The cholesteryl ester transfer protein in Alzheimer's disease

Felix Oestereich

Integrated Program in Neuroscience
McGill University, Montréal, Québec
December 2018

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

Doctor of Philosophy

DEDICATION

To my family in Germany as well as my Montréal family!

Thank you all!


IN DOM INO
CON FI DO
McGill

ACKNOWLEDGEMENTS

I know that these few words can't express how grateful I am for everyone that helped me in any way with my thesis. Your feedback, your stimulating discussions, and your support helped me grow as a scientist over the last 6 years. And all the remarkable friends I made here in Montréal are the reason for some of my fondest memories. You made my time in Montréal special, **THANK YOU!**

Lisa, I will forever be grateful to you for taking me on as a student, guiding my journey from an undergraduate to a doctorate. It has been inspirational having you as my mentor. Not only did you sculpt me into the scientist I am today, you always gave everything to nurture a productive, stimulating, engaging and fun atmosphere in the lab. I could not have wished for a better supervisor! Now, that I am sitting here, realizing that my time in your lab is coming to an end, I feel incredibly sad but also excited at the same time. I will truly miss my time here!

I would also like to thank my committee members ***Dr. Dusica Maysinger*** and ***Dr. Edith Hamel*** for their continuous feedback, support, and encouragement! Your interest in my project and inspiring discussions had a shaped my project considerably.

Additionally, I am incredibly grateful for all the collaborators that helped me throughout my Ph.D. (in no particular order): ***Dr. Pierre Chaurand, Ethan Yang, Elizabeth-Ann Kranjec, Dr. Claus Pietrzik, Dr. Richard Morton, Dr. Rainer Haag, Dr. Scott Kiss***, and ***Dr. Claudio Cuello***.

Sandra, THANK YOU for proof-reading my thesis, your continuous help, your feedback, and supply of only the finest German chocolate! You were always available when I needed an extra pair of hands. I can't thank all of my students enough. Thank you, ***Sasen*** for jumping on the CETP project and helping me out countless times. Additionally, I would not have been able to finish my thesis without the help of my students ***Lotus, Leo, Sebastian*** and especially ***Helen*** who helped me carry-

ing out and analyzing experiments. Next, I want to thank the rest of the Munter lab. ***Sherilyn*** and ***Jackie***, I am especially thankful for your help during my dissection sessions. Also, thank you ***Mehdi*** and ***Erik H.*** for helping me get this project started and ***Mei***, ***Jing***, and ***Paoula*** for managing the lab and making sure everything runs smoothly!

While not directly working with me, I have to thank everyone on the Bellini ground floor. Thank you, ***Filip, Shireen, Rob, James, Irem, Phoebe, Can, Adeola*** and ***Ryan, Erik, Martina, Amanda*** and everyone else from on the ground floor for Pho-Fridays, lots of stimulating Friday-evening discussions, and the great atmosphere you all helped to create. A special thank you to ***Adamo*** for proof-reading my thesis.

Outside of the lab, great friends in Montreal made sure I enjoy my life to the fullest. ***Doug***, I did not expect to find one of my best friends when I moved into the De Bullion apartment, thanks for being a reliable friend. Thank you ***Andy*** & ***Sanne*** for amazing BBQs, lots of ski trips and thank you, ***Natasha***, ***Ryan***, and ***Evan*** for being amazing friends.

Some of my best memories over the last few years are from bike rides with all my amazing two-wheeled friends. Thank you ***Adithya*** for always staying in the red, ***Nick*** for being you, ***Dylan*** for inspiring conversations, ***Ollie*** for allowing me the true Quebecois cottage life. I will always cherish our incredible rides in Vermont and South Carolina, crazy race weekends, silly photo shoots, and all the pain and suffering.

Finally, I want to thank my family for supporting me in every way you could! I would not have been able to be here without your constant support! Thank you, ***Mama***, for continuously sending me German goodies despite some of them getting confiscated at the Canadian Border. And ***Lisa,*** you are a great source of inspiration. ***Linus, Moritz*** & ***Sandra***, thank you for being an inspiration and supporting me. And ***Papa***, thank you for your support.

You are the BEST!

TABLE OF CONTENTS

DEDICATION 3

ACKNOWLEDGEMENTS 6

TABLE OF CