receptor antagonists, angiotensin-converting-enzyme inhibitors, and β_1 receptor blockers) and lipid-lowering agents (i.e., fenofibrate, statins)[352-355]. A meta-analysis reported a significant elevation in circulating adiponectin levels following statin therapy, particularly in cases where statins were used for a duration ≥12 weeks[355]. However, subgroup analyses demonstrated that statin-induced augmentation of adiponectin was dependent on the type of statin used[355]. Along with increasing the cholesterol efflux process, apoA-I mimetic peptides (such as L-4F) have also been found to increase adiponectin concentrations[356].

Adiponectin Receptors

Under conditions of adiponectin resistance, more useful therapeutic strategies would be to enhance the action of adiponectin by increasing the expression and/or activation of the adiponectin receptors, rather than upregulate circulating adiponectin levels.

AdipoR expression has been reported, particularly in adipose tissue and in monocytes/macrophages, to be transcriptionally induced by nuclear hormone receptors; PPAR-α/γ can positively regulate AdipoR2 expression, while LXRs can stimulate both AdipoR1 and AdipoR2[317,357]. Thus, AdipoR expression can be modulated therapeutically by various nuclear hormone receptor agonists to enhance the actions of adiponectin. In fact, induction of AdipoR2 via PPAR-α activation was capable of potentiating adiponectin's actions in macrophages by having an additive effect on reducing intracellular cholesterol ester content[317]. Furthermore, dual activation of PPAR-α and PPAR-γ had a greater effect on improving insulin resistance in obese diabetic KKAy mice than single drug treatment due to increases in both adiponectin levels and AdipoR expression[357].

Activation of AdipoRs using small-molecule agonists that can mimic the effects of adiponectin may also act as an important therapeutic approach for the prevention/treatment of cardiometabolic disorders. Through screening of compound chemical libraries, Okada-Iwabu *et al*. identified the first orally-active synthetic small molecule (named AdipoRon) to bind and activate both AdipoR1 and AdipoR2[358]. Its effects are similar to those of adiponectin, where AdipoRon was reported to activate AMPK and PPAR-α signalling pathways to ameliorate insulin resistance, dyslipidemia, and glucose intolerance in obese diabetic mice[358]. Furthermore, *in vitro* and *in vivo* studies have demonstrated that AdipoRon possesses anti-inflammatory, anti-oxidative, and anti-apoptotic properties, in addition to attenuating vascular SMC proliferation[359,360]. However, its direct and beneficial effects on lipid metabolism and atherosclerosis development have yet to be investigated. Overall, these findings suggest that AdipoRon may be a promising new therapeutic agent for the treatment of obesity-related disorders, but its efficacy and safety have yet to be tested in humans. With the recent crystallization of the AdipoR structure, this can help optimize the interaction between AdipoRon and AdipoR as well as identify novel agonists of the AdipoR pathway[306].

1.11 Circulating Adiponectin and Carotid Intima-media Thickness: A Systematic Review and Meta-analysis

Adapted from: *Gasbarrino K, *Gorgui J, Nauche B, Côté R, Daskalopoulou SS. *Metabolism*. 2016;65(7):968-986[284]

*Share co-first authorship

1.11.1 Abstract

Background: Adiponectin is an adipokine with insulin-sensitizing, anti-inflammatory, and vasculoprotective properties. Hypoadiponectinemia has been linked with disease states, such as obesity, T2DM, and CVD. cIMT is a strong and independent predictor of both coronary and cerebrovascular events and has been used as a surrogate marker of subclinical atherosclerosis. The aim of this report is to systematically review the evidence on the relationship between adiponectin and cIMT in a wide range of individuals.

Materials and methods: Medline, Embase, Biosis, Scopus, Web of Science, and Pubmed were searched for published studies and conference abstracts. The "sign test" and "vote count" methods were used to estimate the direction and significance of the relationship between adiponectin and cIMT. The quality of the eligible studies was evaluated using an adapted version of the New Castle Ottawa quality assessment scale.

Results: Fifty-five articles fulfilled the inclusion criteria, comprised of only cross-sectional studies, including healthy subjects, general population, and individuals with metabolic, inflammatory, or other chronic diseases. Most associations between adiponectin and cIMT followed a negative direction in the healthier and general populations, and also in cohorts with metabolic disorders and other chronic diseases, but not in those with inflammatory diseases (sign test). These associations were generally found to be weak or non-significant among all cohort groups studied (vote count).

Conclusion: Our results are suggestive but not conclusive for an inverse association between adiponectin levels and cIMT in diseased and non-diseased populations.

1.11.2 Introduction

Atherosclerosis is the main underlying cause of CVD, leading to the occurrence of MI and ischemic stroke. Large epidemiological studies, such as The Atherosclerosis Risk in Communities study and The Cardiovascular Healthy Study, have established cIMT to be a strong and independent predictor of cerebral ischemic and coronary events in individuals free of overt CVD[361,362]. In particular, increased cIMT has been demonstrated to predict the occurrence of ischemic stroke events, independent of the traditional risk factors represented in the Framingham Stroke Risk Score[363]. Thus, cIMT has become widely used as an imaging surrogate marker of subclinical atherosclerosis, as well as a surrogate end-point for clinical vascular outcomes[364].

Adiponectin, the most abundant adipose tissue-secreted adipokine, circulates at high concentrations in healthy individuals. Experimental evidence has shown adiponectin to possess insulin-sensitizing, anti-inflammatory, and anti-atherogenic properties[365,366]. In humans, low circulating levels of adiponectin are associated with obesity, T2DM, as well as with CVD[251,264,366,367]. However, in relation to cIMT, conflicting results have been reported, with studies demonstrating either a significant or non-significant association between hypoadiponectinemia and severity of cIMT[279,368]. These disagreements may in part be the result of heterogeneity in the studied populations. Therefore, the objective of our systematic review and restricted meta-analysis was to summarize and critique the existing evidence (observational studies) with regards to the association between circulating adiponectin levels and cIMT (subclinical atherosclerosis) in a wide range of populations, including healthy subjects of varying ages, individuals from the general population, and subjects with metabolic, inflammatory, or other chronic diseases.

1.11.3 Methods

General Search Strategy

A general search strategy was developed by a librarian (BN) for Ovid Medline (1946 to Present) including Ovid Medline In-Process & Other Non-Indexed Citations and Ovid OLDMedline, Ovid Embase Classic and Embase (1947 to Present), Ovid Biosis Previews (1969 to Present), Wiley Cochrane Library, Scopus, Web of Science and PubMed. Search strategies were peer-reviewed by a second librarian. The search strategy was designed to answer four specific research questions relating adiponectin levels to (a) cIMT, (b) carotid atherosclerotic plaques, (c) ischemic stroke,

and (d) mortality due to ischemic stroke. Herein, the results pertaining to the *first research question* are presented. Results were restricted to human and adult (>19 years old) studies. No language restrictions were applied. See **Appendix A** for Ovid Medline search strategy details. The searches were run May 23rd, 2013. Conference abstracts were retrieved via Embase and congresses not covered by Embase between 2008 and 2012 were searched manually for "adiponectin" in June 2013. Two trial registries were also searched (clinicaltrials.gov and the WHO International Clinical Trials Registry Platform) along with grey literature sources. On February 8th 2016 the full search strategy was re-run to retrieve new studies published since the initial search. Reference lists of eligible studies were also hand-searched, from which no additional studies were identified; thus validating our search strategy. In addition, investigators were contacted through email for missing baseline population data or clarifications on adiponectin values and units.

Eligibility Criteria

Original studies reporting on the association between circulating adiponectin levels and cIMT were considered eligible. No restriction in regard to population cohort was applied. Abstracts prior to 2011 or abstracts resulting in published work were excluded. Although the search strategy did not restrict articles based on language, at the screening level of potentially eligible articles, we included only English or French records.

Selection Process

A flow–chart illustrating the review process is presented in **Figure 1.7**. From the initial 3565 studies retrieved (after duplicate removal), 111 original articles and abstracts were independently identified and evaluated in detail by the two first authors (KG and JG), of which 72 pertained to cIMT as an outcome. Discrepancies were resolved through consensus with the corresponding author (SSD). Of those, 26 were excluded due to reasons outlined in **Figure 1.7**, while 9 more articles were included following the updated search, resulting in 55 included articles. All of these articles were written in the English language.

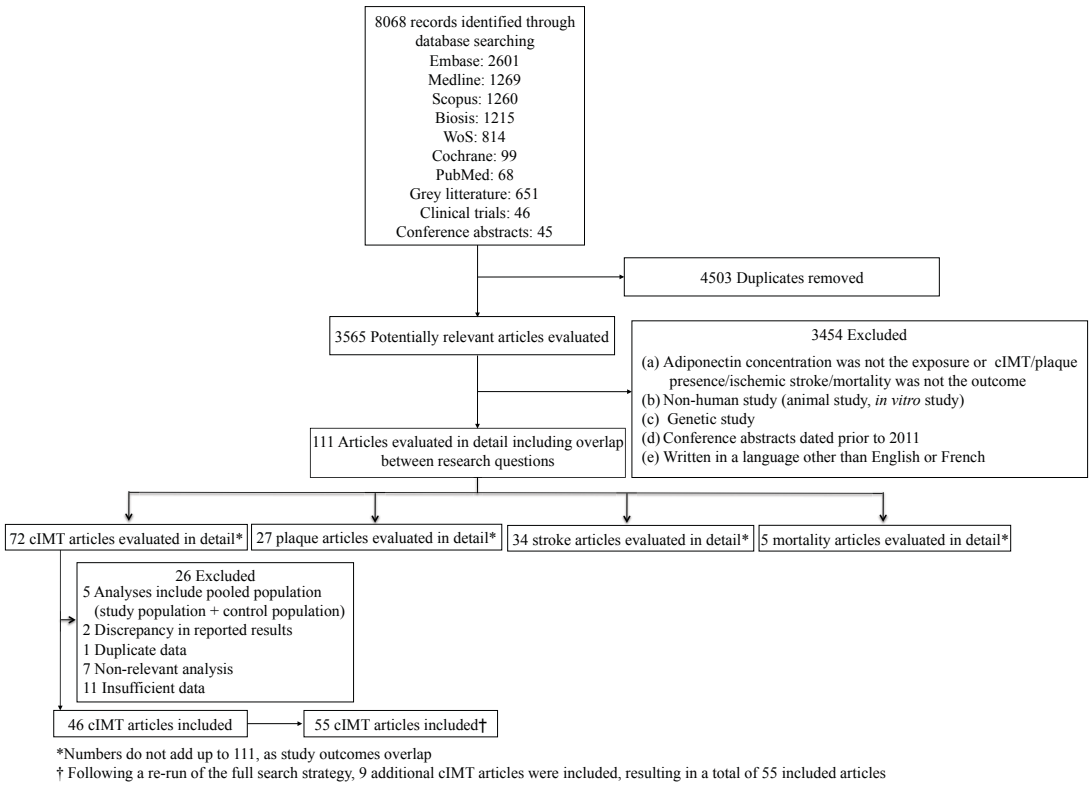


Figure 1.7 Flow-chart of review process for circulating adiponectin and carotid intima-media thickness.[284]

Data Extraction

Our systematic review and restrictive meta-analysis was performed in accordance with the MOOSE (Meta-analysis of Observational Studies in Epidemiology) guidelines. Data extraction was performed independently (KG and JG). Table I of Appendix B illustrates a summary of the baseline population characteristics for each included study (country, population, sample size, percent men, age, body mass index, and cIMT measurements). The population from the different studies were categorized into five groups (healthy subjects, general population, and individuals with metabolic, inflammatory, or other chronic diseases). Healthy subjects were defined as having no medical history of disease, while the general population were representative of subjects with and without comorbidities or disease. Data extraction included parameter effect estimates and covariates used for multivariate analyses (Table II of Appendix B).

Data-analysis Methods

The "sign test" and "vote count" methods were used to provide a crude estimation of the general trend of results for the total population and analyzed by the different population subgroups. The "sign test" allowed us to estimate the proportion of negative versus positive associations, regardless of their significance. The "vote count" method counts the number of significant negative/positive associations with two-sided p-value <0.05[369-371]. The results of these analyses are presented in **Table 1.3**.

Quality Assessment Score

The quality of the included studies was evaluated independently by KG and JG based on an adapted version of the 9-item New Castle Ottawa Quality Assessment Scale for cohort and case-control studies[372]. Since eligible studies were derived from cross-sectional analyses and to ensure quality comparison among studies, the cohort subscale of the New Castle Ottawa Quality Assessment Scale was used, following exclusion of questions 4 (from 'population selection'), 8 (from 'comparability of results'), and 9 (from 'ascertainment of outcome'). Abstracts were not given a quality score. Studies were considered of good quality if the total score was ≥5. However, no studies were excluded from data-analysis, based on the quality score grading.

1.11.4 Results

A systematic review of the literature yielded 52 original articles and 3 conference abstracts assessing the relationship between circulating adiponectin levels and cIMT measurements. Fifty-six of the 67 performed associations were negative (sign test $P<0.001$), 27 of the negative associations had a $P<0.05$ (vote count, $P=0.894$), while no positive associations had a $P<0.05$ (vote count, $P=0.001$). Included studies were conducted in various populations, which have been grouped herein as healthy subjects with a wide age range, general population, and subjects with metabolic, inflammatory, and other chronic disorders. A summary of relevant results for each study is presented in Table II of Appendix B. Vote-counting and sign-test analyses for each population are presented in **Table 1.3**.

Population Description

Pooled population baseline characteristics are presented in Table III of Appendix B. A total of 30,281 (47.26% men) subjects were included, of whom 12,810 were healthy, 9381 represented the general population, 2813 had a metabolic disease, 555 had an inflammatory disease, and 576 had other chronic but non-inflammatory and non-metabolic disorders. The representation of men among the different cohorts was above 40%, except for the inflammatory disease population, where only 18.4% of subjects were men. The mean age (and standard deviation [SD]) of the total population was 53.77±6.15 years. However, the mean age was higher in diseased groups than in their healthy counterparts (healthy population: 50.55±6.05 years; general population: 63.39±5.46 years; metabolic disorders: 54.60±8.48 years; inflammatory diseases: 58.55±3.50 years; other chronic diseases: 57.10±8.77 years). The mean BMI of the total population was 25.85±3.25 $kg.m^{-2}$, which was similar across the population groups. Furthermore, the mean cIMT of the total population was 0.74±0.12 mm, and the mean adiponectin level was 9.58±2.23 μg/mL. Among all the different subgroups, subjects with metabolic and other chronic diseases, as well as the general population had the greatest cIMT measurements, while subjects with metabolic diseases and inflammatory diseases had the lowest adiponectin levels compared with the healthy subjects. Interestingly, subjects with other chronic disorders had significantly higher adiponectin levels than their healthy counterparts.

cIMT Measurements in Healthy Cohorts

In healthy cohorts, 16 of the 17 associations were negative (sign test, $P<0.001$), 8 of the negative associations had a $P<0.05$ (vote count, $P=1.000$), while no positive associations had a $P<0.05$ (vote count, $P=1.000$).

Several studies performed in healthy cohorts demonstrated a significant inverse association between adiponectin and cIMT, following adjustments for metabolic and cardiovascular risk factors, such as age, smoking, blood pressure, BMI, and lipid profile[373-379]. Nishida *et al.* found a similar association in men, but not in women[380]. Additionally, Yoon *et al.* found that in a healthy Korean cohort, cIMT measurements were significantly higher in the lowest tertile of adiponectin in men and women, separately[381]. However, a few studies found no significant relationship between adiponectin levels and cIMT[368,382-384].

Particularly, a significant inverse association was found between adiponectin levels and cIMT in younger individuals with a mean age of 31.7±5 years, which was maintained in sub-analyses according to weight and sex following adjustments for cardiovascular and metabolic confounders[378]. In addition, in a young female population, cIMT measurements were significantly higher in the lowest adiponectin quartile (<8.3 μg/mL) when compared with the highest (≥ 13.9 μg/mL)[385].

However, the majority of studies performed in older populations (mean age: 70.2±0.2 years) found no significant relationship between adiponectin levels and cIMT[386,387]. Nevertheless, Lim *et al.* found a significant inverse association between adiponectin levels and cIMT in an older population with a mean age of 76.0±8.7 years[376].

cIMT Measurements in General Population

In studies representing the general population, all 8 associations were negative (sign test, P=0.008), 6 of the negative associations had a P<0.05 (vote count, P=0.688).

Most studies have demonstrated a significant inverse association between adiponectin levels and cIMT, including cohorts in a multi-ethnic community, members of a German primary health care service, and Japanese outpatients[388-395]. However, no significant association was observed in a Japanese cohort with a high prevalence of T2DM[396], while other studies in the general population[397], or in subjects with increased CV risk[394] demonstrated a significant inverse association in men but not in women.

cIMT Measurements in Subjects with Metabolic Disorders

In cohorts with various metabolic disorders, 19 of the 23 associations were negative (sign test, P=0.003), 7 of the negative associations had a P<0.05 (vote count, P=0.359), while no positive associations had a P<0.05 (vote count, P=0.125). In the control counter-parts, all of the 8 associations were negative (sign test, P=0.008), 4 of these associations had a P<0.05 (vote count, P=1.000).

While some studies found no significant association between adiponectin and cIMT measurements in populations with the metabolic syndrome, obesity, and hypothyroidism[398-402], other studies involving similar populations, as well as subjects with type 1 diabetes, reported a significant inverse association[279,403-407]. In subjects with varying degree of glucose tolerance,

ranging from normal glucose tolerance, to impaired glucose tolerance, to T2DM, low adiponectin levels were significantly associated with high cIMT measurements[279,407-409]. However, in the majority of studies reporting parameter estimates in the subjects with T2DM alone, this negative association was not found to be significant[408-414]. Dullart *et al*. and Gardener *et al.* were the only studies to note an independent inverse association between adiponectin levels and cIMT in both subjects with T2DM and their healthy non-T2DM counterparts[279,407]. Of interest, Yano *et al.* noted that in hypertensive subjects, HMW adiponectin was positively associated with cIMT[415].

cIMT Measurements in Subjects with Inflammatory Disease

No significant association between adiponectin levels and cIMT was noted in studies with subjects with rheumatoid arthritis. Three of the 4 associations were positive (sign test, P=0.625), no negative associations had a P<0.05 (vote count, P=1.000), and no positive associations had a P<0.05 (vote count, P=0.250)[416-419]. In the control counter-parts, both associations were positive (sign test, P=0.500), while none had a P<0.05 (vote count, P=0.500).

cIMT Measurements in Subjects with Chronic Disease

In cohorts with other chronic disorders, 4 of the 5 associations were negative (sign test, P=0.375), 2 of the negative associations had a P<0.05 (vote count, P=1.000), while no positive associations had a P<0.05 (vote count, P=1.000).

A significant negative association between adiponectin levels and cIMT was reported in populations undergoing coronary artery bypass, and in those with severe chronic renal disease[420,421]. Although the univariate analysis in Wang *et al.* demonstrated a significant inverse association between adiponectin levels and cIMT, significance was not maintained following adjustments for cardiovascular and metabolic confounders[422]. Interestingly, Hayashi *et al.* reports significantly higher cIMT measurements in the low adiponectin group (<12.3 μg/mL) compared with the high adiponectin group (≥12.3 μg/mL) in patients with chronic kidney disease[423]. Others, on the other hand, have not found significant associations in similar populations[424,425].

Table 1.3 Overview of the association between adiponectin levels and cIMT measurements within population type[284]

	Number of associations between adiponectin levels and cIMT measurements						
	Positive			Negative			
Population type	Significant*	Non-Significant	Vote-count	Significant*	Non-Significant	Vote-count	Sign test
Healthy	0	1	1.000	8	9	<0.05	<0.001
General	0	0	-	6	2	0.688	0.008
Metabolic disorders							
Cases	0	4	0.125	7	12	0.359	0.003
Controls	0	0	-	4	4	1.000	0.008
Inflammatory Disease							
Cases	0	3	0.250	0	1	1.000	0.625
Controls	0	2	0.500	0	0	-	0.500
Other chronic disease	0	1	1.000	2	2	1.000	0.375
Overall	0	11	0.001	27	29	0.894	<0.001

*Associations were considered significant if the P-value was below 0.05

1.11.5 Discussion

An Inverse Association Between Adiponectin and cIMT

This systematic review and restricted meta-analysis synthesize the results assessing the relationship between adiponectin levels and cIMT in various populations. Results were pooled from larger cohorts grouping healthy subjects or individuals representing the general population, as well as smaller cohorts of diseased subjects (metabolic, inflammatory and other chronic diseases). Mean adiponectin levels were relatively similar across the healthy, general, metabolic, and inflammatory disease population groups, with slightly lower levels observed in the subjects with metabolic and inflammatory diseases. Interestingly, subjects with other chronic diseases had significantly elevated adiponectin levels than the other populations. The majority of studies involving subjects with other chronic diseases include disorders such as chronic kidney disease and end-stage renal disease. Since adiponectin is primarily eliminated from the circulation by the kidneys, adiponectin's accumulation in the circulation of subjects with chronic kidney and end-stage renal disease may be due to impaired biodegradation and elimination by the kidneys[426].

As a general trend, mostly observed in the larger cohorts, studies demonstrated that subjects with adiponectin levels in the lowest quartile or tertile had significantly greater cIMT than subjects with higher adiponectin levels[381,385,395,397,423]. According to the performed sign test, most associations between adiponectin and cIMT (significant or not) followed a negative direction in the larger, healthier, and general populations. The sign test also demonstrated the direction of the association to be inverse in cohorts with metabolic disorders and their control counterparts, and with other chronic diseases, but not in subjects with inflammatory diseases. Results were highly variable among the smaller and more diseased cohorts possibly due to the presence of cardiovascular risk factors (i.e. T2DM, dyslipidemia, higher BMI, increased inflammatory markers). Although the majority of the studies reported an inverse association between adiponectin and cIMT, based on vote counting these associations were generally found to be weak or non-significant among all cohort groups studied.

The inverse association between adiponectin and subclinical atherosclerosis identified herein is in line with previous clinical and experimental findings. Adiponectin is an adipose tissue-secreted anti-inflammatory protein that circulates at low concentrations in individuals suffering from obesity, T2DM, and CAD[251,264,366,367]. Experimental evidence suggests that adiponectin can play a protective role against the initiation and development of atherosclerosis. Particularly,

adiponectin can regulate endothelial function by up-regulating the activity of the eNOS and increasing NO production[366,427]. Animal studies have also highlighted the anti-atherogenic properties of adiponectin in a preclinical *in vivo* model[286,428].

Diabetes and Adiponectin

Insulin resistance is an established risk factor for CVD[429]. Subjects with T2DM have greater cIMT than non-diabetics[430]. Furthermore, decreased adiponectin levels are significantly associated with insulin resistance and T2DM[251,264,366]. In fact, experimental evidence exists to strongly support adiponectin's role as an endogenous insulin-sensitizer[431,432]. Although it has been hypothesized that lower adiponectin levels may be partly involved in enhanced subclinical atherosclerosis in T2DM, interestingly, this was not confirmed in our systematic review. On the other hand, adiponectin levels were significantly and inversely associated with cIMT in healthy individuals and in subjects with varying degree of glucose tolerance. Thus, in relatively healthy subjects, adiponectin seems to play a role in the early pathophysiological changes associated with atherosclerosis. However, since T2DM is a complex disorder and is governed by a variety of atherogenic risk factors[433], this may partly explain lack of a significant association between adiponectin and cIMT in subjects with T2DM. In addition, medications for T2DM can affect both adiponectin levels and cIMT measurements[347,434,435], potentially contributing further to the lack of a relationship between adiponectin and cIMT. Particularly, TZDs (PPAR-γ agonists) have been reported to cause regression of cIMT and elevation of adiponectin levels in the circulation[347,434,435]. Interestingly, a significant correlation between increased adiponectin levels and reduced cIMT measurements was noted in subjects with T2DM following TZD treatment, despite observing no correlation between the two factors at baseline prior to the initiation of TZD therapy[410].

Age-and Sex-Specific Results

Age has been acknowledged as an important risk factor for CVD and it has been noted that older subjects have increased cIMT[436]. On the other hand, adiponectin levels have been shown to be positively associated with increased age[437]. Among the studies included in this systematic review, only Dessein *et al*. performed age-stratified analyses, evaluating the association between adiponectin and cIMT in subjects >55 years old and ≤55 years old[418]. Interestingly, no significant association was observed for either age category[418]. The pooled mean age across all population

groups ranged from 50-63 years old. However, particularly in the healthy cohort, studies with a younger and older population than the pooled mean age have been included. Studies including younger individuals (mean age: 31.7±5 years) demonstrated a stronger association between adiponectin and cIMT than studies with older subjects (mean age: 70.2±0.2 years)[376,378,385-387].

Sex is also another critical variable in the consideration of cardiovascular risk[436]. Some of the studies included in the systematic review were sex-stratified allowing the assessment of potential sex differences. Although limited evidence is available, we noted that this inverse association between adiponectin levels and cIMT was maintained in men but not in women following adjustments. However, this was mainly observed in the healthy and general populations and not in the diseased populations. Interestingly, studies including subjects with inflammatory diseases predominantly consisted of women; only 18.4% of subjects included were men. Thus, the lack of an inverse association between adiponectin and cIMT among the inflammatory disease population may be partly explained by the over-representation of women in this population.

cIMT: A Surrogate Marker of CVD and Outcome of Early Atherosclerosis

Atherosclerosis is a progressive disease that can remain asymptomatic for years before manifesting as a major cardiovascular event. Understanding the relationship between adiponectin and cIMT is clinically relevant as cIMT is a known surrogate marker for generalized atherosclerosis and clinical vascular outcomes[364]. cIMT is also an outcome on its own as it represents subclinical atherosclerosis and early stages of atherosclerotic disease. It can be determined non-invasively by using a B-mode ultrasound, which allows for direct visualization of the carotid arterial wall[438]. The results obtained herein are suggestive but not conclusive of an inverse association between cIMT and adiponectin in non-diseased and diseased populations.

Adiponectin and cIMT Progression (longitudinal evidence)

Hypoadiponectinemia was noted to independently predict the early progression of carotid atherosclerosis. Post-menopausal women with baseline adiponectin levels in the lowest quartile (≤5.1 μg/mL) had a 3-fold risk of cIMT progression over 1 year when compared to women with adiponectin levels in the higher quartiles (≥5.2 μg/mL)[384]. Furthermore, in a 5-year prospective study of a population-based cohort free of overt CVD at baseline, adiponectin was inversely associated with a greater increase in cIMT, independently of traditional cardiovascular risk

factors[391]. Thus, adiponectin has the potential to serve as a marker of cIMT progression and help identify early on those individuals who are at high risk of developing carotid atherosclerotic disease.

Limitations/Strengths

It is noteworthy that only 18 of all studies assessed herein had performed adjustments for at least two covariates. If all studies had systematically adjusted for all relevant risk factors when appropriate, we may have been able to describe the relationship between adiponectin and cIMT more adequately. A number of articles performed stratified analyses to describe associations according to disease severity, however without further adjustments.

To counteract the lack of adjustments in the extracted results, we presented our results divided by population cohort. This allowed us to minimize the heterogeneity of the population and the presence of potential confounding factors namely age, medication use, and past medical history of CVD. By comparing relatively similar population groups, we aimed to mimic a stratification of confounding parameters in order to be able to at least estimate the direction and significance of the association between plasma adiponectin and cIMT within specific population cohorts through the 'sign' and 'vote' counting methods. Furthermore, cIMT measurement methods have been performed using manual calculations in some studies, while others performed automated software applications, which could have led to disparate results. However, as comparison studies using both cIMT measurement methods were not performed, we cannot quantify (or adjust for) the potential discrepancy between these two methods.

Given that it was difficult to obtain subject level data from this large number of studies, and that their performed analyses were mainly correlations or associations (presented as point estimates without 95% CIs), we were unable to perform an extensive meta-analysis of pooled results. Since we could not transform our data and pool them to reflect sample and effect size, we used the "sign test" and "vote counting" meta-analysis methods, which allowed us to report the total number of studies showing negative, positive or no association between adiponectin levels and cIMT measurements. These methods are not without limitations. The "sign test" allowed us to determine how many studies are going in one direction or the other, and if this is significant based on the number of studies. However, it does not take the significance of results for each study into account. To assess the significance of the results we used the "vote counting" method. This

method is limited by the fact that it places equal weight to all studies without considering the population size. It will only reflect the probability of an association in a positive or negative direction, and if these results were significant or not. In systematic reviews, publication bias is a concern, however in our study publication bias is unlikely for two main reasons: a) many studies had associations that were not significant and b) many of these results were simple correlations that were reported in descriptive tables, in which case, results would be published regardless of significance.

As described in the methodology section, we used the Newcastle-Ottawa scale to assess the quality of included studies[372]. No studies were excluded based on these grading scores. The overall grading for quality assessment of the articles included in our systematic review is 4.40/6. Only 48% of the selected articles received a score of ≥5, which we consider to be of good quality. Points were lost mainly in the categories of "representativeness of the exposed cohort" and "comparability of cohorts on the basis of the design or analysis, including adjustments for potential covariates". This could be contributing to the weak or non-significant associations observed in smaller cohorts, and in the overall results.

Due to the abundance of data obtained from our search strategy, only the association between adiponectin levels and cIMT was presented herein. The relationship between adiponectin levels and carotid plaques, as well as risk of ischemic stroke and mortality will be presented in a future systematic report.

1.11.6 Conclusion

Following a systematic review of the literature to evaluate the relationship between circulating adiponectin levels and cIMT measurements, we have outlined an inverse association between these two variables in healthy subjects, individuals from the general population, in subjects with metabolic disorders, and in other chronic diseases, but not in those suffering from inflammatory diseases. Discrepant results observed among the majority of the studies focusing on the diseased populations may be the result of the relatively smaller number of patients and presence of confounding cardiovascular risk factors that may govern these complex disorders. Our study did not demonstrate a significant and strong inverse relationship between adiponectin levels and cIMT possibly due to the cross-sectional design of the selected studies, the heterogeneity of the populations, small sample size of some studies, and the lack of appropriate analyses or adjustments

for potential covariates. Future population studies using a prospective design and appropriate methodology, as well as animal studies, are needed to provide more definitive conclusions for the association between adiponectin levels and cIMT.

1.11.7 Translational potential

Considering the large amount of knowledge that exists underlining adiponectin's anti-inflammatory and vasculo-protective properties, our study aimed to synthesize the evidence that supports the popular belief that adiponectin has a protective role in the early development of atherosclerosis. Herein, despite the methodological limitations, overall an association was noted between low adiponectin levels and high cIMT measurements, suggesting that adiponectin is a potential risk factor for subclinical atherosclerosis. However, associations were not observed among all populations studied, particularly including subjects with inflammatory diseases. Thus, adiponectin may provide cardio-protective benefits in selected populations, which needs to be addressed in future, properly designed studies.

1.12 Circulating Adiponectin Levels in Relation to Carotid Atherosclerotic Plaque Presence, Ischemic Stroke Risk, and Mortality: Systematic Review and Meta-analyses

Adapted from: *Gorgui J, *Gasbarrino K, Georgakis MK, Karalexi MA, Petridou ET, Daskalopoulou SS. *Metabolism*. 2017;69:51-66[285]

*Share co-first authorship

1.12.1 Abstract

Background: Low circulating levels of adiponectin, an anti-inflammatory and vasculoprotective adipokine, are associated with obesity, T2DM, and atherosclerotic disease. Presence of unstable plaques in the carotid artery is a known etiological factor causing ischemic strokes. Herein, we systematically reviewed the association between circulating adiponectin and progression of carotid atherosclerotic disease, particularly evaluating the occurrence of (1) carotid atherosclerotic plaques, (2) ischemic stroke, and (3) mortality in subjects who suffered a previous ischemic stroke.

Methods: Medline, Embase, Biosis, Scopus, Web of Science, and Pubmed were searched for published studies and conference abstracts. The effect size and 95% CIs of the individual studies were pooled using fixed-effect or random-effect models. The quality of the eligible studies was evaluated using the Newcastle–Ottawa quality assessment scale. Sensitivity, subgroup, and meta-regression analyses were performed to address the impact of various risk factors on the association between adiponectin and ischemic stroke risk.

Results: Twelve studies fulfilled the inclusion criteria for 3 independent meta-analyses. The association of increasing circulating adiponectin levels (5 μg/mL-increment) with presence of carotid plaque was not conclusive (n = 327; OR: 1.07; 95% CI: 0.85–1.35; 2 studies), whereas high adiponectin levels showed a significant 8% increase in risk of ischemic stroke (n = 13,683; 7 studies), with a more sizable association observed among men compared to women. HDL was observed to have a marginal effect on the association between adiponectin and ischemic stroke, while other evaluated parameters were not found to be effect modifiers. A non-significant association of adiponectin with mortality was yielded (n = 663; OR: 2.58; 95% CI: 0.69–9.62; 3 studies). Although no publication bias was evident, there was significant between-study heterogeneity in most analyses.

Conclusion: It appears that the direction of the relationship between adiponectin and carotid atherosclerotic plaque presence is dependent on the duration, severity, and nature of the underlying disease, while increased adiponectin levels were associated with an increase in risk for ischemic stroke. Lastly, the results from the mortality meta-analysis remain inconclusive. Future properly designed studies are necessary to further elucidate the role of adiponectin on atherosclerotic plaque development, and its related outcomes.

1.12.2 Introduction

Atherosclerotic disease is characterized by plaque formation within the arterial wall. The presence of unstable atherosclerotic plaques in the carotid artery is a known etiological factor causing cerebrovascular events, such as an ischemic stroke, TIA, or AF[439]. Thus, carotid atherosclerotic plaque represents an important disease burden, as stroke is one of the leading causes of death worldwide and a major cause of long-term disability[440].

Adiponectin is the most abundantly secreted adipokine from adipose tissue that circulates at high concentrations in healthy individuals mainly as 3 isoforms: LMW trimer, MMW hexamer,

and as a HMW 12-18 mer. The HMW multimer accounts for approximately 50% of the total adiponectin in humans[441], and has been shown to be the active form[442-444]. Adiponectin is known to possess insulin-sensitizing, anti-inflammatory, and anti-atherogenic properties[365,366]. In humans, it has been consistently shown that low circulating levels of adiponectin are associated with obesity, T2DM, as well as with CVD[251,264,366,367]. Furthermore, given that inflammatory processes play a fundamental role in cerebrovascular events and ischemic stroke occurrence, circulating adiponectin has been noted to be lower in people with carotid and coronary atherosclerosis[284,442,443]. However, given that studies performed in different populations have shown controversial results it is important to analyze the evidence and synthesize it. In fact, we have previously demonstrated in a systematic review and meta-analysis that circulating adiponectin levels are inversely associated with cIMT, a strong and independent predictor of both coronary and cerebrovascular events, in diseased and non-diseased populations[284].

In the current systematic review and meta-analysis we, therefore, aimed to systematically review and quantitatively synthesize existing evidence regarding the association between circulating adiponectin levels and the progression of carotid artery disease, particularly evaluating the occurrence of carotid atherosclerotic plaques, ischemic stroke, and cerebrovascular mortality, as well as all-cause mortality in subjects who suffered an ischemic stroke.

1.12.3 Methods

Search Strategy

The search strategy is detailed in section 1.11.3. The search strategy was designed to investigate the following research objectives regarding the potential association of adiponectin levels with (1) carotid atherosclerotic plaque presence, (2) ischemic stroke, and (3) mortality (cerebrovascular and all-cause) among patients with history of ischemic stroke.

Eligibility Criteria

Cohort, case-control, and cross-sectional studies involving adults (> 19 years) and exploring the association between circulating adiponectin levels (all isoforms) and (1) carotid atherosclerotic plaque presence, (2) ischemic stroke, and (3) mortality within an ischemic stroke population (cerebrovascular-related and all-cause mortality) were considered eligible. No restrictions on language or study design were initially applied. To standardize the definition of carotid

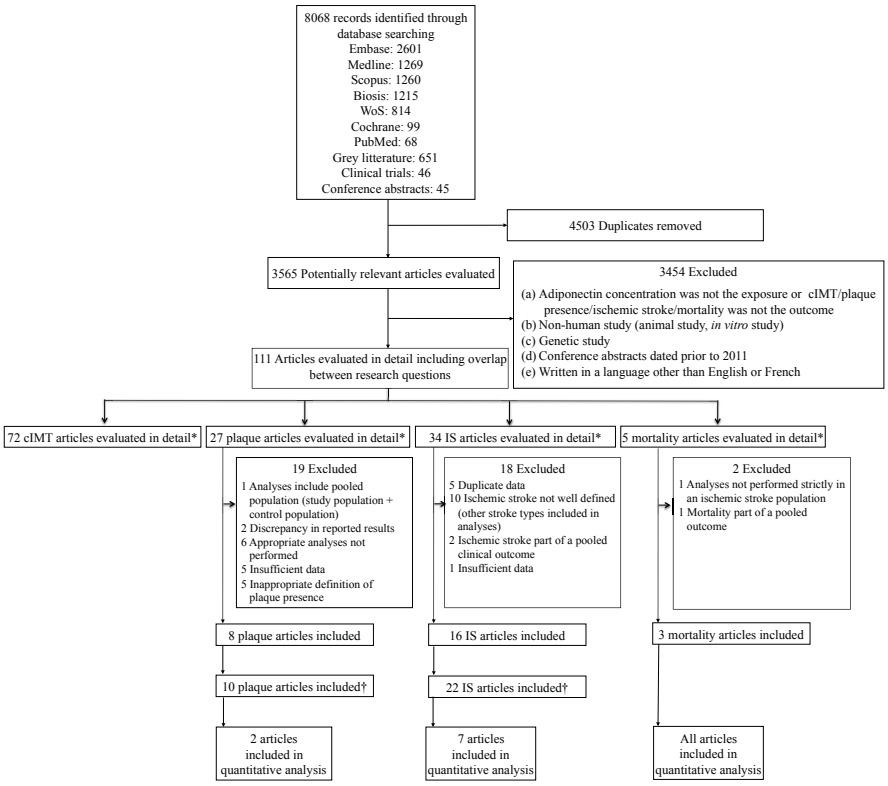
atherosclerotic plaques, studies not describing plaque presence, according to the Mannheim plaque consensus, were excluded[445]. Studies with a strict outcome of ischemic stroke were included, whereas studies pertaining to undetermined or non-carotid sources of ischemic symptoms, such as cardio-embolic causes, intracerebral hemorrhage, intracranial disease, lacunar infarction, and aortic arch disease were excluded. To standardize the definition of ischemic stroke, the outcome had to be ascertained by a medical team through the review of medical charts, hospital/death records, or neuroimaging reports (i.e., computed tomography scans, magnetic resonance imaging). Abstracts prior to 2011 or abstracts resulting in published work were excluded. Although the search strategy did not initially restrict articles based on language, at the screening level of potentially eligible articles, we included only English or French records. Authors of studies not quantifying the association of interest were contacted through email to provide appropriate analyses, potential clarifications, or missing data.

For the 3 independent meta-analyses, the included studies were those that reported an effect measure of risk between adiponectin levels (continuous or binary) and 1) carotid plaque presence, 2) first-ever ischemic stroke, 3) mortality. Studies including populations with prevalent CVD were excluded from the ischemic stroke meta-analysis. Cross-sectional studies were also excluded from the ischemic stroke meta-analysis because of the acute nature of ischemic stroke; adiponectin levels had to be measured prior to the ischemic stroke event. This criterion was not applied to the plaque meta-analysis because plaque development is a chronic process and occurs over a long period of time; thus, it would not influence adiponectin levels abruptly in the same way as an ischemic event would. All studies not involving a population that previously suffered an ischemic stroke were excluded from the mortality meta-analysis.

Selection Process

A flow chart illustrating the review process is presented in **Figure 1.8**. From the initial 3565 studies retrieved, 111 original articles and abstracts were independently and blindly identified and evaluated in detail by two authors (KG and JG), out of which 27 pertained to carotid atherosclerotic plaque presence, 34 to ischemic stroke, and 5 to mortality. Disagreements were resolved through team consensus. Nineteen plaque, 18 ischemic stroke, and 2 mortality studies were excluded due to specific reasons as outlined in **Figure 1.8**. Following a re-run of the full search strategy on October 26, 2016, 2 additional plaque and 6 additional ischemic stroke studies were included in

the systematic review, resulting in the inclusion of a total of 10 plaque[280,418,422,446-452], 22 ischemic stroke[453-474], and 3 mortality studies[475-477]. All included articles were full-length original articles written in the English language. Twelve of these studies (2 for plaque, 7 for ischemic stroke, and 3 for mortality) also fulfilled the inclusion criteria for the 3 independent meta-analyses.



cIMT, carotid intima-media thickness; IS, ischemic stroke
* Numbers do not add up to 111, as study outcomes overlap
† Following a re-run of the full search strategy, 2 additional plaque articles and 6 additional IS articles were included, resulting in a total of 10 and 22 included articles, respectively

Figure 1.8 Flow-chart of review process for circulating adiponectin and carotid atherosclerotic plaque presence, ischemic stroke risk, and mortality.[285]

Data Extraction

This systematic review and meta-analysis was performed in accordance with the MOOSE (Meta-analysis of Observational Studies in Epidemiology) guidelines[478]. Data extraction was performed independently by two authors (KG and JG). Table IV of Appendix B illustrates study characteristics (year, country, design, study period, and duration of follow-up) and characteristics of study participants (type of population, sample size, percentage of men, age, BMI, percentage of plaque prevalence, and ischemic stroke /mortality outcome). For each study included, parameter effect estimates and covariates used for multivariate analyses are presented in Tables V-VIII

(Appendix B) for studies pertaining to carotid plaque presence, ischemic stroke, and mortality, respectively.

Quality Assessment Score

The quality of the included studies was evaluated independently by KG and JG based on the 9-item Newcastle-Ottawa Quality Assessment Scale for cohort and case-control studies[372]. Three parameters were evaluated: (1) population selection, (2) comparability of results, and (3) ascertainment of exposure (case-control) or outcome (cohort studies or cross-sectional studies). For the studies that had a cross-sectional design, the cohort subscale of the Newcastle-Ottawa Quality Assessment Scale was used, following exclusion of questions 4 (from 'population selection'), 8 (from 'comparability of results'), and 9 (from 'ascertainment of outcome'). For comparability reasons, age was set as the most important matching or adjustment factor, while the cut-off point for follow-up time was set at 1 year. Studies were considered of high quality if the total score was ≥5/6 (for cross-sectional) or ≥7/9 (for cohort and case-control studies). However, no studies were excluded based on the quality score grading.

Statistical Analyses

The ORs or hazard ratios (HRs) and 95% CIs of the individual studies were pooled using fixed-effect (Mantel-Haenszel)[479] or random-effect (DerSimonian–Laird) models[480]. Between-studies heterogeneity was measured by I^2 and Cochran Q[481]; significance level was set at $P<0.10$. In case of statistically significant between-studies heterogeneity, random-effects models were applied[481]. For the overall effect, the statistical significance level was set at $P<0.05$. Analyses were conducted separately for the 3 dependent variables of interest, namely risk of carotid plaque presence, risk of ischemic stroke, and risk of mortality, whereas the independent variable, namely levels of circulating adiponectin, was assessed as continuous with 5 μg/ml increments. Studies reporting only category-specific ORs and their 95% CIs were also included after estimation of the log-linear trend, using the generalized least squares approach[482]. This method was feasible in all studies, since the number of cases and controls by category of exposure and the presence of at least 3 levels of exposure (baseline included) were available. Sensitivity and subgroup analyses were performed for the ischemic stroke meta-analysis, stratifying studies by study design (prospective cohort and nested case-control), population under study (community-based or patient-based), level of

adjustment, and sex (men and women). Meta-regression analysis was performed to assess the effect of confounding variables on the relationship between adiponectin levels and ischemic stroke (i.e., age, sex, hypertension, T2DM, current smoking status, HDL-C levels, triglyceride levels, BMI, follow-up duration). Publication bias was assessed by Egger's formal statistical test in analyses including at least 9 study arms[483]. Statistical significance level was set at $P<0.05$. All statistical analyses for meta-analysis were performed using STATA Software, version 11.1 (STATA Corporation, College Station, TX, USA).

1.12.4 Results

Circulating Adiponectin and Carotid Atherosclerotic Plaque Presence

A total of 2 cross-sectional studies were included in the meta-analysis assessing the association between circulating adiponectin levels and carotid atherosclerotic plaque presence[280,418]. According to the Mannheim guidelines, presence of carotid atherosclerotic plaques was assessed using validated ultrasonography techniques[445]. Carotid atherosclerosis was generally defined as a focal structure (plaque) encroaching the arterial lumen of at least 50% of the surrounding cIMT value or a cIMT value ≥ 1.5 mm[445]. Following meta-analysis of individual effect estimates (**Figure 1.9**), the association of circulating adiponectin with the presence of carotid plaque was not conclusive (n= 327; summary OR: 1.07; 95% CI: 0.85-1.35; heterogeneity I^2= 70.6%; P = 0.065). Publication bias was not assessed as less than 9 studies were included, which could potentially hamper the power of this test.

All studies in the meta-analysis included a population with chronic inflammatory disease. Dessein *et al.* showed that low adiponectin is an independent predictor of carotid atherosclerosis; specifically demonstrating for the first time that both HMW and total adiponectin levels were independently associated with reduced carotid plaque prevalence in a large cohort of rheumatoid arthritis subjects with abdominal obesity[418]. However, similar associations were not observed in rheumatoid arthritis patients without abdominal obesity[418]. Contrary to the previous findings, systemic lupus erythematosus patients with carotid plaques had higher levels of adiponectin than those without plaques. In fact, Reynolds *et al.* reported an 80% increased risk for plaque per 10-unit increase in adiponectin (95% CI: 1.1-3.0)[280]. Although the results were not included in the meta-analyses, Grönwall *et al.* also observed a similar positive association between adiponectin levels and ischemic stroke.

Two studies not included in the meta-analysis (due to no reported effect measure of risk between adiponectin levels and carotid plaque presence) found subjects with carotid atherosclerosis to have lower circulating levels of HMW and total adiponectin than those without carotid plaques, in a cohort consisting of middle-aged Japanese men without cardiovascular risk factors and in patients with ambulatory peritoneal disease, respectively[447,452]. On the other hand, five studies with cohorts including middle-aged subjects, subjects with systemic lupus erythematosus, T2DM and stable peritoneal disease, failed to find a relationship between adiponectin levels and carotid atherosclerosis[422,448-451].

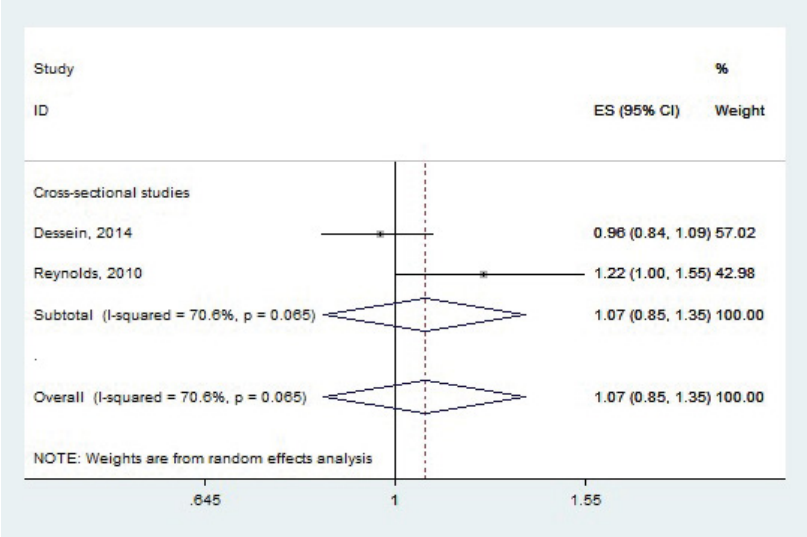


Figure 1.9 Forest plot describing the association of adiponectin levels and risk for carotid plaque presence (2 studies). *Circulating adiponectin levels were assessed as continuous with 5 µg/mL increments. ORs of individual studies are indicated by the data markers; shaded boxes around data markers reflect the statistical weight of the study; 95% CIs are indicated by the error bars; summary-effect estimates with its 95% CIs are depicted as a diamond.*[285]

Circulating Adiponectin and Ischemic Stroke Risk

Overall analysis: A total of 7 eligible studies examining the association between adiponectin levels and first-ever ischemic stroke risk were included, of which 3 were prospective cohort studies and 4 were nested case-control studies with prospective follow-up[453,455,462,465,470-472]. Ischemic stroke was confirmed on the basis of neuroimaging techniques, such as computed tomography or magnetic resonance imaging. Pooled population characteristics are presented in Table IX

(Appendix B). A total of 13,683 (33.3% men) study subjects were included (mean age±SD: 56.9±8.3 years, mean BMI: 26.2±2.8 kg.m^{-2}, and mean total adiponectin levels: 10.95±1.54 μg/mL), with cases presenting higher mean adiponectin levels compared to controls.

Pooling of individual effect estimates of the 9 study arms (**Figure 1.10**), which corresponded to 7 studies (due to analyses being split by sex, 2 of the studies had 2 study arms each), showed a marginally significant association of increasing circulating adiponectin (per 5 μg/mL) with ischemic stroke risk (n= 13,683; summary OR: 1.08; 95% CI: 1.00-1.18; heterogeneity I^2= 66.7%; P = 0.002), which was strengthened when using the most adjusted estimates (n=13,683; summary OR: 1.15; 95% CI: 1.03-1.28; heterogeneity I^2= 71.2%; P=0.002). No publication bias was noted (P = 0.06, Egger test).

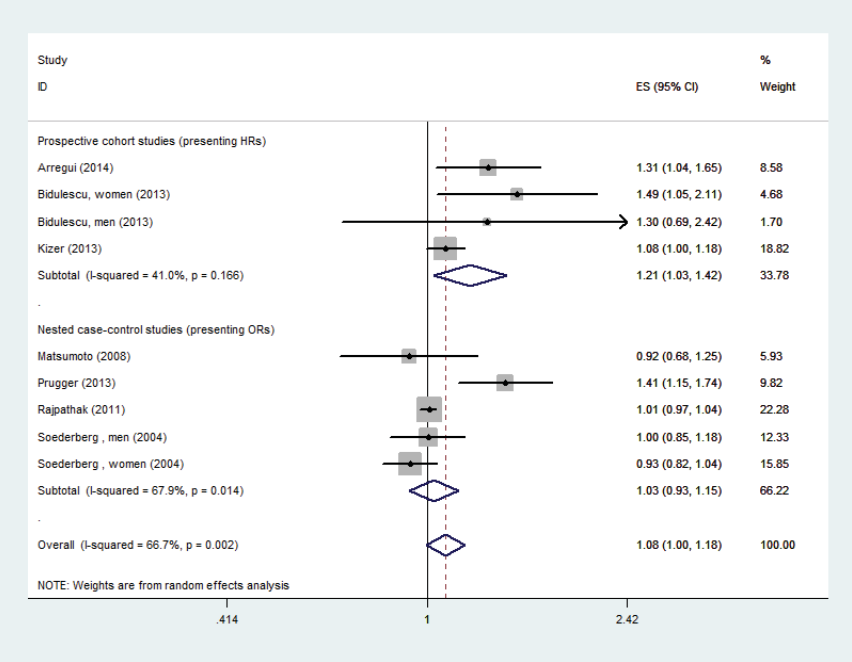


Figure 1.10 Forest plot describing the association of adiponectin levels and risk for ischemic stroke (9 study arms). *Circulating adiponectin levels were assessed as continuous with 5 μg/mL increments. ORs of individual studies are indicated by the data markers; shaded boxes around data markers reflect the statistical weight of the study; 95% CIs are indicated by the error bars; summary-effect estimates with its 95% CIs are depicted as a diamond.*[285]

Study design analysis: Analyses by study design highlighted a significant association between adiponectin levels and ischemic stroke among prospective cohort studies (n = 10,016; summary HR: 1.21; 95% CI: 1.03–1.42; heterogeneity I^2 = 41.0%, P = 0.17) but not in nested case–control studies (n = 3667; summary OR: 1.03; 95% CI: 0.93–1.15; heterogeneity I^2 = 67.9%, P = 0.01). This discrepancy may be due to the fact that prospective cohort studies had a larger sample size and were less heterogeneous as opposed to the nested case-control studies (**Table 1.4**).

Sex-specific analysis: Analyses by sex indicated a men-specific significant association of high adiponectin levels with risk of ischemic stroke (n= 3174; summary OR: 1.15; 95% CI: 1.02-1.30; heterogeneity I^2= 54.1%; P = 0.09) but not among women (n= 6623; summary OR: 1.07; 95% CI: 0.94-1.23; heterogeneity I^2= 71.8%; P = 0.01) (**Table 1.4**). In a large European cohort (EPIC-Potsdam), adjustments including metabolic confounders (e.g., HDL, high-sensitivity CRP, triglycerides, T2DM) yielded a HR of 1.30 (95% CI, 1.02-1.67) for ischemic stroke risk per 5 μg/mL increase in adiponectin in women[453], which was not observed in men. Lack of adjustment or adjustments excluding metabolic confounders resulted in non-significant HRs for both men and women[453]. In the largest community-based cohort of African Americans, a 1 SD increase in adiponectin levels was associated with a significant increase of 41% in risk for ischemic stroke in women, but not in men, following adjustments for various confounders[455]. In contrast, 2 studies using the same cohort of post-menopausal women (HaBPs) demonstrated that total adiponectin but not its HMW isoform was inversely associated with the risk of ischemic stroke, following adjustments for age and ethnicity[468,471]. However, after accounting for obesity and other cardiovascular confounders, no independent association was found[468,471]. A Swedish case-control study failed to demonstrate a significant association between adiponectin and ischemic stroke risk in men and in women[472]. In contrast, the PRIME study, including European middle-aged men, yielded a hazard ratio of 2.78 (95% CI, 1.74-4.44) for ischemic stroke risk per 1 SD increase in plasma adiponectin levels after adjustments for similar metabolic and cardiovascular confounders as above[470].

Table 1.4 (A) Sensitivity and subgroup analyses by study and population characteristics for the effect of circulating adiponectin levels on the risk of ischemic stroke. (B) Meta-regression analysis on the modifying effect of study population characteristics on the association between adiponectin levels and risk of ischemic stroke[285]

Analysis by 5 μg/mL-increment of serum adiponectin levels		**Ischemic stroke**	
(A) Sensitivity and subgroup analyses	$k^{§}$	ES (95% CI)	Heterogeneity I^2, p
Overall analysis	9	1.08 (1.00-1.18)	66.7%, 0.002
Study design			
Prospective cohort studies	4	1.21 (1.03-1.42)	41.0%, 0.17
Nested case-control studies	5	1.03 (0.93-1.15)	67.9%, 0.01
Community-based studies*	9	1.08 (1.00-1.18)	66.7%, 0.002
Adjusted effect sizes**	7	1.15 (1.03-1.28)	71.2%, 0.002
Sex			
Men	4	1.15 (1.02-1.30)	54.1%, 0.09
Women	4	1.07 (0.94-1.23)	71.8%, 0.01
(B) Meta-regression analyses	$k^{§}$	Exponentiated coefficient (95% CI) ***	P-value
Mean age (1-year increment)	8	0.95 (0.87-1.05)	0.27
Male sex (5%-increment of males)	9	1.01 (0.99-1.02)	0.43
Hypertension (5%-increment)	8	1.02 (0.94-1.09)	0.64
Diabetes mellitus (5%-increment)	6	0.90 (0.69-1.19)	0.37
Current smoking (5%-increment)	7	1.02 (0.98-1.05)	0.34
HDL cholesterol levels (5 mg/dL-increment)	7	0.88 (0.78-1.00)	0.05
Triglycerides levels (5 mg/dL-increment)	6	0.98 (0.96-1.01)	0.10
BMI (5 kg/m^2-increment)	8	1.24 (0.88-1.74)	0.17
Mean study follow-up duration (1-year increment)	7	1.01 (0.92-1.10)	0.89

ES: effect size, HDL: high-density lipoprotein, BMI: body mass index.
§ Number of study arms.
*All studies were community-based.
**All studies, besides the study by Söderberg *et al.* (2004) adjusted their analyses at least for age, gender, hypertension (blood pressure levels, presence of hypertension, use of antihypertensive medications), diabetes mellitus, or measurements of insulin resistance, dyslipidemia, and current smoking status.
*** Higher exponentiated coefficient values indicate that increase of the examined factor strengthens the association between increasing adiponectin levels and risk of ischemic stroke.

Meta-regression analysis: Results from the meta-regression analyses for age, sex, hypertension, T2DM, current smoking status, HDL, triglycerides, BMI, follow-up duration, are presented in **Table 1.4**. HDL was the only variable to have a marginal effect on the association between adiponectin levels and ischemic stroke (exponentiated coefficient = 0.88; 95% CI: 0.78–1.00; P = 0.05). The other evaluated parameters are not to be considered as effect modifiers for the association studied herein.

Changes in Circulating Adiponectin Post-Ischemic Stroke

Fifteen studies were retrieved from the systematic review process, but not included in the meta-analysis, as they all were of cross-sectional design, whereby circulating adiponectin levels were assessed post- ischemic stroke[454,456-461,463,464,466-469,473,474]. The majority of these studies reported significantly lower adiponectin levels in subjects who suffered an ischemic stroke in comparison to age- and sex-matched controls[454,457,459-461,463,464,474], as well as following stratification based on T2DM[457], except for 2 studies yielding non-significant differences in adiponectin levels between ischemic stroke subjects and non-matched controls[456,458].

Marousi *et al.* conducted a case-control study matching each ischemic stroke case with a control subject for cardiovascular risk factors (i.e., hypertension, T2DM, ischemic heart disease, atrial fibrillation, hyperlipidemia) and found a significant association between low adiponectin levels and risk for ischemic stroke (OR: 0.87; 95% CI: 0.78-0.97)[464]. Interestingly, when analyses were performed separately for men and women, significant associations remained solely in men-specific analysis, revealing a potential male contribution to the overall cohort effect[464]. Increasing levels of adiponectin were also found to be significantly protective against ischemic stroke in Chen *et al.*'s, with the results remaining unchanged after multiple adjustments for metabolic and cardiovascular confounders (OR: 0.83; 95% CI: 0.78-0.88)[457]. Others have also found similar results between adiponectin levels and risk for ischemic stroke (OR: 0.56; 95% CI: 0.32-0.98)[461].

Additionally, a study found that with increasing levels of ischemic stroke, the proportion of LAA strokes (subtype of ischemic stroke) decreased (P<0.01)[461]. Following adjustments for metabolic and cardiovascular confounders, it was demonstrated that a 1 μg/mL increase in adiponectin levels significantly decreased the risk of LAA strokes (OR: 0.79; 95% CI: 0.64-0.98)[461]. Similarly, an inverse association was identified between reduced levels of adiponectin and LAA stroke risk, even after adjustments for age, sex, and other confounders[474]. On the other

hand, others only reported a significant correlation between adiponectin levels and LAA strokes in men (P=0.003), but not in women[460].

Interestingly, studies assessing circulating adiponectin levels in subjects with high-grade carotid artery stenosis found no significant differences between symptomatic (ischemic stroke, TIA, AF) and asymptomatic subjects[466,467,469]. Given that both groups (symptomatic versus asymptomatic) have severe carotid atherosclerosis, these non-significant results do not necessarily oppose an association between adiponectin and ischemic stroke risk, but rather demonstrate no association between adiponectin and plaque rupture.

Marousi *et al.* detected no significant changes between samples obtained at 0-24h, 24-48h, or 48-72h after ischemic stroke onset[464]. In addition, samples measured at 6 months after ischemic stroke did not differ from their acute phase values, suggesting marked stability over time. This implies that adiponectin levels are suppressed at the early stages of stroke and remain suppressed during the course of the disease. In contrast, Kuwashiro *et al*. showed a progressive yet temporary decrease in adiponectin levels during the first 7 days after atherothrombotic stroke onset[463]. However, during the time frame between 14 and 90 days post-ischemic stroke, adiponectin returned to its baseline level (day 0) after ischemic stroke, while at 90 days, adiponectin levels were higher than those during the acute phase[463]. Additionally, adiponectin levels were significantly lower in the atherothrombotic stroke subjects compared with age- and sex-matched controls at 0, 3, 7 and 14 days after ischemic stroke onset, but not at 90 days[463].

Circulating Adiponectin and Mortality Risk in Ischemic Stroke Populations

Three studies were included in the meta-analysis referring to the association between circulating adiponectin levels and overall mortality, among patients who suffered a previous ischemic stroke [475-477]. A total of 663 (63.5% men) subjects were included with mean age of 69.0 years (SD: ±11.9), mean BMI of 27.9 $kg.m^{-2}$ (SD: ±5.2), and mean total adiponectin levels of 9.96 μg/mL (SD: ±7.14) (Table VIII - Appendix B). Non-significant associations of circulating adiponectin with mortality (**Figure 1.11**) were yielded (n= 663; summary OR: 2.58; 95% CI: 0.69-9.62; heterogeneity I^2 = 84.8%; P =0.001).

A higher probability of all-cause 5-year mortality has been observed in first-ever ischemic stroke patients with adiponectin levels <4 μg/mL[475]. There was a significant 8-fold and 4-fold increase in the relative risk for death within 5 years following a first-ever ischemic stroke when

the lowest and middle tertiles were compared with the highest tertile of adiponectin levels, respectively[475]. In a study with a shorter post-ischemic stroke observation window of 6 months, no associations were found between adiponectin levels and vascular mortality[476]. In contrast, Nagasawa *et al.* found a significant 6-fold increase in stroke mortality with higher levels of adiponectin, but no significant associations with all-cause mortality[477].

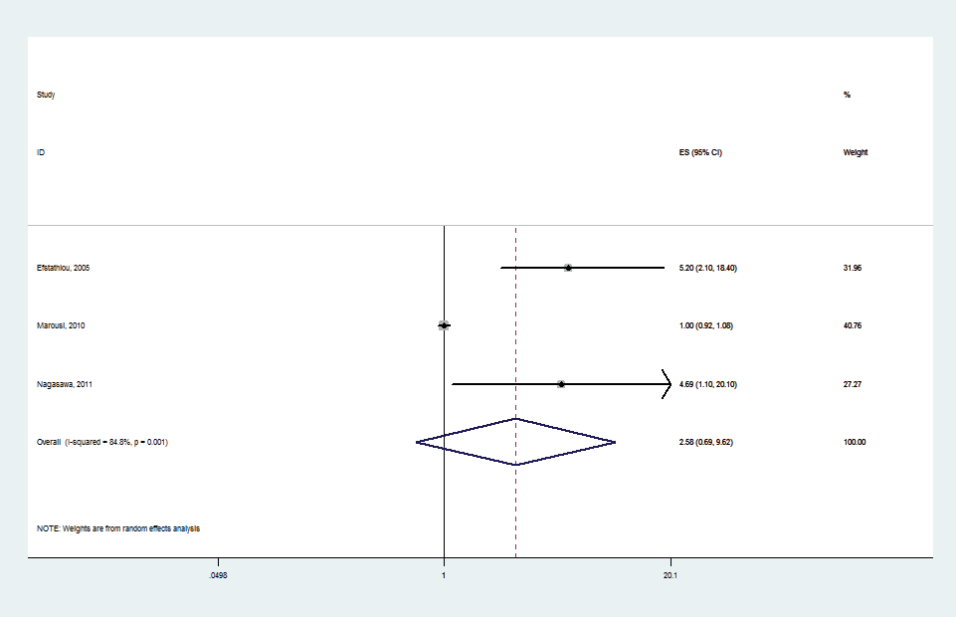


Figure 1.11 Forest plot describing the association of adiponectin levels and overall mortality among patients with history of ischemic stroke (3 studies). *Circulating adiponectin levels were assessed as continuous with 5 µg/mL increments. ORs of individual studies are indicated by the data markers; shaded boxes around data markers reflect the statistical weight of the study; 95% CIs are indicated by the error bars; summary-effect estimates with its 95% CIs are depicted as a diamond.*[285]

1.12.5 Discussion

This is the first systematic review and meta-analysis focused on the association between adiponectin levels and progression of carotid artery disease from carotid atherosclerotic plaque presence, to ischemic stroke risk, to risk of mortality within an ischemic stroke population. This review comes as a continuation to our previously published systematic review and meta-analysis

assessing the association between adiponectin levels and subclinical atherosclerosis (cIMT), thus providing an overview of the full spectrum of carotid artery disease in relation with adiponectin.

Adiponectin and Carotid Atherosclerotic Plaque Presence

Through the meta-analysis, we aimed to assess the association between carotid atherosclerotic plaque presence and circulating adiponectin levels, which yielded a non-significant association. All included studies in the meta-analysis referred to subjects with a high inflammatory profile (rheumatoid arthritis and systemic lupus erythematosus)[280,418]. These subjects are known to have higher levels of adiponectin at baseline due to their disease state compared with healthy controls. However, in the presence of carotid plaque, adiponectin levels were shown to be higher than in their non-plaque counterparts. Thus, we believe that plaque presence contributes to the elevation in adiponectin levels. On the other hand, studies not included in the meta-analysis demonstrated an inverse association between adiponectin levels and carotid plaque presence. These studies included relatively healthy subjects without cardiovascular risk factors or subjects with peritoneal disease[447,452]. In these populations, plaque presence may contribute to a decrease in adiponectin levels. The overall direction of these results mirrors those observed in our previous review and meta-analysis assessing the association between adiponectin levels and cIMT, a surrogate for subclinical atherosclerosis[284]. Thus, these results suggest that adiponectin is a marker of inflammation rather than a precursor of plaque presence. However, plaque presence seems to lead to changes in adiponectin levels depending on the duration, severity, and nature of the underlying disease.

Paradoxical Increase in Adiponectin Levels in Relation to Ischemic Stroke

The meta-analysis of published studies focusing on first-ever ischemic stroke showed a significant association of high adiponectin levels at baseline and risk for ischemic stroke occurrence during prospective follow-up, whereas fully adjusted individual analyses for cardiovascular and metabolic confounders strengthened the aforementioned association[453,455,462,470]. Similar results have been previously reported regarding the association of adiponectin and risk for total stroke (including hemorrhagic stroke) or coronary heart disease[453,484]; yet, the previously published results remain overall contradictory, since numerous studies have pointed to a protective effect of high adiponectin levels against vascular disease.

Several plausible mechanisms have been proposed to explain the paradoxical association of high adiponectin levels with adverse cardiovascular outcomes. High adiponectin levels may reflect the phenomenon of adiponectin resistance (decreased signalling efficacy) in response to atherosclerotic disease progression. Furthermore, high levels of adiponectin may also be attributed to a progressive compensatory response to vascular inflammation in the ischemic stroke subgroup in order to offset this deleterious process[485-487]. Indeed, in studies including data on the inflammatory state at baseline (5-10 years pre- ischemic stroke), higher levels of inflammatory markers (i.e., E-selectin, CRP, IL-6, and RANTES) were noted in the ischemic stroke group compared to non-ischemic stroke subjects[455,470]. Lastly, adiponectin may also possess pro-inflammatory properties in individuals at high-risk for ischemic stroke regardless of its well-known protective actions[488].

There are several limitations in the designs/protocols of the included studies, which prevent us from drawing clear conclusions. The studies comprising the meta-analysis were either nested case-control or prospective cohort studies with a follow-up ranging from 5 to 10 years. Study designs were robust, however, relevant information on plaque presence at the time of recruitment was missing. As we know, atherosclerotic plaque development occurs over the course of several years, and thus, the process may have been initiated for some subjects before adiponectin levels were measured. Additionally, none of the studies reported time to event (time between recruitment/baseline and ischemic stroke occurrence/diagnosis). These variables could be potential effect modifiers.

Sex-specific Associations between Adiponectin and Ischemic Stroke Risk

Given that circulating adiponectin and ischemic stroke risk are independently associated with sex, it was not surprising to find our overall results pointing to a sex-specific effect of adiponectin on ischemic stroke risk. In particular, subgroup analyses showed a significant association between high adiponectin levels and increased risk (14%) for ischemic stroke solely in men, but not in women. Although the 2 largest studies included in the women-only meta-analysis reported an increased risk for ischemic stroke with higher adiponectin[453,455], these estimates have a high variance, which in turn decreased the weights they were assigned.

Adiponectin Levels Post-Ischemic Stroke

Adiponectin levels measured 5-10 years prior to ischemic stroke occurrence were higher in the ischemic stroke subgroup compared to their non- ischemic stroke counterparts[453,455,462]. However, when adiponectin levels were measured in the acute phase following ischemic stroke, the majority of studies showed that adiponectin levels were lower[454,456,457,459-461,464].

Although adiponectin is known for its' anti-inflammatory and vasculoprotective properties, and can suppress various pro-inflammatory cytokines, these cytokines can in turn modulate adiponectin levels resulting in a deleterious state of hypoadiponectinemia[300,489,490]. Particularly, the transient increase in TNF-α and IL-6 is thought to be a distinctive feature of the acute inflammatory response to ischemic stroke[459,491-493]. Thus, the elevation of these markers could be contributing to the observed decrease of adiponectin levels directly following ischemic stroke compared with non-ischemic stroke controls. Furthermore, considering that carotid plaques are crucial and major determinants of cerebrovascular disease, the low levels in adiponectin observed among the ischemic stroke subjects may also be due to the contribution of plaque (as mentioned above in the plaque meta-analysis), since the presence of carotid atherosclerotic plaques was not determined in the non-ischemic stroke controls.

Interestingly, these results were not observed when symptomatic (carotid atherosclerotic plaque related symptoms) and asymptomatic patients with high-grade carotid artery stenosis were compared[466,467,469]. In fact, adiponectin levels remained similar between these 2 groups[466,467,469]. It should be noted that in this comparison, we are observing two populations that have the same potential for ischemic stroke development (or associated symptomatology) triggered by the rupture of the carotid atherosclerotic plaque[439], unlike in the findings above where adiponectin levels were compared between subjects who had ischemic stroke and relatively healthy non-ischemic stroke controls. Thus, these results are not representative of an association between adiponectin and ischemic stroke risk but rather portray the lack of association between adiponectin and plaque rupture.

Adiponectin Isoforms

Although the HMW isoform of adiponectin is known to have strong vasculoprotective and insulin-sensitizing properties and is believed to be the most biologically active form of adiponectin[441-444], the majority of studies measured total circulating adiponectin levels; only 2 plaque studies[418,447]

and 3 ischemic stroke studies assessed HMW adiponectin levels[454,462,468]. Nonetheless the relationships observed with HMW adiponectin reflected those seen with total adiponectin, suggesting that total and HMW levels may be highly correlated. However, stronger associations were detected with total adiponectin and not with HMW adiponectin.

Discrepancy Among Mortality Studies

Given the paucity of published studies, no robust conclusions can be drawn between adiponectin levels post-ischemic stroke and vascular or all-cause mortality[475-477]. The significant between-study heterogeneity might explain the discrepancies observed, mostly owing to: (1) differences in adiponectin measurements; of note, in 2 studies, bloods were drawn acutely between 24 and 72 hours post-admission for ischemic stroke[475,476], whereas in Nagasawa *et al.*, bloods were drawn 2 weeks post-ischemic stroke [477]; and, (2) different follow-up periods ranging from 1-60 months.

Overall Strengths and Limitations

The assessment of the association of adiponectin strictly with the ischemic stroke subtype allowed us to draw more robust conclusions regarding the potential underlying inflammatory-mediated mechanisms; this is considered the major shortcoming of previous meta-analyses focused on the association between adiponectin and all stroke types combined[453,494]. Despite the extensive search strategy in order to pool all relevant studies, certain limitations should be acknowledged. Of note is the significant between-study heterogeneity in most analyses, possibly attributed to differences in study designs (cohort or case-control versus cross-sectional), levels of adjustment, and study populations. Furthermore, scarce were the studies prospectively assessing changes in adiponectin levels throughout atherosclerotic plaque development, to occurrence of ischemic stroke, and ultimately, mortality in an ischemic stroke population.

As described in the methodology section, we used the Newcastle-Ottawa scale to assess the quality of included studies[372]. No studies were excluded based on the grading scores. Quality assessment demonstrated a higher grading score among articles included in the meta-analyses than those that were not included. Particularly, an overall score of 94.4% and 90.5% was given to articles included in the plaque and ischemic stroke meta-analyses, respectively, compared to 72.2% and 65.5% for the non-included plaque and stroke studies, respectively. All mortality studies were included in the meta-analysis and given an overall score of 88.9%. The high score assigned to the

articles included in the meta-analyses suggest that these studies were well designed, and their findings can be interpreted with some certainty.

In order to evaluate the contribution of various modifiable and non-modifiable risk factors, we first created a directed acyclic graph illustrated in **Figure 1.12**. This directed acyclic graph demonstrates the complex relationship between adiponectin (exposure), carotid plaque presence (outcome 1), and ischemic stroke (outcome 2), with known risk factors. Furthermore, adding to the strength of our review, we performed extensive sensitivity, subgroup, and meta-regression analyses, which showed these parameters to be confounding variables and not effect modifiers. However, these analyses could only be performed for the ischemic stroke outcome due to the limitation in the number of studies available for plaque presence and mortality. These analyses justified the importance of including these parameters in adjusted models assessing the relationship between adiponectin and carotid plaque or ischemic stroke, without biasing the estimate. While the majority of included studies primarily adjusted for metabolic risk factors (i.e., hypertension, T2DM, obesity, dyslipidemia) as well as age and sex, inflammatory markers were not properly considered, which we believe to be a major limitation of the included studies. We hypothesize through the results obtained in this review that these markers are important contributors (and potential colliders) to the relationship between adiponectin and the full spectrum of carotid atherosclerotic disease.

Knowledge Gaps

Further prospective studies are needed to assess the levels of adiponectin at various time-points throughout the follow-up time period, in order to recognise potential variations in adiponectin levels over time. Furthermore, future datasets should comprise clinical data on cIMT measurements and morphological characteristics of plaques via ultrasonic investigations, in order to assess plaque progression over time, in relation to adiponectin levels. Lastly, adiponectin levels measured at time-points later than 6 months following ischemic stroke could help determine if adiponectin levels remain suppressed in the long term.

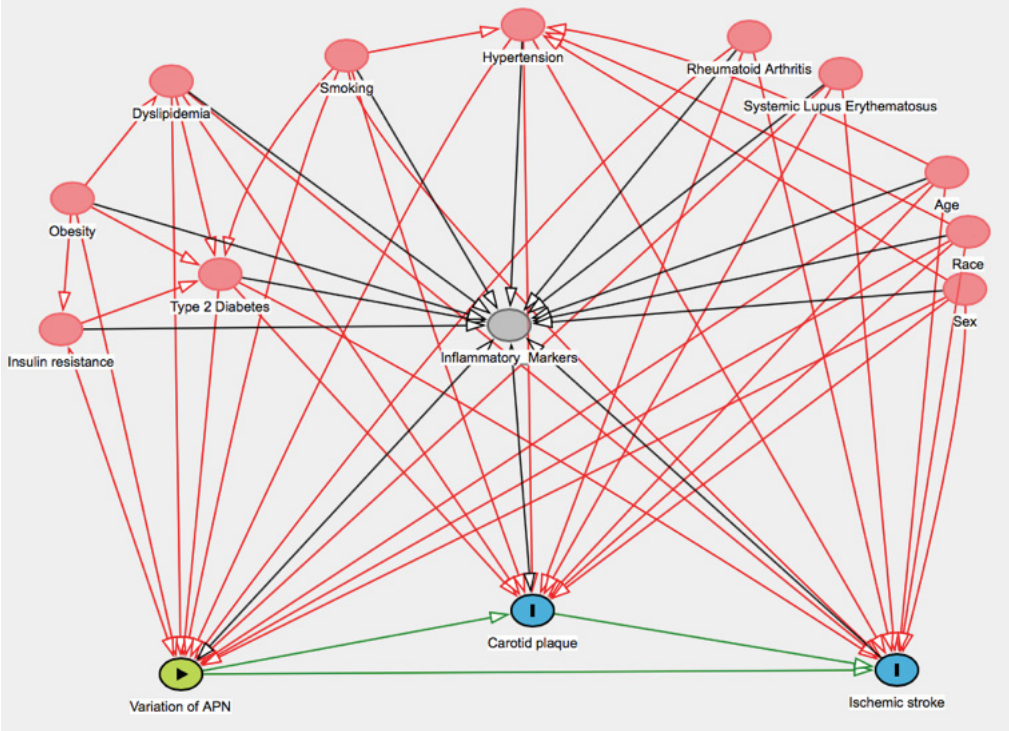


Figure 1.12 Directed acyclic graph describing the complex relationship between adiponectin (exposure), carotid plaque presence (outcome 1), and ischemic stroke (outcome 2), with known risk factors. *Risk factors (shown in red) are 'confounding variables' as they are associated with both the exposure and the outcome (red biasing paths) and should be included in the adjusted models. On the other hand, inflammatory markers (shown in gray) can be potential 'colliders', which would introduce 'collider bias' if not properly taken into consideration in the analyses. APN indicates adiponectin.*[285]

1.12.6 Conclusion

These systematic review and meta-analyses along with our previously published review on cIMT constitute the first comprehensive assessment of the association between adiponectin and the full spectrum of carotid artery disease, from subclinical atherosclerosis (cIMT), to plaque presence, to ischemic stroke risk, to risk of mortality in an ischemic stroke population. We previously observed an association between low adiponectin levels and high cIMT measurements, thus suggesting that adiponectin is a potential risk factor for subclinical atherosclerosis. However, this inverse association was not observed among all studied populations, particularly including those with inflammatory diseases. These results mirror those observed in the assessment of adiponectin and

plaque presence herein. In fact, it appears that the direction of the relationship between adiponectin and carotid atherosclerotic plaque presence is dependent on the duration, severity, and nature of the underlying disease. Indeed, increased adiponectin levels were linked to patients with a highly inflammatory disease status, contrary to lower levels among patients with obesity-related disorders, reflecting a lower degree of inflammation. Furthermore, increased levels of adiponectin were associated with an 8% increase in risk for ischemic stroke in subjects without clinically manifested CVD, with a more sizable association observed among men compared to women. However, adiponectin levels were noted to be suppressed at the acute phase of ischemic stroke and remained suppressed up to 6 months post-ischemic stroke. It remains to be investigated whether changes in adiponectin levels pre- and post-ischemic stroke could be used as a clinical marker of ischemic stroke. Lastly, the results from the mortality meta-analysis remain inconclusive. Our findings, overall, stress the need for future studies to address the limitations identified in this review in order to reach definite conclusions regarding the association between circulating adiponectin levels and carotid atherosclerotic disease-related outcomes.

1.13 Research Objectives

Despite tremendous efforts to understand and treat carotid atherosclerotic disease, its management remains suboptimal in women and in men and the mechanisms leading to plaque instability and ultimately stroke are poorly defined. Recently, it has been demonstrated that adipose tissue can participate in the regulation of vascular function via the secretion of immunomodulatory proteins, such as adiponectin. We hypothesized that adiponectin and its receptors are associated with carotid atherosclerotic plaque instability and that impairment in this pathway, particularly in the monocyte-macrophage lineage will contribute to plaque instability. Hence, the primary goal of this thesis was to 1) determine the contribution of the adiponectin-AdipoR pathway in carotid atherosclerotic plaque instability and 2) explore the effect of modulating this pathway in the monocyte-macrophage lineage. Moreover, we aimed to identify sex-specific signatures associated with the adiponectin-AdipoR pathway and plaque instability. More specifically, as outlined below, four major objectives were pursued herein,

Objective 1 – To determine the association between carotid atherosclerotic plaque instability and the adiponectin–AdipoR pathway at the level of the circulation and at the level of the plaque

(Chapters 3 and 4),

Objective 2 – To study the role of the adiponectin-AdipoR pathway in macrophage-mediated cholesterol efflux and HDL biogenesis to promote plaque stability (Chapter 5),

Objective 3 – To investigate whether statin therapy can modulate adiponectin-AdipoR expression and function in the monocyte-macrophage lineage (Chapter 6),

Objective 4 – To identify sex-specific adiponectin-AdipoR signatures in men and women with stable versus unstable carotid atherosclerotic plaques (Chapter 7).

CHAPTER 2: GENERAL METHODOLOGY

A large ongoing bio-bank of human blood and carotid plaque specimens has been created and used in the majority of the studies presented herein, to act as a translational platform optimized to address the current knowledge gaps pertaining to carotid plaque stabilization. General methodology concerning patient recruitment, blood sample and carotid plaque specimen collection and processing is presented below (**Figure 2.1**).

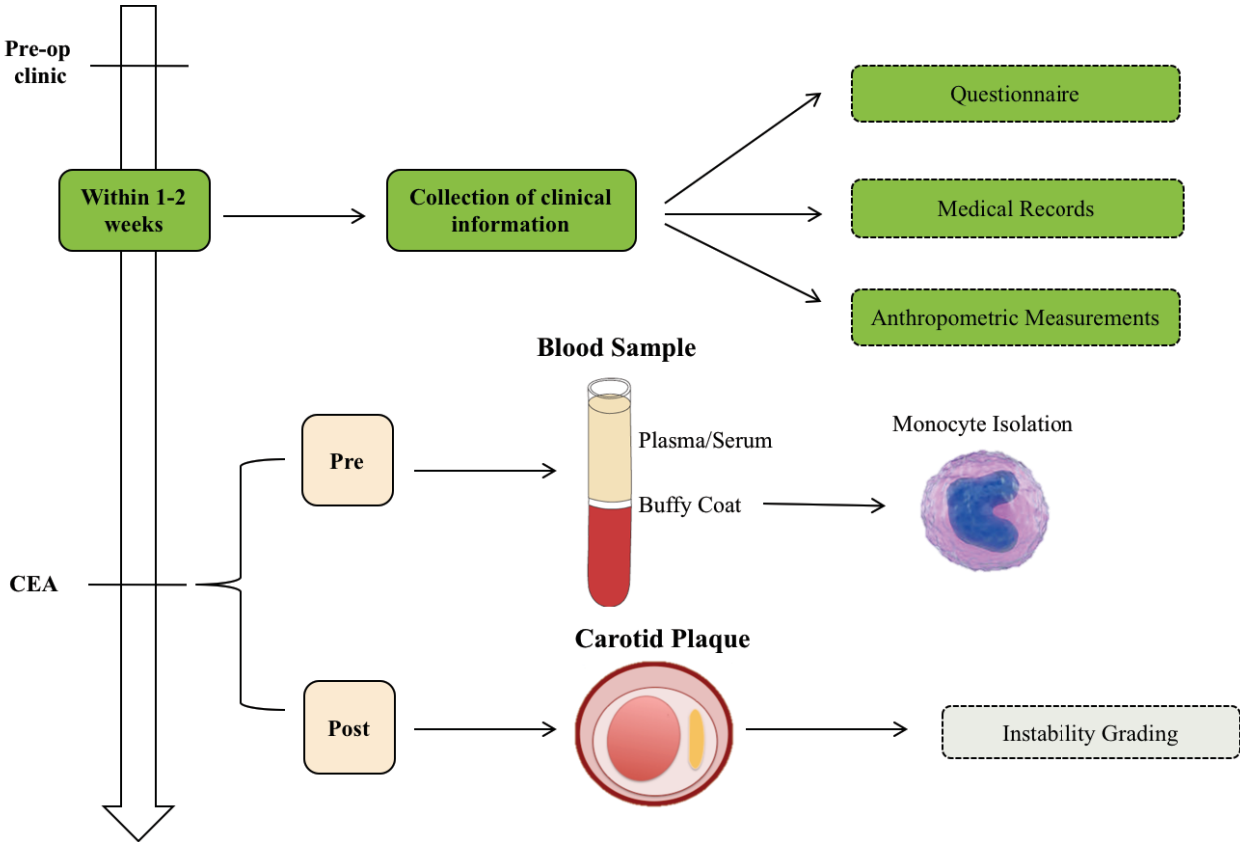


Figure 2.1 Schematic diagram of the patient recruitment and sample collection process.

2.1 Patient Recruitment

Consecutive eligible, neurologically symptomatic and asymptomatic patients with moderate- to high-grade carotid artery stenosis (≥50%), scheduled for a CEA due to clinical indications, were recruited from the Vascular Surgery pre-operative clinics at the McGill University Health Centre and the Jewish General Hospital. In the preoperative clinics, to confirm that carotid artery disease was the cause of stroke in symptomatic patients, other potential etiologies of cerebrovascular events were assessed and ruled out through systematic clinical assessments. The degree of carotid

artery stenosis severity was determined according to the NASCET criteria[150]. Exclusion criteria for CEA (as per clinical indications) include non-carotid sources of ischemic symptoms, such as cardioembolic causes (e.g., atrial fibrillation, recent MI [<3 months], endocarditis), hemorrhagic strokes, intracranial disease, lacunar infarction, and aortic arch disease. Excluded from the study were patients who have had previous interventions on the same carotid artery undergoing surgery (i.e., CEA or CAS), as the histopathology of the re-stenotic plaque may be altered[495,496]. The McGill University's Institutional Review Board granted ethics approval (A12-M145-09B), and written informed consent was obtained from all study participants.

2.2 Clinical Information and Anthropometric Measurements

Patient demographics and clinical information (cerebrovascular symptomatology, past medical and family history, medication use, and lifestyle habits) were obtained and cross-matched through various sources: 1) patient interview, 2) questionnaire, and 3) medical records. Appropriate clinical examinations were performed in the preoperative clinics, to assess the symptomatic status of patients. Patients were defined as 'symptomatic' if they had recently (<6 months prior to CEA) developed cerebrovascular symptomatology (i.e., ischemic stroke, TIA, AF) ipsilateral to the internal carotid artery stenosis. Patients who never reported neurological symptoms of cerebral ischemia from the left or right carotid vascular territories or who experienced symptoms ≥6 months prior to CEA were classified as 'asymptomatic'[151]. Using resources from the outpatient clinics, brachial blood pressure (HEM-705CP, Omron Corp.) was measured according to guidelines[497], and anthropometric measurements (height and weight) were obtained to calculate BMI (kg/m^2), using standardized methods.

2.3 Blood Collection and Measurements

A total of 60 mL of fasting blood was drawn from each patient pre-operatively on the day of the CEA. The blood samples (30 mL) were centrifuged at 3000 rpm for 10 min at 4°C to obtain plasma and serum and rapidly stored at −80°C in 0.5mL aliquots for subsequent analysis. Serum lipid profile (total cholesterol, HDL-C, triglycerides, apoA-I, apoB), high-sensitivity CRP, and glucose levels, as well as complete blood count (including white blood cell counts, red blood cell counts, hemoglobin and hematocrit levels, and platelet counts) were measured in the McGill University

Health Centre central biochemistry labs. LDL-C levels were calculated using the Friedewald equation[498]. The remaining blood (30 mL) was used for monocyte extraction.

2.4 Peripheral Blood Monocyte Isolation

Whole blood was diluted with an equal volume of phosphate buffered saline (PBS) + 0.5% bovine serum albumin (BSA). Peripheral blood mononuclear cells (PBMCs) were then isolated from the diluted blood sample using Ficoll-Paque density gradient centrifugation (1800 rpm for 33 min at room temperature, with brake off). The interphase layer containing the PBMCs was collected and washed twice with PBS + 0.5% BSA (3000 rpm for 8 min at 4°C). Total monocytes were then isolated from the PBMCs by positive selection with $CD14^+$ Human MicroBeads (Miltenyi Biotec, Germany), using a Magnetic Activated Cell-Sorting technique (autoMACS Pro Separator, Miltenyi Biotec). This method of isolation yielded a monocyte population with a purity of >98%, as assessed by flow cytometry staining for CD14. For subsequent RNA extraction, isolated monocytes were centrifuged and flash-frozen with liquid nitrogen.

2.5 Plaque Processing

Human carotid atherosclerotic plaque specimens were obtained from each patient immediately following surgical resection. Plaques were dissected into transverse segments of approximately 3-4 mm in thickness (**Figure 2.2**).

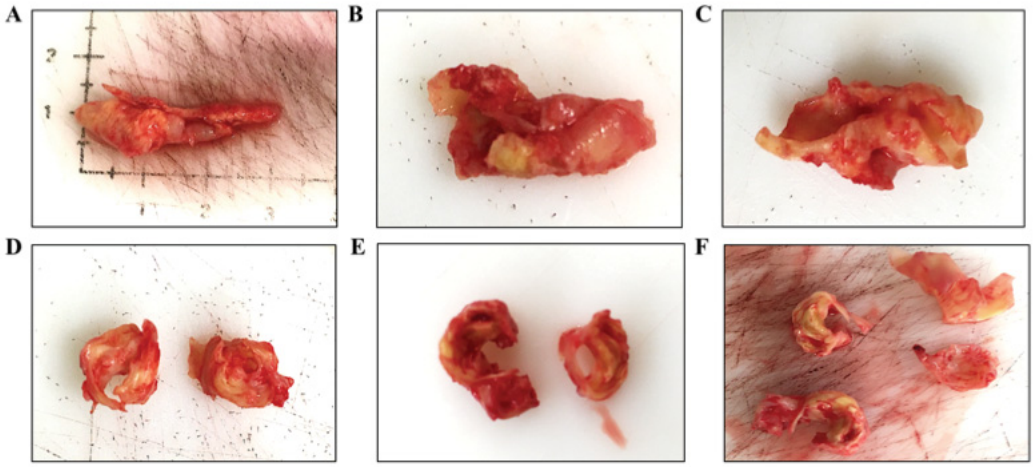


Figure 2.2 Representative longitudinal (A-C) and cross-sectional (D-F) images of fresh carotid plaque specimens obtained from patients who underwent a carotid endarterectomy.

Plaque segments with the area of maximal stenosis and largest plaque burden were dedicated to histological/immunohistochemical and RNA/protein analyses. For histology/immunohistochemistry, plaque segments were fixed in 10% formalin, decalcified (Surgipath Decalcifier I, Leica Microsystems Inc., Buffalo Grove, IL, USA), and then processed for paraffin embedment. For RNA/protein analyses, plaque segments were flash-frozen with liquid nitrogen.

2.6 Histological Assessment of Plaque Stability

A 4-μm section from the plaque segment was stained with hematoxylin and eosin. In addition, four other consecutive 4-μm sections were immunostained for markers of lymphocytes, macrophages, SMCs, and neovascularization (CD3, CD68, α-SMC actin, von Willebrand factor, respectively). Two vascular pathologists independently performed the histological assessments of the plaque specimens. In case of disagreement, a consensus was reached. The pathologists were blinded to patient clinical information and study outcomes. The plaques were characterized according to two gold-standard classifications of plaque instability: 1) AHA histological classification by Stary *et al*[176] (**Table 1.1 – Chapter 1**), and 2) semi-quantitative scale by Lovett *et al*[177,178] (**Table 1.2 – Chapter 1**). The AHA classification ranges from Type I, an initial endothelial lesion, to Type VIII, a predominantly fibrotic plaque[176]. Type VI is the most complicated lesion, which is characterized by the presence of a hematoma, haemorrhage, or thrombus. Clinical outcomes are most often associated with type V and type VI plaques, while plaques beyond type VI represent more fibrotic and calcific lesions that mainly occur following lipid regression. The specimens obtained from the CEAs were all representative of advanced atherosclerotic lesions (Type V-VIII). According to the semi-quantitative scale, individual features of the plaque were classified on a 3- or 4-grade scale, including presence/absence of intraplaque haemorrhage, thrombus, foam cells, lipid core size, proportion of fibrous tissue, neovascularization, calcification, cap rupture, overall inflammation, and infiltration of the fibrous cap with inflammatory cells (inflammation graded according to the number of macrophages and lymphocytes present)[177,178]. Based on the presence of a combination of these features, the overall instability of the plaque was characterized as definitely stable, probably stable, probably unstable, or definitely unstable (**Figure 2.3**). Generally, unstable plaques were either ruptured or rupture-prone, with a large lipid core and thin inflamed cap, while stable plaques had a thick fibrous cap, which protects them from rupturing, and small or no lipid core.

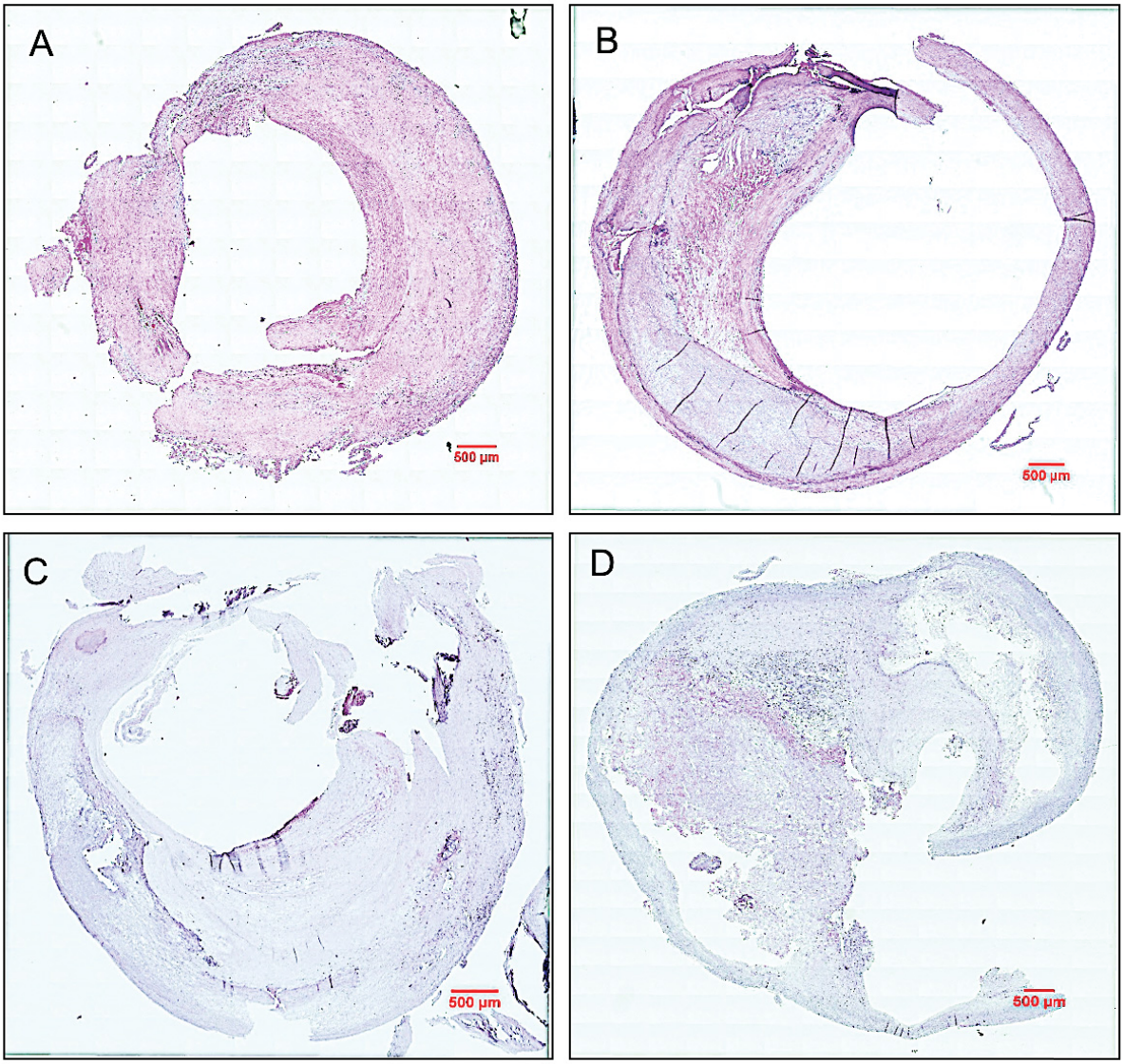


Figure 2.3 Representative photomicrographs of carotid atherosclerotic plaques*. Hematoxylin and eosin staining show* ***A)*** *a definitely stable plaque,* ***B)*** *a probably stable plaque,* ***C)*** *a probably unstable plaque, and* ***D)*** *a definitely unstable plaque. Images were photographed at x20 original magnification.*

CHAPTER 3: CIRCULATING CHEMERIN, BUT NOT ADIPONECTIN, IS ASSOCIATED WITH CAROTID PLAQUE INSTABILITY, WHEREAS RESISTIN IS RELATED TO CEREBROVASCULAR SYMPTOMATOLOGY

Karina Gasbarrino, Christos Mantzoros, Jessica Gorgui, John P. Veinot, Chi Lai, Stella S. Daskalopoulou

Published in and adapted from[1]:
Gasbarrino K, Mantzoros C, Gorgui J, Veinot JP, Lai C, Daskalopoulou SS. *Arterioscler Thromb Vasc Biol*. 2016;36(8):1670-1678.

3.1 Abstract

Objective: The rupture of unstable carotid atherosclerotic plaques is one of the main causes of cerebrovascular ischemic events. There is need for circulating markers that can predict plaque instability and risk of stroke. Pro-inflammatory chemerin, leptin, and resistin, along with anti-inflammatory adiponectin, are adipokines with direct influence on vascular function. We investigated the association of circulating adipokines with carotid plaque instability and cerebrovascular symptomatology.

Approach and Results: Neurologically symptomatic and asymptomatic patients (n=165) scheduled for CEA were recruited. Fasting blood samples were collected pre-operatively; adiponectin and leptin levels were determined by radioimmunoassay, chemerin and resistin levels were measured by enzyme-linked immunosorbent assays. The instability of plaque specimens was assessed using gold-standard histological classifications. Chemerin was significantly associated with plaque instability. The fully adjusted model, accounting for age, sex, BMI, high-sensitivity CRP, T2DM, and circulating adiponectin, leptin, and resistin, yielded an OR of 0.991 (95% CI: 0.985-0.998) for plaque instability per unit increase in chemerin. High leptin levels were significantly associated with presence of specific features of plaque instability. In subjects with T2DM, resistin levels were significantly elevated in symptomatic when compared to asymptomatic subjects (P=0.001) and increased the risk of cerebrovascular symptomatology (adjusted OR: 1.264, 95% CI: 1.004-1.594).

Conclusions: Low chemerin and high resistin levels were associated with carotid disease severity, suggesting that these adipokines may act as potential markers for plaque instability and stroke risk. Future studies are needed to assess causation between circulating adipokines and plaque instability.

3.2 Introduction

The development of unstable atherosclerotic plaques, primarily in the internal carotid artery, but also in the common carotid artery, is a major etiological factor known to cause ischemic strokes[439]. According to the 'traditional concept of plaque vulnerability', histologically, an unstable plaque is characterized by a large lipid-rich core, a thin fibrous cap with dense inflammatory cell infiltration, ulceration, thrombosis, and intraplaque hemorrhage[93]. The degree of carotid artery stenosis is a well-established risk factor of plaque instability, and forms the basis for the guidelines for CEA, a common surgical procedure recommended for stroke prevention[150]. However, more recently, carotid artery stenosis has proven to be an incomplete determinant of plaque instability, since even moderate or low-grade stenoses can be unstable and produce potentially life-threatening strokes[174,499]. Although several circulating markers (e.g., high-sensitivity CRP and IL-6) have emerged as tools for stroke risk assessment in patients with severe carotid atherosclerosis, their ability to reliably and specifically predict plaque instability is suboptimal[499,500]. Thus, there is a need for novel markers that can accurately identify plaque instability and risk of stroke[499,500].

Adipose tissue is a dynamic endocrine organ, which directly regulates vascular function through the secretion of immunomodulatory proteins, termed adipokines[246]. Adipokines, such as chemerin, leptin, and resistin, correlate positively with markers of subclinical atherosclerosis, such as cIMT, and may exert actions that are potentially atherogenic[246,402]. In contrast, adiponectin, the most abundantly secreted adipokine, exhibits anti-inflammatory and vasculo-protective properties, and is inversely associated with cIMT, CAD, and carotid plaque presence[265,280,365,366,402]. As a result, adipokines may serve as potential markers of carotid atherosclerotic disease presence. However, their relationship with plaque instability remains questionable. Therefore, the primary objective of this study was to evaluate the association between circulating levels of adiponectin, chemerin, leptin, and resistin, and carotid plaque instability in patients with moderate- to high-grade carotid artery stenosis scheduled for CEA. As a secondary objective, we evaluated the association between these adipokines and cerebrovascular symptomatology. We hypothesize that adiponectin will be inversely associated with carotid plaque instability and cerebrovascular symptomatology, while the pro-inflammatory adipokines, chemerin, leptin, and resistin, will have a direct association with both of these outcomes.

3.3 Methods

3.3.1 Study population

Consecutive neurologically symptomatic and asymptomatic patients with moderate- to high-grade carotid artery stenosis (≥50%) scheduled for CEA were recruited from the Vascular Surgery pre-operative clinics at the McGill University Health Centre and the Jewish General Hospital, Montreal, Canada[108]. Using a Philips iU22 (Andover, United States) Doppler ultrasound machine and a linear 9-3 MHz probe, the degree of carotid artery stenosis was determined according to NASCET criteria[150].

Exclusion criteria comprised non-carotid sources of ischemic symptoms, such as cardioembolic causes, previous interventions on the same carotid artery, intracerebral hemorrhage, intracranial disease, lacunar infarction, and aortic arch disease[108]. The McGill University's Institutional Review Board granted ethics approval (A12-M145-09B), and written informed consent was obtained from all study participants.

Patient demographics and clinical information (cerebrovascular symptomatology, past medical history, medication use, and lifestyle habits) were obtained and cross-matched through various sources: 1) patient interview, 2) questionnaire, and 3) medical records[108]. Patients were defined as 'symptomatic' if they had recently (<6 months prior to CEA) developed cerebrovascular symptomatology (i.e., stroke, TIA, AF) ipsilateral to the internal carotid artery stenosis. Patients who never experienced symptoms of cerebral ischemia from the left or right carotid vascular territories or who experienced symptoms ≥6 months prior to CEA were classified as 'asymptomatic'[151]. All subjects with T2DM were diagnosed prior to the CEA. No subjects with newly diagnosed diabetes were identified. Diagnosis was confirmed during the pre-operative evaluation, based on anti-diabetic treatment or blood tests of fasting glucose and/or glycated hemoglobin (HbA_{1C}) values[501]. Using resources from the outpatient clinics, brachial blood pressure (HEM-705CP, Omron Corp.) was measured according to guidelines[497], and anthropometric measurements (height and weight) were obtained to calculate BMI (kg/m^2).

3.3.2 Laboratory assays

Fasting blood samples were collected from each participant the morning of the CEA immediately prior to the surgical intervention. The blood samples were centrifuged to obtain plasma and serum and rapidly stored at −80°C for subsequent analysis. Serum lipid profile (triglycerides, total cholesterol, LDL-C, and HDL-C) and high-sensitivity CRP levels were measured in the central

biochemistry laboratories of the McGill University Health Centre using standardized methods and protocols. Glycated hemoglobin A1C (HbA_{1C}) was measured using high-performance liquid chromatography. Total adiponectin and leptin plasma levels were determined by radioimmunoassay (EMD Millipore, Billerica, MA, USA), as previously described[502], while chemerin, and resistin plasma levels were measured by enzyme-linked immunosorbent assays (Human Chemerin ELISA, Biovendor, Brno, Czech Republic; Human Resistin ELISA, EMD Millipore, MA, USA). The lower limits of detection were 0.9375 ng/mL for adiponectin, 0.1 ng/mL for chemerin, and 0.02 ng/mL for resistin. All samples were run in duplicate with quality controls, and inter- and intra-assay coefficients of variation were <10%. Profiling of collected sera for pro-inflammatory, cytokine, chemokine, angiogenesis, and vascular injury markers was performed using the V-PLEX Human Biomarker 40-Plex Kit (MSD, Rockville, MD, USA) in a representative subset of our participants. Selected markers that are related to carotid atherosclerotic disease are presented herein (IL-4, -6, -10, -13, TNF-α, interferon-γ, macrophage inflammatory protein-1 α (MIP-1α), vascular endothelial growth factor, serum amyloid A)[503,504]. The lower limits of detection for these markers were 0.010, 0.026, 0.017, 0.292, 0.023, 0.137, 1.110, 0.369, and 19.100 pg/mL, respectively. Inter-assay coefficients of variation were <15%, while intra-assay coefficients of variation were <7%.

3.3.3 Surgical specimens

Human carotid atherosclerotic plaques were obtained immediately after surgical resection. Plaques were dissected into transverse segments of approximately 3-4 mm in thickness, fixed in 10% formalin, decalcified (Surgipath Decalcifier I, Leica Microsystems Inc., Buffalo Grove, IL, USA), and then processed for paraffin embedment, as previously described[108].

3.3.4 Histological characterization of carotid atherosclerotic plaques

The plaque segment with the area of maximum stenosis or with the largest plaque burden (if different from the area of maximum stenosis) was used for histological analysis. A 4-µm section from this segment was stained with hematoxylin and eosin. In addition, four other consecutive 4-µm sections were immunostained for markers of lymphocytes, macrophages, SMCs, and neovascularization (CD3, CD68, α-SMC actin, von Willebrand factor, respectively). Two vascular pathologists (JV and CL) independently performed the histological assessments of the plaque specimens. In case of disagreement, a consensus was reached. The pathologists were blinded for

patient clinical information and study outcomes. The plaques were characterized according to two gold-standard classifications of plaque instability: 1) AHA histological classification by Stary *et al*[176], and 2) semi-quantitative scale by Lovett *et al*[177,178]. The AHA classification ranges from Type I, an initial endothelial lesion, to Type VIII, a predominantly fibrotic plaque[176]. Type VI is the most complicated lesion, which is characterized by the presence of a hematoma, haemorrhage, or thrombus. Clinical outcomes are most often associated with type V and type VI plaques, while plaques beyond type VI represent more fibrotic and calcific lesions that mainly occur following lipid regression. The specimens obtained from the CEAs were all representative of advanced atherosclerotic lesions (Type V-VIII). According to the semi-quantitative scale, individual features of the plaque were classified on a 3- or 4-grade scale, including presence/absence of intraplaque haemorrhage, thrombus, foam cells, lipid core size, proportion of fibrous tissue, neovascularization, calcification, cap rupture, overall inflammation, and infiltration of the fibrous cap with inflammatory cells (inflammation graded according to the number of macrophages and lymphocytes present)[177,178]. Based on the presence of a combination of these features, the overall instability of the plaque was characterized as definitely stable, probably stable, probably unstable, or definitely unstable. Generally, unstable plaques were either ruptured or rupture-prone, with a large lipid core and thin inflamed cap, while stable plaques had a thick fibrous cap, which protects them from rupturing, and small or no lipid core. **Table 3.1** summarizes the prevalence of histological features of the plaque according to plaque instability.

3.3.5 Statistical analyses

The definitely stable and probably stable plaque groups, classified by the vascular pathologists, were combined and labelled as 'stable', while the probably unstable and definitely unstable plaque groups were combined and labelled as 'unstable' to be used in the statistical analyses. In addition to being clinically relevant, combining instability groups led to increased statistical power. Chi-square (χ^2), independent sample t-test (parametric test) or Mann-Whitney (non-parametric test), as appropriate, were performed to assess the differences in baseline characteristics between patients with stable and unstable plaques. Mann-Whitney was also performed to evaluate the differences in adipokine levels between patient groups based on plaque instability, AHA plaque classification, carotid artery stenosis, and cerebrovascular symptomatology, while the Kruskal-Wallis test was used to assess differences in adipokine levels among the types of cerebrovascular symptomatology. Partial Spearman rank correlation coefficients were used to assess correlations between circulating

adipokine levels, clinical variables, and inflammatory and vascular markers. Step-wise logistic regression analyses were performed to estimate the association between adipokine levels and plaque instability (stable versus unstable), carotid artery stenosis (50-79% versus 80-99%), AHA plaque classification (Type VII/VIII versus Type V), cerebrovascular symptomatology (asymptomatic versus symptomatic), and specific features of plaque instability (plaque inflammation, presence of lipid core, and cap infiltration). ORs are presented with 95% CIs. Multivariate analyses for carotid plaque instability and cerebrovascular symptomatology were presented as 6 adjusted models: a partially adjusted model for age, sex, and BMI (Model 1), a model additionally adjusted for high-sensitivity CRP and T2DM (Model 2), and models (Model 3, Model 4, Model 5, and Model 6 [fully-adjusted model]) adjusting for adiponectin, chemerin, leptin, and resistin, respectively. Traditional risk factors included in the regression models (age, sex, BMI, high-sensitivity CRP, and T2DM) were chosen based on their significance in univariate analyses, their well-established role in plaque instability and symptomatology, and their ability to affect/confound the exposure of interest (circulating adipokines). Extensive modelling was performed including various other variables, such as smoking, hypertension, hypercholesterolemia, and plasma levels of triglycerides and HDL. However, since these variables did not affect the estimates, they were excluded from the adjusted models. Thus, the risk factors included in the multivariate analyses were age, sex, BMI, high-sensitivity CRP, and T2DM.

In a sub-analysis, differences in associations between circulating adipokines, inflammatory markers, and histological parameters of instability were examined in patients who had a CEA within one month versus greater than one month from the cerebrovascular event. This was performed to evaluate whether plaque remodeling modified the overall observed associations.

All statistical analyses were performed in SPSS, Version 20 (IBM, Armonk, New York, United States). Values of $P<0.05$ (2-tailed) were considered significant. The degree of significance for the correlation matrix was adjusted using Bonferroni correction. The Bonferroni adjusted alpha level used was $P<0.003$.

Table 3.1 Prevalence of histological features of the plaque according to plaque instability[1]

Population Characteristic	Total Patients (n=165)	Patients with Stable Plaque Phenotype (n=48)	Patients with Unstable Plaque Phenotype (n=117)	*P-Value
AHA Plaque Classification				**<0.001**
Type V, %	18.2	2.1	24.8	
Type VI, %	33.9	6.2	45.3	
Type VII, %	34.5	54.2	26.5	
Type VIII, %	13.3	37.5	3.4	
Haemorrhage				**0.023**
No hemorrhage, %	80.6	93.8	75.2	
Small hemorrhage, %	18.8	6.2	23.9	
Large hemorrhage, %	0.6	0.0	0.9	
Thrombus				**<0.001**
No thrombus, %	81.2	100.0	73.5	
Small thrombus, %	17.6	0.0	24.8	
Large thrombus, %	1.2	0.0	1.7	
Lipid Core				**<0.001**
No lipid core, %	20.0	60.4	3.4	
Small lipid core, %	19.4	35.4	12.8	
Large lipid core, %	60.6	4.2	83.8	
Fibrous Tissue				**<0.001**
Very little fibrous tissue, %	5.5	0.0	7.7	
~50% fibrous tissue, %	55.2	2.1	76.9	
Predominantly fibrous, %	39.4	97.9	15.4	
Foam Cells				**<0.001**
None, %	43.6	83.3	27.4	
<50 cells, %	17.6	8.3	21.4	
At least 50 cells, %	38.8	8.3	51.3	
New Vessels				0.919
None, %	7.3	6.2	7.7	
<10 per section, %	40.6	39.6	41.0	
At least 10 per section, %	52.1	54.2	51.3	
Calcification				**0.009**
None, %	10.3	10.4	10.3	
Stippling only, %	20.0	6.2	25.6	
Calcified nodules, %	69.7	83.3	64.1	
Inflammatory Cells				**<0.001**
None, %	5.5	16.7	0.9	
Occasional cells, %	32.1	60.4	20.5	

2-5 groups of >50 cells, %	55.8	22.9	69.2	
>5 groups of >50 cells, %	6.7	0.0	9.4	
Cap Infiltration				**<0.001**
None, %	25.5	70.8	6.8	
<10 cells in cap, %	17.6	12.5	19.7	
10-50 cells in cap, %	32.1	12.5	40.2	
>50 cells in cap, %	24.8	4.2	33.3	
Rupture				**0.001**
Intact cap, %	77.6	95.8	70.1	
Probably intact, %	3.0	4.2	2.6	
Probably ruptured, %	12.7	0.0	17.9	
Definitely ruptured, %	6.7	0.0	9.4	
Overall Instability				
Definitely stable, %	13.3	-	-	
Probably stable, %	15.8	-	-	
Probably unstable, %	51.5	-	-	
Definitely unstable, %	19.4	-	-	

AHA, American Heart Association
*P-value indicates significance between patients with stable and patients with unstable plaques

3.4 Results

A total of 165 subjects were recruited. Baseline characteristics of the total study population, and of the subjects with stable and unstable plaques, separately are presented in **Tables 3.2** and **3.3**. Subjects with unstable plaques were more likely to be men (76.1%) than subjects with stable plaques (52.1%, $[\chi^2]=9.17$, $P=0.002$; **Table 3.2**). No other significant differences in terms of patient demographics, clinical variables, adipokine levels (**Table 3.2**), and levels of inflammatory and vascular markers were noted between patients with stable and unstable plaques (**Table 3.3**).

In the non-adjusted Spearman correlation model, age was positively correlated with adiponectin ($P=0.002$) and resistin levels ($P<0.001$), while BMI correlated negatively with adiponectin ($P<0.001$) and positively with leptin ($P<0.001$) (**Table 3.4A-B**). When the correlation model was adjusted for age, sex, and BMI, adiponectin was positively correlated with HDL and chemerin was positively correlated with high-sensitivity CRP and negatively with HDL (**Table 3.5A**). No significant correlations were found between levels of adiponectin and the other adipokines. However, chemerin was correlated positively with leptin and resistin (**Table 3.5B**). Correlations between circulating adipokines and inflammatory and vascular markers are reported in **Table 3.6**. Most of the above correlations were weak to moderate.

Table 3.2 Population demographic and clinical characteristics[1]

Population Characteristic	Total Patients (n=165)	Patients with Stable Plaque Phenotype (n=48)	Patients with Unstable Plaque Phenotype (n=117)	*P-Value
Age, y	69.81±9.48	69.46±8.60	69.95±9.85	0.764
Sex, % men	69.1	52.1	76.1	**0.002**
BMI, kg/m^2	26.87±4.40	27.18±4.14	26.74±4.51	0.591
Ever Smoker, %	80.2	78.7	80.9	0.756
Carotid Artery Stenosis, 50-79%/80-99%	22.6/77.4	22.9/77.1	22.4/77.6	0.944
Cerebrovascular Symptomatology, %	72.7	64.6	76.1	0.132
Amaurosis Fugax, %	20.2	23.3	19.1	0.617
Transient Ischemic Attack, %	39.5	50.0	36.0	0.174
Stroke, %	40.3	26.7	44.9	0.078
CAD, %	40.6	45.8	38.5	0.381
Systolic Blood Pressure, mmHg	138±18	139±20	137±18	0.678
Diastolic Blood Pressure, mmHg	70±10	70±8	70±11	0.875
Hypertension, %	83.6	85.4	82.9	0.692
Antihypertensive medication, %	80.0	81.2	79.5	0.797
†HbA_{1C}, %	6.00 [5.70-6.70]	6.10 [5.68-6.58]	5.95 [5.70-7.05]	0.763
T2DM, %	32.7	31.2	33.3	0.796
Antihyperglycemic medication, %	27.3	29.2	26.5	0.726
Insulin Injections, %	26.7	35.7	22.6	0.356
Biguanides, %	82.2	78.6	83.9	0.667
Sulphonylureas, %	48.9	57.1	45.2	0.457
Thiazolidinedione, %	2.2	7.1	0.0	0.132
Hypercholesterolemia, %	83.6	79.2	85.5	0.320
Statin use, %	69.1	70.8	68.4	0.756
Total cholesterol, mmol/L	3.38 [2.85-3.88]	3.41 [2.87-3.94]	3.28 [2.83-3.85]	0.509
HDL, mmol/L	0.95 [0.75-1.14]	0.93 [0.71-1.12]	0.95 [0.77-1.17]	0.870
LDL, mmol/L	1.69 [1.29-2.21]	1.64 [1.30-2.27]	1.71 [1.28-2.12]	0.799
Triglycerides, mmol/L	1.51 [1.08-1.87]	1.63 [1.28-2.08]	1.45 [1.03-1.80]	0.090
hsCRP, mg/L	1.45 [0.62-3.41]	1.27 [0.69-2.89]	1.63[0.58-3.47]	0.550
Adiponectin, µg/mL	10.40 [5.25-15.35]	10.16 [5.81-15.94]	10.42 [5.11-15.27]	0.838
Chemerin, ng/mL	209.64 [178.54-255.53]	227.17 [192.60-264.83]	206.5 [173.19-253.44]	0.100
Leptin, ng/mL	9.88 [4.55-17.05]	9.51 [4.58-23.05]	10.08 [4.45-16.08]	0.986
Resistin, ng/mL	12.53 [9.66-17.16]	11.97 [9.84-16.96]	12.59 [9.48-17.34]	0.782

Normally distributed data: values represent mean±SD; Non-normally distributed data: values represent median [interquartile range]
BMI indicates body mass index; CAD, coronary artery disease; HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; T2DM, type 2 diabetes mellitus.
*P-value indicates significance between patients with stable and patients with unstable plaques.
†Data for HbA_{1C} is reported for n=64 subjects (n=14 subjects with stable phenotype and n=50 subjects with unstable phenotype).

Table 3.3 Circulating inflammatory and vascular markers[1]

	Total Patients (n=54)	Patients with Stable Plaque Phenotype (n=22)	Patients with Unstable Plaque Phenotype (n=32)	*P-Value
IL-4 (pg/mL)	0.014 [0.004-0.022]	0.012 [0.007-0.018]	0.015 [0.002-0.025]	0.955
IL-6 (pg/mL)	0.54 [0.29-1.00]	0.35 [0.28-0.76]	0.57 [0.32-1.12]	0.238
IL-10 (pg/mL)	0.13 [0.08-0.21]	0.13 [0.09-0.19]	0.12 [0.05-0.21]	0.433
IL-13 (pg/mL)	0.28 [0.16-0.48]	0.20 [0.09-0.53]	0.29 [0.15-0.48]	0.911
TNF-α (pg/mL)	1.73 [1.39-2.03]	1.73 [1.41-2.14]	1.72 [1.24-1.95]	0.283
IFN-γ (pg/mL)	3.58 [1.81-5.77]	4.29 [1.79-5.90]	3.37 [1.81-5.65]	0.379
MIP1-α (pg/mL)	10.16 [8.01-13.16]	8.88 [7.96-12.96]	10.64 [8.24-13.62]	0.313
VEGF (pg/mL)	171.19 [99.04-379.60]	171.69 [93.89-397.47]	171.19 [103.86-366.48]	0.888
SAA (µg/mL)	3.36 [1.66-6.63]	3.16 [1.92-6.68]	3.44 [1.62-6.66]	0.958

IFN indicates interferon; IL, interleukin; MIP, macrophage inflammatory protein; SAA, serum amyloid A; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
*P-value indicates significance between patients with stable and patients with unstable plaques

When adipokine levels were analyzed according to sex and T2DM status, circulating adiponectin and leptin were significantly higher in women than in men (13.10 [7.72-18.36] µg/mL vs. 10.31 [4.74-14.02] µg/mL, *P*=0.011; 24.37 [4.19-44.11] ng/mL vs. 12.72 [4.57-14.49] ng/mL, *P*=0.038, respectively). T2DM subjects, when compared to non-diabetics, had decreased adiponectin levels (9.25 [3.77-13.28] µg/mL vs. 12.11 [6.15-17.93] µg/mL, *P*=0.007) and increased chemerin levels (250.96 [198.25-283.75] ng/mL vs. 213.32 [169.62-240.70] ng/mL, *P*=0.002). HbA_{1c} (glycated hemoglobin) levels were found to be significantly and positively associated with chemerin (r=0.345, *P*=0.005) but not adiponectin (r=-0.140, *P*=0.270). However, following adjustments for HbA_{1c}, the associations between diabetes, and adiponectin and chemerin remained significant (*P*=0.019 and *P*=0.007, respectively), suggesting that these associations are not dependent on the levels of glucose.

Table 3.4 A) Non-adjusted spearman correlation coefficients between circulating adipokines and metabolic and cardiovascular risk factors[1]

	Adiponectin		Chemerin		Leptin		Resistin	
	r	P-value	r	P-value	r	P-value	r	P-value
Age	**0.24**	**0.002**	0.16	0.038	0.04	0.625	**0.35**	**<0.001**
BMI	**-0.31**	**<0.001**	0.13	0.119	**0.35**	**<0.001**	-0.13	0.117
hsCRP	-0.15	0.055	**0.25**	**0.001**	0.15	0.058	0.17	0.025
Total Cholesterol	**0.27**	**<0.001**	-0.09	0.244	0.07	0.348	-0.13	0.096
Triglycerides	**-0.30**	**<0.001**	**0.26**	**0.001**	**0.28**	**<0.001**	0.08	0.301
HDL	**0.44**	**<0.001**	-0.23	0.003	-0.002	0.979	-0.15	0.064
LDL	0.21	0.009	-0.13	0.098	-0.01	0.913	-0.11	0.182
SBP	0.12	0.160	-0.04	0.634	0.05	0.566	0.06	0.448
DBP	-0.08	0.316	-0.20	0.016	0.04	0.660	-0.06	0.435

BMI, body mass index; hsCRP, high sensitivity C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure

Table 3.4 B) Non-adjusted spearman correlation coefficients among circulating adipokines[1]

	Adiponectin		Chemerin		Leptin		Resistin	
	r	P-value	r	P-value	r	P-value	r	P-value
Adiponectin	-	-	-0.06	0.440	-0.11	0.170	0.09	0.230
Chemerin	-0.06	0.440	-	-	0.10	0.216	**0.34**	**<0.001**
Leptin	-0.11	0.170	0.10	0.216	-	-	0.03	0.723
Resistin	0.09	0.230	**0.34**	**<0.001**	0.03	0.723	-	-

Table 3.5 A) Adjusted spearman partial correlation coefficients between circulating adipokines and metabolic and cardiovascular risk factors[1]

	Adiponectin		Chemerin		Leptin		Resistin	
	r	P-value	r	P-value	r	P-value	r	P-value
hsCRP	-0.04	0.694	**0.32**	**<0.001**	0.08	0.390	0.23	0.011
Total Cholesterol	0.15	0.102	-0.13	0.137	-0.08	0.389	-0.09	0.319
Triglycerides	-0.24	0.007	0.20	0.023	0.07	0.434	0.04	0.626
HDL	**0.32**	**<0.001**	**-0.31**	**<0.001**	-0.08	0.368	-0.20	0.026
LDL	0.14	0.118	-0.12	0.189	-0.08	0.356	-0.05	0.569
SBP	0.11	0.235	-0.05	0.541	0.01	0.899	0.04	0.676
DBP	0.06	0.500	-0.15	0.083	0.01	0.935	-0.01	0.953

DBP indicates diastolic blood pressure; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; SBP, systolic blood pressure.
Adjustments were performed for age, sex, and body mass index

Table 3.5 B) Adjusted spearman partial correlation coefficients among circulating adipokines[1]

	Adiponectin		Chemerin		Leptin		Resistin	
	r	P-value	r	P-value	r	P-value	r	P-value
Adiponectin	-	-	-0.03	0.701	-0.02	0.801	0.06	0.534
Chemerin	-0.03	0.701	-	-	**0.32**	**<0.001**	**0.53**	**<0.001**
Leptin	-0.02	0.801	**0.32**	**<0.001**	-	-	0.21	0.016
Resistin	0.06	0.534	**0.53**	**<0.001**	0.21	0.016	-	-

Adjustments were performed for age, sex, and body mass index

Table 3.6 Spearman correlation coefficients between circulating adipokines and inflammatory and vascular markers[1]

	Adiponectin		Chemerin		Leptin		Resistin	
	r	P-value	r	P-value	r	P-value	r	P-value
IL-4	0.39	0.013	0.08	0.630	0.04	0.815	0.21	0.187
IL-6	-0.16	0.242	0.28	0.040	0.28	0.042	0.17	0.234
IL-10	0.39	0.006	0.29	0.040	0.05	0.761	0.21	0.149
IL-13	-0.09	0.670	0.41	0.055	-0.03	0.909	0.51	0.013
TNF-α	-0.14	0.327	0.36	0.008	0.12	0.378	0.22	0.109
IFN-γ	-0.04	0.790	0.23	0.101	0.27	0.048	-0.25	0.071
MIP1-α	0.08	0.620	**0.50**	**0.001**	0.02	0.898	0.24	0.114
VEGF	-0.09	0.527	0.31	0.022	0.12	0.408	0.14	0.299
SAA	-0.32	0.019	0.33	0.016	0.36	0.008	0.02	0.894

IFN indicates interferon; IL, interleukin; MIP, macrophage inflammatory protein; SAA, serum amyloid A; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

3.4.1 Association of circulating adipokines and carotid plaque instability

Adiponectin, leptin, and resistin were not significantly associated with plaque instability, neither in the univariate nor in the multivariate models (**Table 3.7**). Regarding chemerin, the model accounting for age, sex, BMI, CRP, and T2DM, yielded an OR of 0.942 (95% CI: 0.895-1.000) for plaque instability per 10 ng/mL increase in chemerin levels, which corresponds to a 5.8% decrease in the odds of having an unstable plaque. The c-statistic associated with this model is 0.685. This association remained significant even following adjustments for circulating adipokine levels, increasing the c-statistic to 0.705. Furthermore, in the fully adjusted model (Model 6), there was also a significant 3.0-fold decrease in the OR for plaque instability when the highest tertile of chemerin levels was compared with the lowest (**Table 3.7**).

Table 3.7 Univariate and multivariate logistic regression analyses of carotid plaque instability (stable [n=48] vs. unstable [n=117]) for each circulating adipokine[1]

	Univariate	Multivariate					
		Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Adiponectin (µg/mL)							
Continuous	0.997 (0.948-1.049)	1.002 (0.944-1.064)	1.005 (0.944-1.068)	-	1.008 (0.947-1.072)	1.009 (0.948-1.074)	1.007 (0.945-1.073
Tertile 1	Reference	Reference	Reference	-	Reference	Reference	Reference
Tertile 2	0.863 (0.359-2.074)	1.145 (0.440-2.981)	1.059 (0.396-2.835)	-	1.595 (0.483-5.270)	1.438 (0.429-4.827)	1.388 (0.408-4.721)
Tertile 3	1.094 (0.442-2.706)	1.372 (0.498-3.783)	1.369 (0.481-3.901)	-	3.521 (0.462-26.833)	3.001 (0.385-23.410)	2.858 (0.354-23.059)
Chemerin (ng/mL)							
Continuous	0.997 (0.993-1.001)	0.997 (0.992-1.001)	**0.994 (0.989-1.000)**	**0.994 (0.989-1.000)**	-	**0.993 (0.987-0.999)**	**0.991 (0.985-0.998)**
Tertile 1	Reference	Reference	Reference	Reference	-	Reference	Reference
Tertile 2	0.637 (0.257-1.578)	0.700 (0.276-1.778)	0.662 (0.256-1.714)	0.667 (0.257-1.730)	-	0.663 (0.255-1.726)	0.667 (0.257-1.736)
Tertile 3	0.543 (0.220-1.341)	0.492 (0.191-1.269)	0.376 (0.133-1.060)	0.374 (0.133-1.057)	-	0.352 (0.122-1.013)	**0.332 (0.114-0.967)**
Leptin (ng/mL)							
Continuous	0.996 (0.977-1.016)	1.008 (0.984-1.031)	1.006 (0.982-1.029)	1.006 (0.982-1.029)	1.017 (0.992-1.043)	-	1.017 (0.990-1.044)

Tertile 1	Reference	Reference	Reference	Reference	Reference	-	Reference
Tertile 2	1.071 (0.449-2.557)	1.045 (0.428-2.550)	1.082 (0.437-2.676)	1.100 (0.440-2.750)	0.989 (0.388-2.518)	-	1.079 (0.417-2.787)
Tertile 3	1.120 (0.463-2.710)	1.504 (0.561-4.032)	1.468 (0.548-3.937)	1.491 (0.551-4.036)	1.633 (0.588-4.535)	-	1.647 (0.583-4.651)
Resistin (ng/mL)							
Continuous	1.033 (0.970-1.099)	1.028 (0.962-1.099)	1.024 (0.954-1.100)	1.024 (0.954-1.099)	1.065 (0.985-1.152)	1.062 (0.981-1.149)	-
Tertile 1	Reference	Reference	Reference	Reference	Reference	Reference	-
Tertile 2	0.680 (0.286-1.614)	0.740 (0.301-1.818)	0.772 (0.308-1.937)	0.775 (0.307-1.957)	0.809 (0.315-2.077)	0.850 (0.327-2.210)	-
Tertile 3	0.993 (0.397-2.481)	0.868 (0.328-2.299)	0.854 (0.316-2.303)	0.852 (0.315-2.300)	1.307 (0.439-3.888)	1.271 (0.424-3.806)	-

CI indicates confidence interval; OR, odds ratio.
Continuous values are presented as per unit increase
Adiponectin: Tertile 1 = 4.09 [3.25-5.25] µg/mL; Tertile 2 = 10.40 [8.54-11.76] µg/mL; Tertile 3 = 18.37 [15.24-20.78] µg/mL
Chemerin: Tertile 1 = 163.19 [140.77-178.65] ng/mL; Tertile 2 = 209.64 [200.91-226.79] ng/mL; Tertile 3 = 282.21 [255.12-346.24] ng/mL
Leptin: Tertile 1 = 3.74 [2.81-4.58] ng/mL; Tertile 2 = 9.88 [7.95-12.08] ng/mL; Tertile 3 = 31.47 [16.57-47.44] ng/mL
Resistin: Tertile 1 = 8.11 [6.60-9.72]; Tertile 2 = 12.53 [11.56-13.37] ng/mL; Tertile 3 = 19.44 [17.14-24.21] ng/mL
Model 1 = Adjusted for age, sex, and body mass index
Model 2 = Model 1 + type 2 diabetes mellitus, and high-sensitivity C-reactive protein
Model 3 = Model 2 + adiponectin
Model 4 = Model 3 + chemerin
Model 5 = Model 4 + leptin
Model 6 = Model 5 + resistin

No significant associations were found between adipokine levels and AHA plaque classification (Type V vs. Type VI vs. Type VII vs. Type VIII) (**Table 3.8**). However, decreased chemerin levels increased the odds of having a Type V (fibroatheroma with a large lipid core) versus a Type VII/Type VIII (predominantly fibrotic/calcific) plaque following adjustments in Model 1 (**Table 3.9**). This association remained significant after additional adjustments for adiponectin, leptin and resistin levels (**Table 3.9**).

Table 3.8 Circulating adipokine levels between Type V, Type VI, Type VII, and Type VIII plaques according to the American Heart Association classification[1]

	AHA Plaque Classification (n=165)				
	Type V (n=31)	**Type VI (n=56)**	**Type VII (n=56)**	**Type VIII (n=22)**	***P-value**
Adiponectin (µg/mL)	9.70 [4.95-14.80]	9.48 [4.97-14.98]	11.73 [5.84-17.73]	10.34 [4.50-14.67]	0.455
Chemerin (ng/mL)	198.29 [152.91-236.26]	210.35 [179.70-271.63]	222.43 [181.12-254.42]	200.25 [175.19-240.11]	0.251
Leptin (ng/mL)	7.09 [4.51-13.10]	10.60 [4.92-27.26]	9.98 [3.58-16.44]	12.77 [5.15-28.48]	0.535
Resistin (ng/mL)	12.53 [9.96-17.14]	12.53 [9.14-16.20]	12.52 [9.82-18.37]	13.56 [8.58-18.05]	0.917

AHA, American Heart Association
*P-value indicates significance among all AHA classifications

Table 3.9 Univariate and multivariate logistic regression analyses for the association of chemerin with the American Heart Association plaque classification (fibrotic/calcific plaques [Type VII/VIII] [n=78] vs. fibroatheroma plaques [Type V] [n=31])[1]

	Univariate	**Multivariate**			
		Model 1	**Model 2**	**Model 3**	**Model 4**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Chemerin (ng/mL)					
Continuous	0.993 (0.986-1.001)	**0.991 (0.982-0.999)**	**0.991 (0.982-0.999)**	**0.991 (0.982-0.999)**	**0.991 (0.982-1.000)**
Tertile 1	Reference	Reference	Reference	Reference	Reference
Tertile 2	0.833 (0.301-2.309)	0.937 (0.313-2.803)	0.903 (0.298-2.742)	0.848 (0.275-2.614)	0.848 (0.275-2.616)
Tertile 3	0.436 (0.132-1.440)	0.270 (0.071-1.033)	0.287 (0.075-1.099)	0.284 (0.073-1.107)	0.292 (0.073-1.165)

OR, odds ratio; CI, confidence interval
Tertile 1: 163.19 [140.77-178.65], Tertile 2: 209.64 [200.91-226.79], Tertile 3: 282.21 [255.12-346.24]
Model 1 = Adjusted for age, sex, and body mass index
Model 2 = Model 1 + adiponectin
Model 3 = Model 2 + leptin
Model 4 = Model 3 + resistin

Adipokine levels were not associated with the degree of carotid artery stenosis (**Table 3.10**) or with plaque inflammation (data not shown). However, in the multivariate logistic regression models (Models 1-6), increased leptin levels were significantly associated with lipid core presence and with increased presence of inflammatory cell cap infiltration (**Table 3.10**).

Table 3.10 Univariate and multivariate logistic regression analyses for the association of adipokine levels with cerebrovascular symptomatology (asymptomatic [n=46] vs. symptomatic [n=119]), carotid artery stenosis (moderate-grade [50-79%] [n=37] vs. high-grade [80-99%] [n=127] stenosis), and features of plaque instability (no lipid core presence [n=33] vs. lipid core presence [n=132] and low cap infiltration [n=71] vs. high cap infiltration [n=94])[1]

	Symptomatology	Carotid Artery Stenosis	Lipid Core Presence	High Cap Infiltration
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Adiponectin				
Univariate	1.006 (0.955-1.059)	0.976 (0.925-1.030)	1.007 (0.951-1.066)	1.044 (0.993-1.097)
Model 1	0.966 (0.907-1.029)	0.988 (0.930-1.050)	1.019 (0.951-1.092)	1.028 (0.971-1.088)
Model 2	*0.969 (0.908-1.034)	†0.988 (0.929-1.050)	‡1.035 (0.961-1.114)	§1.033 (0.973-1.098)
Model 3	-	-	-	-
Model 4	0.969 (0.908-1.034)	0.987 (0.929-1.049)	1.033 (0.960-1.112)	1.033 (0.972-1.098)
Model 5	0.968 (0.906-1.034)	0.987 (0.929-1.049)	1.034 (0.960-1.114)	1.037 (0.976-1.103)
Model 6	0.967 (0.904-1.033)	0.987 (0.929-1.049)	1.034 (0.960-1.114)	1.037 (0.975-1.103)
Chemerin				
Univariate	1.002 (0.997-1.007)	1.001 (0.996-1.005)	1.000 (0.995-1.005)	1.000 (0.996-1.004)
Model 1	1.002 (0.997-1.006)	1.001 (0.996-1.006)	1.000 (0.995-1.005)	1.000 (0.996-1.004)
Model 2	*1.000 (0.995-1.006)	†1.001 (0.996-1.007)	‡0.997 (0.991-1.003)	§1.000 (0.995-1.006)
Model 3	1.000 (0.995-1.006)	1.001 (0.996-1.007)	0.997 (0.992-1.003)	1.000 (0.995-1.005)
Model 4	-	-	-	-
Model 5	1.002 (0.996-1.008)	1.001 (0.996-1.007)	0.995 (0.988-1.002)	0.998 (0.992-1.003)
Model 6	1.001 (0.995-1.008)	1.002 (0.996-1.008)	0.995 (0.988-1.003)	0.998 (0.992-1.004)
Leptin				
Univariate	1.000 (0.980-1.021)	1.006 (0.983-1.030)	1.012 (0.986-1.038)	1.013 (0.993-1.033)
Model 1	0.990 (0.966-1.015)	1.002 (0.974-1.031)	**1.038 (1.005-1.072)**	**1.031 (1.005-1.059)**
Model 2	*0.987 (0.963-1.012)	†1.002 (0.974-1.031)	‡**1.033 (1.000-1.067)**	§**1.032 (1.004-1.060)**
Model 3	0.988 (0.964-1.012)	1.002 (0.974-1.031)	**1.034 (1.000-1.068)**	**1.032 (1.005-1.061)**
Model 4	0.984 (0.957-1.011)	1.001 (0.972-1.031)	**1.036 (1.003-1.070)**	**1.036 (1.007-1.065)**
Model 5	-	-	-	-
Model 6	0.982 (0.955-1.010)	1.002 (0.972-1.032)	**1.037 (1.003-1.071)**	**1.036 (1.007-1.065)**
Resistin				
Univariate	1.048 (0.980-1.120)	0.978 (0.925-1.035)	1.022 (0.956-1.094)	1.031 (0.976-1.089)
Model 1	1.024 (0.960-1.092)	0.991 (0.934-1.052)	1.015 (0.944-1.091)	1.013 (0.959-1.071)
Model 2	*1.015 (0.945-1.091)	†0.995 (0.936-1.058)	‡0.998 (0.922-1.081)	§1.022 (0.955-1.093)
Model 3	1.018 (0.946-1.094)	0.996 (0.936-1.058)	0.997 (0.920-1.080)	1.020 (0.953-1.091)
Model 4	1.018 (0.941-1.103)	0.984 (0.917-1.056)	1.010 (0.928-1.100)	1.021 (0.950-1.097)
Model 5	1.027 (0.947-1.114)	0.983 (0.916-1.056)	0.992 (0.906-1.087)	1.006 (0.936-1.080)
Model 6	-	-	-	-

OR, odds ratio; CI, confidence interval
ORs from logistic regression analysis per unit increase in adipokine levels
Model 1 = Adjusted for age, sex, and body mass index
Model 2 = *Model 1 + type 2 diabetes mellitus, and high-sensitivity C-reactive protein
†Model 1 + high-sensitivity C-reactive protein
‡Model 1 + high-sensitivity C-reactive protein
§Model 1 + type 2 diabetes mellitus, high-sensitivity C-reactive protein, and coronary artery disease
Model 3 = Model 2 + adiponectin
Model 4 = Model 3 + chemerin
Model 5 = Model 4 + leptin
Model 6 = Model 5 + resistin

3.4.2 Association of circulating adipokines and cerebrovascular symptomatology

In the total population, symptomatic subjects were significantly older (71.49±9.74 vs. 65.31±7.06 years, *P*<0.001) and had higher CRP levels (3.63 [0.69-3.92] vs. 1.91 [0.48-2.00] mg/L, *P*=0.021) than asymptomatic subjects. In addition, there was a strong trend toward significance for higher resistin levels in symptomatic (12.87 [9.96-17.87] ng/mL) versus asymptomatic subjects (11.16 [7.80-15.76] ng/mL; *P*=0.056; **Table 3.11** and **3.12**). No association was noted between adipokine levels and cerebrovascular symptomatology in the univariate and multivariate regression analyses in the total population (**Table 3.10**). However, among subjects with T2DM, resistin levels were significantly elevated in symptomatic versus asymptomatic subjects (*P*=0.001; **Table 3.11**) and increased the risk of cerebrovascular symptomatology by 29% (95% CI: 1.072-1.561; **Table 3.13**). Resistin remained significantly associated with cerebrovascular symptomatology (OR: 1.264, 95% CI: 1.004-1.594), in subjects with T2DM but not in subjects without T2DM, even following adjustments for age, sex, BMI, and chemerin levels (**Table 3.13**). However, significance was lost after additional adjustment for leptin (**Table 3.13**). Furthermore, resistin levels were also noted to be marginally associated with types of cerebrovascular events (P=0.05; **Table 3.12**), particularly in subjects without T2DM, with highest levels present in patients who suffered from a stroke and lowest levels in those who had an AF (P=0.014; **Table 3.12**).

When comparing patients who underwent CEA within one month (14.0 [7.0-21.0] days), versus greater than one month (63.0 [42.0-100.5] days*)* from the cerebrovascular event, no significant differences were observed between circulating adipokines, inflammatory markers, and histological parameters of instability (data not shown).

Table 3.11 Circulating resistin levels in relation to cerebrovascular symptomatology[1]

	Symptomatic Status		
	Asymptomatic (n=46)	**Symptomatic (n=119)**	***P-value**
Resistin (ng/mL)			
Total Subjects (n=165)	11.16 [7.80-15.76]	12.87 [9.96-17.87]	0.056
With T2DM (n=54)	10.43 [7.15-12.88]	14.21 [11.13-20.39]	0.001
Without T2DM (n=111)	12.76 [8.45-16.69]	12.49 [9.42-16.60]	0.936

T2DM indicates type 2 diabetes mellitus

*P-value indicates significance between asymptomatic and symptomatic subjects

Table 3.12 Circulating resistin levels in relation to types of cerebrovascular symptomatology[1]

	Types of Symptomatology			
	AF (n=24)	**TIA (n=47)**	**Stroke (n=48)**	***P-value**
Resistin (ng/mL)				
Total Subjects (n=119)	10.74 [9.10-15.37]	12.52 [10.21-18.14]	13.87 [11.45-25.37]	0.054
With T2DM (n=37)	14.24 [10.63-23.48]	13.11 [10.71-21.90]	14.19 [12.72-20.01]	0.887
Without T2DM (n=82)	9.80 [7.79-12.82]	12.52 [9.48-17.14]	†13.59 [10.22-19.28]	0.050

AF indicates amaurosis fugax; T2DM, type 2 diabetes mellitus; TIA, transient ischemic attack
*P-value indicates significance among all types of cerebrovascular symptomatology
†Tukey's multiple comparison test demonstrates a significance among resistin levels between non-diabetics with AF and with stroke (P=0.014)

Table 3.13 Univariate and multivariate logistic regression analyses for the association of resistin with cerebrovascular symptomatology (asymptomatic vs. symptomatic) in subjects with or without type 2 diabetes mellitus[1]

	Univariate	**Multivariate**		
		Model 1	**Model 2**	**Model 3**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Resistin (per ng/mL increase)				
With T2DM (n=54)	**1.293 (1.072-1.561)**	**1.260 (1.034-1.534)**	**1.265 (1.004-1.594)**	1.251 (0.989-1.583)
Without T2DM (n=111)	0.991 (0.927-1.059)	0.979 (0.914-1.049)	0.973 (0.894-1.059)	0.978 (0.898-1.065)

CI indicates confidence interval; OR, odds ratio; T2DM, type 2 diabetes mellitus.
Model 1 = Adjusted for age, sex, and body mass index
Model 2 = Model 1 + chemerin
Model 3 = Model 2 + leptin

3.5 Discussion

In this study including subjects with moderate- to high-grade carotid artery stenosis scheduled for CEA, we observed that low plasma chemerin levels were associated with increased odds of having an unstable carotid plaque. This association was independent of potential cardiovascular and metabolic confounders. Although not associated with overall plaque instability, leptin levels were increased with the presence of specific histological instability features. Furthermore, in T2DM subjects, high resistin levels were significantly associated with cerebrovascular symptomatology. In contrast, no such associations were observed with the anti-inflammatory protein, adiponectin.

3.5.1 Dual action of chemerin

Chemerin is a pro-inflammatory adipokine, whose circulating levels are associated with inflammation and the metabolic syndrome[504-506]. In line with others, we found high chemerin levels to correlate with T2DM and low HDL[505,506]. Furthermore, a positive correlation was observed between chemerin and MIP-1α, which is known to be involved in atherogenesis[504]. Although chemerin is less studied than other adipokines in atherosclerosis, a positive and independent association was noted between chemerin and CAD[507]. Furthermore, a recent study by Zhao *et al*. demonstrated in a Chinese population, that subjects with acute ischemic stroke had higher circulating levels of chemerin than those without stroke, and that high levels of chemerin were associated with carotid plaque instability[508]. However, in this smaller study (n=70), carotid plaque instability was determined using Doppler ultrasound. On the other hand, our study, which used the gold-standard histological plaque classifications[176-178], is the first to show an independent inverse association between circulating chemerin and carotid plaque instability in CEA subjects with severe carotid stenosis, and no significant association with cerebrovascular symptomatology. Although this finding seems to be in contrast with our hypothesis and with previous evidence[507,508], it could be partly explained by the dual action (pro-inflammatory and anti-inflammatory) of chemerin. In addition to adipose tissue, chemerin is also expressed by a number of cell types involved in innate and adaptive immunity and serves as a chemoattractant promoting recruitment of immune cells to sites of injury[509]. However, beyond its pro-inflammatory role, chemerin also possesses anti-inflammatory properties, by inhibiting the production of inflammatory mediators and preventing monocyte adhesion to the vascular endothelium[510]. Chemerin requires cleavage by serine (secreted by neutrophils) or cysteine proteases (released by activated macrophages) to generate a potent and active pro-inflammatory or anti-inflammatory peptide, respectively[510,511]. Since neutrophils are the first acting cells at sites of injury or infection, it is likely that pro-inflammatory chemerin is generated prior to its anti-inflammatory counterpart. Plaque instability is governed by an increased presence of activated macrophages[439]. Thus, anti-inflammatory chemerin may be produced to control the severity of inflammatory responses associated with unstable plaques, while pro-inflammatory chemerin may be involved in the initial formation and development of atherosclerotic plaques.

3.5.2 Leptin's pro-inflammatory effects on vascular cells

Leptin has been demonstrated in experimental studies to play a pathogenic role in atherosclerotic plaque development[246]. Nevertheless, several large prospective epidemiological studies have either demonstrated moderate associations between leptin and coronary heart disease or failed to show an association with incident cardiovascular events[233,512]. Herein, although circulating leptin levels were not significantly associated with cerebrovascular symptomatology, we reported a positive association between leptin levels and lipid core presence and increased inflammatory cell cap infiltration. In support of this finding, leptin can lead to the up-regulation of inflammatory markers, as well as induce the expression of endothelial adhesion molecules, which promote the recruitment, adhesion, and transmigration of leukocytes into the vascular wall[513]. Furthermore, leptin can facilitate the formation of lipid-laden foam cells, which are major contributors of the lipid core[514].

3.5.3 Proatherogenic actions of resistin

Evidence links elevated resistin levels with atherosclerotic disease. Not only is resistin positively associated with severity of CAD and calcification, but can also independently predict major cardiovascular events, including ischemic stroke and cardiovascular mortality[231,515-517]. Herein, as expected, we identified a significant and independent association between increased resistin levels and cerebrovascular symptomatology. However, this relationship was observed only among T2DM subjects. The positive link between resistin and T2DM has previously been suggested[518-520], where T2DM has been reported to strengthen the association between increased ischemic stroke risk and high resistin levels[521,522]. Resistin was marginally associated with types of cerebrovascular events, particularly in subjects without T2DM and not in subjects with T2DM, where differences were driven by the AF group, with highest resistin levels present in stroke subjects and lowest in patients who suffered from an AF. Considering that we are the first to report resistin levels among subjects who have suffered an AF, it is difficult to explain the differences observed among diabetics and non-diabetics. Since resistin is secreted by macrophages in atherosclerotic plaques, it mediates its pro-atherogenic effects directly on various cells present in the arterial wall by promoting the expression of inflammatory cytokines and adhesion molecules in the endothelium[523-525].

3.5.4 Adiponectin's vasculoprotective properties

Adiponectin plays an important role in lipid and glucose metabolism, improving insulin sensitivity[251]. Furthermore, adiponectin is involved in inflammatory regulation and possesses anti-atherosclerotic and vasculoprotective properties[365,366]. In this context, we found an inverse association between adiponectin and BMI, and detected lower levels of circulating adiponectin in T2DM versus non-T2DM subjects. Although we expected to observe a similar association between adiponectin and plaque instability in our patient population, as reported in two smaller studies ($n \leq 50$)[281,526], we instead noted no differences in adiponectin levels between patients with stable and unstable carotid plaques. Sawada *et al.* suggested that low adiponectin was the strongest predictor of thin-cap fibroatheroma in men with CAD[281], while *Shido et el.* showed that subjects with vulnerable carotid plaques had significantly lower adiponectin compared to subjects with stable plaques[526]. In both studies, plaque vulnerability was assessed by imaging methods, and not by using the 'gold-standard' histological classifications as in our study. In the circulation, adiponectin forms a trimer, a hexamer, and a HMW isoform; the latter is suggested to be the biologically active form, and thus, its measurement may provide a more valuable assessment of the association between adiponectin and plaque instability[444]. Nonetheless, there are several studies that have failed to demonstrate the superiority of HMW over total adiponectin in predicting insulin resistance or atherosclerotic disease[527,528].

Along with others, we also detected no significant differences in adiponectin levels between symptomatic versus asymptomatic subjects with moderate- to high-grade carotid artery stenosis[529,530]. Interestingly, it was recently reported that perivascular adiponectin expression, rather than circulating adiponectin, is significantly associated with symptomatology in patients undergoing CEA. PVAT surrounding symptomatic carotid plaques expressed 1.9-fold higher levels of adiponectin than asymptomatic plaques[530]. This suggests that it may be essential to study adiponectin's local tissue expression rather than its circulating levels in the context of carotid plaque instability and symptomatology.

3.5.5 Traditional concept of plaque vulnerability

The definition of plaque instability used herein, where macrophage-rich plaques characterized by a thin fibrous cap and large lipid-rich core are associated with plaque rupture and subsequent ischemic events, is increasingly becoming recognized as the 'traditional' concept of vulnerability. This is due to evidence demonstrating changes in the morphology and phenotype of the plaque

over the past decade[180,181]. Interestingly, atherosclerotic plaques obtained from recent CEAs were observed to possess more stable features, such as more fibrous tissue and less inflammation, than plaques obtained over a decade ago, despite the significant increase in the proportion of CEAs performed on symptomatic subjects[181]. These changes are partly believed to be associated with improvements in risk factor management. In our own study, plaque instability was not associated with cerebrovascular symptomatology; a relatively high proportion of stable plaques were found to be symptomatic (64.6%), only about 10% lower than unstable plaques (76.1). This cannot be attributed to the timing of the CEA, given that the vast majority of the subjects (60.7%) underwent a CEA within the first month after the event (14.0 [7.0-21.0] days). Of note, most subjects who had symptomatic stable plaques experienced symptoms caused by a TIA, while a greater majority of subjects who had symptomatic unstable plaques suffered from a stroke rather than a TIA. Nevertheless, it is important to keep in mind for future studies that the current concept of plaque vulnerability may need to evolve with the recent observed changes in the histopathologic features of plaques.

3.5.6 Limitations

Our study has certain limitations. Due to the cross-sectional design of this study, the direction of causality between the circulating adipokines and plaque instability or cerebrovascular symptomatology cannot be inferred. However, since specimens are necessary for the assessment of plaque instability, we need to obtain tissue from patients with moderate- to high-grade carotid artery stenosis, which can only be obtained at a single time-point, at the time of CEA. While we acknowledge that the lack of prospective data limits the establishment of the true diagnostic potential of circulating chemerin and resistin in predicting plaque instability or stroke risk, our study acts as the first phase (proof-of-concept phase) in the evaluation of novel biomarkers.

Thus, in the future, it would be necessary to perform prospective studies following patients with early carotid plaques to determine whether circulating adipokines are associated with the development of unstable carotid plaques. Furthermore, in our study we only assessed the independence of individual adipokines in predicting plaque instability and cerebrovascular symptomatology. However, the identification of a panel of markers may provide greater predictive value and reliability than a single marker[499,500]. Secondly, although not the aim of our study, we did not provide any insights into the biological significance of the adipokines in question, in relation to atherosclerotic lesions, which would have greatly increased the impact of the study.

However, our next steps will focus on addressing potential mechanisms in a future experimental study. Lastly, the semi-quantitative analysis of the carotid plaques as stable or unstable is a limitation of the study. However, Stary and Lovett's histological classifications[176-178] were used since they are validated scales that are widely established and represent the gold-standard for classifying the instability of atherosclerotic plaques. Classifications using quantitative image analysis are promising methods for categorization of plaque instability, however, they need to be validated for wide-spread use against the gold-standard methods used herein.

3.6 Conclusion

To the best of our knowledge, this study including a large number of CEAs was the first to assess the relationship between circulating adipokine levels and carotid plaque instability as evaluated via 'gold standard' histological classifications. Our study highlights an association between chemerin and plaque instability in CEA subjects with moderate- to high-grade carotid artery stenosis, while high leptin levels were associated with certain histological instability features. Although no relationship was observed between resistin and plaque instability, among T2DM subjects, high resistin levels were associated with cerebrovascular symptomatology. Future studies are needed to elucidate the additive role of chemerin and resistin to that of traditional risk factors and the degree of carotid artery stenosis in the prediction of plaque instability and in the development of cerebrovascular symptomatology. Furthermore, assessment of the local plaque expression of these adipokines may provide evidence on the human pathophysiological mechanisms linking adipokines and plaque instability.

3.7 Acknowledgments

We are grateful to the vascular surgeons (Drs. Steinmetz, Mackenzie, Corriveau, and O'Brand) at the McGill University Health Centre and the Jewish General Hospital, as well as Yessica Haydee-Gomez for her help with subject recruitment. Furthermore, we would like to thank Dr. Jean-François Cailhier and Dr. Patrick Laplante at the Centre de Recherche du Centre hospitalier de l'Université de Montréal for their assistance with the cytokine/chemokine profiling of collected sera using the V-PLEX Human Biomarker 40-Plex Kit. We would also like to thank Dr. Elham Rahme (Research Institute of the McGill University Health Centre) for her statistical advice, and Dr. Huaien Zheng and Dr. Robert J. Doonan for their assistance with the histological staining of the carotid plaque specimens.

3.8 Funding Sources

The study was funded by a Fonds de la recherche en santé du Québec (FRSQ) grant (#20058). SS Daskalopoulou (Chercheur-Boursier Clinicien) is supported by FRSQ. K Gasbarrino is supported by a Canadian Institutes of Health Research Doctoral Studentship. The funding sources had no involvement in the study.

3.9 Disclosures

None

3.10 References

93. Shah PK. Mechanisms of plaque vulnerability and rupture. *J Am Coll Cardiol*. 2003:41:15S-22S.
108. Doonan RJ, Hafiane A, Lai C, Veinot JP, Genest J, Daskalopoulou SS. Cholesterol efflux capacity, carotid atherosclerosis, and cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2014:34:921-926.
150. Barnett HJM, Taylor DW, Haynes RB, Sackett DL, Peerless SJ, Ferguson GG, Fox AJ, Rankin RN, Hachinski VC, Wiebers DO, Eliasziw M. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. *N Engl J Med*. 1991:325:445-453.
151. Halliday A, Mansfield A, Marro J, Peto C, Peto R, Potter J, Thomas D. Prevention of disabling and fatal strokes by successful carotid endarterectomy in patients without recent neurological symptoms: Randomised controlled trial. *Lancet*. 2004:363:1491-1502.
174. Saam T, Underhill HR, Chu B, Takaya N, Cai J, Polissar NL, Yuan C, Hatsukami TS. Prevalence of american heart association type vi carotid atherosclerotic lesions identified by magnetic resonance imaging for different levels of stenosis as measured by duplex ultrasound. *J Am Coll Cardiol*. 2008:51:1014-1021.
176. Stary HC. Natural history and histological classification of atherosclerotic lesions: An update. *Arterioscler Thromb Vasc Biol*. 2000:20:1177-1178.
177. Lovett JK, Gallagher PJ, Hands LJ, Walton J, Rothwell PM. Histological correlates of carotid plaque surface morphology on lumen contrast imaging. *Circulation*. 2004:110:2190-2197.
178. Lovett JK, Gallagher PJ, Rothwell PM. Reproducibility of histological assessment of carotid plaque: Implications for studies of carotid imaging. *Cerebrovasc Dis*. 2004:18:117-123.
180. Libby P, Pasterkamp G. Requiem for the 'vulnerable plaque'. *Eur Heart J*. 2015:36:2984-2987.
181. van Lammeren GW, den Ruijter HM, Vrijenhoek JE, van der Laan SW, Velema E, de Vries JP, de Kleijn DP, Vink A, de Borst GJ, Moll FL, Bots ML, Pasterkamp G. Time-dependent changes in atherosclerotic plaque composition in patients undergoing carotid surgery. *Circulation*. 2014:129:2269-2276.
231. Muse ED, Feldman DI, Blaha MJ, Dardari ZA, Blumenthal RS, Budoff MJ, Nasir K, Criqui MH, Cushman M, McClelland RL, Allison MA. The association of resistin with cardiovascular disease in the multi-ethnic study of atherosclerosis. *Atherosclerosis*. 2015:239:101-108.

233. Sattar N, Wannamethee G, Sarwar N, Chernova J, Lawlor DA, Kelly A, Wallace AM, Danesh J, Whincup PH. Leptin and coronary heart disease: Prospective study and systematic review. *J Am Coll Cardiol*. 2009:53:167-175.
246. Maresca F, Di Palma V, Bevilacqua M, Uccello G, Taglialatela V, Giaquinto A, Esposito G, Trimarco B, Cirillo P. Adipokines, vascular wall, and cardiovascular disease: A focused overview of the role of adipokines in the pathophysiology of cardiovascular disease. *Angiology*. 2015:66:8-24.
251. Nigro E, Scudiero O, Monaco ML, Palmieri A, Mazzarella G, Costagliola C, Bianco A, Daniele A. New insight into adiponectin role in obesity and obesity-related diseases. *Biomed Res Int*. 2014:2014:658913.
265. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y, Osaka CADSGCad. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol*. 2003:23:85-89.
280. Reynolds HR, Buyon J, Kim M, Rivera TL, Izmirly P, Tunick P, Clancy RM. Association of plasma soluble e-selectin and adiponectin with carotid plaque in patients with systemic lupus erythematosus. *Atherosclerosis*. 2010:210:569-574.
281. Sawada T, Shite J, Shinke T, Otake H, Tanino Y, Ogasawara D, Kawamori H, Kato H, Miyoshi N, Yoshino N, Kozuki A, Hirata K. Low plasma adiponectin levels are associated with presence of thin-cap fibroatheroma in men with stable coronary artery disease. *Int J Cardiol*. 2010:142:250-256.
365. Ohashi K, Ouchi N, Matsuzawa Y. Anti-inflammatory and anti-atherogenic properties of adiponectin. *Biochimie*. 2012:94:2137-2142.
366. Ebrahimi-Mamaeghani M, Mohammadi S, Arefhosseini SR, Fallah P, Bazi Z. Adiponectin as a potential biomarker of vascular disease. *Vasc Health Risk Manag*. 2015:11:55-70.
402. Rubio-Guerra AF, Cabrera-Miranda LJ, Vargas-Robles H, Maceda-Serrano A, Lozano-Nuevo JJ, Escalante-Acosta BA. Correlation between levels of circulating adipokines and adiponectin/resistin index with carotid intima-media thickness in hypertensive type 2 diabetic patients. *Cardiology*. 2013:125:150-153.
439. Lee RT, Libby P. The unstable atheroma. *Arterioscler Thromb Vasc Biol*. 1997:17:1859-1867.
444. Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, Imai Y, Nagai R, Kadowaki T. Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. *Diabetes Care*. 2006:29:1357-1362.
497. Daskalopoulou SS, Rabi DM, Zarnke KB et.al. The 2015 canadian hypertension education program recommendations for blood pressure measurement, diagnosis, assessment of risk, prevention, and treatment of hypertension. *Can J Cardiol*. 2015:31:549-568.
499. Ammirati E, Moroni F, Norata GD, Magnoni M, Camici PG. Markers of inflammation associated with plaque progression and instability in patients with carotid atherosclerosis. *Mediators Inflamm*. 2015:2015:718329.
500. Avgerinos ED, Kadoglou NP, Moulakakis KG, Giannakopoulos TG, Liapis CD. Current role of biomarkers in carotid disease: A systematic review. *Int J Stroke*. 2011:6:337-345.
501. Committee CDACPGE. Canadian diabetes association 2013 clinical practice guidelines for the prevention and management of diabetes in canada. *Can J Diabetes*. 2013:37 S1-S212.

502. Hwang JJ, Chan JL, Ntali G, Malkova D, Mantzoros CS. Leptin does not directly regulate the pancreatic hormones amylin and pancreatic polypeptide: Interventional studies in humans. *Diabetes Care*. 2008:31:945-951.
503. Ait-Oufella H, Taleb S, Mallat Z, Tedgui A. Recent advances on the role of cytokines in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2011:31:969-979.
504. Tedgui A, Mallat Z. Cytokines in atherosclerosis: Pathogenic and regulatory pathways. *Physiol Rev*. 2006:86:515-581.
505. Lehrke M, Becker A, Greif M, Stark R, Laubender RP, von Ziegler F, Lebherz C, Tittus J, Reiser M, Becker C, Goke B, Leber AW, Parhofer KG, Broedl UC. Chemerin is associated with markers of inflammation and components of the metabolic syndrome but does not predict coronary atherosclerosis. *Eur J Endocrinol*. 2009:161:339-344.
506. Li Y, Shi B, Li S. Association between serum chemerin concentrations and clinical indices in obesity or metabolic syndrome: A meta-analysis. *PLoS One*. 2014:9:e113915.
507. Xiaotao L, Xiaoxia Z, Yue X, Liye W. Serum chemerin levels are associated with the presence and extent of coronary artery disease. *Coron Artery Dis*. 2012:23:412-416.
508. Zhao D, Bi G, Feng J, Huang R, Chen X. Association of serum chemerin levels with acute ischemic stroke and carotid artery atherosclerosis in a chinese population. *Med Sci Monit*. 2015:21:3121-3128.
509. Hart R, Greaves DR. Chemerin contributes to inflammation by promoting macrophage adhesion to vcam-1 and fibronectin through clustering of vla-4 and vla-5. *J Immunol*. 2010:185:3728-3739.
510. Cash JL, Hart R, Russ A, Dixon JP, Colledge WH, Doran J, Hendrick AG, Carlton MB, Greaves DR. Synthetic chemerin-derived peptides suppress inflammation through chemr23. *J Exp Med*. 2008:205:767-775.
511. Yoshimura T, Oppenheim JJ. Chemerin reveals its chimeric nature. *J Exp Med*. 2008:205:2187-2190.
512. Martin SS, Blaha MJ, Muse ED, Qasim AN, Reilly MP, Blumenthal RS, Nasir K, Criqui MH, McClelland RL, Hughes-Austin JM, Allison MA. Leptin and incident cardiovascular disease: The multi-ethnic study of atherosclerosis (mesa). *Atherosclerosis*. 2015:239:67-72.
513. Adya R, Tan BK, Randeva HS. Differential effects of leptin and adiponectin in endothelial angiogenesis. *J Diabetes Res*. 2015:2015:648239.
514. Maya-Monteiro CM, Almeida PE, D'Avila H, Martins AS, Rezende AP, Castro-Faria-Neto H, Bozza PT. Leptin induces macrophage lipid body formation by a phosphatidylinositol 3-kinase- and mammalian target of rapamycin-dependent mechanism. *J Biol Chem*. 2008:283:2203-2210.
515. Sinan UY, Canbolat IP, Baydar O, Oktay V, Imre G, Kocas C, Abaci O, Coskun U, Bostan C, Kilickesmez KO, Yildiz A, Kaya A, Gurmen T, Yigit Z. Relationship between increased serum resistin level and severity of coronary artery disease. *Angiology*. 2014:65:239-242.
516. Prugger C, Luc G, Haas B, Morange PE, Ferrieres J, Amouyel P, Kee F, Ducimetiere P, Empana JP. Multiple biomarkers for the prediction of ischemic stroke: The prime study. *Arterioscler Thromb Vasc Biol*. 2013:33:659-666.
517. Efstathiou SP, Tsiakou AG, Tsioulos DI, Panagiotou TN, Pefanis AV, Achimastos AD, Mountokalakis TD. Prognostic significance of plasma resistin levels in patients with atherothrombotic ischemic stroke. *Clin Chim Acta*. 2007:378:78-85.

518. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. *Nature*. 2001:409:307-312.
519. Wang H, Chu WS, Hemphill C, Elbein SC. Human resistin gene: Molecular scanning and evaluation of association with insulin sensitivity and type 2 diabetes in caucasians. *J Clin Endocrinol Metab*. 2002:87:2520-2524.
520. Gharibeh MY, Al Tawallbeh GM, Abboud MM, Radaideh A, Alhader AA, Khabour OF. Correlation of plasma resistin with obesity and insulin resistance in type 2 diabetic patients. *Diabetes Metab*. 2010:36:443-449.
521. Osawa H, Doi Y, Makino H, Ninomiya T, Yonemoto K, Kawamura R, Hata J, Tanizaki Y, Iida M, Kiyohara Y. Diabetes and hypertension markedly increased the risk of ischemic stroke associated with high serum resistin concentration in a general japanese population: The hisayama study. *Cardiovasc Diabetol*. 2009:8:60.
522. Tsukahara T, Nakashima E, Watarai A, Hamada Y, Naruse K, Kamiya H, Nakamura N, Kato N, Hamajima N, Sekido Y, Niwa T, Tomita M, Oiso Y, Nakamura J. Polymorphism in resistin promoter region at -420 determines the serum resistin levels and may be a risk marker of stroke in japanese type 2 diabetic patients. *Diabetes Res Clin Pract*. 2009:84:179-186.
523. Burnett MS, Lee CW, Kinnaird TD et.al. The potential role of resistin in atherogenesis. *Atherosclerosis*. 2005:182:241-248.
524. Jung HS, Park KH, Cho YM, Chung SS, Cho HJ, Cho SY, Kim SJ, Kim SY, Lee HK, Park KS. Resistin is secreted from macrophages in atheromas and promotes atherosclerosis. *Cardiovasc Res*. 2006:69:76-85.
525. Cho Y, Lee SE, Lee HC et.al. Adipokine resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery. *J Am Coll Cardiol*. 2011:57:99-109.
526. Shindo A, Tanemura H, Yata K, Hamada K, Shibata M, Umeda Y, Asakura F, Toma N, Sakaida H, Fujisawa T, Taki W, Tomimoto H. Inflammatory biomarkers in atherosclerosis: Pentraxin 3 can become a novel marker of plaque vulnerability. *PLoS One*. 2014:9:e100045.
527. Bluher M, Brennan AM, Kelesidis T, Kratzsch J, Fasshauer M, Kralisch S, Williams CJ, Mantzoros CS. Total and high-molecular weight adiponectin in relation to metabolic variables at baseline and in response to an exercise treatment program: Comparative evaluation of three assays. *Diabetes Care*. 2007:30:280-285.
528. Pischon T, Hu FB, Girman CJ, Rifai N, Manson JE, Rexrode KM, Rimm EB. Plasma total and high molecular weight adiponectin levels and risk of coronary heart disease in women. *Atherosclerosis*. 2011:219:322-329.
529. Musialek P, Tracz W, Tekieli L, Pieniazek P, Kablak-Ziembicka A, Przewlocki T, Stepien E, Kapusta P, Motyl R, Stepniewski J, Undas A, Podolec P. Multimarker approach in discriminating patients with symptomatic and asymptomatic atherosclerotic carotid artery stenosis. *J Clin Neurol*. 2013:9:165-175.
530. Sharma G, Tao M, Ding K, Yu D, King W, Deyneko G, Wang X, Longchamp A, Schoen FJ, Ozaki CK, Semel ME. Perivascular adipose adiponectin correlates with symptom status of patients undergoing carotid endarterectomy. *Stroke*. 2015:46:1696-1699.

Connecting Text

Through the work done in the previous chapter, no significant associations were observed between circulating levels of adiponectin and plaque instability and cerebrovascular symptomatology. Considering the large amount of evidence that exists highlighting adiponectin's anti-inflammatory and vasculo-protective properties, we hypothesized that total levels of adiponectin would be inversely associated with carotid plaque instability and cerebrovascular symptomatology. However, despite adiponectin's known beneficial and protective effects, there still remains contradictory data surrounding this adipokine. We have performed two systematic reviews and meta-analyses, as outlined in the introduction section of the thesis, which evaluated existing evidence with regards to the association between circulating adiponectin levels and cIMT (subclinical atherosclerosis) and the risk of carotid plaque presence and ischemic stroke risk[284,285]. Interestingly, an adiponectin paradox was identified where depending on the population studied, either a negative or positive association was noted between adiponectin and cIMT and carotid plaque presence. A negative association was observed in obesity-associated inflammatory conditions (i.e., subjects with T2DM, metabolic syndrome, or CVD), while a positive association was noted in classic chronic inflammatory/autoimmune conditions (i.e., subjects with rheumatoid arthritis or systemic lupus erythematosus). However, these associations were either weak or non-significant. Furthermore, adiponectin was found to be an independent and direct predictor of ischemic stroke risk in subjects without clinically manifest CVD, while its levels were suppressed in the acute stage following an ischemic stroke. Thus, explanations remain to be identified in order to fully understand this paradox.

Interestingly, it was recently reported that adiponectin's expression in PVAT, rather than circulating adiponectin, is significantly associated with cerebrovascular symptomatology in patients undergoing a CEA[530]. Since adiponectin is almost exclusively produced and secreted by adipose tissue, the type of adipose tissue from which adiponectin in the plasma and adiponectin in the tissue (i.e., blood vessel or atherosclerotic plaque) is derived from may be of importance. Subcutaneous and visceral adiposity are known to be important determinants of circulating adiponectin levels in humans, which can be influenced by various systemic factors. On the other hand, PVAT is the most likely source of adiponectin in blood vessels or plaques. While visceral adiposity can adversely affect the vasculature by causing a dysregulation in the production of adiponectin, PVAT may play a more direct role in atherosclerosis development as it envelopes the blood vessel. Therefore, we believe that adiponectin's expression in plaques may provide better

evidence of a possible link between adiponectin and plaque instability and cerebrovascular symptomatology than circulating levels of adiponectin. The following chapter investigates whether the plaque expression of adiponectin and its receptors is associated with atherosclerotic plaque instability.

CHAPTER 4: DECREASED ADIPONECTIN-MEDIATED SIGNALING THROUGH THE ADIPOR2 PATHWAY IS ASSOCIATED WITH CAROTID PLAQUE INSTABILITY

Karina Gasbarrino, Huaien Zheng, Anouar Hafiane, John P. Veinot, Chi Lai, Stella S. Daskalopoulou

Published in and adapted from[2]:
Gasbarrino K, Zheng H, Hafiane A, Veinot JP, Lai C, Daskalopoulou SS. *Stroke*. 2017;48(4):915-924.

4.1 Abstract

Background and Purpose: Adiponectin, the most abundantly secreted anti-inflammatory adipokine, protects against all stages of atherosclerotic plaque formation by acting on its receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). Through binding of AdipoR1, adiponectin leads to the activation of the AMPK pathway, whereas stimulation of PPAR-α is attributed to the binding of AdipoR2. However, the role of adiponectin and its receptors in plaque instability remains to be characterized. Thus, we aimed to investigate whether the adiponectin–AdipoR pathway is associated with carotid atherosclerotic plaque instability.

Methods: The instability of plaque specimens obtained from patients who underwent a CEA (n=143) was assessed using gold standard histological classifications.

Results: Using immunohistochemistry, we showed that adiponectin and AdipoR1/AdipoR2 are expressed in human carotid plaques and that their expression was localized most abundantly in areas of macrophage and foam cell accumulation. Unstable plaques expressed more adiponectin protein (Western blot, $P<0.05$) and less AdipoR2 mRNA (2.11-fold decrease, $P<0.05$) than stable plaques, whereas AdipoR1 expression remained similar between stable and unstable plaques. Beyond AdipoR1/AdipoR2 expression, a graded decrease in PPAR-α protein levels was observed in relation to carotid plaque instability ($P<0.001$), whereas AMPK phosphorylation was increased ($P<0.05$). Our in vitro model of plaque instability, involving the induction of foam cells from human Tamm–Horsfall protein 1 (THP-1) macrophages treated with acetylated LDL, supported our in vivo conclusions.

Conclusions: An overall abundance of adiponectin with a decrease in AdipoR2 expression and activity was observed in unstable plaques, suggesting that reduced signaling through the AdipoR2 pathway, and not through AdipoR1, may contribute to plaque instability.

4.2 Introduction

Carotid atherosclerotic plaque rupture is a major cause of acute cerebrovascular ischemic events, such as stroke[439]. Plaque morphology and composition, in addition to progressive stenosis of the vessel lumen, are major determinants for plaque instability and rupture[439].

Adipokines, immunomodulatory proteins secreted by adipose tissue, can regulate vascular homeostasis[188]. The majority of adipokines are pro-inflammatory and exert adverse effects on the vasculature. However, the most abundantly secreted adipokine is adiponectin, which is widely recognized for its anti-inflammatory, anti-atherogenic, and vascular protective properties[188,290]. Hypoadiponectinemia is associated with obesity, T2DM, CAD, and progression of carotid atherosclerosis[265,266,269]. The atheroprotective effects of adiponectin have been demonstrated at all stages in atherosclerotic plaque formation, from endothelial dysfunction, plaque initiation and progression, to plaque rupture and thrombosis; these effects are attributed to adiponectin's actions on all major cell types present in the vasculature, including macrophages, endothelial, and SMCs[290]. Adiponectin exerts its effects via two transmembrane receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2)[305], which play a key role in glucose and energy metabolism through potentially distinct pathways[313,531]. Adiponectin mainly leads to the activation of the AMPK pathway through binding of AdipoR1, whereas AdipoR2 stimulates the PPAR-α pathway[313]. However, the precise physiological role of these receptors in the vasculature and in plaque infiltrating cells remains to be clarified.

Due to its anti-atherosclerotic activities, it is reasonable to hypothesize that a decrease in plaque adiponectin-mediated signalling through the AdipoR1/AdipoR2 pathways may reflect plaque instability in humans. Therefore, the aim of this study was to investigate whether the adiponectin-AdipoR pathway is associated with plaque instability in humans.

4.3 Methods

4.3.1 Study population

Consecutive patients with high-grade carotid stenosis, scheduled for a CEA, were recruited from the Vascular Surgery pre-operative clinics at the McGill University Health Centre and the Jewish General Hospital in Montreal, Canada, as described previously[1]. Patients were excluded from participation in the study if they had previous interventions on the same carotid artery (CEA and/or CAS). Patient demographics and clinical information (cerebrovascular symptomatic status, past medical history, medication use, and lifestyle habits) were obtained from all recruited subjects who

underwent a CEA. The information collected was confirmed through various sources: 1) patient interview, 2) a detailed questionnaire, and 3) medical records. Blood pressure (HEM-705CP, Omron Corp.) and anthropometric measurements (height and weight) were obtained. BMI (kg/m^2) was calculated.

The McGill University's Institutional Ethics Review Board and Transplant Quebec granted ethics approval (A12-M145-09B) for this study. All subjects provided written informed consent prior to study participation.

4.3.2 Blood collection, processing, and measurements

Fasting blood samples were collected from each subject the day of the CEA before surgical intervention. Plasma and serum were obtained from whole blood and rapidly stored at −80°C for subsequent analysis. Measurements for serum lipid profile (total cholesterol, triglycerides, and HDL-C) and high-sensitivity CRP were performed in the central biochemistry laboratories of the McGill University Health Centre. LDL-C was calculated using the Friedewald formula[498]. Plasma total adiponectin levels were determined by radioimmunoassay (Human Adiponectin RIA Kit, EMD Millipore, Billerica, MA, USA). The intra- and inter-assay coefficients of variation ranged from 1.78-3.59% and 6.90-9.25%, respectively, and the lower limit of detection was 0.94 ng/mL.

4.3.3 Surgical sample collection and processing

Human carotid atherosclerotic plaques were obtained immediately after surgical resection and processed. To serve as healthy control tissue, carotid artery specimens free of atherosclerosis (termed herein as *healthy carotid arteries*) were obtained from recently deceased organ transplant donors, who consented for research through Transplant Quebec.

Human carotid atherosclerotic plaques were dissected into transverse segments of approximately 3-4 mm in thickness. Plaque segments with the area of maximal stenosis and largest plaque burden were dedicated to histological/immunohistochemical and RNA/protein analyses, as described below. In order to mirror the same layers present in the carotid plaques and to avoid contamination of tissue expressing adiponectin, PVAT and adventitia was carefully removed surrounding the healthy donor carotid arteries. Once removed of their surrounding adventitial and adipose tissue layers, the healthy carotid arteries then underwent the same histological/immunohistochemical and RNA/protein analyses as the carotid plaque specimens.

4.3.4 Staining procedures

Histology

Consecutive 4-µm sections from paraffin-embedded plaque segments were stained with hematoxylin and eosin. Two vascular pathologists (JV and CL) independently characterized the instability of the plaques, according to gold-standard histological classifications: 1) AHA classification by Stary *et al*[176], and 2) semi-quantitative scale by Lovett *et al*[177,178]. They were blinded to the patients' clinical status. In case of disagreement, a consensus was reached. Accordingly, plaques were defined as: definitely stable, probably stable, probably unstable, and definitely unstable. Furthermore, individual features of the plaque were classified on a 3- or 4-grade scale, including presence/absence of intraplaque hemorrhage, thrombus, foam cells, lipid core size, proportion of fibrous tissue, neovascularization, calcification, cap rupture, overall inflammation, and infiltration of the fibrous cap with inflammatory cells (inflammation graded according to the number of macrophages and lymphocytes present)[177,178].

Immunohistochemistry

Four µm sections from the maximal area of the plaque and from the healthy carotid arteries were stained with rabbit anti-human antibodies for adiponectin (1:200, ab25891, abcam, Cambridge, MA, USA), AdipoR1 (1:500, H-001-44, Phoenix Pharmaceuticals, Burlingame, CA, USA), or AdipoR2 (1:500, H-001-23, Phoenix Pharmaceuticals), for 30 minutes at 37°C. Staining was performed using the EnVision+ System-HRP kit (Dako, Carpinteria, CA, USA). Plaque and healthy carotid artery sections were also stained with specific antibody markers to represent macrophages, SMCs, and endothelial cells (CD68, α-SMC actin, and von Willebrand factor, respectively).

Immunofluorescence

Double immunofluorescence staining was performed. Sections were incubated with either rabbit anti-human AdipoR1 (1:100; Phoenix Pharmaceuticals) and mouse anti-human CD68 (1:50; ab955, abcam), or anti-AdipoR2 (1:100; Phoenix Pharmaceuticals) and anti- CD68 (1:50; ab955, abcam) antibodies. Donkey anti-rabbit IgG conjugated Alexa 594 (1:100; Life Technologies Inc, Burlington, ON, Canada) and goat anti-mouse IgG conjugated Alexa 488 (1:100; ab150117, abcam) were used as secondary antibodies. Cell nuclei were counterstained with DAPI (Life

Technologies Inc). Olympus BX61VS microscope equipped with a VS110 slide scanner linked to OlyVia image viewer software was used for image analysis.

4.3.5 RNA and protein isolation

Total RNA and protein were simultaneously extracted from the whole plaque area and healthy carotid arteries using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA), and treated with RNase-free DNase I (RNase-Free DNase Set, Qiagen) to remove contamination with genomic DNA. Total RNA concentration was measured by absorbance at 260 nm using a spectrophotometer (Ultrospec 2100 pro, GE Healthcare Life Sciences) and its quality and purity were determined using the ratio of absorbance 260/280 and 260/230, respectively. Total protein concentration was measured using the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

4.3.6 Quantitative real-time polymerase chain reaction

cDNA was synthesized from 1 μg of total RNA by the Eppendorf Mastercycler RT system using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Life Technologies Inc). The resulting cDNA samples were then used to assess mRNA expression of adiponectin, AdipoR1, and AdipoR2, via SYBR Green-based quantitative real-time polymerase chain reaction (qRT-PCR). All samples were run in triplicate with the StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA, USA), using PerfeCTa SYBR Green SuperMix (QUANTA Biosciences Inc., Gaithersburg, MD, USA). Primer sequences for the targets (adiponectin, AdipoR1, and AdipoR2) as well as the reference gene (β-actin) were designed using NCBI/Primer-Blast. The following designed primer sets (Alpha DNA, Montreal, QC, Canada) were used:

human adiponectin – reverse 5'-TGGGGATAGTAACGTAAGTCTCC-3' and forward 5'-GGCTTTCCGGGAATCCAAGG-3';

human AdipoR1 – reverse 5'-GGTTGGCGATTACCCGTTTG-3' and forward 5'-TCCTGCCAGTAACAGGGAAG-3';

human AdipoR2 – reverse 5'-TGGGCTTGTAAGAGAGGGGAC-3' and forward 5'-CTGGATGGTACACGAAGAGGT-3';

human β-actin – reverse 5'-TAGAAGCATTTGCGGTGGACGATGGA-3' and forward 5'-GGCACCCAGCACAATGAAGATCAA-3'.

The resultant quantitative cycle (C_q) values for the target genes were normalized to the respective β-actin values. Relative mRNA abundance was calculated using the $2^{-\Delta\Delta Cq}$ method.

4.3.7 Western blot analysis

Homogenate protein extracts from healthy carotid arteries were pooled at the same concentration and served as a standard control across all gels. An optimum of 14 µg of protein obtained from definitely stable and definitely unstable plaques were mixed with an equal volume of 1x SDS sample buffer (10% 2-mercaptoethanol), boiled for 10 minutes at 95°C, and analyzed in duplicate by 8-28% SDS-PAGE. Samples were transferred to 0.45 µm nitrocellulose membrane (Bio-Rad) and immunoblotted overnight at 4°C by rabbit polyclonal anti-human adiponectin (1:1500, ab25891, abcam), PPAR-α (1:1000, ab3484), AMPK-phosphorylated (1:1000, ab131357, abcam), or AMPK-total (1:4000, ab32047). Membranes were stripped and re-probed by rabbit anti-β-actin (1:2500, ab75186, abcam), as a loading-control. Bands were visualized by enhanced chemiluminescence (ZmTech Scientifique, Montreal, QC, Canada) and band densities were evaluated with AlphaImager HP Imaging Densitometer with the Multi-Analyst software (Alpha Innotech Corporation, USA). To correct for inter-assay variation across all gels, the pooled control standard was included on each gel in triplicate and used for normalization of test sample values.

4.3.8 In vitro model of plaque instability

We developed an *in vitro* model of plaque instability, using THP-1 cells, a human monocytic cell line (American Type Tissue Culture Collection, Camden, NJ), to confirm our *in vivo* findings. These cells were first differentiated into macrophages and then transformed into foam cells using human acetylated LDL.

THP-1 cells were plated in a 6 well-plate at a density of 0.5×10^6 cells/0.5mL in RPMI-1640 medium supplemented with 100 IU/mL of penicillin/streptomycin, 50 µmol/L β-mercaptoethanol, and 10% fetal bovine serum (FBS) and differentiated into macrophages by the addition of 200 nM phorbol myristyl acetate (PMA) for 72 hours[532]. Over an additional period of 72 hours, macrophages were transformed into foam cells by labeling with ^{3}[H]-cholesterol (2 µCi/mL) in the presence of 100 µg/mL of acetylated LDL in serum-free RPMI-1640 medium containing 1% BSA. Following foam cell formation, cells were incubated for 24 hours with 5 µg/mL of human recombinant adiponectin (R&D Systems, Minneapolis, MN, USA). The oligomeric structure of recombinant adiponectin was explored by native-PAGE gel analysis (**Figure 4.1**).

Cells were lysed at 4°C in the presence of protease inhibitors (Roche Diagnostic, Basel, Switzerland) with buffer containing 20 mmol/L Tris, 5 mmol/L EDTA, 5 mmol/L EGTA, and

0.5% *n*-dodecylmaltoside. AdipoR1, AdipoR2, and their respective signaling proteins (AMPK, PPAR-α) were analyzed by SDS-PAGE (8-28%).

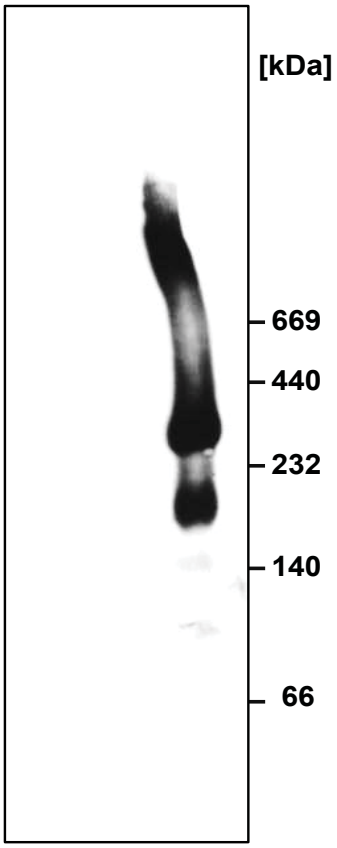


Figure 4.1 Characterization of the oligomeric structure of human recombinant adiponectin. *Recombinant adiponectin (20ug/ml) was separated by non-denaturing gradient gel electrophoresis (4-15%). Adiponectin oligomers were detected by adiponectin antibody. A representative immunoblot demonstrates that the recombinant adiponectin used in the in vitro study is composed of the hexameric (~180kDa) and HMW multimers (>360kDA).*[2]

4.3.9 Foam cell formation

Acetylated Low-density Lipoprotein Preparation

Acetylated LDL was prepared according to Basu *et al*[533]. LDL (density 1.019 to 1.063 g/ml) was isolated from pooled normolipidemic plasma obtained from healthy subjects by sequential ultracentrifugation. LDL preparation was desalted by extensive dialysis using 0.15 mol/L NaCl and 0.3 mmol/L EDTA, pH 7.4, and final protein concentrations were determined by Bradford protein assay (Bio-Rad). Dialyzed LDL was added to an equal volume of saturated sodium acetate. Then small aliquots of acetic anhydride (2 μL) were added over the course of 1 hour with continuous stirring, leading to the addition of a total mass of acetic anhydride equal to 1.5 times the mass of LDL protein used. The resulting solution was then dialyzed with 0.15 mol/L NaCl and 0.3 mmol/L EDTA, pH 7.4, for 24 hours at 4 °C. Native agarose gel electrophoresis (0.8%), stained with Sudan black, was applied for quality control analysis of the prepared acetylated LDL[534] (**Figure 4.2**).

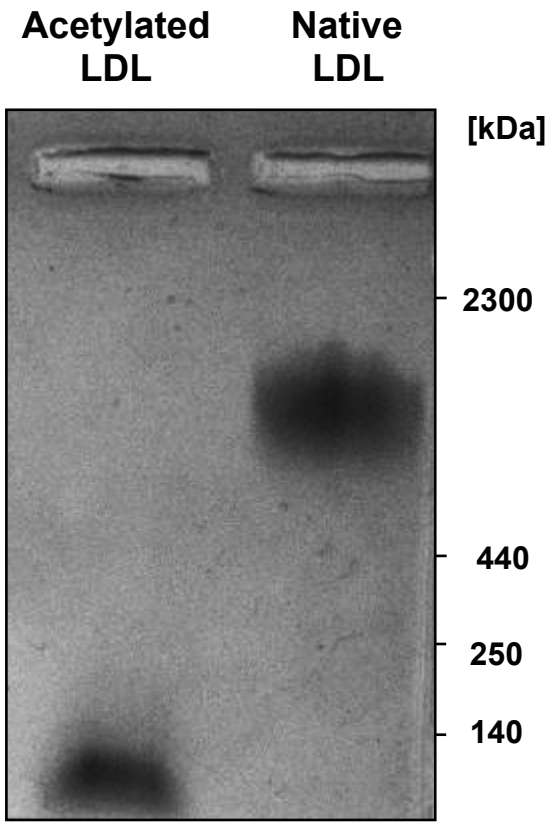


Figure 4.2 Migration pattern of low-density lipoproteins. *Detection of LDL by native agarose gel electrophoresis stained with Sudan black (lipid stain). 40 µL of native LDL or acetylated LDL were stained at 4°C overnight with 20 µL of 1% Sudan black previously diluted in 30% ethanol. Samples were then loaded onto a 0.8% agarose gel with Tris glycine buffer (pH 8.3), thereby allowing visualization of lipid components as black bands. HMW protein mixture (GE Healthcare, UK) was run as a standard on the gel. Molecular weight markers were revealed by Coomassie Blue stain. Following acetylation, acetylated LDL has a LMW than native LDL.*[2]

The identity of transformed foam cells was quantitatively and qualitatively validated by:

1) Quantification of Intracellular Lipid Content: Lipids were extracted from the protein lysates using Folch solution (chloroform: methanol, 2:1, vol./vol.). Samples were loaded in triplicate onto Silica Gel (Analtech, Newark, DE, USA) thin layer chromatography plates[532]. Bands corresponding to ^{3}[H]-cholesterol ester were located by exposure to iodine vapour and were

scraped off the plate into liquid scintillating vials and assayed for radioactivity. Transformed foam cells were observed to have greater accumulation of intracellular cholesterol ester content than macrophages (P<0.05; **Figure 4.3A**).

2) Oil Red O Staining: Cells were fixed with 4% paraformaldehyde for 30 minutes and stained with Oil Red O dye (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 30 minutes. After removing dye, the cells were washed with PBS and counter-stained with hematoxylin. Representative images were obtained using the Zeiss Imager Microscope. Transformed foam cells were observed to have greater Oil Red O staining of lipid droplets than macrophages (**Figure 4.3B**).

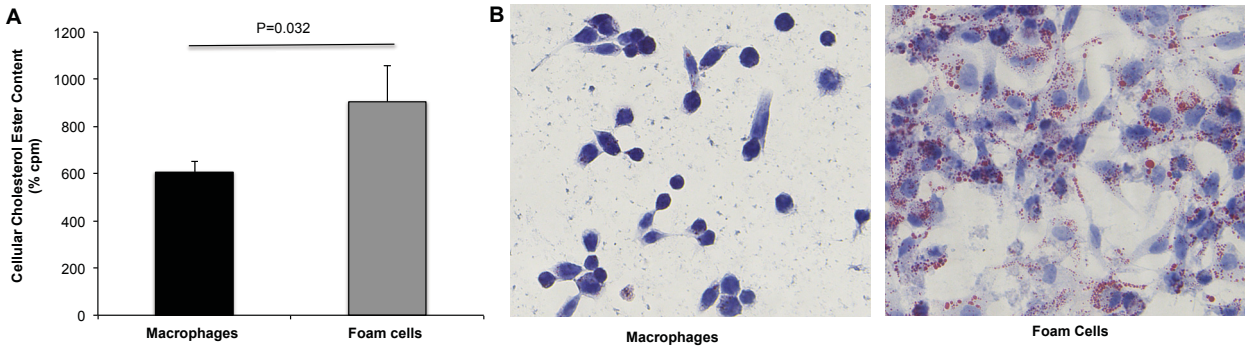


Figure 4.3 Confirmation of in vitro foam cell formation in human THP-1 cells*. THP-1 cells were incubated for 3 days in growth medium containing 2μCi of ^{3}[H]-free cholesterol with or without the presence of acetylated LDL (100 μg/mL), resulting in the formation of foam cells or macrophages, respectively.* ***A)*** *Cholesterol ester content within foam cells and macrophages was quantified using thin layer chromatography. Samples were analyzed in triplicate. Analysis comparing the cholesterol ester content within foam cells and macrophages was determined by independent T-test (P<0.05).* ***B)*** *Representative photomicrographs demonstrating Oil Red O staining in foam cells and macrophages fixed in 4% paraformaldehyde. Images were photographed at magnification x400.*[2]

4.3.10 Statistical analyses

Two vascular pathologists graded the instability of the carotid plaques according to gold-standard histological classifications[176-178] (details above) and classified them into 4 groups: 1) 'definitely stable', 2) 'probably stable', 3) 'probably unstable', and 4) 'definitely unstable'. The 'probably stable' and 'probably unstable' plaque groups were combined and labelled as 'intermediate stable'

group to be used in the statistical analyses. In addition to being clinically relevant, combining these instability groups led to increased statistical power. Descriptive statistics were performed as appropriate to summarize the baseline characteristics between patients with definitely stable, intermediate stable, and definitely unstable plaques. Partial Spearman rank correlations were performed between adiponectin circulating levels, AdipoR1, AdipoR2, and clinical variables. Parametric (independent T-test, ANOVA) and non-parametric tests (Mann-Whitney, Kruskal-Wallis) were used, as appropriate, to analyze differences in adiponectin circulating levels, plaque adiponectin, AdipoR1, AdipoR2, PPAR-α, and AMPK expression, between definitely stable, intermediate stable, and definitely unstable plaques. Post-hoc analyses were performed following significance with the ANOVA or Kruskal-Wallis test. All statistical analyses were executed using SPSS, version 20.0 (IBM Corporation). A P-value of <0.05 (2-tailed) was considered significant. The degree of significance for the correlation matrix was adjusted using Bonferroni correction. The Bonferroni adjusted alpha level used was $P<0.005$.

4.4 Results

4.4.1 Patient characteristics

Demographic, clinical, and biological parameters of the total CEA population (n=143), as well as of the population split according to the plaque instability classification (definitely stable, intermediate stable, and definitely unstable) are presented in **Table 4.1**. The total population had a mean age (± SD) of 69.1±9.0 years, was predominantly male (70.6%), and had a median circulating adiponectin level of 8.54 [4.28-13.92] µg/mL. The proportion of patients with CAD differed significantly across all plaque groups ($P=0.044$), with the definitely stable group having the greatest proportion of patients with CAD than the intermediate stable and definitely unstable group. Differences in sex and ever smoker status were approaching significance ($P=0.058$ and $P=0.056$, respectively), with a greater proportion of women (52.9%) within the definitely stable group and a greater proportion of ever smokers (96.7%) within the definitely unstable group.

Circulating adiponectin levels did not differ significantly among the plaque groups (**Table 4.1**). However, adiponectin was positively correlated with HDL-C (r=0.413, $P<0.001$), and negatively with BMI (r=-0.366, $P<0.001$), and triglycerides (r=-0.286, $P=0.001$).

4.4.2 Symptomatology and plaque instability

The overall relationship between cerebrovascular symptomatology and plaque stability was not significant (**Table 4.1**, *P*=0.246), suggesting that symptomatology is not a good indicator of the stability of a plaque. In addition, the nature of the cerebrovascular event (AF, TIA, or stroke) as well as the average time between the cerebrovascular event and the CEA, did not differ significantly in relation to plaque stability (**Table 4.1**).

Among the CEA patients who experienced a cerebrovascular ischemic event (AF, TIA, or stroke), 23% had their CEA within the acute phase (≤7 days). The time since event ranged from 1 to 169 days, with an average time of 5.0 [3.0-6.0] days for subjects who underwent a CEA within the acute phase (≤7 days) and 30.0 [20.0-60.5] days for subjects who underwent a CEA following the acute phase (>7 days). However, the nature of the event (AF versus TIA versus stroke) remained similar between the two groups (acute: 13%/52.5%/34.8%; non-acute: 20.8%/41.6%/37.7%; *P*=0.591). In addition, the stability of the plaque (definitely stable versus intermediate stable versus definitely unstable) in relation to acute and non-acute cerebrovascular ischemic events did not differ (acute: 8.7%/69.6%/21.7%; non-acute: 9.1%/66.2%/24.7%; P=0.953), even when the relationship was assessed according to the nature of the event.

4.4.3 Graded expression of adiponectin in carotid atherosclerotic plaques in relation to plaque instability

Using qRT-PCR, adiponectin mRNA was not detected in carotid plaques or in healthy carotid arteries, suggesting that adiponectin mRNA transcripts are not produced by cells present in the plaque or in the intimal and medial layer of the healthy vasculature (**Figure 4.4**). In contrast, using Western Blot, adiponectin protein was detected in both atherosclerotic plaques and in healthy carotid arteries and a graded increase in adiponectin expression was noted from healthy carotid arteries, to definitely stable plaques, to definitely unstable plaques (*P*=0.023; **Figure 4.5**).

The associations between plaque adiponectin protein expression and plaque instability, according to specific clinical covariates (sex, smoking, diabetes, CAD, hypertension, symptomatology, and statin use), are presented in **Table 4.2**. Furthermore, **Table 4.3** presents differences in plaque adiponectin protein levels in relation to these clinical variables.

Table 4.1 Population demographic and clinical characteristics[2]

Population Characteristic	Total Patients (n=143)	Definitely Stable Patients (n=17)	Intermediate Stable Patients (n=94)	Definitely Unstable Patients (n=32)	*P-Value
Age, y	69.1±9.0	69.2±8.4	69.4±9.0	68.5±9.4	0.897
Sex, % men	70.6	47.1	75.5	68.8	0.058
BMI, kg/m^2	27.0±4.7	28.5±3.9	26.6±4.3	27.4±5.9	0.310
Ever Smoker, %	82.2	82.4	77.3	96.7	0.056
CAS, 50-79%/80-99%	23.1/76.9	18.8/81.2	23.3/76.7	25.0/75.0	0.892
Neurologically Symptomatic, %	69.9	52.9	71.3	75.0	0.246
AF/TIA/Stroke, %	19.0/44.0/37.0	22.2/44.4/33.4	19.4/43.3/37.3	16.7/45.8/37.5	0.996
Timing (Event-CEA), days	22.5 [9.3-49.3]	15.0 [7.5-32.5]	21.0 [8.0-47]	28.0 [10.5-72.5]	0.513
CAD, %	39.6	64.7	38.9	28.1	**0.044**
SBP, mmHg	138±19	146±24	137±18	135±18	0.136
DBP, mmHg	71±10	73±10	71±10	73±11	0.519
Hypertension, %	80.6	94.1	81.1	71.9	0.169
Anti-hypertensive medication, %	77.7	88.2	78.9	68.8	0.267
Diabetes mellitus, %	36.0	29.4	34.4	43.8	0.535
Anti-hyperglycemic medication, %	30.9	23.5	31.1	34.4	0.735
Hypercholesterolemia, %	84.9	82.4	86.7	81.2	0.727
Statin use, %	70.5	70.6	71.1	68.8	0.969
Total cholesterol, mmol/L	3.24[2.80-3.81]	3.24[2.79-4.08]	3.22[2.78-3.79]	3.29[2.95-3.81]	0.715
LDL, mmol/L	1.64[1.19-2.08]	1.73[1.19-2.24]	1.63[1.18-2.54]	1.61[1.21-2.23]	0.921
HDL, mmol/L	0.95[0.75-1.13]	1.06[0.81-1.15]	0.95[0.74-1.12]	0.96[0.73-1.18]	0.649
Triglycerides, mmol/L	1.55[1.06-1.89]	1.55[1.10-1.81]	1.55[0.99-1.84]	1.57[1.08-2.00]	0.808
hs-CRP, mg/L	1.45[0.58-3.51]	0.91[0.48-1.61]	1.65[0.66-3.64]	1.09[0.39-5.39]	0.130
Adiponectin, µg/mL	8.54[4.28-13.92]	10.19[5.94-15.06]	8.32 [4.25-14.46]	8.47 [2.93-13.34]	0.563

Normally distributed data: values represent mean±SD; Non-normally distributed data: values represent median [interquartile range]
BMI, body mass index; CAS, carotid artery stenosis; AF, amaurosis fugax; TIA, transient ischemic attack; CEA, carotid endarterectomy; CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein.
*P-value indicates significance among the three instability groups: definitely stable, intermediate unstable, definitely unstable (analysis was performed by ANOVA or Kruskall-Wallis, as appropriate).

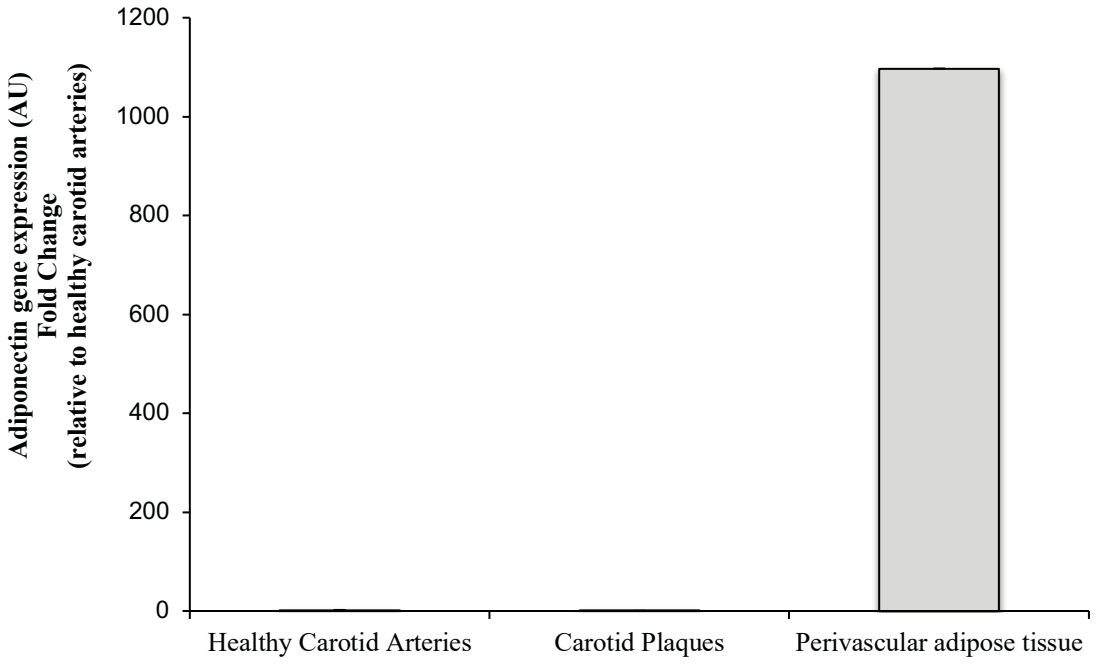


Figure 4.4 Adiponectin mRNA expression in various tissues*. Adiponectin mRNA was not expressed in healthy carotid arteries or in carotid atherosclerotic plaques. PVAT that was surrounding the healthy carotid arteries was used as a positive control, demonstrating 1100-fold higher adiponectin mRNA expression than in healthy carotid arteries and in carotid plaques.*[2]

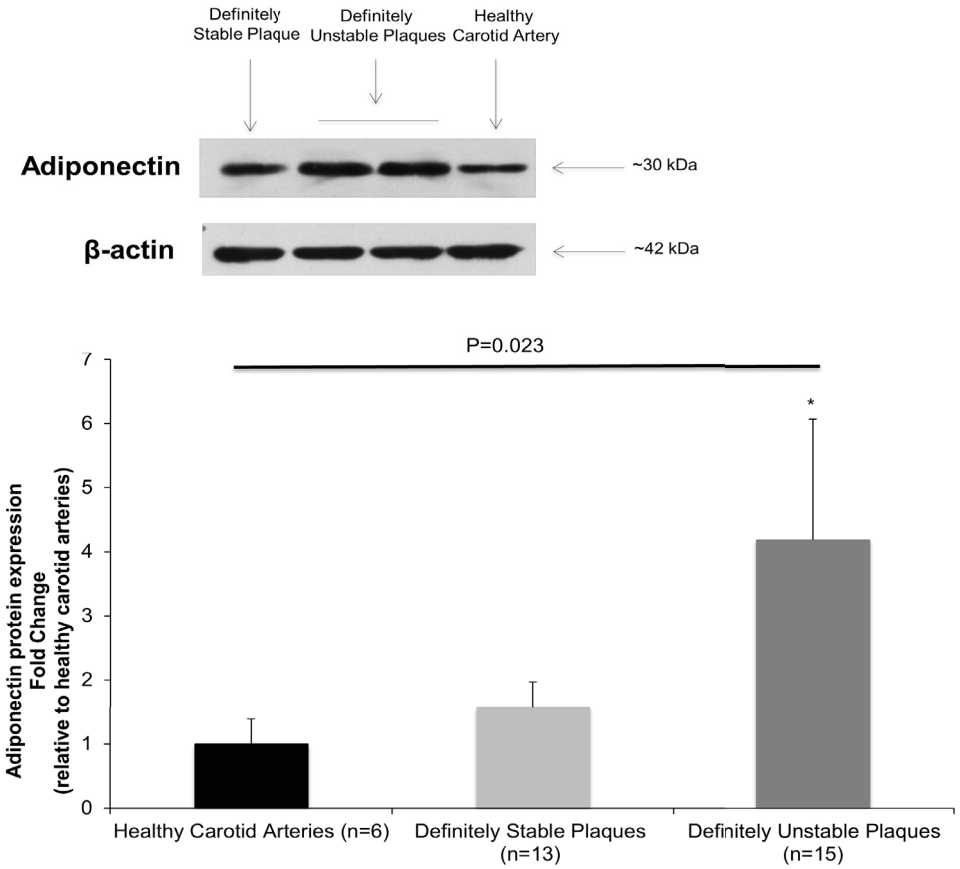


Figure 4.5 Western Blot analyses of plaque adiponectin in relation to plaque instability. *Representative Western Blot showing expression of adiponectin in human healthy carotid arteries (n=6) and carotid atherosclerotic plaques (definitely stable [n=13] and definitely unstable plaques [n=15]). Changes in adiponectin protein expression were determined by normalizing against the densitometric intensity of β-actin. Data represent mean ± SD. Analysis comparing groups was performed by ANOVA. *P<0.05 (Tukey's multiple comparisons test between definitely unstable plaques and definitely stable plaques, and between definitely unstable plaques and healthy carotid arteries).*[2]

Table 4.2 Adiponectin protein and AdipoR2 mRNA levels in relation with plaque instability according to specific clinical covariate analyses[2]

Characteristics	Adiponectin protein			AdipoR2 mRNA			
	Definitely Stable ([N], fold change ± SD)	Definitely Unstable ([N], fold change ± SD)	†P-value	Definitely Stable ([N], $2^{(-\Delta\Delta Cq)}$ ± SD)	Intermediate Stable ([N], $2^{(-\Delta\Delta Cq)}$ ± SD)	Definitely Unstable ([N], $2^{(-\Delta\Delta Cq)}$ ± SD)	†P-value
All	[13], 1.77±0.40	[15], 4.31±1.90	0.028	[17], 0.96±0.40	[94], 0.57±0.43	[32], 0.46±0.39	0.019
Sex							
Men	[7], 1.93±0.43	[12], 5.10±2.11	0.057	[8], 1.03±0.49	[71], 0.61±0.41	[22], 0.40±0.36	0.035
Women	[6], 1.59±0.38	[3], 3.13±0.32	0.042	[9], 0.90±0.33	[23], 0.48±0.53	[10], 0.59±0.46	0.134
Smoking							
Yes	[10], 1.81±0.45	[14], 4.36±1.88	0.060	[14], 1.08±0.42	[68], 0.57±0.41	[29], 0.46±0.37	0.017
No	[3], 1.66±0.19	[1], 9.78±(-)	-	[3], 0.55±0.34	[20], 0.58±0.45	[1], 0.30±(-)	0.737
Diabetes							
Yes	[4], 1.57±0.37	[7], 4.28±1.67	0.132	[5], 0.99±0.36	[31], 0.74±0.47	[14], 0.46±0.40	0.118
No	[9], 1.86±0.42	[8], 5.08±2.18	0.128	[12], 0.95±0.40	[59], 0.51±0.41	[18], 0.45±0.37	0.072
Coronary Artery Disease							
Yes	[9], 1.71±0.38	[6], 7.59±2.43	0.057	[11], 0.83±0.36	[35], 0.63±0.48	[9], 0.41±0.55	0.150
No	[4], 1.88±0.49	[9], 2.80±0.92	0.463	[6], 1.24±0.52	[55], 0.55±0.40	[23], 0.47±0.34	0.089
Symptomatology							
Yes	[7], 1.90±0.38	[12], 4.94±2.01	0.056	[9], 1.04±0.41	[69], 0.55±0.45	[25], 0.43±0.38	0.034
No	[6], 1.61±0.44	[3], 3.80±1.67	0.446	[8], 0.87±0.38	[25], 0.64±0.39	[7], 0.54±0.37	0.594
Hypertension							
Yes	[12], 1.67±0.38	[11], 5.69±2.08	0.024	[16], 1.02±0.41	[73], 0.59±0.41	[23], 0.46±0.52	0.018
No	[1], 2.98±(-)	[4], 2.02±0.35	-	[1], 0.36±(-)	[17], 0.53±0.49	[9], 0.43±0.22	0.867
Statin Use							
Yes	[10], 1.92±0.33	[10], 3.83±1.24	0.078	[12], 1.00±0.39	[64], 0.61±0.42	[22], 0.54±0.42	0.129
No	[3], 1.27±0.62	[5], 6.48±2.86	0.169	[5], 0.86±0.41	[26], 0.52±0.43	[10], 0.31±0.34	0.117

SD, standard deviation
For adiponectin levels, the fold change is relative to healthy control arteries
$2^{(-\Delta\Delta Cq)}$ values presented are normalized to healthy control arteries
†P-value indicates significance between the stability groups

Table 4.3 Adiponectin protein and AdipoR2 mRNA levels in relation to patient characteristics[2]

Characteristics	Adiponectin protein		AdipoR2 mRNA	
	[N], mean±SD (AU)	P-value	[N], ΔΔCq±SD (AU)	P-value
Sex				
Men	[19], 1.63±1.79	0.096	[101]; 0.79±1.34	0.983
Women	[9], 0.87±0.47		[42], 0.79±1.16	
Smoking				
Yes	[24], 1.36±1.54	0.841	[111], 0.78±1.35	0.825
No	[4], 1.53±1.69		[24], 0.84±1.15	
Diabetes				
Yes	[11], 1.37±1.43	0.958	[50], 0.59±1.22	0.212
No	[17], 1.40±1.63		[89], 0.88±1.34	
Coronary Artery Disease				
Yes	[15], 1.69±1.93	0.250	[55], 0.68±1.15	0.497
No	[13], 1.04±0.81		[84], 0.84±1.40	
Symptomatology				
Yes	[19], 1.59±1.71	0.328	[103], 0.86±1.26	0.278
No	[9], 0.97±1.01		[40], 0.60±1.36	
Hypertension				
Yes	[23], 1.49±1.67	0.457	[112], 0.71±1.26	0.239
No	[5], 0.92±0.35		[27], 1.04±1.48	
Statin Use				
Yes	[20], 1.19±0.97	0.466	[98], 0.67±1.27	0.119
No	[8], 1.88±2.46		[41], 1.04±1.36	

SD, standard deviation; AU, arbitrary units

4.4.4 Differential expression of AdipoR1/AdipoR2 in carotid atherosclerotic plaques in relation to plaque instability

AdipoR1 and AdipoR2 mRNA transcripts were present in both human atherosclerotic lesions and healthy carotid arteries. AdipoR1 expression did not differ between atherosclerotic plaques and healthy arteries (1.092±0.270 arbitrary units [AU] versus 1.000±0.158 AU, respectively, *P*=0.870; **Figure 4.6A**), while there was a trend for a 1.73-fold reduction of AdipoR2 expression in atherosclerotic plaques compared to healthy carotid arteries (0.578±0.349 AU versus 1.000±0.305 AU, respectively, *P*=0.293; **Figure 4.6B**). AdipoR1 expression remained similar across all grades of instability (*P*=0.659; **Figure 4.6C**), whereas AdipoR2 differed significantly (*P*=0.019; **Figure 4.6D**). Specifically, there was a graded decrease in AdipoR2 expression from definitely stable to intermediate stable to definitely unstable plaques. When compared to definitely stable plaques, there was a 1.68-fold and a 2.11-fold decrease in AdipoR2 expression in intermediate stable

(0.596±0.345 AU versus 1.000±0.382 AU; *P*=0.068) and definitely unstable plaques (0.474±0.342 AU versus 1.000±0.382 AU; *P*=0.014), respectively. Furthermore, in plaques that had a high presence of foam cells (≥50 cells), AdipoR2 mRNA expression decreased significantly by 1.37-fold compared to plaques with a lower presence of foam cells (<50 cells) (0.729±0.332 AU versus 1.000±0.364 AU, respectively, *P*=0.033; **Figure 4.7**). **Table 4.4** presents AdipoR1 and AdipoR2 mRNA expression in relation to other plaques features, including infiltration of inflammatory cells in the cap, which was found to be trending in association with lower AdipoR2 expression.

Circulating adiponectin levels were negatively correlated with AdipoR1 mRNA expression in the plaque (r=-0.243, *P*=0.004) but not with AdipoR2 mRNA (r=-0.056, *P*=0.508). Neither AdipoR1 nor AdipoR2 mRNA were correlated with clinical variables (**Table 4.3**). **Table 4.2** presents the association between plaque AdipoR2 mRNA expression and plaque instability, according to specific clinical covariates (sex, smoking, diabetes, CAD, hypertension, symptomatology, and statin use).

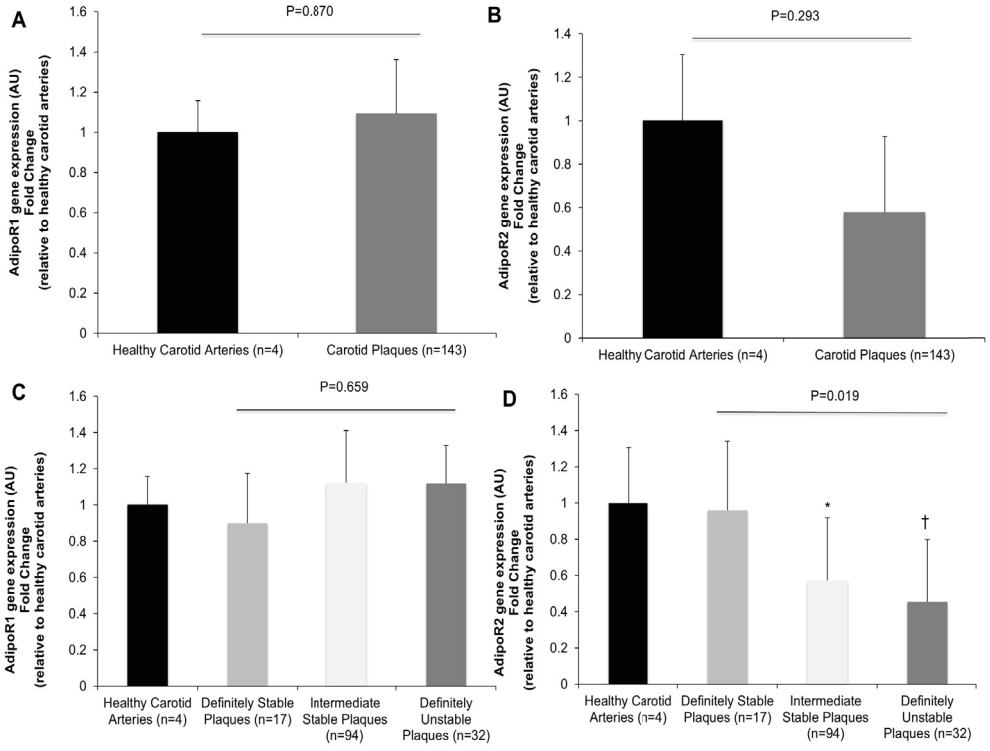


Figure 4.6 Quantitative real-time polymerase chain reaction analyses of plaque AdipoR1 and AdipoR2 in relation to plaque instability*. Bar graphs illustrate qRT-PCR data for AdipoR1 and AdipoR2 mRNA levels in relation to plaque instability (n=143). Data represent mean ± SD.* ***A*** *and* ***B****, Analysis comparing carotid plaques to healthy carotid arteries was performed by independent*

t test. ***C*** *and* ***D****, Analysis comparing groups of instability was performed by ANOVA. Post-hoc analysis was performed when overall trend was found to be significant. *P=0.068 between intermediate stable plaques and definitely stable plaques. †P=0.014 between definitely unstable plaques and definitely stable plaques.*[2]

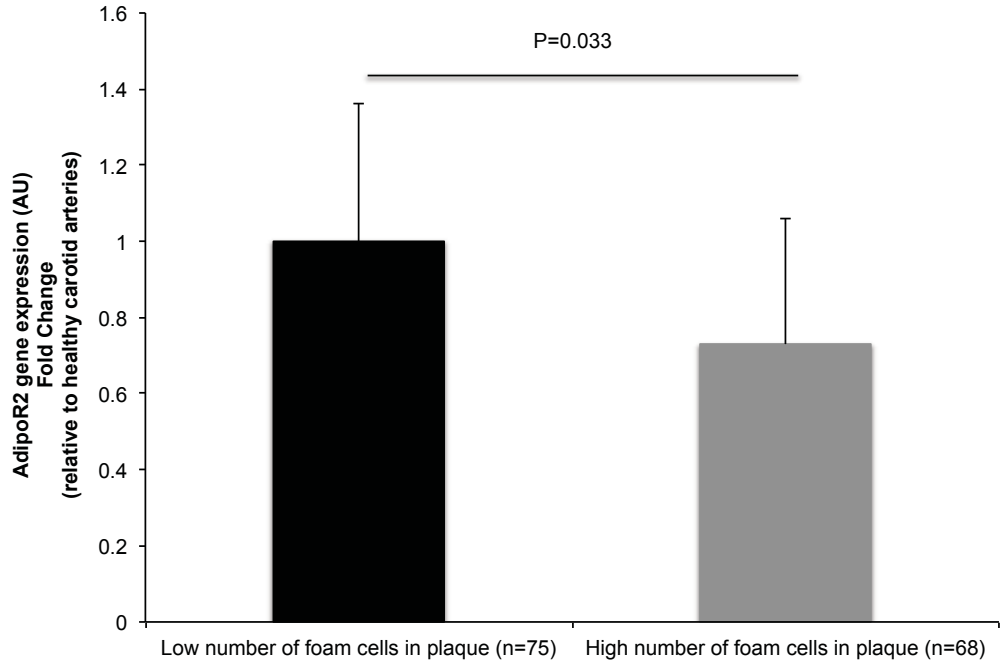


Figure 4.7 Quantitative real-time polymerase chain reaction analyses of plaque AdipoR2 in relation to foam cell presence*. Bar graph illustrates qRT-PCR data for AdipoR2 mRNA levels in relation to foam cell presence (n=143). Data represent mean ± SD. Analysis comparing high (≥50 cells) versus low presence of foam cells (<50 cells) was performed by independent t test.*[2]

Table 4.4 AdipoR mRNA levels in relation with histological features of the plaque[2]

Histological Features	AdipoR1		AdipoR2	
	*$2^{(-\Delta\Delta Cq)}$ AU	†P-value	*$2^{(-\Delta\Delta Cq)}$ AU	†P-value
Hemorrhage				
None (n=112)	1.08±0.40		0.62±0.42	
Small (n=29	1.05±0.37		0.45±0.37	
Large (n=2)	3.17±0.66	0.259	0.53±0.90	0.216
Thrombus				
None (n=109)	1.11±0.38		0.57±0.42	
Small (n=32)	1.00±0.48		0.62±0.36	
Large (n=2)	2.06±0.36	0.547	0.56±0.97	0.875
Lipid Core				
None (n=21)	0.96±0.35		0.71±0.40	
Small (n=30)	0.99±0.43		0.55±0.37	
Large (n=92)	1.16±0.40	0.550	0.56±0.42	0.516
Fibrous Tissue				
≤50% (n=93)	1.15±0.40		0.56±0.42	
>50% (n=50)	0.99±0.39	0.345	0.70±0.38	0.554
Foam Cells				
<50 (n=75)	1.05±0.39		0.67±0.38	
≥50 (n=68)	1.14±0.40	0.565	0.49±0.46	0.033
New Vessels				
None (n=16)	1.18±0.33		0.49±0.45	
<10 (n=53)	0.97±0.42		0.55±0.37	
≥10 (n=74)	1.17±0.39	0.497	0.62±0.43	0.571
Calcification				
None (n=20)	0.92±0.48		0.47±0.38	
Stippling only (n=76)	1.06±0.40	0.448	0.57±0.40	0.386
Calcified nodules (n=47)	1.24±0.36		0.65±0.44	
‡Inflammatory Cells				
≤5 groups of >50 cells (n=50)	1.14±0.36		0.62±0.41	
>5 groups of >50 cells or 1 group >500 cells (n=93)	1.07±0.41	0.680	0.56±0.41	0.471
‡Cap Infiltration				
None	1.13±0.34		0.68±0.43	
≤50 cells in cap	1.05±0.41		0.63±0.38	
>50 cells in cap	1.13±0.41	0.912	0.45±0.49	0.078
Rupture				
Intact cap (n=105)	1.01±0.43		0.60±0.42	
Probable rupture (n=29)	1.36±0.32		0.49±0.36	
Definite rupture (n=9)	1.34±0.38	0.241	0.61±0.40	0.498

AU, arbitrary units
*$2^{(-\Delta\Delta Cq)}$ values presented are normalized to healthy controls
†P-value indicates significance across all groups within a specific histological feature
‡Inflammation graded according to the number of macrophages and lymphocytes present

4.4.5 Adiponectin-mediated signalling in relation to plaque instability

A graded decrease in PPAR-α protein levels was observed in relation to carotid plaque instability (P<0.001; **Figure 4.8B**). A 1.92- and 1.84-fold reduction in PPAR-α was noted in definitely unstable plaques when compared to healthy carotid arteries and definitely stable plaques, respectively (P<0.001; **Figure 4.8B**), while AMPK phosphorylation (activity) was significantly increased in definitely unstable plaques (P<0.05; **Figure 4.8C**).

4.4.6 Cellular localization of adiponectin, AdipoR1, and AdipoR2

Healthy carotid arteries. Using immunohistochemistry, adiponectin was highly detected in the endothelial layer and to a lower extent in the SMCs. AdipoR1 and AdipoR2 were also found expressed in endothelial cells and SMCs (**Figure 4.9**).

Carotid atherosclerotic plaques. Adiponectin, AdipoR1, and AdipoR2 were expressed in endothelial cells (**Figure 4.10**) and vascular SMCs (**Figure 4.11**). Importantly, expression of adiponectin, AdipoR1, and AdipoR2 was most abundant in macrophage and foam cell-rich areas in the fibrous cap and surrounding the lipid core (**Figure 4.12**). Immunofluorescence staining demonstrated colocalization of AdipoR1 and AdipoR2 with CD68 (**Figures 4.13** and **4.14**), a marker for macrophage/foam cells, indicating that both receptors are expressed on macrophages/foam cells in human atherosclerotic plaques.

4.4.7 In vitro model of plaque instability

THP-1 macrophages upon treatment with adiponectin resulted in a significant up-regulation in AdipoR2 and PPAR-α protein expression (P<0.01; **Figure 4.15B**) compared with non-treated macrophages, while no change in expression was observed among foam cells treated with adiponectin (**Figure 4.15C**). However, adiponectin treatment of both macrophages and foam cells did not result in a significant change in AdipoR1 expression or activity (AMPK phosphorylation) (**Figure 4.15B** and **4.15C**). **Figure 4.15D** presents the absolute change in expression of AdipoR1, AdipoR2, AMPK-phosphorylated, and PPAR-α between treated and non-treated macrophages and foam cells. Furthermore, in the presence of adiponectin, foam cells had significantly lower AdipoR2 and PPAR-α expression than macrophages (P<0.001; **Figure 4.15E**), while in the absence of adiponectin, foam cells and macrophages demonstrated comparable AdipoR2 and PPAR-α protein levels than macrophages (**Figure 4.15F**). However, AMPK phosphorylation was observed to be significantly higher in foam cells treated with adiponectin when compared to

macrophages treated with adiponectin (P<0.05; **Figure 4.15E**), whereas no significant change in AdipoR1 was observed. We noted a similar significant increase in AMPK phosphorylation in foam cells not treated with adiponectin when compared to macrophages not treated with adiponectin (P<0.05; **Figure 4.15F**), with no observed changes in AdipoR1 expression. A summary diagram of these results is presented in **Figure 4.16**.

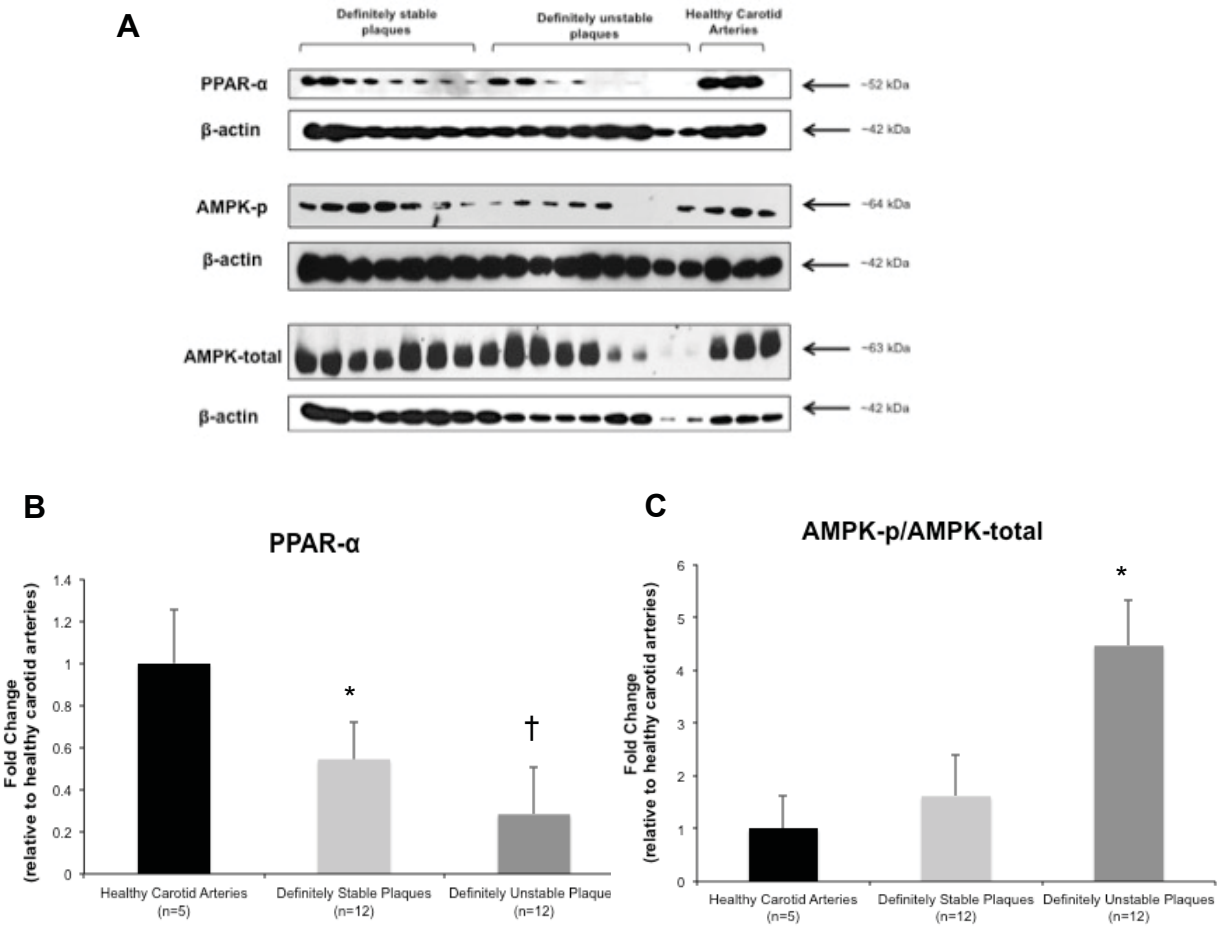


***Figure 4.8 Western Blot analyses of adiponectin-mediated signalling pathways**. **A)** Representative Western Blot showing expression of PPAR-α, AMPK-phosphorylated, and AMPK-total in human healthy carotid arteries (n=5) and carotid atherosclerotic plaques (definitely stable [n=12] and definitely unstable plaques [n=12]). **B)** Changes in PPAR-α protein expression were determined by normalizing against the densitometric intensity of β-actin. Data represent mean ± SD. Analysis comparing groups was performed by ANOVA. *P<0.001 (Tukey's multiple comparisons test between definitely stable plaques and healthy carotid arteries). †P<0.001 (between definitely unstable plaques and definitely stable plaques, and between definitely unstable plaques and healthy carotid arteries). **C)** Changes in AMPK activity were determined using the*

*ratio of AMPK-phosphorylated to AMPK-total protein expression. Data represent mean ± SD. Analysis comparing groups was performed by ANOVA. *P<0.05 (Tukey's multiple comparisons test between definitely unstable plaques and definitely stable plaques, and between definitely unstable plaques and healthy carotid arteries).*[2]

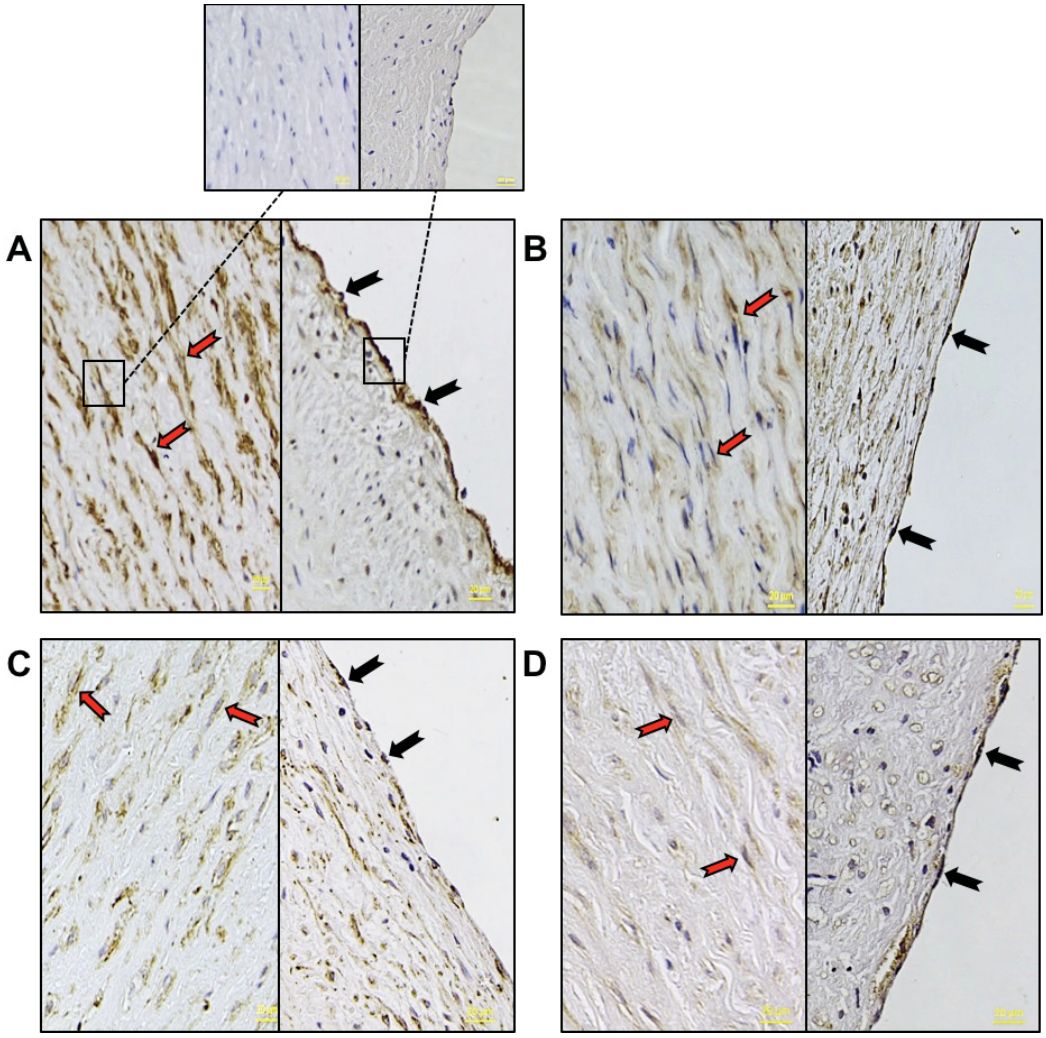


Figure 4.9 Cellular localization of adiponectin, AdipoR1, and AdipoR2 in sections of healthy human carotid arteries*. Representative photomicrographs demonstrating immunohistochemical staining for adiponectin, AdipoR1, and AdipoR2, visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, brown). Images were photographed at magnification x400. Inlets: close-up images were taken at magnification x400.* ***A)*** *Left panel = α-SMC actin (SMC marker), right panel = von Willebrand factor (endothelial cell marker); Inlet represents negative control;* ***B)*** *adiponectin;* ***C)*** *AdipoR1;* ***D)*** *AdipoR2. Black arrows represent positive staining on endothelial cells; red arrows represent positive staining on SMCs. Scale bar represents 20μm.*[2]

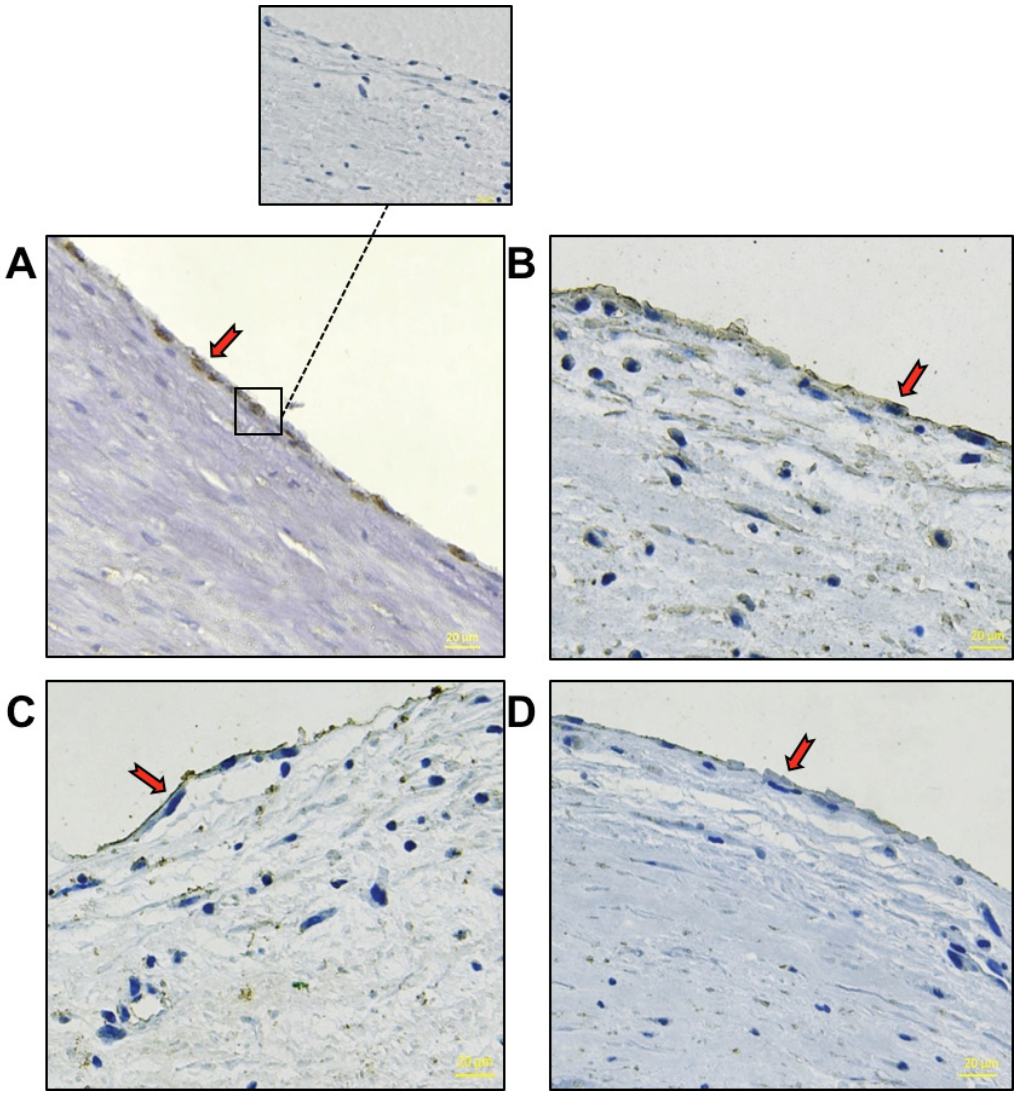


Figure 4.10 Cellular localization of adiponectin, AdipoR1, and AdipoR2 in human carotid atherosclerotic plaques – endothelial staining*. Representative photomicrographs demonstrating IHC staining on endothelial cells for adiponectin, AdipoR1, and AdipoR2 in sections of human carotid atherosclerotic plaques, visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, brown). Images were photographed at magnification x400.* ***A)*** *von Willebrand factor (endothelial cell marker); inlet represents negative control;* ***B)*** *adiponectin;* ***C)*** *AdipoR1;* ***D)*** *AdipoR2. Red arrows represent positive staining on endothelial cells. Scale bar represents 20µm.*[2]

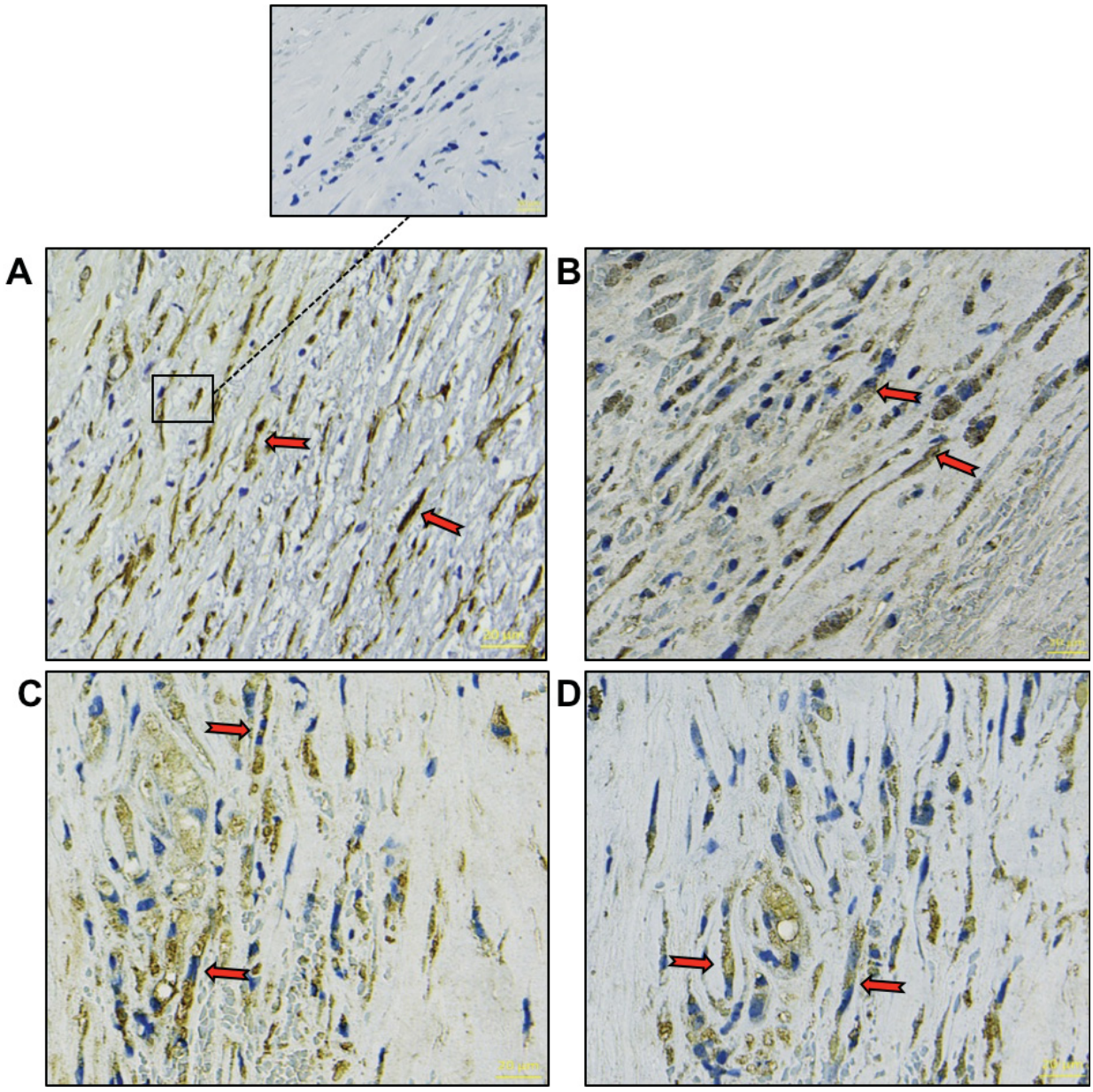


Figure 4.11 Cellular localization of adiponectin, AdipoR1, and AdipoR2 in human carotid atherosclerotic plaques – smooth muscle cell staining*. Representative photomicrographs demonstrating IHC staining on vascular smooth muscle cells for adiponectin, AdipoR1, and AdipoR2 in sections of human carotid atherosclerotic plaques, visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, brown). Images were photographed at magnification x400.* ***A)*** *α-smooth muscle cell actin (smooth muscle cell marker); inlet represents negative control;* ***B)*** *adiponectin;* ***C)*** *AdipoR1;* ***D)*** *AdipoR2. Red arrows represent positive staining on smooth muscle cells. Scale bar represents 20µm.*[2]

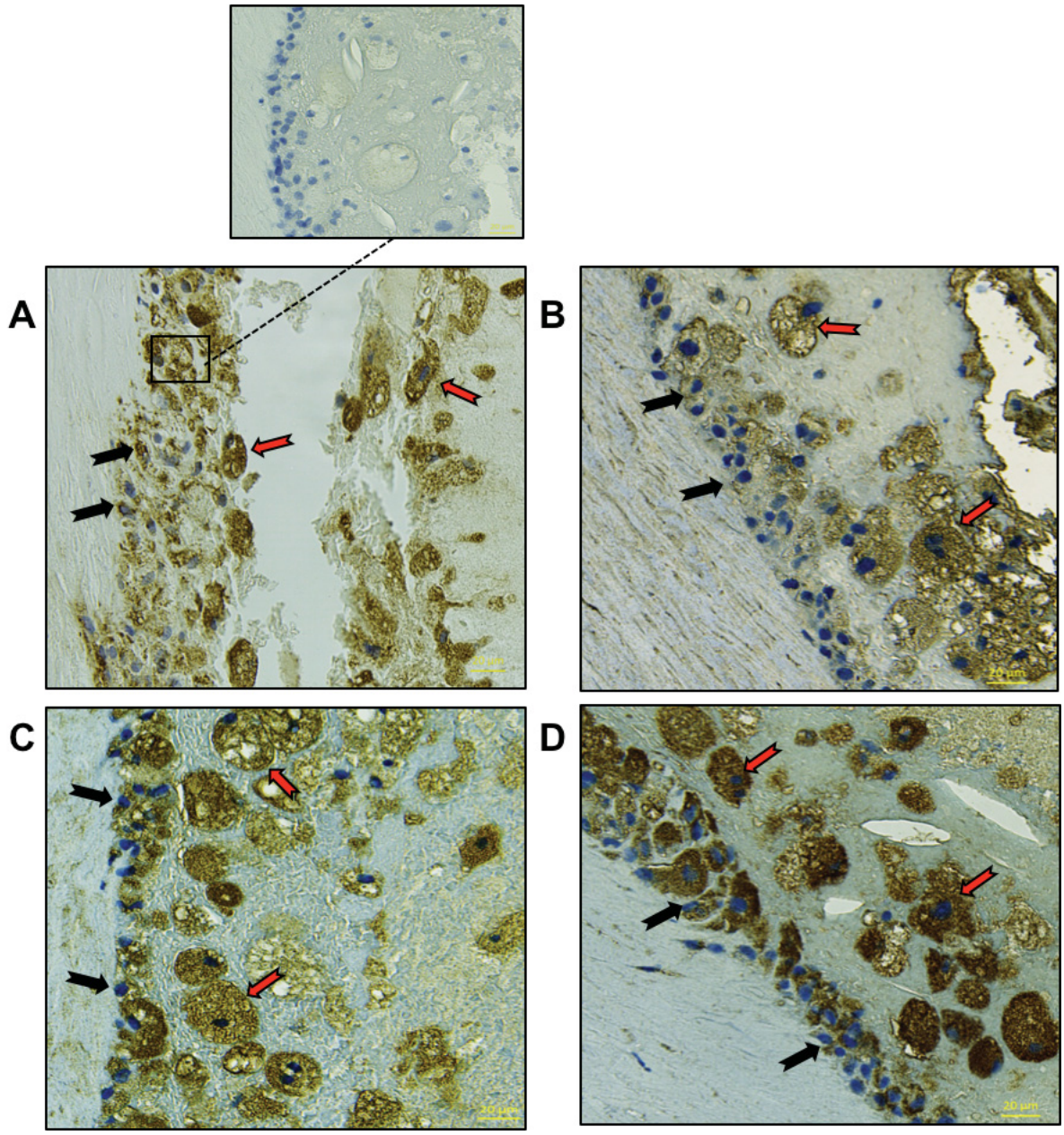


Figure 4.12 Cellular localization of adiponectin, AdipoR1, and AdipoR2 in human carotid atherosclerotic plaques – macrophage/foam cell staining. *Representative photomicrographs demonstrating immunohistochemical staining for adiponectin, AdipoR1, and AdipoR2, visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, brown). Images were photographed at magnification x400.* ***A)*** *CD68 (macrophage/foam cell marker); inlet represents negative control;* ***B)*** *adiponectin;* ***C)*** *AdipoR1;* ***D)*** *AdipoR2. Black arrows represent positive staining on macrophages; red arrows represent positive staining on foam cells. Scale bar represents 20µm.*[2]

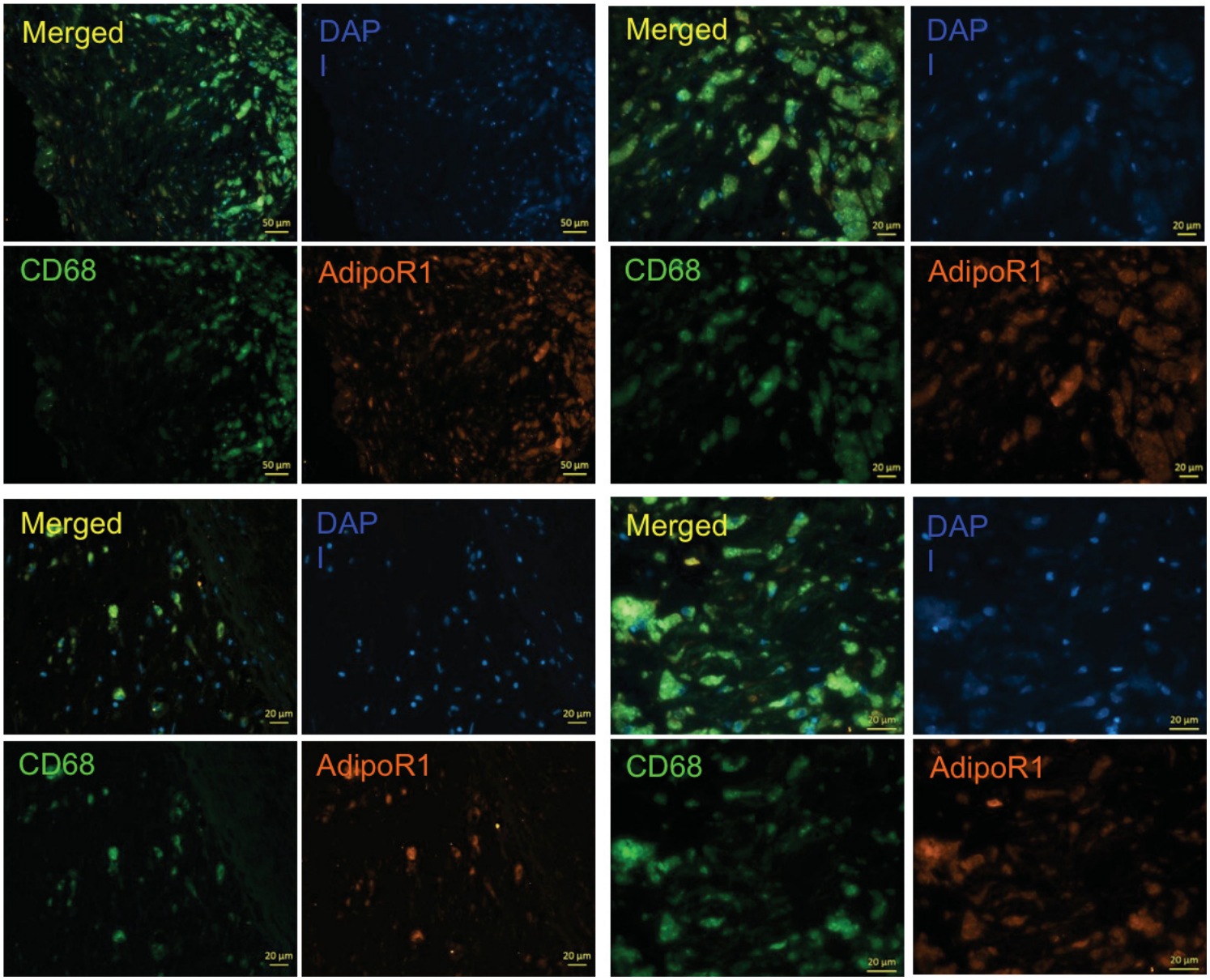


Figure 4.13 Colocalization of AdipoR1 and CD68 (yellow). *Representative immunofluorescent micrographs demonstrating staining for AdipoR1 (orange) and CD68 – macrophage marker (green) with DAPI-stained nuclei (blue) in carotid atherosclerotic plaques. Images were photographed at magnification x200, x400, or x630.*[2]

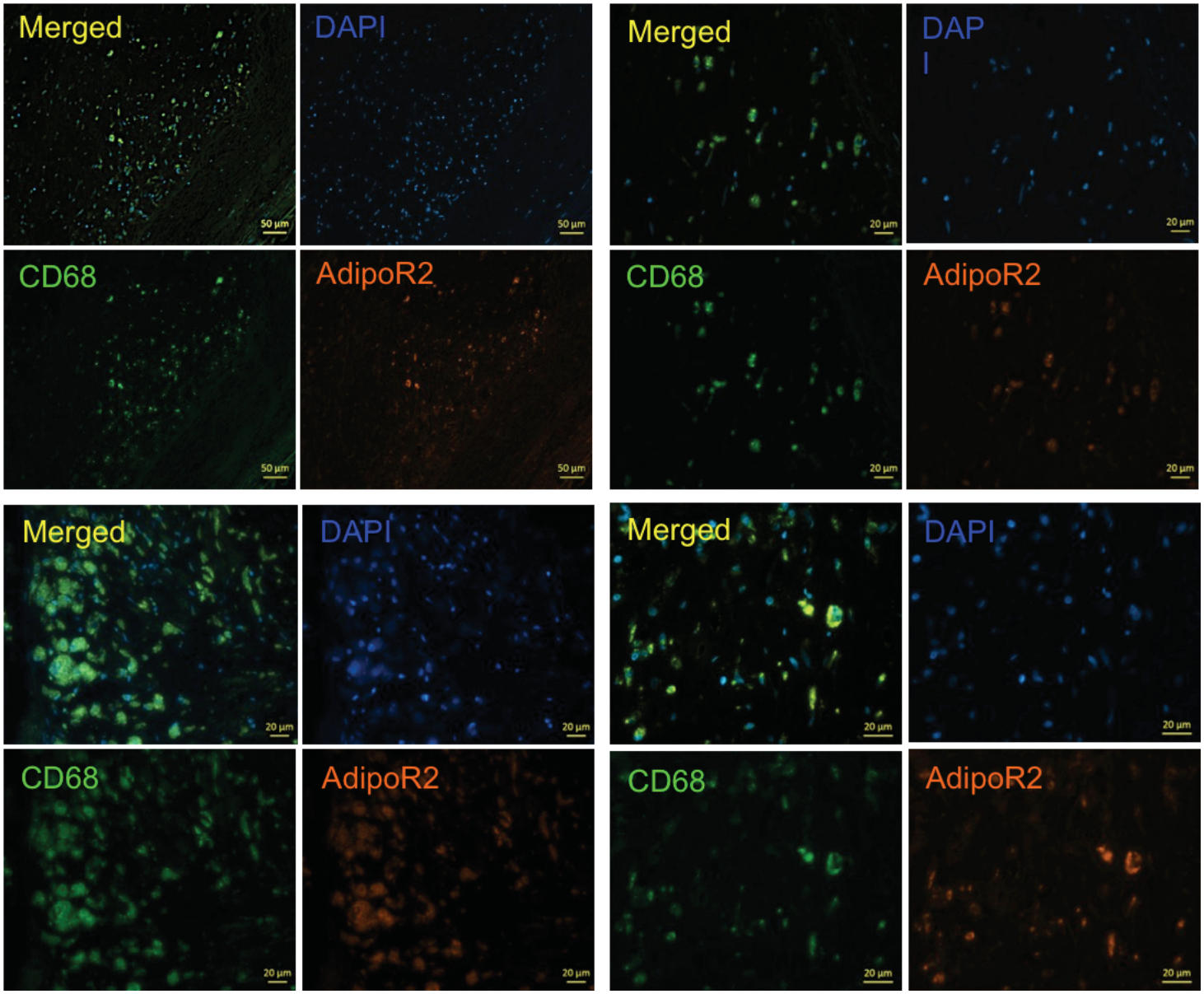


Figure 4.14 Colocalization of AdipoR2 and CD68 (yellow). *Representative immunofluorescent micrographs demonstrating staining for AdipoR2 (orange) and CD68 – macrophage marker (green) with DAPI-stained nuclei (blue) in carotid atherosclerotic plaques. Images were photographed at magnification x200, x400, or x630.*[2]

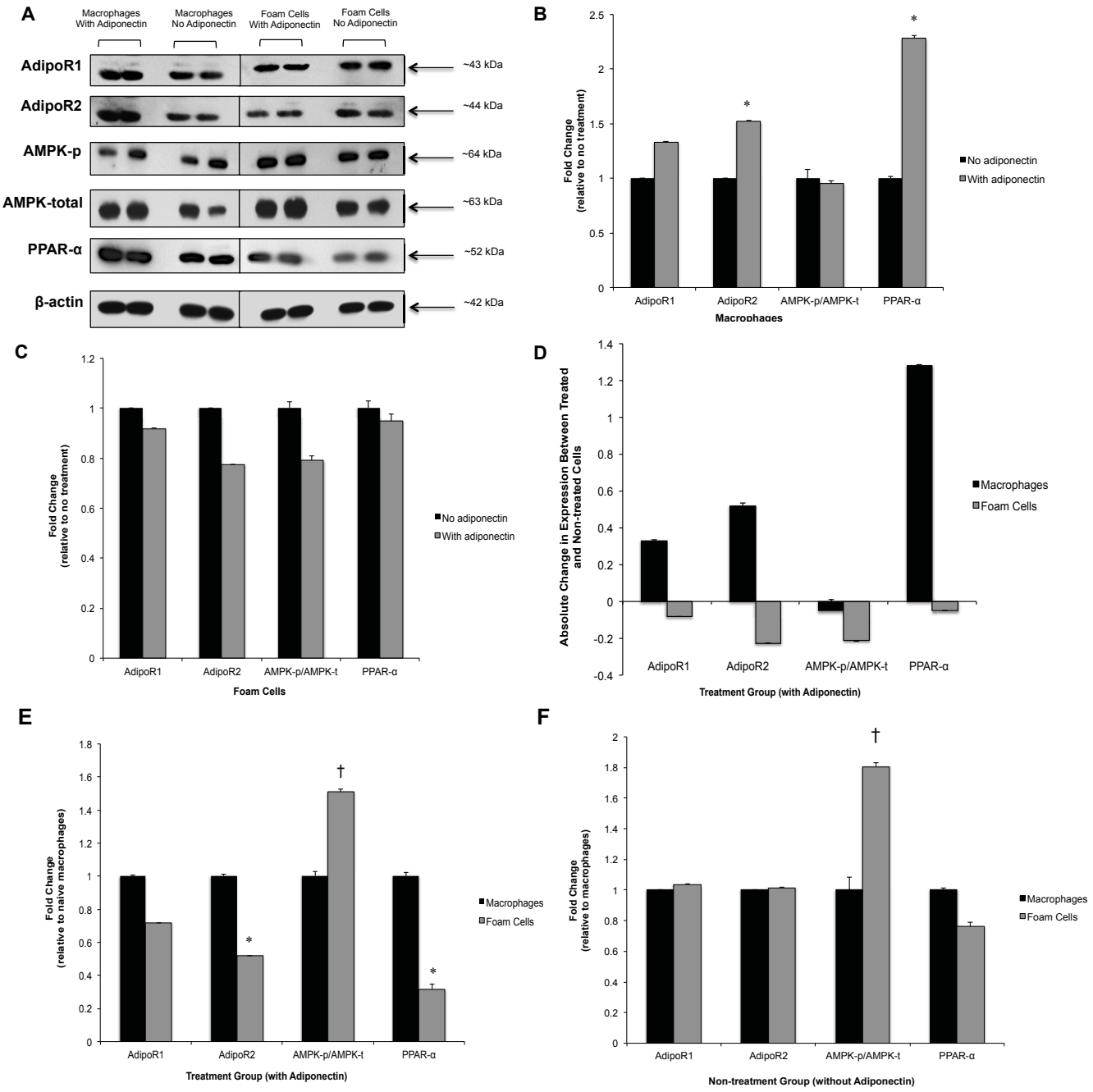


Figure 4.15 In vitro model of plaque instability*. **A)** Representative Western Blot showing expression of AdipoR1, AdipoR2, AMPK-phosphorylated, AMPK-total, and PPAR-α, in THP-1 macrophages or THP-1 derived foam cells incubated in the presence or absence of adiponectin. Samples were analyzed in duplicate. The dividing lines illustrate the grouping of images from different parts of the same gel. **B)** Comparing changes in protein expression between macrophages treated and non-treated with adiponectin, which were determined by normalizing against the*

*densitometric intensity of β-actin. Changes in AMPK activity were determined using the ratio of AMPK-phosphorylated to AMPK-total protein expression. Data represent mean ± SD. Analysis comparing treatment versus non-treatment groups was determined by independent t test. *P<0.01.* ***C)*** *Comparing changes in protein expression between foam cells treated and non-treated with adiponectin. Data represent mean ± SD. Analysis comparing treatment versus non-treatment groups was determined by independent t test.* ***D)*** *Bar graphs represent absolute changes in protein expression between adiponectin treated and non-treated macrophages and foam cells. Data represent mean ± SD.* ***E)*** *Comparing changes in protein expression between foam cells and macrophages treated with adiponectin. Data represent mean ± SD. Analysis comparing foam cells versus macrophages was determined by independent t test.* ***F)*** *Comparing changes in protein expression between foam cells and macrophages not treated with adiponectin. Data represent mean ± SD. Analysis comparing foam cells versus macrophages was determined by independent t test. *P<0.001; †P<0.05.*[2]

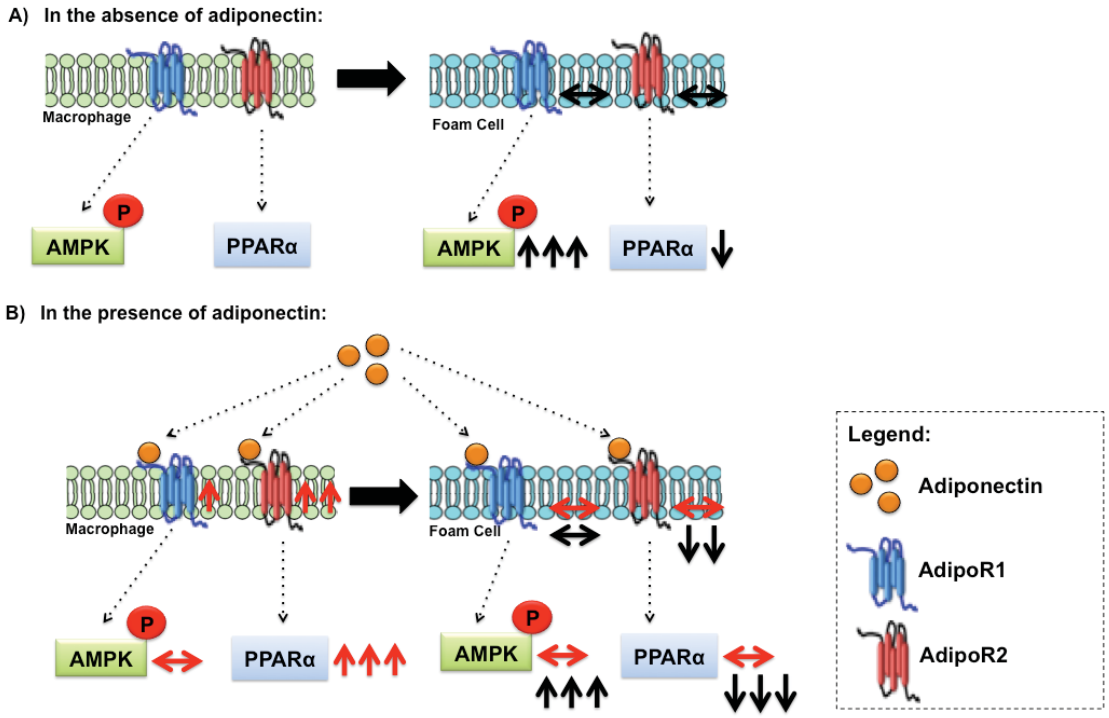


Figure 4.16 Summary diagram for in vitro model of plaque instability*. In panel A, the black arrows depict the direction in the change of expression for AdipoR1/AdipoR2 and its downstream signalling factors, AMPK/PPAR-α, following the transformation of macrophages into foam cells in the absence of adiponectin. The number of arrows represents the strength of the change in*

expression. AdipoR1 and AdipoR2 expression did not differ, while there was a slight decrease in PPAR-α and a significant increase in AMPK phosphorylation. In panel B, the black arrows depict the direction in the change of expression for AdipoR1/AdipoR2 and its downstream signalling factors, AMPK/PPAR-α, following the transformation of macrophages into foam cells in the presence of adiponectin. No change in expression was observed for AdipoR1, while a significant decrease was noted for AdipoR2 expression and PPAR-α and an increase for AMPK phosphorylation. The red arrows depict the direction in the change of expression for AdipoR1/AdipoR2 and its downstream signalling factors, AMPK/PPAR-α, on macrophages or foam cells upon treatment with adiponectin. The number of arrows represents the strength of the change in expression. Adiponectin treatment of macrophages led to a significant increase in AdipoR2 and PPAR-α expression, a slight increase in AdipoR1, and no change in AMPK phosphorylation. On the other hand, adiponectin treatment of foam cells resulted in no change in expression of AdipoR1, AdipoR2, AMPK, or PPAR-α.[2]

4.5 Discussion

This study represents an essential step in identifying a novel association between decreased adiponectin-mediated signaling through the AdipoR2 pathway and plaque instability. We identified that definitely unstable plaques express more adiponectin and less overall AdipoR2 than definitely stable plaques, while AdipoR1 expression remained similar among all groups of plaque instability. In parallel, we noted a decrease in AdipoR2 activity, specifically through PPAR-α, in definitely unstable plaques, whereas an up-regulation was observed in AMPK phosphorylation, which is a downstream target of AdipoR1. Furthermore, we created an *in vitro* model of plaque instability, using human THP-1 macrophages, which supported our *in vivo* hypotheses.

Adiponectin exerts atheroprotective and anti-inflammatory effects on the vasculature[290]. It can attenuate monocyte attachment and migration into the intima, suppress macrophage transformation into foam cells, and induce the expression of TIMP-1, ultimately reducing the risk of a rupture in the fibrous cap[293,296,300]. Its deficiency, both clinically and experimentally, plays a significant role in the development of various vascular complications. Hypoadiponectinemia is associated with endothelial dysfunction, CAD, and progression of carotid atherosclerosis. In animal models, adiponectin deficiency enhances neointimal formation in response to acute vascular damage and impaired endothelium-dependent vasodilation, whereas treatment with

adiponectin decreases atherosclerotic lesion formation in apoE$^{-/-}$ mice and in a rabbit model of abdominal aortic atherosclerosis[265,266,286,288,535,536].

Adiponectin protein has been detected in the vasculature of normal and atherosclerotic mice[319]. We demonstrated that adiponectin protein is also present in healthy human carotid arteries, as well as human carotid atherosclerotic plaques. Interestingly, we noted a lack of adiponectin mRNA in healthy arteries (comprised of only the intimal and medial layer) and carotid plaques. This indicates that adiponectin protein in the plaque area or in the intimal and medial layer of healthy vasculature is not because of de novo cellular expression. Instead, adiponectin must enter these layers from outside sources[251,319,530]. Indeed, one murine study assessing the ultrastructural localization of adiponectin within endothelial cells of the aortic wall, observed the presence of adiponectin in endocytic vesicles, suggesting that adiponectin undergoes endocytosis from the circulation into the endothelial cells; however the mechanism through which adiponectin endocytosis occurs is currently unknown[319]. Others have suggested that adiponectin in the vascular wall is derived from the adventitia and surrounding PVAT, an abundant producer and secretor of adiponectin[251,530]. Herein, we have confirmed the abundant expression of adiponectin in PVAT (surrounding healthy carotid arteries), using qRT-PCR and immunohistochemical analyses (**Figures 4.4** and **4.17**).

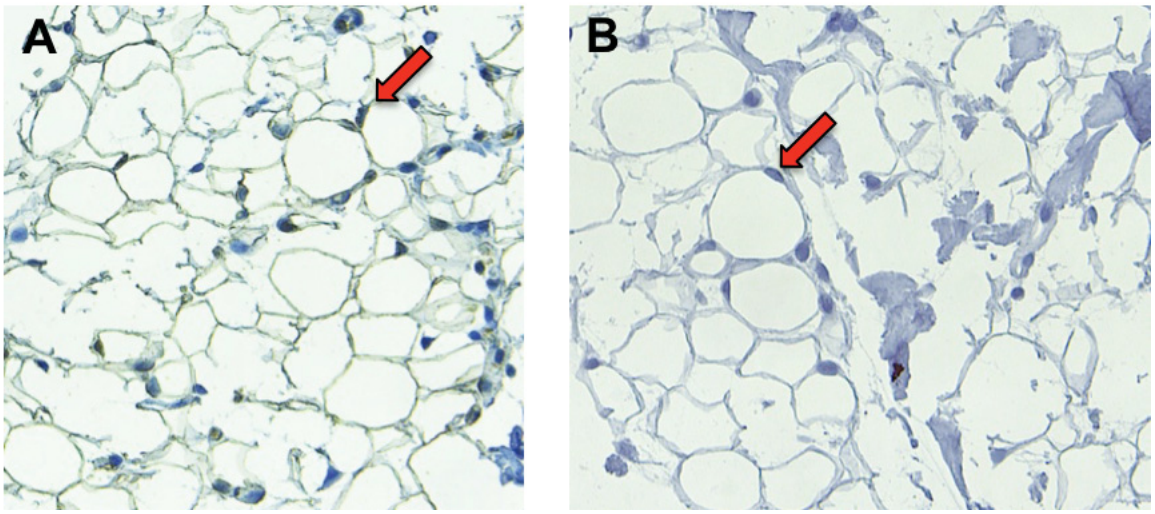


Figure 4.17 Adiponectin protein expression in human perivascular adipose tissue*. Representative photomicrographs demonstrating immunohistochemical staining on adipocytes from PVAT (that was removed from healthy carotid arteries).* ***A)*** *Immunohistochemical staining for adiponectin,* ***B)*** *Negative control. Staining was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, brown). Red arrows represent adipocytes.*[2]

Furthermore, in our study, immunohistochemical staining of healthy carotid arteries demonstrated that adiponectin protein is highly localized to the endothelial layer and to a lower extent on SMCs, whereas in carotid plaques, adiponectin was mainly detected in areas of high macrophage and foam cell abundance. Differences in the localization pattern of adiponectin have also been detected within the aortic wall of normal versus atherosclerotic mice[319]. We are the first to observe an increase in adiponectin expression within atherosclerotic plaques that exhibit unstable features when compared to definitely stable plaques or healthy arteries. Lending support to the hypothesis that the origin of adiponectin in the plaque may be derived from the surrounding PVAT, a recent study demonstrated higher adiponectin expression in the PVAT of neurologically symptomatic patients versus asymptomatic patients who underwent a CEA[530]. Our findings, along with previous experimental evidence[296,537], suggest that adiponectin may accumulate in unstable lesions or in the damaged vascular wall as a protective mechanism in response to injury, or because of increased endothelial permeability that is associated with vascular damage and atherosclerotic lesion progression[538].

The beneficial effects of adiponectin are mediated through its receptors, AdipoR1 and AdipoR2[305,313], which we observed to be expressed most abundantly on macrophages/foam cells, and to a lower extent on endothelial and SMCs in atherosclerotic plaques. An overall decrease in AdipoR2 expression was noted in definitely unstable plaques. Furthermore, AdipoR2 signaling through PPAR-α was significantly impaired in definitely unstable plaques, suggesting not only a decrease in AdipoR2 expression but also in its activity. In contrast, AdipoR1 expression remained unchanged between definitely stable and definitely unstable plaques. However, a significant up-regulation in AMPK-phosphorylated activity was observed in association with greater plaque instability. Although PPAR-α and AMPK are main downstream signaling components of AdipoR1 and AdipoR2, respectively, they are not limited to these receptors, and can also be triggered by other factors present in carotid lesions[539,540]. Thus, we used the *in vitro* model of foam cell formation (a critical process of plaque instability), to confirm our *in vivo* observations.

Upon treatment with recombinant adiponectin, a significant up-regulation in AdipoR2 and PPAR-α protein expression was observed in macrophages but resulted in no change in AdipoR1 expression and AMPK activity. The recombinant adiponectin used was primarily composed of the HMW isoforms of adiponectin (**Figure 4.1**), thus, it was not surprising to observe significant changes in the AdipoR2 pathway but not in AdipoR1 since AdipoR1 possesses lower affinity than AdipoR2 for the HMW isoforms of adiponectin[305]. AdipoR2 or PPAR-α expression was not up-

regulated in adiponectin-treated foam cells, suggesting impairment in the response of AdipoR2 to its ligand and a resulting loss in its downstream signaling activity. The baseline values (i.e., in the absence of adiponectin) of AdipoR2 and PPAR-α remained unchanged following the transformation of macrophages into foam cells. However, the response of foam cells to adiponectin was impaired upon transformation from macrophages, resulting in decreased AdipoR2 expression and activity (through PPAR-α) compared with adiponectin-treated macrophages (summary diagram, **Figure 4.18**), which mirrors the decrease in expression noted between definitely unstable plaques (i.e., contains greater presence of foam cells), definitely stable plaques, and healthy carotid arteries. We hypothesize that decreased signaling downstream of AdipoR2 may impair the atheroprotective actions of adiponectin and cause adiponectin resistance, thereby contributing to plaque instability and an accumulation of adiponectin in vulnerable lesions. In contrast, AMPK activity was up-regulated in adiponectin-treated foam cells compared to macrophages, whereas no changes in AdipoR1 were noted, as similarly observed in definitely unstable versus definitely stable plaques. However, this increase in AMPK activity was also noted between foam cells and macrophages not treated with adiponectin. Thus, we think that the increase in AMPK activity associated with foam cell induction, as well as plaque instability, is independent of the adiponectin-AdipoR1 pathway.

Interestingly, previous evidence supports a differential role of the two receptors. In mice, the two receptors demonstrated opposite effects on glucose tolerance and energy expenditure, where AdipoR2 deficiency promoted diabetes[531,541]. However, sufficient evidence remains lacking to elucidate their independent role in atherosclerotic disease.

Our study has several strengths, including the use of human carotid arteries, pointing to its translational aspect, the combination of *in vivo* and *in vitro* experiments to unravel the observed associations, and the novelty of the topic in the area of atherosclerotic disease. We acknowledge that our study also contains limitations. No causative associations can be determined; since the study analyzed carotid specimens, a cross-sectional design was inevitable. Furthermore, as we used human data, adiponectin and its receptors can be influenced by several factors. However, since the stable and unstable patient groups were similar in terms of patient comorbidities and clinical characteristics, and analyses performed according to specific clinical covariates did not affect the significance of the results, we are confident that the observed differences are not because of inter-individual variability. Furthermore, our *in vitro* experiments led support to our *in vivo* findings.

Additionally, although beyond the scope of this study, another limitation is that the origin of adiponectin in the plaque could not be determined, as it is not feasible to collect PVAT from our CEA subjects. A recently published article demonstrated higher adiponectin expression in the PVAT (surrounding the carotid plaques) of neurologically symptomatic patients versus asymptomatic patients who underwent a CEA[530], thus mirroring the elevation in adiponectin protein levels we observed in unstable versus stable carotid plaques versus healthy carotid arteries. To provide some supporting evidence, we performed qRT-PCR analysis on the removed PVAT that was surrounding the healthy carotid arteries and demonstrated that the adiponectin mRNA expression was a 1100-fold higher than in healthy carotid arteries and in carotid plaques (**Figure 4.4**), confirming that adiponectin is highly expressed by PVAT and not by plaque tissue or the healthy vasculature that has been stripped of its surrounding adventitial and adipose tissue layers. We also performed immunohistochemical analyses of the removed PVAT, which demonstrated adiponectin protein to be expressed by the adipocytes (**Figure 4.17**).

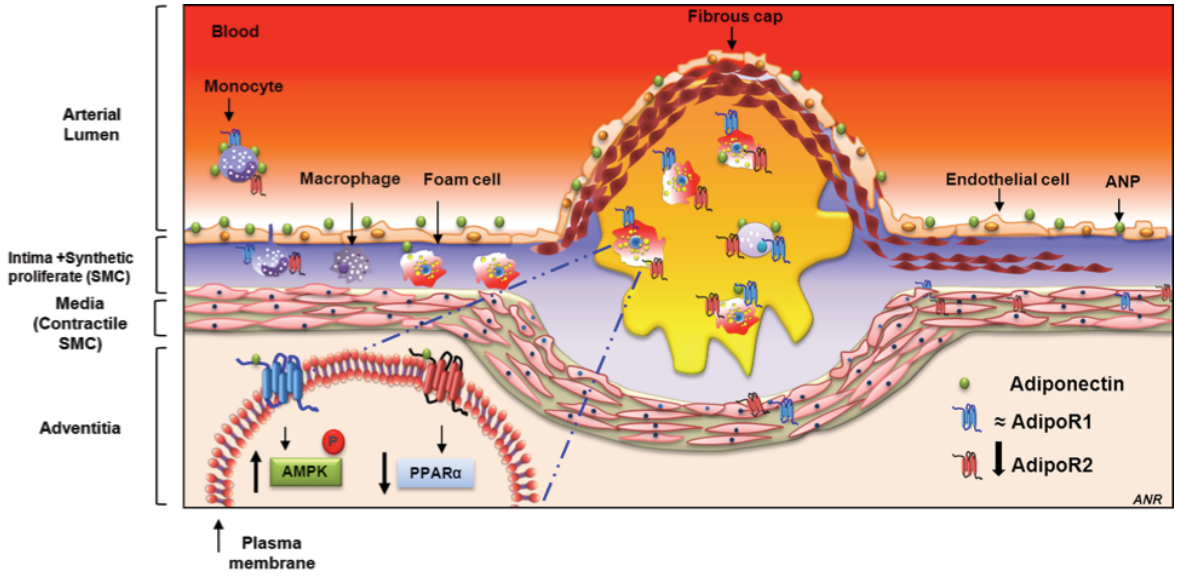


Figure 4.18 Summary model of the adiponectin-AdipoR1 and -AdipoR2 signaling pathway in an unstable carotid plaque*. To summarize, unstable carotid plaques had less AdipoR2 expression than stable plaques, while AdipoR1 expression remained similar. Furthermore, a graded decrease in PPAR-α protein levels was observed in relation to carotid plaque instability, while AMPK phosphorylation was increased. Adiponectin, and AdipoR1/R2 protein expression was mainly detected in endothelial cells, intimal SMCs, and macrophages and foam cells.*[2]

4.6 Conclusion

Our findings suggest that a decrease in AdipoR2 signaling in unstable plaques may define a state of adiponectin resistance, which partly explains the compensatory elevation in adiponectin levels observed in unstable plaques. This study including a large number of carotid plaque specimens provided human evidence that AdipoR2 may be a key player in the context of plaque instability, while the adiponectin-AdipoR1 pathway plays no significant role (summary model in **Figure 4.18**). However, as this is a relatively untapped area of research, investigations are ongoing to further elucidate the distinct effect of AdipoR2 on plaque instability.

4.7 Acknowledgements

We are grateful to the vascular surgeons (Drs Steinmetz, MacKenzie, Corriveau, and Obrand) at the McGill University Health Centre and Jewish General Hospital, in Montreal, Canada, as well as Ms Gorgui and Ms Gomez for their help with subject recruitment.

4.8 Funding Sources

The work was supported by the Fonds de recherche du Québec-Santé (FRQ-S) [20058] and the Research Institute of the McGill University Health Centre, as well as the Canadian Institutes of Health Research (CIHR) Catalyst Grant [360043] and the CIHR Project Scheme Grant [367403]. Dr Daskalopoulou is a Chercheur-Boursier Clinicien supported by the FRQ-S. K Gasbarrino is supported by a CIHR Doctoral Studentship. The funding sources had no involvement in the study.

4.9 Disclosures

None

4.10 References

1. Gasbarrino K, Mantzoros C, Gorgui J, Veinot JP, Lai C, Daskalopoulou SS. Circulating chemerin is associated with carotid plaque instability, whereas resistin is related to cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2016

176. Stary HC. Natural history and histological classification of atherosclerotic lesions: An update. *Arterioscler Thromb Vasc Biol*. 2000:20:1177-1178.

177. Lovett JK, Gallagher PJ, Hands LJ, Walton J, Rothwell PM. Histological correlates of carotid plaque surface morphology on lumen contrast imaging. *Circulation*. 2004:110:2190-2197.

178. Lovett JK, Gallagher PJ, Rothwell PM. Reproducibility of histological assessment of carotid plaque: Implications for studies of carotid imaging. *Cerebrovasc Dis*. 2004:18:117-123.
188. Tilg H, Moschen AR. Adipocytokines: Mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol*. 2006:6:772-783.
251. Nigro E, Scudiero O, Monaco ML, Palmieri A, Mazzarella G, Costagliola C, Bianco A, Daniele A. New insight into adiponectin role in obesity and obesity-related diseases. *Biomed Res Int*. 2014:2014:658913.
265. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y, Osaka CADSGCad. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol*. 2003:23:85-89.
266. Hui E, Xu A, Chow WS, Lee PC, Fong CH, Cheung SC, Tse HF, Chau MT, Cheung BM, Lam KS. Hypoadiponectinemia as an independent predictor for the progression of carotid atherosclerosis: A 5-year prospective study. *Metab Syndr Relat Disord*. 2014:12:517-522.
269. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA. Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab*. 2001:86:1930-1935.
286. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y. Adiponectin reduces atherosclerosis in apolipoprotein e-deficient mice. *Circulation*. 2002:106:2767-2770.
288. Kubota N, Terauchi Y, Yamauchi T et.al. Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem*. 2002:277:25863-25866.
290. Zhu W, Cheng KK, Vanhoutte PM, Lam KS, Xu A. Vascular effects of adiponectin: Molecular mechanisms and potential therapeutic intervention. *Clin Sci (Lond)*. 2008:114:361-374.
293. Ouedraogo R, Gong Y, Berzins B, Wu X, Mahadev K, Hough K, Chan L, Goldstein BJ, Scalia R. Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo. *J Clin Invest*. 2007:117:1718-1726.
296. Ouchi N, Kihara S, Arita Y et.al. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class a scavenger receptor expression in human monocyte-derived macrophages. *Circulation*. 2001:103:1057-1063.
300. Kumada M, Kihara S, Ouchi N, Kobayashi H, Okamoto Y, Ohashi K, Maeda K, Nagaretani H, Kishida K, Maeda N, Nagasawa A, Funahashi T, Matsuzawa Y. Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. *Circulation*. 2004:109:2046-2049.
305. Yamauchi T, Kamon J, Ito Y et.al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature*. 2003:423:762-769.
313. Yamauchi T, Nio Y, Maki T et.al. Targeted disruption of adipor1 and adipor2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med*. 2007:13:332-339.
319. Mori T, Koyama Y, Maeda N, Nakamura Y, Fujishima Y, Matsuda K, Funahashi T, Shimada S, Shimomura I. Ultrastructural localization of adiponectin protein in vasculature of normal and atherosclerotic mice. *Sci Rep*. 2014:4:4895.
439. Lee RT, Libby P. The unstable atheroma. *Arterioscler Thromb Vasc Biol*. 1997:17:1859-1867.

498. Fukuyama N, Homma K, Wakana N, Kudo K, Suyama A, Ohazama H, Tsuji C, Ishiwata K, Eguchi Y, Nakazawa H, Tanaka E. Validation of the friedewald equation for evaluation of plasma ldl-cholesterol. *J Clin Biochem Nutr*. 2008:43:1-5.
530. Sharma G, Tao M, Ding K, Yu D, King W, Deyneko G, Wang X, Longchamp A, Schoen FJ, Ozaki CK, Semel ME. Perivascular adipose adiponectin correlates with symptom status of patients undergoing carotid endarterectomy. *Stroke*. 2015:46:1696-1699.
531. Bjursell M, Ahnmark A, Bohlooly YM, William-Olsson L, Rhedin M, Peng XR, Ploj K, Gerdin AK, Arnerup G, Elmgren A, Berg AL, Oscarsson J, Linden D. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes*. 2007:56:583-593.
532. Hafiane A, Bielicki JK, Johansson JO, Genest J. Novel apo e-derived abca1 agonist peptide (cs-6253) promotes reverse cholesterol transport and induces formation of prebeta-1 hdl in vitro. *PLoS One*. 2015:10:e0131997.
533. Basu SK, Goldstein JL, Anderson GW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A*. 1976:73:3178-3182.
534. Vera A, Recabal A, Saldivia N, Stanic K, Torrejon M, Montecinos H, Caprile T. Interaction between sco-spondin and low density lipoproteins from embryonic cerebrospinal fluid modulates their roles in early neurogenesis. *Front Neuroanat*. 2015:9:72.
535. Shimabukuro M, Higa N, Asahi T, Oshiro Y, Takasu N, Tagawa T, Ueda S, Shimomura I, Funahashi T, Matsuzawa Y. Hypoadiponectinemia is closely linked to endothelial dysfunction in man. *J Clin Endocrinol Metab*. 2003:88:3236-3240.
536. Li CJ, Sun HW, Zhu FL, Chen L, Rong YY, Zhang Y, Zhang M. Local adiponectin treatment reduces atherosclerotic plaque size in rabbits. *J Endocrinol*. 2007:193:137-145.
537. Okamoto Y, Arita Y, Nishida M, Muraguchi M, Ouchi N, Takahashi M, Igura T, Inui Y, Kihara S, Nakamura T, Yamashita S, Miyagawa J, Funahashi T, Matsuzawa Y. An adipocyte-derived plasma protein, adiponectin, adheres to injured vascular walls. *Horm Metab Res*. 2000:32:47-50.
538. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*. 2004:109:Iii27-32.
539. Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *J Adv Pharm Technol Res*. 2011:2:236-240.
540. Jeon SM. Regulation and function of ampk in physiology and diseases. *Exp Mol Med*. 2016:48:e245.
541. Liu Y, Michael MD, Kash S, Bensch WR, Monia BP, Murray SF, Otto KA, Syed SK, Bhanot S, Sloop KW, Sullivan JM, Reifel-Miller A. Deficiency of adiponectin receptor 2 reduces diet-induced insulin resistance but promotes type 2 diabetes. *Endocrinology*. 2007:148:683-692.

Connecting Text

Our results from Chapter 4 demonstrated that the local presence of adiponectin at the level of the plaque plays a more important role in the context of plaque instability than circulating levels of adiponectin (as noted in Chapter 3), as greater plaque adiponectin expression was observed in more unstable vs stable plaques. Furthermore, we identified a novel association between decreased adiponectin-mediated signalling through the AdipoR2 pathway and plaque instability. These *in vivo* observations and *in vitro* findings led us to hypothesize that defective signalling downstream of AdipoR2 may impair the atheroprotective actions of adiponectin and cause adiponectin resistance, thereby contributing to an accumulation of adiponectin in more vulnerable lesions. Since the expression of adiponectin and its receptors were observed in our study to be highly expressed in plaque macrophages, we also hypothesized that the impairment in adiponectin-AdipoR2 activity in unstable plaques occurs at the level of the macrophage.

To test these hypotheses, our next chapter investigates how modulation of the adiponectin-AdipoR pathway in the monocyte-macrophage lineage can affect the response of macrophages to adiponectin and promote plaque stability/instability. Specifically, this will be achieved by determining the role of the adiponectin-AdipoR pathway in macrophage-mediated cholesterol efflux and HDL biogenesis, under conditions that enhance or impair AdipoR activity in monocyte-derived macrophages. We have selected to use cholesterol efflux and HDL biogenesis as a proxy mechanism of plaque stability, as removal of excess cholesterol from peripheral cells (i.e., macrophages) via the RCT pathway is known to be a major protective system against the development of atherosclerotic plaques. We have previously shown in our lab that cholesterol efflux capacity is inversely associated with more severe carotid stenosis and more unstable carotid plaque morphology, suggesting that cholesterol efflux capacity may be a biomarker for atherosclerotic plaque severity and instability[108]. Importantly, circulating adiponectin levels have been strongly associated with increased cholesterol efflux capacity[295], while *in vitro* adiponectin treatment of human monocyte-derived macrophages has led to an increase in apoA-I-mediated cholesterol efflux by up-regulating ABCA1 expression, with similar findings observed in mice[295,332,340]. To further expand knowledge in this domain, our study (presented in the following chapter) aims to provide evidence to link these associations with the AdipoR pathway.

CHAPTER 5: ADIPONECTIN-ADIPOR PATHWAY STIMULATES CHOLESTEROL EFFLUX EFFICIENTLY IN HUMAN MACROPHAGES AND MODULATES HDL-APOA-I BIOGENESIS

Karina Gasbarrino, Anouar Hafiane, Huaien Zheng, Stella S. Daskalopoulou

Preliminary work

Data concerning adiponectin's effects on cholesterol efflux kinetics and HDL biogenesis were presented at Arteriosclerosis, Thrombosis, and Vascular Scientific Sessions 2016, and abstract was published in *Arterioscler Thromb Vasc Biol* **36**,1 Suppl: A28, 2016)[3]

5.1 Abstract

Introduction: Adiponectin is an anti-inflammatory and anti-atherogenic adipokine that is strongly and positively correlated with circulating HDL levels. Adiponectin exerts its effects through two receptors, AdipoR1 and AdipoR2, which are found highly expressed on macrophages. Recently, we demonstrated that decreased adiponectin-AdipoR activity is associated with atherosclerotic plaque instability. However, the role of this pathway in macrophage-mediated cholesterol efflux, a crucial process in atherogenesis, remains poorly investigated. Herein, we examined the effect of the adiponectin-AdipoR pathway on cholesterol efflux from human THP-1 macrophages, elucidated its kinetics, and investigated its role in HDL biogenesis.

Methods: Adiponectin dose-dependent (0.5 to 60 μg/mL) and time-dependent (0.5 to 24 hours) cholesterol efflux studies were performed in ^{3}[H]-cholesterol labeled human THP-1 macrophages in the presence of apoA-I. Intracellular cholesterol species were measured by thin layer chromatography. Following efflux studies, the HDL fractions within media were concentrated (10kDa cut-off filter) and subjected to analytical fast-performance liquid chromatography (FPLC) and 2D-PAGE analysis to reveal HDL species. Lentiviral short hairpin RNA (shRNA) experiments were performed to determine the contribution of the adiponectin receptors in promoting cholesterol removal.

Results: Adiponectin, in the presence of apoA-I, stimulated a specific increase in macrophage-mediated cholesterol efflux in a dose-dependent and time-dependent manner. Kinetics analysis revealed that increased doses of apoA-I incubated in the presence of adiponectin resulted in higher cholesterol efflux with greater velocity than apoA-I alone. This was associated with a significant decrease in intracellular free cholesterol and cholesterol ester content in THP-1 macrophages in the presence of adiponectin and apoA-I when compared to apoA-I alone. The FPLC cholesterol profiles and 2D-PAGE analysis demonstrated that in the presence of adiponectin and apoA-I there was increased generation of nascent HDL particles (pre-β and α-HDL species), compared to apoA-I alone. Activation of the adiponectin receptor pathway via treatment with AdipoRon, led to a similar significant increase in apoA-I-mediated cholesterol efflux as adiponectin. On the other hand, downregulation of AdipoR1 or AdipoR2 expression resulted in significant reductions in cholesterol efflux in macrophages incubated in the presence of both apoA-I and adiponectin.

Conclusions: In addition to promoting the rate-limiting step of the cholesterol efflux process**,** adiponectin can modulate HDL-apoA-I biogenesis, by increasing the generation of nascent HDL particles. AdipoR1 and AdipoR2 both play major contributions in mediating adiponectin's effects

on cholesterol efflux and HDL biogenesis. These findings suggest that combining adiponectin and apoA-I may be of potential therapeutic value in modulating HDL's protective role in atherosclerosis. Moreover, the AdipoR pathway may serve as a novel target for promoting cholesterol efflux activity.

5.2 Introduction

Atherosclerosis is a disease of the arterial wall that is characterized by the progressive accumulation of lipids, chronic inflammation, cellular debris, and fibrous connective tissue. The rupture and/or erosion of unstable atherosclerotic plaques often leads to the formation of a thrombus that can cause acute occlusion of blood flow resulting in clinical complications such as a MI or stroke. The RCT pathway is a multi-step process that plays a critical role in protecting against the development of atherosclerotic plaques. It involves the removal of excess cholesterol from peripheral tissues and orchestrates its return, via HDL particles, to the liver for excretion in the bile[102,106,107]. Our group has previously demonstrated cholesterol efflux, the initial step in the RCT pathway, to have strong, inverse associations with increased carotid artery stenosis and advanced plaque morphology in patients with severe carotid atherosclerosis[108].

Macrophages are major cellular effectors of the initiation, progression, and complication of atherosclerotic lesions[93]. Following excess cholesterol uptake and intracellular cholesterol ester accumulation, macrophages can transform into foam cells, which serve as a hallmark of atherosclerotic lesion formation. However, macrophage-specific cholesterol efflux is an atheroprotective process, which can retard and/or reverse foam cell formation[103]. Macrophages express a variety of lipid transporters implicated in cholesterol efflux, such as ABCA1, ABCG1, and SR-BI[111]. ABCA1 preferentially interacts with lipid-poor or lipid-free apoA-I, which absorbs the excess lipids, to form small nascent HDL particles[111,115]. This interaction is defined as the initial rate-limiting step of the RCT pathway. On the other hand, ABCG1 and SR-BI are responsible for mediating cholesterol efflux to already lipidated HDL particles[115]. There are a number of studies linking impaired macrophage cholesterol efflux with cardiometabolic diseases. Thus, modification/enhancement of this process may serve as a novel therapeutic approach for the prevention of atherosclerosis and its associated complications.

Adiponectin is an anti-inflammatory and anti-atherogenic adipokine that exerts its effects through two receptors, AdipoR1 and AdipoR2, which are found highly expressed on macrophages in the plaque. We have previously demonstrated that decreased adiponectin-AdipoR activity is

associated with atherosclerotic plaque instability[2]. Experimental evidence suggests that adiponectin can significantly decrease cholesterol accumulation in macrophages, thereby suppressing macrophage-to-foam cell transformation[295]. A proposed mechanism by which adiponectin attains lower intracellular cholesterol levels is by promoting an increase in cholesterol efflux capacity. Circulating adiponectin has been reported to be a strong and independent predictor of high circulating HDL levels and increased cholesterol efflux capacity[295,323-327]. Importantly, adiponectin has been previously shown to affect the RCT pathway both at the level of the liver and of the macrophage. In adiponectin knock-out mice the expression levels of apoA-I and ABCA1 in the liver were reduced[338]. Furthermore, *in vitro* treatment of human monocyte-derived macrophages with adiponectin led to a significant increase in apoA-I-mediated cholesterol efflux by up-regulating ABCA1 expression[332,340]. However, there remains limited knowledge concerning the efficiency and therapeutic potential of combining adiponectin with apoA-I to promote cholesterol efflux and HDL biogenesis. Moreover, AdipoR's contribution to adiponectin's effects on the RCT system remains unclear. Thus, herein, we aimed to examine the role of the adiponectin-AdipoR pathway in macrophage-mediated cholesterol efflux and HDL biogenesis, and elucidate the kinetics associated with this process.

5.3 Materials and Methods

THP-1 monocytes were purchased from the American Type Culture Collection (ATCC, Camden, NJ, USA). The following materials were used: ^{3}[H]-free cholesterol (Perkin Elmer, Waltham, MA, USA); human recombinant adiponectin (R&D Systems, Minneapolis, MN, USA); human recombinant apoA-I (Meridian Life Science, Cincinnati, OH, USA); AdipoRon (Cayman Chemical, Ann Arbor, MI, USA); polyclonal antibodies against human apoA-I (1:1000, K45252P, Meridian Life Science), AdipoR1 (1:1000, ab126611, abcam, Cambridge, MA, USA), AdipoR2 (1:650, ab113538, abcam), PPAR-α (1:1000, ab3484, abcam), AMPK-phosphorylated (1:1000, ab131357, abcam), AMPK-total (1:4000, ab32047, abcam), and β-actin (1:2500, ab75186, abcam); PMA (InvivoGen, San Diego, CA, USA); scintillation liquid (MP Biomedicals, Santa Ana, CA, USA); Silica Gel thin layer chromatography plates (Analtech, Newark, DE, USA); miRNeasy Mini Kit (Qiagen, Valencia, CA, USA); High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA); Human AdipoR1 PrimePCR SYBR Green Assay (Unique Assay ID: qHsaCID0018245, Bio-Rad Laboratories, Hercules, CA, USA); Human AdipoR2 PrimePCR SYBR Green Assay (Unique Assay ID:

qHsaCID0011851, Bio-Rad Laboratories), and Human B2M PrimeTime qPCR Primers (Hs.PT.58v.18759587, Integrated DNA Technologies, Coralville, IA, USA).

5.3.1 Cell culture systems

Human THP-1 monocytes were grown as previously described, in RPMI-1640 medium containing 100 IU/mL of penicillin/streptomycin, 10% FBS, and 50 µmol/L β-mercaptoethanol, and maintained at 37°C in a humidified atmosphere of 5% CO_2[2]. Prior to cholesterol efflux experiments, monocytes were fully differentiated into macrophages by the addition of 200 nM PMA for 48-72 hours in RPMI-1640 medium supplemented with 100 IU/mL of penicillin/streptomycin and 1% FBS.

THP-1 monocytes were stably transduced with lentiviral vectors containing shRNA constructs against AdipoR1 or AdipoR2, or mock-transduced with a lentiviral vector containing scrambled shRNA (used as a negative control). Prior to experiments, transduced monocytes were also differentiated into macrophages, as detailed above.

5.3.2 Lentiviral shRNA vector production against AdipoR1 and AdipoR2

293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS and 100 IU/mL of penicillin/streptomycin. 293FT cells were 85-95% confluent at the time of transfection. TRC shRNA constructs against human AdipoR1 (#TRCN0000063046; mature antisense: AAACAGCACGAAACCAAGCAG) and AdipoR2 (#TRCN0000060570; mature antisense: TAGGAATGATTCCACTCAGGC) expressed in pLKO.1 HIV-based lentiviral expression plasmids (**Figure 5.1**) were obtained from Dharmacon, Inc. (Lafayette, CO, USA). Transfection of 293T cells (with lentiviral expression plasmids plus packaging plasmids) was accomplished using Lipofectamine 2000 reagent (Thermo Fisher Scientific). Two days following transfection, the viral supernatant was collected and used to infect THP-1 monocytes. To increase the efficiency of the infection, a spinoculation step was performed to concentrate the virus at the surface of the cell (900g for 1.5 hours at 32ºC). Infection occurred over a period of 12-14 hours. Four days after infection, successfully infected cells were selected by culturing in 1 µg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) until the non-transduced negative cells have died, as determined by Trypan Blue staining.

To determine successful down-regulation of AdipoR1 and AdipoR2 expression, total RNA and protein was extracted from mock-, shRNA AdipoR1-, and shRNA AdipoR2-transduced THP-

1 macrophages using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). mRNA expression of AdipoR1 and AdipoR2 was assessed via SYBR Green-based qRT-PCR, using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). All measurements were performed in duplicate and C_q values obtained were normalized to the housekeeping gene, β-2 microglobulin. Relative mRNA abundance was calculated using the $2^{-\Delta\Delta Cq}$ method. Protein expression of AdipoR1 and AdipoR2 was determined by SDS-PAGE (10%), as previously described[2]. β-actin was used as a loading control.

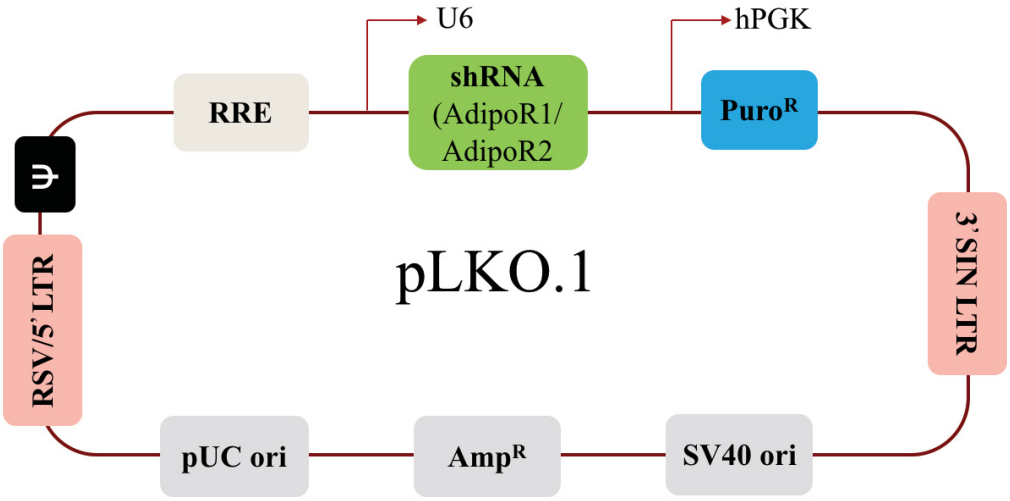


Figure 5.1 The pLKO.1 vector backbone used. *The lentiviral vectors used contain shRNA constructs against AdipoR1 or AdipoR2 under the human U6 (RNA polymerase III) promoter, and a puromycin resistance gene (Puro^R; mammalian selection marker), under the human phosphoglycerate kinase promoter (hPGK). The vector also contains a Rev response element (RRE), a Psi packaging sequence (Ψ), an RSV promoter/5' long terminal repeat (RSV/5'LTR), a 3' self-inactivating long terminal repeat (3' SIN LTR), and an ampicillin resistance gene (Amp^R; bacterial selection marker).*

5.3.3 Cholesterol efflux assays

Studies were carried out on standard 24-well plates (0.5×10^6 cells/well). Human THP-1 macrophages (transduced, or non-transduced) were labelled with 2 µCi/mL of ^{3}[H]-free cholesterol in the presence of 1% FBS and incubated for 72 hours. Cells were washed gently (2-3x) with PBS before exposure to cholesterol efflux acceptors (i.e., apoA-I, adiponectin, and/or AdipoRon) in medium supplemented with 0.1% essentially fatty-acid free BSA. Various doses of acceptor at various time-points were tested. Media were collected, centrifuged at 1500*g* for 10 min at room

temperature to remove cellular debris, and then counted for radioactivity by beta-scintillation. Cells were lysed in 0.5 mL of 0.1N NaOH overnight at room temperature with constant shaking. Cell associated-^{3}[H]-cholesterol was measured the following day by liquid scintillation counting. Cholesterol efflux was calculated according to the following formula as the percentage of ^{3}[H]-cpm medium/(^{3}[H]-cpm medium + ^{3}[H]-cpm cells) x 100%[542]. Percent efflux from cells in the absence of the acceptor was subtracted from % efflux in the presence of the acceptor to determine the % specific efflux.

5.3.4 Thin layer chromatography

To measure intracellular cholesterol levels following the cholesterol efflux process, lipids were extracted from THP-1 macrophages using a mixture of hexane and isopropanol (HIP; 3:2 vol./vol.). Lipid extracts were dried under N_2 gas and then reconstituted in Folch solution (chloroform: methanol, 2:1, vol./vol.). Samples were loaded in triplicate onto Silica Gel thin layer chromatography plates, as previously reported[2]. Bands corresponding to ^{3}[H]-free cholesterol and ^{3}[H]-cholesterol ester were located by exposure to iodine vapor and were scraped off the plate into liquid scintillating vials and assayed for radioactivity by beta-scintillation.

5.3.5 Fast-performance liquid chromatography (FPLC) analysis

HDL species released into the media during the efflux process were characterized using FPLC separation with a Superose 6B size-exclusion column (GE Healthcare, Madison, WI). Media containing ^{3}[H]-cholesterol was collected and centrifuged (1500g, 10min, room temperature) to remove cellular debris. HDL particles within the media were concentrated by ultrafiltration using a 10 kDa centrifugal filter (Amicon Ultra Centrifugal Filter Unit, MilliporeSigma, Burlington, MA, USA), loaded onto the column, and eluted with 50 mM Tris, 0.15 M NaCl, pH 7.4 at a constant flow rate of 0.35 mL/min. Radioactivity associated with each fraction eluted was determined by beta-scintillation.

5.3.6 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

FPLC fractions 30-60, corresponding to elution peaks, were pooled and analyzed by 2D-PAGE to determine the integrity and quality of apoA-I-containing HDL particles. Samples (50 µL of pooled FPLC fractions) were separated in the first dimension according to their isoelectric point (0.75% agarose gel electrophoresis, horizontal axis, 100V, 3 hours, 4°C) and in the second dimension

according to their molecular weight (5% to 15% polyacrylamide gradient gel electrophoresis, vertical axis, 125V, 24 hours, 4°C). Samples were then transferred onto nitrocellulose membranes (30V, 24 hours, 4°C). Membranes were incubated with goat polyclonal anti-human apoA-I antibody. Bands were detected by chemiluminescence using the SuperSignal West Pico substrate (Thermo Fisher Scientific) and band densities were quantified using ImageStudioLite software (LI-COR Biosciences, Lincoln, NE, USA). High molecular weight protein mixture (4-17 nm; GE Healthcare, UK) was run as a standard on each gel. Molecular weight markers were revealed by Ponceau S sodium salt.

5.3.7 Statistical analysis

Data are presented as mean ± SD. Results were compared statistically by independent-samples t-test. In order to determine the kinetic parameters for cholesterol efflux, we used the Michaelis-Menten equation (K_m = Michealis constant; V_{max} = maximum velocity, V_{max}/K_m = relative catalytic efficiency). All statistical analyses were executed using Graph-Pad Prism 6 software (GraphPad Software, Inc. La Jolla, CA). A P-value of <0.05 (2-tailed) was considered significant.

5.4 Results

5.4.1 Effect of adiponectin treatment on apoA-I-mediated cholesterol efflux and HDL biogenesis

Cholesterol efflux studies were performed in ^{3}[H]-cholesterol labeled human THP-1 macrophages in the presence of apoA-I. Adiponectin, in the presence of apoA-I, stimulated a specific increase in macrophage-mediated cholesterol efflux in a dose-dependent and time-dependent manner (**Figure 5.2**). When increased doses of adiponectin were incubated for 6 hours with an optimal and fixed dose of apoA-I (10 µg/mL), apoA-I-mediated cholesterol efflux was stimulated with high-efficiency in a dose-dependent manner with a Michaelis-Menten constant (K_m) value of 0.26±0.24 µg/mL and a V_{max} of 7.25±0.78 %efflux/6h (**Figure 5.2A**). Adiponectin reached saturation or its maximal effect on efflux at a rather low dose of about 5 µg/mL; further increasing adiponectin concentrations against a fixed dose of apoA-I did not result in any further increase in cholesterol efflux. Thus, 5 µg/mL of adiponectin was chosen as its' optimal dose for the following experiments. Importantly, adiponectin, without the presence of apoA-I, was unable to stimulate cholesterol efflux. The level of adiponectin efflux on its own was similar to the level of diffusional or passive efflux when macrophages were incubated without the presence of any acceptor (media alone). Furthermore, adiponectin and apoA-I combined stimulated cholesterol efflux in a time-

dependent manner, generating greater efflux than apoA-I alone, across all time-points after ~3 hours of incubation (**Figure 5.2B**). Adiponectin + apoA-I-mediated cholesterol efflux reached saturation after 24 hours of incubation, while apoA-I-mediated cholesterol efflux reached saturation more rapidly at ~16 hours of incubation. Kinetics analysis revealed that when increased concentrations of apoA-I were incubated in the presence of a fixed dose of adiponectin (5 µg/mL) for 24 hours, this resulted in higher cholesterol efflux with increased kinetic efficiency (V_{max}/K_m) and greater velocity (K_m=1.76±0.80 µg/mL, V_{max}=9.76±1.17 %efflux/24h) when compared to apoA-I alone (K_m=4.32±1.41 µg/mL, V_{max}=8.54±0.98 %efflux/24h) (**Figure 5.3A**). The kinetic efficiency of adiponectin + apoA-I in promoting cholesterol efflux from THP-1 macrophages was 2.8-fold higher than apoA-I alone. This significant increase in specific apoA-I-mediated cholesterol efflux in the presence of adiponectin (P<0.05; **Figure 5.3B**) was associated with a significant decrease in intracellular free cholesterol (P<0.01) and cholesterol ester content (P<0.05), as measured by thin layer chromatography, in macrophages incubated for 24 hours in the presence of both adiponectin and apoA-I when compared to apoA-I alone (**Figure 5.3C** and **D**, respectively).

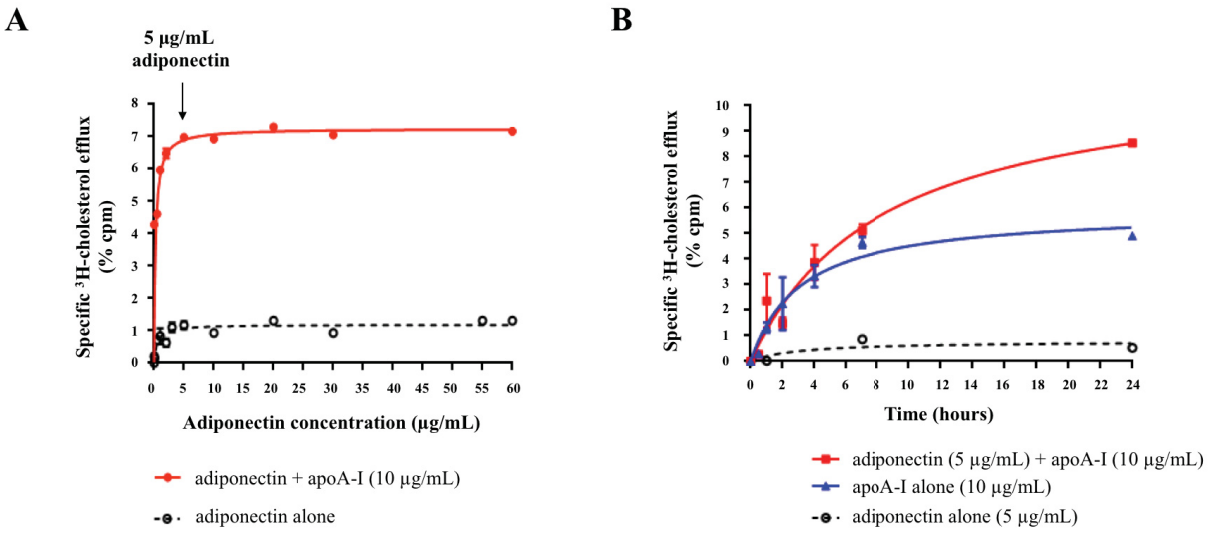


Figure 5.2 Adiponectin promotes apoA-I-mediated cholesterol efflux in a dose-dependent and time-dependent manner. A) *Dose-dependent macrophage cholesterol efflux: ^{3}[H]-cholesterol-labeled THP-1 macrophages were incubated for 6 hours with increased doses of adiponectin (0, 0.5, 1, 2.5, 5, 10, 20, 30, 55, 60 µg/mL) in the absence (dotted black line) or presence (red line) of a fixed dose of apoA-I (10 µg/mL). Medium- and cell-associated ^{3}[H]-radioactivity was counted. Specific cholesterol efflux was calculated and expressed as % cpm. Each data-point represents the*

mean of triplicates ± SD. Kinetic parameters for adiponectin + apoA-I-mediated cholesterol efflux are as follows: K_m = 0.26±0.24 µg/mL, V_{max} = 7.25±0.78 %efflux/6h, and relative catalytic efficiency (V_{max}/K_m) = 27.88. ***B)*** *Time-course activity of macrophage cholesterol efflux to 1) adiponectin (5 µg/mL) + apoA-I (10 µg/mL), 2) apoA-I alone (10 µg/mL), or 3) adiponectin alone (5 µg/mL). Specific cholesterol efflux, expressed as % cpm, was determined at the indicated time-points (0, 30 min, 1, 2, 4, 7, and 24 hours). Each data-point represents the mean of triplicates ± SD.*

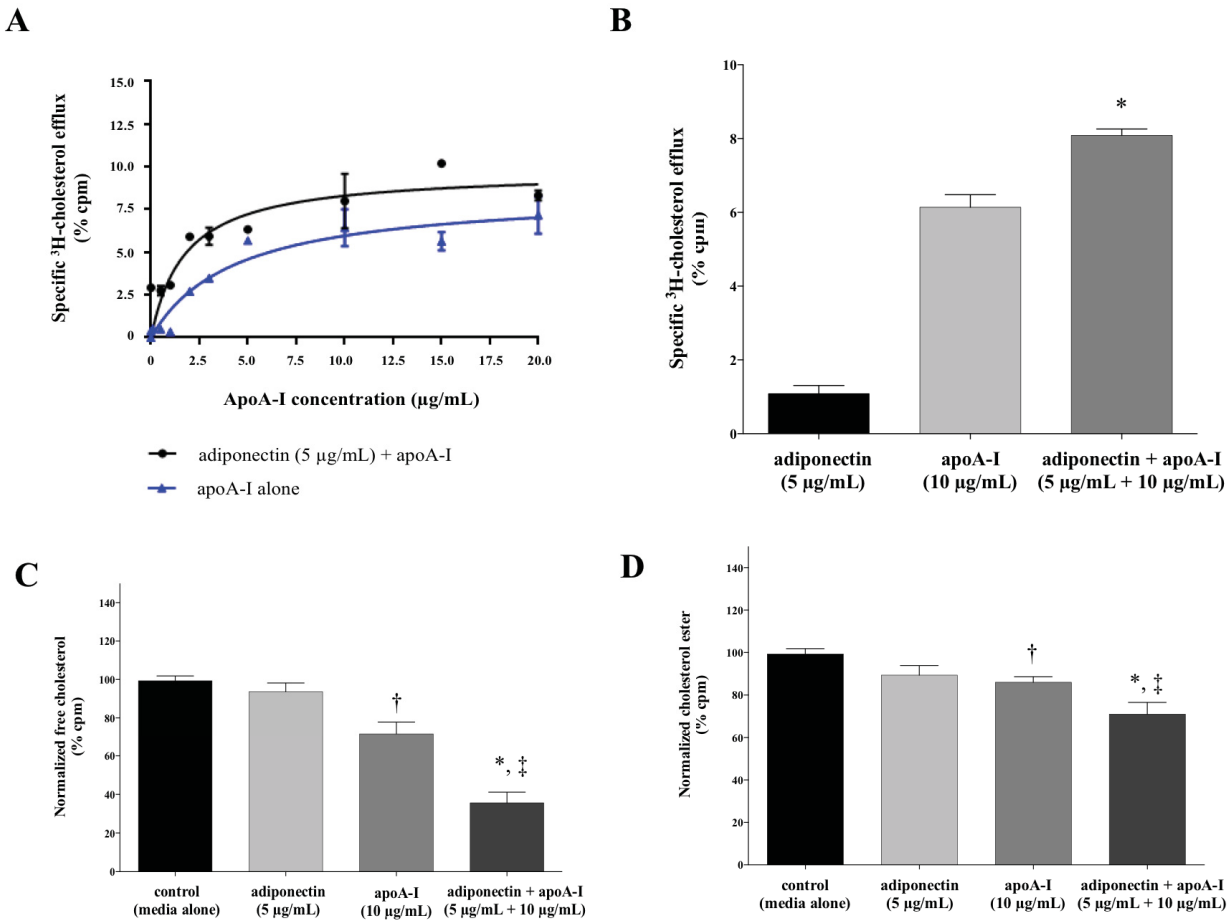


Figure 5.3 Effect of adiponectin treatment on cholesterol efflux kinetic parameters and on intracellular cholesterol species. A) *^{3}[H]-cholesterol-labeled THP-1 macrophages were incubated for 24 hours with increased doses of apoA-I (0, 0.5, 1, 2, 3, 5, 10, 15, 20 µg/mL) in the absence (blue line) or presence (black line) of a fixed dose of adiponectin (5 µg/mL). Medium- and cell-associated ^{3}[H]-radioactivity was counted. Specific cholesterol efflux was calculated and expressed as % cpm. Each data-point represents the mean of triplicates ± SD. Kinetic parameters*

for cholesterol efflux to: <u>adiponectin + apoA-I:</u> K_m = 1.76±0.80 µg/mL, V_{max} = 9.76±1.17 %efflux/24h, and relative catalytic efficiency (V_{max}/K_m) = 5.55; <u>apoA-I alone:</u> K_m = 4.32±1.41 µg/mL, V_{max} = 8.54±0.98 %efflux/24h, and relative catalytic efficiency (V_{max}/K_m) = 1.98. ***B)*** *^{3}[H]-cholesterol-labeled THP-1 macrophages were incubated for 24 hours with 1) adiponectin (5 µg/mL) + apoA-I (10µg/mL), 2) apoA-I alone (10 µg/mL), and 3) adiponectin alone (5 µg/mL). Bar graphs represent specific cholesterol efflux expressed as % cpm. Data presented as mean ± SD. *P<0.001 vs. macrophages treated with apoA-I alone (independent-samples t-test). Results shown are representative of three independent experiments.* ***C)*** *Bar graphs represent intracellular free cholesterol measured by thin layer chromatography. Radioactivity (^{3}[H]-free cholesterol) was measured by beta-scintillation, divided by cellular protein content (cpm/µg), and expressed as a percentage of control (100%, in the absence of acceptor). Data presented as mean ± SD. *P<0.01 vs. macrophages treated with apoA-I alone; †P<0.01 vs. control macrophages; ‡P<0.0001 vs. control macrophages (independent-samples t-test). Results shown are representative of three independent experiments.* ***D)*** *Bar graphs represent intracellular cholesterol ester measured by thin layer chromatography. Radioactivity (^{3}[H]-cholesterol ester) was measured by beta-scintillation, divided by cellular protein content (cpm/µg), and expressed as a percentage of control (100%, in the absence of acceptor). Data presented as mean ± SD. *P<0.05 vs. macrophages treated with apoA-I alone; †P<0.05 vs. control macrophages; ‡P<0.01 vs. control macrophages (independent-samples t-test). Results shown are representative of three independent experiments.*

5.4.2 Effect of adiponectin treatment on HDL biogenesis

The cholesterol content within HDL particles generated in the cell culture media during the cholesterol efflux process was characterized by FPLC analysis (**Figure 5.4A-C**). The FPLC elution profiles of ^{3}[H]-cholesterol are shown in **Figure 5.4A**. Upon treatment of macrophages with adiponectin + apoA-I for 24 hours, larger elution peaks were produced between fractions ~30-60 compared to treatment with apoA-I alone, demonstrating a significant increase in lipidated nascent HDL particle formation (pre-β and α-HDL species) during the process of cholesterol efflux that occurred in the presence of adiponectin (**Figures 5.4A-C**). The nature of the apoA-I-containing HDL species generated during the cholesterol efflux process was further investigated by 2D-PAGGE analyses, where bands were detected in the pre-β and α regions both in the presence and absence of adiponectin (**Figure 5.4D**). However, in the presence of adiponectin + apoA-I, an

increase was found in pre-β HDL by 19.7% and in α-HDL by 49.8% compared to HDL particles formed in the presence of apoA-I alone.

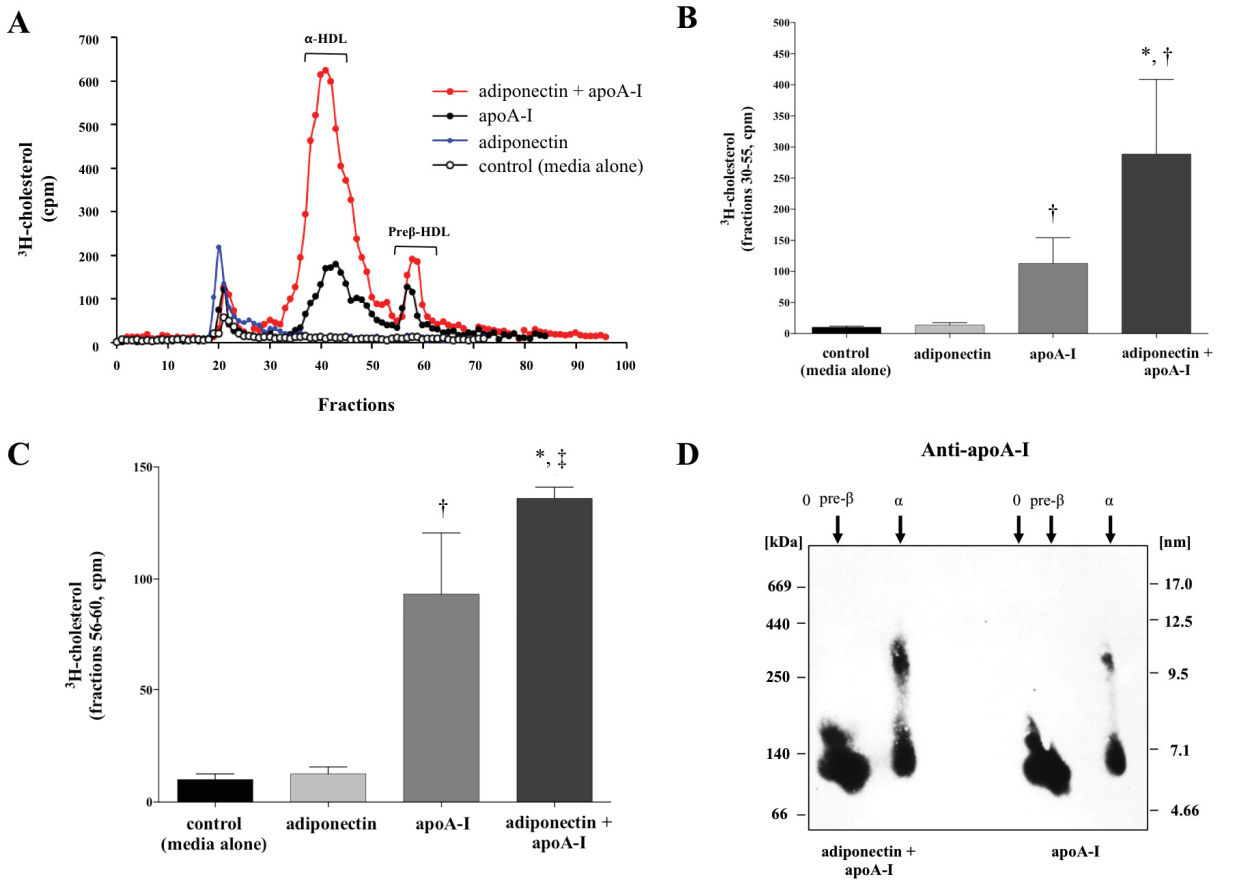


Figure 5.4 Characterization of lipidated nascent HDL species generated in the cell culture media during apoA-I-mediated cholesterol efflux in the presence or absence of adiponectin. A) *Representative FPLC elution profiles of ^{3}[H]-cholesterol. 3[H]-cholesterol-labeled THP-1 macrophages were incubated for 24 hours with 1) adiponectin (5 µg/mL) + apoA-I (10 µg/mL), 2) apoA-I alone (10 µg/mL), 3) adiponectin alone (5 µg/mL), 4) no acceptor (media alone). Media was collected, concentrated by ultrafiltration, and then subjected to FPLC separation, using a Superose 6B size-exclusion column where larger molecules are eluted first. Radioactivity (^{3}[H]-cholesterol) associated with each fraction was determined and expressed as cpm.* ***B)*** *Bar graphs represent ^{3}[H]-cholesterol within FPLC fractions ~30-55 corresponding to the α-HDL peak. Data presented as mean ± SD. *P<0.0001 vs. macrophages treated with apoA-I alone (independent-samples t-test); †P<0.0001 vs. control macrophages. Results shown are representative of three independent experiments.* ***C)*** *Bar graphs represent ^{3}[H]-cholesterol within FPLC fractions ~56-60 corresponding to the pre-β HDL peak. Data presented as mean ± SD. *P<0.05 vs. macrophages*

treated with apoA-I alone; †P<0.001 vs. control macrophages; ‡P<0.0001 vs. control macrophages (independent-samples t-test). Results shown are representative of three independent experiments. ***D)*** *Representative Western blot of native 2D-PAGE of apoA-I containing nascent HDL species generated by incubation of THP-1 macrophages with apoA-I in the absence or presence of adiponectin. FPLC fractions ~30-60 corresponding to the elution peaks were pooled and analyzed by 2D-PAGE. Molecular weight markers were revealed by Ponceau S. Molecular size markers are indicated (diameter in nm and kDa).*

5.4.3 Modulation of adiponectin receptors and its effect on apoA-I-mediated cholesterol efflux

AdipoRon is a synthetic small-molecule that binds to and activates the adiponectin receptors, as demonstrated by a dose-dependent increase in the expression of AMPK and PPAR-α, which are well-known downstream signalling pathways of AdipoR1 and AdipoR2, respectively (**Figure 5.5A**). Treatment of THP-1 macrophages with AdipoRon demonstrated similar effects in stimulating apoA-I-mediated cholesterol efflux as treatment with adiponectin (**Figure 5.5B**). AdipoRon, on its own, acted as a poor acceptor, generating a passive efflux of <1%. However, in the presence of apoA-I, AdipoRon promoted an increase in cholesterol efflux in a dose-dependent manner. Treatment with higher doses of AdipoRon (5 and 10 μM) significantly increased apoA-I-mediated cholesterol efflux compared to treatment in the absence of AdipoRon (P<0.05, **Figure 5.5B**).

Lentiviral shRNA experiments were performed to determine the contribution of each adiponectin receptor in promoting cholesterol removal from macrophages. Following infection of THP-1 macrophages with shRNA constructs against AdipoR1 or AdipoR2, the expression levels of AdipoR1 and AdipoR2 mRNA and protein were significantly decreased in shRNA AdipoR1- and AdipoR2-transduced macrophages, respectively, relative to mock-transduced macrophages (**Figure 5.6A-D**). Following knockdown of AdipoR1 or AdipoR2 expression, apoA-I-mediated cholesterol efflux in the presence of adiponectin was significantly decreased by 66.48% and 55.73% in shRNA AdipoR1- and AdipoR2-transduced macrophages, respectively, compared to mock transduction (**Figure 5.6E**).

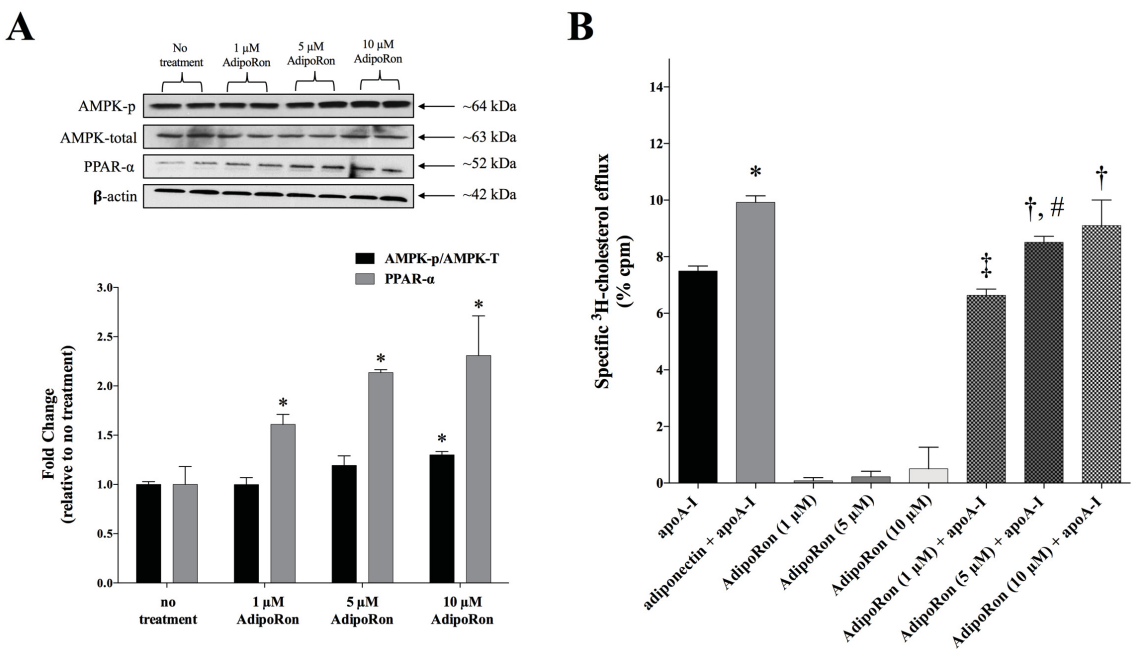


Figure 5.5 Activation of adiponectin receptors via treatment with AdipoRon stimulates apoA-I-mediated cholesterol efflux in a dose-dependent manner. A) *Representative Western blot showing expression of AMPK-phosphorylated, AMPK-total, and PPAR-α, in THP-1 macrophages treated and non-treated with increasing doses of AdipoRon. Three independent determinations were performed, and similar results were observed. Samples were analyzed in duplicate. Bar graphs represent changes in protein expression between macrophages treated and non-treated with AdipoRon. Data presented as fold change ± SD. Protein expression was determined by normalizing against the densitometric intensity of β-actin. Changes in AMPK activity were determined using the ratio of AMPK-phosphorylated to AMPK-total protein expression. *P<0.05 vs. untreated macrophages (independent-samples t-test).* ***B)*** *^{3}[H]-cholesterol-labeled THP-1 macrophages were incubated for 24 hours with increased doses of AdipoRon (1, 5, 10 μM) in the absence or presence of a fixed dose of apoA-I (10 μg/mL). Macrophages were also treated with adiponectin (5 μg/mL) + apoA-I (10 μg/mL) or with apoA-I alone (10 μg/mL). Bar graphs represent specific cholesterol efflux expressed as % cpm. Data presented as mean ± SD. *P<0.01 vs. macrophages treated with apoA-I alone; †P<0.05 vs. macrophages treated with apoA-I alone; ‡P<0.01 vs. macrophages treated with adiponectin + apoA-I; #P<0.05 vs. macrophages treated with adiponectin + apoA-I (independent-samples t-test). Results shown are representative of two independent experiments.*

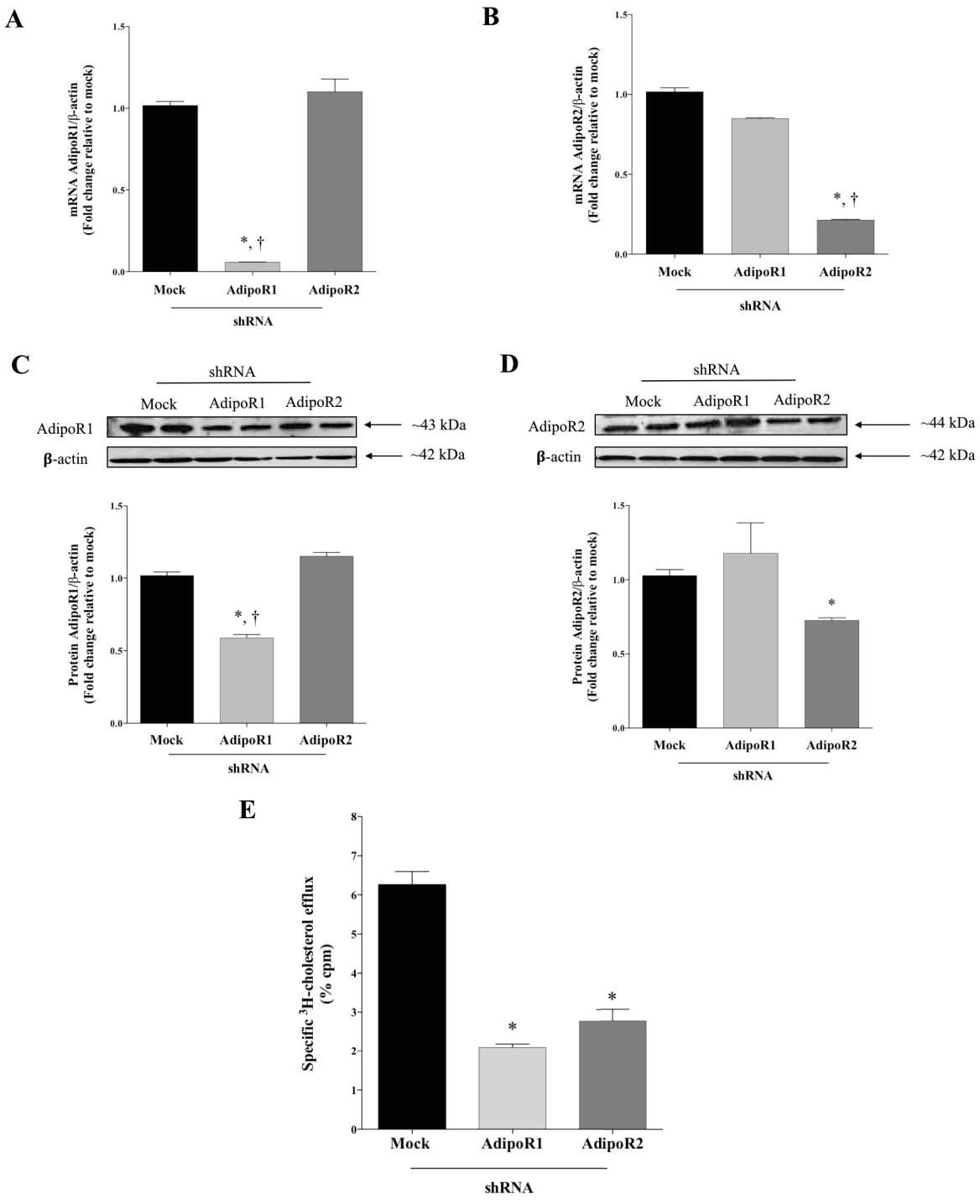


Figure 5.6 AdipoR1 and AdipoR2 expression and apoA-I-mediated cholesterol efflux in mock, shRNA AdipoR1-, and shRNA AdipoR2-transduced THP-1 macrophages. *Comparing changes in the levels of* ***A)*** *AdipoR1 and* ***B)*** *AdipoR2 mRNA between shRNA AdipoR1- or shRNA AdipoR2-transduced THP-1 macrophages and mock-transduced macrophages. Bar graphs represent qRT-PCR data for AdipoR1 and AdipoR2 mRNA levels in macrophages. Data presented as fold change ± SD. *P<0.001 vs. mock macrophages; †P<0.001 vs. shRNA AdipoR1- or shRNA AdipoR2-*

transduced macrophages (independent-samples t-test). Comparing changes in the levels of ***C)*** *AdipoR1 and* ***D)*** *AdipoR2 protein between shRNA AdipoR1- or shRNA AdipoR2-transduced THP-1 macrophages and mock-transduced macrophages. Representative Western Blot images are shown for AdipoR1 and AdipoR2. Three independent determinations were performed, and similar results were observed. Samples were analyzed in duplicate. Protein expression was determined by normalizing against the densitometric intensity of β-actin. Data presented as fold change ± SD. *P<0.01 vs. mock macrophages; †P<0.01 vs. shRNA AdipoR2-transduced macrophages (independent-samples t-test).* ***E)*** *^{3}[H]-cholesterol-labeled mock-, shRNA AdipoR1-, and shRNA AdipoR2-transduced THP-1 macrophages were incubated for 24 hours with adiponectin (5 µg/mL) + apoA-I (10 µg/mL). Bar graphs represent specific cholesterol efflux expressed as % cpm. Data presented as mean ± SD. *P<0.01 vs. mock macrophages (independent-samples t-test). Results shown are representative of two independent experiments.*

5.5 Discussion

Our work revealed that treatment of macrophages with apoA-I in combination with adiponectin results in efficient stimulation of cholesterol efflux and greater HDL particle formation than treatment with apoA-I alone. Moreover, we demonstrated that adiponectin's effects on cholesterol efflux and HDL biogenesis are mediated via its receptors, AdipoR1 and AdipoR2. Activation of the adiponectin receptor pathway via treatment with AdipoRon, led to a similar significant increase in apoA-I-mediated cholesterol efflux as adiponectin, while downregulation of AdipoR1 or AdipoR2 expression resulted in significant reductions in cholesterol efflux in macrophages incubated in the presence of both apoA-I and adiponectin.

5.5.1 Adiponectin's effects on cholesterol efflux and HDL biogenesis

Epidemiological studies have consistently demonstrated that low plasma HDL levels are associated with increased risk of CVD[543]. However, these associations have failed to translate into clinical evidence that raising HDL levels pharmacologically can reduce the risk of cardiac and stroke events and prevent CVD[544-546]. It has been suggested that measures of HDL functionality, rather than its absolute cholesterol mass, may be more accurate indicators of cardiovascular risk[106]. HDL particles possess a variety of pleiotropic effects; they are known to mediate cellular cholesterol efflux, have antioxidant properties, and modulate vascular inflammation and vasomotor function and thrombosis[106,547]. Thus, perhaps more relevant to human physiology

would be to develop efficacious drugs as well as novel approaches not only to raise HDL levels but more importantly to modulate and increase its pleiotropic functions. Our group has previously reported that patients with more severe carotid artery stenosis and more advanced/unstable carotid plaque morphology have significantly lower cholesterol efflux from macrophages than patients with less severe and more stable atherosclerotic plaque burden[108]. Therefore, cholesterol efflux, particularly from macrophages, serves as an important protective mechanism against atherosclerosis development and severity and as a potential target for novel therapies.

Previous evidence has shown that adiponectin, an adipose tissue secreted adipokine with anti-inflammatory and vasculo-protective properties, affects the RCT pathway at the level of the liver and of the macrophage, by increasing ABCA1 expression and enhancing HDL/apoA-I mediated cholesterol efflux[332,338,340]. In line with previous evidence, herein, we have shown that adiponectin requires the presence of apoA-I (which is the primary acceptor of cholesterol and the preferred substrate of ABCA1) to stimulate an increase in macrophage-mediated cholesterol efflux in a dose-dependent and time-dependent manner. Importantly, adiponectin specifically promotes active efflux, without affecting the level of diffusional or passive efflux (efflux without the presence of any acceptor). It is critical that the level of passive efflux remains low, as higher levels can cause cell lysis and toxicity. Even at elevated doses, adiponectin does not exhibit unwanted toxicity, thereby depicting its therapeutic utility. Moreover, our study expands on previous knowledge, by demonstrating that the kinetics associated with the interaction between adiponectin and apoA-I in promoting cholesterol efflux was highly efficient (relative catalytic efficiency was almost 3-fold higher) compared to apoA-I alone. This suggests that therapeutically combining adiponectin with apoA-I may help improve the cholesterol efflux process in patients with advanced atherosclerotic disease, where this system may be impaired.

We are the first to demonstrate that adiponectin modulates HDL-apoA-I biogenesis, by increasing the generation of nascent HDL particles in an apoA-I-dependent process. HDL is a collection of subspecies with different compositions and properties. The impact of adiponectin on HDL subspecies remains elusive. However, our data showed that adiponectin can increase the generation of both preβ and α-HDL species. Preβ-HDL is an immature form of HDL, which acts as both a product and substrate in the ABCA1-mediated cholesterol efflux process[548]. On the other hand, α-HDL species represent more mature forms of HDL and high levels of the larger α-species are associated with decreased cardiovascular risk[114]. Our findings suggest that adiponectin is efficient in maintaining cholesterol efflux by promoting the generation of a continuous pool of

HDL particles that act as acceptors, while also promoting HDL maturation. Our future work will focus on elucidating the functional properties of these HDL particles generated in the presence of both adiponectin and apoA-I, particularly its effects on mediating ABCG1-dependent cholesterol efflux, as well as effects on inflammation and oxidation.

5.5.2 Contribution of the AdipoR pathway

To date, there exists limited studies investigating the potential involvement of the adiponectin receptors (AdipoR1 and AdipoR2) in mediating adiponectin's effects on apoA-I-dependent cholesterol efflux and HDL biogenesis. Overexpression and downregulation of AdipoR1 and AdipoR2 in HEK293T human kidney cells provided the first evidence that both receptors are positively involved in the cholesterol efflux process via upregulation of ABCA1 expression and activity[331]. However, there exists conflicting evidence concerning the importance of each receptor in macrophage lipid metabolism. Downregulation of the AdipoR1 signalling pathway (but not AdipoR2) blunted adiponectin's effects on cholesterol efflux in macrophages isolated from diabetic patients[295]. On the other hand, Li *et al*. demonstrated that small interfering RNA-mediated downregulation of AdipoR2 in THP-1 macrophages enhanced apoA-I- and HDL-mediated cholesterol efflux, while the contribution of the AdipoR1 pathway was not investigated[334]. However, Li's efflux experiments were performed in the absence of adiponectin. Therefore, a firm conclusion regarding the direct impact of AdipoR2 gene silencing on adiponectin's effects on cholesterol efflux cannot be established. In fact, the upregulation observed in cholesterol efflux may be attributed to off-target effects of downregulating AdipoR2 on the expression of ABCA1, ABCG1, LXR-α, PPAR-γ. In light of these conflicting results, herein, we performed AdipoR activation and silencing experiments to clarify the existing evidence and further elucidate whether the adiponectin-induced effects on cholesterol efflux were AdipoR-dependent. Treatment of THP-1 macrophages with AdipoRon, a synthetic small-molecule AdipoR agonist, replicated the effects of adiponectin on increasing apoA-I-mediated cholesterol efflux, while 94% reduction in the AdipoR1 gene (42% in protein) and 80% reduction in the AdipoR2 gene (28% in protein) led to a significant 66.48% and 55.73% decrease, respectively, in cholesterol efflux in the presence of both apoA-I and adiponectin. These results suggest that both receptors are needed for adiponectin to fulfill its role on cellular cholesterol efflux and that the expression of one receptor is not sufficient to compensate for lack of the other. One major limitation of the study is that the percentage of AdipoR1/R2 protein downregulation was not efficient enough to determine the precise

contribution that each receptor plays. Knock-out strategies using CRISPR-Cas9 technology may prove to be a more successful approach than shRNA-mediated gene silencing. It is suggested that AdipoR1 and AdipoR2 play a key role in mediating adiponectin's effects on lipid metabolism by inducing a cascade of signalling pathways particularly involving the AMPK and PPAR-α molecules[549], which in turn may activate PPAR-γ and LXR-α, i.e., major pathways through which ABCA1 and ABCG1 transporter expression can be upregulated[550,551]. Future studies are needed to confirm the role and interaction of these pathways in the efflux system.

5.6 Conclusion

Our study provided preliminary evidence that adiponectin can improve the efficiency of the rate-limiting step of the cholesterol efflux process and promote nascent HDL biogenesis via activation of both the AdipoR1 and AdipoR2 receptors. Moreover, our study suggests that combining adiponectin and apoA-I may have therapeutic potential in modulating HDL's protective role in atherosclerosis. Since adiponectin, on its own, exerts beneficial effects leading to increased insulin sensitivity, decreased inflammation, as well as vasculo-protection, we expect that adiponectin in combination with apoA-I will result in "super-HDL" particles with greater functionality than those generated from apoA-I alone therapies that are currently under investigation. Therefore, combining adiponectin and apoA-I therapeutically may serve as a promising HDL-targeted therapy for the prevention and treatment of atherosclerotic disease and for the stabilization of plaques. Alternatively, specific activation of the AdipoR pathway may serve as a novel and potent therapeutic strategy in enhancing the action of adiponectin and promoting cholesterol efflux activity without the need to target/increase adiponectin levels.

5.7 Funding Sources

This work was supported by the Canadian Institutes of Health Research [CIHR, PJT-148966] and the Heart & Stroke Foundation of Canada [G-17-0018755]. SS Daskalopoulou is a Senior Chercheur-Boursier Clinicien supported by the Fonds de recherche du Québec – Santé. K Gasbarrino is supported by a CIHR Doctoral Studentship. The funding sources had no involvement in the study.

5.8 Disclosures

None

5.9 References

2. Gasbarrino K, Zheng H, Hafiane A, Veinot JP, Lai C, Daskalopoulou SS. Decreased adiponectin-mediated signaling through the adipor2 pathway is associated with carotid plaque instability. *Stroke*. 2017:48:915-924.
93. Shah PK. Mechanisms of plaque vulnerability and rupture. *J Am Coll Cardiol*. 2003:41:15S-22S.
102. Ohashi R, Mu H, Wang X, Yao Q, Chen C. Reverse cholesterol transport and cholesterol efflux in atherosclerosis. *Qjm*. 2005:98:845-856.
103. Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: Key to the regression of atherosclerosis? *Circulation*. 2006:113:2548-2555.
106. Hafiane A, Genest J. Hdl, atherosclerosis, and emerging therapies. *Cholesterol*. 2013:2013:891403.
107. Yokoyama S. Assembly of high density lipoprotein by the abca1/apolipoprotein pathway. *Curr Opin Lipidol*. 2005:16:269-279.
108. Doonan RJ, Hafiane A, Lai C, Veinot JP, Genest J, Daskalopoulou SS. Cholesterol efflux capacity, carotid atherosclerosis, and cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2014:34:921-926.
111. Wang X, Collins HL, Ranalletta M, Fuki IV, Billheimer JT, Rothblat GH, Tall AR, Rader DJ. Macrophage abca1 and abcg1, but not sr-bi, promote macrophage reverse cholesterol transport in vivo. *J Clin Invest*. 2007:117:2216-2224.
114. Asztalos BF, Roheim PS, Milani RL, Lefevre M, McNamara JR, Horvath KV, Schaefer EJ. Distribution of apoa-i-containing hdl subpopulations in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol*. 2000:20:2670-2676.
115. Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, Cartland S, Packianathan M, Kritharides L, Jessup W. Abca1 and abcg1 synergize to mediate cholesterol export to apoa-i. *Arterioscler Thromb Vasc Biol*. 2006:26:534-540.
295. Wang M, Wang D, Zhang Y, Wang X, Liu Y, Xia M. Adiponectin increases macrophages cholesterol efflux and suppresses foam cell formation in patients with type 2 diabetes mellitus. *Atherosclerosis*. 2013:229:62-70.
323. Yamamoto Y, Hirose H, Saito I, Tomita M, Taniyama M, Matsubara K, Okazaki Y, Ishii T, Nishikai K, Saruta T. Correlation of the adipocyte-derived protein adiponectin with insulin resistance index and serum high-density lipoprotein-cholesterol, independent of body mass index, in the japanese population. *Clin Sci (Lond)*. 2002:103:137-142.
324. Schulze MB, Rimm EB, Shai I, Rifai N, Hu FB. Relationship between adiponectin and glycemic control, blood lipids, and inflammatory markers in men with type 2 diabetes. *Diabetes Care*. 2004:27:1680-1687.
325. Marsche G, Zelzer S, Meinitzer A, Kern S, Meissl S, Pregartner G, Weghuber D, Almer G, Mangge H. Adiponectin predicts hdl cholesterol efflux capacity in adults irrespective of body mass index and fat distribution. *J Clin Endocrinol Metab*. 2017
326. Posadas-Sanchez R, Posadas-Romero C, Mendoza-Perez E, Caracas-Portilla NA, Cardoso-Saldana G, Medina-Urrutia A, Jorge-Galarza E, Juarez-Rojas JG. Cholesterol efflux and metabolic abnormalities associated with low high-density-lipoprotein-cholesterol and high triglycerides in statin-treated coronary men with low-density lipoprotein-cholesterol <70 mg/dl. *Am J Cardiol*. 2012:109:636-641.
327. Xu M, Zhou H, Wang J, Li C, Yu Y. The expression of atp-binding cassette transporter a1 in chinese overweight and obese patients. *Int J Obes (Lond)*. 2009:33:851-856.
331. Kitajima K, Miura S, Yamauchi T, Uehara Y, Kiya Y, Rye KA, Kadowaki T, Saku K. Possibility of increasing cholesterol efflux by adiponectin and its receptors through the

atp binding cassette transporter a1 in hek293t cells. *Biochem Biophys Res Commun*. 2011:411:305-311.
332. Liang B, Wang X, Guo X, Yang Z, Bai R, Liu M, Xiao C, Bian Y. Adiponectin upregulates abca1 expression through liver x receptor alpha signaling pathway in raw 264.7 macrophages. *Int J Clin Exp Pathol*. 2015:8:450-457.
334. Li J, Zhang S. Microrna-150 inhibits the formation of macrophage foam cells through targeting adiponectin receptor 2. *Biochem Biophys Res Commun*. 2016:476:218-224.
338. Oku H, Matsuura F, Koseki M et.al. Adiponectin deficiency suppresses abca1 expression and apoa-i synthesis in the liver. *FEBS Lett*. 2007:581:5029-5033.
340. Tsubakio-Yamamoto K, Matsuura F, Koseki M et.al. Adiponectin prevents atherosclerosis by increasing cholesterol efflux from macrophages. *Biochem Biophys Res Commun*. 2008:375:390-394.
542. Hafiane A, Genest J. Hdl-mediated cellular cholesterol efflux assay method. *Ann Clin Lab Sci*. 2015:45:659-668.
543. Gordon DJ, Rifkind BM. High-density lipoprotein--the clinical implications of recent studies. *N Engl J Med*. 1989:321:1311-1316.
544. Schwartz GG, Olsson AG, Abt M et.al. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *N Engl J Med*. 2012:367:2089-2099.
545. Hps2-thrive randomized placebo-controlled trial in 25 673 high-risk patients of er niacin/laropiprant: Trial design, pre-specified muscle and liver outcomes, and reasons for stopping study treatment. *Eur Heart J*. 2013:34:1279-1291.
546. Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K, McBride R, Teo K, Weintraub W. Niacin in patients with low hdl cholesterol levels receiving intensive statin therapy. *N Engl J Med*. 2011:365:2255-2267.
547. Lund-Katz S, Phillips MC. High density lipoprotein structure-function and role in reverse cholesterol transport. *Subcell Biochem*. 2010:51:183-227.
548. Duong PT, Weibel GL, Lund-Katz S, Rothblat GH, Phillips MC. Characterization and properties of pre beta-hdl particles formed by abca1-mediated cellular lipid efflux to apoa-i. *J Lipid Res*. 2008:49:1006-1014.
549. Ruan H, Dong LQ. Adiponectin signaling and function in insulin target tissues. *J Mol Cell Biol*. 2016:8:101-109.
550. Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the abc1 promoter by the liver x receptor/retinoid x receptor. *J Biol Chem*. 2000:275:28240-28245.
551. Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P. A ppar gamma-lxr-abca1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell*. 2001:7:161-171.

Connecting Text

Stabilization of a vulnerable plaque is the ultimately clinically relevant goal. The results from the previous chapter provided preliminary evidence supporting the adiponectin-AdipoR pathway as a potential therapeutic target for plaque stabilization. A therapy aimed at modulating specifically AdipoR expression and/or activity may have the potential to promote a more stable macrophage phenotype, and ultimately more plaque stability by increasing cholesterol efflux and HDL biogenesis.

In the following chapter (Chapter 6) we aimed to determine whether currently used cardio-metabolic medications can positively modulate the adiponectin-AdipoR pathway in the monocyte-macrophage lineage. We took a particular interest in statin therapy since it is widely used for the primary and secondary prevention of CVD and there is evidence for off-target anti-inflammatory effects, where it can modulate immune responses by altering cytokine levels and affect the function of cells involved in both innate and adaptive responses[552,553]. Moreover, statins have been shown to promote plaque regression and decrease atherosclerotic plaque burden[143,144].

A meta-analysis reported a significant elevation in circulating adiponectin levels following statin therapy[355]. However, this statin-induced augmentation of adiponectin was dependent on the type of statin used[355]. Herein, through a comprehensive approach, including *in vivo* (cross-sectional and longitudinal) and *in vitro* (in human THP-1 monocyte-derived macrophages) studies, we assessed the effects of various doses of statins as well as the duration of statin use on the expression and function of the adiponectin-AdipoR pathway, which has not been previously explored. We focused on specifically testing the impact of atorvastatin and rosuvastatin on the adiponectin-AdipoR pathway, as these are known to be the most effective, widely used, and clinically relevant statins.

CHAPTER 6: INTENSIVE STATIN THERAPY COMPROMISES THE ADIPONECTIN-ADIPOR PATHWAY IN THE HUMAN MONOCYTE-MACROPHAGE LINEAGE

Karina Gasbarrino, Anouar Hafiane, Huaien Zheng, Stella S. Daskalopoulou

6.1 Abstract

Statins are widely used for primary and secondary prevention of CVD through cholesterol lowering and anti-inflammatory effects. Adiponectin, an anti-inflammatory adipokine, acts via two receptors, AdipoR1 and AdipoR2, to exert protective effects on the vasculature. We aimed to investigate whether statins can modulate the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage. AdipoR1 and AdipoR2 mRNA expression on circulating monocytes from patients with severe carotid atherosclerosis, was significantly lower by 1.36- and 1.17-fold, respectively, in statin users *vs.* statin-naïve patients. Specifically, patients on high doses of atorvastatin (40-80 mg) or rosuvastatin (20-40 mg), had significantly lower AdipoR expression compared to statin-naïve patients. Similarly, in a longitudinal *in vivo* study, longer treatment of atorvastatin or rosuvastatin (≥5 months following statin initiation) in patients with cardiovascular risk factors resulted in lower AdipoR1 and AdipoR2 expression on circulating monocytes compared to pre-statin levels. *In vitro*, THP-1 macrophages were treated with increasing doses of atorvastatin or rosuvastatin for 24 or 72 hours. Higher statin doses and longer exposure resulted in a greater decrease in AdipoR mRNA expression and signalling through AMPK and PPAR-α, and greater macrophage secretion of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α. High doses of statins also significantly reduced adiponectin's capacity to suppress intracellular cholesterol ester levels in oxidized LDL-loaded macrophages, with rosuvastatin exhibiting higher potency than atorvastatin. Our *in vivo* (cross-sectional and longitudinal) and *in vitro* studies identified a novel pleiotropic property of statins in modulating the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage, where intensive statin therapy compromised the expression and function of adiponectin and its receptors.

6.2 Introduction

Atherosclerosis is a chronic and progressive inflammatory disease of the arterial wall that is characterized by the cross-talk between excessive inflammation and lipid accumulation. Circulating monocytes, which migrate to the arterial wall, and tissue-resident macrophages are instrumental in the development of human atherosclerotic plaques and are heavily involved in their progression and destabilization[90].

Statins are widely used for primary and secondary prevention of CVD[554]. Beyond lowering cholesterol levels, which is their main beneficial effect, statins also possess pleiotropic actions, including the improvement of endothelial function, modulation of immune activation, and reduction of platelet aggregation and thrombus formation[141,142]. Furthermore, statins have direct favorable effects on atherosclerotic plaque burden by promoting plaque regression[143,144]. They possess anti-inflammatory effects which can affect the plaque by 1) decreasing monocyte recruitment to the plaque[146], 2) reducing macrophage accumulation in the plaque[147], and 3) inhibiting the production of MMPs by activated macrophages[147], which are enzymes that degrade and destabilize the fibrous cap of the plaque.

Adiponectin, an abundant adipose tissue-secreted protein, is involved in glucose regulation, promotes insulin sensitivity, and possesses anti-inflammatory, anti-atherogenic, and vasculo-protective properties[307]. In humans, low levels of circulating total and HMW adiponectin are associated with cardiovascular risk factors, such as obesity, diabetes, hypertension, and low levels of HDL-C, as well as a higher prevalence of CAD[265,269,555]. Adiponectin acts via two receptors, AdipoR1 and AdipoR2, to reduce inflammation and exert protective effects on the vasculature by 1) decreasing the expression of adhesion molecules on the surface of the vascular endothelium, resulting in an attenuation of monocyte attachment to endothelial cells and their migration into the intimal layer of the arterial wall[293], and 2) suppressing foam cell formation[296]. Recently, we demonstrated that adiponectin receptors are highly expressed on macrophages present in the plaque and that decreased AdipoR2 expression and signalling is associated with atherosclerotic plaque instability in patients with moderate-to-high-grade carotid artery stenosis[2].

Both statins and adiponectin share several beneficial effects on inflammation and atherosclerosis, thus reducing cardiovascular risk. While several studies have reported an increase in circulating adiponectin levels following statin therapy, others have demonstrated a decrease, which could be dependent on the type of statin used[355]. We hypothesize that along with changes in adiponectin levels, statins can also affect AdipoR activity, which may be a novel pleiotropic

action of statins. Thus, the aim of this study was to investigate whether statins can modulate the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage.

6.3 Methods

6.3.1 Cross-sectional study population

Consecutive patients with severe carotid atherosclerosis, scheduled for a CEA, were recruited from the Vascular Surgery pre-operative clinics at the McGill University Health Centre and the Jewish General Hospital in Montreal, Canada, as previously described[2,556].

Clinical information concerning statin use (statin type and dose prescribed) prior to CEA was obtained and cross-matched through various sources: 1) patient interview, 2) a detailed questionnaire, and 3) medical records. Subjects who were only prescribed statins following their CEA were considered to be statin-naïve. Sociodemographic and other clinical information (i.e., cerebrovascular symptomatic status, carotid artery stenosis, past medical history, other medication use, and lifestyle habits) were also obtained.

Fasting blood samples were collected from each subject the day of the CEA prior to surgical intervention. Blood samples were used for obtaining plasma and serum for subsequent biochemical analyses, and for peripheral blood monocyte isolation. Carotid plaque specimens were obtained immediately following surgical resection.

6.3.2 Longitudinal study population

Patients with various cardiovascular risk factors, including hypercholesterolemia, were recruited from the Vascular Health Clinic at the McGill University Health Centre. All subjects had clinical indication for statin therapy, were statin naïve, and were prescribed a low dose statin at the baseline visit, which was titrated until LDL levels were at target (<2 mmol/L). Blood samples were collected from each patient on the day of recruitment (baseline visit [pre-statins]) and at each follow-up (f/u) visit (early f/u: <5 months after baseline, late f/u: ≥5 months after baseline) for biochemical analyses and peripheral blood monocyte isolation. Sociodemographic and clinical information were obtained from each patient as detailed in section 6.3.1.

6.3.3 Ethics approval

The cross-sectional and longitudinal studies fully comply with the Declaration of Helsinki; the McGill University's Institutional Ethics Review Board granted ethics approval (A12-M145-09B) and all subjects provided written informed consent prior to inclusion in the study.

6.3.4 Blood laboratory assays

Serum lipid profile (total cholesterol, HDL-C, triglycerides, apoA-I, apoB, high-sensitivity CRP, and glucose levels, as well as white blood cell count were measured at the McGill University Health Centre central labs. LDL-C levels were calculated using the Friedewald equation[498]. Plasma total and HMW adiponectin levels were determined by enzyme-linked immunosorbent assays (Human Total Adiponectin/Acrp30 Quantikine ELISA Kit, R&D Systems, Minneapolis, MN, USA; Human HMW Adiponectin/Acrp30 Quantikine ELISA Kit, R&D Systems). All samples were run in duplicate and the lower limits of detection were 0.891 ng/ml for total adiponectin and 0.989 ng/mL for HMW adiponectin. Inter-assay coefficients of variation were <7% and <9% for total and HMW adiponectin, respectively, while intra-assay coefficients of variation were <5%.

6.3.5 Histological assessment of carotid atherosclerotic plaque instability

The carotid plaque segment (approximately 3-4 mm in thickness) with the area of maximal stenosis and largest plaque burden was processed for histological assessment, as described previously[2,556]. Two vascular pathologists (JV and CL) independently and blindly (unaware of patients' clinical status) characterized the instability of the plaques, according to two gold-standard classifications: 1) AHA classification by Stary *et al*.[176], and 2) semi-quantitative scale by Lovett *et al*[177,178]. According to the AHA classification, plaques in our study were either classified as a Type V (fibroatheroma with a large lipid or necrotic core), Type VI (complex plaque with possible surface defect, hemorrhage, or thrombus), Type VII (calcified plaque), or Type VIII plaque (fibrotic plaque without lipid core), with Type V and VI plaques considered unstable[176]. According to the semi-quantitative scale, individual features of the plaque (i.e., inflammation, lipid core size, fibrous tissue content, etc.) were graded on a scale of 3 or 4. Based on the presence of a combination of these features, the overall instability of the plaque was defined as: definitely stable, probably stable, probably unstable, or definitely unstable[177,178].

6.3.6 Peripheral blood monocyte isolation

PBMCs were isolated from whole blood using Ficoll gradient centrifugation. Total monocytes were then isolated from PBMCs by positive selection with $CD14^+$ Human MicroBeads (Miltenyi Biotec, Germany), using a Magnetic Activated Cell-Sorting technique (autoMACS Pro Separator, Miltenyi Biotec).

RNA Isolation

Total RNA was extracted from monocytes using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA), and treated with RNase-free DNase I (RNase-Free DNase Set, Qiagen) to remove contamination with genomic DNA. Total RNA concentration was measured by absorbance at 260 nm using the BioDrop µLITE spectrophotometer (Montreal Biotech Inc., Dorval, QC, Canada) and its quality and purity were determined using the ratio of absorbance 260/280 and 260/230, respectively.

Quantitative Real-Time Polymerase Chain Reaction

cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA), as described previously[2]. mRNA expression of AdipoR1 (Human AdipoR1 PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCID0018245, Bio-Rad Laboratories, Hercules, CA, USA) and AdipoR2 (Human AdipoR2 PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCID0011851, Bio-Rad Laboratories) was assessed via SYBR Green-based qRT-PCR, using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). All measurements were performed in duplicate and C_q values obtained were normalized to the housekeeping gene, β-2 microglobulin (Human B2M PrimeTime qPCR Primers, Hs.PT.58v.18759587, Integrated DNA Technologies, Coralville, IA, USA). Relative mRNA abundance was calculated using the $2^{-\Delta\Delta Cq}$ method.

6.3.7 In vitro statin treatment of human macrophages

THP-1 cells, a human monocytic cell line (American Type Tissue Culture Collection, Camden, NJ, USA) were differentiated into macrophages by the addition of 200 nM PMA (InvivoGen, San Diego, CA, USA) for 48 hours in RPMI-1640 medium supplemented with 100 IU/mL of penicillin/streptomycin and 1% FBS. Macrophages were then treated with atorvastatin or rosuvastatin to determine their effects on cell viability, AdipoR expression, cytokine secretion, and

adiponectin-mediated signalling and function, as detailed below. In accordance with our longitudinal and cross-sectional *in vivo* studies, atorvastatin and rosuvastatin were chosen to test *in vitro*; these statins are the most clinically relevant and widely used in patients at risk for CVD as well as in patients with established CVD.

Cell Viability Assay

To determine the effect of various statin doses on cell viability, an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)-based assay was used (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit, Promega Corporation, Madison, WI, USA).

RNA Analyses

THP-1 monocytes were seeded in a 24 well-plate at a density of 1.0 x 10^6 cells/mL. Following differentiation into macrophages, cells were treated in triplicate with atorvastatin or rosuvastatin (Cayman Chemical, Ann Arbor, MI, USA) at increased doses (1 μM, 10 μM, 60 μM) for either 24 or 72 hours, in serum-free RPMI-1640 medium containing 0.5% BSA. Following statin treatment, the cells were collected for RNA extraction and the culture media for cytokine profiling. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) and qRT-PCR analysis of AdipoR1 and AdipoR2 mRNA expression was performed as above.

Cytokine Profiling

Cell culture media were profiled for macrophage secretion of various cytokines, IL-1β, IL-6, IL-10, and TNF-α, using a V-PLEX Human custom-made kit (MSD, Rockville, MD, USA). A 5-fold dilution of samples was performed, and all samples were run in duplicate. The lower limits of detection for IL-1β, IL-6, IL-10, and TNF-α were 0.035, 0.045, 0.030, and 0.094 pg/mL, respectively. Inter- and intra-assay coefficients of variation were <15% and <7%, respectively.

Protein Analyses – THP-1 monocytes were seeded in a 6 well-plate at a density of 1.0 x 10^6 cells/mL. Following differentiation into macrophages, cells were incubated for 24 hours with 5 μg/mL of human recombinant adiponectin (R&D Systems, Minneapolis, MN, USA) in serum-free RPMI-1640 medium containing 0.5% BSA. Cells were then treated in triplicate with atorvastatin or rosuvastatin at various doses (10 μM and 60 μM) for either 24 or 72 hours.

Following treatment, cells were lysed at 4°C with RIPA Lysis Buffer (Alpha Diagnostic International, San Antonio, TX, USA) in the presence of protease inhibitors. AMPK and PPAR-α were analyzed by SDS-PAGE, as described previously[2]. Fifteen µg of protein isolated from *in vitro* statin-treated monocyte-derived macrophages was mixed with an equal volume of Laemmli sample buffer (2X), boiled for 10 minutes at 95°C, and analyzed in duplicate by 10% SDS-PAGE. Samples were transferred to 0.45 µm nitrocellulose membrane, blocked with 5% non-fat milk in Tris-buffered saline (TBS) for 1 hour at room temperature and then incubated overnight at 4°C with rabbit polyclonal anti-human PPAR-α (1:1000 in 1% non-fat milk, ab3484, abcam, Cambridge, MA, USA), AMPK-phosphorylated (1:1000 in 1% non-fat milk, ab131357, abcam), or AMPK-total (1:4000 in 1% non-fat milk, ab32047, abcam). Rabbit anti-β-actin (1:2500 in 1% non-fat milk, ab75186, abcam) was used as a loading control. After washing in 0.1% Tween 20-TBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:6000 in 1% non-fat milk; Jackson ImmunoResearch, West Grove, PA, USA) for 2 hours at room temperature. Bands were detected by chemiluminescence using the SuperSignal West Pico substrate (Thermo Fisher Scientific) and band densities were quantified using ImageStudioLite software (LI-COR Biosciences, Lincoln, NE, USA).

Oxidized Low-density Lipoprotein Loading

THP-1 monocyte-derived macrophages (seeded in a 24 well-plate at a density of 1.0 x 10^6 cells/mL) were treated in duplicate for 24 hours in the presence of 5 µg/mL of human recombinant adiponectin and 60 µM of statin (atorvastatin or rosuvastatin) in serum-free RPMI-1640 medium containing 0.5% BSA. Following treatment, cells were loaded for 48 hours with oxidized LDL-containing radiolabeled cholesterol. Oxidized LDL-containing radiolabeled cholesterol was prepared by incubating 100 µg/mL of oxidized LDL (Alfa Aesar, Haverhill, MA, USA) with 4 µCi/mL of ^{3}[H]-free cholesterol (PerkinElmer, Waltham, MA, USA) at 37°C for 4 hours. The ^{3}[H]-cholesterol-oxidized LDL mixture was separated from the unconjugated ^{3}[H]-free cholesterol by using a 100 kDa centrifugal filter (Amicon Ultra-15 Centrifugal Filter Unit, MilliporeSigma, Burlington, MA, USA). Following oxidized LDL loading, cells were either used for 1) the visualization of intracellular neutral lipid droplets by fluorescent microscopy or 2) the quantification of intracellular cholesterol ester content by thin layer chromatography.

Visualization of Intracellular Neutral Lipids

Cells were stained using the neutral lipid droplet-specific fluorophore 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY^{TM} 493/503, Thermo Fisher Scientific) as described by Qiu *et al*[557]. Cells were incubated with 4μM BODIPY staining solution for 15 min at 37°C. Cell nuclei were counterstained with DAPI (Thermo Fisher Scientific). Bodipy fluorescence was visualized using the Zeiss Axio Imager M2 imaging system (Carl Zeiss Canada Ltd).

Quantification of Intracellular Cholesterol Ester Content

Lipids were extracted from cells using a mixture of hexane and isopropanol (HIP; 3:2 vol./vol.). Lipid extracts were dried under N_2 gas and then reconstituted in Folch solution (chloroform: methanol, 2:1, vol./vol.). Samples were loaded in triplicate onto Silica Gel (Analtech, Newark, DE, USA) thin layer chromatography plates, as previously reported[532]. Bands corresponding to ^{3}[H]-cholesterol ester were located by exposure to iodine vapour and were scraped off the plate into liquid scintillating vials and assayed for radioactivity by beta-scintillation. The results were normalized to total cellular protein content.

6.3.8 Statistics

Descriptive statistics were performed as appropriate to summarize the baseline characteristics between statin-naïve patients and statin users. Pearson and Spearman correlations were performed, as appropriate, between circulating adiponectin levels, circulating monocyte AdipoR1 and AdipoR2 mRNA expression, and clinical and biochemical variables. Parametric (independent T-test or ANOVA) and non-parametric tests (Mann-Whitney or Kruskal-Wallis) were used, as appropriate, to analyze differences in circulating adiponectin levels, other biochemical variables, and AdipoR1 and AdipoR2 mRNA expression on circulating monocytes between statin-naïve patients and statin users. Following significance with the ANOVA or Kruskal-Wallis test, post-hoc analyses were performed. Sub-analyses were performed based on the type and dose of statin used. For the *in vitro* analyses, independent-samples t-test was performed to determine changes in AdipoR expression, cytokine secretion, adiponectin-mediated signalling, and cholesterol ester content between monocyte-derived macrophages treated and non-treated with varying doses of atorvastatin and rosuvastatin. All statistical analyses were executed using SPSS, version 20.0 (IBM Corporation). A P-value of <0.05 (2-tailed) was considered significant. The degree of significance for the correlation matrix was adjusted using Bonferroni correction. The Bonferroni adjusted alpha

level used was P<0.003.

6.4 Results

6.4.1 Cross-sectional study results

Patient Characteristics and Lipid Profiles

Eighty-one percent of the patients recruited to undergo a CEA were statin users (n=157), where a majority of the population was either on atorvastatin (67.5%) or rosuvastatin therapy (26.8%). The remaining patients were either taking simvastatin (3.2%), pravastatin (1.9%), or fluvastatin (0.6%). Demographic and clinical parameters of 1) all statin users, 2) patients on atorvastatin therapy, 3) patients on rosuvastatin therapy, and 4) statin-naïve patients are presented in **Table 6.1**, while biochemical variables are presented in **Table 6.2**. A significantly greater proportion of patients on statin therapy (41.0%), specifically on atorvastatin (45.3%), had a history of CAD compared to the statin-naïve group (18.9%). Differences in the degree of carotid artery stenosis were approaching significance, with a smaller proportion of statin users (62.8%) having severe 80-99% stenosis than statin-naïve patients (80.0%).

Patients on atorvastatin or rosuvastatin therapy had significantly lower circulating levels of total cholesterol, LDL-C, HDL-C, apoA-I, apoB, and high-sensitivity CRP than statin-naïve patients (**Table 6.2**). Generally, greater reductions were observed with higher doses of statins used (atorvastatin: 40-80 mg; rosuvastatin: 20-40 mg) than low doses (atorvastatin: 10-20 mg; rosuvastatin: 5-10 mg) (**Table 6.3**).

Total and HMW Adiponectin Levels in Relation to Statin Use

Patients on rosuvastatin, but not atorvastatin, had significantly lower total and HMW adiponectin levels than statin-naïve patients (P<0.05; **Table 6.2**). Significantly lower adiponectin levels were observed in patients on high doses of rosuvastatin (20-40 mg) but not on low doses (5-10 mg) (**Table 6.3**).

Significant correlations were observed between circulating adiponectin levels and various clinical and biochemical variables; total and HMW adiponectin was highly and positively correlated with HDL-C (r=0.454, P<0.001; r=0.432, P<0.001) and apoA-I (r=0.342, P<0.001; r=0.337, P<0.001) and negatively correlated with BMI (r=-0.249, P=0.002; r=-0.263, P=0.001), triglycerides (r=-0.428, P<0.001; r=-0.411, P<0.001), and glucose levels (r=-0.236, P=0.001; r=-0.225, P=0.002).

Table 6.1 Cross-sectional Study: Population demographic and clinical characteristics in relation to statin use

Population Characteristic	Statin-naïve (n=37)	*Statin users (n=157)		Atorvastatin (n=106)		Rosuvastatin (n=42)	
		Values	P-value	Values	P-value	Values	P-value
Age, y	71.5±9.2	71.3±9.2	0.895	71.4±9.2	0.948	71.3±9.0	0.905
Sex, % men	59.5	72.0	0.137	71.7	0.168	76.2	0.111
BMI, kg/m^2	26.2±4.5	27.6±4.6	0.135	27.9±5.0	0.100	27.2±3.9	0.386
Ever smoker, %	75.7	64.3	0.188	59.6	0.081	78.0	0.804
CAS, 50-79%/80-99%	20.0/80.0	37.2/62.8	0.054	36.6/63.4	0.070	37.5/62.5	0.097
Cerebrovascular symptomatology, %	81.1	79.6	0.842	80.2	0.906	78.6	0.782
AF/TIA/stroke, %	20.0/40.0/40.0	19.5/43.1/37.4	0.951	20.5/38.6/41.0	0.990	15.2/54.5/30.3	0.514
CAD, %	18.9	41.0	**0.012**	45.3	**0.005**	31.7	0.196
SBP, mmHg	140±19	138±21	0.691	138±22	0.649	139±19	0.827
DBP, mmHg	77±10	75±10	0.470	75±11	0.568	76±9	0.800
Hypertension, %	73.0	85.9	0.057	86.8	0.053	85.4	0.176
Anti-hypertensive medication, %	85.2	94.8	0.072	94.6	0.105	94.3	0.229
T2DM, %	24.3	31.6	0.386	33.0	0.324	34.1	0.342
Anti-diabetic medication, %	88.9	95.9	0.381	94.3	0.567	100.0	0.202
Hypercholesterolemia, %	27.0	94.9	**<0.001**	94.3	**<0.001**	95.1	**<0.001**

Values are either represented as mean ± standard deviation (for continuous data) or as percentages (for nominal data).
BMI indicates body mass index; CAS, carotid artery stenosis; AF, amaurosis fugax; TIA, transient ischemic attack; CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2DM, type 2 diabetes mellitus.
*Includes patients on all types of statin therapy (atorvastatin [n=106], rosuvastatin [n=42], simvastatin [n=5], pravastatin [n=3], and fluvastatin [n=1])
P-value indicates comparison between statin-naïve patients and statin users (all types, atorvastatin, or rosuvastatin) (analysis was performed by independent-samples t-test, or Chi-square ($\chi 2$), as appropriate).

Table 6.2 Cross-sectional Study: Biochemical analyses in relation to statin use

Biochemical Analyte	Statin-naïve (n=37)	*Statin users (n=157)		Atorvastatin (n=106)		Rosuvastatin (n=42)	
		Values	P-value	Values	P-value	Values	P-value
Total cholesterol, mmol/L	3.86 [3.29-4.34]	3.05 [2.69-3.47]	**<0.001**	3.09 [2.69-3.43]	**<0.001**	2.93 [2.50-3.45]	**<0.001**
LDL-C, mmol/L	2.01 [1.62-2.43]	1.37 [1.04-1.69]	**<0.001**	1.40 [1.06-1.68]	**<0.001**	1.23 [0.89-1.53]	**<0.001**
HDL-C, mmol/L	1.08 [0.92-1.37]	0.93 [0.80-1.11]	**0.004**	0.96 [0.82-1.13]	**0.013**	0.87 [0.77-1.08]	**0.002**
Triglycerides, mmol/L	1.23 [0.93-1.62]	1.42 [1.09-1.85]	0.116	1.36 [0.98-1.71]	0.370	1.57 [1.28-1.95]	**0.011**
ApoA-I, g/L	1.24±0.26	1.13±0.19	**0.018**	1.13±0.18	**0.021**	1.12±0.21	**0.031**
ApoB, g/L	0.80±0.22	0.67±0.18	**<0.001**	0.66±0.18	**<0.001**	0.67±0.15	**0.002**
Glucose, mmol/L	6.10 [5.35-6.90]	5.70 [5.10-6.80]	0.195	5.70 [5.35-6.90]	0.192	5.80 [5.10-7.10]	0.585
hsCRP, mg/L	3.20 [1.65-9.00]	1.60 [0.90-4.25]	**0.006**	1.55 [0.90-4.09]	**0.005**	2.10 [0.68-4.63]	**0.037**
Total adiponectin, µg/mL	5.64 [3.60-9.91]	4.85 [3.21-7.68]	0.142	5.01 [3.47-7.78]	0.308	4.10 [2.60-6.27]	**0.017**
HMW adiponectin, µg/mL	3.82 [1.87-6.49]	3.03 [1.83-4.45]	0.175	3.11 [2.02-4.58]	0.334	2.51 [1.23-4.05]	**0.035**
HMW:Total adiponectin	0.57±0.14	0.58±0.16	0.685	0.59±0.15	0.518	0.56±0.17	0.838

Normally distributed data: values represent mean ± standard deviation; non-normally distributed data: values represent median [interquartile range].
LDL-C indicates low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoA-I, apolipoprotein AI; ApoB, apolipoprotein B; hsCRP, high-sensitivity C-reactive protein; HMW, high-molecular weight.
*Includes patients on all types of statin therapy (atorvastatin [n=106], rosuvastatin [n=42], simvastatin [n=5], pravastatin [n=3], and fluvastatin [n=1])
P-value indicates comparison between statin-naïve patients and statin users (all types, atorvastatin, or rosuvastatin) (analysis was performed by independent-samples t-test or Mann-Whitney U test, as appropriate).

Table 6.3 Cross-sectional Study: Biochemical analyses in relation to statin dose

Biochemical Analyte	Statin-naïve (n=37)	Atorvastatin (n=104)			Rosuvastatin (n=42)		
		10-20 mg (n=38)	40-80 mg (n=66)	P-value	5-10 mg (n=18)	20-40 mg (n=24)	P-value
Total cholesterol, mmol/L	3.86 [3.29-4.34]	3.20 [2.64-3.59]†	3.05 [2.69-3.43]‡	**<0.001**	3.04 [2.64-3.53]*	2.88 [2.48-3.39]‡	**<0.001**
LDL-C, mmol/L	2.01 [1.62-2.43]	1.46 [1.15-1.69]‡	1.33 [1.01-1.69]‡	**<0.001**	1.33 [1.05-1.75]*	1.08 [0.81-1.41]‡	**<0.001**
HDL-C, mmol/L	1.08 [0.92-1.37]	0.95 [0.82-1.22]	0.97 [0.84-1.12]	0.060	0.85 [0.73-1.18]	0.91 [0.78-1.04]*	**0.008**
Triglycerides, mmol/L	1.23 [0.93-1.62]	1.36 [1.04-1.68]	1.33 [0.93-1.76]	0.753	1.44 [1.28-1.89]	1.67 [1.24-2.14]*	**0.033**
ApoA-I, g/L	1.24±0.26	1.15±0.26	1.13±0.16*	**0.026**	1.11±0.25	1.13±0.19	0.096
ApoB, g/L	0.80±0.22	0.67±0.17†	0.64±0.21†	**0.001**	0.70±0.14	0.64±0.15†	**0.006**
Glucose, mmol/L	6.10 [5.35-6.90]	5.60 [5.10-6.58]	5.80 [5.10-6.60]	0.355	6.60 [5.40-8.75]	5.45 [4.93-6.70]	0.054
hsCRP, mg/L	3.20 [1.65-9.00]	1.50 [0.98-3.38]*	1.55 [0.88-4.53]*	**0.017**	2.57 [1.05-4.63]	2.01 [0.53-4.60]	0.089
Total adiponectin, µg/mL	5.64 [3.60-9.91]	4.53 [3.49-7.88]	5.58 [3.68-7.82]	0.473	3.93 [2.52-6.97]	4.43 [2.71-5.86]*	**0.018**
HMW adiponectin, µg/mL	3.82 [1.87-6.49]	3.11 [2.07-4.25]	3.14 [2.07-4.93]	0.643	2.48 [1.13-5.33]	2.51 [1.26-3.60]*	**0.025**
HMW:Total adiponectin	0.57±0.14	0.62±0.16	0.58±0.14	0.312	0.59±0.21	0.54±0.13	0.574

Normally distributed data: values represent mean ± standard deviation; non-normally distributed data: values represent median [interquartile range].
LDL-C indicates low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoA-I, apolipoprotein AI; ApoB, apolipoprotein B; hsCRP, high-sensitivity C-reactive protein; HMW, high-molecular weight.
Atorvastatin 30 mg (n=2) was excluded from the statin dose analyses, as the dose did not fall within the low (10-20 mg) or high (40-80 mg) atorvastatin dose groups.
P value indicates overall significance among atorvastatin high and low dose groups and statin-naïve group or among rosuvastatin high and low dose groups and statin-naïve group (analysis was performed by ANOVA or Kruskal–Wallis, as appropriate).
‡P<0.001 (Tukey multiple comparisons test between atorvastatin/rosuvastatin dose groups and statin-naïve group)
†P<0.01 (Tukey multiple comparisons test between atorvastatin/rosuvastatin dose groups and statin-naïve group)
*P≤0.05 (Tukey multiple comparisons test between atorvastatin/rosuvastatin dose groups and statin-naïve group)

AdipoR Expression on Circulating Monocytes in Relation to Statin Use

Total circulating white blood cell count, as well as absolute number and proportion of monocytes in the circulation, did not differ between statin users and statin-naïve patients (**Table 6.4**).

AdipoR1 and AdipoR2 mRNA expression on circulating monocytes was significantly lower in patients on statin therapy compared to statin-naïve patients (AdipoR1: 0.733±0.263 AU versus 1.000±0.174 AU; AdipoR2: 0.856±0.196 AU versus 1.000±0.110 AU; **Figure 6.1A**); this difference was specifically driven by rosuvastatin. Patients on high doses of atorvastatin (40-80 mg) had significantly lower AdipoR1 and AdipoR2 levels (by 1.51- and 1.23-fold, respectively) compared to statin-naïve patients, as well as significantly lower AdipoR2 expression compared to patients on low doses of atorvastatin (10-20 mg, $P<0.05$; **Figure 6.1B**). Similarly, high doses of rosuvastatin (20-40 mg) resulted in significantly lower AdipoR1 and AdipoR2 expression (by 1.80- and 1.28-fold, respectively) compared to statin-naïve patients ($P<0.05$; **Figure 6.1B**).

AdipoR1 and AdipoR2 mRNA expression profiles on circulating monocytes were not significantly correlated with circulating total and HMW adiponectin levels but were positively correlated with the HMW to total adiponectin ratio ($r=0.345$, $P<0.001$; $r=0.209$, $P=0.003$, respectively). In terms of other clinical and biochemical variables, serum concentrations of CRP were significantly correlated with monocyte AdipoR2 mRNA levels ($r=0.216$, $P=0.002$). Furthermore, AdipoR1 and AdipoR2 mRNA expression did not differ according to the following clinical covariates (smoking, carotid artery stenosis, cerebrovascular symptomatology, CAD, hypertension, T2DM, and carotid plaque stability; data not shown).

Carotid plaque specimens were characterized as either ‘stable’ or ‘unstable’. There were no observed differences in carotid plaque instability and in histological features of the plaque between statin users and statin-naïve subjects (**Table 6.5**). Among subjects with stable carotid plaques, AdipoR1 and AdipoR2 mRNA expression did not differ significantly between those taking versus not taking statins (**Figure 6.1C**). However, among subjects with unstable plaques, AdipoR1 and AdipoR2 mRNA levels were significantly lower in statin users versus statin-naïve subjects (1.80- and 1.55-fold lower, respectively; $P<0.05$; **Figure 6.1D**); this difference was specifically driven by rosuvastatin (2.39- and 1.49-fold lower, respectively; $P<0.05$; **Figure 6.1D**).

Table 6.4 White blood cell count and differential in relation to statin use

	Statin-naïve (n=34)	*Statin users (n=148)		Atorvastatin (n=102)		Rosuvastatin (n=38)	
		Values	P-value	Values	P-value	Values	P-value
WBCs, 10^9/L	7.31 [6.07-9.32]	8.14 [6.73-9.88]	0.069	8.14 [6.70-9.84]	0.109	7.75 [6.40-9.67]	0.169
Lymphocytes, 10^9/L	1.61 [1.27-2.12]	1.88 [1.37-2.30]	0.098	1.80 [1.30-2.25]	0.351	1.90 [1.45-2.58]	**0.032**
Lymphocyte:WBC, %	21.88 [17.80-26.58]	23.18 [18.30-29.05]	0.385	22.40 [16.28-28.56]	0.900	23.91 [20.18-29.55]	0.083
Monocytes, 10^9/L	0.61 [0.45-0.80]	0.65 [0.53-0.82]	0.137	0.66 [0.55-0.82]	0.125	0.62 [0.50-0.82]	0.364
Monocyte:WBC, %	7.99 [6.26-9.68]	8.19 [6.78-10.00]	0.575	8.33 [7.02-10.01]	0.423	8.31 [6.32-10.25]	0.791
Neutrophils, 10^9/L	4.77 [4.01-5.67]	5.22 [4.20-6.39]	0.250	5.34 [4.22-6.60]	0.193	4.75 [4.10-6.27]	0.817
Neutrophil:WBC, %	66.89 [60.16-70.76]	64.67 [59.21-71.29]	0.571	65.75 [59.40-72.76]	0.980	63.00 [58.85-69.69]	0.172
Eosinophils, 10^9/L	0.16 [0.09-0.24]	0.17 [0.10-0.25]	0.491	0.17 [0.10-0.26]	0.604	0.18 [0.10-0.24]	0.270
Eosinophil:WBC, %	2.14 [1.08-3.67]	2.19 [1.24-3.13]	0.977	2.23 [1.18-3.17]	0.930	2.36 [1.59-2.96]	0.648
Basophils, 10^9/L	0.02 [0.00-0.06]	0.04 [0.00-0.09]	0.356	0.03 [0.00-0.08]	0.515	0.04 [0.00-0.09]	0.503
Basophil:WBC, %	0.22 [0.00-0.91]	0.50 [0.00-0.93]	0.551	0.36 [0.00-0.89]	0.709	0.58 [0.00-0.95]	0.645

Values are represented as median [interquartile range].
WBC indicates white blood cell.
*Includes patients on all types of statin therapy (atorvastatin [n=102], rosuvastatin [n=38], simvastatin [n=4], pravastatin [n=3], and fluvastatin [n=1])
P-value indicates comparison between statin-naïve patients and statin users (all types, atorvastatin, or rosuvastatin) (analysis was performed by Mann-Whitney U test).

Table 6.5 Prevalence of histological features of the plaque according to statin use

Histological Plaque Features	Statin-naïve (n=37)	*Statin users (n=157)		Atorvastatin (n=106)		Rosuvastatin (n=42)	
		Values	P-value	Values	P-value	Values	P-value
Overall Instability			0.429		0.537		0.145
Definitely Stable, %	16.2	24.8		27.4		16.7	
Probably Stable, %	40.5	29.9		31.1		26.2	
Probably Unstable, %	18.9	14.6		17.0		9.5	
Definitely Unstable, %	24.3	30.6		24.5		47.6	
AHA Plaque Classification			0.694		0.795		0.360
Type V, %	35.1	26.1		27.4		19.0	
Type VI, %	29.7	36.9		34.9		45.2	
Type VII, %	27.0	29.9		31.1		26.2	
Type VIII, %	8.1	7.0		6.6		9.5	
Hemorrhage			0.931		1.000		0.440
No hemorrhage, %	56.8	54.1		56.6		45.2	
Small hemorrhage, %	24.3	24.2		24.5		23.8	
Large hemorrhage, %	18.9	21.7		18.9		31.0	
Thrombus			0.940		0.815		0.744
No thrombus, %	78.4	80.3		83.0		71.4	
Small thrombus, %	18.9	17.8		15.1		26.2	
Large thrombus, %	2.7	1.9		1.9		2.4	

Lipid Core			0.130		0.252	0.111
No lipid core, %	18.9	14.0		15.1	14.3	
Small lipid core, %	27.0	45.2		42.5	50.0	
Large lipid core, %	54.1	40.8		42.5	35.7	
Fibrous Tissue			0.352		0.500	0.201
Very little fibrous tissue, %	16.2	8.9		10.4	4.8	
~50% fibrous tissue, %	62.2	62.4		60.4	64.3	
Predominantly fibrous, %	21.6	28.7		29.2	31.0	
Foam Cells			0.086		0.205	0.179
None, %	27.0	38.2		37.7	33.3	
<50 cells, %	32.4	38.9		36.8	45.2	
At least 50 cells, %	40.5	22.9		25.5	21.4	
New Vessels			0.587		0.638	0.508
None, %	35.1	38.9		40.6	28.6	
<10 per section, %	32.4	36.9		34.9	45.2	
At least 10 per section, %	32.4	24.2		24.5	26.2	
Calcification			0.434		0.587	0.169
None, %	24.3	14.0		17.9	7.1	
Stippling only, %	24.3	28.7		25.5	38.1	
Calcified nodules, %	51.3	57.4		56.6	54.8	
Inflammatory Cells			0.209		0.168	0.231
None, %	24.3	28.0		34.0	11.9	

Occasional cells, %	35.1	48.4		44.3	57.1	
2-5 groups of >50 cells, %	32.4	18.5		17.0	23.8	
>5 groups of >50 cells, %	8.1	5.1		4.7	7.1	
Cap Infiltration			0.568	0.661		0.898
None, %	43.2	55.6		54.3	51.3	
<10 cells in cap, %	27.0	20.9		21.0	25.6	
10-50 cells in cap, %	16.2	11.1		11.4	12.8	
>50 cells in cap, %	13.5	12.4		13.3	10.3	
Rupture			0.993	0.961		0.446
Intact cap, %	54.1	53.9		56.2	50.0	
Probably intact, %	27.0	26.6		28.6	17.5	
Probably ruptured, %	13.5	14.9		10.5	27.5	
Definitely ruptured, %	5.4	4.5		4.8	5.0	

AHA indicates American Heart Association.
Plaque and cap inflammation were graded according to the number of macrophages and lymphocytes present.
*Includes patients on all types of statin therapy (atorvastatin [n=106], rosuvastatin [n=42], simvastatin [n=5], pravastatin [n=3], and fluvastatin [n=1]).
P-value indicates comparison between statin-naïve patients and statin users (all types, atorvastatin, or rosuvastatin) (analysis was performed by Chi-square [$\chi 2$]).

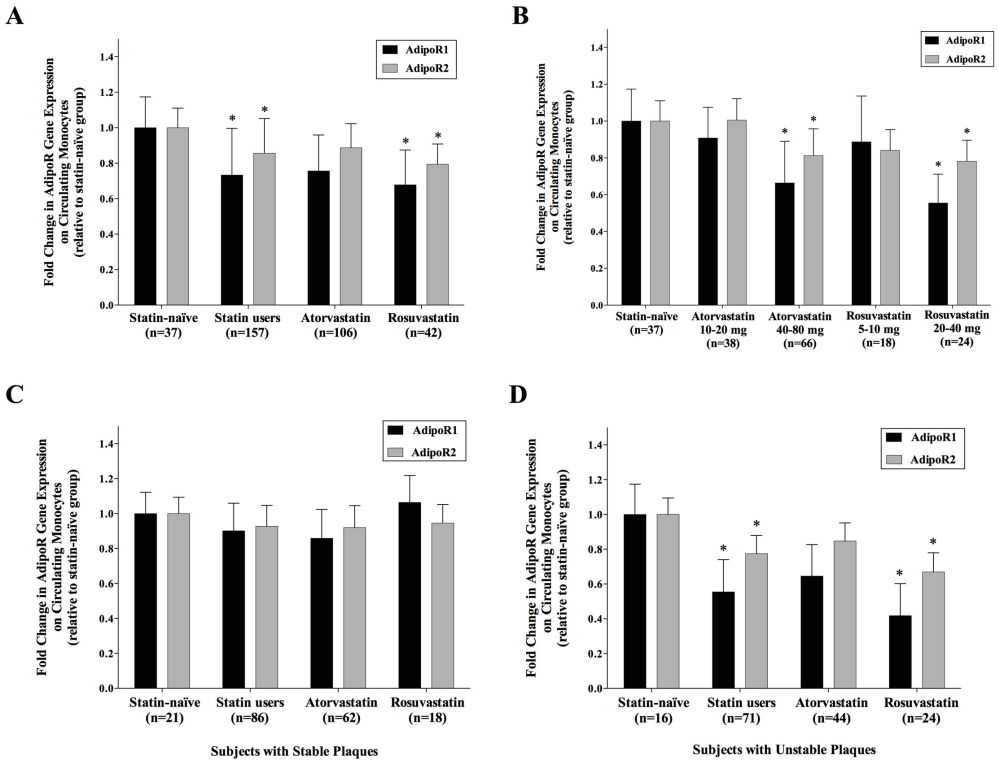


Figure 6.1 Cross-sectional study: AdipoR1 and AdipoR2 gene expression on circulating monocytes in relation to statin use and dose. *Bar graphs represent qRT-PCR data for AdipoR1 and AdipoR2 mRNA levels on circulating monocytes. Data presented as mean ± SD.* ***A)*** *Analysis comparing CEA patients on 1) all types of statin therapy (atorvastatin [n=106], rosuvastatin [n=42], simvastatin [n=5], pravastatin [n=3], and fluvastatin [n=1]), 2) atorvastatin therapy, or 3) rosuvastatin therapy, to statin-naïve patients was performed by independent-samples t-test. *P<0.05.* ***B)*** *Analysis comparing patients on 1) low and high doses of atorvastatin (low: 10-20 mg, high: 40-80 mg) or 2) low and high doses of rosuvastatin (low: 5-10 mg, high: 20-40 mg), to statin-naïve patients was performed by ANOVA. *P<0.05 (Tukey multiple comparisons test between patients on high doses of atorvastatin and statin-naïve patients, and between patients on high doses of rosuvastatin and statin-naïve patients).* ***C)*** *Analysis comparing subjects with stable plaques on 1) all types of statin therapy (atorvastatin [n=62], rosuvastatin [n=18], simvastatin [n=4], pravastatin [n=1], and fluvastatin [n=1]), 2) atorvastatin therapy, or 3) rosuvastatin therapy, to statin-naïve subjects with stable plaques was performed by independent-samples t-test. *P<0.05.* ***D)*** *Analysis comparing subjects with unstable plaques on 1) all types of statin therapy (atorvastatin [n=44], rosuvastatin [n=24], simvastatin [n=1], and pravastatin [n=2]), 2) atorvastatin therapy, or 3) rosuvastatin therapy, to statin-naïve subjects with unstable plaques was performed by independent-samples t-test. *P<0.05.*

6.4.2 Longitudinal study results

Patient Characteristics, Lipid Profiles, and Circulating Adiponectin Levels

The baseline demographic and clinical characteristics of the 17 subjects initiated on statin therapy (atorvastatin or rosuvastatin) are presented in **Table 6.6**. All subjects were hypercholesterolemic at the baseline visit (pre-statins) and presented with additional cardiovascular risk factors, such as smoking (46.7%), hypertension (64.7%), and T2DM (23.5%). At the early and late f/u visits after statin initiation, total cholesterol, LDL-C, and apoB levels were significantly lower compared to pre-statin levels (all $P<0.001$, **Table 6.7** and **Figure 6.2C**). Atorvastatin and rosuvastatin significantly reduced total cholesterol and LDL-C levels at both the early and late f/u visits (**Figure 6.2D**). Circulating total and HMW adiponectin levels were not significantly different before and after statin initiation, although there was a trend towards lower levels following rosuvastatin therapy (but not atorvastatin therapy), particularly at the late f/u visit (**Table 6.8**).

Table 6.6 Longitudinal Study: Baseline population demographic and clinical characteristics

Population Characteristic	Baseline visit (n=17)
Age, y	74.1±17.0
Sex, % men	46.7
BMI, kg/m^2	28.5±4.6
Ever smoker, %	46.7
Carotid artery disease, %	35.3
Cerebrovascular symptomatology, %	5.9
CAD, %	12.5
SBP, mmHg	127±19
DBP, mmHg	74±9
Hypertension, %	64.7
T2DM, %	23.5
Hypercholesterolemia, %	100.0

Values are either represented as mean ± standard deviation (for continuous data) or as percentages (for nominal data).
BMI indicates body mass index; CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2DM, type 2 diabetes mellitus.

Table 6.7 Longitudinal Study: Lipid and inflammatory profile before and after statin use

Biochemical Analyte	Pre-Statins (n=17)	Early f/u, <5 Mo. (n=17)	Late f/u, ≥5 Mo. (n=17)	P-Value
Total cholesterol, mmol/L	5.77±0.64	*4.23±0.42	*3.83±0.51	**<0.001**
LDL-C, mmol/L	3.77±0.56	*2.26±0.38	*†1.93±0.26	**<0.001**
HDL-C, mmol/L	1.48±0.50	1.45±0.42	1.38±0.44	0.680
Triglycerides, mmol/L	1.05 [0.81-1.23]	1.00 [0.78-1.41]	0.92 [0.71-1.34]	0.613
ApoA-I, g/L	1.68±0.37	1.67±0.40	1.63±0.56	0.780
Apo-B, g/L	1.14±0.15	*0.80±0.15	*0.73±0.07	**<0.001**
hsCRP, mg/L	3.25±2.59	3.09±2.83	3.45±2.83	0.842

Normally distributed data: values represent mean ± standard deviation; non-normally distributed data: values represent median [interquartile range]
f/u indicates follow-up; Mo., months; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; ApoA-I, apolipoprotein A1; Apo-B, apolipoprotein B; hsCRP, high-sensitivity C-reactive protein
P-value indicates significance among the three measurement groups: pre-statins, early f/u, late f/u (analysis was performed by one-way ANOVA with repeated measures or Friedman test, as appropriate, depending if data were normally distributed)
*P<0.001 vs. pre-statins
†P<0.05 vs. early f/u

Table 6.8 Longitudinal Study: Circulating adiponectin levels before and after statin use

	Pre-Statins	Early f/u, <5 Mo.	Late f/u, ≥5 Mo.	P-Value
Statins combined (n=17)				
Total adiponectin, µg/mL	9.28 [4.01-17.55]	8.64 [3.98-19.97]	6.63 [3.38-9.15]	0.273
HMW adiponectin, µg/mL	6.66 [1.84-12.39]	6.52 [1.93-13.67]	4.31 [2.73-6.38]	0.368
HMW:Total adiponectin	0.67±0.18	0.64±0.11	0.67±0.12	0.410
Atorvastatin alone (n=9)				
Total adiponectin, µg/mL	5.38 [3.18-9.28]	6.61 [3.20-11.91]	6.22 [4.11-7.48]	0.779
HMW adiponectin, µg/mL	3.24 [1.46-6.66]	3.90 [1.50-8.58]	4.22 [2.47-4.65]	0.936
HMW:Total adiponectin	0.61±0.13	0.63±0.12	0.64±0.12	0.910
Rosuvastatin alone (n=8)				
Total adiponectin, µg/mL	13.06 [7.59-20.35]	15.75 [6.93-21.46]	7.98 [3.04-16.71]	0.311
HMW adiponectin, µg/mL	10.51 [6.23-14.91]	9.44 [5.33-15.14]	5.35 [2.29-12.58]	0.115
HMW:Total adiponectin	0.74±0.21	0.66±0.11	0.69±0.13	0.379

Normally distributed data: values represent mean ± standard deviation; non-normally distributed data: values represent median [interquartile range]
HMW indicates high-molecular weight
P-value indicates significance among the three measurement groups: pre-statins, early f/u, late f/u (analysis was performed by one-way ANOVA with repeated measures or Friedman test, as appropriate, depending if data were normally distributed)

AdipoR Expression on Circulating Monocytes Before and After Statin Use

AdipoR1 and AdipoR2 mRNA expression on circulating monocytes was decreased at the late f/u visit (≥5 months after statin initiation) (AdipoR1: 0.64±0.19 AU versus 1.00±0.19 AU; AdipoR2: 0.83±0.17 AU versus 1.00±0.16 AU; **Figure 6.2A**), while no differences were observed at the early f/u visit. This was consistent regardless of the type of statin used (atorvastatin or rosuvastatin) (**Figure 6.2B**). AdipoR1 and AdipoR2 mRNA expression profiles on circulating monocytes were not correlated with the changes observed in total and LDL-C levels at the early and late f/u visits (data not shown).

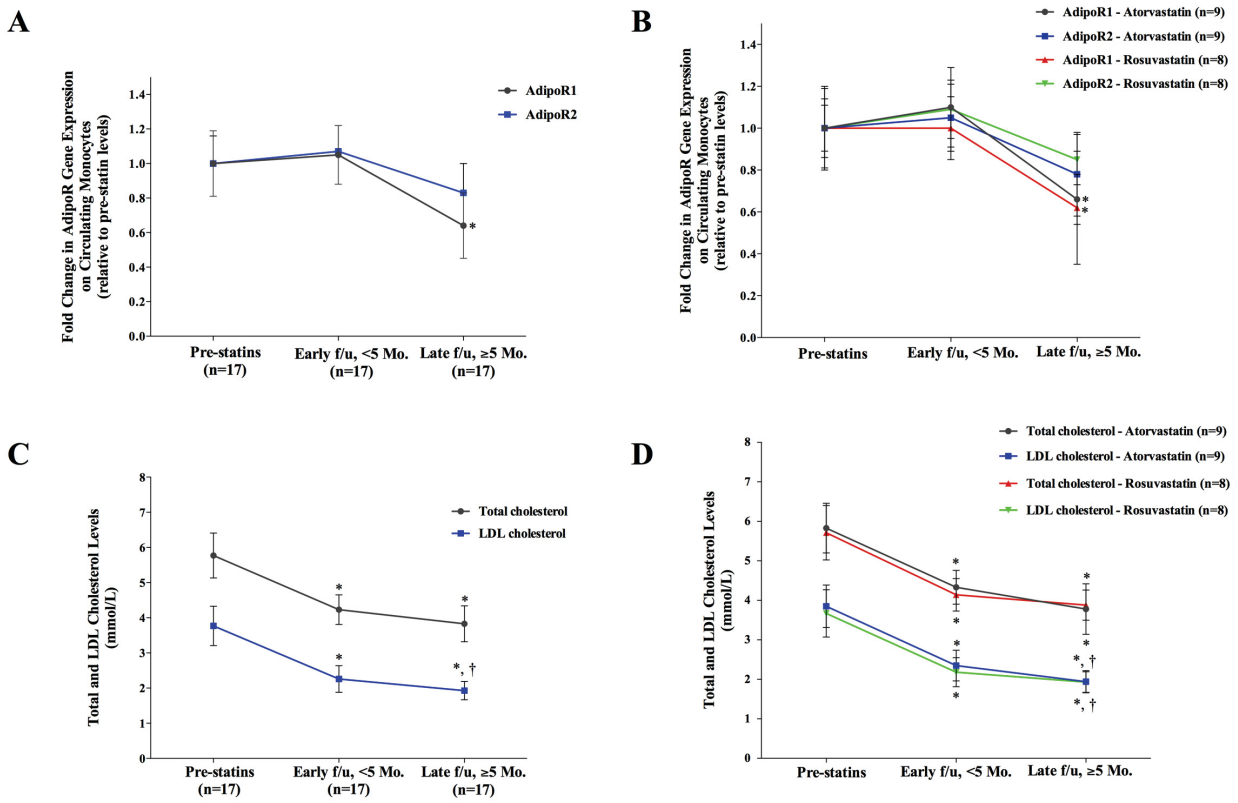


Figure 6.2 Longitudinal study: AdipoR1 and AdipoR2 gene expression on circulating monocytes and lipid profiles before and after statin use. ***A-B)*** *Data represent qRT-PCR data for AdipoR1 and AdipoR2 mRNA levels on circulating monocytes and are presented as mean ± SD. Analysis comparing expression levels at early f/u and late f/u visits to pre-statin levels was performed by one-way ANOVA with repeated measures.* ***A)*** *AdipoR1: P<0.01; AdipoR2: P=0.084. *P<0.05 vs. pre-statins and early f/u.* ***B)*** *AdipoR1 - Atorvastatin: P<0.05; AdipoR2 - Atorvastatin: P>0.05; AdipoR1 - Rosuvastatin: P<0.05; AdipoR2 - Rosuvastatin: P>0.05. *P<0.05 vs. pre-*

statins and early f/u. ***C-D)*** *Data represent circulating total and LDL-C levels and are presented as mean ± SD. Analysis comparing levels at early f/u and late f/u visits to pre-statin levels was performed by one-way ANOVA with repeated measures.* ***C)*** *Total cholesterol: P<0.001; LDL-C: P<0.001. *P<0.001 vs. pre-statins; †P<0.05 vs. early f/u.* ***D)*** *Total cholesterol - Atorvastatin: P<0.001; LDL-C - Atorvastatin: P<0.001; Total cholesterol - Rosuvastatin: P<0.001; LDL cholesterol - Rosuvastatin: P<0.001. *P<0.001 vs. pre-statins; †P<0.05 vs. early f/u.*

6.4.3 In vitro statin treatment of human monocyte-derived macrophages

Treatment of monocyte-derived macrophages with atorvastatin or rosuvastatin for 24 and 72 hours did not affect cell viability compared to untreated macrophages. The cell viability remained >95% for 1 μM, 10 μM, and 60 μM of atorvastatin and rosuvastatin tested (**Figure 6.3**).

Statins' Effect on AdipoR Expression

When human monocyte-derived macrophages were treated for 24 hours with varying doses of atorvastatin and rosuvastatin, lower doses (1 μM and 10 μM) resulted in a significant upregulation in AdipoR2 but not AdipoR1 mRNA expression in comparison to no statin treatment, with the 10 μM dose having the greatest effect (**Figure 6.4A**). In contrast, 60 μM atorvastatin led to a significant 1.27-fold reduction in AdipoR1 expression. AdipoR1 expression was also decreased with 60 μM rosuvastatin but non-significantly (**Figure 6.4A**). Following 72-hour statin treatment, AdipoR1 levels were reduced significantly by 10 and 60 μM atorvastatin (by 1.22- and 1.39-fold, respectively), as well as 60 μM rosuvastatin (by 1.40-fold), compared to no statin treatment (**Figure 6.4B**). Furthermore, 60 μM rosuvastatin also led to a significant reduction in AdipoR2 (**Figure 6.4B**). Overall, longer exposure of macrophages to atorvastatin or rosuvastatin resulted in lower AdipoR expression levels at all doses tested than 24-hour statin treatment (**Figure 6.5**).

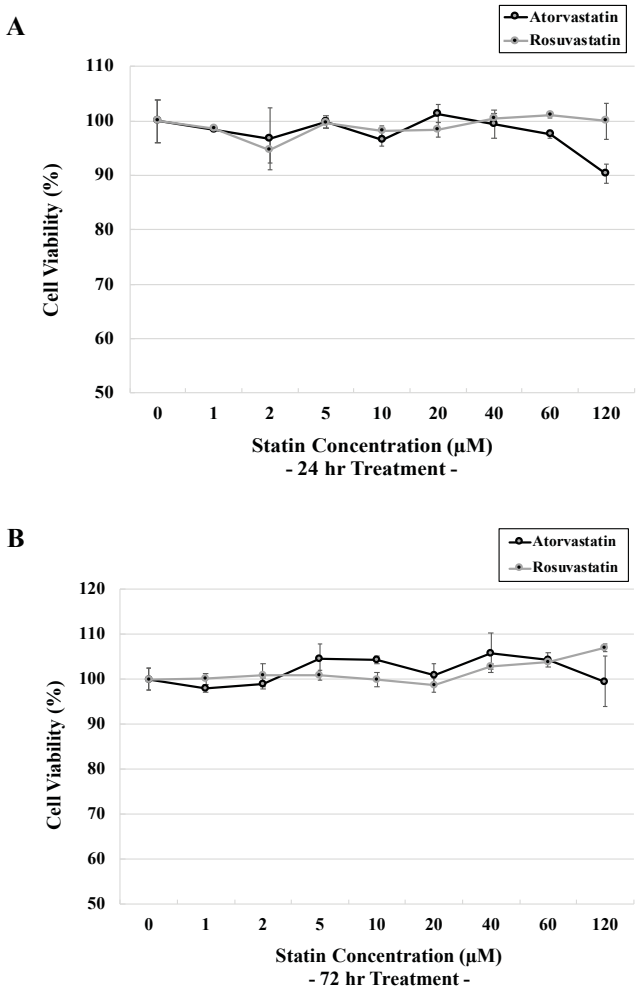


Figure 6.3 Statins' effect on cell viability. *THP-1 monocyte-derived macrophages were seeded into 96-well plates at a density of 1.5×10^4 per well (100 µL). Cells were treated in triplicate with increasing doses (1.25, 2.5, 5, 10, 20, 40, 60, and 120 µM) of atorvastatin and rosuvastatin in serum-free RPMI-1640 medium containing 0.5% BSA for* ***A)*** *24 hours and* ***B)*** *72 hours. 20 µL of MTS reagent was added into each well and cells were incubated for 2 hours at 37˚C in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was recorded using an ELISA plate reader. Each point represents the mean ± SD of 3 replicates and is expressed as % live cells relative to non-treated group. Background absorbance was measured from control wells containing medium without cells and subtracted from these data.*

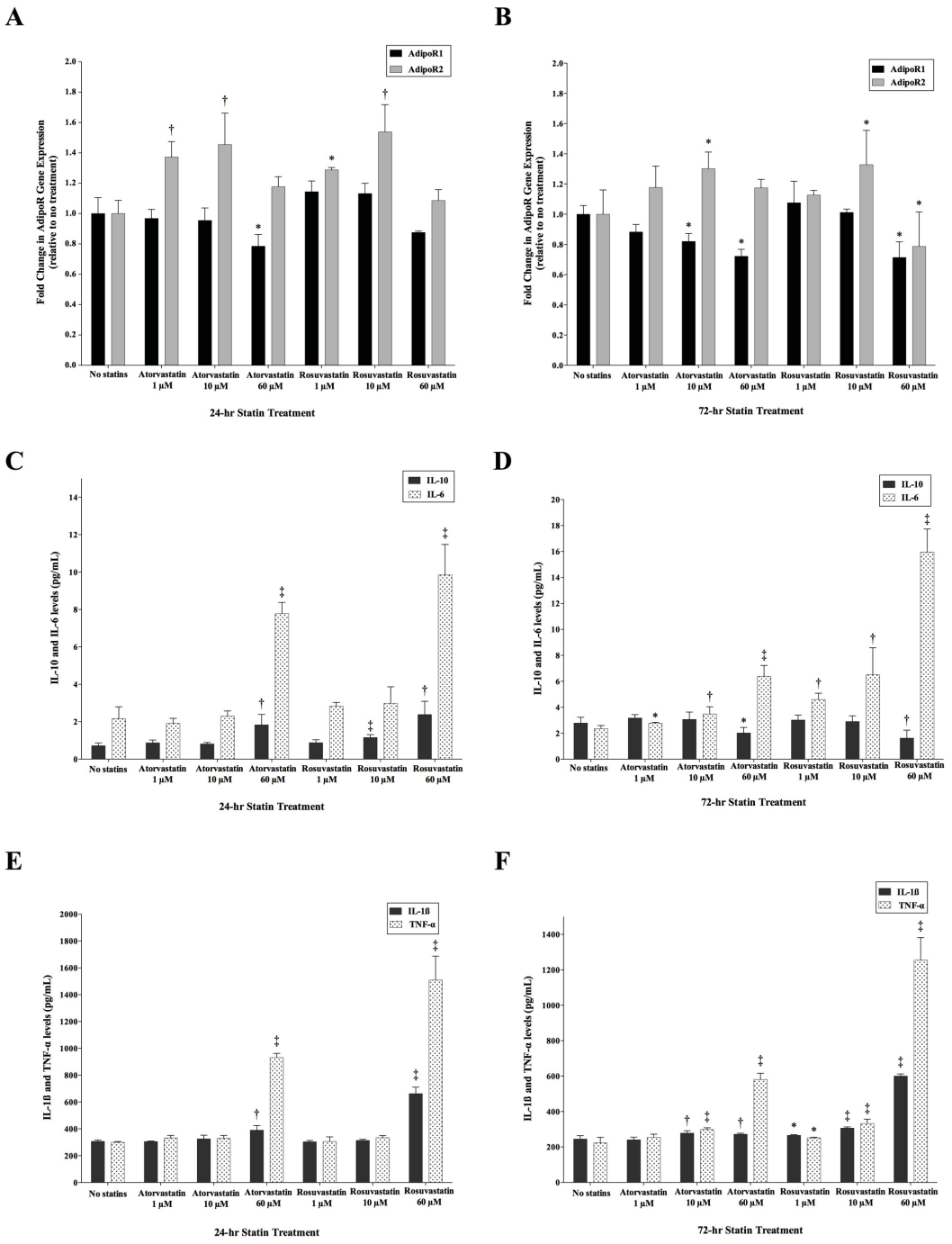


Figure 6.4 Effects of in vitro statin treatment on AdipoR expression and cytokine secretion in monocyte-derived macrophages. ***A-B*** *Comparing changes in AdipoR1 and AdipoR2 gene expression between macrophages treated and non-treated with increasing doses of atorvastatin and rosuvastatin (1 μM, 10 μM, and 60 μM) for* ***A)*** *24 or* ***B)*** *72 hours. Bar graphs represent qRT-PCR data for AdipoR1 and AdipoR2 mRNA levels on macrophages. Data presented as mean ± SD. *P<0.05, †P<0.01 vs. untreated macrophages (independent-samples t-test). Experiment was repeated three times.* ***C-F*** *Comparing changes in IL-10, IL-6, IL-1β, and TNF-α cytokine secretion*

from macrophages treated and non-treated with increasing doses of atorvastatin and rosuvastatin (1 µM, 10 µM, and 60 µM) for ***C & E)*** *24, or* ***D & F)*** *72 hours. Data presented as mean ± SD. *P<0.05, †P<0.01, ‡P<0.001 vs. untreated macrophages (independent-samples t-test). Experiment was repeated three times.*

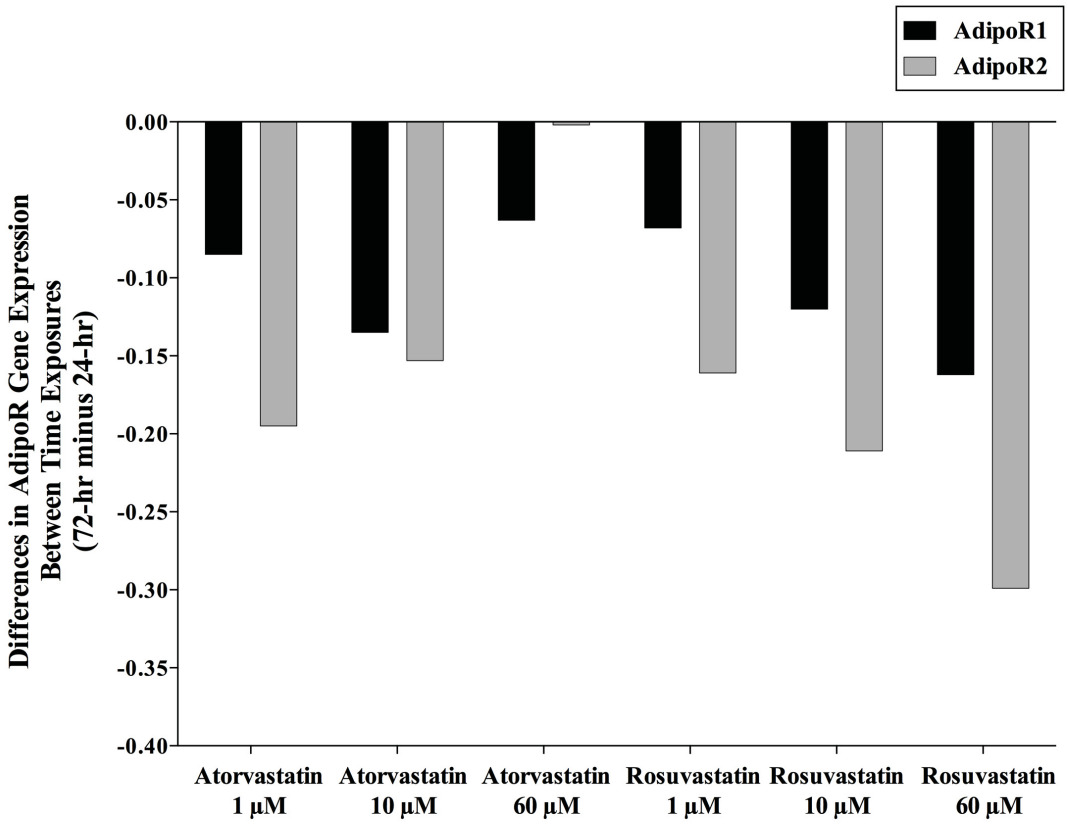


Figure 6.5 Differences in AdipoR1 and AdipoR2 gene expression between different time exposures*. Bar graphs represent the change in AdipoR expression on monocyte-derived macrophages treated with increasing doses of atorvastatin and rosuvastatin between different time exposures (72-hour minus 24-hour statin treatment).*

Statins' Effect on Cytokine Secretion

Twenty-four-hour treatment of monocyte-derived macrophages with 60 µM atorvastatin or rosuvastatin led to a significant increase in the secretion of the anti-inflammatory cytokine, IL-10, and pro-inflammatory cytokines, IL-6, IL-1ß, and TNF-α (**Figures 6.4C** and **6.4E**). However, treatment with 10 µM rosuvastatin only led to a significant increase in IL-10 secretion (P<0.001; **Figure 6.4C**). Long-term treatment (72 hours) with 60 µM atorvastatin or rosuvastatin resulted in

a significant reduction in IL-10 levels, while increasing doses of atorvastatin or rosuvastatin promoted a progressive and significant increase in IL-6, IL-1ß, and TNF-α secretion (**Figures 6.4D** and **6.4F**). Overall, 24- and 72-hour treatment with rosuvastatin had a greater effect on cytokine secretion than atorvastatin.

Statins' Effect on Adiponectin-mediated Signalling

Adiponectin binding to AdipoR1 and AdipoR2 triggers the activation of AMPK and PPAR-α signalling, respectively. Following 24-hour treatment with 10 μM atorvastatin or rosuvastatin, AMPK activity was significantly increased in adiponectin-treated macrophages, while 60 μM rosuvastatin led to a significant decrease in its activity, compared to control macrophages (treated only with adiponectin) (**Figure 6.6B**). Similarly, PPAR-α expression in adiponectin-treated macrophages was significantly increased with a 10 μM dose of rosuvastatin and decreased when a higher dose (60 μM) was used (**Figure 6.6B**). Seventy-two-hour statin treatment of macrophages with atorvastatin or rosuvastatin had no effect on adiponectin-mediated AMPK activity, but significantly reduced PPAR-α expression when 60 μM dose was used (**Figure 6.6C**).

Statins' Effect on Adiponectin's Capacity to Suppress Cholesterol Ester Formation in Oxidized LDL-loaded Human Monocyte-derived Macrophages

Adiponectin has the capacity to significantly reduce cholesterol ester formation in oxidized LDL-loaded macrophages, thereby suppressing foam cell formation ($P<0.001$, **Figure 6.7**). We investigated whether intensive statin therapy can antagonize adiponectin's beneficial effects on lipid accumulation. Following the various treatment conditions, lipid droplet accumulation was visualized using BODIPY 493/503 (**Figure 6.7A**). Statin-treated oxidized LDL-loaded macrophages exhibited more neutral lipid droplets than oxidized LDL-loaded macrophages alone or those treated with adiponectin (**Figure 6.7A**). Furthermore, oxidized LDL-loaded macrophages treated with both adiponectin and 60 μM atorvastatin or rosuvastatin had significantly higher levels of intracellular cholesterol ester content, compared to oxidized LDL-loaded macrophages treated only with adiponectin ($P<0.001$, **Figure 6.7B**). Rosuvastatin demonstrated higher potency than atorvastatin in raising intracellular cholesterol ester levels (3.52-fold *vs.* 1.98-fold increase, respectively). Moreover, cholesterol ester levels within oxidized LDL-loaded macrophages treated with both adiponectin and statins were significantly lower compared to levels within oxidized LDL-loaded macrophages treated with statins alone ($P<0.05$, **Figure 6.7B**). These results suggest

that statin therapy did not fully abolish adiponectin's capacity to suppress cholesterol ester formation, but rather mitigated its protective effects.

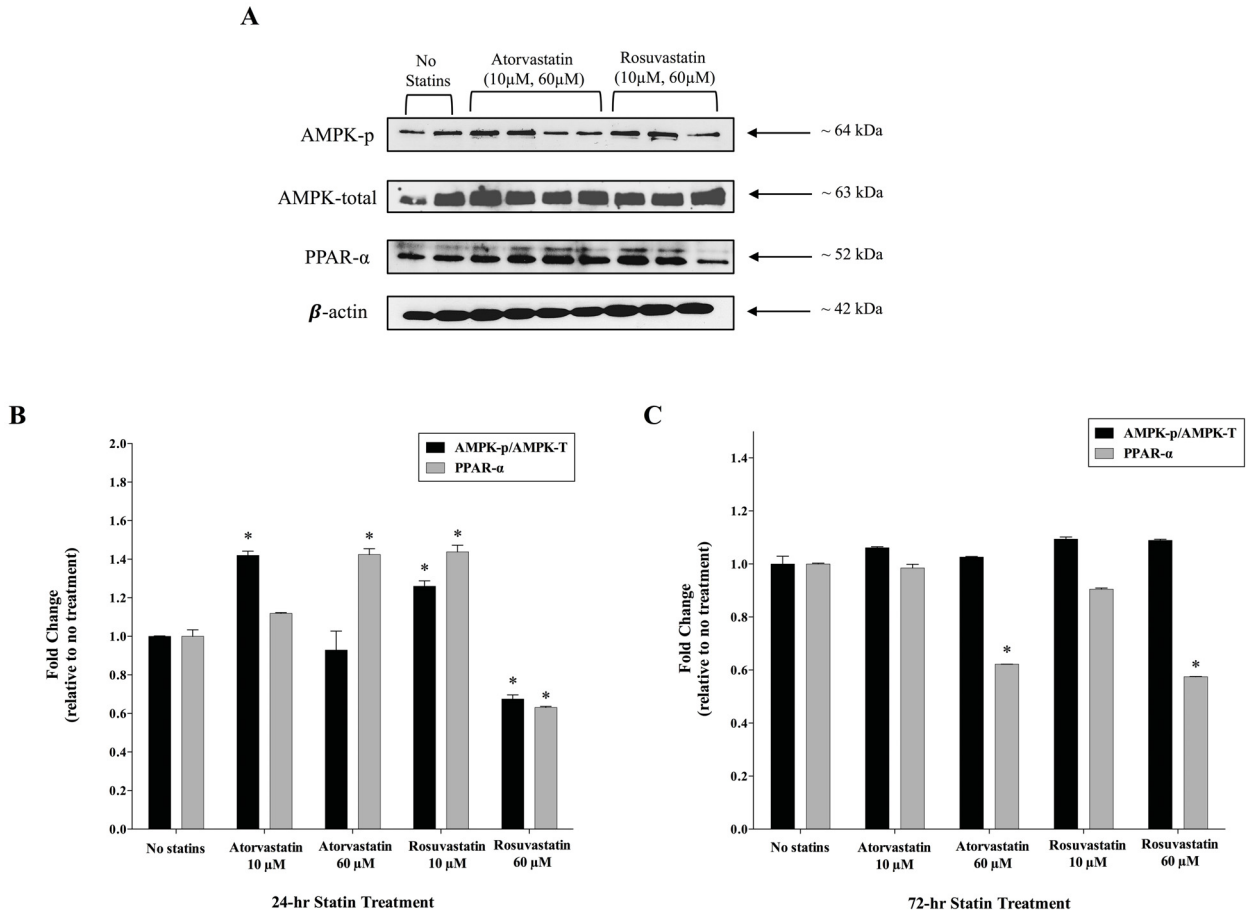


Figure 6.6 Effects of in vitro statin treatment on adiponectin-mediated signalling in monocyte-derived macrophages. ***A)*** *Representative Western blot showing expression of AMPK-phosphorylated, AMPK-total, and PPAR-α in macrophages treated and non-treated with 10 μM and 60 μM atorvastatin and rosuvastatin. All macrophages treated or non-treated with statins were pre-treated with 5 μg/mL of adiponectin. Samples were analyzed in duplicate. Comparing changes in protein expression between macrophages treated and non-treated with atorvastatin and rosuvastatin for* ***B)*** *24 or* ***C)*** *72 hours. Protein expression was determined by normalizing against the densitometric intensity of β-actin. Changes in AMPK activity were determined using the ratio of AMPK-phosphorylated to AMPK-total protein expression. Data presented as mean ± SD. *P<0.05 vs. untreated macrophages (independent-samples t-test). Experiment was repeated three times.*

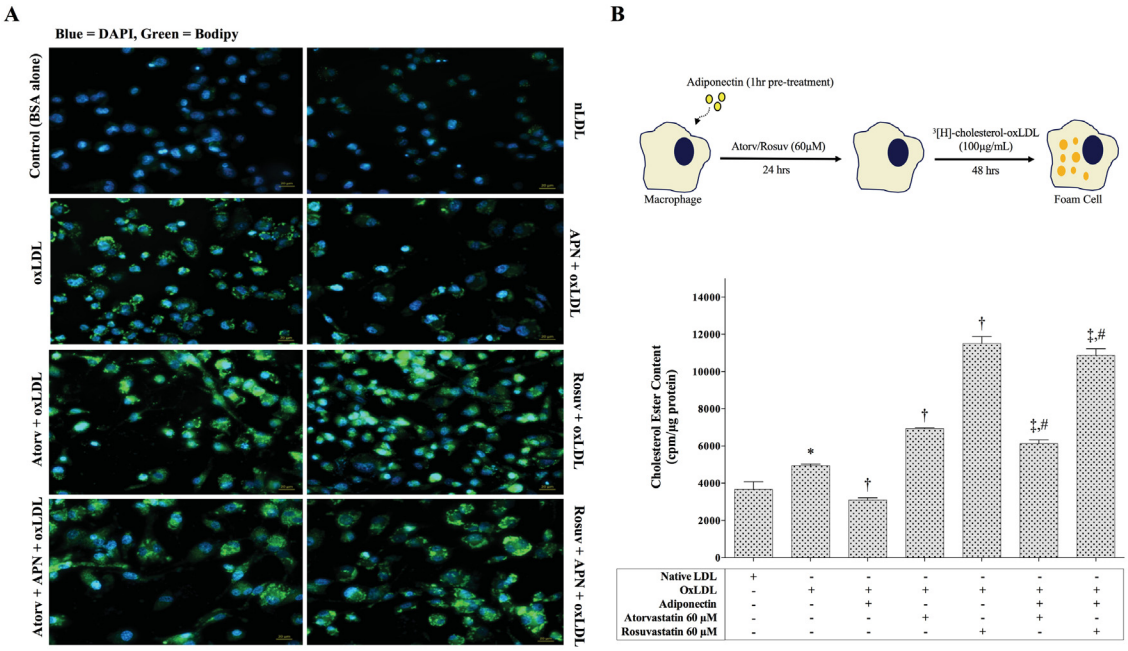


Figure 6.7 Statin treatment of oxidized LDL-loaded human monocyte-derived macrophages mitigated adiponectin's capacity to suppress cholesterol ester formation. ***A)*** *Cells under the following treatment conditions: (a) 0.5% BSA (control), (b) native LDL, (c) oxidized LDL, (d) adiponectin + oxidized LDL, (e) atorvastatin + oxidized LDL, (f) rosuvastatin + oxidized LDL, (g) atorvastatin + adiponectin + oxidized LDL, (h) rosuvastatin + adiponectin + oxidized LDL, were stained with 4 μM BODIPY 493/503 (green) to label intracellular neutral lipid droplets. Nuclei were stained with DAPI (blue). Scale bar, 20 μm.* ***B)*** *Bar graphs represent cholesterol ester content measured by thin layer chromatography. Radioactivity (^{3}[H]-cholesterol ester) was measured by beta-scintillation and divided by cellular protein content (cpm/μg). Data presented as mean ± SD. *P<0.01 vs. control macrophages loaded with native LDL; †P<0.001 vs. oxidized LDL-loaded macrophages; ‡P<0.001 vs. oxidized LDL-loaded macrophages treated with adiponectin; #P<0.05 vs. oxidized LDL-loaded macrophages treated with statins (independent-samples t-test). Experiment was repeated three times.*

6.5 Discussion

Our study identified a novel pleiotropic property of statins in modulating the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage. We demonstrated that intensive statin therapy can compromise the expression and function of adiponectin and its receptors (see Graphical Abstract – **Figure 6.8**). *In vivo*, patients on high doses of atorvastatin or rosuvastatin resulted in significantly lower AdipoR expression on circulating monocytes than statin-naïve

patients. Furthermore, ≥5 months of statin treatment resulted in lower AdipoR1 and AdipoR2 expression compared to pre-statin levels. *In vitro*, higher doses and longer exposure of macrophages to atorvastatin or rosuvastatin resulted in a greater decrease in AdipoR mRNA expression and signalling through AMPK and PPAR-α, and greater secretion of pro-inflammatory cytokines. Furthermore, statins reduced adiponectin's capacity to suppress intracellular cholesterol ester formation.

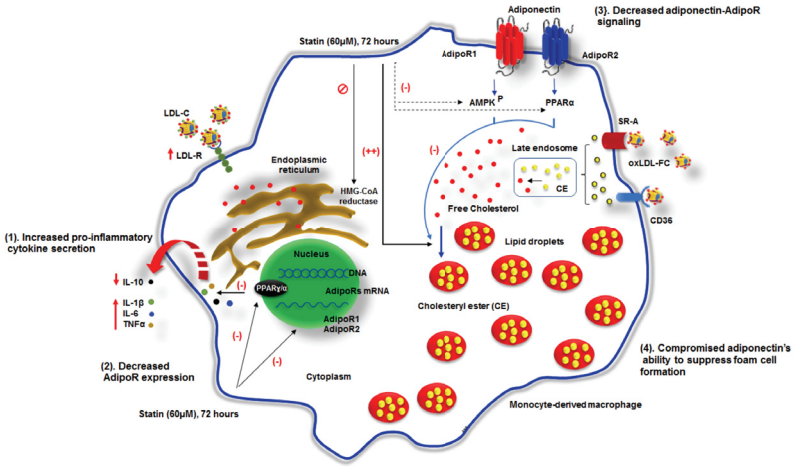


Figure 6.8 Graphical abstract. *Intensive statin therapy (high dose, long-term treatment) was observed both in vivo and in vitro to compromise the expression and function of the adiponectin-AdipoR pathway in the monocyte-macrophage lineage and to promote a more pro-inflammatory profile.*

6.5.1 Statins' effect on atherosclerotic plaques

Statins significantly reduce cholesterol biosynthesis via inhibition of the HMG-CoA reductase enzyme. Additionally, statins possess anti-inflammatory, immune-modulatory, and atheroprotective pleiotropic properties. Evidence suggests that statins can promote plaque regression by reducing plaque volume[143,144]. However, as demonstrated in a meta-analysis, this decrease in plaque volume is due to a significant reduction in fibrous tissue but no changes in fibro-fatty/necrotic tissue volumes; thereby statins may not significantly alter the instability features of the plaque[558]. Likewise, our study demonstrated no differences in plaque instability between statin users and statin-naïve patients. To this end, our *in vitro* experiments showed that oxidized LDL-loaded macrophages treated with high doses of statins can promote intracellular cholesterol ester accumulation. These results are in accordance with studies showing that statins

reduce macrophage-mediated cholesterol efflux[559-561]. These adverse properties of statins may partly explain the associated 'residual cholesterol risk' and 'residual inflammatory risk'[149]. Indeed, despite statin therapy, patients in our cohort had similar incidence and severity of cerebrovascular events compared to statin-naïve patients.

6.5.2 Statins and the adiponectin-AdipoR pathway

Adiponectin and the AdipoR pathway exert several protective effects on monocytes/macrophages in the vasculature leading to a reduction in atherosclerotic plaque development and instability[2]. Herein, we observed that intensive statin treatment decreased the expression and function of the adiponectin-AdipoR pathway *in vivo* and *in vitro*.

While we are the first to demonstrate an effect of statins specifically on the adiponectin-AdipoR pathway in the monocyte-macrophage lineage, others have evaluated the effects of statin therapy on circulating adiponectin levels. The association between statin therapy and adiponectin levels is dependent on statin type[355]. A meta-analysis demonstrated pitavastatin to be the only statin to significantly increase plasma adiponectin concentrations, particularly when used for ≥12 weeks, while atorvastatin and rosuvastatin either showed no effect or significantly reduced adiponectin levels, respectively[355]. Similarly, we demonstrated that patients on high doses of rosuvastatin had significantly lower circulating adiponectin levels than statin-naïve patients.

We identified that statins' effect on lowering AdipoR expression was dose- and time-dependent. In our cross-sectional *in vivo* study, we showed that patients on high doses of atorvastatin or rosuvastatin, but not on low doses, had significantly lower AdipoR1 and AdipoR2 gene expression on their monocytes compared to statin-naïve patients. Interestingly, this significant lowering in AdipoR expression by statins was specifically present only in CEA patients with unstable plaques. This suggests that unstable plaques create a microenvironment that triggers the AdipoR pathway to respond to statin modulation. Through our longitudinal *in vivo* study, we assessed the effect of statin duration on AdipoR expression. We observed that longer treatment with statins resulted in lower AdipoR1 and AdipoR2 expression on circulating monocytes compared to pre-statin levels, while a shorter duration in treatment did not affect expression levels. This observation was independent of the changes in total and LDL cholesterol levels observed at the early and late f/u visits. In our *in vitro* study we further assessed the effects of various doses of statins and shorter (24 hrs) and longer (72 hrs) statin treatment on the AdipoR pathway. In line with the *in vivo* results, high doses of statins (60 μM) and longer treatment led to lower AdipoR

expression, as well as decreased adiponectin-mediated signalling via the AMPK and PPAR-α pathways, and promoted pro-inflammatory cytokine IL-6, TNF-α, and IL-1β secretion. On the other hand, lower statin doses had more favorable effects on the AdipoR pathway and on cytokine secretion.

Statins are widely considered to be anti-inflammatory; they can skew immune responses toward an anti-inflammatory phenotype and reduce the production and secretion of CRP[562]. Furthermore, several studies suggest a positive interplay between statins and the nuclear transcription factors, PPARs, which may mediate the anti-inflammatory effects of statins[563,564]. However, it is noteworthy that these observations were mainly seen with low statin doses (≤10 μM) for an incubation length up to 24 hours. A series of studies, including our own, have demonstrated that statins can promote in a dose-dependent manner, an increase in pro-inflammatory IL-1β secretion from macrophages, which is characteristic of NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome pathway activation[565,566]. IL-1β contributes critically to atherosclerosis and its stimulation can in turn promote the expression and secretion of other pro-inflammatory cytokines, such as IL-6 and TNF-α[567-569].

We also demonstrated that a reduction in AdipoR expression and downstream signalling upon treatment with high doses of statins may be associated with a significant decrease in adiponectin's capacity to suppress foam cell formation. This suggests that statins atheroprotective properties may not be mediated through the adiponectin-AdipoR pathway.

6.5.3 Atorvastatin versus rosuvastatin

In our longitudinal *in vivo* study and *in vitro* experiments, we focused on specifically testing the impact of atorvastatin and rosuvastatin on the adiponectin-AdipoR pathway, as these are known to be the most effective and clinically relevant statins and were the most widely used among our CEA population (in our cross-sectional *in vivo* study). Rosuvastatin's effects were more potent than atorvastatin in modulating the adiponectin-AdipoR pathway. Furthermore, unlike atorvastatin, rosuvastatin was associated with lower circulating adiponectin levels compared to statin-naïve patients. Rosuvastatin, a third-generation hydrophilic statin, is considered the most effective in lowering LDL-C and total cholesterol levels[570]. However, despite its hydrophilicity and its high selectivity for hepatocytes, rosuvastatin also exerts potent pleiotropic actions[570]. For example, the SATURN study, which compared the effects of rosuvastatin versus atorvastatin on the progression of coronary atherosclerosis, demonstrated that a higher percentage of rosuvastatin-

treated patients (68.5%) showed plaque regression than atorvastatin-treated patients (63.2%)[143]. Rosuvastatin's higher potency compared to atorvastatin and other statins, may be explained by its greater number of binding interactions with HMG-CoA reductase due to its molecule containing an unique polar methyl sulfonamide group that facilitates interaction with the enzyme[571]. Thus, no matter which cell it enters it will exhibit a higher affinity for the enzyme's active site. Furthermore, rosuvastatin has greater systemic bioavailability than atorvastatin due to its slow metabolism and longer half-life[570].

6.5.4 Adverse effects of statins

Statins can elicit severe adverse effects, which are often associated with long-term treatment and higher doses. Interestingly, statins can elevate lipoprotein(a) levels, an independent risk factor for CVD and calcific aortic valve stenosis, possibly contributing partly to the 'residual risk' observed among statin-treated individuals[572]. Furthermore, statins have been associated with T2DM development[573]. According to a large meta-analysis, statins have been linked with a significant 9% increase in new-onset T2DM[574], while another demonstrated that this increased risk of T2DM is associated with higher statin doses[575]. One possible mechanism linking statin therapy to T2DM risk is activation of the NLRP3 inflammasome pathway, due to statin inhibition of the mevalonate pathway[576]. NLRP3 inflammasome activation leads to IL-1β production, which is involved in the inhibition of insulin signalling, thereby contributing to insulin resistance. In contrast, the adiponectin-AdipoR pathway plays a positive role in insulin sensitivity, and adiponectin production has previously been observed to be decreased by IL-1β[432,577]. Thus, statins' negative effects on the expression and function of the adiponectin-AdipoR in our study may be the result of statins' activation of the inflammasome-IL-1β pathway.

6.5.5 Strengths and limitations

Using a combination of both clinical and experimental data, our study was the first to comprehensively explore the modulation of the adiponectin-AdipoR pathway by statin therapy. The cross-sectional component of our study provided the opportunity to assess the effect of various doses of statins, while the longitudinal component provided insight on the effects of statin duration. Our *in vitro* experiments were then able to dissect further the associations observed in humans.

While our study has various strengths, we acknowledge that it also contains limitations. The statin dose employed for the *in vitro* experiments appear to be higher than the mean statin

concentration in human serum[578]. However, humans are not subjected only to a single dose of statins but rather continuous dosing over long periods of time. Thus, in this context, treating *in vitro* with higher concentrations may assimilate the low and sustained circulating levels of statins *in vivo*. Furthermore, the intracellular statin concentration in monocytes or macrophages is unknown and its accumulation may be higher than what is present in the circulation.

6.6 Conclusion

Both statins and the adiponectin-AdipoR pathway share several beneficial effects on inflammation, endothelial dysfunction, and atherosclerosis, thus reducing cardiovascular risk. However, our study demonstrated that statins' known atheroprotective properties may not be mediated through the adiponectin-AdipoR pathway, as intensive statin therapy was observed *in vivo* and *in vitro* to be associated with a significant reduction in the expression and function of this pathway. Although statins remain the mainstay of lipid management for cardiovascular risk reduction and its benefits are indisputable, they do not fully abolish the cardiovascular risk. Our discovery provides some insight into the potential mechanisms associated with statins' adverse effects. Moreover, our findings may partly explain the residual cardiovascular risk in individuals treated with statins, which stresses the need for new therapies, potentially those that can positively modulate the adiponectin-AdipoR pathway.

6.7 Acknowledgements

We are grateful to the vascular pathologists (Drs Veinot and Lai) for their histological assessments of plaque instability. Furthermore, we thank the vascular surgeons (Drs Steinmetz, MacKenzie, Corriveau, Obrand, Gill, and Bayne) at the McGill University Health Centre and Jewish General Hospital, as well as Ms Haydee-Gomez and Ms Gorgui for their help with subject recruitment.

6.8 Funding Sources

This work was supported by the Canadian Institutes of Health Research [CIHR, PJT-148966] and the Heart & Stroke Foundation of Canada [G-17-0018755]. SS Daskalopoulou is a Senior Chercheur-Boursier Clinicien supported by the Fonds de recherche du Québec – Santé. K Gasbarrino is supported by a CIHR Doctoral Studentship. The funding sources had no involvement in the study.

6.9 Disclosures

None

6.10 References

2. Gasbarrino K, Zheng H, Hafiane A, Veinot JP, Lai C, Daskalopoulou SS. Decreased adiponectin-mediated signaling through the adipor2 pathway is associated with carotid plaque instability. *Stroke*. 2017:48:915-924.
90. Woollard KJ, Geissmann F. Monocytes in atherosclerosis: Subsets and functions. *Nat Rev Cardiol*. 2010:7:77-86.
141. Zhou Q, Liao JK. Statins and cardiovascular diseases: From cholesterol lowering to pleiotropy. *Curr Pharm Des*. 2009:15:467-478.
142. Antonopoulos AS, Margaritis M, Lee R, Channon K, Antoniades C. Statins as anti-inflammatory agents in atherogenesis: Molecular mechanisms and lessons from the recent clinical trials. *Curr Pharm Des*. 2012:18:1519-1530.
143. Nicholls SJ, Ballantyne CM, Barter PJ, Chapman MJ, Erbel RM, Libby P, Raichlen JS, Uno K, Borgman M, Wolski K, Nissen SE. Effect of two intensive statin regimens on progression of coronary disease. *N Engl J Med*. 2011:365:2078-2087.
144. Nissen SE, Nicholls SJ, Sipahi I et.al. Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: The asteroid trial. *Jama*. 2006:295:1556-1565.
146. Nie P, Li D, Hu L, Jin S, Yu Y, Cai Z, Shao Q, Shen J, Yi J, Xiao H, Shen L, He B. Atorvastatin improves plaque stability in apoe-knockout mice by regulating chemokines and chemokine receptors. *PLoS One*. 2014:9:e97009.
147. Crisby M, Nordin-Fredriksson G, Shah PK, Yano J, Zhu J, Nilsson J. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: Implications for plaque stabilization. *Circulation*. 2001:103:926-933.
149. Ridker PM. Residual inflammatory risk: Addressing the obverse side of the atherosclerosis prevention coin. *Eur Heart J*. 2016:37:1720-1722.
176. Stary HC. Natural history and histological classification of atherosclerotic lesions: An update. *Arterioscler Thromb Vasc Biol*. 2000:20:1177-1178.
177. Lovett JK, Gallagher PJ, Hands LJ, Walton J, Rothwell PM. Histological correlates of carotid plaque surface morphology on lumen contrast imaging. *Circulation*. 2004:110:2190-2197.
178. Lovett JK, Gallagher PJ, Rothwell PM. Reproducibility of histological assessment of carotid plaque: Implications for studies of carotid imaging. *Cerebrovasc Dis*. 2004:18:117-123.
265. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y, Osaka CADSGCad. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol*. 2003:23:85-89.
269. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA. Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab*. 2001:86:1930-1935.
293. Ouedraogo R, Gong Y, Berzins B, Wu X, Mahadev K, Hough K, Chan L, Goldstein BJ, Scalia R. Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo. *J Clin Invest*. 2007:117:1718-1726.

296. Ouchi N, Kihara S, Arita Y et.al. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class a scavenger receptor expression in human monocyte-derived macrophages. *Circulation*. 2001:103:1057-1063.
307. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev*. 2005:26:439-451.
355. Chrusciel P, Sahebkar A, Rembek-Wieliczko M et.al. Impact of statin therapy on plasma adiponectin concentrations: A systematic review and meta-analysis of 43 randomized controlled trial arms. *Atherosclerosis*. 2016:253:194-208.
432. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest*. 2006:116:1784-1792.
498. Fukuyama N, Homma K, Wakana N, Kudo K, Suyama A, Ohazama H, Tsuji C, Ishiwata K, Eguchi Y, Nakazawa H, Tanaka E. Validation of the friedewald equation for evaluation of plasma ldl-cholesterol. *J Clin Biochem Nutr*. 2008:43:1-5.
532. Hafiane A, Bielicki JK, Johansson JO, Genest J. Novel apo e-derived abca1 agonist peptide (cs-6253) promotes reverse cholesterol transport and induces formation of prebeta-1 hdl in vitro. *PLoS One*. 2015:10:e0131997.
554. Ziaeian B, Fonarow GC. Statins and the prevention of heart disease. *JAMA Cardiol*. 2017:2:464.
555. Kim DH, Kim C, Ding EL, Townsend MK, Lipsitz LA. Adiponectin levels and the risk of hypertension: A systematic review and meta-analysis. *Hypertension*. 2013:62:27-32.
556. Gasbarrino K, Mantzoros C, Gorgui J, Veinot JP, Lai C, Daskalopoulou SS. Circulating chemerin is associated with carotid plaque instability, whereas resistin is related to cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2016:36:1670-1678.
557. Qiu B, Simon MC. Bodipy 493/503 staining of neutral lipid droplets for microscopy and quantification by flow cytometry. *Bio Protoc*. 2016:6
558. Banach M, Serban C, Sahebkar A, Mikhailidis DP, Ursoniu S, Ray KK, Rysz J, Toth PP, Muntner P, Mosteoru S, Garcia-Garcia HM, Hovingh GK, Kastelein JJ, Serruys PW. Impact of statin therapy on coronary plaque composition: A systematic review and meta-analysis of virtual histology intravascular ultrasound studies. *BMC Med*. 2015:13:229.
559. Wong J, Quinn CM, Gelissen IC, Jessup W, Brown AJ. The effect of statins on abca1 and abcg1 expression in human macrophages is influenced by cellular cholesterol levels and extent of differentiation. *Atherosclerosis*. 2008:196:180-189.
560. Ronsein GE, Hutchins PM, Isquith D, Vaisar T, Zhao XQ, Heinecke JW. Niacin therapy increases high-density lipoprotein particles and total cholesterol efflux capacity but not abca1-specific cholesterol efflux in statin-treated subjects. *Arterioscler Thromb Vasc Biol*. 2016:36:404-411.
561. Wong J, Quinn CM, Brown AJ. Statins inhibit synthesis of an oxysterol ligand for the liver x receptor in human macrophages with consequences for cholesterol flux. *Arterioscler Thromb Vasc Biol*. 2004:24:2365-2371.
562. Tuomisto TT, Lumivuori H, Kansanen E, Hakkinen SK, Turunen MP, van Thienen JV, Horrevoets AJ, Levonen AL, Yla-Herttuala S. Simvastatin has an anti-inflammatory effect on macrophages via upregulation of an atheroprotective transcription factor, kruppel-like factor 2. *Cardiovasc Res*. 2008:78:175-184.
563. Yano M, Matsumura T, Senokuchi T, Ishii N, Murata Y, Taketa K, Motoshima H, Taguchi T, Sonoda K, Kukidome D, Takuwa Y, Kawada T, Brownlee M, Nishikawa T, Araki E. Statins activate peroxisome proliferator-activated receptor gamma through

extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase-dependent cyclooxygenase-2 expression in macrophages. *Circ Res*. 2007:100:1442-1451.
564. Grip O, Janciauskiene S, Lindgren S. Atorvastatin activates ppar-gamma and attenuates the inflammatory response in human monocytes. *Inflamm Res*. 2002:51:58-62.
565. Henriksbo BD, Lau TC, Cavallari JF, Denou E, Chi W, Lally JS, Crane JD, Duggan BM, Foley KP, Fullerton MD, Tarnopolsky MA, Steinberg GR, Schertzer JD. Fluvastatin causes nlrp3 inflammasome-mediated adipose insulin resistance. *Diabetes*. 2014:63:3742-3747.
566. Kuijk LM, Mandey SH, Schellens I, Waterham HR, Rijkers GT, Coffer PJ, Frenkel J. Statin synergizes with lps to induce il-1beta release by thp-1 cells through activation of caspase-1. *Mol Immunol*. 2008:45:2158-2165.
567. Libby P. Interleukin-1 beta as a target for atherosclerosis therapy: Biological basis of cantos and beyond. *J Am Coll Cardiol*. 2017:70:2278-2289.
568. Ridker PM. From c-reactive protein to interleukin-6 to interleukin-1: Moving upstream to identify novel targets for atheroprotection. *Circ Res*. 2016:118:145-156.
569. Eskan MA, Benakanakere MR, Rose BG, Zhang P, Zhao J, Stathopoulou P, Fujioka D, Kinane DF. Interleukin-1beta modulates proinflammatory cytokine production in human epithelial cells. *Infect Immun*. 2008:76:2080-2089.
570. McTaggart F. Comparative pharmacology of rosuvastatin. *Atheroscler Suppl*. 2003:4:9-14.
571. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of hmg-coa reductase. *Science*. 2001:292:1160-1164.
572. Arsenault BJ, Petrides F, Tabet F, Bao W, Hovingh GK, Boekholdt SM, Ramin-Mangata S, Meilhac O, DeMicco D, Rye KA, Waters DD, Kastelein JJP, Barter P, Lambert G. Effect of atorvastatin, cholesterol ester transfer protein inhibition, and diabetes mellitus on circulating proprotein subtilisin kexin type 9 and lipoprotein(a) levels in patients at high cardiovascular risk. *J Clin Lipidol*. 2018:12:130-136.
573. Brault M, Ray J, Gomez YH, Mantzoros CS, Daskalopoulou SS. Statin treatment and new-onset diabetes: A review of proposed mechanisms. *Metabolism*. 2014:63:735-745.
574. Sattar N, Preiss D, Murray HM et.al. Statins and risk of incident diabetes: A collaborative meta-analysis of randomised statin trials. *Lancet*. 2010:375:735-742.
575. Preiss D, Seshasai SR, Welsh P et.al. Risk of incident diabetes with intensive-dose compared with moderate-dose statin therapy: A meta-analysis. *Jama*. 2011:305:2556-2564.
576. Henriksbo BD, Schertzer JD. Is immunity a mechanism contributing to statin-induced diabetes? *Adipocyte*. 2015:4:232-238.
577. Lagathu C, Yvan-Charvet L, Bastard JP, Maachi M, Quignard-Boulange A, Capeau J, Caron M. Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. *Diabetologia*. 2006:49:2162-2173.
578. Bjorkhem-Bergman L, Lindh JD, Bergman P. What is a relevant statin concentration in cell experiments claiming pleiotropic effects? *Br J Clin Pharmacol*. 2011:72:164-165.

Connecting Text

For decades there has been a long-standing lack of representation of women in stroke research, which has shaped current guidelines for carotid disease management and stroke prevention that are widely being used for both sexes. Fortunately, we have reached an era where the scientific and medical community has realized that this 'one size fits all' approach has led women to be under-diagnosed, under-treated, under-supported, and under-aware. The Heart and Stroke's 2018 report sheds light on the importance of women-specific research in the context of CVD and states that "women's hearts are victims of a system ill-equipped to diagnose, treat, and support them"[579].

In our recruited study population of men and women who underwent a CEA, we performed analyses looking at the association between patient demographic and clinical characteristics and plaque stability. We consistently demonstrated that a significantly greater proportion of men have unstable plaques than women, while more women have stable plaques (presented in Chapter 3 – Table 3.2 and Chapter 4 – Table 4.1). Considering that women benefit less from CEA than men and are at an increased risk for periprocedural stroke following a CEA, the degree of carotid artery stenosis may be a poor indicator of plaque instability and an incomplete determinant of stroke risk specifically in women. This suggests that the current guidelines for carotid disease management should not be based solely on the degree of carotid artery stenosis. Thus, our results, along with the pressing need for more sex-specific research on stroke, sparked interest in exploring circulating markers that can reflect sex-specific differences in the plaque for better prediction of stroke risk in women and in men. Among various lipid and immune markers, we also investigated sex-specific adiponectin-AdipoR signatures in men and women with stable versus unstable carotid plaques.

CHAPTER 7: SEX DIFFERENCES IN THE ADIPOKINE, LIPID, AND IMMUNE PROFILES OF MEN AND WOMEN WITH SEVERE CAROTID ATHEROSCLEROSIS

Karina Gasbarrino, Huaien Zheng, Ed Daly, John P Veinot, Chi Lai, Stella S. Daskalopoulou

Preliminary work

Part of the data was presented at the Organization for the Study of Sex Differences in 2017 and at the International Symposium of Atherosclerosis in 2018, and abstract was published in *Atherosclerosis Suppl.* **32**:13-14, 2018)[4]

7.1 Abstract

Introduction: Despite men exhibiting greater atherosclerotic plaque instability, women have increased mortality risk due to stroke. This paradox points towards a lack in a sex-specific approach for carotid disease management. Thus, circulating markers that reflect sex-specific features in the plaque should be explored for better prediction of stroke risk in women and in men. Herein we investigated sex differences in the adipokine, lipid, and immune profiles of patients with severe carotid atherosclerosis.

Methods: Pre-operative plasma/sera samples were collected from consecutive men and women scheduled for a CEA, to perform adipokine, lipid, and immune profiling. Peripheral blood monocytes were isolated from whole blood and their adiponectin and sex hormone receptor (AdipoR1/AdipoR2, estrogen receptor [ER]-α, ER-β, G protein-coupled estrogen receptor 1 [GPER], androgen receptor) gene expression was assessed via qRT-PCR. Plaque stability was determined by two gold-standard histological classifications. A new method was developed to measure a panel of sex hormones using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Results: Twice as many men underwent a CEA compared to women (70.5 *vs.* 29.5%). Men had more unstable plaques than women (P<0.001), exhibiting greater plaque hemorrhage, less fibrous tissue, larger lipid core, and greater number of foam cells and inflammation (P<0.001). Men also had a greater proportion of circulating monocyte to white blood cell counts, as well as significantly higher levels of pro-inflammatory cytokines and chemokines, IL-6, TNF-α, MIP1-α, and sVCAM-1 (P=0.031), compared to women. HDL-C, apoA-I, and total and HMW adiponectin levels were significantly higher in women than in men (P<0.001). With regards to plaque stability, women with stable plaques had significantly higher HMW:total adiponectin ratio, and higher AdipoR1 and ER-α gene expression on circulating monocytes compared to men with stable plaques. However, in cases of unstable plaques, no significant differences were observed between men and women in HMW:total adiponectin ratio and AdipoR1 expression, while ER-α was higher in men versus women.

Conclusion: Our study demonstrated that men and women who underwent a CEA based on current guidelines, exhibited clear differences at the level of the plaque and at the level of the circulation, with women exhibiting more favourable adipokine, lipid, and immune profiles compared to men. Furthermore, our study revealed that decreased HMW:total adiponectin ratio may contribute or act as a potential marker of plaque instability specifically in women. Measurement of circulating sex

hormones using our newly developed method will provide insight on how these hormones can influence the adipokine, lipid, and immune profiles of men and women with stable vs unstable plaques and may help identify sex-specific signatures associated with atherosclerotic plaque instability.

7.2 Introduction

Stroke remains one of the leading causes of mortality and disability worldwide[5]. Although stroke incidence rates are higher among men than women, it has been reported that women have worse recovery and higher mortality and disability rates than men post-stroke[15-17]. The vast majority of strokes are caused by cerebral ischemia, which can result from the rupture of unstable atherosclerotic plaques that form in the internal carotid artery that feeds blood to the brain. Unstable plaques are characterized by a large lipid-rich core, a thin fibrous cap, ulceration, thrombosis, and intraplaque hemorrhage, as well as an abundance of inflammatory cells[93]. It is well established that differences in plaque morphology and composition exist among men and women, where men are more likely to develop unstable and complex plaques with more inflammatory features than women, while women's plaques are characterized by more stable histological features and a higher-grade stenosis[169,183]. Although these sex disparities have led to an increased interest in understanding the mechanisms underlying the atherosclerotic process in women versus men, gaps in optimal clinical practice still exist, such as the lack of sex-specific guidelines for carotid atherosclerotic disease management.

Current guidelines recommend a CEA for stroke prevention in patients with symptomatic and asymptomatic moderate-to-high-grade carotid artery stenosis[134,138]. However, these guidelines were based off of studies where women were under-represented, and the evidence was mainly extrapolated from research performed in men. As a result, women were reported to benefit less from having a CEA than men, as their stroke relative risk reduction following a CEA was significantly lower compared to that in men (only 4% in women compared to 51% in men)[168]. Thus, there is a need to explore circulating markers that reflect sex-specific features in the plaque for better prediction of stroke risk in women and in men. The aim of this study is to investigate differences in the lipid, immune, and adipokine circulating profiles of men and women with stable versus unstable atherosclerotic plaques. A better understanding of differences between men and women with severe carotid atherosclerosis will allow for more appropriate and tailored prevention, diagnosis, and treatment for women and men.

7.3 Methods

7.3.1 Study population

Consecutive men and women with moderate- to high-grade carotid artery stenosis (≥50%) and who were scheduled to undergo a CEA, according to current guidelines, were recruited from the preoperative clinics of the McGill University Health Centre and the Jewish General Hospital, as described previously[1,2]. In the preoperative clinics, extensive assessments were performed to rule out non-carotid causes of cerebrovascular events in symptomatic patients. Our study complies with the Declaration of Helsinki. Ethics approval has been granted by the McGill University's Institutional Ethics Review Board (A12-M145-09B), and all study participants provided written informed consent. All women included in the study were post-menopausal and not currently on hormone replacement therapy.

Sociodemographic and clinical patient information (i.e., cerebrovascular symptomatic status, carotid artery stenosis, past medical history, medication use, and lifestyle habits) were obtained and cross-matched through various sources: 1) patient interview, 2) a detailed questionnaire, and 3) medical records.

7.3.2 Blood collection/measurements

Blood sample collection and analysis methods have been previously reported[1,2]. Fasting blood samples were collected from each subject preoperatively on the morning of the CEA and were partly used for obtaining plasma and serum for subsequent biochemical analyses, and partly for peripheral blood monocyte isolation.

Biochemical Analyses

Serum lipid profile (triglycerides, total cholesterol, HDL-C, apoA-I, and apoB), high-sensitivity CRP, and glucose levels, as well as a complete blood count (including white blood cell counts, red blood cell counts, hemoglobin and hematocrit levels, and platelet counts) were measured at the McGill University Health Centre central labs. LDL-C levels were calculated using the Friedewald formula. Circulating plasma leptin levels were measured by radioimmunoassay (EMD Millipore, Billerica, MA, USA), while chemerin, resistin, and total and HMW adiponectin levels were determined by enzyme-linked immunosorbent assays (Human Chemerin ELISA, Biovendor, Brno, Czech Republic; Human Resistin ELISA, EMD Millipore; Human Total Adiponectin/Acrp30 Quantikine ELISA Kit, R&D Systems, Minneapolis, MN, USA; Human HMW

Adiponectin/Acrp30 Quantikine ELISA Kit, R&D Systems). All samples were run in duplicate and the lower limits of detection were 0.1 ng/mL for chemerin, 0.02 ng/mL for resistin, and 0.891 ng/mL and 0.989 ng/mL for total and HMW adiponectin, respectively. Inter- and intra-assay coefficients of variation were <10%. Profiling of collected sera for pro-inflammatory, cytokine, chemokine, angiogenesis, and vascular injury markers was performed using the V-PLEX Human Biomarker 40-Plex Kit (MSD, Rockville, MD, USA) in a representative subset of our participants. Inter-assay coefficients of variation were <15%, while intra-assay coefficients of variation were <7%.

Monocyte Isolation and mRNA Analyses

Whole blood was used to isolate PBMCs by Ficoll gradient centrifugation. Total monocytes were then isolated from PBMCs by positive selection with $CD14^+$ Human MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), using a Magnetic Activated Cell-Sorting technique (autoMACS Pro Separator, Miltenyi Biotec). Total RNA was extracted from monocytes, as previously described, using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). SYBR Green-based qRT-PCR was performed, using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) to determine the mRNA expression of AdipoR1 (Human AdipoR1 PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCID0018245, Bio-Rad Laboratories), AdipoR2 (Human AdipoR2 PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCID0011851, Bio-Rad Laboratories), ER-α (Human ESR1 PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCED0033920, Bio-Rad Laboratories), ER-β (Human ESR2 PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCED0044944, Bio-Rad Laboratories), GPER (Human GPER PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCEP0033079, , Bio-Rad Laboratories), and androgen receptor (Human AR PrimePCR SYBR Green Assay, Unique Assay ID: qHsaCID0007155, Bio-Rad Laboratories). All measurements were performed in duplicate and C_q values obtained were normalized to the housekeeping gene, β-2 microglobulin (Human B2M PrimeTime qPCR Primers, Hs.PT.58v.18759587, Integrated DNA Technologies, Coralville, IA, USA). Relative mRNA abundance was calculated using the $2^{-\Delta\Delta Cq}$ method.

7.3.3 Histological classification of carotid atherosclerotic plaques

Carotid plaque specimens were obtained immediately following surgical resection and were processed for histological analyses, as previously described[1,2]. Four micrometer sections from the plaque segment with the area of maximal stenosis and largest plaque burden were stained with hematoxylin and eosin, and for markers of lymphocytes, macrophages, SMCs, and neovessels (CD3, CD68, α-SMC actin, von Willebrand factor, respectively). Plaque morphology and composition were characterized by vascular pathologists (JV and CL), according to two gold-standard classifications of plaque instability: 1) AHA classification by Stary *et al*.[176], and 2) semi-quantitative scale by Lovett *et al*[177,178], as previously described[1,2]. The pathologists were blinded to the patient's cerebrovascular symptomatic status.

7.3.4 Sex hormone measurements

Serum levels of total testosterone, androstenedione, estradiol (E2), and dehydroepiandrosterone (DHEA) will be measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Preparation of Calibration Standards and Internal Standards for LC-MS/MS

Primary stock solutions of 1 mg/mL of testosterone, androstenedione, E2, and DHEA were prepared in ethanol. Sub-stock solutions of 10 µg/mL were also prepared in ethanol. Working calibration solutions were prepared in 10/90 ethanol/0.1% formic acid to a final concentration of 100 ng/mL, 10 ng/mL, and 1 ng/mL. From the working solutions a series of calibration standards were prepared in 1 mL 10/90 ethanol/0.1% formic acid at concentrations 0.025-5 ng/mL for testosterone, 0.025-5 ng/mL for androstenedione, 0.025-2.5 ng/mL for E2, and 0.025-2.5 ng/mL for DHEA, and spiked with 10 µL of 100 ng/mL of deuterated internal standard (for a final concentration of 1 ng/mL). The analytical curve was spiked into solution because of elevated levels of steroid hormones detected in commercial blank human serum. Unacceptable levels of steroid hormones were also detected in lots of commercial charcoal stripped human serum as well as steroid hormone depleted commercial human serum. However, inclusion of deuterated steroids (d3-testosterone, d7-androstenedione, d4-E2, and d6-DHEA [C/D/N Isotopes Inc., Pointe-Claire, QC, Canada]) as internal standards enabled absolute quantification of the steroid hormones by correcting for procedural losses or ion suppression caused by matrix effects. A blank solvent was injected after the highest calibrant to evaluate carryover potential of the method.

Serum Sample Preparation

Aliquots of 100 µL of patient serum were extracted with 0.5 mL of methyl *tert*-butyl ether (MTBE). The aqueous layer was extracted a second time with 0.5 mL of MTBE. The two organic layers were combined and then evaporated at room temperature using a SpeedVac. The dried residues were reconstituted with derivatizing agent (1,2-dimethylimidazole-5-sulfonyl chloride [DMIS] for E2 or Girard's P Reagent for DHEA) and then incubated in a heating block at 70°C for 30-45 minutes. Following incubation and evaporation, 100 µL of 10/90 ethanol/0.1% formic acid was added to each sample and then mixed with 10 µL of 10 ng/mL of deuterated internal standard (for a final concentration of 1 ng/mL) prior to analysis by LC-MS/MS. In the case of testosterone and androstenedione, where no derivatizing agent was needed, dried residues were immediately reconstituted in 10/90 ethanol/0.1% formic acid plus deuterated internal standard prior to analysis. Testosterone and androstenedione were analyzed together by LC-MS/MS, while E2 and DHEA were analyzed separately due to differing derivatization protocols.

LC-MS/MS Conditions

The analytical instrumentation consisted of a Thermo Scientific™ ISQ Quantiva™ Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific) incorporating a heated electrospray ionization (HESI) source coupled with a Thermo Scientific UltiMate™ 3000 UHPLC system (including an UltiMate™ 3000 RS autosampler with cooled tray holder). The MS instrument was operated in selected reaction-monitoring, positive ion detection mode at optimized collision energies. Mass transitions were monitored for each target analyte and its internal standard in extracted human serum and are presented in **Table 7.1**.

UHPLC Parameters for Testosterone, Androstenedione, and DHEA Analysis: The chromatography was resolved by gradient elution of a binary solvent system using an Agilent Eclipse Plus™ C-18 analytical column (50 mm X 2.1 mm) with high-resolution 1.8 µm particles (Agilent Technologies, Santa Clara, CA, USA). The mobile phase for the separation consisted of water plus 0.1% formic acid (bottle A) and acetonitrile plus 0.1% formic acid (bottle B). The mobile phase was delivered at a flow rate of 200 µL/min with a Reverse Phase elution gradient: 95% A + 5% B for 0.5 minutes, linear gradient to 95% B between 0.5 and 5 minutes, step gradient to 95% B between 5.1 and 8 minutes, followed by column re-equilibration to initial conditions (95% A + 5% B) after 8 minutes.

UHPLC Parameters for E2 Analysis: The chromatography was resolved by gradient elution of a binary solvent system using a Thermo Hypersil GOLD PFP HPLC column (150 mm X 2.1 mm) with high-resolution 3 μm particles (Thermo Fisher Scientific). The mobile phase for the separation consisted of 5 mM NH_4OAc (bottle A) and 100% MeOH (bottle B). The mobile phase was delivered at a flow rate of 200 μL/min with a Reverse Phase elution gradient: 70% A + 30% B for 0.5 minutes, linear gradient to 90% B between 0.5 and 9 minutes, step gradient to 90% B between 9.1 and 10 minutes, followed by column re-equilibration to initial conditions (70% A + 30% B) after 10 minutes.

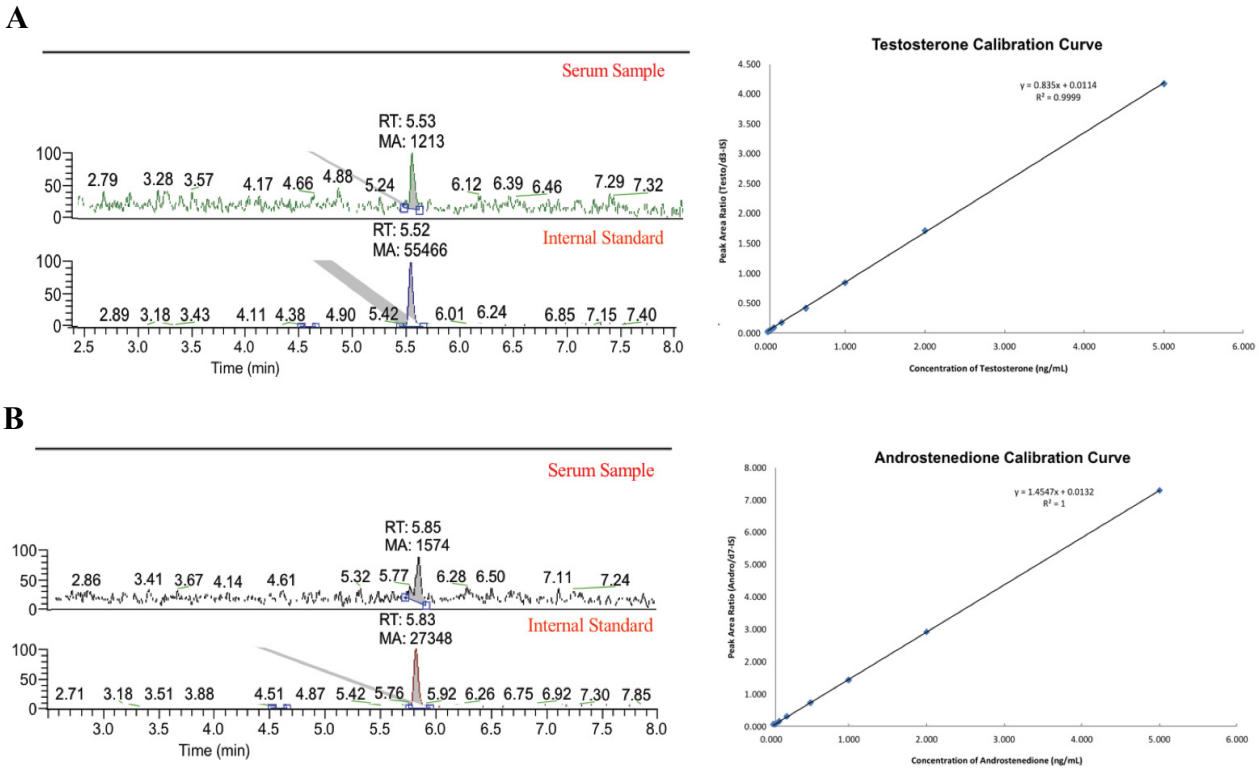
Table 7.1 Mass Spectrometer parameters

	Polarity[1]	Precursor (*m/z*)[2]	Product (*m/z*)[3]	Collision Energy (V)[4]
Testosterone	+	289.2	97.1	20.7
d3-Testo (IS)	+	292.2	97.0	21.4
Androstenedione	+	287.2	97.2	19.7
d7-Andro (IS)	+	294.21	113.2	21.4
E2-DMIS	+	431.2	96.1	34
	+	431.2	367.1	27
d4-E2 (IS)	+	435.2	96.1	33
	+	435.2	371.2	28
DHEA-GP	+	422.1	343.1	22.2
	+	422.1	253.2	26.6
d6-DHEA (IS)	+	428.1	349.2	23.5
	+	428.1	259.2	31.2

[1]Ion Detection Mode: the mass spectrometer was operated in Positive Ion Detection Mode (analyzing singly charged, positive ions).
[2]Precursor Ions: represented by the symbol $[M+H]^+$ meaning the protonated molecule mass of an analyte of interest plus the mass of a proton and described using the mass to charge ratio (*m/z*).
[3]Product Ions: protonated mass of the ion fragments of an analyte of interest also described using the mass to charge ratio (*m/z*).
[4]Collision Energy: represents the amount of energy in the collision cell used to fragment analytes of interest measured in volts
E2-DMIS = Estradiol derivatized with 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS).
DHEA-GP = DHEA derivatized with Girard's P Reagent (GP).

Data Analysis

The Thermo Xcalibur Quantitative Analysis Software Version 3.0 (Thermo Fisher Scientific) was employed for data acquisition and peak-area integration of analyte and internal standard. Using linear regression, calibration standard curves for each analyte were constructed by plotting the peak area ratio of the target analyte to its internal standard versus the concentration of the target analyte. The standard curve was used for the quantification of the target analyte in unknown serum samples. The lower limit of quantification (LLOQ) was determined as the lowest concentration at which chromatographic peaks of the analytes were present, distinct, and chromatographically resolved at the expected retention times with a signal/noise ratio >3. The LLOQ was 0.025 ng/mL for testosterone, androstenedione, DMIS-derivatized E2, and Girard's P-derivatized DHEA representing 0.25 pg on column with a 10 µL injection volume. Typical standard curves for testosterone, androstenedione, E2, and DHEA are illustrated in **Figure 7.1** along with representative selected reaction monitoring ion chromatograms of each target analyte and its internal standard from an extracted patient serum sample.



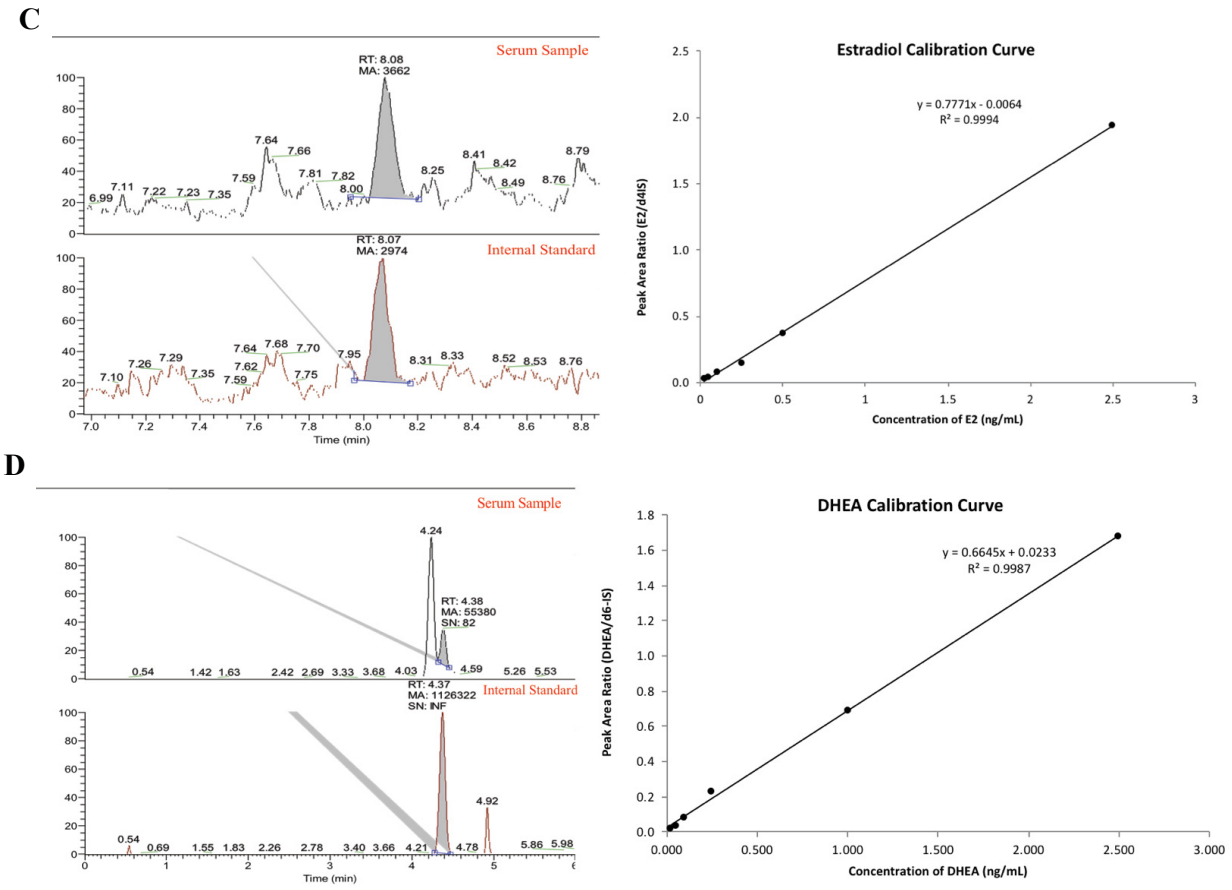


Figure 7.1 Representative calibration standard curves and selected reaction monitoring ion chromatograms of each target analyte and its internal standard from an extracted patient serum sample. *(A) testosterone and d3-testo; (B) androstenedione and d7-andro; (C) DMIS-derivatized E2 and d4-E2; (D) Girard's P-derivatized DHEA and d6-DHEA. The general retention time of testosterone was* t_R *= 5.53 min, androstenedione* t_R *= 5.85 min, E2* t_R *= 8.08 min, and DHEA* t_R *= 4.38 min.*

7.3.5 Statistical analyses

Chi-square ($\chi 2$), independent sample t-test (parametric test) or Mann-Whitney (non-parametric test), were used as appropriate, to assess the differences in clinical characteristics, plaque stability, cerebrovascular symptomatology, histological features of the plaque, circulating lipid, immune, and adipokine markers, blood count parameters, and adiponectin and sex hormone receptor expression on circulating monocytes between men and women with carotid plaques who underwent a CEA. Sub-analyses were performed based on the stability of the plaques. The definitely stable and probably stable plaque groups, classified by the vascular pathologists

according to Lovett's semi-quantitative scale, were combined and labeled as 'stable', while the probably unstable and definitely unstable plaque groups were combined and labeled as 'unstable' to be used in the statistical analyses. In addition to being clinically relevant, combining instability groups led to increased statistical power.

Logistic regression analyses were performed to estimate the association between sex and plaque instability (stable versus unstable), as well as between HMW:total adiponectin ratio and plaque instability (stable versus unstable). ORs are presented with 95% CIs. Multivariate models were adjusted for BMI, history of smoking, CAD, and carotid artery stenosis. All statistical analyses were performed in SPSS, Version 20 (IBM, Armonk, New York, United States). Values of $P<0.05$ (2-tailed) were considered significant.

7.4 Results

7.4.1 Sex differences in clinical characteristics

In our study population over twice as many men underwent a CEA than women (29.5% women/70.5% men). **Table 7.2** summarizes the baseline clinical characteristics of the study population between men and women. Age, BMI and blood pressure were similar between men and women. Both men and women suffered from a high presence of comorbidities, such as hypertension, hypercholesterolemia, and T2DM. However, a greater proportion of men had a history of smoking and CAD than women ($P<0.05$). Medication use (statins, anti-hypertensive and anti-hyperglycemic medication) did not differ between men and women. **Table 7.3** summarizes the baseline clinical characteristics of men and women who underwent a CEA based on their plaque stability. No differences in clinical characteristics were observed between men and women with stable plaques, while women with unstable plaques had a significantly smaller BMI than men with unstable plaques. Furthermore, women with unstable plaques were significantly thinner than women with stable plaques (BMI: 25.8 ± 4.3 kg/m^2 vs. 27.8 ± 5.2 kg/m^2; $P= 0.039$).

Table 7.2 Sex-specific differences in patient clinical characteristics

Clinical Characteristics	Women (n=116)	Men (n=277)	P-value
Age, y	70.7±8.9	70.4±10.1	0.798
BMI, kg/m^2	26.9±4.9	27.3±4.3	0.484
Ever smoker, %	65.5	76.1	**0.033**
CAD, %	31.0	42.2	**0.039**
Carotid Artery Stenosis, 50-79%/80-99%	24.6/75.4	31.2/68.8	0.192
SBP, mmHg	138±22	138±18	0.869
DBP, mmHg	71±11	73±10	0.076
Hypertension, %	82.8	84.0	0.762
Antihypertensive medication, %	95.8	94.8	0.695
Glucose, mmol/L	6.36 [5.50-7.65]	6.30 [5.50-7.60]	0.900
T2DM, %	28.7	33.8	0.324
Antihyperglycemic medication, %	97.0	89.2	0.177
Hypercholesterolemia, %	83.6	84.0	0.926
Statin use, %	75.9	76.8	0.840
hsCRP, mg/L	1.73 [0.70-4.62]	1.79 [0.90-4.30]	0.958

Values represented as mean ± standard deviation or median [interquartile range] (for continuous data) or as percentages (for nominal data).
BMI indicates body mass index; CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2DM, type 2 diabetes mellitus; hsCRP, high-sensitivity C-reactive protein.
P-value indicates comparison between women and men (analysis was performed by independent-samples t-test, Mann-Whitney U test, or Chi-square (χ2), as appropriate).

Table 7.3 Sex-specific differences in patient clinical characteristics in relation to plaque stability

Clinical Characteristics	Stable Plaques		P-value	Unstable Plaques		P-value
	Women (n=68)	Men (n=96)		Women (n=48)	Men (n=181)	
Age, y	71.4±8.2	70.7±9.1	0.593	69.8±9.7	70.3±10.7	0.737
BMI, kg/m^2	27.8±5.2	26.9±3.7	0.272	*25.8±4.3	27.5±4.6	**0.029**
Ever smoker, %	59.7	70.5	0.152	73.9	79.1	0.449
CAD, %	32.4	46.9	0.062	29.2	39.7	0.182
Carotid Artery Stenosis, 50-79%/80-99%	27.3/72.7	30.9/69.1	0.625	20.8/79.2	31.4/68.6	0.154
SBP, mmHg	140±23	138±18	0.661	136±21	137±19	0.605
DBP, mmHg	72±10	74±10	0.374	70±12	73±10	0.071
Hypertension, %	82.4	87.5	0.358	83.3	82.1	0.845
Antihypertensive medication, %	96.4	96.4	1.000	95.0	93.9	0.789
Glucose, mmol/L	6.00 [5.30-7.26]	6.20 [5.40-7.40]	0.759	6.60 [5.54-7.71]	6.39 [5.51-7.65]	0.483
T2DM, %	31.3	37.5	0.417	25.0	31.8	0.360
Antihyperglycemic medication, %	100.0	94.4	0.272	91.7	86.0	0.594
Hypercholesterolemia, %	79.4	85.4	0.314	89.6	83.2	0.280
Statin use, %	72.1	82.3	0.119	81.2	73.9	0.292
hsCRP, mg/L	1.65 [0.73-4.54]	1.54 [1.00-4.17]	0.943	1.99 [0.61-4.75]	2.00 [0.79-4.35]	0.973

Values represented as mean ± standard deviation or median [interquartile range] (for continuous data) or as percentages (for nominal data).
BMI indicates body mass index; CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2DM, type 2 diabetes mellitus; hsCRP, high-sensitivity C-reactive protein.
P-value indicates comparison between women and men (analysis was performed by independent-samples t-test, Mann-Whitney U test, or Chi-square (χ2), as appropriate).
*P<0.05 *vs.* women with stable plaques (independent-samples t-test).

7.4.2 Sex differences in plaque stability and clinical presentation

According to Lovett's semi-quantitative scale of plaque instability, women had a significantly greater proportion of definitely stable and probably stable plaques, while men had a greater proportion of definitely unstable and probably unstable plaques (P<0.001; **Figure 7.2A**). Classifying plaques according to the AHA histological classification further confirmed these results; women had a significantly greater proportion of Type VII and VIII plaques than men, while men had a greater proportion of Type V and Type VI plaques (P<0.001; **Figure 7.2B**). Univariate logistic regression analyses demonstrated that being a man increased the odds of having an unstable plaque by 167% (OR [95% CI]: 2.67 [1.71-4.17]; P<0.001) compared to women. This association remained significant after adjustments for BMI, history of smoking and CAD, and carotid artery stenosis (1.97 [1.20-3.23]; P=0.007). Looking at the individual features of the plaque, men's plaques were significantly characterized by more unstable histological features than women, such as a larger hemorrhage, less fibrous tissue, more foam cells, a larger lipid core, and more inflammation (macrophage and lymphocyte infiltration) overall in the plaque as well as in the fibrous cap of the plaque (**Table 7.4**). However, despite these differences in plaque composition, a similar proportion of men and women were symptomatic and experienced similar types of ischemic events (**Figure 7.2C-D**).

7.4.3 Sex differences in circulating lipid, immune, and adipokine profiles

Tables 7.5-7.7 summarize the differences in lipid, immune and adipokine markers, and blood count parameters between men and women. Total cholesterol levels were significantly higher in women compared with men, which was mainly driven by the significantly higher HDL-C levels also observed among women (**Table 7.5**). As a result of these higher HDL-C levels, apoA-I levels were also significantly higher among women compared to men (**Table 7.5**). Whether plaques were classified as stable or unstable, total cholesterol, HDL-C, and apoA-I levels remained significantly higher in women compared to men (**Table 7.6**).

The total white blood cell count was observed to be significantly higher in men versus women when plaques were stable (**Table 7.6**). However, the opposite was true when plaques were classified as unstable. Interestingly, the total white blood cell count was significantly increased in women with unstable vs stable plaques (P=0.017) but was decreased in men with unstable vs stable plaques (P<0.001) (**Table 7.6**). Women were characterized by a significantly greater proportion of lymphocyte to white blood cell counts, while men had a greater proportion of monocyte to white

blood cell counts (**Table 7.5** and **7.6**). Furthermore, men had significantly higher levels of circulating pro-inflammatory cytokines and chemokines, and vascular injury markers, IL-6, TNF-α, MIP1-α, and sVCAM-1 than women (**Table 7.7**). The total red blood cell count, along with hemoglobin and hematocrit levels were significantly higher in men compared with women (**Table 7.5**). This difference remained significant whether plaques were classified as stable or unstable (**Table 7.6**). The platelet count, on the other hand, was significantly higher among women with unstable plaques compared with men with unstable plaques but remained similar among men and women with stable plaques (**Table 7.6**). Interestingly, men with unstable plaques had significantly lower total platelet counts versus men with stable plaques (P=0.002), while no differences were observed among women with unstable versus stable plaques (**Table 7.6**).

Women had significantly higher circulating leptin and total and HMW adiponectin levels compared with men, while chemerin and resistin levels remained similar (**Table 7.5**). The proportion of HMW to total adiponectin levels was also higher among women than men (**Table 7.5**). HMW and total adiponectin levels remained significantly different between men and women when their plaques were categorized as stable or unstable (**Table 7.6**). On the other hand, while the proportion of HMW to total adiponectin was significantly higher in women versus men with stable plaques, no significant difference in the ratio was observed between men and women who had unstable plaques (**Table 7.6**). Interestingly, women with unstable plaques had significantly lower HMW:total adiponectin than women with stable plaques (**Table 7.6**). Logistic regression analyses revealed a significant association specifically in women but not in men between decreased HMW:total adiponectin ratio and greater plaque instability (**Table 7.8**).

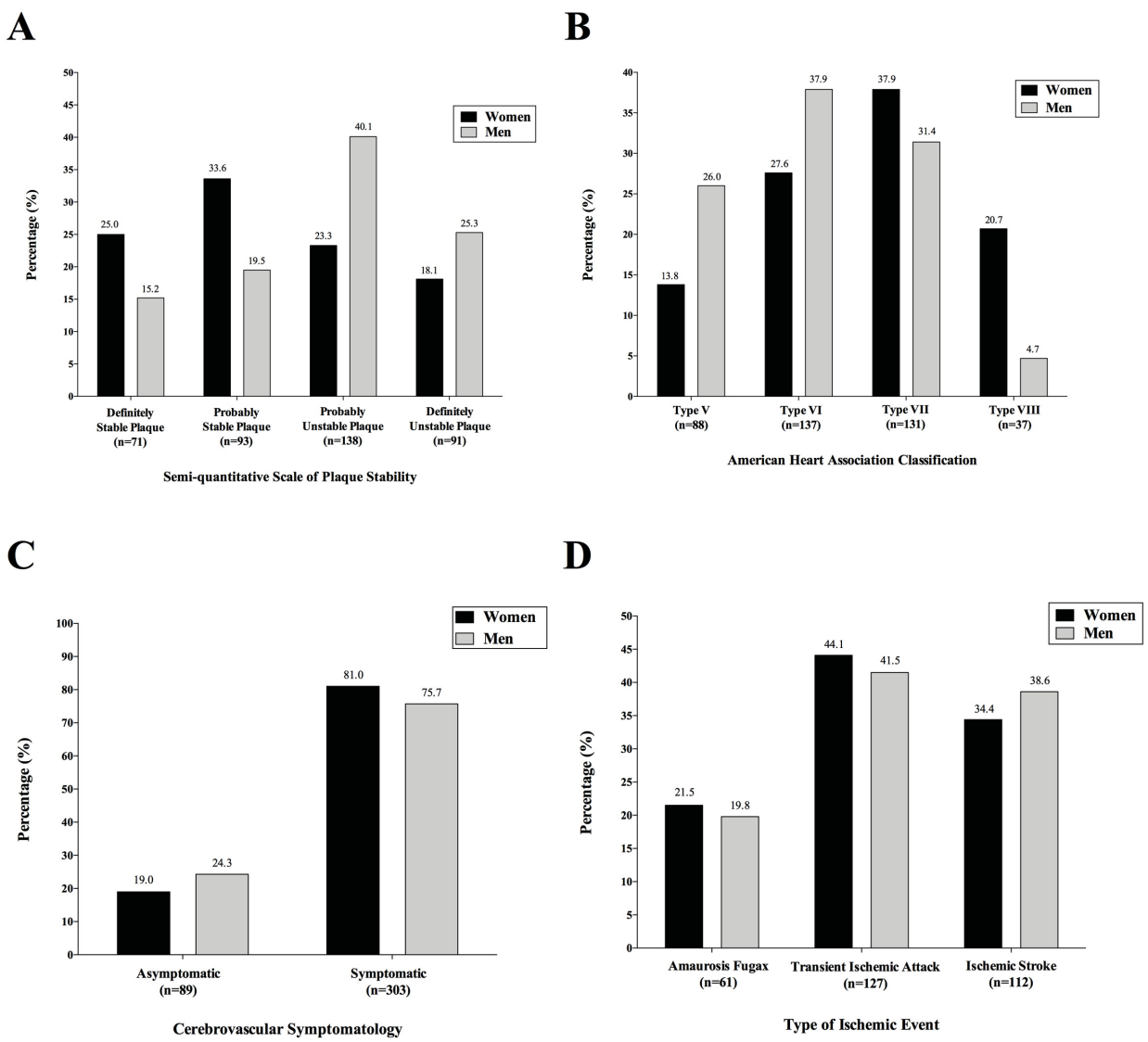


Figure 7.2 Sex-specific differences in plaque stability and cerebrovascular symptomatology in CEA population. **A)** Bar graphs represent the percentage of women and men with stable or unstable plaques according to Lovett's semi-quantitative scale of plaque stability, P<0.001 (Chi-square test). **B)** Bar graphs represent the percentage of women and men with Type V, VI, VII, or VIII plaques according to the AHA classification, P<0.001 (Chi-square test). **C)** Bar graphs represent the percentage of women and men who were symptomatic or asymptomatic, P=0.252 (Chi-square test). **D)** Bar graphs represent the percentage of symptomatic women and men according to the type of cerebrovascular event they suffered, P=0.780 (Chi-square test).

Table 7.4 Sex-specific differences in the prevalence of histological features of the plaque

Histological Plaque Features	N	Women (n=116)	Men (n=277)	P-value
Hemorrhage, %				
No hemorrhage	261	76.7	62.1	
Small hemorrhage	87	18.1	23.8	
Large hemorrhage	45	5.2	14.1	**0.009**
Thrombus, %				
No thrombus	320	82.8	80.9	
Small thrombus	65	16.4	16.6	
Large thrombus	8	0.9	2.5	0.562
Fibrous tissue, %				
Very little fibrous tissue	32	5.2	9.4	
~50% fibrous tissue	235	42.2	67.1	
Predominantly fibrous	126	52.6	23.5	**<0.001**
Foam cells, %				
None	150	50.0	33.2	
<50 cells	105	24.1	27.8	
At least 50 cells	138	25.9	39.0	**0.005**
New vessels, %				
None	92	26.7	22.0	
<10 per section	147	31.0	40.1	
At least 10 per section	154	42.2	37.9	0.229
Calcification, %				
None	51	7.8	15.2	
Stippling only	240	60.4	61.4	
Calcified nodules	102	31.9	23.5	0.097
Lipid core, %				
No lipid core	65	30.2	10.8	
Small lipid core	118	34.5	28.2	
Large lipid core	210	35.3	61.0	**<0.001**
Inflammatory cells, %				
None	62	24.1	12.3	
Occasional cells	155	43.1	37.9	
2-5 groups of >50 cells	152	30.2	42.2	
>5 groups of >50 cells	24	2.6	7.6	**0.003**
***Cap infiltration, %**				
None	156	54.3	34.1	
<10 cells in cap	74	15.5	20.5	
10-50 cells in cap	84	13.8	24.9	
>50 cells in cap	75	16.4	20.5	**0.002**

***Cap rupture, %**				
Intact cap	252	68.1	63.4	
Probably intact	66	16.4	17.2	
Probably ruptured	50	12.9	12.8	
Definitely ruptured	21	2.6	6.6	0.434

Plaque and cap inflammation were graded according to the number of macrophages and lymphocytes present.
*N=389 (for cap infiltration and cap rupture) because plaque had no detectable cap.
P-value indicates comparison between women and men (analysis was performed by Chi-square [$\chi 2$]).

Table 7.5 Sex-specific differences in circulating lipid and adipokine markers, and blood count parameters

Circulating Markers	Women	Men	P-value
Lipid Markers			
Total cholesterol, mmol/L	116, 3.44 [2.96-4.08]	277, 3.13 [2.70-3.68]	**<0.001**
HDL cholesterol, mmol/L	116, 1.09 [0.91-1.23]	277, 0.91 [0.74-1.07]	**<0.001**
LDL cholesterol, mmol/L	116, 1.62 [1.23-2.28]	277, 1.53 [1.15-1.92]	0.055
Triglycerides, mmol/L	116, 1.41 [1.10-1.80]	277, 1.45 [1.07-1.88]	0.928
ApoA1, g/L	116, 1.26 [1.12-1.46]	277, 1.12 [0.99-1.27]	**<0.001**
ApoB, g/L	116, 0.70 [0.59-0.86]	277, 0.67 [0.56-0.79]	0.056
Blood Count Parameters			
WBCs, 10^9/L	104, 7.96 [6.23-9.58]	244, 7.80 [6.44-9.49]	0.661
Lymphocyte:WBC, %	104, 24.96 [19.84-30.58]	244, 22.27 [17.08-27.93]	**0.001**
Monocyte:WBC, %	104, 7.39 [6.12-8.54]	244, 8.38 [6.82-9.97]	**0.001**
Neutrophil:WBC, %	104, 63.57 [58.46-69.72]	244, 65.40 [59.38-71.97]	0.092
Eosinophil:WBC, %	104, 2.23 [1.25-3.19]	244, 2.36 [1.40-3.55]	0.206
Basophil:WBC, %	104, 0.00 [0.00-0.87]	244, 0.00 [0.00-0.93]	0.296
RBCs, 10^{12}/L	104, 4.24 [3.98-4.54]	244, 4.55 [4.15-4.90]	**<0.001**
Hemoglobin, g/L	104, 131.50 [119.25-141.00]	244, 139.00 [126.00-150.00]	**<0.001**
Hematocrit, L/L	104, 0.39 [0.36-0.42]	244, 0.42 [0.38-0.45]	**<0.001**
Platelets, 10^9/L	104, 247.50 [196.25-291.75]	244, 200.00 [171.00-249.75]	**<0.001**
Adipokine Markers			
Chemerin, ng/mL	49, 219.62 [182.39-244.44]	114, 208.64 [177.19-260.23]	0.654
Leptin, ng/mL	49, 13.81 [4.39-45.07]	114, 9.43 [4.56-14.49]	**0.031**
Resistin, ng/mL	49, 12.46 [10.01-16.08]	114, 12.88 [9.69-17.63]	0.947
Total adiponectin, µg/mL	116, 8.01 [5.42-12.01]	277, 5.19 [3.36-8.85]	**<0.001**
HMW adiponectin, µg/mL	116, 4.48 [3.02-6.99]	277, 2.86 [1.58-4.65]	**<0.001**
HMW:Total adiponectin	116, 0.58±0.12	277, 0.52±0.14	**<0.001**

HMW indicates high-molecular weight; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; WBC, white blood cell; RBC, red blood cell; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2DM, type 2 diabetes mellitus.
Data is presented as N, median [interquartile range] or N, mean ± standard deviation
P-value indicates comparison between women and men (analysis was performed by independent-samples t-test or Mann-Whitney U test, as appropriate).

Table 7.6 Sex-specific differences in circulating lipid and adipokine markers, and blood count parameters, in relation to plaque stability

Circulating Markers	Stable Plaques		P-value	Unstable Plaques		P-value
	Women	Men		Women	Men	
Lipid Markers						
Total cholesterol, mmol/L	68, 3.44 [2.94-4.15]	96, 3.12 [2.74-3.64]	**0.003**	48, 3.43 [3.10-3.93]	181, 3.16 [2.66-3.69]	**0.023**
HDL cholesterol, mmol/L	68, 1.09 [0.92-1.31]	96, 0.92 [0.75-1.11]	**<0.001**	48, 1.07 [0.87-1.18]	181, 0.91 [0.74-1.06]	**0.001**
LDL cholesterol, mmol/L	68, 1.55 [1.21-2.28]	96, 1.42 [1.13-1.77]	0.058	48, 1.66 [1.25-2.25]	181, 1.58 [1.16-2.00]	0.230
Triglycerides, mmol/L	68, 1.41 [1.12-1.79]	96, 1.51 [1.16-1.88]	0.562	48, 1.42 [1.07-1.82]	181, 1.43 [0.99-1.87]	0.882
ApoA1, g/L	68, 1.25 [1.10-1.43]	96, 1.12 [1.01-1.26]	**<0.001**	48, 1.26 [1.12-1.50]	181, 1.12 [0.98-1.27]	**<0.001**
ApoB, g/L	68, 0.70 [0.58-0.87]	96, 0.64 [0.56-0.76]	0.075	48, 0.71 [0.61-0.83]	181, 0.68 [0.56-0.81]	0.248
Blood Count Parameters						
WBCs, 10^9/L	62, 7.52 [6.07-9.02]	85, 8.30 [7.09-10.13]	**0.009**	*42, 8.50 [7.38-10.06]	†159, 7.33 [6.00-8.94]	**0.003**
Lymphocyte:WBC, %	62, 24.48 [19.92-30.00]	85, 22.05 [16.28-26.82]	**0.020**	42, 26.01 [19.55-31.88]	159, 22.38 [17.38-28.71]	**0.018**
Monocyte:WBC, %	62, 7.33 [6.07-8.86]	85, 8.07 [6.57-9.46]	0.120	42, 7.53 [6.58-8.46]	159, 8.58 [6.92-10.19]	**0.008**
Neutrophil:WBC, %	62, 64.06 [58.99-70.50]	85, 65.89 [60.87-73.73]	0.160	42, 62.87 [57.86-68.74]	159, 65.32 [59.23-71.38]	0.141
Eosinophil:WBC, %	62, 2.08 [1.22-3.00]	85, 1.96 [1.17-3.24]	0.913	42, 2.45 [1.45-3.74]	†159, 2.49 [1.67-3.84]	0.452
Basophil:WBC, %	62, 0.00 [0.00-0.75]	85, 0.55 [0.00-0.94]	**0.004**	42, 0.00 [0.00-0.94]	†159, 0.00 [0.00-0.093]	0.355
RBCs, 10^{12}/L	62, 4.31 [3.98-4.55]	85, 4.59 [4.19-4.90]	**0.001**	42, 4.20 [3.96-4.51]	159, 4.51 [4.15-4.90]	**0.004**
Hemoglobin, g/L	62, 132.00 [118.00-141.25]	85, 140.00 [128.50-151.00]	**0.001**	42, 129.00 [119.75-139.50]	159, 139.00 [125.00-150.00]	**0.004**
Hematocrit, L/L	62, 0.40 [0.36-0.42]	85, 0.42 [0.38-0.45]	**0.001**	42, 0.38 [0.36-0.41]	159, 0.41 [0.37-0.44]	**0.003**
Platelets, 10^9/L	62, 239.50 [192.75-289.25]	85, 223.00 [189.00-266.50]	0.283	42, 249.50 [196.75-300.00]	†159, 192.00 [163.00-239.00]	**<0.001**
Adipokine Markers						
Chemerin, ng/mL	23, 225.85 [181.02-255.12]	25, 230.60 [199.91-273.72]	0.516	26, 210.84 [182.88-241.53]	89, 202.41 [165.19-257.56]	0.552
Leptin, ng/mL	23, 9.61 [3.53-44.11]	25, 9.41 [5.49-15.02]	0.741	26, 13.96 [7.26-46.21]	89, 9.45 [4.36-14.53]	**0.011**
Resistin, ng/mL	23, 12.78 [10.21-15.68]	25, 10.84 [9.60-18.16]	0.657	26, 12.22 [9.44-18.19]	89, 13.05 [9.71-17.58]	0.792
Total adiponectin, µg/mL	68, 7.87 [5.66-12.30]	96, 4.94 [3.09-7.81]	**<0.001**	48, 8.20 [5.04-10.40]	181, 5.57 [3.61-9.25]	**0.002**
HMW adiponectin, µg/mL	68, 4.70 [3.26-7.50]	96, 2.85 [1.38-4.29]	**<0.001**	48, 4.37 [2.60-6.25]	181, 2.89 [1.75-4.89]	**0.002**
HMW:Total adiponectin	68, 0.60±0.12	96, 0.53±0.15	**0.001**	*48, 0.54±0.11	181, 0.52±0.13	0.233

HMW indicates high-molecular weight; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; WBC, white blood cell; RBC, red blood cell; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2DM, type 2 diabetes mellitus.
Data is presented as N, median [interquartile range] or N, mean ± standard deviation
P-value indicates comparison between women and men (analysis was performed by independent-samples t-test or Mann-Whitney U test, as appropriate).
*P<0.05 *vs.* women with stable plaques (Mann-Whitney U test)
†P<0.05 *vs.* men with stable plaques (Mann-Whitney U test)

Table 7.7 Sex-specific differences in circulating pro-inflammatory, cytokine, chemokine, angiogenesis, and vascular injury markers

Circulating Markers	Women	Men	P-value
Pro-inflammatory Markers			
IFN-γ, pg/mL	28, 3.47 [1.81-6.16]	46, 3.61 [1.99-5.42]	0.973
IL-4, pg/mL	28, 0.01 [0.00-0.02]	46, 0.01 [0.00-0.03]	0.250
IL-6, pg/mL	28, 0.31 [0.26-0.93]	46, 0.62 [0.33-1.15]	**0.039**
IL-8, pg/mL	28, 7.55 [5.95-13.37]	46, 7.90 [6.60-11.75]	0.850
IL-10, pg/mL	28, 0.13 [0.08-0.18]	46, 0.09 [0.06-0.21]	0.613
IL-12p70, pg/mL	28, 0.04 [0.02-0.07]	46, 0.03 [0.01-0.08]	0.400
IL-13, pg/mL	28, 0.30 [0.15-0.50]	46, 0.30 [0.18-0.49]	0.938
TNF-α, pg/mL	28, 1.65 [1.17-1.96]	46, 1.79 [1.47-2.51]	**0.041**
Cytokines			
GM-CSF, pg/mL	28, 0.03 [0.01-0.08]	46, 0.05 [0.02-0.07]	0.356
IL-5, pg/mL	28, 0.13 [0.05-0.86]	46, 0.24 [0.07-0.52]	0.636
IL-7, pg/mL	28, 11.12 [8.41-17.59]	46, 14.00 [10.59-18.84]	0.084
IL-12/IL-23p40, pg/mL	28, 99.11 [48.53-125.75]	46, 79.67 [49.21-157.81]	0.860
IL-15, pg/mL	28, 1.22 [1.03-1.53]	46, 1.27 [1.10-1.62]	0.422
IL-16, pg/mL	28, 137.15 [104.06-188.04]	46, 168.03 [126.85-188.85]	0.084
IL-17A, pg/mL	28, 0.43 [0.32-1.01]	46, 0.51 [0.31-0.89]	0.730
TNF-β, pg/mL	28, 0.10 [0.07-0.13]	46, 0.09 [0.07-0.13]	0.828
Chemokines			
Eotaxin, pg/mL	28, 84.15 [68.02-154.31]	46, 87.39 [65.97-122.95]	0.677
Eotaxin-3, pg/mL	28, 3.92 [2.56-5.70]	46, 4.68 [2.59-8.35]	0.313
TARC, pg/mL	28, 241.03 [158.05-460.85]	46, 205.67 [117.23-324.91]	0.430
IP-10, pg/mL	28, 174.57 [90.28-230.57]	46, 160.30 [120.66-262.24]	0.567
MIP-1α, pg/mL	28, 9.64±3.54	46, 12.98±5.51	**0.009**
MIP-1β, pg/mL	28, 84.74±45.18	46, 80.19±29.40	0.656
MCP-1, pg/mL	28, 296.43 [228.16-346.01]	46, 297.07 [235.78-363.00]	0.362
MCP-4, pg/mL	28, 119.48 [88.52-162.37]	46, 114.31 [80.75-147.10]	0.678
MDC, pg/mL	28, 956.29±388.28	46, 794.14±350.24	0.088
Angiogenesis Markers			
VEGF-A, pg/mL	28, 228.80 [101.85-406.70]	46, 185.86 [121.47-308.85]	0.600
VEGF-C, pg/mL	28, 230.27 [177.45-361.07]	46, 230.45 [187.33-323.01]	0.929
VEGF-D, pg/mL	28, 617.55 [416.50-766.31]	46, 428.81 [344.08-525.18]	**0.033**
TIE-2, pg/mL	28, 2132.18±439.83	46, 2157.11±502.80	0.829
FLT-1, pg/mL	28, 41.66 [32.14-54.94]	46, 44.39 [37.41-51.99]	0.397
PIGF, pg/mL	28, 12.87±3.01	46, 13.34±2.56	0.478
FGF, pg/mL	28, 1.62 [0.90-2.36]	46, 1.45 [0.78-2.29]	0.902
Vascular Injury Markers			
SAA, pg/mL	28, 269.90 [166.37-738.43]	46, 349.55 [151.84-678.78]	0.858
sICAM-1, ng/mL	28, 326.49 [252.60-392.86]	46, 328.87 [299.45-453.23]	0.275
sVCAM-1, ng/mL	28, 570.14 [491.19-686.64]	46, 673.00 [572.43-797.09]	**0.031**

IFN indicates interferon; IL, interleukin; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TARC, thymus and activation-regulated chemokine; IP, interferon gamma-inducible protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; MDC, monodansylcadaverine; VEGF, vascular endothelial growth factor; TIE, tyrosine kinase with immunoglobulin-like and EGF-like domains; FLT, fms related tyrosine kinase; PIGF, placental growth factor; FGF, fibroblast growth factor; SAA, serum amyloid A; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule

Data is presented as N, median [interquartile range] or N, mean ± standard deviation

P-value indicates comparison between women and men (analysis was performed by independent-samples t-test or Mann-Whitney U test, as appropriate).

Table 7.8 Logistic regression analyses for the association of HMW:total adiponectin with carotid plaque instability

	Univariate	*Multivariate
	OR (95% CI)	**OR (95% CI)**
All patients	0.114 (0.025-0.526)	0.159 (0.026-0.968)
Men only	0.459 (0.076-2.764)	0.739 (0.092-5.923)
Women only	0.009 (0.000-0.343)	0.003 (0.000-0.307)

OR indicates odds ratio; CI, confidence interval
ORs from logistic regression analysis per unit increase in marker levels
*Adjustments for body mass index, history of smoking, coronary artery disease, and carotid artery stenosis

7.4.4 Sex differences in adiponectin and sex hormone receptor expression on circulating monocytes

AdipoR1 gene expression on circulating monocytes was observed to be significantly lower (1.58-fold lower, P<0.01) in men with stable plaques versus women with stable plaques (**Figure 7.3A**). However, this difference was not apparent when men and women with unstable plaques were compared. Although the effect was attenuated, AdipoR2 followed a similar pattern of expression as AdipoR1; in this case a trend for lower AdipoR2 gene expression was only noted between men and women with stable plaques (**Figure 7.3B**). Interestingly, men with unstable plaques had significantly greater AdipoR1 expression (1.44-fold higher, P<0.05) than their counterparts with stable plaques, while no difference in expression was observed between women with stable versus unstable plaques (**Figure 7.3A**). ER-α gene expression on circulating monocytes was 1.57-fold lower in men versus women when their plaques were stable but 1.88-fold higher in men when plaques were unstable (P<0.05, **Figure 7.3C**). Women with unstable plaques had significantly lower ER-α expression (1.75-fold lower) than women with stable plaques (P<0.05, **Figure 7.3C**). The opposite was reported in men who had 1.66-fold higher ER-α expression when their plaques were unstable versus when their plaques were stable (P<0.05, **Figure 7.3C**). Although no significant differences were observed, GPER followed a similar pattern of expression as ER-α (**Figure 2D**). ER-β was expressed to such a low degree on circulating monocytes that differences among patients were non-quantifiable (**Figure 7.4**). Androgen receptor, on the other hand, was not found to be expressed on circulating monocytes (**Figure 7.4**).

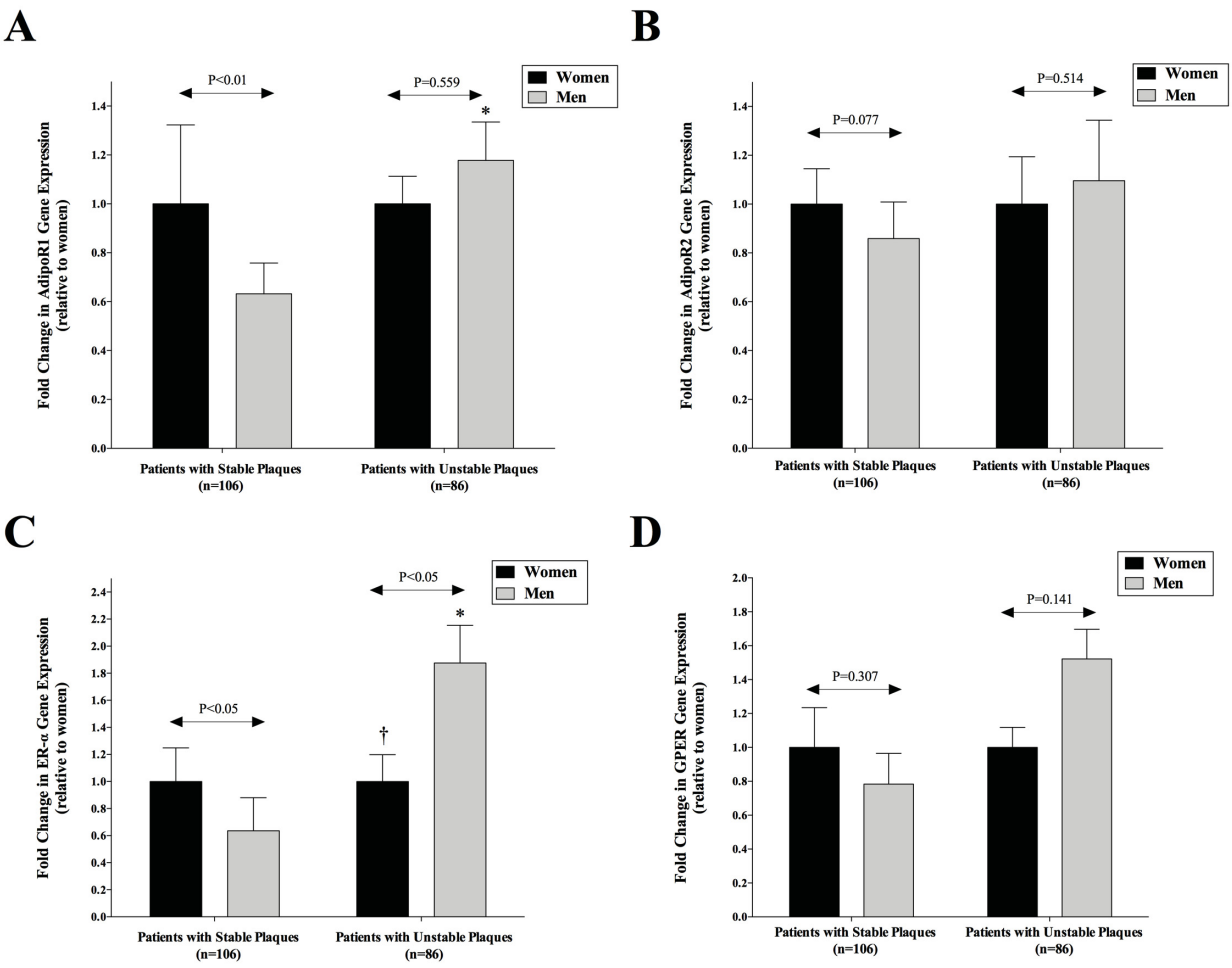


Figure 7.3 Sex-specific differences in AdipoR1, AdipoR2, ER-α, and GPER gene expression on circulating monocytes in CEA population. Bar graphs represent qRT-PCR data. Data presented as mean ± SD. **A)** P-values represent analysis comparing AdipoR1 gene expression on circulating monocytes between women and men with stable or unstable plaques (independent-samples t-test). *P<0.05 vs. men with stable plaques (independent-samples t-test) **B)** P-values represent analysis comparing AdipoR2 gene expression on circulating monocytes between women and men with stable or unstable plaques (independent-samples t-test). **C)** P-values represent analysis comparing ER-α gene expression on circulating monocytes between women and men with stable or unstable plaques (independent-samples t-test). *P<0.05 vs. men with stable plaques (independent-samples t-test); †P<0.05 vs. women with stable plaques (independent-samples t-test). **D)** P-values represent analysis comparing GPER gene expression on circulating monocytes between women and men with stable or unstable plaques (independent-samples t-test).

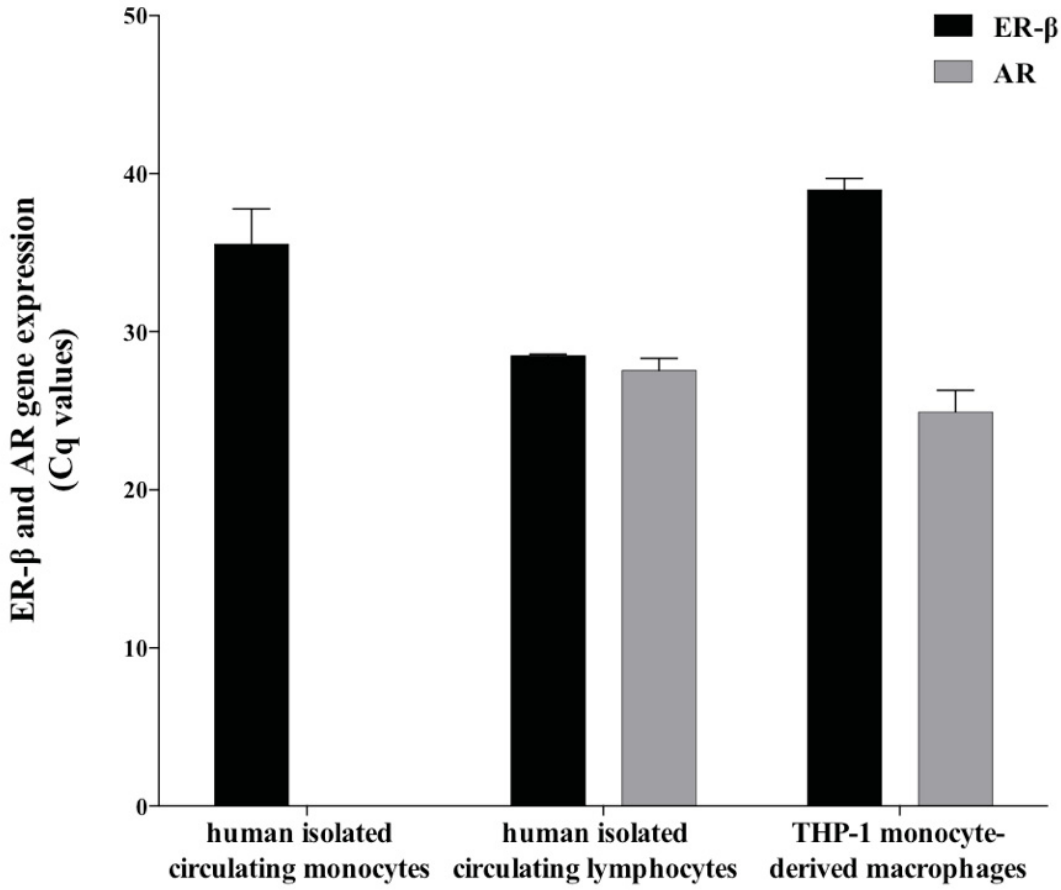


Figure 7.4 ER-β and androgen receptor mRNA expression in various cell types. *ER-β mRNA was expressed to a low degree in human isolated circulating monocytes (Cq value = ~35), while androgen receptor was not expressed in monocytes (no Cq value detectable). Human isolated circulating lymphocytes and THP-1 monocyte-derived macrophages were used as a positive control, where ER-β mRNA was observed to be expressed in lymphocytes, while androgen receptor was expressed in both lymphocytes and monocyte-derived macrophages. Cq > 35 defined as no expression.*

7.5 Discussion

Our study provided a comprehensive characterization of sex differences that exist between men and women with severe carotid atherosclerosis who underwent a CEA based on the current guidelines for carotid disease management. We demonstrated that men and women exhibited clear differences not only at the level of the plaque but also at the level of the circulation, with women displaying more favourable adipokine, lipid, and immune profiles compared to men. These favourable profiles among women may partly explain why women possessed more histologically

stable plaques than men. Furthermore, our study was the first to reveal that a decrease in the HMW:total adiponectin ratio may contribute or act as a potential marker of plaque instability specifically in women but not in men. Lastly, we presented a newly developed and optimized method to measure a panel of sex hormones using LC-MS/MS, which will overcome the limitations of currently used assays that lack sensitivity and specificity.

7.5.1 Sex differences in plaque instability and cerebrovascular symptomatology

In our study, sex was observed to be a strong and independent determinant of plaque instability, where men exhibited more unstable plaques than women, predominantly composed of a large lipid core, intraplaque hemorrhage, and a greater presence of inflammation and cap infiltration, while women exhibited stable plaques which were composed predominantly of fibrous tissue. Our findings support other lines of evidence demonstrating clear differences in plaque morphology and composition between men and women regardless of the degree of carotid artery stenosis, where men possessed higher risk carotid plaque features than women[169,183]. It is suggested that these differences in plaque composition may help explain why women benefit less from carotid revascularization than men and have increased perioperative stroke risk[155,167,168]. As a result, less women are selected to undergo a CEA than men as there exists much uncertainty in deciding which treatment may be appropriate for the management of carotid disease in women. In our own study, women comprised only 30% of the total CEA population, who are consecutively recruited (acceptance rate: 99%). Thus, clinical selection bias represents a limitation of our study, as the vascular surgeons are more likely to select, based on the current guidelines, women who they believe represent the most severe cases to be operated on and are the most likely to benefit from surgical intervention. In the majority of cases, these are women who suffered a symptomatic event. This explains why a similar proportion of men and women were observed to be symptomatic in our study population, and experienced similar types of ischemic events, despite stroke incidence being higher among men than women in the general population. Interestingly, despite the majority of women being symptomatic, their plaques exhibited minimal features of plaque instability. This may partly be due to delays in surgical treatment. Although not directly assessed in our study, the duration from event to CEA has been reported to be longer in women compared to men (median time to CEA: 18 days in men and 35 days in women), providing sufficient time for the plaque to remodel and stabilize[172,179]. Secondly, it has been suggested that thrombi overlying fibrotic atherosclerotic plaques in women may likely form due to surface endothelial erosion, rather than

rupture of the fibrous cap[580]. Thrombosis in such cases are often triggered by an enhanced systemic thrombogenic state promoted by traditional risk factors, such as hypertension, smoking, and hypercholesterolemia[581].

7.5.2 Sex differences in circulating lipid, immune, and adipokine profiles

Significant sex differences observed at the level of the plaque led us to hypothesize that significant differences between men and women would also be observed at the level of the circulation. Therefore, we explored various circulating lipid, immune, and adipokine markers that could potentially reflect the sex-specific features in the plaque. Women had a more anti-atherogenic plasma lipid profile than men. Specifically, higher concentrations of circulating HDL-C and apoA-I were detected in women compared to men. Although these findings have been consistently documented in other studies, particularly when comparing premenopausal women to age-matched men, the mechanism for this sex-specific difference remains not well understood[582]. It is suggested that women may have a greater HDL-apoA-I synthesis rate than men, which is associated with enhanced cholesterol efflux capacity[583].

Sex is also a biological variable that affects the functions of the immune system[584]. Men were observed to have a greater presence of inflammation in their circulation than women, reflecting the larger abundance of inflammation also observed at the level of their plaques. Specifically, men had more innate immune activation than women, where a greater proportion of circulating monocyte to white blood cell counts was detected along with higher levels of pro-inflammatory cytokines, chemokines, and soluble adhesion molecules, such as IL-6, TNF-alpha, MIP1-alpha, and sVCAM-1. These factors are crucial for the development of atherosclerosis; they are either expressed by monocytes or promote monocyte adhesion to the vascular endothelium and their migration into the atherosclerotic plaque[585]. Sex hormones are believed to regulate the phenotypic and functional differences in innate immune cells documented between men and women[586]. Sex hormones mediate their effects through various estrogen (ER-α, ER-β, GPER) and androgen receptors. Interestingly, in our study, only ER-α and GPER gene expression were detected in circulating monocytes, while androgen receptor was expressed in fully differentiated macrophages. ER-α expression on monocytes was significantly lower in women with unstable versus stable plaques, while in contrast, its expression was significantly higher in men with unstable versus stable plaques. This resulted in men having significantly higher ER-α expression than women when plaques were unstable, while the opposite held true when plaques were stable.

These findings suggest that estrogens might differentially affect the function of innate immune cells in women and in men, where its actions may be protective in women yet deleterious in men.

Sex differences in adipose tissue biology also exist, with respect to the release of adipokines. In our study population, women had significantly higher circulating total and HMW adiponectin levels than men, as well as higher HMW:total adiponectin ratio. These findings are in line with multiple studies that have established higher circulating adiponectin levels among women compared to men[587,588]. However, we are the first to report that a decrease in the HMW:total adiponectin ratio, also referred to as the adiponectin sensitivity index, is a strong and independent predictor of greater plaque instability specifically in women but not in men. Therefore, this ratio may have the potential to be used as a marker that may help identify women (with 'unstable' profiles similar to men) who may be better suited for surgical intervention. Adiponectin is the most abundantly secreted adipose tissue-derived protein, which exerts insulin-sensitizing, anti-inflammatory, and vasculoprotective properties. The HMW isoform is considered the biologically active form of adiponectin and its secretion has been reported to be selectively inhibited by testosterone[252]. Adiponectin is known to mediate its actions via two transmembrane receptors, AdipoR1 and AdipoR2. Herein, we are the first to report a sexual pattern in the distribution of the adiponectin receptors on circulating monocytes, where men with stable plaques had lower AdipoR gene expression than women with stable plaques. Thus, lower circulating HMW adiponectin and less adiponectin receptor expression may partially explain why men have a higher risk of developing atherosclerotic disease than women.

7.5.3 Sex hormones

As mentioned above, we believe that sex hormones play a crucial role in delineating the differences observed herein between men and women at the level of the plaque and at the level of the circulation, as estrogens and androgens have been suggested to have a direct impact on the vascular system and on the cells of which it is comprised. Exposure to high levels of estrogens are believed to retard the atherogenic process in premenopausal women placing these women at decreased risk for CVD or stroke compared to men of similar age[122]. Menopause due to a decrease in estrogen levels, diminishes the 'protection' in women and contributes to an adverse impact on cardiovascular risk variables[21]. Interestingly, in our study population, women were observed to have more favorable lipid, immune, and adipokine profiles than men despite being post-menopausal for many years (mean age: 70.7 years). These findings suggest that 'residual'

protective effects of estrogens may persist even after menopause. Therefore, measurement of sex hormones will provide insight on how these hormones (including E2) can influence the adipokine, lipid, and immune profiles of men and women with stable versus unstable plaques. Currently, there exists lack of a highly sensitive and specific assay to measure circulating sex hormones, thus making it cumbersome and impractical to study these hormones in their entirety and their implication in the atherosclerotic process and plaque instability. Analysis of estrogens and androgens in biological samples has been commonly performed using immunoassays for many years[589]. However, these methods are suboptimal, as they include cross-reactivity with similar analytes and have moderate specificity and sensitivity, especially when it comes to detecting estrogen levels in men and in post-menopausal women, where circulating endogenous levels are extremely low[590]. Thus, mass spectrometry–based methods are becoming the preferred method for measurement of these sex hormones because of better specificity, more sensitivity, and the potential for simultaneous detection of multiple analytes within the same assay[589,590]. Herein, we developed a method for the measurement of endogenous concentrations of various important sex hormones (i.e. E2, testosterone, androstenedione, DHEA), using LC-MS/MS. Thus, 1) allowing comparability between hormones since the same method of measurement is used and 2) detecting levels below the normal limits of detection when using immunoassays. Our future work will focus on using our newly developed method to measure the endogenous levels of sex hormones in our CEA population. In women and in men, we will be able to evaluate the association of these hormone levels with lipid, immune, and adiponectin-AdipoR pathway markers to identify sex-specific signatures of plaque instability.

7.6 Conclusion

To conclude, our study provided much-needed evidence that sex differences do exist in carotid atherosclerosis. These findings place importance on the fact that the ‘one size fits all’ approach should not be applied in carotid disease management and that there is a dire need for sex-specific guidelines. Unfortunately, the current guidelines are lacking sex-specific orientation and are based solely on the degree of carotid artery stenosis, which has been proven to be an incomplete determinant of plaque instability, especially in women who tend to have plaques causing higher stenoses. Since women have more favorable adipokine, lipid, and immune profiles, the severity of their disease may be masked, resulting in women being mis-classified or not properly selected for a CEA or even for medical management, based on the current guidelines; this may be contributing

to the higher stroke death rates reported among women compared to men. Therefore, we need to do a better job at screening and identifying those women who are at risk of having a stroke and providing them with the proper treatment they need. Herein, we identified the HMW:total adiponectin ratio as a potential marker of plaque instability specifically in women. With the measurement of circulating sex hormones using our newly developed assay, our future work will encompass both a translational and mechanistic approach, to determine the best predictive models of plaque instability specifically in men and women with carotid atherosclerotic disease and to understand the sex-specific mechanisms associated with the adiponectin-AdipoR pathway and plaque instability.

7.7 Acknowledgements

We are grateful to the vascular pathologists (Drs Veinot and Lai) for their histological assessments of plaque instability. Furthermore, we are thankful to the vascular surgeons (Drs Steinmetz, MacKenzie, Corriveau, Obrand, Gill, and Bayne) at the MUHC and JGH for their help with subject recruitment. We would also like to thank Dr. Jean-François Cailhier and Dr. Patrick Laplante at the Centre de Recherche du Centre hospitalier de l'Université de Montréal for their assistance with the cytokine/chemokine profiling of collected sera using the V-PLEX Human Biomarker 40-Plex Kit and members of the Proteomics Platform of the Research Institute of McGill University (Ed Daly, Lorne Taylor, Amy Wong) for their expertise in the development and optimization of the novel method to analyze endogenous levels of sex hormones.

7.8 Funding Sources

This work was supported by the Canadian Institutes of Health Research [CIHR, PJT-148966 & FRN 145589] and the Heart & Stroke Foundation of Canada [G-17-0018755]. The funding sources had no involvement in the study.

7.9 Disclosures

None

7.10 References

1. Gasbarrino K, Mantzoros C, Gorgui J, Veinot JP, Lai C, Daskalopoulou SS. Circulating chemerin is associated with carotid plaque instability, whereas resistin is related to cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2016
2. Gasbarrino K, Zheng H, Hafiane A, Veinot JP, Lai C, Daskalopoulou SS. Decreased adiponectin-mediated signaling through the adipor2 pathway is associated with carotid plaque instability. *Stroke*. 2017:48:915-924.
5. Organization WH. The top 10 causes of death. 2018
15. Persky RW, Turtzo LC, McCullough LD. Stroke in women: Disparities and outcomes. *Curr Cardiol Rep*. 2010:12:6-13.
16. Benjamin EJ, Virani SS, Callaway CW et.al. Heart disease and stroke statistics-2018 update: A report from the american heart association. *Circulation*. 2018:137:e67-e492.
17. Reeves MJ, Bushnell CD, Howard G, Gargano JW, Duncan PW, Lynch G, Khatiwoda A, Lisabeth L. Sex differences in stroke: Epidemiology, clinical presentation, medical care, and outcomes. *Lancet Neurol*. 2008:7:915-926.
21. Lisabeth L, Bushnell C. Stroke risk in women: The role of menopause and hormone therapy. *Lancet Neurol*. 2012:11:82-91.
93. Shah PK. Mechanisms of plaque vulnerability and rupture. *J Am Coll Cardiol*. 2003:41:15S-22S.
122. Joakimsen O, Bonaa KH, Stensland-Bugge E, Jacobsen BK. Age and sex differences in the distribution and ultrasound morphology of carotid atherosclerosis: The tromso study. *Arterioscler Thromb Vasc Biol*. 1999:19:3007-3013.
134. Brott TG, Halperin JL, Abbara S et.al. 2011 asa/accf/aha/aann/aans/acr/asnr/cns/saip/scai/sir/snis/svm/svs guideline on the management of patients with extracranial carotid and vertebral artery disease: Executive summary. A report of the american college of cardiology foundation/american heart association task force on practice guidelines, and the american stroke association, american association of neuroscience nurses, american association of neurological surgeons, american college of radiology, american society of neuroradiology, congress of neurological surgeons, society of atherosclerosis imaging and prevention, society for cardiovascular angiography and interventions, society of interventional radiology, society of neurointerventional surgery, society for vascular medicine, and society for vascular surgery. *Circulation*. 2011:124:489-532.
138. Wein T, Lindsay MP, Cote R et.al. Canadian stroke best practice recommendations: Secondary prevention of stroke, sixth edition practice guidelines, update 2017. *Int J Stroke*. 2018:13:420-443.
155. Endarterectomy for asymptomatic carotid artery stenosis. Executive committee for the asymptomatic carotid atherosclerosis study. *Jama*. 1995:273:1421-1428.
167. Alamowitch S, Eliasziw M, Barnett HJ. The risk and benefit of endarterectomy in women with symptomatic internal carotid artery disease. *Stroke*. 2005:36:27-31.
168. Chambers BR, Donnan GA. Carotid endarterectomy for asymptomatic carotid stenosis. *Cochrane Database Syst Rev*. 2005:Cd001923.
169. Sangiorgi G, Roversi S, Biondi Zoccai G, Modena MG, Servadei F, Ippoliti A, Mauriello A. Sex-related differences in carotid plaque features and inflammation. *J Vasc Surg*. 2013:57:338-344.
172. Poisson SN, Johnston SC, Sidney S, Klingman JG, Nguyen-Huynh MN. Gender differences in treatment of severe carotid stenosis after transient ischemic attack. *Stroke*. 2010:41:1891-1895.

176. Stary HC. Natural history and histological classification of atherosclerotic lesions: An update. *Arterioscler Thromb Vasc Biol*. 2000:20:1177-1178.
177. Lovett JK, Gallagher PJ, Hands LJ, Walton J, Rothwell PM. Histological correlates of carotid plaque surface morphology on lumen contrast imaging. *Circulation*. 2004:110:2190-2197.
178. Lovett JK, Gallagher PJ, Rothwell PM. Reproducibility of histological assessment of carotid plaque: Implications for studies of carotid imaging. *Cerebrovasc Dis*. 2004:18:117-123.
179. Redgrave JN, Lovett JK, Gallagher PJ, Rothwell PM. Histological assessment of 526 symptomatic carotid plaques in relation to the nature and timing of ischemic symptoms: The oxford plaque study. *Circulation*. 2006:113:2320-2328.
183. Ota H, Reeves MJ, Zhu DC, Majid A, Collar A, Yuan C, DeMarco JK. Sex differences of high-risk carotid atherosclerotic plaque with less than 50% stenosis in asymptomatic patients: An in vivo 3t mri study. *AJNR Am J Neuroradiol*. 2013:34:1049-1055, s1041.
252. Xu A, Chan KW, Hoo RL, Wang Y, Tan KC, Zhang J, Chen B, Lam MC, Tse C, Cooper GJ, Lam KS. Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. *J Biol Chem*. 2005:280:18073-18080.
580. Libby P. The molecular mechanisms of the thrombotic complications of atherosclerosis. *J Intern Med*. 2008:263:517-527.
581. Corti R, Fuster V, Badimon JJ. Pathogenetic concepts of acute coronary syndromes. *J Am Coll Cardiol*. 2003:41:7s-14s.
582. Wang X, Magkos F, Mittendorfer B. Sex differences in lipid and lipoprotein metabolism: It's not just about sex hormones. *J Clin Endocrinol Metab*. 2011:96:885-893.
583. Palmisano BT, Zhu L, Eckel RH, Stafford JM. Sex differences in lipid and lipoprotein metabolism. *Mol Metab*. 2018:15:45-55.
584. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol*. 2016:16:626-638.
585. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002:105:1135-1143.
586. Fairweather D. Sex differences in inflammation during atherosclerosis. *Clin Med Insights Cardiol*. 2014:8:49-59.
587. Boyne MS, Bennett NR, Cooper RS, Royal-Thomas TY, Bennett FI, Luke A, Wilks RJ, Forrester TE. Sex-differences in adiponectin levels and body fat distribution: Longitudinal observations in afro-jamaicans. *Diabetes Res Clin Pract*. 2010:90:e33-36.
588. Ohman-Hanson RA, Cree-Green M, Kelsey MM, Bessesen DH, Sharp TA, Pyle L, Pereira RI, Nadeau KJ. Ethnic and sex differences in adiponectin: From childhood to adulthood. *J Clin Endocrinol Metab*. 2016:101:4808-4815.
589. Taylor AE, Keevil B, Huhtaniemi IT. Mass spectrometry and immunoassay: How to measure steroid hormones today and tomorrow. *Eur J Endocrinol*. 2015:173:D1-12.
590. Handelsman DJ, Wartofsky L. Requirement for mass spectrometry sex steroid assays in the journal of clinical endocrinology and metabolism. *J Clin Endocrinol Metab*. 2013:98:3971-3973.

CHAPTER 8: GENERAL DISCUSSION AND CONTRIBUTIONS TO KNOWLEDGE

Atherosclerotic disease and its thrombotic complications remain one of the leading causes of morbidity and mortality worldwide[5]. The acute rupture of atherosclerotic plaques causes local thrombosis, leading to partial or total occlusion of the affected artery as well as embolism to distal arteries resulting in clinical manifestations, such as a stroke[71,72]. Plaque instability is an important causative factor of these complications. Stroke alone kills ~ 13,000 Canadians per year, while over 400,000 stroke survivors in Canada are living with its disabling effects[6,7]. However, in addition to its huge impact at the level of the patient, stroke also causes a deep burden on the health care system, costing the Canadian economy $3.6 billion per year[8]. Thus, the exploration and identification of novel strategies that can improve our ability to detect, prevent, and treat carotid atherosclerotic disease would prove to be of clinical and economical benefit to our society at large.

Management of carotid atherosclerosis involves aggressive medical therapy and surgical intervention (i.e., CEA or CAS). Accurate stratification of patients' stroke risk is clinically relevant as it can guide appropriate disease management. However, the current guidelines used to inform decision for surgical intervention in patients with symptomatic or asymptomatic carotid disease are limited in stratifying patients' stroke risk as they are based solely on the degree of carotid artery stenosis[134,138]. While carotid stenosis is a strong predictor of stroke risk, as portrayed by large carotid stenosis trials, it has also been proven to be an incomplete determinant of a patient's risk as it does not entirely reflect how unstable a plaque truly is and its likelihood to rupture[150,151,153-155,174]. As a result, many people with unstable plaques remain undiagnosed or untreated. Women in particular have suffered from suboptimal prevention and/or management of strokes. Since women have been reported to benefit less from carotid revascularization than men and have increased risk for perioperative stroke and death[155,167,168], much uncertainty exists in deciding which treatment may be appropriate for the management of carotid disease in women. Thus, there is a dire need for sex-specific guidelines to properly inform these clinical decisions. Optimal medical management can lead to large reductions in primary and secondary stroke-related events, and therefore is important for the treatment of all patients with carotid artery disease, regardless of the degree of stenosis and whether surgical intervention is planned. Treatment includes lifestyle modifications as well as pharmacological interventions that either prevent thrombosis, as do antiplatelet regimens, or target individual vascular risk factors, such as anti-hypertensive therapy or lipid-lowering agents[132,134,138]. Some of these agents, such as statins, may also possess pleiotropic properties that help reduce atherosclerotic plaque burden[141-144]. However, a considerable amount of 'residual risk' still exists among these patients despite aggressive medical

treatment[149]. The problem lies in the fact that the majority of these drugs target the risk factors/mediators and not the actual atherosclerotic disease. Thus, major limitations exist in the current guidelines for carotid disease management: 1) effective methods to accurately identify vulnerable patients with unstable plaques who are at high risk for stroke are lacking, 2) there are no therapies aimed at specifically targeting and stabilizing atherosclerotic plaques, and 3) there is need for inclusion of sex-specific recommendations into clinical guidelines. Despite tremendous efforts to understand atherosclerotic plaque development, there still remain major knowledge gaps in what causes certain plaques (even highly stenotic ones), to remain stable and others to become unstable and rupture. Thus, it is imperative to identify potential mechanisms that underlie the atherosclerotic process and contribute to plaque instability in men and in women, as it may lead to more accurate patient risk stratification, may pave the way for novel therapeutic targets aiming to stabilize vulnerable plaques, and may help improve the current recommendations for carotid disease management.

The overarching aims of the series of studies presented in this thesis were to address current knowledge gaps and investigate a potential and novel mechanism of carotid atherosclerotic plaque instability that involves the adiponectin-AdipoR pathway and to explore the effect of modulating this pathway in the monocyte-macrophage lineage. The majority of human studies have defined plaque instability based on cerebrovascular symptomatology. However, symptomatology is in fact a poor proxy of plaque instability as it is a binary outcome that does not reflect the broad spectrum of the disease. Instead, our approach overcomes these limitations by defining plaque instability based on plaque morphology and composition, according to gold-standard histological classifications[175-178], which are recognized as more accurate and reliable indicators of plaque instability. This was made possible by creating a large ongoing bio-bank of human blood and carotid plaque specimens from patients undergoing a CEA. Unfortunately, the understanding of mechanisms leading to atherosclerotic plaque destabilization has been greatly impeded by the lack of a single, reliable, gold-standard animal model of plaque instability/plaque rupture that mimics the process in humans[591]. Therefore, following the associations and relationships we observed in humans, we decided to test/confirm these mechanistic hypotheses in a controlled *in vitro* manner, enhancing the translational, patient-centred nature of the project. By combining *ex vivo* observations and *in vitro* experiments, we determined the contribution of the adiponectin-AdipoR pathway in carotid atherosclerotic plaque instability, which led to its identification as a novel target for plaque stabilization and as a potential sex-specific marker of plaque instability.

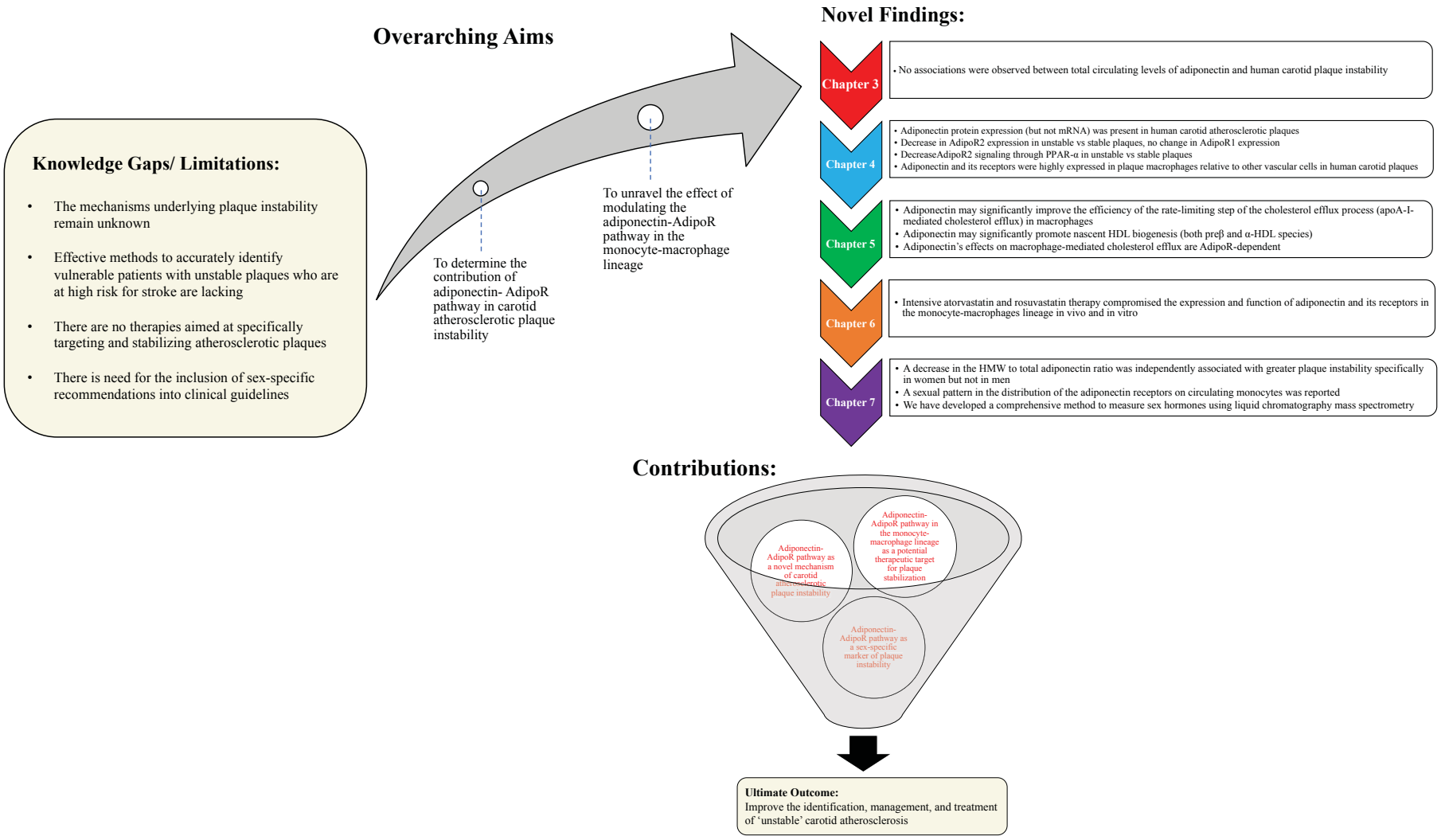


Figure 8.1 Summary diagram of knowledge gaps, aims, novel findings, and contributions to knowledge.

8.1 Contribution 1 – Adiponectin-AdipoR pathway as a novel mechanism of carotid atherosclerotic plaque instability

Traditionally, adipose tissue was recognized simply as a storage depot for excess energy, however more recently it has emerged as a highly dynamic endocrine organ, which produces and secretes a variety of adipokines and cytokines that can influence vascular function[184,186-188]. Since PVAT surrounds blood vessels, it may affect the pathogenesis of atherosclerosis more severely than other adipose tissue depots[209,225,226]. Under physiological conditions, healthy PVAT possesses anti-atherogenic properties. On the contrary, under pathological conditions, such as obesity, dysfunctional PVAT leads to an imbalance in the production and release of adipokines, thereby promoting the development of atherosclerosis[209,225,226,229]. Although various studies have tried to disentangle the role of these adipokines in atherosclerosis, in Chapter 3, we were the first to investigate the relationship between circulating adipokine levels (chemerin, resistin, leptin, and adiponectin) and carotid atherosclerotic plaque instability in humans[1]. We observed low levels of chemerin to be associated with the increased odds of having an unstable plaque, leptin levels to be increased with the presence of specific histological instability features, and high resistin levels to be associated with cerebrovascular symptomatology. In contrast, no such associations were observed with adiponectin. The latter seemed paradoxical considering the strong experimental evidence (*in vitro* and animal studies) that exists highlighting adiponectin's anti-inflammatory and vasculoprotective properties. However, we performed meta-analyses evaluating the existing clinical evidence with regards to the association between circulating adiponectin levels and cIMT (subclinical atherosclerosis) and the risk of carotid plaque presence and ischemic stroke risk, which demonstrated that the relationship between circulating adiponectin and carotid atherosclerotic disease in humans is conflicting and complex[284,285]. This may be due to the fact that various isoforms of adiponectin exist, which contribute to total adiponectin levels in the circulation[254-256]. While HMW adiponectin is believed to be the most active form of adiponectin, the precise biological role of these differing isoforms remains to be identified[254,256]. Moreover, circulating adiponectin, which is mainly derived from subcutaneous and visceral adipose tissue, can be influenced by various systemic factors, disease states, and therapeutic agents. Therefore, to understand the true contribution of adiponectin in the context of plaque instability, it may be more important to study the expression of adiponectin in the plaque rather than in the circulation.

While adiponectin protein has been detected in the vasculature of normal and atherosclerotic mice, in Chapter 4 we were the first to show that adiponectin protein expression

(but not mRNA) is also present in healthy human carotid arteries as well as in human carotid atherosclerotic plaques[2]. Moreover, we observed an increase in adiponectin expression within atherosclerotic plaques that exhibit unstable features when compared to stable plaques or healthy arteries. This suggests that adiponectin's presence in the vascular wall and plaque is derived from an outside source, most likely the surrounding PVAT, and that greater plaque instability may trigger an increase in its production and release. Interestingly, these observations are in support with a study that demonstrated higher adiponectin expression in the PVAT of neurologically symptomatic patients versus asymptomatic patients who underwent a CEA[530]. Moreover, our meta-analysis revealed adiponectin to be an independent and direct predictor of ischemic stroke risk[285]. Therefore, overproduction of adiponectin in these cases may serve as a protective mechanism in response to vascular injury[562]. To further understand the elevation in adiponectin levels in unstable vs. stable lesions, we also investigated the expression of the AdipoR pathway. In contrast to adiponectin, a decrease in AdipoR2 expression was noted in unstable vs stable plaques, while no change in AdipoR1 expression was observed. Furthermore, AdipoR2 signaling through PPAR-α was significantly impaired in unstable plaques, indicating not only a decrease in AdipoR2 expression but also in its activity. These human *in vivo* observations were confirmed using an *in vitro* model of plaque instability. Decreased signaling downstream of AdipoR2 may impair the atheroprotective actions of adiponectin and cause adiponectin resistance, thereby contributing to plaque instability and an accumulation of adiponectin in vulnerable lesions. While previous evidence does point towards an important involvement of both AdipoR1 and AdipoR2 in processes related to atherosclerosis development[320], our own evidence reveals that AdipoR2 may also play a crucial role at the plaque destabilization stage. Interestingly, it was recently noted that AdipoR2 deficiency (but not AdipoR1) led to a reduction in the size of brachiocephalic atherosclerotic plaques in $apoE^{-/-}$ mice; however, these plaques contained a higher degree of macrophages, less collagen content, and no clear fibrous cap, compared with $AdipoR2^{+/+}apoE^{-/-}$ mice[321]. Therefore, this evidence also suggests that AdipoR2 may be protective against atherosclerotic plaque instability.

Overall, Chapter 4 not only contributed evidence of a novel mechanism of atherosclerotic plaque instability but also highlighted the importance of performing studies that focus on investigating the interactions between the PVAT and the vasculature. While adiponectin itself is the most abundantly-secreted adipokine, there exists a multitude of immunomodulatory proteins

secreted by PVAT, which can positively or negatively influence the vessel wall, atherosclerosis development, and plaque instability. This will be a future direction of this study.

8.2 Contribution 2 – Adiponectin-AdipoR pathway in the monocyte-macrophage lineage as a potential therapeutic target for plaque stabilization

Macrophages are major effector cells of plaque development, progression, and instability due to their influence on intra-plaque cholesterol homeostasis, inflammation, necrotic core initiation and expansion, and fibrous cap degradation[93-95]. Adiponectin's atheroprotective effects on macrophages have been established *in vivo* and *in vitro* and include suppression of macrophage-to-foam cell transformation[295], promotion of macrophage polarization towards an anti-inflammatory M2 macrophage phenotype[298,299], and stimulation of the production of TIMP-1 by macrophages, which helps prevent fibrous cap thinning and rupture[300]. Therefore, our observation that adiponectin and its receptors are highly expressed in plaque macrophages relative to other vascular cells in human carotid atherosclerotic plaques falls in line with this previous evidence (Chapter 4)[2]. This led us to hypothesize that the impairment observed in adiponectin-AdipoR2 activity in unstable plaques (Chapter 4) occurs most importantly at the level of the macrophage.

In Chapter 5 we performed *in vitro* experiments where we used macrophage-mediated cholesterol efflux and HDL biogenesis as a proxy mechanism of plaque stability to explore how modulation of the adiponectin-AdipoR pathway in macrophages can affect the response of macrophages to adiponectin and promote plaque stability/instability. In a previous study we have shown that cholesterol efflux from macrophages is decreased/impaired in patients with more severe carotid artery stenosis and more advanced/unstable carotid plaque morphology compared to patients with less severe and more stable atherosclerotic plaque burden[108]. Chapter 5 presents preliminary results suggesting that the adiponectin-AdipoR pathway might serve as a potential therapeutic target for plaque stabilization that will be further studied in our lab. The preliminary evidence we generated indicates that adiponectin may improve the efficiency of the rate-limiting step of the cholesterol efflux process and promote nascent HDL biogenesis via activation of both the AdipoR1 and AdipoR2 receptors. Specifically, we demonstrated that the kinetics associated with the interaction between adiponectin and apoA-I in promoting cholesterol efflux was highly efficient (relative catalytic efficiency was almost 3-fold higher) compared to apoA-I alone. Secondly, we are the first to demonstrate that adiponectin modulates HDL-apoA-I biogenesis, by increasing the generation of both preβ and α-HDL species in an apoA-I-dependent process. Our

findings suggest that adiponectin is efficient in maintaining cholesterol efflux by promoting the generation of a continuous pool of HDL particles that act as acceptors, while also promoting HDL maturation. If these results are proven further, therapeutically combining adiponectin with apoA-I may help improve the cholesterol efflux process in patients with advanced atherosclerotic disease, where this system may be impaired, and may help serve as a promising therapy for the stabilization of atherosclerotic plaques. Future work may focus on testing this combination therapy in an animal model of atherosclerotic disease. The route of administration of adiponectin would be an important factor to consider. As we pointed out in Chapter 3, circulating levels of adiponectin were not associated with plaque stability. Thus, raising circulating levels of adiponectin may not directly affect the stabilization of the plaque. Instead, adiponectin at the plaque level, may better protect the plaque from becoming unstable.

However, under conditions of adiponectin resistance, upregulating the local plaque expression of adiponectin may not be a useful therapeutic strategy without also enhancing the expression and/or activation of its receptors. In Chapter 4 we identified that decreased adiponectin-mediated signalling through the AdipoR2 pathway in unstable lesions is associated with adiponectin resistance and an over-accumulation of adiponectin in the plaque[2]. In Chapter 5, we demonstrated that the adiponectin-induced effects on macrophage-mediated cholesterol efflux were AdipoR-dependent and that impairments in AdipoR1 or AdipoR2 expression on macrophages via shRNA-mediated gene silencing, resulted in significant reductions in cholesterol efflux in the presence of both apoA-I and adiponectin. Thus, specific activation of the AdipoR pathway in macrophages may serve as a useful and novel therapeutic strategy in enhancing the action of adiponectin and promoting cholesterol efflux activity without having to target/increase adiponectin levels. Through screening of compound chemical libraries, Okada-Iwabu *et al*. identified the first orally-active synthetic small molecule (named AdipoRon) to bind and activate both AdipoR1 and AdipoR2[358]. Ongoing investigations have shown that AdipoRon may be used for the treatment of obesity-related disorders[358]. However, we are the first to provide evidence that AdipoRon may also exert direct and beneficial effects on atherosclerosis development and promote a more stable macrophage phenotype by mimicking the effects of adiponectin on increasing apoA-I-mediated cholesterol efflux in macrophages (Chapter 5). While AdipoRon may act as a promising new therapeutic agent for plaque stabilization, in a future study we will perform high-content screening[592], with a small diversity set of compounds that uses antibodies against the two

receptors, to identify other novel agonists that may have a more specific and potent effect on modulating the AdipoR1 or AdipoR2 pathways in macrophages.

Following the identification of the adiponectin-AdipoR pathway as a potential therapeutic target for plaque stabilization, we aimed to determine whether currently used cardio-metabolic medications, like statins, can positively modulate the adiponectin-AdipoR pathway in the monocyte-macrophage lineage (Chapter 6). A particular interest was taken in statin therapy since it is widely used for the primary and secondary prevention of CVD and there is evidence for off-target anti-inflammatory effects[552,553]. Moreover, statins have been shown to promote plaque regression and decrease atherosclerotic plaque burden[143,144]. Although our *in vivo* (cross-sectional and longitudinal) and *in vitro* studies identified a novel pleiotropic property of statins in modulating the adiponectin-AdipoR pathway in the human monocyte-macrophage lineage, in contrary to our hypotheses, intensive atorvastatin and rosuvastatin therapy compromised the expression and function of adiponectin and its receptors. Our findings may partly explain the residual cardiovascular risk observed among individuals treated with statins[149]. Despite its many pleiotropic actions, the limitation of statins (and of many other pharmacological interventions for CVD) is that they primarily target the individual vascular risk factors (i.e., hypercholesterolemia) rather than the actual established disease (i.e., atherosclerosis). Therefore, our study further stresses the need to develop novel therapies or strategies aimed at specifically targeting and stabilizing atherosclerotic plaques, potentially those that can positively modulate the adiponectin-AdipoR pathway.

In our last chapter (Chapter 7), we were the first to report a sexual pattern in the distribution of the adiponectin receptors on circulating monocytes. While these were simply preliminary analyses and further work is needed to disentangle the sex-specific mechanisms associated with the adiponectin-AdipoR pathway and plaque instability, these results may have implications for future adiponectin-AdipoR targeted therapy in women and in men. Since women and men may respond differently to such therapies, it is important to obtain sex-specific information concerning pharmacodynamics and pharmacokinetics, in order to include sex-specific recommendations into clinical practice guidelines.

8.3 Contribution 3 – Adiponectin-AdipoR pathway as a sex-specific marker of plaque instability

The degree of carotid artery stenosis is a well-established risk factor of plaque instability and forms the basis for the guidelines for carotid disease management and stroke prevention[134,138]. To date, measurement of stenosis via Doppler ultrasound is the only widely used method in clinical practice to detect 'vulnerable' patients who are at increased risk of having a stroke. However, more recently, carotid artery stenosis has been proven to be an inadequate predictor of plaque instability, since even moderate or low-grade stenoses can be unstable and produce potentially life-threatening strokes, and it is increasingly recognized that plaque composition plays a pivotal role in plaque instability[174]. Although several circulating markers (e.g., high-sensitivity CRP and IL-6) have emerged as potential tools for stroke risk assessment in patients with severe carotid atherosclerosis, their ability to reliably and specifically predict plaque instability is suboptimal, stressing a need for novel markers that can accurately identify plaque instability and risk of stroke[499,500]. CRP for example, is an acute phase protein produced and secreted by liver, and thus represents a marker of systemic inflammation that increases in response to various types of injury, infection, and inflammatory stimuli.

In Chapter 3 we aimed to evaluate the association between circulating levels of total adiponectin and carotid plaque instability in patients with moderate- to high-grade carotid artery stenosis scheduled to undergo a CEA[1]. However, despite adiponectin's well-known anti-inflammatory and vasculoprotective properties, no differences in total adiponectin levels were observed between patients with stable and unstable carotid plaques. However, given that sex disparities exist in atherosclerosis development, plaque morphology and composition, stroke risk, and adiponectin production and secretion, we re-explored the relationship between adiponectin and plaque instability in a sex-specific manner. In Chapter 7, we demonstrated that women and men who underwent a CEA, exhibit clear differences not only at the level of the plaque (i.e., plaque composition) but also at the level of the circulation (i.e., adipokine, lipid, and immune profiles). Specifically, we identified that a decrease in the HMW to total adiponectin ratio was independently associated with greater plaque instability specifically in women but not in men. This ratio, also referred to as the adiponectin sensitivity index, appeared to be a better predictive measure than circulating levels of HMW and total adiponectin levels independently, as the ratio takes into account changes in the levels of HMW adiponectin (considered to be the bioactive form) relative to changes in the levels of total adiponectin.

We believe that the HMW to total adiponectin ratio may have the potential to be used as a clinically useful marker that may help identify women (with 'unstable profiles' similar to men) who may be better suited for surgical intervention, as its measurement is minimally invasive (blood test), not complex, and cost-effective. According to a well-established framework for biomarker development, as outlined by Hlatky *et al.*, there exists different phases of evaluation of a novel biomarker[593]. The first phase, proof-of-concept, aims to investigate whether novel marker levels differ between participants with and without the outcome of interest. The following phases consist of prospective validation studies (where the predictive value of the novel marker is assessed) and studies involving comparisons with already established biomarkers. Our study, proposed herein, was designed as the key first step in exploring associations between sex-specific adiponectin-AdipoR signatures and carotid plaque instability, an investigation that has never been performed before. Although using human tissue as a measure of plaque instability is a strength of our study, we also acknowledge the limitations of performing a study with a cross-sectional design, as it limits the establishment of the true diagnostic potential of the HMW to total adiponectin ratio in predicting plaque instability/stroke risk in women. However, we believe that our study acts as the first phase (proof-of-concept phase) in the evaluation of novel biomarkers and justifies our next step, which is to validate our results in a large prospective follow-up study. We are aware that the development of unstable atherosclerotic plaques is a complex process, thus a combination of factors, rather than a single marker, will likely improve our ability to not only predict, but also ultimately, to prevent strokes. As we continue the exploration of other potential and novel markers that can reflect the sex-specific features in the plaque and plaque instability (i.e., measurement of circulating sex hormones using our newly developed LC-MS/MS method), future studies will then be needed to elucidate the additive role of these novel markers to traditional risk factors and the degree of carotid artery stenosis to generate the best predictive model for plaque instability and stroke risk specifically in women and in men.

8.4 Conclusion

Through the series of studies presented herein, we have contributed to many novelties that will advance the field of atherosclerosis and will direct our future studies: provided evidence for the adiponectin-AdipoR pathway as a (a) novel mechanism of plaque instability, (b) potential therapeutic target for plaque stabilization and ultimately, stroke prevention, and (c) probable sex-specific marker of plaque instability. Given the knowledge gaps and limitations that exist

surrounding the current guidelines for carotid disease management, we hope that our efforts will serve as a stepping-stone for future work aimed at improving the identification, management, and treatment of carotid atherosclerotic plaque instability that will benefit the society, as well as the health care systems in Canada and world-wide.

REFERENCES

1. Gasbarrino K, Mantzoros C, Gorgui J, Veinot JP, Lai C, Daskalopoulou SS. Circulating chemerin is associated with carotid plaque instability, whereas resistin is related to cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2016
2. Gasbarrino K, Zheng H, Hafiane A, Veinot JP, Lai C, Daskalopoulou SS. Decreased adiponectin-mediated signaling through the adipor2 pathway is associated with carotid plaque instability. *Stroke*. 2017:48:915-924.
3. Gasbarrino K, Hafiane A, Genest J, Daskalopoulou SS. Abstract 28: Adiponectin stimulates cholesterol efflux efficiently in human thp-1 macrophages and modulates hdl-apoa-i biogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2016:36:A28-A28.
4. Gasbarrino K, Zheng H, Veinot J, SS D. Sex differences in the adipokine, lipid, and immune profiles of men and women with severe carotid atherosclerosis. *Atherosclerosis Supplements*. 2018:32:13-14.
5. Organization WH. The top 10 causes of death. 2018
6. Canada HSFo. Mind the connection: Preventing stroke and dementia - 2016 stroke report. 2016
7. Canada HSFo. Different strokes: Recovery triumphs and challenges at any age - 2017 stroke report. 2017
8. Canada PHAo. Tracking heart disease and stroke in canada - stroke highlights 2011. 2011
9. Camici GG, Savarese G, Akhmedov A, Luscher TF. Molecular mechanism of endothelial and vascular aging: Implications for cardiovascular disease. *Eur Heart J*. 2015:36:3392-3403.
10. Sun Z. Aging, arterial stiffness, and hypertension. *Hypertension*. 2015:65:252-256.
11. Mattace-Raso FU, van der Cammen TJ, Hofman A, van Popele NM, Bos ML, Schalekamp MA, Asmar R, Reneman RS, Hoeks AP, Breteler MM, Witteman JC. Arterial stiffness and risk of coronary heart disease and stroke: The rotterdam study. *Circulation*. 2006:113:657-663.
12. van Popele NM, Grobbee DE, Bots ML, Asmar R, Topouchian J, Reneman RS, Hoeks AP, van der Kuip DA, Hofman A, Witteman JC. Association between arterial stiffness and atherosclerosis: The rotterdam study. *Stroke*. 2001:32:454-460.
13. Mitchell GF, Hwang SJ, Vasan RS, Larson MG, Pencina MJ, Hamburg NM, Vita JA, Levy D, Benjamin EJ. Arterial stiffness and cardiovascular events: The framingham heart study. *Circulation*. 2010:121:505-511.
14. Seshadri S, Beiser A, Kelly-Hayes M, Kase CS, Au R, Kannel WB, Wolf PA. The lifetime risk of stroke: Estimates from the framingham study. *Stroke*. 2006:37:345-350.
15. Persky RW, Turtzo LC, McCullough LD. Stroke in women: Disparities and outcomes. *Curr Cardiol Rep*. 2010:12:6-13.
16. Benjamin EJ, Virani SS, Callaway CW et.al. Heart disease and stroke statistics-2018 update: A report from the american heart association. *Circulation*. 2018:137:e67-e492.
17. Reeves MJ, Bushnell CD, Howard G, Gargano JW, Duncan PW, Lynch G, Khatiwoda A, Lisabeth L. Sex differences in stroke: Epidemiology, clinical presentation, medical care, and outcomes. *Lancet Neurol*. 2008:7:915-926.
18. Canada HSFo. Lives disrupted: The impact of stroke on women - 2018 stroke report. 2018
19. Di Carlo A, Lamassa M, Baldereschi M, Pracucci G, Basile AM, Wolfe CD, Giroud M, Rudd A, Ghetti A, Inzitari D. Sex differences in the clinical presentation, resource use, and 3-month outcome of acute stroke in europe: Data from a multicenter multinational hospital-based registry. *Stroke*. 2003:34:1114-1119.

20. Niewada M, Kobayashi A, Sandercock PA, Kaminski B, Czlonkowska A. Influence of gender on baseline features and clinical outcomes among 17,370 patients with confirmed ischaemic stroke in the international stroke trial. *Neuroepidemiology*. 2005:24:123-128.
21. Lisabeth L, Bushnell C. Stroke risk in women: The role of menopause and hormone therapy. *Lancet Neurol*. 2012:11:82-91.
22. Flossmann E, Schulz UG, Rothwell PM. Systematic review of methods and results of studies of the genetic epidemiology of ischemic stroke. *Stroke*. 2004:35:212-227.
23. Choi JC, Lee JS, Kang SY, Kang JH, Bae JM. Family history and risk for ischemic stroke: Sibling history is more strongly correlated with the disease than parental history. *J Neurol Sci*. 2009:284:29-32.
24. Kulshreshtha A, Vaccarino V, Goyal A, McClellan W, Nahab F, Howard VJ, Judd SE. Family history of stroke and cardiovascular health in a national cohort. *J Stroke Cerebrovasc Dis*. 2015:24:447-454.
25. Schulz UG, Flossmann E, Rothwell PM. Heritability of ischemic stroke in relation to age, vascular risk factors, and subtypes of incident stroke in population-based studies. *Stroke*. 2004:35:819-824.
26. Meschia JF, Bushnell C, Boden-Albala B et.al. Guidelines for the primary prevention of stroke: A statement for healthcare professionals from the american heart association/american stroke association. *Stroke*. 2014:45:3754-3832.
27. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R. Age-specific relevance of usual blood pressure to vascular mortality: A meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet*. 2002:360:1903-1913.
28. MacMahon S, Peto R, Cutler J, Collins R, Sorlie P, Neaton J, Abbott R, Godwin J, Dyer A, Stamler J. Blood pressure, stroke, and coronary heart disease. Part 1, prolonged differences in blood pressure: Prospective observational studies corrected for the regression dilution bias. *Lancet*. 1990:335:765-774.
29. Psaty BM, Lumley T, Furberg CD, Schellenbaum G, Pahor M, Alderman MH, Weiss NS. Health outcomes associated with various antihypertensive therapies used as first-line agents: A network meta-analysis. *Jama*. 2003:289:2534-2544.
30. Randomised trial of a perindopril-based blood-pressure-lowering regimen among 6,105 individuals with previous stroke or transient ischaemic attack. *Lancet*. 2001:358:1033-1041.
31. Gordon T, Kannel WB, Castelli WP, Dawber TR. Lipoproteins, cardiovascular disease, and death. The framingham study. *Arch Intern Med*. 1981:141:1128-1131.
32. Castelli WP. Cholesterol and lipids in the risk of coronary artery disease--the framingham heart study. *Can J Cardiol*. 1988:4 Suppl A:5a-10a.
33. Stamler J, Wentworth D, Neaton JD. Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the multiple risk factor intervention trial (mrfit). *Jama*. 1986:256:2823-2828.
34. Shahar E, Chambless LE, Rosamond WD, Boland LL, Ballantyne CM, McGovern PG, Sharrett AR. Plasma lipid profile and incident ischemic stroke: The atherosclerosis risk in communities (aric) study. *Stroke*. 2003:34:623-631.
35. Amarenco P, Labreuche J. Lipid management in the prevention of stroke: Review and updated meta-analysis of statins for stroke prevention. *Lancet Neurol*. 2009:8:453-463.
36. Cholesterol, diastolic blood pressure, and stroke: 13,000 strokes in 450,000 people in 45 prospective cohorts. Prospective studies collaboration. *Lancet*. 1995:346:1647-1653.

37. Chan DK, O'Rourke F, Shen Q, Mak JC, Hung WT. Meta-analysis of the cardiovascular benefits of intensive lipid lowering with statins. *Acta Neurol Scand*. 2011:124:188-195.
38. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R, Simes R. Efficacy and safety of cholesterol-lowering treatment: Prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet*. 2005:366:1267-1278.
39. Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J, Barnes EH, Voysey M, Gray A, Collins R, Baigent C. The effects of lowering ldl cholesterol with statin therapy in people at low risk of vascular disease: Meta-analysis of individual data from 27 randomised trials. *Lancet*. 2012:380:581-590.
40. Mills EJ, O'Regan C, Eyawo O, Wu P, Mills F, Berwanger O, Briel M. Intensive statin therapy compared with moderate dosing for prevention of cardiovascular events: A meta-analysis of >40 000 patients. *Eur Heart J*. 2011:32:1409-1415.
41. De Caterina R, Scarano M, Marfisi R, Lucisano G, Palma F, Tatasciore A, Marchioli R. Cholesterol-lowering interventions and stroke: Insights from a meta-analysis of randomized controlled trials. *J Am Coll Cardiol*. 2010:55:198-211.
42. Kannel WB, McGee DL. Diabetes and cardiovascular risk factors: The framingham study. *Circulation*. 1979:59:8-13.
43. Leon BM, Maddox TM. Diabetes and cardiovascular disease: Epidemiology, biological mechanisms, treatment recommendations and future research. *World J Diabetes*. 2015:6:1246-1258.
44. Woo CH, Shishido T, McClain C, Lim JH, Li JD, Yang J, Yan C, Abe J. Extracellular signal-regulated kinase 5 sumoylation antagonizes shear stress-induced antiinflammatory response and endothelial nitric oxide synthase expression in endothelial cells. *Circ Res*. 2008:102:538-545.
45. Chait A, Bornfeldt KE. Diabetes and atherosclerosis: Is there a role for hyperglycemia? *J Lipid Res*. 2009:50 Suppl:S335-339.
46. Kannel WB, McGee DL. Diabetes and cardiovascular disease. The framingham study. *Jama*. 1979:241:2035-2038.
47. Abbott RD, Donahue RP, MacMahon SW, Reed DM, Yano K. Diabetes and the risk of stroke. The honolulu heart program. *Jama*. 1987:257:949-952.
48. Burchfiel CM, Curb JD, Rodriguez BL, Abbott RD, Chiu D, Yano K. Glucose intolerance and 22-year stroke incidence. The honolulu heart program. *Stroke*. 1994:25:951-957.
49. Banerjee C, Moon YP, Paik MC, Rundek T, Mora-McLaughlin C, Vieira JR, Sacco RL, Elkind MS. Duration of diabetes and risk of ischemic stroke: The northern manhattan study. *Stroke*. 2012:43:1212-1217.
50. Sui X, Lavie CJ, Hooker SP, Lee DC, Colabianchi N, Lee CD, Blair SN. A prospective study of fasting plasma glucose and risk of stroke in asymptomatic men. *Mayo Clin Proc*. 2011:86:1042-1049.
51. Brown CD, Higgins M, Donato KA, Rohde FC, Garrison R, Obarzanek E, Ernst ND, Horan M. Body mass index and the prevalence of hypertension and dyslipidemia. *Obes Res*. 2000:8:605-619.
52. Ford ES, Williamson DF, Liu S. Weight change and diabetes incidence: Findings from a national cohort of us adults. *Am J Epidemiol*. 1997:146:214-222.
53. Kurth T, Gaziano JM, Berger K, Kase CS, Rexrode KM, Cook NR, Buring JE, Manson JE. Body mass index and the risk of stroke in men. *Arch Intern Med*. 2002:162:2557-2562.

54. Strazzullo P, D'Elia L, Cairella G, Garbagnati F, Cappuccio FP, Scalfi L. Excess body weight and incidence of stroke: Meta-analysis of prospective studies with 2 million participants. *Stroke*. 2010:41:e418-426.
55. Rexrode KM, Hennekens CH, Willett WC, Colditz GA, Stampfer MJ, Rich-Edwards JW, Speizer FE, Manson JE. A prospective study of body mass index, weight change, and risk of stroke in women. *Jama*. 1997:277:1539-1545.
56. Whitlock G, Lewington S, Sherliker P, Clarke R, Emberson J, Halsey J, Qizilbash N, Collins R, Peto R. Body-mass index and cause-specific mortality in 900 000 adults: Collaborative analyses of 57 prospective studies. *Lancet*. 2009:373:1083-1096.
57. Suk SH, Sacco RL, Boden-Albala B, Cheun JF, Pittman JG, Elkind MS, Paik MC. Abdominal obesity and risk of ischemic stroke: The northern manhattan stroke study. *Stroke*. 2003:34:1586-1592.
58. Walker SP, Rimm EB, Ascherio A, Kawachi I, Stampfer MJ, Willett WC. Body size and fat distribution as predictors of stroke among us men. *Am J Epidemiol*. 1996:144:1143-1150.
59. Sjostrom L. Review of the key results from the swedish obese subjects (sos) trial - a prospective controlled intervention study of bariatric surgery. *J Intern Med*. 2013:273:219-234.
60. Wolf PA, D'Agostino RB, Kannel WB, Bonita R, Belanger AJ. Cigarette smoking as a risk factor for stroke. The framingham study. *Jama*. 1988:259:1025-1029.
61. Rodriguez BL, D'Agostino R, Abbott RD, Kagan A, Burchfiel CM, Yano K, Ross GW, Silbershatz H, Higgins MW, Popper J, Wolf PA, Curb JD. Risk of hospitalized stroke in men enrolled in the honolulu heart program and the framingham study: A comparison of incidence and risk factor effects. *Stroke*. 2002:33:230-236.
62. Shinton R, Beevers G. Meta-analysis of relation between cigarette smoking and stroke. *Bmj*. 1989:298:789-794.
63. Bhat VM, Cole JW, Sorkin JD, Wozniak MA, Malarcher AM, Giles WH, Stern BJ, Kittner SJ. Dose-response relationship between cigarette smoking and risk of ischemic stroke in young women. *Stroke*. 2008:39:2439-2443.
64. He Y, Lam TH, Jiang B, Wang J, Sai X, Fan L, Li X, Qin Y, Hu FB. Passive smoking and risk of peripheral arterial disease and ischemic stroke in chinese women who never smoked. *Circulation*. 2008:118:1535-1540.
65. Iribarren C, Darbinian J, Klatsky AL, Friedman GD. Cohort study of exposure to environmental tobacco smoke and risk of first ischemic stroke and transient ischemic attack. *Neuroepidemiology*. 2004:23:38-44.
66. Song YM, Cho HJ. Risk of stroke and myocardial infarction after reduction or cessation of cigarette smoking: A cohort study in korean men. *Stroke*. 2008:39:2432-2438.
67. Kool MJ, Hoeks AP, Struijker Boudier HA, Reneman RS, Van Bortel LM. Short- and long-term effects of smoking on arterial wall properties in habitual smokers. *J Am Coll Cardiol*. 1993:22:1881-1886.
68. Howard G, Wagenknecht LE, Burke GL, Diez-Roux A, Evans GW, McGovern P, Nieto FJ, Tell GS. Cigarette smoking and progression of atherosclerosis: The atherosclerosis risk in communities (aric) study. *Jama*. 1998:279:119-124.
69. Albers GW, Caplan LR, Easton JD, Fayad PB, Mohr JP, Saver JL, Sherman DG. Transient ischemic attack--proposal for a new definition. *N Engl J Med*. 2002:347:1713-1716.
70. Gautier JC. Amaurosis fugax. *N Engl J Med*. 1993:329:426-428.

71. Mohr JP, Albers GW, Amarenco P, Babikian VL, Biller J, Brey RL, Coull B, Easton JD, Gomez CR, Helgason CM, Kase CS, Pullicino PM, Turpie AG. American heart association prevention conference. Iv. Prevention and rehabilitation of stroke. Etiology of stroke. *Stroke*. 1997:28:1501-1506.
72. Adams HP, Jr., Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE, 3rd. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. Toast. Trial of org 10172 in acute stroke treatment. *Stroke*. 1993:24:35-41.
73. VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis: Site-selective responses to atherosclerotic modulators. *Arterioscler Thromb Vasc Biol*. 2004:24:12-22.
74. Kronzon I, Tunick PA. Aortic atherosclerotic disease and stroke. *Circulation*. 2006:114:63-75.
75. Petty GW, Brown RD, Jr., Whisnant JP, Sicks JD, O'Fallon WM, Wiebers DO. Ischemic stroke subtypes: A population-based study of incidence and risk factors. *Stroke*. 1999:30:2513-2516.
76. Chugh SS, Havmoeller R, Narayanan K, Singh D, Rienstra M, Benjamin EJ, Gillum RF, Kim YH, McAnulty JH, Jr., Zheng ZJ, Forouzanfar MH, Naghavi M, Mensah GA, Ezzati M, Murray CJ. Worldwide epidemiology of atrial fibrillation: A global burden of disease 2010 study. *Circulation*. 2014:129:837-847.
77. Morillo CA, Banerjee A, Perel P, Wood D, Jouven X. Atrial fibrillation: The current epidemic. *J Geriatr Cardiol*. 2017:14:195-203.
78. Anderson DC, Kappelle LJ, Eliasziw M, Babikian VL, Pearce LA, Barnett HJ. Occurrence of hemispheric and retinal ischemia in atrial fibrillation compared with carotid stenosis. *Stroke*. 2002:33:1963-1967.
79. Lin HJ, Wolf PA, Kelly-Hayes M, Beiser AS, Kase CS, Benjamin EJ, D'Agostino RB. Stroke severity in atrial fibrillation. The framingham study. *Stroke*. 1996:27:1760-1764.
80. Cairns JA, Lewis HD, Jr., Meade TW, Sutton GC, Theroux P. Antithrombotic agents in coronary artery disease. *Chest*. 1995:108:380s-400s.
81. Gimbrone MA, Jr., Garcia-Cardena G. Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. *Cardiovasc Pathol*. 2013:22:9-15.
82. Lusis AJ. Atherosclerosis. *Nature*. 2000:407:233-241.
83. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011:473:317-325.
84. Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: Update and therapeutic implications. *Circulation*. 2007:116:1832-1844.
85. Boren J, Olin K, Lee I, Chait A, Wight TN, Innerarity TL. Identification of the principal proteoglycan-binding site in ldl. A single-point mutation in apo-b100 severely affects proteoglycan interaction without affecting ldl receptor binding. *J Clin Invest*. 1998:101:2658-2664.
86. Steinberg D, Witztum JL. Oxidized low-density lipoprotein and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2010:30:2311-2316.
87. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell*. 1998:2:275-281.
88. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of p- and e-selectins in atherosclerosis. *J Clin Invest*. 1998:102:145-152.

89. Shih PT, Brennan ML, Vora DK, Territo MC, Strahl D, Elices MJ, Lusis AJ, Berliner JA. Blocking very late antigen-4 integrin decreases leukocyte entry and fatty streak formation in mice fed an atherogenic diet. *Circ Res*. 1999:84:345-351.
90. Woollard KJ, Geissmann F. Monocytes in atherosclerosis: Subsets and functions. *Nat Rev Cardiol*. 2010:7:77-86.
91. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science*. 2010:327:656-661.
92. Swirski FK, Pittet MJ, Kircher MF, Aikawa E, Jaffer FA, Libby P, Weissleder R. Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease. *Proc Natl Acad Sci U S A*. 2006:103:10340-10345.
93. Shah PK. Mechanisms of plaque vulnerability and rupture. *J Am Coll Cardiol*. 2003:41:15S-22S.
94. Shah PK, Falk E, Badimon JJ, Fernandez-Ortiz A, Mailhac A, Villareal-Levy G, Fallon JT, Regnstrom J, Fuster V. Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture. *Circulation*. 1995:92:1565-1569.
95. Johnson JL, Newby AC. Macrophage heterogeneity in atherosclerotic plaques. *Curr Opin Lipidol*. 2009:20:370-378.
96. Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF, Freeman MW. Scavenger receptors class a-i/ii and cd36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem*. 2002:277:49982-49988.
97. Suzuki H, Kurihara Y, Takeya M et.al. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature*. 1997:386:292-296.
98. Febbraio M, Podrez EA, Smith JD, Hajjar DP, Hazen SL, Hoff HF, Sharma K, Silverstein RL. Targeted disruption of the class b scavenger receptor cd36 protects against atherosclerotic lesion development in mice. *J Clin Invest*. 2000:105:1049-1056.
99. Seimon TA, Nadolski MJ, Liao X, Magallon J, Nguyen M, Feric NT, Koschinsky ML, Harkewicz R, Witztum JL, Tsimikas S, Golenbock D, Moore KJ, Tabas I. Atherogenic lipids and lipoproteins trigger cd36-tlr2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress. *Cell Metab*. 2010:12:467-482.
100. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2005:25:1256-1261.
101. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*. 2010:10:36-46.
102. Ohashi R, Mu H, Wang X, Yao Q, Chen C. Reverse cholesterol transport and cholesterol efflux in atherosclerosis. *Qjm*. 2005:98:845-856.
103. Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: Key to the regression of atherosclerosis? *Circulation*. 2006:113:2548-2555.
104. van der Velde AE, Brufau G, Groen AK. Transintestinal cholesterol efflux. *Curr Opin Lipidol*. 2010:21:167-171.
105. van der Veen JN, van Dijk TH, Vrins CL, van Meer H, Havinga R, Bijsterveld K, Tietge UJ, Groen AK, Kuipers F. Activation of the liver x receptor stimulates trans-intestinal excretion of plasma cholesterol. *J Biol Chem*. 2009:284:19211-19219.
106. Hafiane A, Genest J. Hdl, atherosclerosis, and emerging therapies. *Cholesterol*. 2013:2013:891403.

107. Yokoyama S. Assembly of high density lipoprotein by the abca1/apolipoprotein pathway. *Curr Opin Lipidol*. 2005:16:269-279.
108. Doonan RJ, Hafiane A, Lai C, Veinot JP, Genest J, Daskalopoulou SS. Cholesterol efflux capacity, carotid atherosclerosis, and cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2014:34:921-926.
109. Saleheen D, Scott R, Javad S, Zhao W, Rodrigues A, Picataggi A, Lukmanova D, Mucksavage ML, Luben R, Billheimer J, Kastelein JJ, Boekholdt SM, Khaw KT, Wareham N, Rader DJ. Association of hdl cholesterol efflux capacity with incident coronary heart disease events: A prospective case-control study. *Lancet Diabetes Endocrinol*. 2015:3:507-513.
110. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, Neeland IJ, Yuhanna IS, Rader DR, de Lemos JA, Shaul PW. Hdl cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med*. 2014:371:2383-2393.
111. Wang X, Collins HL, Ranalletta M, Fuki IV, Billheimer JT, Rothblat GH, Tall AR, Rader DJ. Macrophage abca1 and abcg1, but not sr-bi, promote macrophage reverse cholesterol transport in vivo. *J Clin Invest*. 2007:117:2216-2224.
112. Brownell N, Rohatgi A. Modulating cholesterol efflux capacity to improve cardiovascular disease. *Curr Opin Lipidol*. 2016:27:398-407.
113. de Haan W, Bhattacharjee A, Ruddle P, Kang MH, Hayden MR. Abca1 in adipocytes regulates adipose tissue lipid content, glucose tolerance, and insulin sensitivity. *J Lipid Res*. 2014:55:516-523.
114. Asztalos BF, Roheim PS, Milani RL, Lefevre M, McNamara JR, Horvath KV, Schaefer EJ. Distribution of apoa-i-containing hdl subpopulations in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol*. 2000:20:2670-2676.
115. Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, Cartland S, Packianathan M, Kritharides L, Jessup W. Abca1 and abcg1 synergize to mediate cholesterol export to apoa-i. *Arterioscler Thromb Vasc Biol*. 2006:26:534-540.
116. Ji Y, Jian B, Wang N, Sun Y, Moya ML, Phillips MC, Rothblat GH, Swaney JB, Tall AR. Scavenger receptor bi promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem*. 1997:272:20982-20985.
117. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. *Annu Rev Physiol*. 2012:74:13-40.
118. Kolodgie FD, Burke AP, Farb A, Gold HK, Yuan J, Narula J, Finn AV, Virmani R. The thin-cap fibroatheroma: A type of vulnerable plaque: The major precursor lesion to acute coronary syndromes. *Curr Opin Cardiol*. 2001:16:285-292.
119. Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of cd40: Induction of collagenase, stromelysin, and tissue factor. *Circulation*. 1997:96:396-399.
120. Fernandez-Ortiz A, Badimon JJ, Falk E, Fuster V, Meyer B, Mailhac A, Weng D, Shah PK, Badimon L. Characterization of the relative thrombogenicity of atherosclerotic plaque components: Implications for consequences of plaque rupture. *J Am Coll Cardiol*. 1994:23:1562-1569.
121. Rautou PE, Vion AC, Amabile N, Chironi G, Simon A, Tedgui A, Boulanger CM. Microparticles, vascular function, and atherothrombosis. *Circ Res*. 2011:109:593-606.

122. Joakimsen O, Bonaa KH, Stensland-Bugge E, Jacobsen BK. Age and sex differences in the distribution and ultrasound morphology of carotid atherosclerosis: The tromso study. *Arterioscler Thromb Vasc Biol*. 1999:19:3007-3013.
123. Arnold AP, Cassis LA, Eghbali M, Reue K, Sandberg K. Sex hormones and sex chromosomes cause sex differences in the development of cardiovascular diseases. *Arterioscler Thromb Vasc Biol*. 2017:37:746-756.
124. Wassertheil-Smoller S, Hendrix SL, Limacher M, Heiss G, Kooperberg C, Baird A, Kotchen T, Curb JD, Black H, Rossouw JE, Aragaki A, Safford M, Stein E, Laowattana S, Mysiw WJ. Effect of estrogen plus progestin on stroke in postmenopausal women: The women's health initiative: A randomized trial. *Jama*. 2003:289:2673-2684.
125. Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med*. 2000:133:933-941.
126. Glisic M, Mujaj B, Rueda-Ochoa OL, Asllanaj E, Laven JSE, Kavousi M, Ikram MK, Vernooij MW, Ikram MA, Franco OH, Bos D, Muka T. Associations of endogenous estradiol and testosterone levels with plaque composition and risk of stroke in subjects with carotid atherosclerosis. *Circ Res*. 2018:122:97-105.
127. Zarins CK, Giddens DP, Bharadvaj BK, Sottiurai VS, Mabon RF, Glagov S. Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. *Circ Res*. 1983:53:502-514.
128. Ku DN, Giddens DP, Zarins CK, Glagov S. Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis*. 1985:5:293-302.
129. Traub O, Berk BC. Laminar shear stress: Mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol*. 1998:18:677-685.
130. Kvickstrom P, Lindblom B, Bergstrom G, Zetterberg M. Amaurosis fugax: Risk factors and prevalence of significant carotid stenosis. *Clin Ophthalmol*. 2016:10:2165-2170.
131. Sobieszczyk P, Beckman J. Carotid artery disease. *Circulation*. 2006:114:e244-247.
132. Ricotta JJ, Aburahma A, Ascher E, Eskandari M, Faries P, Lal BK. Updated society for vascular surgery guidelines for management of extracranial carotid disease. *J Vasc Surg*. 2011:54:e1-31.
133. Mughal MM, Khan MK, DeMarco JK, Majid A, Shamoun F, Abela GS. Symptomatic and asymptomatic carotid artery plaque. *Expert Rev Cardiovasc Ther*. 2011:9:1315-1330.
134. Brott TG, Halperin JL, Abbara S et.al. 2011 asa/accf/aha/aann/aans/acr/asnr/cns/saip/scai/sir/snis/svm/svs guideline on the management of patients with extracranial carotid and vertebral artery disease: Executive summary. A report of the american college of cardiology foundation/american heart association task force on practice guidelines, and the american stroke association, american association of neuroscience nurses, american association of neurological surgeons, american college of radiology, american society of neuroradiology, congress of neurological surgeons, society of atherosclerosis imaging and prevention, society for cardiovascular angiography and interventions, society of interventional radiology, society of neurointerventional surgery, society for vascular medicine, and society for vascular surgery. *Circulation*. 2011:124:489-532.
135. Mineva PP, Manchev IC, Hadjiev DI. Prevalence and outcome of asymptomatic carotid stenosis: A population-based ultrasonographic study. *Eur J Neurol*. 2002:9:383-388.

136. Jacobowitz GR, Rockman CB, Gagne PJ, Adelman MA, Lamparello PJ, Landis R, Riles TS. A model for predicting occult carotid artery stenosis: Screening is justified in a selected population. *J Vasc Surg*. 2003:38:705-709.
137. Nederkoorn PJ, van der Graaf Y, Hunink MG. Duplex ultrasound and magnetic resonance angiography compared with digital subtraction angiography in carotid artery stenosis: A systematic review. *Stroke*. 2003:34:1324-1332.
138. Wein T, Lindsay MP, Cote R et.al. Canadian stroke best practice recommendations: Secondary prevention of stroke, sixth edition practice guidelines, update 2017. *Int J Stroke*. 2018:13:420-443.
139. Stancu C, Sima A. Statins: Mechanism of action and effects. *J Cell Mol Med*. 2001:5:378-387.
140. Bibbins-Domingo K, Grossman DC, Curry SJ et.al. Statin use for the primary prevention of cardiovascular disease in adults: Us preventive services task force recommendation statement. *Jama*. 2016:316:1997-2007.
141. Zhou Q, Liao JK. Statins and cardiovascular diseases: From cholesterol lowering to pleiotropy. *Curr Pharm Des*. 2009:15:467-478.
142. Antonopoulos AS, Margaritis M, Lee R, Channon K, Antoniades C. Statins as anti-inflammatory agents in atherogenesis: Molecular mechanisms and lessons from the recent clinical trials. *Curr Pharm Des*. 2012:18:1519-1530.
143. Nicholls SJ, Ballantyne CM, Barter PJ, Chapman MJ, Erbel RM, Libby P, Raichlen JS, Uno K, Borgman M, Wolski K, Nissen SE. Effect of two intensive statin regimens on progression of coronary disease. *N Engl J Med*. 2011:365:2078-2087.
144. Nissen SE, Nicholls SJ, Sipahi I et.al. Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: The asteroid trial. *Jama*. 2006:295:1556-1565.
145. Nissen SE, Tuzcu EM, Schoenhagen P, Brown BG, Ganz P, Vogel RA, Crowe T, Howard G, Cooper CJ, Brodie B, Grines CL, DeMaria AN. Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: A randomized controlled trial. *Jama*. 2004:291:1071-1080.
146. Nie P, Li D, Hu L, Jin S, Yu Y, Cai Z, Shao Q, Shen J, Yi J, Xiao H, Shen L, He B. Atorvastatin improves plaque stability in apoe-knockout mice by regulating chemokines and chemokine receptors. *PLoS One*. 2014:9:e97009.
147. Crisby M, Nordin-Fredriksson G, Shah PK, Yano J, Zhu J, Nilsson J. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: Implications for plaque stabilization. *Circulation*. 2001:103:926-933.
148. Komukai M, Wajima YS, Tashiro J, Shinomiya M, Saito Y, Morisaki N. Carvastatin suppresses intimal thickening of rabbit carotid artery after balloon catheter injury probably through the inhibition of vascular smooth muscle cell proliferation and migration. *Scand J Clin Lab Invest*. 1999:59:159-166.
149. Ridker PM. Residual inflammatory risk: Addressing the obverse side of the atherosclerosis prevention coin. *Eur Heart J*. 2016:37:1720-1722.
150. Barnett HJM, Taylor DW, Haynes RB, Sackett DL, Peerless SJ, Ferguson GG, Fox AJ, Rankin RN, Hachinski VC, Wiebers DO, Eliasziw M. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. *N Engl J Med*. 1991:325:445-453.
151. Halliday A, Mansfield A, Marro J, Peto C, Peto R, Potter J, Thomas D. Prevention of disabling and fatal strokes by successful carotid endarterectomy in patients without recent neurological symptoms: Randomised controlled trial. *Lancet*. 2004:363:1491-1502.

152. Ricotta JJ, Schenck EA, Hassett JM, DeWeese JA. Lesion width as a discriminator of plaque characteristics. *Cardiovasc Surg*. 1996:4:124-129.
153. Barnett HJ, Taylor DW, Eliasziw M, Fox AJ, Ferguson GG, Haynes RB, Rankin RN, Clagett GP, Hachinski VC, Sackett DL, Thorpe KE, Meldrum HE, Spence JD. Benefit of carotid endarterectomy in patients with symptomatic moderate or severe stenosis. North american symptomatic carotid endarterectomy trial collaborators. *N Engl J Med*. 1998:339:1415-1425.
154. Randomised trial of endarterectomy for recently symptomatic carotid stenosis: Final results of the mrc european carotid surgery trial (ecst). *Lancet*. 1998:351:1379-1387.
155. Endarterectomy for asymptomatic carotid artery stenosis. Executive committee for the asymptomatic carotid atherosclerosis study. *Jama*. 1995:273:1421-1428.
156. Rothwell PM, Gibson RJ, Slattery J, Sellar RJ, Warlow CP. Equivalence of measurements of carotid stenosis. A comparison of three methods on 1001 angiograms. European carotid surgery trialists' collaborative group. *Stroke*. 1994:25:2435-2439.
157. Jonas DE, Feltner C, Amick HR, Sheridan S, Zheng ZJ, Watford DJ, Carter JL, Rowe CJ, Harris R. Screening for asymptomatic carotid artery stenosis: A systematic review and meta-analysis for the u.S. Preventive services task force. *Ann Intern Med*. 2014:161:336-346.
158. Spence JD, Song H, Cheng G. Appropriate management of asymptomatic carotid stenosis. *Stroke Vasc Neurol*. 2016:1:64-71.
159. Anderson DC. Acp journal club: Review: Screening and treating asymptomatic adults with carotid artery stenosis lead to no net benefit. *Ann Intern Med*. 2014:161:Jc6.
160. Mott M, Koroshetz W, Wright CB. Crest-2: Identifying the best method of stroke prevention for carotid artery stenosis: National institute of neurological disorders and stroke organizational update. *Stroke*. 2017:48:e130-e131.
161. Bonati LH, Dobson J, Featherstone RL, Ederle J, van der Worp HB, de Borst GJ, Mali WP, Beard JD, Cleveland T, Engelter ST, Lyrer PA, Ford GA, Dorman PJ, Brown MM. Long-term outcomes after stenting versus endarterectomy for treatment of symptomatic carotid stenosis: The international carotid stenting study (icss) randomised trial. *Lancet*. 2015:385:529-538.
162. Brott TG, Hobson RW, 2nd, Howard G et.al. Stenting versus endarterectomy for treatment of carotid-artery stenosis. *N Engl J Med*. 2010:363:11-23.
163. Moresoli P, Habib B, Reynier P, Secrest MH, Eisenberg MJ, Filion KB. Carotid stenting versus endarterectomy for asymptomatic carotid artery stenosis: A systematic review and meta-analysis. *Stroke*. 2017:48:2150-2157.
164. Murad MH, Shahrour A, Shah ND, Montori VM, Ricotta JJ. A systematic review and meta-analysis of randomized trials of carotid endarterectomy vs stenting. *J Vasc Surg*. 2011:53:792-797.
165. Bonati LH, Lyrer P, Ederle J, Featherstone R, Brown MM. Percutaneous transluminal balloon angioplasty and stenting for carotid artery stenosis. *Cochrane Database Syst Rev*. 2012:Cd000515.
166. Rothwell PM, Eliasziw M, Gutnikov SA, Warlow CP, Barnett HJ. Endarterectomy for symptomatic carotid stenosis in relation to clinical subgroups and timing of surgery. *Lancet*. 2004:363:915-924.
167. Alamowitch S, Eliasziw M, Barnett HJ. The risk and benefit of endarterectomy in women with symptomatic internal carotid artery disease. *Stroke*. 2005:36:27-31.
168. Chambers BR, Donnan GA. Carotid endarterectomy for asymptomatic carotid stenosis. *Cochrane Database Syst Rev*. 2005:Cd001923.

169. Sangiorgi G, Roversi S, Biondi Zoccai G, Modena MG, Servadei F, Ippoliti A, Mauriello A. Sex-related differences in carotid plaque features and inflammation. *J Vasc Surg*. 2013:57:338-344.
170. Rothwell PM, Slattery J, Warlow CP. Clinical and angiographic predictors of stroke and death from carotid endarterectomy: Systematic review. *Bmj*. 1997:315:1571-1577.
171. Rothwell PM, Eliasziw M, Gutnikov SA, Warlow CP, Barnett HJ. Sex difference in the effect of time from symptoms to surgery on benefit from carotid endarterectomy for transient ischemic attack and nondisabling stroke. *Stroke*. 2004:35:2855-2861.
172. Poisson SN, Johnston SC, Sidney S, Klingman JG, Nguyen-Huynh MN. Gender differences in treatment of severe carotid stenosis after transient ischemic attack. *Stroke*. 2010:41:1891-1895.
173. Reeves M, Bhatt A, Jajou P, Brown M, Lisabeth L. Sex differences in the use of intravenous rt-pa thrombolysis treatment for acute ischemic stroke: A meta-analysis. *Stroke*. 2009:40:1743-1749.
174. Saam T, Underhill HR, Chu B, Takaya N, Cai J, Polissar NL, Yuan C, Hatsukami TS. Prevalence of american heart association type vi carotid atherosclerotic lesions identified by magnetic resonance imaging for different levels of stenosis as measured by duplex ultrasound. *J Am Coll Cardiol*. 2008:51:1014-1021.
175. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the committee on vascular lesions of the council on arteriosclerosis, american heart association. *Arterioscler Thromb Vasc Biol*. 1995:15:1512-1531.
176. Stary HC. Natural history and histological classification of atherosclerotic lesions: An update. *Arterioscler Thromb Vasc Biol*. 2000:20:1177-1178.
177. Lovett JK, Gallagher PJ, Hands LJ, Walton J, Rothwell PM. Histological correlates of carotid plaque surface morphology on lumen contrast imaging. *Circulation*. 2004:110:2190-2197.
178. Lovett JK, Gallagher PJ, Rothwell PM. Reproducibility of histological assessment of carotid plaque: Implications for studies of carotid imaging. *Cerebrovasc Dis*. 2004:18:117-123.
179. Redgrave JN, Lovett JK, Gallagher PJ, Rothwell PM. Histological assessment of 526 symptomatic carotid plaques in relation to the nature and timing of ischemic symptoms: The oxford plaque study. *Circulation*. 2006:113:2320-2328.
180. Libby P, Pasterkamp G. Requiem for the 'vulnerable plaque'. *Eur Heart J*. 2015:36:2984-2987.
181. van Lammeren GW, den Ruijter HM, Vrijenhoek JE, van der Laan SW, Velema E, de Vries JP, de Kleijn DP, Vink A, de Borst GJ, Moll FL, Bots ML, Pasterkamp G. Time-dependent changes in atherosclerotic plaque composition in patients undergoing carotid surgery. *Circulation*. 2014:129:2269-2276.
182. Iemolo F, Martiniuk A, Steinman DA, Spence JD. Sex differences in carotid plaque and stenosis. *Stroke*. 2004:35:477-481.
183. Ota H, Reeves MJ, Zhu DC, Majid A, Collar A, Yuan C, DeMarco JK. Sex differences of high-risk carotid atherosclerotic plaque with less than 50% stenosis in asymptomatic patients: An in vivo 3t mri study. *AJNR Am J Neuroradiol*. 2013:34:1049-1055, s1041.
184. Giamila Fantuzzi TM. *Adipose tissue and adipokines in health and disease*. Totowa, New Jersey: Humana Press; 2007.

185. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol*. 2011:11:85-97.
186. Cao H. Adipocytokines in obesity and metabolic disease. *J Endocrinol*. 2014:220:T47-59.
187. Ouchi N, Ohashi K, Shibata R, Murohara T. Adipocytokines and obesity-linked disorders. *Nagoya J Med Sci*. 2012:74:19-30.
188. Tilg H, Moschen AR. Adipocytokines: Mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol*. 2006:6:772-783.
189. Flier JS, Cook KS, Usher P, Spiegelman BM. Severely impaired adipsin expression in genetic and acquired obesity. *Science*. 1987:237:405-408.
190. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*. 1995:269:543-546.
191. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994:372:425-432.
192. Xu A, Vanhoutte PM. Adiponectin and adipocyte fatty acid binding protein in the pathogenesis of cardiovascular disease. *Am J Physiol Heart Circ Physiol*. 2012:302:H1231-1240.
193. Hauner H. Secretory factors from human adipose tissue and their functional role. *Proc Nutr Soc*. 2005:64:163-169.
194. Trayhurn P, Drevon CA, Eckel J. Secreted proteins from adipose tissue and skeletal muscle - adipokines, myokines and adipose/muscle cross-talk. *Arch Physiol Biochem*. 2011:117:47-56.
195. Mancuso P. The role of adipokines in chronic inflammation. *Immunotargets Ther*. 2016:5:47-56.
196. Bluher M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. *Best Pract Res Clin Endocrinol Metab*. 2013:27:163-177.
197. Harman-Boehm I, Bluher M, Redel H, Sion-Vardy N, Ovadia S, Avinoach E, Shai I, Kloting N, Stumvoll M, Bashan N, Rudich A. Macrophage infiltration into omental versus subcutaneous fat across different populations: Effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab*. 2007:92:2240-2247.
198. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*. 2003:112:1796-1808.
199. Pajvani UB, Trujillo ME, Combs TP, Iyengar P, Jelicks L, Roth KA, Kitsis RN, Scherer PE. Fat apoptosis through targeted activation of caspase 8: A new mouse model of inducible and reversible lipoatrophy. *Nat Med*. 2005:11:797-803.
200. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. Mcp-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest*. 2006:116:1494-1505.
201. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A*. 2003:100:7265-7270.
202. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res*. 2005:46:2347-2355.
203. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science*. 1993:259:87-91.

204. Zou C, Shao J. Role of adipocytokines in obesity-associated insulin resistance. *J Nutr Biochem*. 2008:19:277-286.
205. Fantuzzi G. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol*. 2005:115:911-919; quiz 920.
206. Baranova A, Collantes R, Gowder SJ, Elariny H, Schlauch K, Younoszai A, King S, Randhawa M, Pusulury S, Alsheddi T, Ong JP, Martin LM, Chandhoke V, Younossi ZM. Obesity-related differential gene expression in the visceral adipose tissue. *Obes Surg*. 2005:15:758-765.
207. Das UN, Rao AA. Gene expression profile in obesity and type 2 diabetes mellitus. *Lipids Health Dis*. 2007:6:35.
208. Lee YH, Nair S, Rousseau E, Allison DB, Page GP, Tataranni PA, Bogardus C, Permana PA. Microarray profiling of isolated abdominal subcutaneous adipocytes from obese vs non-obese pima indians: Increased expression of inflammation-related genes. *Diabetologia*. 2005:48:1776-1783.
209. van Dam AD, Boon MR, Berbee JFP, Rensen PCN, van Harmelen V. Targeting white, brown and perivascular adipose tissue in atherosclerosis development. *Eur J Pharmacol*. 2017:816:82-92.
210. Fantuzzi G, Mazzone T. Adipose tissue and atherosclerosis: Exploring the connection. *Arterioscler Thromb Vasc Biol*. 2007:27:996-1003.
211. Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, Eckel RH. Obesity and cardiovascular disease: Pathophysiology, evaluation, and effect of weight loss: An update of the 1997 american heart association scientific statement on obesity and heart disease from the obesity committee of the council on nutrition, physical activity, and metabolism. *Circulation*. 2006:113:898-918.
212. Adams KF, Schatzkin A, Harris TB, Kipnis V, Mouw T, Ballard-Barbash R, Hollenbeck A, Leitzmann MF. Overweight, obesity, and mortality in a large prospective cohort of persons 50 to 71 years old. *N Engl J Med*. 2006:355:763-778.
213. Donohoe CL, Doyle SL, Reynolds JV. Visceral adiposity, insulin resistance and cancer risk. *Diabetol Metab Syndr*. 2011:3:12.
214. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, Vasan RS, Murabito JM, Meigs JB, Cupples LA, D'Agostino RB, Sr., O'Donnell CJ. Abdominal visceral and subcutaneous adipose tissue compartments: Association with metabolic risk factors in the framingham heart study. *Circulation*. 2007:116:39-48.
215. Fujioka S, Matsuzawa Y, Tokunaga K, Tarui S. Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity. *Metabolism*. 1987:36:54-59.
216. Alvehus M, Buren J, Sjostrom M, Goedecke J, Olsson T. The human visceral fat depot has a unique inflammatory profile. *Obesity (Silver Spring)*. 2010:18:879-883.
217. Vohl MC, Sladek R, Robitaille J, Gurd S, Marceau P, Richard D, Hudson TJ, Tchernof A. A survey of genes differentially expressed in subcutaneous and visceral adipose tissue in men. *Obes Res*. 2004:12:1217-1222.
218. Bluher M. Adipose tissue dysfunction in obesity. *Exp Clin Endocrinol Diabetes*. 2009:117:241-250.
219. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology*. 2004:145:2273-2282.

220. Ebbert JO, Jensen MD. Fat depots, free fatty acids, and dyslipidemia. *Nutrients*. 2013:5:498-508.
221. Yu YH, Ginsberg HN. Adipocyte signaling and lipid homeostasis: Sequelae of insulin-resistant adipose tissue. *Circ Res*. 2005:96:1042-1052.
222. Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: Role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol*. 2005:25:2062-2068.
223. Hufnagel B, Dworak M, Soufi M, Mester Z, Zhu Y, Schaefer JR, Klumpp S, Krieglstein J. Unsaturated fatty acids isolated from human lipoproteins activate protein phosphatase type 2cbeta and induce apoptosis in endothelial cells. *Atherosclerosis*. 2005:180:245-254.
224. Steinberg HO, Tarshoby M, Monestel R, Hook G, Cronin J, Johnson A, Bayazeed B, Baron AD. Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. *J Clin Invest*. 1997:100:1230-1239.
225. Tanaka K, Sata M. Roles of perivascular adipose tissue in the pathogenesis of atherosclerosis. *Front Physiol*. 2018:9:3.
226. Britton KA, Fox CS. Perivascular adipose tissue and vascular disease. *Clin Lipidol*. 2011:6:79-91.
227. Takaoka M, Nagata D, Kihara S, Shimomura I, Kimura Y, Tabata Y, Saito Y, Nagai R, Sata M. Periadventitial adipose tissue plays a critical role in vascular remodeling. *Circ Res*. 2009:105:906-911.
228. Ketonen J, Shi J, Martonen E, Mervaala E. Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese c57bl/6 mice. *Circ J*. 2010:74:1479-1487.
229. Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur Heart J*. 2008:29:2959-2971.
230. Yannakoulia M, Yiannakouris N, Bluher S, Matalas AL, Klimis-Zacas D, Mantzoros CS. Body fat mass and macronutrient intake in relation to circulating soluble leptin receptor, free leptin index, adiponectin, and resistin concentrations in healthy humans. *J Clin Endocrinol Metab*. 2003:88:1730-1736.
231. Muse ED, Feldman DI, Blaha MJ, Dardari ZA, Blumenthal RS, Budoff MJ, Nasir K, Criqui MH, Cushman M, McClelland RL, Allison MA. The association of resistin with cardiovascular disease in the multi-ethnic study of atherosclerosis. *Atherosclerosis*. 2015:239:101-108.
232. Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ. Resistin is an inflammatory marker of atherosclerosis in humans. *Circulation*. 2005:111:932-939.
233. Sattar N, Wannamethee G, Sarwar N, Chernova J, Lawlor DA, Kelly A, Wallace AM, Danesh J, Whincup PH. Leptin and coronary heart disease: Prospective study and systematic review. *J Am Coll Cardiol*. 2009:53:167-175.
234. Verma S, Li SH, Wang CH, Fedak PW, Li RK, Weisel RD, Mickle DA. Resistin promotes endothelial cell activation: Further evidence of adipokine-endothelial interaction. *Circulation*. 2003:108:736-740.
235. Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ. Human resistin stimulates the pro-inflammatory cytokines tnf-alpha and il-12 in macrophages by nf-kappab-dependent pathway. *Biochem Biophys Res Commun*. 2005:334:1092-1101.
236. Xu W, Yu L, Zhou W, Luo M. Resistin increases lipid accumulation and cd36 expression in human macrophages. *Biochem Biophys Res Commun*. 2006:351:376-382.
237. Bouloumie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. *Faseb j*. 1999:13:1231-1238.

238. Quehenberger P, Exner M, Sunder-Plassmann R, Ruzicka K, Bieglmayer C, Endler G, Muellner C, Speiser W, Wagner O. Leptin induces endothelin-1 in endothelial cells in vitro. *Circ Res*. 2002:90:711-718.
239. Trovati M, Doronzo G, Barale C, Vaccheris C, Russo I, Cavalot F. Leptin and vascular smooth muscle cells. *Curr Pharm Des*. 2014:20:625-634.
240. Anderson HD, Rahmutula D, Gardner DG. Tumor necrosis factor-alpha inhibits endothelial nitric-oxide synthase gene promoter activity in bovine aortic endothelial cells. *J Biol Chem*. 2004:279:963-969.
241. Yoshizumi M, Perrella MA, Burnett JC, Jr., Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mrna by shortening its half-life. *Circ Res*. 1993:73:205-209.
242. Barter PJ, Kastelein JJ. Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease. *J Am Coll Cardiol*. 2006:47:492-499.
243. Rajman I, Maxwell S, Cramb R, Kendall M. Particle size: The key to the atherogenic lipoprotein? *Qjm*. 1994:87:709-720.
244. Garg N, Goyal N, Strawn TL, Wu J, Mann KM, Lawrence DA, Fay WP. Plasminogen activator inhibitor-1 and vitronectin expression level and stoichiometry regulate vascular smooth muscle cell migration through physiological collagen matrices. *J Thromb Haemost*. 2010:8:1847-1854.
245. Sobel BE, Taatjes DJ, Schneider DJ. Intramural plasminogen activator inhibitor type-1 and coronary atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2003:23:1979-1989.
246. Maresca F, Di Palma V, Bevilacqua M, Uccello G, Taglialatela V, Giaquinto A, Esposito G, Trimarco B, Cirillo P. Adipokines, vascular wall, and cardiovascular disease: A focused overview of the role of adipokines in the pathophysiology of cardiovascular disease. *Angiology*. 2015:66:8-24.
247. Hu E, Liang P, Spiegelman BM. Adipoq is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem*. 1996:271:10697-10703.
248. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K. Cdna cloning and expression of a novel adipose specific collagen-like factor, apm1 (adipose most abundant gene transcript 1). *Biochem Biophys Res Commun*. 1996:221:286-289.
249. Nakano Y, Tobe T, Choi-Miura NH, Mazda T, Tomita M. Isolation and characterization of gbp28, a novel gelatin-binding protein purified from human plasma. *J Biochem*. 1996:120:803-812.
250. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to c1q, produced exclusively in adipocytes. *J Biol Chem*. 1995:270:26746-26749.
251. Nigro E, Scudiero O, Monaco ML, Palmieri A, Mazzarella G, Costagliola C, Bianco A, Daniele A. New insight into adiponectin role in obesity and obesity-related diseases. *Biomed Res Int*. 2014:2014:658913.
252. Xu A, Chan KW, Hoo RL, Wang Y, Tan KC, Zhang J, Chen B, Lam MC, Tse C, Cooper GJ, Lam KS. Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. *J Biol Chem*. 2005:280:18073-18080.
253. Shapiro L, Scherer PE. The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr Biol*. 1998:8:335-338.
254. Wang Y, Lam KS, Yau MH, Xu A. Post-translational modifications of adiponectin: Mechanisms and functional implications. *Biochem J*. 2008:409:623-633.
255. Suzuki S, Wilson-Kubalek EM, Wert D, Tsao TS, Lee DH. The oligomeric structure of high molecular weight adiponectin. *FEBS Lett*. 2007:581:809-814.

256. Hada Y, Yamauchi T, Waki H, Tsuchida A, Hara K, Yago H, Miyazaki O, Ebinuma H, Kadowaki T. Selective purification and characterization of adiponectin multimer species from human plasma. *Biochem Biophys Res Commun*. 2007:356:487-493.
257. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF. Proteolytic cleavage product of 30-kda adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A*. 2001:98:2005-2010.
258. Waki H, Yamauchi T, Kamon J, Kita S, Ito Y, Hada Y, Uchida S, Tsuchida A, Takekawa S, Kadowaki T. Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line thp-1. *Endocrinology*. 2005:146:790-796.
259. Wang Y, Lam KS, Chan L, Chan KW, Lam JB, Lam MC, Hoo RC, Mak WW, Cooper GJ, Xu A. Post-translational modifications of the four conserved lysine residues within the collagenous domain of adiponectin are required for the formation of its high molecular weight oligomeric complex. *J Biol Chem*. 2006:281:16391-16400.
260. Wang ZV, Schraw TD, Kim JY, Khan T, Rajala MW, Follenzi A, Scherer PE. Secretion of the adipocyte-specific secretory protein adiponectin critically depends on thiol-mediated protein retention. *Mol Cell Biol*. 2007:27:3716-3731.
261. Arita Y, Kihara S, Ouchi N et.al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun*. 1999:257:79-83.
262. Saito K, Tobe T, Yoda M, Nakano Y, Choi-Miura NH, Tomita M. Regulation of gelatin-binding protein 28 (gbp28) gene expression by c/ebp. *Biol Pharm Bull*. 1999:22:1158-1162.
263. Kappes A, Loffler G. Influences of ionomycin, dibutyryl-cycloamp and tumour necrosis factor-alpha on intracellular amount and secretion of apm1 in differentiating primary human preadipocytes. *Horm Metab Res*. 2000:32:548-554.
264. Hotta K, Funahashi T, Arita Y et.al. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol*. 2000:20:1595-1599.
265. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y, Osaka CADSGCad. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol*. 2003:23:85-89.
266. Hui E, Xu A, Chow WS, Lee PC, Fong CH, Cheung SC, Tse HF, Chau MT, Cheung BM, Lam KS. Hypoadiponectinemia as an independent predictor for the progression of carotid atherosclerosis: A 5-year prospective study. *Metab Syndr Relat Disord*. 2014:12:517-522.
267. Yamauchi T, Kamon J, Waki H et.al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med*. 2001:7:941-946.
268. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE. The adipocyte-secreted protein acrp30 enhances hepatic insulin action. *Nat Med*. 2001:7:947-953.
269. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA. Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab*. 2001:86:1930-1935.
270. Spranger J, Kroke A, Mohlig M, Bergmann MM, Ristow M, Boeing H, Pfeiffer AF. Adiponectin and protection against type 2 diabetes mellitus. *Lancet*. 2003:361:226-228.
271. Hara K, Boutin P, Mori Y et.al. Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the japanese population. *Diabetes*. 2002:51:536-540.

272. Kondo H, Shimomura I, Matsukawa Y, Kumada M, Takahashi M, Matsuda M, Ouchi N, Kihara S, Kawamoto T, Sumitsuji S, Funahashi T, Matsuzawa Y. Association of adiponectin mutation with type 2 diabetes: A candidate gene for the insulin resistance syndrome. *Diabetes*. 2002:51:2325-2328.
273. Kissebah AH, Sonnenberg GE, Myklebust J, Goldstein M, Broman K, James RG, Marks JA, Krakower GR, Jacob HJ, Weber J, Martin L, Blangero J, Comuzzie AG. Quantitative trait loci on chromosomes 3 and 17 influence phenotypes of the metabolic syndrome. *Proc Natl Acad Sci U S A*. 2000:97:14478-14483.
274. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial nf-kappab signaling through a camp-dependent pathway. *Circulation*. 2000:102:1296-1301.
275. Qi Y, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, Scherer PE, Ahima RS. Adiponectin acts in the brain to decrease body weight. *Nat Med*. 2004:10:524-529.
276. Kubota N, Yano W, Kubota T et.al. Adiponectin stimulates amp-activated protein kinase in the hypothalamus and increases food intake. *Cell Metab*. 2007:6:55-68.
277. Dalamaga M, Diakopoulos KN, Mantzoros CS. The role of adiponectin in cancer: A review of current evidence. *Endocr Rev*. 2012:33:547-594.
278. Sattar N, Wannamethee G, Sarwar N, Tchernova J, Cherry L, Wallace AM, Danesh J, Whincup PH. Adiponectin and coronary heart disease: A prospective study and meta-analysis. *Circulation*. 2006:114:623-629.
279. Gardener H, Sjoberg C, Crisby M, Goldberg R, Mendez A, Wright CB, Elkind MS, Sacco RL, Rundek T. Adiponectin and carotid intima-media thickness in the northern manhattan study.[erratum appears in stroke. 2012 sep;43(9):E99]. *Stroke*. 2012:43:1123-1125.
280. Reynolds HR, Buyon J, Kim M, Rivera TL, Izmirly P, Tunick P, Clancy RM. Association of plasma soluble e-selectin and adiponectin with carotid plaque in patients with systemic lupus erythematosus. *Atherosclerosis*. 2010:210:569-574.
281. Sawada T, Shite J, Shinke T, Otake H, Tanino Y, Ogasawara D, Kawamori H, Kato H, Miyoshi N, Yoshino N, Kozuki A, Hirata K. Low plasma adiponectin levels are associated with presence of thin-cap fibroatheroma in men with stable coronary artery disease. *Int J Cardiol*. 2010:142:250-256.
282. Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, Rimm EB. Plasma adiponectin levels and risk of myocardial infarction in men. *Jama*. 2004:291:1730-1737.
283. Ai M, Otokozawa S, Asztalos BF, White CC, Cupples LA, Nakajima K, Lamon-Fava S, Wilson PW, Matsuzawa Y, Schaefer EJ. Adiponectin: An independent risk factor for coronary heart disease in men in the framingham offspring study. *Atherosclerosis*. 2011:217:543-548.
284. Gasbarrino K, Gorgui J, Nauche B, Cote R, Daskalopoulou SS. Circulating adiponectin and carotid intima-media thickness: A systematic review and meta-analysis. *Metabolism*. 2016:65:968-986.
285. Gorgui J, Gasbarrino K, Georgakis MK, Karalexi MA, Nauche B, Petridou ET, Daskalopoulou SS. Circulating adiponectin levels in relation to carotid atherosclerotic plaque presence, ischemic stroke risk, and mortality: A systematic review and meta-analyses. *Metabolism*. 2017:69:51-66.
286. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y.

Adiponectin reduces atherosclerosis in apolipoprotein e-deficient mice. *Circulation*. 2002:106:2767-2770.
287. Yamauchi T, Kamon J, Waki H et.al. Globular adiponectin protected ob/ob mice from diabetes and apoe-deficient mice from atherosclerosis. *J Biol Chem*. 2003:278:2461-2468.
288. Kubota N, Terauchi Y, Yamauchi T et.al. Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem*. 2002:277:25863-25866.
289. Matsuda M, Shimomura I, Sata M et.al. Role of adiponectin in preventing vascular stenosis. The missing link of adipo-vascular axis. *J Biol Chem*. 2002:277:37487-37491.
290. Zhu W, Cheng KK, Vanhoutte PM, Lam KS, Xu A. Vascular effects of adiponectin: Molecular mechanisms and potential therapeutic intervention. *Clin Sci (Lond)*. 2008:114:361-374.
291. Chen H, Montagnani M, Funahashi T, Shimomura I, Quon MJ. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J Biol Chem*. 2003:278:45021-45026.
292. Li R, Wang WQ, Zhang H, Yang X, Fan Q, Christopher TA, Lopez BL, Tao L, Goldstein BJ, Gao F, Ma XL. Adiponectin improves endothelial function in hyperlipidemic rats by reducing oxidative/nitrative stress and differential regulation of enos/inos activity. *Am J Physiol Endocrinol Metab*. 2007:293:E1703-1708.
293. Ouedraogo R, Gong Y, Berzins B, Wu X, Mahadev K, Hough K, Chan L, Goldstein BJ, Scalia R. Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo. *J Clin Invest*. 2007:117:1718-1726.
294. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Novel modulator for endothelial adhesion molecules: Adipocyte-derived plasma protein adiponectin. *Circulation*. 1999:100:2473-2476.
295. Wang M, Wang D, Zhang Y, Wang X, Liu Y, Xia M. Adiponectin increases macrophages cholesterol efflux and suppresses foam cell formation in patients with type 2 diabetes mellitus. *Atherosclerosis*. 2013:229:62-70.
296. Ouchi N, Kihara S, Arita Y et.al. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class a scavenger receptor expression in human monocyte-derived macrophages. *Circulation*. 2001:103:1057-1063.
297. Oram JF. Tangier disease and abca1. *Biochim Biophys Acta*. 2000:1529:321-330.
298. Lovren F, Pan Y, Quan A, Szmitko PE, Singh KK, Shukla PC, Gupta M, Chan L, Al-Omran M, Teoh H, Verma S. Adiponectin primes human monocytes into alternative anti-inflammatory m2 macrophages. *Am J Physiol Heart Circ Physiol*. 2010:299:H656-663.
299. Ohashi K, Parker JL, Ouchi N, Higuchi A, Vita JA, Gokce N, Pedersen AA, Kalthoff C, Tullin S, Sams A, Summer R, Walsh K. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J Biol Chem*. 2010:285:6153-6160.
300. Kumada M, Kihara S, Ouchi N, Kobayashi H, Okamoto Y, Ohashi K, Maeda K, Nagaretani H, Kishida K, Maeda N, Nagasawa A, Funahashi T, Matsuzawa Y. Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. *Circulation*. 2004:109:2046-2049.
301. Arita Y, Kihara S, Ouchi N et.al. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-bb-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation*. 2002:105:2893-2898.

302. Wang Y, Lam KS, Xu JY, Lu G, Xu LY, Cooper GJ, Xu A. Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. *J Biol Chem*. 2005:280:18341-18347.
303. Restituto P, Colina I, Varo JJ, Varo N. Adiponectin diminishes platelet aggregation and scd40l release. Potential role in the metabolic syndrome. *Am J Physiol Endocrinol Metab*. 2010:298:E1072-1077.
304. Kato H, Kashiwagi H, Shiraga M et.al. Adiponectin acts as an endogenous antithrombotic factor. *Arterioscler Thromb Vasc Biol*. 2006:26:224-230.
305. Yamauchi T, Kamon J, Ito Y et.al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature*. 2003:423:762-769.
306. Tanabe H, Fujii Y, Okada-Iwabu M et.al. Crystal structures of the human adiponectin receptors. *Nature*. 2015:520:312-316.
307. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev*. 2005:26:439-451.
308. Yamauchi T, Iwabu M, Okada-Iwabu M, Kadowaki T. Adiponectin receptors: A review of their structure, function and how they work. *Best Pract Res Clin Endocrinol Metab*. 2014:28:15-23.
309. Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, Liu F, Dong LQ. Appl1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat Cell Biol*. 2006:8:516-523.
310. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS, Lodish HF. T-cadherin is a receptor for hexameric and high-molecular-weight forms of acrp30/adiponectin. *Proc Natl Acad Sci U S A*. 2004:101:10308-10313.
311. Denzel MS, Scimia MC, Zumstein PM, Walsh K, Ruiz-Lozano P, Ranscht B. T-cadherin is critical for adiponectin-mediated cardioprotection in mice. *J Clin Invest*. 2010:120:4342-4352.
312. Tsuchida A, Yamauchi T, Ito Y et.al. Insulin/foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. *J Biol Chem*. 2004:279:30817-30822.
313. Yamauchi T, Nio Y, Maki T et.al. Targeted disruption of adipor1 and adipor2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med*. 2007:13:332-339.
314. Civitarese AE, Jenkinson CP, Richardson D, Bajaj M, Cusi K, Kashyap S, Berria R, Belfort R, DeFronzo RA, Mandarino LJ, Ravussin E. Adiponectin receptors gene expression and insulin sensitivity in non-diabetic mexican americans with or without a family history of type 2 diabetes. *Diabetologia*. 2004:47:816-820.
315. Debard C, Laville M, Berbe V, Loizon E, Guillet C, Morio-Liondore B, Boirie Y, Vidal H. Expression of key genes of fatty acid oxidation, including adiponectin receptors, in skeletal muscle of type 2 diabetic patients. *Diabetologia*. 2004:47:917-925.
316. Takeuchi S, Wada K, Uozumi Y, Otani N, Osada H, Nagatani K, Mori K. Adiponectin receptor 1 expression is associated with carotid plaque stability. *Neurol India*. 2013:61:249-253.
317. Chinetti G, Zawadski C, Fruchart JC, Staels B. Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors pparalpha, ppargamma, and lxr. *Biochem Biophys Res Commun*. 2004:314:151-158.
318. Pang TT, Narendran P. The distribution of adiponectin receptors on human peripheral blood mononuclear cells. *Ann N Y Acad Sci*. 2008:1150:143-145.

319. Mori T, Koyama Y, Maeda N, Nakamura Y, Fujishima Y, Matsuda K, Funahashi T, Shimada S, Shimomura I. Ultrastructural localization of adiponectin protein in vasculature of normal and atherosclerotic mice. *Sci Rep*. 2014:4:4895.
320. Tian L, Luo N, Zhu X, Chung BH, Garvey WT, Fu Y. Adiponectin-adipor1/2-appl1 signaling axis suppresses human foam cell formation: Differential ability of adipor1 and adipor2 to regulate inflammatory cytokine responses. *Atherosclerosis*. 2012:221:66-75.
321. Lindgren A, Levin M, Rodrigo Blomqvist S, Wikstrom J, Ahnmark A, Mogensen C, Bottcher G, Bohlooly YM, Boren J, Gan LM, Linden D. Adiponectin receptor 2 deficiency results in reduced atherosclerosis in the brachiocephalic artery in apolipoprotein e deficient mice. *PLoS One*. 2013:8:e80330.
322. Schulze MB, Shai I, Rimm EB, Li T, Rifai N, Hu FB. Adiponectin and future coronary heart disease events among men with type 2 diabetes. *Diabetes*. 2005:54:534-539.
323. Yamamoto Y, Hirose H, Saito I, Tomita M, Taniyama M, Matsubara K, Okazaki Y, Ishii T, Nishikai K, Saruta T. Correlation of the adipocyte-derived protein adiponectin with insulin resistance index and serum high-density lipoprotein-cholesterol, independent of body mass index, in the japanese population. *Clin Sci (Lond)*. 2002:103:137-142.
324. Schulze MB, Rimm EB, Shai I, Rifai N, Hu FB. Relationship between adiponectin and glycemic control, blood lipids, and inflammatory markers in men with type 2 diabetes. *Diabetes Care*. 2004:27:1680-1687.
325. Marsche G, Zelzer S, Meinitzer A, Kern S, Meissl S, Pregartner G, Weghuber D, Almer G, Mangge H. Adiponectin predicts hdl cholesterol efflux capacity in adults irrespective of body mass index and fat distribution. *J Clin Endocrinol Metab*. 2017
326. Posadas-Sanchez R, Posadas-Romero C, Mendoza-Perez E, Caracas-Portilla NA, Cardoso-Saldana G, Medina-Urrutia A, Jorge-Galarza E, Juarez-Rojas JG. Cholesterol efflux and metabolic abnormalities associated with low high-density-lipoprotein-cholesterol and high triglycerides in statin-treated coronary men with low-density lipoprotein-cholesterol <70 mg/dl. *Am J Cardiol*. 2012:109:636-641.
327. Xu M, Zhou H, Wang J, Li C, Yu Y. The expression of atp-binding cassette transporter a1 in chinese overweight and obese patients. *Int J Obes (Lond)*. 2009:33:851-856.
328. Chan DC, Barrett PH, Ooi EM, Ji J, Chan DT, Watts GF. Very low density lipoprotein metabolism and plasma adiponectin as predictors of high-density lipoprotein apolipoprotein a-i kinetics in obese and nonobese men. *J Clin Endocrinol Metab*. 2009:94:989-997.
329. Ng TW, Watts GF, Barrett PH, Rye KA, Chan DC. Effect of weight loss on ldl and hdl kinetics in the metabolic syndrome: Associations with changes in plasma retinol-binding protein-4 and adiponectin levels. *Diabetes Care*. 2007:30:2945-2950.
330. Verges B, Petit JM, Duvillard L, Dautin G, Florentin E, Galland F, Gambert P. Adiponectin is an important determinant of apoa-i catabolism. *Arterioscler Thromb Vasc Biol*. 2006:26:1364-1369.
331. Kitajima K, Miura S, Yamauchi T, Uehara Y, Kiya Y, Rye KA, Kadowaki T, Saku K. Possibility of increasing cholesterol efflux by adiponectin and its receptors through the atp binding cassette transporter a1 in hek293t cells. *Biochem Biophys Res Commun*. 2011:411:305-311.
332. Liang B, Wang X, Guo X, Yang Z, Bai R, Liu M, Xiao C, Bian Y. Adiponectin upregulates abca1 expression through liver x receptor alpha signaling pathway in raw 264.7 macrophages. *Int J Clin Exp Pathol*. 2015:8:450-457.
333. Li Y, Qin G, Liu J, Mao L, Zhang Z, Shang J. Adipose tissue regulates hepatic cholesterol metabolism via adiponectin. *Life Sci*. 2014:118:27-33.

334. Li J, Zhang S. Microrna-150 inhibits the formation of macrophage foam cells through targeting adiponectin receptor 2. *Biochem Biophys Res Commun*. 2016:476:218-224.
335. Luo N, Wang X, Chung BH, Lee MH, Klein RL, Garvey WT, Fu Y. Effects of macrophage-specific adiponectin expression on lipid metabolism in vivo. *Am J Physiol Endocrinol Metab*. 2011:301:E180-186.
336. Matsuura F, Oku H, Koseki M et.al. Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. *Biochem Biophys Res Commun*. 2007:358:1091-1095.
337. Neumeier M, Sigruener A, Eggenhofer E, Weigert J, Weiss TS, Schaeffler A, Schlitt HJ, Aslanidis C, Piso P, Langmann T, Schmitz G, Scholmerich J, Buechler C. High molecular weight adiponectin reduces apolipoprotein b and e release in human hepatocytes. *Biochem Biophys Res Commun*. 2007:352:543-548.
338. Oku H, Matsuura F, Koseki M et.al. Adiponectin deficiency suppresses abca1 expression and apoa-i synthesis in the liver. *FEBS Lett*. 2007:581:5029-5033.
339. Tian L, Luo N, Klein RL, Chung BH, Garvey WT, Fu Y. Adiponectin reduces lipid accumulation in macrophage foam cells. *Atherosclerosis*. 2009:202:152-161.
340. Tsubakio-Yamamoto K, Matsuura F, Koseki M et.al. Adiponectin prevents atherosclerosis by increasing cholesterol efflux from macrophages. *Biochem Biophys Res Commun*. 2008:375:390-394.
341. Wang Y, Wang X, Guo Y, Bian Y, Bai R, Liang B, Xiao C. Effect of adiponectin on macrophage reverse cholesterol transport in adiponectin-/- mice and its mechanism. *Exp Ther Med*. 2017:13:2757-2762.
342. Nolan JJ, Ludvik B, Beerdsen P, Joyce M, Olefsky J. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med*. 1994:331:1188-1193.
343. Saltiel AR, Olefsky JM. Thiazolidinediones in the treatment of insulin resistance and type ii diabetes. *Diabetes*. 1996:45:1661-1669.
344. Bowen L, Stein PP, Stevenson R, Shulman GI. The effect of cp 68,722, a thiozolidinedione derivative, on insulin sensitivity in lean and obese zucker rats. *Metabolism*. 1991:40:1025-1030.
345. Yu JG, Javorschi S, Hevener AL, Kruszynska YT, Norman RA, Sinha M, Olefsky JM. The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes*. 2002:51:2968-2974.
346. Kubota N, Terauchi Y, Kubota T et.al. Pioglitazone ameliorates insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. *J Biol Chem*. 2006:281:8748-8755.
347. Riera-Guardia N, Rothenbacher D. The effect of thiazolidinediones on adiponectin serum level: A meta-analysis. *Diabetes Obes Metab*. 2008:10:367-375.
348. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE. Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem*. 2004:279:12152-12162.
349. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y. Ppargamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes*. 2001:50:2094-2099.

350. Combs TP, Wagner JA, Berger J et.al. Induction of adipocyte complement-related protein of 30 kilodaltons by ppargamma agonists: A potential mechanism of insulin sensitization. *Endocrinology*. 2002:143:998-1007.
351. Ogasawara D, Shite J, Shinke T, Watanabe S, Otake H, Tanino Y, Sawada T, Kawamori H, Kato H, Miyoshi N, Hirata K. Pioglitazone reduces the necrotic-core component in coronary plaque in association with enhanced plasma adiponectin in patients with type 2 diabetes mellitus. *Circ J*. 2009:73:343-351.
352. Furuhashi M, Ura N, Higashiura K, Murakami H, Tanaka M, Moniwa N, Yoshida D, Shimamoto K. Blockade of the renin-angiotensin system increases adiponectin concentrations in patients with essential hypertension. *Hypertension*. 2003:42:76-81.
353. Celik T, Iyisoy A, Kursaklioglu H, Kardesoglu E, Kilic S, Turhan H, Yilmaz MI, Ozcan O, Yaman H, Isik E, Fici F. Comparative effects of nebivolol and metoprolol on oxidative stress, insulin resistance, plasma adiponectin and soluble p-selectin levels in hypertensive patients. *J Hypertens*. 2006:24:591-596.
354. Koh KK, Han SH, Quon MJ, Yeal Ahn J, Shin EK. Beneficial effects of fenofibrate to improve endothelial dysfunction and raise adiponectin levels in patients with primary hypertriglyceridemia. *Diabetes Care*. 2005:28:1419-1424.
355. Chrusciel P, Sahebkar A, Rembek-Wieliczko M et.al. Impact of statin therapy on plasma adiponectin concentrations: A systematic review and meta-analysis of 43 randomized controlled trial arms. *Atherosclerosis*. 2016:253:194-208.
356. Van Lenten BJ, Wagner AC, Anantharamaiah GM, Navab M, Reddy ST, Buga GM, Fogelman AM. Apolipoprotein a-i mimetic peptides. *Curr Atheroscler Rep*. 2009:11:52-57.
357. Tsuchida A, Yamauchi T, Takekawa S, Hada Y, Ito Y, Maki T, Kadowaki T. Peroxisome proliferator-activated receptor (ppar)alpha activation increases adiponectin receptors and reduces obesity-related inflammation in adipose tissue: Comparison of activation of pparalpha, ppargamma, and their combination. *Diabetes*. 2005:54:3358-3370.
358. Okada-Iwabu M, Yamauchi T, Iwabu M et.al. A small-molecule adipor agonist for type 2 diabetes and short life in obesity. *Nature*. 2013:503:493-499.
359. Zhang Y, Zhao J, Li R, Lau WB, Yuan YX, Liang B, Li R, Gao EH, Koch WJ, Ma XL, Wang YJ. Adiporon, the first orally active adiponectin receptor activator, attenuates postischemic myocardial apoptosis through both ampk-mediated and ampk-independent signalings. *Am J Physiol Endocrinol Metab*. 2015:309:E275-282.
360. Fairaq A, Shawky NM, Osman I, Pichavaram P, Segar L. Adiporon, an adiponectin receptor agonist, attenuates pdgf-induced vsmc proliferation through inhibition of mtor signaling independent of ampk: Implications toward suppression of neointimal hyperplasia. *Pharmacol Res*. 2017:119:289-302.
361. Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, Clegg LX. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: The atherosclerosis risk in communities (aric) study, 1987-1993. *Am J Epidemiol*. 1997:146:483-494.
362. Gardin JM, Bartz TM, Polak JF, O'Leary DH, Wong ND. What do carotid intima-media thickness and plaque add to the prediction of stroke and cardiovascular disease risk in older adults? The cardiovascular health study. *J Am Soc Echocardiogr*. 2014:27:998-1005.e1002.
363. Prati P, Tosetto A, Vanuzzo D, Bader G, Casaroli M, Canciani L, Castellani S, Touboul PJ. Carotid intima media thickness and plaques can predict the occurrence of ischemic cerebrovascular events. *Stroke*. 2008:39:2470-2476.

364. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: A systematic review and meta-analysis. *Circulation*. 2007:115:459-467.
365. Ohashi K, Ouchi N, Matsuzawa Y. Anti-inflammatory and anti-atherogenic properties of adiponectin. *Biochimie*. 2012:94:2137-2142.
366. Ebrahimi-Mamaeghani M, Mohammadi S, Arefhosseini SR, Fallah P, Bazi Z. Adiponectin as a potential biomarker of vascular disease. *Vasc Health Risk Manag*. 2015:11:55-70.
367. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y. Association of hypoadiponectinemia with coronary artery disease in men. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2003:23:85-89.
368. Liu J, Wang F, Cha Y, Chen ZP, Ding HY. Adiponectin levels in non-obese first-degree relatives of type 2 diabetes patients and non-diabetic subjects: A 5-year follow-up study. *Journal of International Medical Research*. 2010:38:792-802.
369. Hedges LV, Olkin I. Vote-counting methods in research synthesis. *Psychological bulletin*. 1980:88:359.
370. Bushman BJ, Wang MC. Vote-counting procedures in meta-analysis. *The handbook of research synthesis*. 1994:236:193-213.
371. Rothman K, Greenland S. *Modern epidemiology* 1998.
372. Wells G, Shea B, O'Connell D. The newcastle-ottawa scale (nos) for assessing the quality of nonradomized studies in meta-analyses. 2011

373. Ahn M-S, Koh S-B, Kim J-Y, Yoon JH, Sung JK, Youn YJ, Yoo B-S, Lee S-H, Yoon J, Eom A, Park J-K, Choe K-H. The association between serum adiponectin and carotid intima media thickness in community based cohort in korea: The arirang study. *Molecular & Cellular Toxicology*. 2011:7:33-38.
374. Iglseder B, Mackevics V, Stadlmayer A, Tasch G, Ladurner G, Paulweber B. Plasma adiponectin levels and sonographic phenotypes of subclinical carotid artery atherosclerosis: Data from the saphir study. *Stroke*. 2005:36:2577-2582.
375. Jansson PA, Pellme F, Hammarstedt A, Sandqvist M, Brekke H, Caidahl K, Forsberg M, Volkmann R, Carvalho E, Funahashi T, Matsuzawa Y, Wiklund O, Yang X, Taskinen MR, Smith U. A novel cellular marker of insulin resistance and early atherosclerosis in humans is related to impaired fat cell differentiation and low adiponectin. *FASEB Journal*. 2003:17:1434-1440.
376. Lim S, Jin Choi H, Shin H, Khang AR, Kang SM, Yoon JW, Choi SH, Jeong IK, Cho SI, Park KS, Jang HC. Subclinical atherosclerosis in a community-based elderly cohort: The korean longitudinal study on health and aging. *International Journal of Cardiology*. 2012:155:126-133.
377. Lo J, Dolan SE, Kanter JR, Hemphill LC, Connelly JM, Lees RS, Grinspoon SK. Effects of obesity, body composition, and adiponectin on carotid intima-media thickness in healthy women. *Journal of Clinical Endocrinology & Metabolism*. 2006:91:1677-1682.
378. Saarikoski LA, Huupponen RK, Viikari JS, Marniemi J, Juonala M, Kahonen M, Raitakari OT. Adiponectin is related with carotid artery intima-media thickness and brachial flow-mediated dilatation in young adults--the cardiovascular risk in young finns study. *Annals of Medicine*. 2010:42:603-611.
379. Yoon JH, Park JK, Oh SS, Lee KH, Kim SK, Kim JK, Kang HT, Youn YJ, Lee JW, Lee SH, Eom AY, Chung CH, Kim JY, Koh SB. The clustering patterns of metabolic risk

factors and its association with sub-clinical atherosclerosis in korean population. *Annals of Human Biology*. 2011:38:640-646.
380. Nishida M, Moriyama T, Ishii K, Takashima S, Yoshizaki K, Sugita Y, Yamauchi-Takihara K. Effects of il-6, adiponectin, crp and metabolic syndrome on subclinical atherosclerosis. *Clinica Chimica Acta*. 2007:384:99-104.
381. Yoon JH, Kim SK, Choi HJ, Choi SI, Cha SY, Koh SB, Kang HT, Ahn SV. Adiponectin provides additional information to conventional cardiovascular risk factors for assessing the risk of atherosclerosis in both genders. *PLoS One*. 2013:8:e75535.
382. Flyvbjerg A, Astiarraga BD, Glintborg D et.al. The effect of menopause on carotid artery remodeling, insulin sensitivity, and plasma adiponectin in healthy women. *American Journal of Hypertension*. 2009:22:364-370.
383. Patel S, Flyvbjerg A, Kozakova M, Frystyk J, Ibrahim IM, Petrie JR, Avery PJ, Ferrannini E, Walker M, Investigators R. Variation in the adipoq gene promoter is associated with carotid intima media thickness independent of plasma adiponectin levels in healthy subjects. *European Heart Journal*. 2008:29:386-393.
384. Stork S, Bots ML, Angerer P, von Schacky C, Grobbee DE, Angermann CE, Seufert J. Low levels of adiponectin predict worsening of arterial morphology and function. *Atherosclerosis*. 2007:194:e147-153.
385. Miyanaga K, Fukuo K, Akasaka H, Katsuya T, Fukada R, Rakugi H, Kazumi T. C allele of angiotensin ii type 1 receptor gene a1166c polymorphism affects plasma adiponectin concentrations in healthy young japanese women. *Hypertension Research - Clinical & Experimental*. 2009:32:901-905.
386. Gustafsson S, Lind L, Soderberg S, Ingelsson E. Associations of circulating adiponectin with measures of vascular function and morphology. *Journal of Clinical Endocrinology & Metabolism*. 2010:95:2927-2934.
387. Kotani K, Shimohiro H, Sakane N. The relationship between leptin:Adiponectin ratio and carotid intima-media thickness in asymptomatic females. *Stroke*. 2008:39:e32-33; author reply e34.
388. Bevan S, Meidtner K, Lorenz M, Sitzer M, Grant PJ, Markus HS. Adiponectin level as a consequence of genetic variation, but not leptin level or leptin: Adiponectin ratio, is a risk factor for carotid intima-media thickness. *Stroke*. 2011:42:1510-1514.
389. Chaer RA, Kip K, Mulukutla S, Aryan A, Reis S. Association of inflammatory and metabolic serum biomarkers with carotid intima media thickness (cimt) in a community-based population: The heart strategies concentrating on risk evaluation (heart score) study. *Journal of Vascular Surgery*. 2012:56 (3):890-891.
390. Liao H, Li Z, Zheng D, Liu J, Liu Y, Xiao C, Wang H. Increased hs-crp/adiponectin ratio is associated with increase carotid intima-media thickness. *Lipids Health Dis*. 2014:13:120.
391. Hui E, Xu A, Chow WS, Lee PCH, Fong CHY, Cheung SCW, Tse HF, Chau MT, Cheung BMY, Lam KSL. Hypoadiponectinemia as an independent predictor for the progression of carotid atherosclerosis: A 5-year prospective study. *Metabolic Syndrome and Related Disorders*. 2014:12:517-522.
392. Hanada H, Masuda D, Fushimi E, Takehashi M, Mugii S, Okubo M, Maeda I, Suehisa E, Hidaka Y, Yasushi S, Yamashita S. Serum adiponectin levels correlated with serum apolipoprotein b-48 and remnant lipoprotein-cholesterol concentrations and carotid intima-media thickness. *Atherosclerosis*. 2014:235 (2):e170.

393. Rundek T, Blanton SH, Bartels S, Dong C, Raval A, Demmer RT, Cabral D, Elkind MS, Sacco RL, Desvarieux M. Traditional risk factors are not major contributors to the variance in carotid intima-media thickness. *Stroke*. 2013:44:2101-2108.
394. Persson J, Strawbridge RJ, McLeod O et.al. Sex-specific effects of adiponectin on carotid intima-media thickness and incident cardiovascular disease. *J Am Heart Assoc*. 2015:4:e001853.
395. Tahara N, Yamagishi SI, Tahara A, Ishibashi M, Hayabuchi N, Takeuchi M, Imaizumi T. Adiponectin is inversely associated with ratio of serum levels of ages to srage and vascular inflammation. *International Journal of Cardiology*. 2012:158:461-462.
396. Shoji T, Koyama H, Fukumoto S, Maeno T, Yokoyama H, Shinohara K, Emoto M, Shoji T, Yamane T, Hino M, Shioi A, Nishizawa Y. Platelet activation is associated with hypoadiponectinemia and carotid atherosclerosis. *Atherosclerosis*. 2006:188:190-195.
397. Nilsson PM, Engstrom G, Hedblad B, Frystyk J, Persson MM, Berglund G, Flyvbjerg A. Plasma adiponectin levels in relation to carotid intima media thickness and markers of insulin resistance. *Arteriosclerosis, Thrombosis & Vascular Biology*. 2006:26:2758-2762.
398. Hajmohammadi T, Sadeghi M, Dashti M, Hashemi M, Saadatnia M, Soghrati M, Talaei M, Sarrafzadegan N. Relationship between carotid intima-media thickness with some inflammatory biomarkers, ghrelin and adiponectin in iranians with and without metabolic syndrome in isfahan cohort study. *Arya Atherosclerosis*. 2010:6:56-61.
399. Vasilescu R, Ifrim S, Ionescu-Tirgoviste C. Leptin to adiponectin ratio is an independent predictor of common carotid artery intima-media thickness in obese subjects. *Proceedings of the Romanian Academy Series B-Chemistry Life Sciences & Geosciences*. 2010:12:209-217.
400. Sturm W, Sandhofer A, Engl J, Laimer M, Molnar C, Kaser S, Weiss H, Tilg H, Ebenbichler CF, Patsch JR. Influence of visceral obesity and liver fat on vascular structure and function in obese subjects. *Obesity*. 2009:17:1783-1788.
401. Nagasaki T, Inaba M, Hiura Y, Tahara H, Kumeda Y, Shirakawa K, Onoda N, Ishikawa T, Ishimura E, Nishizawa Y. Plasma levels of adiponectin and soluble thrombomodulin in hypothyroid patients with normal thyroid function following levothyroxine replacement therapy. *Biomedicine & Pharmacotherapy*. 2005:59:571-577.
402. Rubio-Guerra AF, Cabrera-Miranda LJ, Vargas-Robles H, Maceda-Serrano A, Lozano-Nuevo JJ, Escalante-Acosta BA. Correlation between levels of circulating adipokines and adiponectin/resistin index with carotid intima-media thickness in hypertensive type 2 diabetic patients. *Cardiology*. 2013:125:150-153.
403. Yazici D, Yavuz D, Ogunc AV, Sirikci O, Toprak A, Deyneli O, Akalin S. Serum adipokine levels in type 1 diabetic patients: Association with carotid intima media thickness. *Metabolic Syndrome & Related Disorders*. 2012:10:26-31.
404. Juonala M, Saarikoski LA, Viikari JS, Oikonen M, Lehtimaki T, Lyytikainen LP, Huupponen R, Magnussen CG, Koskinen J, Laitinen T, Taittonen L, Kahonen M, Kivimaki M, Raitakari OT. A longitudinal analysis on associations of adiponectin levels with metabolic syndrome and carotid artery intima-media thickness. The cardiovascular risk in young finns study. *Atherosclerosis*. 2011:217:234-239.
405. Dullaart RP, Kappelle PJ, Dallinga-Thie GM. Carotid intima media thickness is associated with plasma adiponectin but not with the leptin:Adiponectin ratio independently of metabolic syndrome. *Atherosclerosis*. 2010:211:393-396.
406. Shargorodsky M, Boaz M, Goldberg Y, Matas Z, Gavish D, Fux A, Wolfson N. Adiponectin and vascular properties in obese patients: Is it a novel biomarker of early atherosclerosis? *International Journal of Obesity*. 2009:33:553-558.

407. Dullaart RP, de Vries R, van Tol A, Sluiter WJ. Lower plasma adiponectin is a marker of increased intima-media thickness associated with type 2 diabetes mellitus and with male gender. *European Journal of Endocrinology*. 2007:156:387-394.
408. Gokulakrishnan K, Indulekha K, Ganesan S, Anuradha S, Mohan V. Adiponectin and carotid intimal medial thickness in subjects with and without glucose intolerance (cures-82). *Diabetes Technology & Therapeutics*. 2010:12:109-115.
409. Behre CJ, Brohall G, Hulthe J, Wikstrand J, Fagerberg B. Are serum adiponectin concentrations in a population sample of 64-year-old caucasian women with varying glucose tolerance associated with ultrasound-assessed atherosclerosis? *Journal of Internal Medicine*. 2006:260:238-244.
410. Forst T, Hohberg C, Fuellert SD, Lübben G, Konrad T, Löbig M, Weber MM, Sachara C, Gottschall V, Pfützner A. Pharmacological pparγ stimulation in contrast to beta cell stimulation results in an improvement in adiponectin and proinsulin intact levels and reduces intima media thickness in patients with type 2 diabetes. *Hormone and Metabolic Research*. 2005:37:521-527.
411. Vasilescu R, Ifrim S, Ionescu-Tirgoviste C. Relationship between plasma adipokines, inflammation, insulin resistance and subclinical atherosclerosis in newly diagnosed type 2 diabetes. *Journal of Diabetes Mellitus*. 2011:1:17-25.
412. Mansouri M, Heshmat R, Tabatabaei-Malazy O, Sharifi F, Badamchizadeh Z, Alatab S, Omidfar K, Fakhrzadeh H, Larijani B. The association of carotid intima media thickness with retinol binding protein-4 and total and high molecular weight adiponectin in type 2 diabetic patients. *Journal of Diabetes & Matabolic Disorders*. 2012:11:2.
413. Matsuda M, Kawasaki F, Yamada K, Kanda Y, Saito M, Eto M, Matsuki M, Kaku K. Impact of adiposity and plasma adipocytokines on diabetic angiopathies in japanese type 2 diabetic subjects. *Diabetic Medicine*. 2004:21:881-888.
414. Tavridou A, Georgoulidou A, Roumeliotis A, Roumeliotis S, Giannakopoulou E, Papanas N, Passadakis P, Manolopoulos VG, Vargemezis V. Association of plasma adiponectin and oxidized low-density lipoprotein with carotid intima-media thickness in diabetic nephropathy. *J Diabetes Res*. 2015:2015:507265.
415. Yano Y, Toshinai K, Inokuchi T, Kangawa K, Shimada K, Kario K, Nakazato M. Plasma des-acyl ghrelin, but not plasma hmw adiponectin, is a useful cardiometabolic marker for predicting atherosclerosis in elderly hypertensive patients. *Atherosclerosis*. 2009:204:590-594.
416. Dessein PH, Norton GR, Badenhorst M, Woodiwiss AJ, Solomon A. Rheumatoid arthritis impacts on the independent relationships between circulating adiponectin concentrations and cardiovascular metabolic risk. *Mediators of Inflammation*. 2013:2013:461849.
417. Gonzalez-Gay MA, Gonzalez-Juanatey C, Rodriguez-Rodriguez L, Miranda-Filloy JA, Martin J, Llorca J. Lack of association between adipokines and ghrelin and carotid intima-media thickness in patients with severe rheumatoid arthritis. *Clinical and Experimental Rheumatology*. 2011:29:358-359.
418. Dessein PH, Tsang L, Solomon A, Woodiwiss AJ, Millen AM, Norton GR. Adiponectin and atherosclerosis in rheumatoid arthritis. *Mediators Inflamm*. 2014:2014:358949.
419. Pemberton PW, Ahmad Y, Bodill H, Lokko D, Hider SL, Yates AP, Walker MG, Laing I, Bruce IN. Biomarkers of oxidant stress, insulin sensitivity and endothelial activation in rheumatoid arthritis: A cross-sectional study of their association with accelerated atherosclerosis. *BMC Research Notes*. 2009:2:83.

420. Karakitsos D, De Groot E, Patrianakos AP, Parthenakis F, Boletis J, Karabinis A, Kyriazis J, Vardas P, Daphnis E. Adiponectin and cardiovascular remodeling in end-stage renal disease and co-morbid diabetes mellitus. *American Journal of Nephrology*. 2006:26:340-347.
421. Kiris I, Tekin I, Yesildag A, Vural H, Oyar O, Sirin B, Okutan H, Ibrisim E. Inverse relationship between adiponectin levels and subclinical carotid atherosclerosis in patients undergoing coronary artery bypass grafting. *International Heart Journal*. 2006:47:855-866.
422. Wang AY, Ho SS, Liu EK, Chan IH, Ho S, Sanderson JE, Lam CW. Differential associations of traditional and non-traditional risk factors with carotid intima-media thickening and plaque in peritoneal dialysis patients. *American Journal of Nephrology*. 2007:27:458-465.
423. Hayashi M, Shibata R, Takahashi H, Ishii H, Aoyama T, Kasuga H, Yamada S, Ohashi K, Maruyama S, Matsuo S, Ouchi N, Murohara T, Toriyama T. Association of adiponectin with carotid arteriosclerosis in predialysis chronic kidney disease. *American Journal of Nephrology*. 2011:34:249-255.
424. Ignacy W, Chudek J, Adamczak M, Funahashi T, Matsuzawa Y, Kokot F, Wiecek A. Reciprocal association of plasma adiponectin and serum c-reactive protein concentration in haemodialysis patients with end-stage kidney disease--a follow-up study. *Nephron*. 2005:101:c18-24.
425. Aggarwal A, Jain R, Dwivedi S. Adiponectin, central obesity, carotid intima media thickness and coronary artery disease. *Indian Heart Journal*. 2013:65:S6.
426. Adamczak M, Chudek J, Wiecek A. Adiponectin in patients with chronic kidney disease. *Semin Dial*. 2009:22:391-395.
427. Hattori Y, Suzuki M, Hattori S, Kasai K. Globular adiponectin upregulates nitric oxide production in vascular endothelial cells. *Diabetologia*. 2003:46:1543-1549.
428. Kubota N, Terauchi Y, Yamauchi T et.al. Disruption of adiponectin causes insulin resistance and neointimal formation. *Journal of Biological Chemistry*. 2002:277:25863-25866.
429. Ruige JB, Assendelft WJ, Dekker JM, Kostense PJ, Heine RJ, Bouter LM. Insulin and risk of cardiovascular disease: A meta-analysis. *Circulation*. 1998:97:996-1001.
430. Brohall G, Oden A, Fagerberg B. Carotid artery intima-media thickness in patients with type 2 diabetes mellitus and impaired glucose tolerance: A systematic review. *Diabet Med*. 2006:23:609-616.
431. Yadav A, Kataria MA, Saini V, Yadav A. Role of leptin and adiponectin in insulin resistance. *Clinica Chimica Acta*. 2013:417:80-84.
432. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest*. 2006:116:1784-1792.
433. Martin-Timon I, Sevillano-Collantes C, Segura-Galindo A, Del Canizo-Gomez FJ. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World J Diabetes*. 2014:5:444-470.
434. Lonn EM, Gerstein HC, Sheridan P, Smith S, Diaz R, Mohan V, Bosch J, Yusuf S, Dagenais GR. Effect of ramipril and of rosiglitazone on carotid intima-media thickness in people with impaired glucose tolerance or impaired fasting glucose: Starr (study of atherosclerosis with ramipril and rosiglitazone). *J Am Coll Cardiol*. 2009:53:2028-2035.
435. Mazzone T, Meyer PM, Feinstein SB, Davidson MH, Kondos GT, D'Agostino RB, Sr., Perez A, Provost JC, Haffner SM. Effect of pioglitazone compared with glimepiride on

carotid intima-media thickness in type 2 diabetes: A randomized trial. *Jama*. 2006:296:2572-2581.
436. Juonala M, Kahonen M, Laitinen T, Hutri-Kahonen N, Jokinen E, Taittonen L, Pietikainen M, Helenius H, Viikari JS, Raitakari OT. Effect of age and sex on carotid intima-media thickness, elasticity and brachial endothelial function in healthy adults: The cardiovascular risk in young finns study. *Eur Heart J*. 2008:29:1198-1206.
437. Obata Y, Yamada Y, Takahi Y, Baden MY, Saisho K, Tamba S, Yamamoto K, Umeda M, Furubayashi A, Matsuzawa Y. Relationship between serum adiponectin levels and age in healthy subjects and patients with type 2 diabetes. *Clin Endocrinol (Oxf)*. 2013:79:204-210.
438. Bots M, Grobbee DE. Intima media thickness as a surrogate marker for generalised atherosclerosis. *Cardiovascular Drugs and Therapy*. 2002:16:341-351.
439. Lee RT, Libby P. The unstable atheroma. *Arterioscler Thromb Vasc Biol*. 1997:17:1859-1867.
440. Canada PHAo. Tracking heart disease and stroke in canada. 2009
441. Ebinuma H, Miyazaki O, Yago H, Hara K, Yamauchi T, Kadowaki T. A novel elisa system for selective measurement of human adiponectin multimers by using proteases. *Clin Chim Acta*. 2006:372:47-53.
442. Aso Y, Yamamoto R, Wakabayashi S, Uchida T, Takayanagi K, Takebayashi K, Okuno T, Inoue T, Node K, Tobe T, Inukai T, Nakano Y. Comparison of serum high-molecular weight (hmw) adiponectin with total adiponectin concentrations in type 2 diabetic patients with coronary artery disease using a novel enzyme-linked immunosorbent assay to detect hmw adiponectin. *Diabetes*. 2006:55:1954-1960.
443. Inoue T, Kotooka N, Morooka T, Komoda H, Uchida T, Aso Y, Inukai T, Okuno T, Node K. High molecular weight adiponectin as a predictor of long-term clinical outcome in patients with coronary artery disease. *Am J Cardiol*. 2007:100:569-574.
444. Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, Imai Y, Nagai R, Kadowaki T. Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. *Diabetes Care*. 2006:29:1357-1362.
445. Touboul PJ, Hennerici MG, Meairs S et.al. Mannheim carotid intima-media thickness and plaque consensus (2004-2006-2011). An update on behalf of the advisory board of the 3rd, 4th and 5th watching the risk symposia, at the 13th, 15th and 20th european stroke conferences, mannheim, germany, 2004, brussels, belgium, 2006, and hamburg, germany, 2011. *Cerebrovasc Dis*. 2012:34:290-296.
446. Gronwall C, Reynolds H, Kim JK, Buyon J, Goldberg JD, Clancy RM, Silverman GJ. Relation of carotid plaque with natural igm antibodies in patients with systemic lupus erythematosus. *Clin Immunol*. 2014:153:1-7.
447. Komatsu J, Fujiwara M, Matsuura M, Ito S, Oritsu M. Study on the usefulness of high-molecular-weight (hmw)-adiponectin level check of japanese general population upon health check: Comparison of carotid ultrasonography measurement. *Clinical Biochemistry*. 2012:45:72-76.
448. Kozakova M, Palombo C, Eng MP, Dekker J, Flyvbjerg A, Mitrakou A, Gastaldelli A, Ferrannini E, Investigators R. Fatty liver index, gamma-glutamyltransferase, and early carotid plaques. *Hepatology*. 2012:55:1406-1415.
449. McMahon M, Skaggs BJ, Sahakian L, Grossman J, FitzGerald J, Ragavendra N, Charles-Schoeman C, Chernishof M, Gorn A, Witztum JL, Wong WK, Weisman M, Wallace DJ, La Cava A, Hahn BH. High plasma leptin levels confer increased risk of atherosclerosis

in women with systemic lupus erythematosus, and are associated with inflammatory oxidised lipids. *Annals of the Rheumatic Diseases*. 2011:70:1619-1624.
450. McMahon M, Skaggs BJ, Grossman JM et.al. A panel of biomarkers is associated with increased risk of the presence and progression of atherosclerosis in women with systemic lupus erythematosus. *Arthritis and Rheumatology*. 2014:66:130-139.
451. Pellitero S, Reverter JL, Granada ML, Pizarro E, Pastor MC, Tassies D, Reverter JC, Salinas I, Sanmarti A. Association of the igf1/pregnancy-associated plasma protein-a system and adipocytokine levels with the presence and the morphology of carotid plaques in type 2 diabetes mellitus patients with stable glycaemic control. *European Journal of Endocrinology*. 2009:160:925-932.
452. Yu ZZ, Ni ZH, Gu LY, Lin AW, Fang W, Yao Q, Lindholm B, Qian JQ. Adiponectin is related to carotid artery plaque and a predictor of cardiovascular outcome in a cohort of non-diabetic peritoneal dialysis patients. *Blood Purification*. 2008:26:386-393.
453. Arregui M, Buijsse B, Fritsche A, di Giuseppe R, Schulze MB, Westphal S, Isermann B, Boeing H, Weikert C. Adiponectin and risk of stroke: Prospective study and meta-analysis. *Stroke*. 2014:45:10-17.
454. Baranowska B, Kochanowski J, Grudniak M, Baranowska-Bik A, Wolinska-Witort E, Martynska L, Bik W. Decreased total serum adiponectin and its isoforms in women with acute ischemic stroke. *Neuroendocrinology Letters*. 2011:32:711-715.
455. Bidulescu A, Liu J, Chen Z, Hickson DA, Musani SK, Samdarshi TE, Fox ER, Taylor HA, Gibbons GH. Associations of adiponectin and leptin with incident coronary heart disease and ischemic stroke in african americans: The jackson heart study. *Frontiers in Public Health*. 2013:1
456. Bienek R, Marek B, Kajdaniuk D, Borgiel-Marek H, Piecha T, Nowak M, Sieminska L, Glogowska-Szelag J, Foltyn W, Kos-Kudla B. Adiponectin, leptin, resistin and insulin blood concentrations in patients with ischaemic cerebral stroke. *Endokrynologia Polska*. 2012:63:338-345.
457. Chen MP, Tsai JC, Chung FM, Yang SS, Hsing LL, Shin SJ, Lee YJ. Hypoadiponectinemia is associated with ischemic cerebrovascular disease. *Arteriosclerosis, Thrombosis & Vascular Biology*. 2005:25:821-826.
458. Delva M, Lytvynenko N. Adipokines level and insulin resistance in abdominally obese patients with acute ischemic non-lacunar hemispheric stroke. *Milli Nevrologiya Jurnali*. 2013:70-75.
459. Jaleel A, Aqil S, Jaleel S, Jaleel F. Adipocytokines in subjects with and without ischemic cerebrovascular disease. *Acta Neurologica Belgica*. 2010:110:234-238.
460. Kantorova E, Chomova M, Kurca E, Sivak S, Zelenak K, Kucera P, Galajda P. Leptin, adiponectin and ghrelin, new potential mediators of ischemic stroke. *Neuroendocrinology Letters*. 2011:32:716-721.
461. Kim BJ, Lee SH, Ryu WS, Kim CK, Yoon BW. Adipocytokines and ischemic stroke: Differential associations between stroke subtypes. *Journal of the Neurological Sciences*. 2012:312:117-122.
462. Kizer JR, Benkeser D, Arnold AM, Djousse L, Zieman SJ, Mukamal KJ, Tracy RP, Mantzoros CS, Siscovick DS, Gottdiener JS, Ix JH. Total and high-molecular-weight adiponectin and risk of coronary heart disease and ischemic stroke in older adults. *Journal of Clinical Endocrinology & Metabolism*. 2013:98:255-263.
463. Kuwashiro T, Ago T, Kamouchi M et.al. Significance of plasma adiponectin for diagnosis, neurological severity and functional outcome in ischemic stroke - research for biomarkers in ischemic stroke (rebios). *Metabolism*. 2014:63:1093-1103.

464. Marousi S, Theodorou G, Karakantza M, Papathanasopoulos P, Ellul J. Serum adiponectin acutely after an ischemic stroke: Implications for a long-lasting, suppressed anti-inflammatory role. *Acta Neurologica Scandinavica*. 2010:121:277-284.
465. Matsumoto M, Ishikawa S, Kajii E. Association of adiponectin with cerebrovascular disease: A nested case-control study. *Stroke*. 2008:39:323-328.
466. Montecucco F, Lenglet S, Gayet-Ageron A, Bertolotto M, Pelli G, Palombo D, Pane B, Spinella G, Steffens S, Raffaghello L, Pistoia V, Ottonello L, Pende A, Dallegri F, Mac HF. Systemic and intraplaque mediators of inflammation are increased in patients symptomatic for ischemic stroke. *Stroke*. 2010:41:1394-1404.
467. Musialek P, Tekieli L, Pieniazek P, Kablak-Ziembicka A, Undas A, Stepien E, Stepniewski J, Przewlocki T, Tracz W, Podolec P. Risk factors and risk markers in symptomatic and asymptomatic patients with atherosclerotic carotid artery stenosis. *European Heart Journal*. 2011:32:563.
468. Ogorodnikova AD, Wassertheil-Smoller S, Mancuso P, Sowers MR, Rajpathak SN, Allison MA, Baird AE, Rodriguez B, Wildman RP. High-molecular-weight adiponectin and incident ischemic stroke in postmenopausal women: A women's health initiative study. *Stroke*. 2010:41:1376-1381.
469. Ozturk ZG, Ekmekci H, Ekmekci OB, Atukeren P, Butun I, Gode S, Besirli K, Kokoglu E, Sonmez H. Nontraditional risk factors in carotid artery disease. *Clinical & Applied Thrombosis/Hemostasis*. 2010:16:554-558.
470. Prugger C, Luc G, Haas B, Morange PE, Ferrieres J, Amouyel P, Kee F, Ducimetiere P, Empana JP, Group PS. Multiple biomarkers for the prediction of ischemic stroke: The prime study. *Arteriosclerosis, Thrombosis & Vascular Biology*. 2013:33:659-666.
471. Rajpathak SN, Kaplan RC, Wassertheil-Smoller S, Cushman M, Rohan TE, McGinn AP, Wang T, Strickler HD, Scherer PE, Mackey R, Curb D, Ho GY. Resistin, but not adiponectin and leptin, is associated with the risk of ischemic stroke among postmenopausal women: Results from the women's health initiative. *Stroke*. 2011:42:1813-1820.
472. Soderberg S, Stegmayr B, Stenlund H, Sjostrom LG, Agren A, Johansson L, Weinehall L, Olsson T. Leptin, but not adiponectin, predicts stroke in males. *J Intern Med*. 2004:256:128-136.
473. Stott DJ, Welsh P, Rumley A, Robertson M, Ford I, Sattar N, Westendorp RG, Jukema JW, Cobbe SM, Lowe GD. Adipocytokines and risk of stroke in older people: A nested case-control study. *International Journal of Epidemiology*. 2009:38:253-261.
474. Zhang X, Huang Z, Xie Y, Chen X, Zhang J, Qiu Z, Ma N, Xu G, Liu X. Lower levels of plasma adiponectin and endothelial progenitor cells are associated with large artery atherosclerotic stroke. *Int J Neurosci*. 2016:126:121-126.
475. Efstathiou SP, Tsioulos DI, Tsiakou AG, Gratsias YE, Pefanis AV, Mountokalakis TD. Plasma adiponectin levels and five-year survival after first-ever ischemic stroke. *Stroke*. 2005:36:1915-1919.
476. Marousi SG, Theodorou GL, Karakantza M, Zampakis P, Papathanasopoulos P, Ellul J. Acute post-stroke adiponectin in relation to stroke severity, progression and 6 month functional outcome. *Neurological Research*. 2010:32:841-844.
477. Nagasawa H, Yokota C, Toyoda K, Ito A, Minematsu K. High level of plasma adiponectin in acute stroke patients is associated with stroke mortality. *Journal of the Neurological Sciences*. 2011:304:102-106.
478. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA, Thacker SB. Meta-analysis of observational studies in epidemiology: A

proposal for reporting. Meta-analysis of observational studies in epidemiology (moose) group. *Jama*. 2000:283:2008-2012.
479. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst*. 1959:22:719-748.
480. DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials*. 1986:7:177-188.
481. Higgins J, Green S. Cochrane handbook for systematic reviews of interventions; version 5.1.0. 2011
482. Orsini N, Li R, Wolk A, Khudyakov P, Spiegelman D. Meta-analysis for linear and nonlinear dose-response relations: Examples, an evaluation of approximations, and software. *Am J Epidemiol*. 2012:175:66-73.
483. Egger M, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *Bmj*. 1997:315:629-634.
484. Kizer JR. Adiponectin, cardiovascular disease, and mortality: Parsing the dual prognostic implications of a complex adipokine. *Metabolism*. 2014:63:1079-1083.
485. Rathmann W, Haastert B, Herder C, Hauner H, Koenig W, Meisinger C, Holle R, Giani G. Differential association of adiponectin with cardiovascular risk markers in men and women? The kora survey 2000. *Int J Obes (Lond)*. 2007:31:770-776.
486. Lago F, Dieguez C, Gomez-Reino J, Gualillo O. Adipokines as emerging mediators of immune response and inflammation. *Nat Clin Pract Rheumatol*. 2007:3:716-724.
487. Aprahamian TR, Sam F. Adiponectin in cardiovascular inflammation and obesity. *Int J Inflam*. 2011:2011:376909.
488. Fantuzzi G. Adiponectin and inflammation: Consensus and controversy. *J Allergy Clin Immunol*. 2008:121:326-330.
489. Krakoff J, Funahashi T, Stehouwer CD, Schalkwijk CG, Tanaka S, Matsuzawa Y, Kobes S, Tataranni PA, Hanson RL, Knowler WC, Lindsay RS. Inflammatory markers, adiponectin, and risk of type 2 diabetes in the pima indian. *Diabetes Care*. 2003:26:1745-1751.
490. Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A, Richelsen B. Regulation of adiponectin by adipose tissue-derived cytokines: In vivo and in vitro investigations in humans. *Am J Physiol Endocrinol Metab*. 2003:285:E527-533.
491. Waje-Andreassen U, Krakenes J, Ulvestad E, Thomassen L, Myhr KM, Aarseth J, Vedeler CA. Il-6: An early marker for outcome in acute ischemic stroke. *Acta Neurol Scand*. 2005:111:360-365.
492. Domac FM, Somay G, Misirli H, Erenoglu NY. Tumor necrosis factor alpha serum levels and inflammatory response in acute ischemic stroke. *Neurosciences (Riyadh)*. 2007:12:25-30.
493. Intiso D, Zarrelli MM, Lagioia G, Di Rienzo F, Checchia De Ambrosio C, Simone P, Tonali P, Cioffi Dagger RP. Tumor necrosis factor alpha serum levels and inflammatory response in acute ischemic stroke patients. *Neurol Sci*. 2004:24:390-396.
494. Kanhai DA, Kranendonk ME, Uiterwaal CS, van der Graaf Y, Kappelle LJ, Visseren FL. Adiponectin and incident coronary heart disease and stroke. A systematic review and meta-analysis of prospective studies. *Obes Rev*. 2013
495. Hellings WE, Moll FL, de Vries JP, de Bruin P, de Kleijn DP, Pasterkamp G. Histological characterization of restenotic carotid plaques in relation to recurrence interval and clinical presentation: A cohort study. *Stroke*. 2008:39:1029-1032.

496. Marek JM, Koehler C, Aguirre ML, Westerband A, Gentile AT, Mills JL, Hunter GC. The histologic characteristics of primary and restenotic carotid plaque. *J Surg Res*. 1998:74:27-33.
497. Daskalopoulou SS, Rabi DM, Zarnke KB et.al. The 2015 canadian hypertension education program recommendations for blood pressure measurement, diagnosis, assessment of risk, prevention, and treatment of hypertension. *Can J Cardiol*. 2015:31:549-568.
498. Fukuyama N, Homma K, Wakana N, Kudo K, Suyama A, Ohazama H, Tsuji C, Ishiwata K, Eguchi Y, Nakazawa H, Tanaka E. Validation of the friedewald equation for evaluation of plasma ldl-cholesterol. *J Clin Biochem Nutr*. 2008:43:1-5.
499. Ammirati E, Moroni F, Norata GD, Magnoni M, Camici PG. Markers of inflammation associated with plaque progression and instability in patients with carotid atherosclerosis. *Mediators Inflamm*. 2015:2015:718329.
500. Avgerinos ED, Kadoglou NP, Moulakakis KG, Giannakopoulos TG, Liapis CD. Current role of biomarkers in carotid disease: A systematic review. *Int J Stroke*. 2011:6:337-345.
501. Committee CDACPGE. Canadian diabetes association 2013 clinical practice guidelines for the prevention and management of diabetes in canada. *Can J Diabetes*. 2013:37 S1-S212.
502. Hwang JJ, Chan JL, Ntali G, Malkova D, Mantzoros CS. Leptin does not directly regulate the pancreatic hormones amylin and pancreatic polypeptide: Interventional studies in humans. *Diabetes Care*. 2008:31:945-951.
503. Ait-Oufella H, Taleb S, Mallat Z, Tedgui A. Recent advances on the role of cytokines in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2011:31:969-979.
504. Tedgui A, Mallat Z. Cytokines in atherosclerosis: Pathogenic and regulatory pathways. *Physiol Rev*. 2006:86:515-581.
505. Lehrke M, Becker A, Greif M, Stark R, Laubender RP, von Ziegler F, Lebherz C, Tittus J, Reiser M, Becker C, Goke B, Leber AW, Parhofer KG, Broedl UC. Chemerin is associated with markers of inflammation and components of the metabolic syndrome but does not predict coronary atherosclerosis. *Eur J Endocrinol*. 2009:161:339-344.
506. Li Y, Shi B, Li S. Association between serum chemerin concentrations and clinical indices in obesity or metabolic syndrome: A meta-analysis. *PLoS One*. 2014:9:e113915.
507. Xiaotao L, Xiaoxia Z, Yue X, Liye W. Serum chemerin levels are associated with the presence and extent of coronary artery disease. *Coron Artery Dis*. 2012:23:412-416.
508. Zhao D, Bi G, Feng J, Huang R, Chen X. Association of serum chemerin levels with acute ischemic stroke and carotid artery atherosclerosis in a chinese population. *Med Sci Monit*. 2015:21:3121-3128.
509. Hart R, Greaves DR. Chemerin contributes to inflammation by promoting macrophage adhesion to vcam-1 and fibronectin through clustering of vla-4 and vla-5. *J Immunol*. 2010:185:3728-3739.
510. Cash JL, Hart R, Russ A, Dixon JP, Colledge WH, Doran J, Hendrick AG, Carlton MB, Greaves DR. Synthetic chemerin-derived peptides suppress inflammation through chemr23. *J Exp Med*. 2008:205:767-775.
511. Yoshimura T, Oppenheim JJ. Chemerin reveals its chimeric nature. *J Exp Med*. 2008:205:2187-2190.
512. Martin SS, Blaha MJ, Muse ED, Qasim AN, Reilly MP, Blumenthal RS, Nasir K, Criqui MH, McClelland RL, Hughes-Austin JM, Allison MA. Leptin and incident cardiovascular disease: The multi-ethnic study of atherosclerosis (mesa). *Atherosclerosis*. 2015:239:67-72.

513. Adya R, Tan BK, Randeva HS. Differential effects of leptin and adiponectin in endothelial angiogenesis. *J Diabetes Res*. 2015:2015:648239.
514. Maya-Monteiro CM, Almeida PE, D'Avila H, Martins AS, Rezende AP, Castro-Faria-Neto H, Bozza PT. Leptin induces macrophage lipid body formation by a phosphatidylinositol 3-kinase- and mammalian target of rapamycin-dependent mechanism. *J Biol Chem*. 2008:283:2203-2210.
515. Sinan UY, Canbolat IP, Baydar O, Oktay V, Imre G, Kocas C, Abaci O, Coskun U, Bostan C, Kilickesmez KO, Yildiz A, Kaya A, Gurmen T, Yigit Z. Relationship between increased serum resistin level and severity of coronary artery disease. *Angiology*. 2014:65:239-242.
516. Prugger C, Luc G, Haas B, Morange PE, Ferrieres J, Amouyel P, Kee F, Ducimetiere P, Empana JP. Multiple biomarkers for the prediction of ischemic stroke: The prime study. *Arterioscler Thromb Vasc Biol*. 2013:33:659-666.
517. Efstathiou SP, Tsiakou AG, Tsioulos DI, Panagiotou TN, Pefanis AV, Achimastos AD, Mountokalakis TD. Prognostic significance of plasma resistin levels in patients with atherothrombotic ischemic stroke. *Clin Chim Acta*. 2007:378:78-85.
518. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. *Nature*. 2001:409:307-312.
519. Wang H, Chu WS, Hemphill C, Elbein SC. Human resistin gene: Molecular scanning and evaluation of association with insulin sensitivity and type 2 diabetes in caucasians. *J Clin Endocrinol Metab*. 2002:87:2520-2524.
520. Gharibeh MY, Al Tawallbeh GM, Abboud MM, Radaideh A, Alhader AA, Khabour OF. Correlation of plasma resistin with obesity and insulin resistance in type 2 diabetic patients. *Diabetes Metab*. 2010:36:443-449.
521. Osawa H, Doi Y, Makino H, Ninomiya T, Yonemoto K, Kawamura R, Hata J, Tanizaki Y, Iida M, Kiyohara Y. Diabetes and hypertension markedly increased the risk of ischemic stroke associated with high serum resistin concentration in a general japanese population: The hisayama study. *Cardiovasc Diabetol*. 2009:8:60.
522. Tsukahara T, Nakashima E, Watarai A, Hamada Y, Naruse K, Kamiya H, Nakamura N, Kato N, Hamajima N, Sekido Y, Niwa T, Tomita M, Oiso Y, Nakamura J. Polymorphism in resistin promoter region at -420 determines the serum resistin levels and may be a risk marker of stroke in japanese type 2 diabetic patients. *Diabetes Res Clin Pract*. 2009:84:179-186.
523. Burnett MS, Lee CW, Kinnaird TD et.al. The potential role of resistin in atherogenesis. *Atherosclerosis*. 2005:182:241-248.
524. Jung HS, Park KH, Cho YM, Chung SS, Cho HJ, Cho SY, Kim SJ, Kim SY, Lee HK, Park KS. Resistin is secreted from macrophages in atheromas and promotes atherosclerosis. *Cardiovasc Res*. 2006:69:76-85.
525. Cho Y, Lee SE, Lee HC et.al. Adipokine resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery. *J Am Coll Cardiol*. 2011:57:99-109.
526. Shindo A, Tanemura H, Yata K, Hamada K, Shibata M, Umeda Y, Asakura F, Toma N, Sakaida H, Fujisawa T, Taki W, Tomimoto H. Inflammatory biomarkers in atherosclerosis: Pentraxin 3 can become a novel marker of plaque vulnerability. *PLoS One*. 2014:9:e100045.
527. Bluher M, Brennan AM, Kelesidis T, Kratzsch J, Fasshauer M, Kralisch S, Williams CJ, Mantzoros CS. Total and high-molecular weight adiponectin in relation to metabolic

variables at baseline and in response to an exercise treatment program: Comparative evaluation of three assays. *Diabetes Care*. 2007:30:280-285.
528. Pischon T, Hu FB, Girman CJ, Rifai N, Manson JE, Rexrode KM, Rimm EB. Plasma total and high molecular weight adiponectin levels and risk of coronary heart disease in women. *Atherosclerosis*. 2011:219:322-329.
529. Musialek P, Tracz W, Tekieli L, Pieniazek P, Kablak-Ziembicka A, Przewlocki T, Stepien E, Kapusta P, Motyl R, Stepniewski J, Undas A, Podolec P. Multimarker approach in discriminating patients with symptomatic and asymptomatic atherosclerotic carotid artery stenosis. *J Clin Neurol*. 2013:9:165-175.
530. Sharma G, Tao M, Ding K, Yu D, King W, Deyneko G, Wang X, Longchamp A, Schoen FJ, Ozaki CK, Semel ME. Perivascular adipose adiponectin correlates with symptom status of patients undergoing carotid endarterectomy. *Stroke*. 2015:46:1696-1699.
531. Bjursell M, Ahnmark A, Bohlooly YM, William-Olsson L, Rhedin M, Peng XR, Ploj K, Gerdin AK, Arnerup G, Elmgren A, Berg AL, Oscarsson J, Linden D. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes*. 2007:56:583-593.
532. Hafiane A, Bielicki JK, Johansson JO, Genest J. Novel apo e-derived abca1 agonist peptide (cs-6253) promotes reverse cholesterol transport and induces formation of prebeta-1 hdl in vitro. *PLoS One*. 2015:10:e0131997.
533. Basu SK, Goldstein JL, Anderson GW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A*. 1976:73:3178-3182.
534. Vera A, Recabal A, Saldivia N, Stanic K, Torrejon M, Montecinos H, Caprile T. Interaction between sco-spondin and low density lipoproteins from embryonic cerebrospinal fluid modulates their roles in early neurogenesis. *Front Neuroanat*. 2015:9:72.
535. Shimabukuro M, Higa N, Asahi T, Oshiro Y, Takasu N, Tagawa T, Ueda S, Shimomura I, Funahashi T, Matsuzawa Y. Hypoadiponectinemia is closely linked to endothelial dysfunction in man. *J Clin Endocrinol Metab*. 2003:88:3236-3240.
536. Li CJ, Sun HW, Zhu FL, Chen L, Rong YY, Zhang Y, Zhang M. Local adiponectin treatment reduces atherosclerotic plaque size in rabbits. *J Endocrinol*. 2007:193:137-145.
537. Okamoto Y, Arita Y, Nishida M, Muraguchi M, Ouchi N, Takahashi M, Igura T, Inui Y, Kihara S, Nakamura T, Yamashita S, Miyagawa J, Funahashi T, Matsuzawa Y. An adipocyte-derived plasma protein, adiponectin, adheres to injured vascular walls. *Horm Metab Res*. 2000:32:47-50.
538. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*. 2004:109:Iii27-32.
539. Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *J Adv Pharm Technol Res*. 2011:2:236-240.
540. Jeon SM. Regulation and function of ampk in physiology and diseases. *Exp Mol Med*. 2016:48:e245.
541. Liu Y, Michael MD, Kash S, Bensch WR, Monia BP, Murray SF, Otto KA, Syed SK, Bhanot S, Sloop KW, Sullivan JM, Reifel-Miller A. Deficiency of adiponectin receptor 2 reduces diet-induced insulin resistance but promotes type 2 diabetes. *Endocrinology*. 2007:148:683-692.
542. Hafiane A, Genest J. Hdl-mediated cellular cholesterol efflux assay method. *Ann Clin Lab Sci*. 2015:45:659-668.

543. Gordon DJ, Rifkind BM. High-density lipoprotein--the clinical implications of recent studies. *N Engl J Med*. 1989:321:1311-1316.
544. Schwartz GG, Olsson AG, Abt M et.al. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *N Engl J Med*. 2012:367:2089-2099.
545. Hps2-thrive randomized placebo-controlled trial in 25 673 high-risk patients of er niacin/laropiprant: Trial design, pre-specified muscle and liver outcomes, and reasons for stopping study treatment. *Eur Heart J*. 2013:34:1279-1291.
546. Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K, McBride R, Teo K, Weintraub W. Niacin in patients with low hdl cholesterol levels receiving intensive statin therapy. *N Engl J Med*. 2011:365:2255-2267.
547. Lund-Katz S, Phillips MC. High density lipoprotein structure-function and role in reverse cholesterol transport. *Subcell Biochem*. 2010:51:183-227.
548. Duong PT, Weibel GL, Lund-Katz S, Rothblat GH, Phillips MC. Characterization and properties of pre beta-hdl particles formed by abca1-mediated cellular lipid efflux to apoa-i. *J Lipid Res*. 2008:49:1006-1014.
549. Ruan H, Dong LQ. Adiponectin signaling and function in insulin target tissues. *J Mol Cell Biol*. 2016:8:101-109.
550. Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the abc1 promoter by the liver x receptor/retinoid x receptor. *J Biol Chem*. 2000:275:28240-28245.
551. Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P. A ppar gamma-lxr-abca1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell*. 2001:7:161-171.
552. Smaldone C, Brugaletta S, Pazzano V, Liuzzo G. Immunomodulator activity of 3-hydroxy-3-methilglutaryl-coa inhibitors. *Cardiovasc Hematol Agents Med Chem*. 2009:7:279-294.
553. Methe H, Kim JO, Kofler S, Nabauer M, Weis M. Statins decrease toll-like receptor 4 expression and downstream signaling in human cd14+ monocytes. *Arterioscler Thromb Vasc Biol*. 2005:25:1439-1445.
554. Ziaeian B, Fonarow GC. Statins and the prevention of heart disease. *JAMA Cardiol*. 2017:2:464.
555. Kim DH, Kim C, Ding EL, Townsend MK, Lipsitz LA. Adiponectin levels and the risk of hypertension: A systematic review and meta-analysis. *Hypertension*. 2013:62:27-32.
556. Gasbarrino K, Mantzoros C, Gorgui J, Veinot JP, Lai C, Daskalopoulou SS. Circulating chemerin is associated with carotid plaque instability, whereas resistin is related to cerebrovascular symptomatology. *Arterioscler Thromb Vasc Biol*. 2016:36:1670-1678.
557. Qiu B, Simon MC. Bodipy 493/503 staining of neutral lipid droplets for microscopy and quantification by flow cytometry. *Bio Protoc*. 2016:6
558. Banach M, Serban C, Sahebkar A, Mikhailidis DP, Ursoniu S, Ray KK, Rysz J, Toth PP, Muntner P, Mosteoru S, Garcia-Garcia HM, Hovingh GK, Kastelein JJ, Serruys PW. Impact of statin therapy on coronary plaque composition: A systematic review and meta-analysis of virtual histology intravascular ultrasound studies. *BMC Med*. 2015:13:229.
559. Wong J, Quinn CM, Gelissen IC, Jessup W, Brown AJ. The effect of statins on abca1 and abcg1 expression in human macrophages is influenced by cellular cholesterol levels and extent of differentiation. *Atherosclerosis*. 2008:196:180-189.
560. Ronsein GE, Hutchins PM, Isquith D, Vaisar T, Zhao XQ, Heinecke JW. Niacin therapy increases high-density lipoprotein particles and total cholesterol efflux capacity but not

abca1-specific cholesterol efflux in statin-treated subjects. *Arterioscler Thromb Vasc Biol*. 2016:36:404-411.
561. Wong J, Quinn CM, Brown AJ. Statins inhibit synthesis of an oxysterol ligand for the liver x receptor in human macrophages with consequences for cholesterol flux. *Arterioscler Thromb Vasc Biol*. 2004:24:2365-2371.
562. Tuomisto TT, Lumivuori H, Kansanen E, Hakkinen SK, Turunen MP, van Thienen JV, Horrevoets AJ, Levonen AL, Yla-Herttuala S. Simvastatin has an anti-inflammatory effect on macrophages via upregulation of an atheroprotective transcription factor, kruppel-like factor 2. *Cardiovasc Res*. 2008:78:175-184.
563. Yano M, Matsumura T, Senokuchi T, Ishii N, Murata Y, Taketa K, Motoshima H, Taguchi T, Sonoda K, Kukidome D, Takuwa Y, Kawada T, Brownlee M, Nishikawa T, Araki E. Statins activate peroxisome proliferator-activated receptor gamma through extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase-dependent cyclooxygenase-2 expression in macrophages. *Circ Res*. 2007:100:1442-1451.
564. Grip O, Janciauskiene S, Lindgren S. Atorvastatin activates ppar-gamma and attenuates the inflammatory response in human monocytes. *Inflamm Res*. 2002:51:58-62.
565. Henriksbo BD, Lau TC, Cavallari JF, Denou E, Chi W, Lally JS, Crane JD, Duggan BM, Foley KP, Fullerton MD, Tarnopolsky MA, Steinberg GR, Schertzer JD. Fluvastatin causes nlrp3 inflammasome-mediated adipose insulin resistance. *Diabetes*. 2014:63:3742-3747.
566. Kuijk LM, Mandey SH, Schellens I, Waterham HR, Rijkers GT, Coffer PJ, Frenkel J. Statin synergizes with lps to induce il-1beta release by thp-1 cells through activation of caspase-1. *Mol Immunol*. 2008:45:2158-2165.
567. Libby P. Interleukin-1 beta as a target for atherosclerosis therapy: Biological basis of cantos and beyond. *J Am Coll Cardiol*. 2017:70:2278-2289.
568. Ridker PM. From c-reactive protein to interleukin-6 to interleukin-1: Moving upstream to identify novel targets for atheroprotection. *Circ Res*. 2016:118:145-156.
569. Eskan MA, Benakanakere MR, Rose BG, Zhang P, Zhao J, Stathopoulou P, Fujioka D, Kinane DF. Interleukin-1beta modulates proinflammatory cytokine production in human epithelial cells. *Infect Immun*. 2008:76:2080-2089.
570. McTaggart F. Comparative pharmacology of rosuvastatin. *Atheroscler Suppl*. 2003:4:9-14.
571. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of hmg-coa reductase. *Science*. 2001:292:1160-1164.
572. Arsenault BJ, Petrides F, Tabet F, Bao W, Hovingh GK, Boekholdt SM, Ramin-Mangata S, Meilhac O, DeMicco D, Rye KA, Waters DD, Kastelein JJP, Barter P, Lambert G. Effect of atorvastatin, cholesterol ester transfer protein inhibition, and diabetes mellitus on circulating proprotein subtilisin kexin type 9 and lipoprotein(a) levels in patients at high cardiovascular risk. *J Clin Lipidol*. 2018:12:130-136.
573. Brault M, Ray J, Gomez YH, Mantzoros CS, Daskalopoulou SS. Statin treatment and new-onset diabetes: A review of proposed mechanisms. *Metabolism*. 2014:63:735-745.
574. Sattar N, Preiss D, Murray HM et.al. Statins and risk of incident diabetes: A collaborative meta-analysis of randomised statin trials. *Lancet*. 2010:375:735-742.
575. Preiss D, Seshasai SR, Welsh P et.al. Risk of incident diabetes with intensive-dose compared with moderate-dose statin therapy: A meta-analysis. *Jama*. 2011:305:2556-2564.
576. Henriksbo BD, Schertzer JD. Is immunity a mechanism contributing to statin-induced diabetes? *Adipocyte*. 2015:4:232-238.

577. Lagathu C, Yvan-Charvet L, Bastard JP, Maachi M, Quignard-Boulange A, Capeau J, Caron M. Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. *Diabetologia*. 2006:49:2162-2173.
578. Bjorkhem-Bergman L, Lindh JD, Bergman P. What is a relevant statin concentration in cell experiments claiming pleiotropic effects? *Br J Clin Pharmacol*. 2011:72:164-165.
579. Canada HaSFo. Ms.Understood: Women's hearts are victims of a system that is ill-equipped to diagnose, treat and support them - 2018 stroke report. 2018
580. Libby P. The molecular mechanisms of the thrombotic complications of atherosclerosis. *J Intern Med*. 2008:263:517-527.
581. Corti R, Fuster V, Badimon JJ. Pathogenetic concepts of acute coronary syndromes. *J Am Coll Cardiol*. 2003:41:7s-14s.
582. Wang X, Magkos F, Mittendorfer B. Sex differences in lipid and lipoprotein metabolism: It's not just about sex hormones. *J Clin Endocrinol Metab*. 2011:96:885-893.
583. Palmisano BT, Zhu L, Eckel RH, Stafford JM. Sex differences in lipid and lipoprotein metabolism. *Mol Metab*. 2018:15:45-55.
584. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol*. 2016:16:626-638.
585. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002:105:1135-1143.
586. Fairweather D. Sex differences in inflammation during atherosclerosis. *Clin Med Insights Cardiol*. 2014:8:49-59.
587. Boyne MS, Bennett NR, Cooper RS, Royal-Thomas TY, Bennett FI, Luke A, Wilks RJ, Forrester TE. Sex-differences in adiponectin levels and body fat distribution: Longitudinal observations in afro-jamaicans. *Diabetes Res Clin Pract*. 2010:90:e33-36.
588. Ohman-Hanson RA, Cree-Green M, Kelsey MM, Bessesen DH, Sharp TA, Pyle L, Pereira RI, Nadeau KJ. Ethnic and sex differences in adiponectin: From childhood to adulthood. *J Clin Endocrinol Metab*. 2016:101:4808-4815.
589. Taylor AE, Keevil B, Huhtaniemi IT. Mass spectrometry and immunoassay: How to measure steroid hormones today and tomorrow. *Eur J Endocrinol*. 2015:173:D1-12.
590. Handelsman DJ, Wartofsky L. Requirement for mass spectrometry sex steroid assays in the journal of clinical endocrinology and metabolism. *J Clin Endocrinol Metab*. 2013:98:3971-3973.
591. Cullen P, Baetta R, Bellosta S et.al. Rupture of the atherosclerotic plaque: Does a good animal model exist? *Arterioscler Thromb Vasc Biol*. 2003:23:535-542.
592. Cencic R, Senechal P, Pelletier J. Establishment of a primary screening assay for the dhx9 helicase. *Comb Chem High Throughput Screen*. 2015:18:855-861.
593. Hlatky MA. Framework for evaluating novel risk markers. *Ann Intern Med*. 2012:156:468-469.

APPENDIX A

RELATIONSHIP BETWEEN ADIPONECTIN LEVELS AND CAROTID ARTERY DISEASES – SYSTEMATIC REVIEW SEARCH STRATEGIES

Designed and run by Bénédicte Nauche (MUHC librarian), May 23rd, 2013

Medline and Embase search strategies have been reviewed by Tara Landry (MUHC librarian).

Database: Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R) <1946 to Present>

Search Strategy:

---

1 Adiponectin/ (7419)
2 adiponectin$.mp. (11227)
3 (apm1 or "apm 1").mp. (132)
4 (adipo$ adj5 abundant gene$).mp. (19)
5 (adipo$ adj5 protein adj3 "30").mp. (33)
6 (acrp30 or "acrp 30").mp. (107)
7 adipoq.mp. (1422)
8 (adipo$ adj3 collagen).mp. (143)
9 (gelatin adj3 protein adj3 "28").mp. (9)
10 (gbp28 or "gbp 28").mp. (18)
11 or/1-10 (11476)
12 exp carotid arteries/ (47911)
13 carotid artery diseases/ or carotid artery thrombosis/ or carotid stenosis/ (30286)
14 Carotid Intima-Media Thickness/ (806)
15 tunica intima/ (9736)
16 tunica media/ (4002)
17 (intima$ or media$1 or arter$ or aort$).tw. (1398270)
18 15 or 16 or 17 (1399225)
19 ultrasonography.fs. (186637)
20 ultrasonography/ or ultrasonography, Doppler/ (72034)
21 (ultrasonogra$ or "ultra sonogra$" or doppler or echotomogra$ or "echo tomogra$" or ultrasonic or "ultra sonic" or echogra$ or "echo gra$" or echoscop$ or "echo scop$" or echosound or "echo sound" or sonogra$ or tomogra$ or ultrasound).tw. (513889)
22 19 or 20 or 21 (598898)
23 18 and 22 (140729)
24 12 or 13 or 14 or 23 (192337)
25 Plaque, Atherosclerotic/ (1849)
26 exp Atherosclerosis/ (19455)
27 arteriosclerosis/ or arteriosclerosis obliterans/ (58333)

28 stroke/ (52258)
29 brain ischemia/ or Hypoxia-Ischemia, Brain/ or brain infarction/ or cerebral infarction/ (55708)
30 exp "intracranial embolism and thrombosis"/ (17885)
31 Ischemic Attack, Transient/ (17099)
32 brain/ (366915)
33 blood supply.fs. (293126)
34 32 and 33 (23863)
35 25 or 26 or 27 or 28 or 29 or 30 or 31 or 34 (220470)
36 ischemia/ (41726)
37 Amaurosis Fugax/ (212)
38 blindness/ (16196)
39 vision disorders/ (20990)
40 carotid.tw. (85996)
41 ((aort$ or arter$) adj2 thickness).tw. (1206)
42 ((intimamedia$ or (intima$ adj3 media$)) adj4 thickness).tw. (7399)
43 cIMT.tw. (1129)
44 (ather?sclero$ or ather$ sclero$).tw. (103286)
45 (ather?genes$ or ather$ genes$).tw. (10297)
46 (arteri?sclero$ or arteri$ sclero$).tw. (14140)
47 (fibroatheroma* or atheroma*).tw. (9092)
48 (fatty adj2 arter$).tw. (127)
49 stroke$.tw,in. (144946)
50 (cerebrovascular adj2 (accident$ or event$ or isch?emi$ or infarct$ or occlusion$ or obstruction$ or insult or insufficien$ or attack$ or arrest or disturb$ or lesion$ or circulation or failure$)).tw. (11601)
51 ((neural or brain or cerebr$) adj2 (blood or vascul$ or vessel$1 or arter$) adj2 (accident$ or event$ or isch?emi$ or infarct$ or occlusion$ or obstruction$ or insult or insufficien$ or attack$ or arrest or disturb$ or lesion$ or circulation or failure$ or supply$ or flow)).tw. (36886)
52 ((neural or brain or cerebr$ or hemispher$) adj2 (isch?emi$ or infarct$ or seizure$ or insult or attack$ or occlusion$ or circulation)).tw. (57575)
53 (isch?emi$ adj2 (seizure$ or attack$)).tw. (9955)
54 ((intracranial or cranial or cerebr$ or brain$) adj3 (thromb$ or embol$ or angiopath$ or vasculopath$ or phlebothromb$)).tw. (13194)
55 apoplex$.tw. (2443)
56 (cva or cvas).tw. (1809)
57 ((transien$ adj2 isch?emi$) and (encephal$ or brain$ or cerebr$ or neural or cranial or intracranial)).tw. (8214)
58 (Tia or tias).tw. (5528)
59 (amauros$ adj2 fuga$).tw. (729)
60 ((transien$ or transit$) adj3 blind$).tw. (451)
61 or/36-60 (471133)
62 24 or 35 or 61 (650871)
63 11 and 62 (1550)
64 limit 63 to humans (1264)

65 limit 63 to animals (384)
66 63 not (64 or 65) (122)
67 64 or 66 (1386)
68 limit 67 to "all adult (19 plus years)" (762)
69 limit 67 to "all child (0 to 18 years)" (116)
70 67 not (68 or 69) (563)
71 68 or 70 (1325)
72 remove duplicates from 71 (1269)

Database: Embase Classic+Embase <1947 to 2013 May 22>

1 adiponectin/ (16728)
2 adiponectin$.mp. (18284)
3 (apm1 or "apm 1").mp. (161)
4 (adipo$ adj5 abundant gene$).mp. (22)
5 (adipo$ adj5 protein adj3 "30").mp. (40)
6 (acrp30 or "acrp 30").mp. (164)
7 adipoq.mp. (477)
8 (adipo$ adj3 collagen).tw,kw,ot. (179)
9 (gelatin adj3 protein adj3 "28").mp. (10)
10 (gbp28 or "gbp 28").mp. (24)
11 or/1-10 (18571)
12 exp carotid artery/ (72468)
13 carotid artery disease/ (10675)
14 carotid atherosclerosis/ (2612)
15 carotid artery obstruction/ or carotid artery thrombosis/ or internal carotid artery occlusion/ (25577)
16 brain infarction/ or brain infarction size/ (38620)
17 brain ischemia/ or hypoxic ischemic encephalopathy/ or transient ischemic attack/ (91893)
18 cerebrovascular accident/ or cardioembolic stroke/ (57797)
19 Hypophysis apoplexy/ (1025)
20 brain embolism/ (5842)
21 occlusive cerebrovascular disease/ or middle cerebral artery occlusion/ (17516)
22 atherosclerosis/ or atherogenesis/ or atheroma/ or atheromatosis/ or atherosclerotic cardiovascular disease/ or atherosclerotic plaque/ (133690)
23 arteriosclerosis/ or artery intima proliferation/ (51266)
24 arterial wall thickness/ (7326)
25 or/12-24 (415927)
26 intima/ or tunica media/ or artery wall/ (20838)
27 (intima$ or media$1 or arter$ or aort$).tw,kw. (2069558)
28 26 or 27 (2071456)
29 echography/ or doppler echography/ or gray scale echography/ or intravascular ultrasound/ (255938)
30 (ultrasonogra$ or "ultra sonogra$" or doppler or echotomogra$ or "echo tomogra$" or ultrasonic or "ultra sonic" or echogra$ or "echo gra$" or echoscop$ or "echo scop$" or echosound or "echo sound" or sonogra$ or tomogra$ or ultrasound).tw,kw. (680376)
31 29 or 30 (766847)
32 28 and 31 (182289)
33 transitional blindness/ (1363)
34 carotid.tw,kw. (124225)
35 ((aort$ or arter$) adj2 thickness).tw,kw. (1680)
36 ((intima$ or media$1 or arter$) adj4 thickness).tw,kw. (15663)
37 cIMT.tw,kw. (1969)

38 (ather?sclero$ or ather$ sclero$).tw,kw. (150520)
39 (ather?genes$ or ather$ genes$).tw,kw. (13648)
40 (arteri?sclero$ or arteri$ sclero$).tw,kw. (27166)
41 (fibroatheroma* or atheroma*).tw,kw. (14687)
42 (fatty adj2 arter$).tw,kw. (184)
43 stroke$.tw,kw. (202268)
44 (cerebrovascular adj2 (accident$ or event$ or isch?emi$ or infarct$ or occlusion$ or obstruction$ or insult or insufficien$ or attack$ or arrest or disturb$ or lesion$ or circulation or failure$)).tw,kw. (19146)
45 ((neural or brain or cerebr$) adj2 (blood or vascul$ or vessel$1 or arter$) adj2 (accident$ or event$ or isch?emi$ or infarct$ or occlusion$ or obstruction$ or insult or insufficien$ or attack$ or arrest or disturb$ or lesion$ or circulation or failure$ or supply$ or flow)).tw,kw. (51495)
46 ((neural or brain or cerebr$ or hemispher$) adj2 (isch?emi$ or infarct$ or seizure$ or insult or attack$ or occlusion$ or circulation)).tw,kw. (84877)
47 (isch?emi$ adj2 (seizure$ or attack$)).tw,kw. (14057)
48 ((intracranial or cranial or cerebr$ or brain$) adj3 (thromb$ or embol$ or angiopath$ or vasculopath$ or phlebothromb$)).tw,kw. (20092)
49 apoplex$.tw,kw. (3817)
50 (cva or cvas).tw,kw. (3325)
51 ((transien$ adj2 isch?emi$) and (encephal$ or brain$ or cerebr$ or neural or cranial or intracranial)).tw,kw. (11368)
52 (circulatory adj2 epilepsy).tw,kw. (4)
53 (tia or tias).tw,kw. (9538)
54 (amauros$ adj2 fuga$).tw,kw. (1004)
55 ((transien$ or transit$) adj3 blind$).tw,kw. (669)
56 or/33-55 (572485)
57 25 or 32 or 56 (820156)
58 11 and 57 (3115)
59 limit 58 to animals (310)
60 limit 58 to humans (2422)
61 58 not (59 or 60) (383)
62 60 or 61 (2805)
63 limit 62 to (adult <18 to 64 years> or aged <65+ years>) (1008)
64 limit 62 to (embryo <first trimester> or infant <to one year> or child <unspecified age> or preschool child <1 to 6 years> or school child <7 to 12 years> or adolescent <13 to 17 years>) (177)
65 62 not (63 or 64) (1679)
66 63 or 65 (2687)
67 limit 66 to conference abstract (556)
68 66 not 67 (2131)
69 remove duplicates from 68 (2045)
70 remove duplicates from 67 (556)

PubMed

(((carotid[Title/Abstract]) OR (wall thickness[Title/Abstract]) OR (arter*[Title/Abstract] AND thickness[Title/Abstract]) OR (media*[Title/Abstract] AND thickness[Title/Abstract]) OR (intima*[Title/Abstract] AND thickness[Title/Abstract]) OR (((intima*[Title/Abstract] OR media*[Title/Abstract] OR arter*[Title/Abstract] OR aort*[Title/Abstract])) AND (echogra*[Title/Abstract] OR ultrasonogra*[Title/Abstract] OR echotomogra*[Title/Abstract] OR ultrasonic[Title/Abstract] OR echoscop*[Title/Abstract] OR echosound[Title/Abstract] OR sonogra*[Title/Abstract] OR tomogra*[Title/Abstract] OR ultrasound[Title/Abstract] OR doppler[Title/Abstract] OR echo[Title/Abstract])) OR (cimt[Title/Abstract]) OR (atherosclero*[Title/Abstract] OR (athero[Title/Abstract] AND sclero*[Title/Abstract])) OR (atherogenes*[Title/Abstract] OR (athero[Title/Abstract] AND genes*[Title/Abstract])) OR (arteriosclero*[Title/Abstract] OR (arter*[Title/Abstract] AND sclero*[Title/Abstract])) OR (fibroatheroma*[Title/Abstract] OR atheroma*[Title/Abstract]) OR (fatty[Title/Abstract] AND and arter*[Title/Abstract]) OR (stroke*[Title/Abstract]) OR ((((((((((((((accident*[Title/Abstract]) OR event*[Title/Abstract]) OR ischemi*[Title/Abstract]) OR ischaemi*[Title/Abstract]) OR infarct*[Title/Abstract]) OR occlusion*[Title/Abstract]) OR obstruction*[Title/Abstract]) OR insult[Title/Abstract]) OR insufficien*[Title/Abstract]) OR attack*[Title/Abstract]) OR arrest[Title/Abstract]) OR disturb*[Title/Abstract]) OR lesion*[Title/Abstract]) OR circulation[Title/Abstract]) OR failure*[Title/Abstract]) AND cerebr*[Title/Abstract]) OR ((accident*[Title/Abstract]) OR event*[Title/Abstract] OR ischemi*[Title/Abstract] OR ischaemi*[Title/Abstract] OR infarct*[Title/Abstract] OR occlusion*[Title/Abstract] OR obstruction*[Title/Abstract] OR insult[Title/Abstract] OR insufficien*[Title/Abstract] OR attack*[Title/Abstract] OR arrest[Title/Abstract] OR disturb*[Title/Abstract] OR lesion*[Title/Abstract] OR circulation[Title/Abstract] OR failure*[Title/Abstract] OR supply[Title/Abstract] OR flow[Title/Abstract] AND (neural[Title/Abstract] OR brain*[Title/Abstract]) AND (blood[Title/Abstract] OR vascul*[Title/Abstract] OR vessel*[Title/Abstract] OR arter*[Title/Abstract])) OR ((neural[Title/Abstract] OR brain*[Title/Abstract] OR cerebr*[Title/Abstract] OR hemispher*[Title/Abstract]) AND (ischemi*[Title/Abstract] OR ischaemi*[Title/Abstract] OR infarct*[Title/Abstract] OR seizure*[Title/Abstract] OR insult OR attack*[Title/Abstract] OR occlusion*[Title/Abstract] OR circulation[Title/Abstract])) OR ((ischemi*[Title/Abstract] OR ischaemi*[Title/Abstract]) AND (seizure*[Title/Abstract] OR attack*[Title/Abstract])) OR ((intracranial[Title/Abstract] OR cranial[Title/Abstract] OR cerebr*[Title/Abstract] OR brain*[Title/Abstract]) AND (thromb*[Title/Abstract] OR embol*[Title/Abstract] OR angiopath*[Title/Abstract] OR vasculopath*[Title/Abstract] OR phlebothromb*[Title/Abstract])) OR (apoplex*[Title/Abstract]) OR (cva[Title/Abstract] OR cvas[Title/Abstract]) OR (transien*[Title/Abstract] AND (ischemi*[Title/Abstract] OR ischaemi*[Title/Abstract]) AND (encephal*[Title/Abstract] OR brain*[Title/Abstract] OR cerebr*[Title/Abstract] OR neural[Title/Abstract] OR cranial[Title/Abstract] OR intracranial[Title/Abstract])) OR (circulatory[Title/Abstract] AND epilepsy[Title/Abstract]) OR (tia[Title/Abstract] OR tias[Title/Abstract]) OR

(amauros*[Title/Abstract] AND fuga*[Title/Abstract]) OR ((transien*[Title/Abstract] OR transit*[Title/Abstract]) AND blind*[Title/Abstract]))) AND ((((adiponectin*[Title/Abstract]) OR (apm1[Title/Abstract]) OR ("apm 1"[Title/Abstract]) OR (adipose most abundant gene[Title/Abstract]) OR (adipocyte complement related[Title/Abstract]) OR ((adipocyte*[Title/Abstract]) AND protein 30*[Title/Abstract]) OR (acrp30[Title/Abstract]) OR ("acrp 30"[Title/Abstract]) OR (adipoq[Title/Abstract]) OR ((adipocyte*[Title/Abstract]) AND collagen[Title/Abstract]) OR (((gelatin[Title/Abstract]) AND protein[Title/Abstract]) AND 28[Title/Abstract]) OR (gbp28[Title/Abstract]) OR ("gbp 28"[Title/Abstract]))) AND ((publisher[sb] NOT pmcbook) OR (pubmednotmedline[sb]) OR (oldmedline[sb])))

Database: BIOSIS Previews <1969 to 2013 Week 24>

1 adiponectin$.mp,hw. (12464)
2 (apm1 or "apm 1").mp,hw. (195)
3 (adipo$ adj5 abundant gene$).mp,hw. (20)
4 (adipo$ adj5 protein adj3 "30").mp,hw. (43)
5 (acrp30 or "acrp 30").mp,hw. (155)
6 adipoq.mp,hw. (390)
7 (adipo$ adj3 collagen).mp,hw. (198)
8 (gelatin adj3 protein adj3 "28").mp,hw. (9)
9 (gbp28 or "gbp 28").mp,hw. (26)
10 or/1-9 (12869)
11 carotid.mp,hw. (73193)
12 ((aort$ or arter$) adj2 thickness).mp,hw. (1292)
13 ((intimamedia$ or (intima$ adj3 media$)) adj4 thickness).mp,hw. (7540)
14 cIMT.mp,hw. (759)
15 ((intima$ or media$1 or arter$ or aort$) and (ultrasonogra$ or "ultra sonogra$" or doppler or echotomogra$ or "echo tomogra$" or ultrasonic or "ultra sonic" or echogra$ or "echo gra$" or echoscop$ or "echo scop$" or echosound or "echo sound" or sonogra$ or tomogra$ or ultrasound)).mp,hw. (101958)
16 (ather?sclero$ or ather$ sclero$).mp,hw. (119557)
17 (ather?genes$ or ather$ genes$).mp,hw. (12813)
18 (arteri?sclero$ or arteri$ sclero$).mp,hw. (64272)
19 (fibroatheroma$ or atheroma$).mp,hw. (7768)
20 (fatty adj2 arter$).mp,hw. (130)
21 stroke$.mp,hw,in. (126604)
22 (cerebrovascular adj2 (accident$ or event$ or isch?emi$ or infarct$ or occlusion$ or obstruction$ or insult or insufficien$ or attack$ or arrest or disturb$ or lesion$ or circulation or failure$)).mp,hw. (7923)
23 ((neural or brain or cerebr$) adj2 (blood or vascul$ or vessel$1 or arter$) adj2 (accident$ or event$ or isch?emi$ or infarct$ or occlusion$ or obstruction$ or insult or insufficien$ or attack$ or arrest or disturb$ or lesion$ or circulation or failure$ or supply$ or flow)).mp,hw. (54194)
24 ((neural or brain or cerebr$ or hemispher$) adj2 (isch?emi$ or infarct$ or seizure$ or insult or attack$ or occlusion$ or circulation)).mp,hw. (78032)
25 (isch?emi$ adj2 (seizure$ or attack$)).mp,hw. (9887)
26 ((intracranial or cranial or cerebr$ or brain$) adj3 (thromb$ or embol$ or angiopath$ or vasculopath$ or phlebothromb$)).mp,hw. (14415)
27 apoplex$.mp,hw. (1067)
28 (cva or cvas).mp,hw. (1527)
29 ((transien$ adj2 isch?emi$) and (encephal$ or brain$ or cerebr$ or neural or cranial or intracranial)).mp,hw. (11790)
30 (Tia or tias).mp,hw. (4543)
31 (amauros$ adj2 fuga$).mp,hw. (520)
32 ((transien$ or transit$) adj3 blind$).mp,hw. (274)

33 or/11-32 (460325)
34 10 and 33 (1619)
35 limit 34 to human (1323)
36 limit 34 to animals (1572)
37 34 not (35 or 36) (47)
38 35 or 37 (1370)
39 limit 38 to (adult or aged or "aged/80 and over" or middle age) (707)
40 limit 38 to (adolescent or blastula or child or egg or embryo or fetus or gastrula or immature or infant or larva or neonate or newborn or preadolescent or preschool or seed or seedling or spore or zygote) (100)
41 38 not (39 or 40) (599)
42 39 or 41 (1306)
43 remove duplicates from 42 (1243)
44 (meeting or meeting abstract or meeting paper).pt. (5095093)
45 43 and 44 (428)
46 article.pt. (15044677)
47 43 and 46 (787)

Cochrane Library

ID	Search	Hits
#1	adiponectin*:ti,ab	745
#2	adipoq:ti,ab	8
#3	(apm1 or "apm 1"):ti,ab	2
#4	(adipo* near/5 abundant gene*):ti,ab	0
#5	(adipo* near/5 protein near/3 "30"):ti,ab	0
#6	(acrp30 or "acrp 30"):ti,ab	0
#7	(adipo* near/3 collagen):ti,ab	2
#8	(gelatin near/3 protein near/3 "28"):ti,ab	0
#9	(gbp28 or "gbp 28"):ti,ab	2
#10	594-#9	751
#11	MeSH descriptor: [Carotid Arteries] this term only	581
#12	MeSH descriptor: [Carotid Artery Diseases] this term only	359
#13	MeSH descriptor: [Carotid Artery Thrombosis] this term only	17
#14	MeSH descriptor: [Carotid Stenosis] this term only	535
#15	MeSH descriptor: [Carotid Intima-Media Thickness] this term only	17
#16	MeSH descriptor: [Tunica Intima] explode all trees and with qualifiers: [Ultrasonography - US]	192
#17	MeSH descriptor: [Tunica Media] this term only and with qualifiers: [Ultrasonography - US]	99
#18	(intima* or media? or arter* or aort*):ti,ab and (ultrasonogra* or (ultra next sonogra*) or doppler or echotomogra* or (echo next tomogra*) or ultrasonic or "ultra sonic" or echogra* or (echo next gra*) or echoscop* or (echo next scop*) or echosound or "echo sound" or sonogra* or tomogra* or ultrasound):ti,ab	5820

#19 MeSH descriptor: [Plaque, Atherosclerotic] this term only
27
#20 MeSH descriptor: [Atherosclerosis] explode all trees
501
#21 MeSH descriptor: [Arteriosclerosis] this term only
894
#22 MeSH descriptor: [Arteriosclerosis Obliterans] this term only
72
#23 MeSH descriptor: [Stroke] this term only
3812
#24 MeSH descriptor: [Brain Ischemia] this term only
911
#25 MeSH descriptor: [Hypoxia-Ischemia, Brain] this term only
104
#26 MeSH descriptor: [Brain Infarction] this term only
40
#27 MeSH descriptor: [Cerebral Infarction] this term only
475
#28 MeSH descriptor: [Intracranial Embolism and Thrombosis] explode all trees
239
#29 MeSH descriptor: [Ischemic Attack, Transient] this term only
489
#30 MeSH descriptor: [Brain] this term only and with qualifiers: [Blood supply - BS]
455
#31 MeSH descriptor: [Ischemia] this term only
764
#32 MeSH descriptor: [Amaurosis Fugax] this term only
1
#33 MeSH descriptor: [Blindness] this term only
148
#34 MeSH descriptor: [Vision Disorders] this term only
497
#35 carotid:ti,ab
3199
#36 ((aort* or arter*) near/2 thickness):ti,ab
71
#37 ((intimamedia* or (intima* near/3 media*)) near/4 thickness):ti,ab
623
#38 cimt:ti,ab
122
#39 (ather?sclero* or (ather* next sclero*)):ti,ab
4187
#40 (ather?genes* or (ather* next genes*)):ti,ab
234
#41 (arteri?sclero* or (arteri* next sclero*)):ti,ab
369

#42 (fibroatheroma* or atheroma*):ti,ab
212
#43 (fatty near/2 arter*):ti,ab
3
#44 stroke*:ti,ab
15678
#45 (cerebrovascular near/2 (accident* or event* or isch?emi* or infarct* or occlusion* or obstruction* or insult or insufficien* or attack* or arrest or disturb* or lesion* or circulation or failure*)):ti,ab
692
#46 ((neural or brain or cerebr*) near/2 (blood or vascul* or vessel? or arter*) near/2 (accident* or event* or isch?emi* or infarct* or occlusion* or obstruction* or insult or insufficien* or attack* or arrest or disturb* or lesion* or circulation or failure* or supply* or flow)):ti,ab
1723
#47 ((neural or brain or cerebr* or hemispher*) near/2 (isch?emi* or infarct* or seizure* or insult or attack* or occlusion* or circulation)):ti,ab
2040
#48 (isch?emi* near/2 (seizure* or attack*)):ti,ab
229
#49 ((intracranial or cranial or cerebr* or brain*) near/3 (thromb* or embol* or angiopath* or vasculopath* or phlebothromb*)):ti,ab
441
#50 apoplex*:ti,ab
194
#51 (cva or cvas):ti,ab
106
#52 ((transien* near/2 isch?emi*) and (encephal* or brain* or cerebr* or neural or cranial or intracranial)):ti,ab
98
#53 (Tia or tias):ti,ab
409
#54 (amauros* near/2 fuga*):ti,ab
18
#55 ((transien* or transit*) near/3 blind*):ti,ab
61
#56 (or #11-#55)
32509
#57 #10 and #56
99

Scopus

((TITLE-ABS-KEY(adiponectin* OR apm1 OR "apm 1" OR acrp30 OR "acrp 30" OR gbp28 OR "gbp 28" OR adipoq)) OR (TITLE-ABS-KEY(adipo* W/5 "abundant gene*")) OR (TITLE-ABS-KEY(adipo* W/5 protein W/3 "30")) OR (TITLE-ABS-KEY(adipo* W/5 collagen)) OR (TITLE-ABS-KEY(gelatin W/3 protein W/3 "28"))) AND (((TITLE-ABS-KEY(carotid)) OR (TITLE-ABS-KEY((aort* OR arter*) W/2 thickness)) OR (TITLE-ABS-KEY((intimamedia* OR (intima* W/3 media*)) W/4 thickness)) OR (TITLE-ABS-KEY(cimt)) OR (TITLE-ABS-KEY((carotid OR plaque) AND ((atheroscleros*) OR (ather?sclerosis OR ather?scleroses) OR (ather* sclero*)))) OR (TITLE-ABS-KEY(atherogenes* OR ather?genesis OR ather?geneses OR (ather* genes*))) OR (TITLE-ABS-KEY(arterioscleros* OR arteri?sclerosis OR arteri?scleroses OR (arter* sclero*))) OR (TITLE-ABS-KEY(fibroatheroma* OR atheroma*))) OR ((TITLE-ABS-KEY(fatty W/2 arter*)) OR (TITLE-ABS-KEY(stroke*)) OR (TITLE-ABS-KEY(cerebrovascular W/2 (accident* OR event* OR ischemi* OR ischaemi* OR infarct* OR occlusion* OR obstruction* OR insult OR insufficien* OR attack* OR arrest OR disturb* OR lesion* OR circulation OR failure*))) OR (TITLE-ABS-KEY((neural OR brain OR cerebr*) W/2 (blood OR vascul* OR vessel? OR arter*) W/2 (accident* OR event* OR ischemi* OR ischaemi* OR infarct* OR occlusion* OR obstruction* OR insult OR insufficien* OR attack* OR arrest OR disturb* OR lesion* OR circulation OR failure* OR supply* OR flow))) OR (TITLE-ABS-KEY((neural OR brain OR cerebr* OR hemispher*) W/2 (ischemi* OR ischaemi* OR infarct* OR seizure* OR insult OR attack* OR occlusion* OR circulation))) OR (TITLE-ABS-KEY((ischemi* OR ischaemi*) W/2 (seizure* OR attack*))) OR (TITLE-ABS-KEY((intracranial OR cranial OR cerebr* OR brain*) W/3 (thromb* OR embol* OR angiopath* OR vasculopath* OR phlebothromb*)))) OR ((TITLE-ABS-KEY(apoplex*)) OR (TITLE-ABS-KEY(cva OR cvas)) OR (TITLE-ABS-KEY((transien* W/2 (ischemi* OR ischaemi*)) AND (encephal* OR brain* OR cerebr* OR neural OR cranial OR intracranial))) OR (TITLE-ABS-KEY(tia OR tias)) OR (TITLE-ABS-KEY(amauros* W/2 fuga*)) OR (TITLE-ABS-KEY((transien* OR transit*) W/3 blind*))))
1230 articles
30 conference papers

Web of Science

Databases=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH Timespan=All years

# 1	14,350	TS=(adiponectin* OR apm1 OR "apm 1" OR acrp30 OR "acrp 30" OR gbp28 OR "gbp 28" OR adipoq)
# 2	306	TS=((adipo* NEAR/5 "abundant gene*") OR (adipo* NEAR/5 protein NEAR/3 "30") OR (adipo* NEAR/3 collagen) OR (gelatin NEAR/3 protein NEAR/3 "28"))
# 3	14,550	#2 OR #1
# 4	89,149	TS=carotid
# 5	4,053	TS=((aort* or arter*) NEAR/2 thickness)
# 6	10,973	TS=((intimamedia* OR (intima* NEAR/3 media*)) NEAR/4 thickness)
# 7	1,034	TS=cimt
# 8	27,123	TS=((carotid OR plaque) NEAR (ather*sclero* OR (ather* NEAR sclero*)))
# 9	13,246	TS=(ather*genes* OR (ather* NEAR genes*))
# 10	13,136	TS=(arteri*sclero* OR (arteri* NEAR sclero*))
# 11	8,376	TS=(fibroatheroma* OR atheroma*)
# 12	431	TS=(fatty NEAR/2 arter*)
# 13	176,465	TS=stroke*
# 14	11,656	TS=(cerebrovascular NEAR/2 (accident* OR event* OR isch*emi* OR infarct* OR occlusion* OR obstruction* OR insult OR insufficien* OR attack* OR arrest OR disturb* OR lesion* OR circulation OR failure*))
# 15	56,402	TS=((neural OR brain OR cerebr*) NEAR/2 (blood OR vascul* OR vessel? OR arter*) NEAR/2 (accident* OR event* OR isch*emi* OR infarct* OR occlusion* OR obstruction* OR insult OR insufficien* OR attack* OR arrest OR disturb* OR lesion* OR circulation OR failure* OR supply* OR flow))
# 16	82,581	TS=((neural OR brain OR cerebr* OR hemispher*) NEAR/2 (isch*emi* OR infarct* OR seizure* OR insult OR attack* OR occlusion* OR circulation))
# 17	10,087	TS=(isch*emi* NEAR/2 (seizure* OR attack*))
# 18	16,868	TS=((intracranial OR cranial OR cerebr* OR brain*) NEAR/3 (thromb* OR embol* OR angiopath* OR vasculopath* OR phlebothromb*))
# 19	1,606	TS=apoplex*
# 20	1,992	TS=(cva OR cvas)
# 21	8,252	TS=((transien* NEAR/2 isch*emi*) NEAR (encephal* OR brain* OR cerebr* OR neural OR cranial OR intracranial))
# 22	5,988	TS=(Tia OR tias)
# 23	653	TS=(amauros* NEAR/2 fuga*)
# 24	545	TS=((transien* OR transit*) NEAR/3 blind*)
# 25	394,197	#24 OR #23 OR #22 OR #21 OR #20 OR #19 OR #18 OR #17 OR #16 OR #15 OR #14 OR #13 OR #12 OR #11 OR #10 OR #9 OR #8 OR #7 OR #6 OR #5 OR #4
# 26	799	#25 AND #3

# 28	729	#25 AND #3 Refined by: Document Types=(ARTICLE OR LETTER OR REVIEW OR EDITORIAL MATERIAL OR CORRECTION)
# 29	85	#25 AND #3 Refined by: Document Types=(MEETING ABSTRACT OR PROCEEDINGS PAPER)

Deduping details

		# imported records	# records after dedup TI	# records after dedup AU+YR	# records after dedup YR+VL+PG	# records after dedup JO+PG	# records after dedup TI+YR
Articles	Medline	1269	1260	1260	1260	1260	1260
	Embase	2045	1015	891	885	885	884
	PubMed	68	36	34	34	34	34
	Biosis	787	94	72	70	70	70
	Cochrane	99	1	1	1	1	1
	Scopus	1230	255	217	201	201	201
	WoS	729	207	198	196	195	195
Conferences	Embase	556	499	497	495	495	495
	Biosis	428	317	312	312	312	312
	Scopus	30	11	9	9	9	9
	WoS	85	34	33	33	33	33
TOTAL		7326	3729	3524	3496	3495	3494
Duplicates found			*5485*	*486*	254	*202*	*54*
Duplicates deleted			*3597*	*205*	28	*1*	*1*

APPENDIX B

Table I. Baseline population characteristics

Study	Year	Country	Population	N (% men)	Age (years)	BMI (kg/m^2)	cIMT (mm)
Aggarwal	2013	India	Subjects with coronary artery disease	50 (-)	43.7±8.2	-	0.68±0.10
Ahn	2011	Korea	Healthy subjects	1353 (69.0)	53.8±8.0	24.4±3.1	0.78±0.11
Behre	2006	Sweden	64-year-old women with varying glucose tolerance	NGT: 188 (0)	Not specified (~64)	-	-
				IGT: 204 (0)			
				T2DM: 220 (0)			
Bevan	2011	Germany	Members of a primary healthcare service population	990 (47.4)	53.1±9.0	27.0±4.1	0.74±0.14
Chaer	2012	USA	Community-based cohort	697 (38.0)	range 44-74	29.7	-
Dessein	2014	South Africa	Subjects with RA	208 (-)	-	-	0.71±0.10
Dessein	2013	South Africa	Subjects with RA	119 (10.9)	55.8±10.2	29.3±6.6	0.69±0.10
			Age- + sex-matched non-RA controls	158 (13.9)	56.5±10.9	33.7±8.0	0.70±0.12
Dullaart	2010	Netherlands	Subjects with and without MetS	161 (57.8)	56.9±9.6	27.1±3.8	0.85±0.17

Dullaart	2007	Netherlands	Subjects with T2DM	84 (61.9)	60.0 [52.9-65.8]	28.0 [25.0-31.9]	0.85 [0.76-1.01]
			Non-T2DM controls	85 (54.1)	53.8 [47.8-62.8]	25.3 [23.0-27.3]	0.78 [0.72-0.86]
Flyvbjerg	2009	14 European countries	Healthy subjects	148 (0)	51.0±3.0	25.7±4.7	0.60±0.08
Forst	2005	Germany	Subjects with T2DM	173 (61.80)	62.6±7.9	31.7±4.7	-
Gardener	2012	USA	Subjects with T2DM	298 (-)	-	-	-
			Non-T2DM controls	1224 (-)	-	-	-
Gokulakrishnan	2010	India	Subjects with varying glucose tolerance	NGT: 520 (-)	43.0±12.0	24.0±4.7	0.73±0.16
				IGT : 115 (-)	51.0±10.0	25.9±4.6	0.80±0.16
				T2DM: 540 (-)	51.0±11.0	25.3±4.3	0.86±0.26
Gonzalez-Gay	2011	Spain	Subjects with severe RA	182 (28.0)	61.0 [51.0–70.0]	-	-
Gustafsson	2010	Sweden	70 year old subjects	981 (50.0)	70.2±0.2	27.1±4.3	0.89±0.16
Hajmohammadi	2010	Iran	Subjects with MetS	44 (34.1)	55.6±8.6	30.7±6.2	Right: 0.73±0.14 Left: 0.77±0.19
			Non-MetS controls	44 (65.9)	52.9±7.6	26.0±3.7	Right: 0.68±0.12 Left: 0.68±0.14

Hanada	2014	Japan	Subjects who underwent annual health examinations	569 (72.6)	-	-	-
Hayashi	2011	Japan	Subjects with predialysis CKD	95 (63.5)	69.0±9.0	23.8±3.8	0.81±0.19
Hui	2014	China	Community-based cohort	265 (48.7)	54.6±12.3	24.9±3.7	0.63 [0.52-0.73]
Iglseder	2005	Austria	Healthy subjects	1515 (62.0)	51.9±6.0	26.7±3.9	0.76±0.13
Ignacy	2005	Japan	Hemodialysis subjects with ESRD	80 (42.5)	47.0±2.0	24.0±0.5	0.77±0.01
Jansson	2003	Sweden	Healthy relatives to subjects with T2DM	39 (100)	43.1 [40.3-45.8]	25.4 [24.6-26.2]	-
Juonala	2011	Finland	Subjects with MetS	205 (58.1)	33.5±4.6	30.7±4.9	0.61±0.11
			Non-MetS controls	1488 (44.3)	31.7±5.0	24.2±3.4	0.58±0.09
Karakitsos	2006	Greece	Hemodialysis subjects with stable ESRD	120 (60.0)	60.0±9.3	-	0.82±0.11
Kiris	2006	Turkey	Patients undergoing CABG procedure	84 (82.1)	60.8±10.6	27.6±5.0	CCA: 0.97±0.13 Bulb: 1.01±0.14
Kotani	2008	Japan	Healthy post-menopausal women	129 (0)	73.0±8.0	22.0±3.0	0.95±0.18

Liao	2014	China	Subjects without overt cardiovascular disease	160 (55.0)	55.3±4.8	26.5±4.1	0.69±0.14
Lim	2012	Korea	Community-based elderly cohort	1000 (43.9)	76.0±8.7	24.0±3.4	0.78±0.14
Liu	2010	China	Healthy first-degree relatives to subjects with T2DM	29 (44.8)	53.1±6.2	24.7±3.2	0.83±0.10
			Healthy controls without known family history of T2DM (matched for age, BMI, WHR, blood glucose, and fasting lipids)	20 (35.0)	53.6±9.0	24.5±3.5	0.85±0.15
Lo	2006	USA	Healthy women	100 (0)	40.0 [24.0-59.0]	25.9 [24.0-29.8]	0.61 [0.45-1.11] (n=99)
Mansouri	2012	Iran	Subjects with T2DM	101 (47.5)	53.6±8.4	27.7±4.1	0.80±0.20
			Age- + BMI-matched non-T2DM controls	42 (45.2)	50.1±8.4	28.7±4.6	0.66±0.20
Matsuda	2004	Japan	Subjects with T2DM	231 (58.4)	60.4±12.3	24.8±5.2	0.89±0.18
Miyanaga	2009	Japan	Young healthy female students	416 (0)	20.0 [19.8-21.0]	20.6 [19.4-22.1]	0.41 [0.37-0.45] (n=140)

Nagasaki	2005	Japan	Subjects with hypothyroidism	52 (17.3)	50.2±2.4	22.9±0.4	0.63±0.02
Nilsson	2006	Sweden	Community-based cohort	887 (42.0)	64.6±5.8	27.2±3.8	0.86±0.16
Nishida	2007	Japan	Healthy subjects who underwent health examinations	1078 (66.2)	48.4±5.8	22.9±3.0	0.68±0.19
Patel	2008	14 European countries	Healthy subjects	1306 (45.1)	43.8±8.3	25.6±3.9	0.60 [0.54-0.65]
Pemberton	2009	UK	Subjects with RA	46 (0)	56.0 [49.0-60.0]	25.5 [23.4-29.6]	0.53 [0.47-0.60]
			Age-matched non-RA controls	48 (0)	57.0 [51.5-60.0]	25.2 [23.3-28.8]	0.55 [0.47-0.62]
Persson	2015	Sweden	Subjects with high CVD risk but no prevalent disease	3430 (48.2)	64.6 [59.7-67.2]	26.8 [24.2-29.5]	CCA: 0.72 [0.65-0.80] BIF: 1.06 [0.86-1.34]
Rubio-Guerra	2013	Mexico	Hypertensive subjects with T2DM	30 (53.3)	60.0±9.0	30.4±5.0	1.43±0.43
			Normotensive + non-T2DM age-matched controls	30 (63.3)	58.0±11.0	29.8±6.0	0.59±0.06
Rundek	2013	USA	Multi-ethnic urban population	1790 (40.0)	69.4±9.3	28.2±5.0	0.92±0.09

Saarikoski	2010	Finland	Young healthy adults	2147 (45.3)	31.7±5.0	25.0±4.3	0.58±0.09
Shargorodsky	2009	Israel	Obese subjects	47 (25.5)	58.8±8.7	33.5±7.0	0.63±0.17
Shoji	2006	Japan	Participants of a medical check program	277 (49.1)	60.4±9.8	23.8±3.5	1.08±0.49
Störk	2007	Germany	Healthy post-menopausal women	142 (0)	59.1±4.3	26.4±4.6	1.19 ±0.23
Sturm	2009	Austria	Obese subjects	64 (25.0)	34.9±8.7	42.3±4.3	0.58±0.14
Tahara	2012	Japan	Consecutive outpatients	316 (30.7)	62.0±9.2	23.9±3.0	0.71±0.15
Tavridou	2015	Greece	Subjects with T2DM	119 (41.2)	67.8±3.4	31.0±5.0	0.89 [0.61-1.56]
Vasilescu	2011	Romania	Subjects with newly diagnosed T2DM	50 (50.0)	52.7±8.0	31.0±1.8	0.91±0.16
Vasilescu	2010	Romania	Obese subjects	56 (55.4)	52.9±8.7	34.1±3.3	0.89±0.18
Wang	2007	China	Clinically stable PD subjects	147 (54.4)	55.0±11.0	23.3±3.7	0.93±0.36
Yano	2009	Japan	Ambulatory patients with essential hypertension	263 (37.0)	72.6±8.4	24.4±3.5	0.81±0.17
Yazici	2012	Turkey	Subjects with T1DM	75 (52.0)	29.4±7.8	23.9±3.4	0.53±0.05

Yoon	2013	Korea	Healthy adult population in 5 geographic areas	1033 (43.9)	56.2±7.2	24.5±3.6	0.84 [0.72-0.98]
Yoon	2011	Korea	Healthy adult population in 5 geographic areas	1374 (68.9)	53.7 ± 8.0	24.4±3.1	0.74 [0.64–0.87]

BMI: body mass index; cIMT: carotid intima-media thickness; NGT: normal glucose tolerance; IGT: impaired glucose tolerance; T2DM: type 2 diabetes mellitus; RA: rheumatoid arthritis; MetS: metabolic syndrome; CKD: chronic kidne disease; ESRD: end-stage renal disease; CABG: coronary artery bypass grafting; CCA, common carotid artery; WHR: waist-hip ratio; CVD: cardiovascular disease; BIF: bifurcation; PD: peritoneal dialysis; T1DM, type 1 diabetes mellitus

Table II. Summary of relevant results assessing the relationship between adiponectin and cIMT in various populations

Study	Subgroup	Type of Analysis	Effect Estimates		P-value	Adjustment
Healthy population						
Ahn			r*	-0.07	<0.05	*Sex Model 1: age, sex. Model 2: age, sex, SBP, triglycerides, LDL-C, fasting blood glucose, waist circumference Model 3: age, sex, SBP, triglyceride, LDL-C, fasting blood glucose, WHR, hsCRP, smoking history (pack-years), HOMA-IR.
		Multivariate models				
		Model 1	ß (SE)	-34.4 (9.1)	0.01	
		Model 2	ß (SE)	-21.6 (9.8)	0.03	
		Model 3	ß (SE)	-20.5 (10.2)	0.04	
Flyvbjerg		Correlation between log (APN) and bilateral cIMT	r	-0.08	>0.05	
Gustafsson			ß (95%CI)*	-0.01 (-0.02;0.00)	0.21	*Age and sex †Age, sex, BMI, SBP, antihypertensive, antidiabetic, and lipid lowering treatment, log fasting blood glucose, TC, HDL-C, creatinine, smoking status.
			ß (95%CI)†	0.00 (-0.01;0.01)	0.97	
Iglseder	All (n=1515)		r	-0.065	0.012	
	Men (n=940)		r	-0.092	0.005	
	Women (n=575)		r	-0.086	0.039	
	Men (n=940)	Average cIMT increase (µm) per 1µg/mL decrease in APN		3.48 (1.23-5.73)		
	Women (n=575)	Average cIMT increase (µm) per 1µg/mL decrease in APN		2.39 (0.50-427)		
Jansson		Correlation between APN and bulb cIMT	r	-0.37	<0.05	

Kotani			r ß*	-0.137 -0.078	0.12 0.41	*Age, BMI, SBP, DBP, TC, triglycerides, HDL-C, glucose, insulin, HOMA-IR.
Lim			r	-0.133	<0.01	
Liu	First degree relatives of T2DM patients (n=29) Controls (n=20)		r ß* r ß*	-0.372 -0.319 -0.256 -0.449	0.037 0.059 >0.05 0.052	*Age, WHR, BMI, LDL-C, HDL-C, fasting plasma glucose, 2 h post-load glucose, IMT, EDV, HOMA-IR, HOMA-ß
Lo		Multivariate models Model 1 Model 2	ρ ß (SE) ß	-0.20 -0.0042 (0.0021) -0.0045	0.077 0.045 0.03	Model 1: age, race, smoking pack-years, visceral abdominal fat area, subcutaneous abdominal fat area. Model 2: Model 1 + BMI
Miyanaga		Lowest APN quartile <8.3 (n=40) Highest APN quartile ≥ 13.9 (n=45)	Mean cIMT Mean cIMT	0.45±0.06 0.39±0.06	0.03 0.03	
Nishida	Men Women	Association between log (APN) and cIMT Association between log (APN) and cIMT	r r	-0.199 0.111	0.013 0.27	
Patel	Men (n=589) Women (n=717)	Correlation between log (APN) and log (cIMT) Correlation between log (APN) and log (cIMT)	r r	-0.05 -0.05	0.257 0.258	
Saarikoski	General (n=2147)	Correlation between ln (APN) and cIMT Association between ln (APN) and cIMT	r ß*	-0.16 -0.018±0.005	<0.001 <0.001	*Age, sex, BMI, hsCRP, TC,

	Women (n=174)	Association between ln (APN) and cIMT	ß*	-0.023±0.007	0.003	HDL-C, triglycerides, SBP, glucose, smoking status
	Men (n=973)	Association between ln (APN) and cIMT	ß*	-0.016±0.006	0.01	
	Overweight (n=663)	Association between ln (APN) and cIMT	ß*	-0.017±0.009	0.054	
	Obese (n=261)	Association between ln (APN) and cIMT	ß*	-0.004±0.01	0.003	
Störk		Correlation between APN and mean max cIMT	r	-0.20	0.054	
		Association between APN and mean max cIMT	ß*	-0.013±0.009	0.053	*Age
Yoon, 2011		Multivariate models				Model 1: Age, sex, LDL-C, smoking history (pack-years) Model 2: M1 + BMI, WHR, hsCRP, SBP, DBP, triglycerides, HDL-C, fasting blood glucose and insulin, HOMA-IR. Model 3: M1+ WHR, hsCRP, DBP, triglycerides, HDL-C, fasting blood glucose, HOMA-IR
		Model 1	ß (SE)	-32.3 (9.4)	<0.001	
		Model 2	ß (SE)	-20.6 (10.3)	0.047	
		Model 3	ß (SE)	-21.2 (10.2)	0.039	
Yoon, 2013	Men (n=454)	Tertiles of APN	Mean cIMT			
		1st tertile ≤ 6.25		0.91 (0.76-1.04)		
		2nd tertile > 6.25		0.84 (0.72-1.00)		
		3rd tertile > 9.75		0.84 (0.68-0.99)	0.022	
	Women (n=579)	Tertiles of APN	Mean cIMT			
		1st tertile ≤ 9.45		0.88 (0.75-1.00)		
		2nd tertile > 9.45		0.84 (0.71-0.96)		
		3rd tertile > 13.40		0.80 (0.71-0.92)	0.018	
General population						
Bevan	Men (n=469)		r	-0.069	0.133	
	Women (n=521)		r	0.003	0.945	
	All cases (n=990)		r	-0.079	0.013	
		Multivariate models				Model 1: age and sex Model 2: age, sex, and BMI
		Model 1	r	-0.113	0.001	
		Model 2	r	-0.082	0.016	

Chaer			r	-0.20	<0.001	
Hanada		Quartiles of APN (µg/mL)	Mean cIMT			
		1st quartile, mean = 3.8±0.8	(mm)	0.71±0.14		
		2nd quartile, mean = 6.3±0.7		0.69±0.14		
		3rd quartile, mean = 9.3±1.2		0.68±0.13		
		4th quartile, mean = 16.4±5.3		0.64±0.14	<0.05	
Hui			r*	-0.184	0.003	*Age
Liao			ß	-0.031	0.01	
Nilsson	Men	Quartiles of APN (µg/mL)	Mean cIMT			Model 1: Mean IMT CCA adjusted for age Model 2: Model 1+WHR, smoking, HDL-C, DBP Model 3: Model 2+ HbA_{1c} (log-transformed) + HOMA (log-transformed) + lipid-lowering drugs + blood pressure-lowering drugs
		Unadjusted	(mm)			
	n=94	1st quartile, <5.3		0.92± 0.03		
	n=93	2nd quartile, 5.3-7.02		0.93± 0.02		
	n=93	3rd quartile, 7.02-9.16		0.93± 0.02	0.067	
	n=93	4th quartile, >9.16		0.92± 0.02		
		Model 1				
	n=94	1st quartile, <5.3		0.93± 0.02		
	n=93	2nd quartile, 5.3-7.02		0.89± 0.02		
	n=93	3rd quartile, 7.02-9.16		0.89± 0.02	0.02	
	n=93	4th quartile, >9.16		0.86± 0.02		
		Model 2				
	n=94	1st quartile, <5.3		0.93± 0.02		
	n=93	2nd quartile, 5.3-7.02		0.89± 0.02		
	n=93	3rd quartile, 7.02-9.16		0.90± 0.02	0.04	
	n=93	4th quartile, >9.16		0.86± 0.02		
		Model 3				
	n=94	1st quartile, <5.3		0.92±0.02		
	n=93	2nd quartile, 5.3-7.02		0.89± 0.02		
		3rd quartile, 7.02-9.16		0.89± 0.02	0.17	

	n=93	4th quartile, >9.16		0.87± 0.02		
	n=93					
	Women	Quartiles of APN (µg/mL)	Mean cIMT			
		Unadjusted	(mm)			
	n=129	1st quartile, <7.72		0.86±0.14		
	n=127	2nd quartile, 7.72-10.53		0.83±0.14		
	n=129	3rd quartile, 10.53-14.2		0.85±0.14	0.15	
	n=129	4th quartile, >14.2		0.82±0.13		
		Model 1				
	n=129	1st quartile, <7.72		0.86±0.01		
	n=127	2nd quartile, 7.72-10.53		0.84±0.01		
	n=129	3rd quartile, 10.53-14.2		0.85±0.01	0.11	
	n=129	4th quartile, >14.2		0.82±0.01		
		Model 2				
	n=129	1st quartile, <7.72		0.85±0.01		
	n=127	2nd quartile, 7.72-10.53		0.83±0.01		
	n=129	3rd quartile, 10.53-14.2		0.85±0.01	0.54	
	n=129	4th quartile, >14.2		0.83±0.01		
		Model 3				
	n=129	1st quartile, <7.72		0.85±0.01		
	n=127	2nd quartile, 7.72-10.53		0.83±0.01		
	n=129	3rd quartile, 10.53-14.2		0.85±0.01	0.56	
	n=129	4th quartile, >14.2		0.83±0.01		
Persson	Men	Association between APN and CCA IMT				
		Model 1	ß (95%CI)	-0.007 (-0.012 – -0.002)	0.006	Model 1: age Model 2: age, BMI, T2DM, SBP, current smoking, triglycerides, HDL-C, hs-CRP
		Model 2	ß (95%CI)	-0.003 (-0.008 – 0.002)	0.233	
		Association between APN and BIF IMT				
		Model 1	ß (95%CI)	-0.020 (-0.029 – -0.011)	<0.001	
		Model 2	ß (95%CI)	-0.018 (-0.027 – -0.009)	<0.001	
	Women	Association between APN and CCA IMT				

		Model 1	ß (95%CI)	-0.005 (-0.009 – -0.002)	0.007	
		Model 2	ß (95%CI)	-0.001 (-0.005 – 0.003)	0.644	
		Association between APN and BIF IMT				
		Model 1	ß (95%CI)	-0.013 (-0.021 – -0.005)	0.002	
		Model 2	ß (95%CI)	0.006 (-0.015 – 0.003)	0.185	
Rundek			r*	-0.06	<0.001	*Age
Shoji	All		ß	-0.019	>0.05	
	Men		ß	0.033	>0.05	
	Women		ß	0.046	>0.05	
Tahara		Tertiles of APN (µg/mL)	Mean cIMT			
		1st tertile, mean = 2.31±0.18	(mm)	0.74±0.17		
		2nd tertile, mean = 5.38±0.41		0.69±0.13		
		3rd tertile, mean = 12.58±0.97		0.69±0.14	<0.03	
Metabolic disorders						
Behre	Entire population (n=612)		r	-0.11	0.01	
			r*	-0.09	<0.05	
			r†	-0.09	<0.05	*GT †BMI ‡HDL-C §Triglycerides
			r‡	-0.09	<0.05	
			r§	-0.09	<0.05	
	T2DM (n=220)		r	-0.04	>0.065	
	IGT (n=204)		r	-0.14	0.056	
	NGT (n=188)		r	-0.002	>0.05	
Dullaart, 2007	T2DM and controls (n=169)	Multivariate models between ln (APN) and cIMT				
		Model 1	ß	-0.29	0.002	Model 1: age, T2DM, gender, and adipokines.
		Model 2	ß	-0.14	0.092	Model 2: age, T2DM, gender, and variables with which IMT is independently

	T2DM	Model 3	r	-0.27	0.006	associated as derived from other models Model 3: age, sex Model 4: HOMA-IR
		Model 4	r	-0.22	0.037	
	Controls	Model 3	r	-0.22	0.031	
		Model 4	r	-0.19	0.045	
Dullaart, 2010		Association between ln (APN) and cIMT	ß*	-0.211	0.005	*Age, sex
Forst			r	0.12	0.15	
Gardener	T2DM		ß	-0.014	0.01	
	Controls		ß	-0.005	0.07	
Gokularkrishnan						
	NGT (n=520)		r	-0.122	0.005	
	IGT (n=115)		r	-0.257	0.005	
	T2DM (n=540)		r	-0.043	0.324	
	All patients (n=1175)		ß*	-0.054	<0.001	*Age, gender, BMI, HOMA-IR and the presence of T2DM
Hajmohammadi	All patients	Association between APN and left cIMT	ß	-0.07	0.46	
		Association between APN and right cIMT	ß	-0.16	0.08	
	Ø MetS	Association between APN and left cIMT	ß	0.011	0.94	
		Association between APN and right cIMT	ß	-0.227	0.12	
	MetS	Association between APN and left cIMT	ß	-0.149	0.29	
		Association between APN and right cIMT	ß	-0.113	0.36	
Juonala	Ø MetS		r	-0.12	<0.01	
	MetS		r	-0.21	<0.01	
Mansouri	T2DM (n=101)	Correlation between total APN and cIMT	r	0.125	>0.05	
		Correlation between HMW APN and cIMT	r	0.109	>0.05	
		Association between total APN and cIMT	ß	-0.07	0.58	
		Association between HMW APN and cIMT	ß	0.07	0.53	
	Controls (n=42)	Association between total APN and cIMT	ß	-0.06	0.88	
		Association between HMW APN and cIMT	ß	-0.08	0.07	

Matsuda			r	0.042	>0.05	
Nagasaki			r	0.059	>0.05	
			ß*	-0.088	>0.05	*Thrombomodulin, BMI, MAP
Rubio-Guerra	Hypertensive T2DM		r	-0.11	>0.1	
	Controls		r	-0.39	<0.02	
Shargorodsky			r	-0.369	0.011	
			ß*	-0.41	0.015	*Age, sex, serum glucose, current smoking, MAP, TC, BMI
Sturm			r	0.037	>0.05	
Tavridou			r	0.14	0.13	
Vasilescu, 2010	Obese men		ß (95%CI)*	-0.237 (-0.0024;0.0017)	0.12	*Age, SBP, leptin, leptin/APN ratio. hs-CRP, HOMA-IR, fasting proinsulin, triglycerides, HDL-C, LDL-C, BMI
	Obese women		ß (95%CI)*	-0.138 (-0.0041;0.0025)	0.41	
Vasilescu, 2011	Men	Correlation between log (APN) and cIMT	r	-0.16	0.503	
	Women	Correlation between log (APN) and cIMT	r	-0.19	0.361	
Yano			r	0.122	0.049	
			ß*	-0.010	0.863	*Age, smoking status, 24-h SBP, des-acyl ghrelin
Yazici			r	-0.24	0.04	
Inflammatory disorders						
Dessein, 2013		Multivariate models				Model 1: age, sex Model 2: age, sex, WHR, hsCRP, angiotensin
	øRA (n=77)	Model 1	Partial R	-0.008	1.0	
		Model 2	Partial R	0.008	1.0	

	RA (n=119)	Model 1	Partial R	-0.076	0.4	converting enzyme inhibitor and diuretic use, statin use.
		Model 2	Partial R	-0.096	0.3	
Dessein, 2014	All	Independent relationship between ISD increments of total APN and cIMT	Partial R	0.042	0.6	
	>55yo					
	Yes		Partial R	0.031	0.8	
	No		Partial R	0.023	0.8	
	Obese					
	Yes		Partial R	0.068	0.6	
	No		Partial R	0.040	0.7	
	All	Independent relationship between ISD increments of HMW APN and cIMT	Partial R	-0.008	0.9	
	>55yo					
	Yes		Partial R	-0.057	0.6	
	No		Partial R	0.008	0.9	
	Obese					
	Yes		Partial R	-0.008	1.0	
	No		Partial R	-0.011	0.9	
Gonzalez-Gay			r	0.3946	0.04	
			r*	0.0249	0.91	*Sex, age at study, disease duration, rheumatoid factor status, and classic cardiovascular risk factors
Pemberton	Controls		r	0.065	>0.05	
	RA		r	0.067	>0.05	
Other chronic disorders						
Aggarwal			r	-0.198	0.220	
Hayashi		High APN group ≥12.3 n=50	Mean IMT	0.76±0.14	0.015	
		Low APN <12.3 n=45		0.86±0.23		

Ignacy		Kendal τ-test	0.046	>0.05	
Karakitsos		r	-0.227	0.022	
Kiris	Correlation between APN and				
	CCA cIMT	r	-0.581	<0.01	*BMI, glucose, TC, HDL-C, LDL-C, triglycerides, SBP, DBP, the number of diseased coronary arteries, hsCRP, age
	Bulb cIMT	r	-0.415	<0.01	
	Association between APN and				
	CCA cIMT	ß (95%CI)	-0.572 (-0.020;-0.009)	<0.001	
	Bulb cIMT	ß (95%CI)	-0.558 (-0.022;-0.010)	<0.001	
	CCA cIMT				
	Bulb cIMT				
		ß (SE)*	-0.568 (0.002)	<0.001	
		ß (SE)*	-0.559 (0.003)	<0.001	
Wang		r	-0.188	0.027	
		r*	-0.098	0.29	*Age, sex, DBP, lipoprotein A, smoking history, hemoglobin, T2DM, hsCRP, duration of dialysis, serum fetuin-A, serum albumin, triglycerides.

APN, adiponectin; cIMT, carotid intima-media thickness; SE, standard error; CI, confidence interval; T2DM, type 2 diabetes mellitus; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; MetS, metabolic syndrome; HMW, high-molecular weight; ISD, intra-individual standard deviation; CCA, common carotid artery; SBP, systolic blood pressure; LDL-C, low-density lipoprotein cholesterol; WHR, waist-hip ratio; hsCRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment-estimated insulin resistance; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; BMI, body mass index; DBP, diastolic blood pressure; EDV, end-diastolic volume; $HbA_{1c,}$ glycated haemoglobin A1c; MAP, mean arterial pressure; BIF, bifurcation
When not otherwise mentioned, associations and correlations were performed between non-transformed APN and mean cIMT

Table III. Pooled population baseline characteristics

Population	Pooled n	Pooled % men	Pooled age	Pooled BMI [$kg.m^{-2}$]	Pooled APN [µg/mL]	Pooled cIMT [mm]
Total Population	30281*	47.26	53.77±6.15	25.85±3.25	9.58±2.23	0.74±0.12
Healthy	12810	51.03	50.55±6.05	24.85±3.53	9.06±1.83	0.72±0.09
General Population	9381	46.25	63.39±5.46	27.13±2.35	10.16±2.78	0.80±0.09
Metabolic Disorders	2813	43.76	54.60±8.48	27.75±4.11	8.61±1.80	0.80±0.17
Inflammatory Diseases	555	18.44	58.55±3.50	28.24±4.78	7.01±0.65	0.68±0.09
Other Chronic Diseases	576	59.93	57.10±8.77	24.44±3.36	17.90±7.66	0.85±0.18

Age, BMI, APN, and cIMT are presented as mean±SD

BMI, body mass index; APN, adiponectin; cIMT, carotid intima media thickness

*The sum of "n" from healthy population, general population, and diseased populations (metabolic disorders, inflammatory diseases, and other chronic diseases) does not add up to the pooled "n" reported for the total population; the "n" for total population also includes control subjects (without disease) that were not included in the pooled "n" for the diseased subgroup populations.

Table IV. Baseline population characteristics

Plaque Studies								
Study	**Year**	**Study Design**	**Country**	**Population**	**N (% men)**	**Age (years)**	**BMI (kg/m^2)**	**Plaque Prevalence (%)**
Dessein	2014	Case-control	South Africa	RA	208 (-)	-	-	38.1
Grönwall	2014	Cross-sectional	USA	SLE	105 (7.6)	42.6±11.8	26.1±6.3	41.9
Komatsu	2012	Cross-sectional	Japan	Health check-up subjects	47 (100)	58.3±8.7	-	29.8
Kozakova	2012	Cross-sectional	European countries	RISC study cohort	1012 (42.8)	43.3±8.0	24.9±3.7	5.4
McMahon	2011	Cross-sectional	USA	SLE	250 (0)	42.0±13.1	26.1±6.5	17.2
McMahon	2014	Cross-sectional	USA	SLE	210 (0)	43.2±12.5	26.4±6.2	29.0
				Healthy controls	100 (0)	44.4±11.3	24.1±5.3	28.0
Pellitero	2009	Cross-sectional	Spain	T2DM	125 (56.8)	61.4±7.2	31.4±6.3	60.0
Reynolds	2010	Case-control	USA	SLE	119 (9.0)	42.6±12.4	25.9±6.3	43.0
Wang	2007	Cross-sectional	China	Stable PD	147 (54.4)	55.0±11.0	23.3±3.7	51.0
Yu	2008	Cross-sectional	China	Ambulatory PD	59 (47.5)	49.6±14.1	22.4±3.5	44.4

Ischemic Stroke Studies									
Study	**Year**	**Study Design**	**Country**	**Population**	**Follow-up period**	**N (% men)**	**Age (years)**	**BMI (kg/m^2)**	**IS Outcome (N)**

Arregui	2014	Prospective Case-cohort	Germany	EPIC-Potsdam Cohort	8.2±2.2 years 3(mean)	2155 (37.3)	50.1±0.5	26.1±0.2	170
Baranowska	2011	Cross-sectional	Poland	Subjects with first-ever IS	N/a	38 (0)	76.2±6.3	28.9±4.2	N/a
				Healthy stroke-free controls (matched for age and BMI)	N/a	38 (0)	75.3±5.3	27.6±3.8	N/a
Bidulescu	2013	Prospective Cohort	USA	Jackson Heart Study	6.2 years (mean)	4571 (36.0)	54.0±13.0	-	87
Bienek	2012	Cross-sectional	Poland	Subjects with IS	N/a	69 (46.4)	70.2±13.0	27.4±4.4	N/a
				Stroke-free controls	N/a	26 (46.2)	65.1±13.8	26.0±3.2	N/a
Chen	2005	Cross-sectional	Taiwan	Subjects with IS	N/a	228 (50.0)	69.3±10.0	24.4±3.8	N/a
				Stroke-free controls	N/a	306 (51.0)	62.7±8.6	25.2±1.7	N/a
Delva	2013	Cross-sectional	Ukraine	Subjects with IS	N/a	27 (-)	-	-	N/a
				Stroke-free controls	N/a	18 (-)	-	-	N/a
Jaleel	2010	Cross-sectional	Pakistan	Subjects with IS	N/a	40 (-)	58.2±0.9	-	N/a
				Stroke-free controls (matched for age, sex, and waist hip ratio)	N/a	40 (-)	58.0±0.9	-	N/a

Kantorova	2011	Cross-sectional	Slovak Republic	Subjects with IS	N/a	145 (55.2)	66.7±12.1	-	N/a
				Stroke-free controls (matched for age and sex)	N/a	67 (-)	63.06±11.2	-	N/a
Kim	2012	Cross-sectional	Korea	Subjects with IS	N/a	98 (79.6)	65.2±12.0	23.9±3.0	N/a
				Stroke-free controls	N/a	48 (60.4)	61.9±10.4	24.1±3.1	N/a
Kizer	2013	Prospective Cohort	USA	CHS	10.5 years (median)	3290 (-)	-	-	492
Kuwashiro	2014	Cross-sectional	Japan	Subjects with IS	N/a	171 (67.3)	68.3 ± 10.1	23.6±3.8	N/a
				Stroke-free controls (matched for age and sex)	N/a	171 (67.3)	68.1 ± 10.1	23.3±2.8	N/a
Marousi	2010	Cross-sectional	Greece	Subjects with IS	N/a	82 (64.6)	66.8±13.0	28.0±5.4	N/a
				Stroke-free controls (matched for age and sex)	N/a	30 (63.3)	66.3±13.1	28.8±4.6	N/a
Matsumoto	2008	Retrospective Nested case-control	Japan	JMS Cohort:					
				Subjects with IS	-	116 (-)	-	-	N/a
				Stroke-free controls (matched for age, sex, and community)	10.6±2.6 years	630 (49.4)	65.9±8.6	22.6±3.0	N/a

Montecucco	2010	Cross-sectional	Italy	Symptomatic subjects with severe ICA stenosis	N/a	18 (66.6)	73.5 [67.3-76.3]	-	N/a
				Asymptomatic controls with severe ICA stenosis	N/a	63 (60.3)	70.0 [65.0-75.0]	-	N/a
Musialek	2011	Cross-sectional	Poland	Symptomatic subjects with severe ICA stenosis	N/a	190 (67.4)	66.0 [60.0-72.0]	27.7 [25.5-30.1]	N/a
				Asymptomatic controls with severe ICA stenosis	N/a	110 (59.1)	67.0 [60.0-71.0]	27.8 [25.9-30.2]	N/a
Ogorodnikova	2010	Retrospective Nested case-control	USA	HaBPS Study:					
				Subjects with IS	-	855 (0)	-	-	N/a
				Stroke-free controls (matched for age, race ethnicity, date of entry into cohort, and follow-up time)	-	855 (0)	68.8±6.4	27.0±5.3	N/a
Ozturk	2010	Cross-sectional	Turkey	Symptomatic subjects with severe ICA stenosis	N/a	43 (-)	67.4±10.3	-	N/a
				Asymptomatic controls with severe ICA stenosis	N/a	12 (-)	46.9±12.3	-	N/a

Prugger	2013	Retrospective Nested case-control	France, Ireland	PRIME Cohort:					
				Subjects with first-ever IS	10 years	95 (100)	55.6±2.9	26.9±4.0	N/a
				Stroke-free controls (matched for age, study center, and date of recruitment)	10 years	190 (100)	55.5±2.7	26.6±3.3	N/a
Rajpathak	2011	Retrospective Nested case-control	USA	HaBPS Study:					
				Subjects with IS	-	972 (0)	68.7±6.4	27.7±5.9	N/a
				Stroke-free controls (matched for age, race ethnicity, date of study enrolment, and follow-up time)	-	972 (0)	68.7±6.4	27.0±5.3	N/a
Söderberg	2004	Retrospective Nested case-control	Sweden	MONICA and VIP Cohorts:					
				Subjects with first-ever IS	4.9 years (median)	231 (55.0)	-	-	N/a
				Stroke-free controls (matched for age, sex, type of cohort, and geographical area)	4.9 years (median)	461 (54.9)	-	-	N/a
Stott	2009	Retrospective Nested case-control	Ireland, Netherland, Scotland,	PROSPER Cohort:					
				Subjects with IS		179 (-)	-	-	N/a

				Stroke-free controls (matched for age, sex, and treatment allocated)	3.2 years (mean)	532 (50.8)	75.9±3.6	26.8±4.0	N/a
Zhang	2016	Cross-sectional	China	Subjects with LAA IS	3.2 years (mean) N/a	127 (73.2)	61.3±11.0	25.1±3.0	N/a
				Stroke-free controls (matched for age and sex)	N/a	58 (70.7)	60.9±9.2	24.7±3.1	N/a

Mortality Studies

Study	Year	Study Design	Country	Population	Follow-up period	N (% men)	Age (years)	BMI (kg/m^2)	Mortality Outcome (N)
Efstathiou	2005	Prospective Cohort	Greece	Subjects with first-ever IS	5 years	160 (55.0)	70.2±11.3	27.8±5.1	85
Marousi	2010	Prospective Cohort	Greece	Subjects with IS	6 months	82 (64.6)	66.8±13.0	28.0±5.4	15/76
Nagasawa	2011	Prospective Cohort	Japan	Subjects with IS	17 months (median)	421 (-)	-	-	-

BMI, body mass index; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; RISC, Relationship Between Insulin Sensitivity and Cardiovascular Disease; T2DM, type 2 diabetes mellitus; PD, peritoneal dialysis; IS, ischemic stroke; EPIC, European Prospective Investigation into Cancer; CHS, Cardiovascular Health Study; JMS, Jichi Medical School; ICA, internal carotid artery; HaBPS, Hormones and Biomarkers Predicting Stroke; PRIME, Prospective Epidemiological Study on Myocardial Infarction; MONICA, MONItoring trends and determinants of CArdiovascular disease; VIP, Västerbotten Intervention Program; PROSPER, Prospective Study of Pravastatin in the Elderly; LAA, large artery atherosclerosis

Table V. Summary of relevant results assessing the relationship between adiponectin and carotid plaque presence in various populations

Study	Population	APN isoform	Subgroup			Estimate effect	95 % CI	P value	Adjustments
Dessein	RA	Total	All	1SD increase in APN levels	OR	0.98	0.91-1.05	0.6	age, sex, race, waist circumfrence
			>55 yo		OR	0.98	0.89-1.07	0.07	
			<55 yo		OR	0.92	0.75-1.02	0.08	
			Obese		OR	0.93	0.84-1.02	0.1	
			Not obese		OR	0.99	0.90-1.09	0.9	
			Abdominal obesity		OR	0.87	0.76-0.99	0.02	
			No abdominal obesity		OR	1.08	0.92-1.13	0.7	
		HMW	All	1SD increase in APN levels	OR	0.98	0.91-1.05	0.6	age, sex, race, waist circumfrence
			>55 yo		OR	0.99	0.94-1.04	0.07	
			<55 yo		OR	0.95	0.91-1.01	0.1	
			Obese		OR	0.96	0.90-1.01	0.1	
			Not obese		OR	1.00	0.96-1.05	1.0	
			Abdominal obesity		OR	0.92	0.85-0.99	0.02	
			No abdominal obesity		OR	1.02	0.94-1.06	0.6	
Grönwall	SLE	Total		10-unit increase in APN levels	OR	2.40	1.2-4.9	0.01	age, hypertension, total cholesterol
				redictive value of APN>21µg/mL	OR	2.60	1.0-6.5	0.15	
Reynolds	SLE	Total		10-unit increase in APN levels	OR	1.80	1.1-3	0.02	-
Wang	PD	Total		APN levels	OR	1.00	1.00-1.00	0.12	

RA, rheumatoid arthritis; yo, years old; SD, standard deviation; APN, adiponectin; OR, odds ratio; CI, confidence interval; HMW, high-molecular weight; SLE, systemic lupus erythematosus; PD, peritoneal disease

Table VI. Summary of adiponectin levels in subjects with and without atherosclerotic plaque, when reported

Study	Population	APN isoform	Subgroup	Mean APN	SD	P value
Grönwall	SLE	Total	No plaque	14.6	9.4	0.02
			Plaque	18.7	9.0	
Komatsu	Health check-up	HMW	No plaque	3.18	1.45	0.048
			Plaque	2.28	0.9	
Kozakova	RISC study	Total	No plaque	Median: 8.3	IR: 4.1	ns
			Plaque	Median: 7.9	IR: 4.5	
McMahon, 201	SLE	Total	No plaque	14.60	7.50	ns
			Plaque	17.00	12.30	
McMahon, 2014	Healthy Controls	Total	No plaque	13.30	6.30	ns
			Plaque	13.80	8.00	
	SLE		No plaque	14.30	7.60	ns
			Plaque	16.30	10.20	
Pellitero	T2DM	Total	No plaque	10.97	7.06	-
			Plaque	10.90	5.64	
Reynolds	SLE	Total	No plaque	14.40	9.30	0.003
			Plaque	18.10	8.70	
Yu	Ambulatory PD	Total	No plaque	15.17	7.48	<0.01
			Plaque	9.60	5.09	

SLE, systemic lupus erythematosus; HMW, high-molecular weight; RISC, Relationship Between Insulin Sensitivity and Cardiovascular Disease; IR, interquartile range; T2DM, type 2 diabetes mellitus; PD, peritoneal disease

Table VII. Summary of relevant results assessing the relationship between adiponectin and ischemic stroke in various populations

Study	APN isoform	Subgroup			Estimate effect	95 % CI	P value	Adjustments
Arregui			5µg/mL higher total-APN					M1: stratified for age at baseline and adjusted for sex M2: M1+ waist circumference, smoking status, sports activity, education, alcohol consumption, prevalent HTN M3: M2+fast status, prevalent DM, HDL, TG, hsCRP
	Total	All population	Model 1	HR	1.05	0.85-1.30		
			Model 2	HR	1.10	0.89-1.37		
			Model 3	HR	1.31	1.04-1.64		
	Total	Women	Model 1	HR	1.09	0.85-1.40		
			Model 2	HR	1.17	0.92-1.50		
			Model 3	HR	1.30	1.02-1.67		
	Total	Men	Model 1	HR	0.97	0.69-1.37		
			Model 2	HR	0.93	0.66-1.31		
			Model 3	HR	1.15	0.76-1.73		
Bidulescu	Total	Women	1 SD increase	HR	1 .41	1.04-1.91	0.03	age, BMI, SBP, blood pressure medication, HDL, TG, hsCRP, insulin resistance, smoking, physical activity
	Total	Men	1 SD increase	HR	1.18	0.79-1.74	0.42	
Chen	Total	All population	Univariate	OR	0.86	0.83-0.90	<0.001	Model 1: age, sex, BMI, WHR, fasting glucose, SBP, DBP, total cholesterol, TG, HDL, LDL, uric acid, creatinine, hsCRP, smoking status
			Model 1	OR	0.83	0.78-0.88	<0.001	
Kim	Total	Non-Stroke	Per 1 µg/mL increase in APN	OR	1.00			
		Non-LAA stroke	Per 1 µg/mL increase in APN	OR	0.90	0.79-1.02		
		LAA stroke	Per 1 µg/mL increase in APN	OR	0.79	0.64-0.98		
		Total IS	Per 1 µg/mL increase in APN	OR	0.56	0.56-0.98		
Kizer	Total	All population	Model 1: APN <20 µg/mL	HR	0.97	0.82-1.15	0.719	Model 1: age, sex, race Model 2: M1+ BMI, income, education, center, smoking status, alcohol use, SBP, antihypertensive medication, estrogen replacement therapy, eGFR, aspirin use, health status, and albumin
			Model 1: APN ≥20 µg/mL	HR	1.14	0.97-1.36	0.118	
			Model 2: APN <20 µg/mL	HR	0.91	0.76-1.09	0.309	
			Model 2: APN ≥20 µg/mL	HR	1.13	0.95-1.34	0.177	
			Model 3: APN <20 µg/mL	HR	0.85	0.70-1.05	0.128	
			Model 3: APN ≥20 µg/mL	HR	1.08	0.88-1.33	0.475	
			Model 4: APN <20 µg/mL	HR	0.97	0.81-1.16	0.737	
			Model 4: APN ≥20 µg/mL	HR	1.15	0.97-1.37	0.110	

			Model 5: APN <20 µg/mL	HR	1.07	0.88-1.30	0.475	Model 3: M2 + N-terminal pro-hormone of brain natriuretic peptide Model 4: M2 + subclinical CVD Model 5: M4 + DM, LDL, HDL, TG, hsCRP Model 6: M4 + HOMA-IR, LDL, HDL, TG, hsCRP
			Model 5: APN ≥20 µg/mL	HR	1.15	0.96-1.37	0.126	
			Model 6: APN <20 µg/mL	HR	1.10	0.89-1.35	0.392	
			Model 6: APN ≥20 µg/mL	HR	1.16	0.97-1.38	0.104	
	HMW	All population	Model 1: HMW APN <10 µg/mL	HR	0.96	0.79-1.18	0.712	
			Model 1: HMW APN ≥10 µg/mL	HR	1.09	0.95-1.25	0.237	
			Model 2: HMW APN <10 µg/mL	HR	0.89	0.72-1.11	0.310	
			Model 2: HMW APN ≥10 µg/mL	HR	1.08	0.94-1.24	0.306	
			Model 3: HMW APN <10 µg/mL	HR	0.81	0.64-1.04	0.094	
			Model 3: HMW APN ≥10 µg/mL	HR	1.02	0.86-1.21	0.823	
			Model 4: HMW APN <10 µg/mL	HR	0.96	0.78-1.19	0.735	
			Model 4: HMW APN ≥10 µg/mL	HR	1.10	0.96-1.26	0.189	
			Model 5: HMW APN <10 µg/mL	HR	1.05	0.83-1.32	0.684	
			Model 5: HMW APN ≥10 µg/mL	HR	1.12	0.97-1.29	0.121	
			Model 6: HMW APN <10 µg/mL	HR	1.05	0.82-1.35	0.691	
			Model 6: HMW APN ≥10 µg/mL	HR	1.12	0.97-1.29	0.119	
Marousi	Total		Univariate analysis					
			LAA	ß	-0.049	-	0.665	
			APN levels					
		All population	Model 1	OR	0.92	0.86-0.99	0.027	
			Model 2	OR	0.88	0.79-0.98	0.016	
			Model 3	OR	0.82	0.72-0.94	0.005	
		Women	Model 1	OR	0.99	0.93-1.06	0.751	
			Model 2	OR	0.92	0.78-1.09	0.334	
			Model 3	OR	0.71	0.49-1.02	0.065	
		Men	Model 1	OR	0.88	0.79-0.99	0.029	
			Model 2	OR	0.83	0.70-0.97	0.023	
			Model 3	OR	0.82	0.69-0.98	0.024	
			Per 1 SD increase					
		All population	Model 1	OR	0.62	0.35-1.07	0.086	
			Model 2	OR	0.45	0.22-0.93	0.032	
			Model 3	OR	0.34	0.15-0.79	0.012	
		Women	Model 1	OR	0.96	0.88-1.05	0.394	

			Model 2	OR	0.55	0.16-1.88	0.337	
			Model 3	OR	0.11	0.01-0.40	0.090	
		Men	Model 1	OR	0.91	0.83-1.00	0.050	
			Model 2	OR	0.33	0.11-0.96	0.043	
			Model 3	OR	0.345	0.12-1.02	0.055	
			Per tertile of APN					
			Model 1: low (ref)	OR	1.00			
			Model 1: med	OR	0.14	0.03-0.61	0.009	
			Model 1: high	OR	0.35	0.16-0.78	0.010	
			Model 2: low (ref)	OR	1.00			
			Model 2: med	OR	0.03	0.00-0.31	0.003	
			Model 2: high	OR	0.26	0.09-0.76	0.014	
			Model 3: low (ref)	OR	1.00			
			Model 3: med	OR	0.03	0.00-0.31	0.003	
			Model 3: high	OR	0.19	0.05-0.69	0.011	
Matsumoto			Per 1 SD increase of log APN					M1: age, sex M2: M1 + HDL, triglyceride, BMI M3: M2+ current smoking, SBP, hs-CRP
			Model 1	OR	0.88	0.71-1.09	0.23	
			Model 2	OR	0.92	0.72-1.18	0.50	
			Model 3	OR	0.98	0.76-1.27	0.90	
Montecucco	Total		APN and risk of symptomatic stroke	OR	0.87	0.66-1.14	0.306	
Ogorodnikova	HMW		APN quartiles					Model 1: age, race/ethnicity = matching factors Model 2: Model 1 + BMI groups, DM2, HTN, smoking, LDL, HDL, metabolic equivalents, hs-CRP, aspirin use
			Model 1					
			< 5.2 µg/mL (ref)	OR	1.00			
			5.2-11.0 µg/mL	OR	1.02	0.78-1.35		
			11.1-18.8 µg/mL	OR	1.03	0.78-1.37		
			> 18.8 µg/mL	OR	0.96	0.71-1.29	0.71	
			Model 2					
			< 5.2 µg/mL (ref)	OR	1.00			
			5.2-11.0 µg/mL	OR	0.99	0.71-1.37		
			11.1-18.8 µg/mL	OR	1.37	0.99-1.91		
			> 18.8 µg/mL	OR	1.25	0.88-1.79	0.14	
Prugger			Univariate	OR	2.40	1.64-3.25	<0.001	Univariate analysis : age, study center, and date of recruitment
			Multivariate	OR	2.78	1.74-4.44	<0.001	

			Mutually adjusted	OR	1.82	1.04-3.19	0.036	Multivariate analysis : SBP, antihypertensive treatment, cig smoking, alcohol drinking, total cholesterol, hdl, waist circumference, DM *Model was fitted using stepwise backward selection from traditional risk factors and subsequently, including eligible biomarker
Rajpathak			Per APN quartiles					Model 1: Age + ethnicity Model 2: Age + ethnicity + BMI Model 3: Age + ethnicity + current smoking, physical activity, nonsteroidal anti-inflammatory drug use, hypertension medication use, SBP, history of coronary and artery diseases, HDL, TG,DM, WC
			Model 1					
			Median: 14.8 µg/mL (ref)	OR	1.00			
			Median: 24.0 µg/mL	OR	0.91	0.71-1.17		
			Median: 33.0 µg/mL	OR	0.70	0.54-0.91		
			Median: 46.0 µg/mL	OR	0.77	0.59-1.01	0.023	
			Model 2					
			Median: 14.8 µg/mL (ref)	OR	1.00			
			Median: 24.0 µg/mL	OR	0.92	0.71-1.19		
			Median: 33.0 µg/mL	OR	0.72	0.55-0.94		
			Median: 46.0 µg/mL	OR	0.81	0.61-1.08	0.068	
			Model 3					
			Median: 14.8 µg/mL (ref)	OR	1.00			
			Median: 24.0 µg/mL	OR	1.08	0.81-1.43		
			Median: 33.0 µg/mL	OR	0.89	0.65-1.22		
			Median: 46.0 µg/mL	OR	1.16	0.82-1.63	0.588	
Söderberg	Total	Women	14.5-27.6 µg/mL	OR	0.78	0.41-1.45	0.4	
		Men	8.3-17.3 µg/mL	OR	0.90	0.50-1.61	1.0	
Stott			Placebo and Pravastatin	OR	0.79	0.63-0.99	0.042	Adjusting for TNF-α, IL-18, log CRP, log IL-6, and fibrinogen
			Placebo only	OR	0.79	0.58-1.07	0.13	
			Pravastatin only	OR	0.81	0.58-1.13	0.22	

APN, adiponectin; BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HMW, high molecular weight; HOMA-IR, homeostatic model assessment - estimated insulin resistance; HR, hazard ratio; hsCRP, high-sensitivity C-reactive protein; HTN, hypertension; LAA, large artery atherosclerosis; LDL, low-density lipoprotein; OR, odds ratio; SBP, systolic blood pressure; SD, standard deviation; TG, triglycerides; WC, waist circumference; WHR, waist-hip ratio.

Table VIII. Summary of relevant results assessing the relationship between adiponectin and mortality in various populations

Study	APN isoform			Estimate effect	95 % CI	P value	Adjustments
Efstathiou		Death within 5 years of stroke					
	Total	Lowest vs highest tertile of APN	RR	8.1	3.1-24.5	<0.001	
		Middle vs highest tertile of APN	RR	4.3	1.5-14.6	<0.01	
		Death within 5 years of stroke	OR	3.8	1.6-9.1	-	
		APN <4 μg/mL	HR	5.2	2.1-18.4	<0.001	
Marousi		Acute post-stroke APN and vascular death	OR	1.00	0.92-1.08		Age, gender, presence of DM, HTN, AFib, hyperlipidemia, smoking, and TOAST classification of disease, neurological severity on admission (sum of scandinavian stroke scale) and pre-stroke modified ranking scale
	Total					0.912	
Nagasawa	Total	Highest vs middle + lowest APN tertiles					
		Stroke mortality	HR	6.90	1.34-35.5		
		All-cause mortality	HR	1.90	0.87-4.14		

AFib, atrial fibrillation; APN, adiponectin; DM, diabetes mellitus; HR, hazard ratio; HTN, hypertension; OR, odds ratio; RR, risk ratio.

Table IX. Pooled baseline characteristics for ischemic stroke and mortality articles included in meta-analyses

Meta-analysis	Pooled n	Pooled % men	Pooled age [years]	Pooled BMI [kg.m^{-2}]	Pooled APN [µg/mL]
Ischemic Stroke	13683	33.3	56.9±8.3	26.2±2.8	10.95±1.54
*Cases	1414	10.9	67.5±6.1	27.9±5.8	24.91±0.95
*Controls	6283	33.4	66.3±6.8	25.4±4.3	18.72±0.56
Mortality	663	63.5	69.0±11.9	27.9±5.2	9.96±7.14

Age, BMI, and APN are presented as mean±SD
BMI, body mass index; APN, adiponectin
Pooling was not performed for plaque studies, since baseline information was only available for one of the two studies included in the meta-analysis.
*Includes only the studies where baseline information is available for cases and controls, separately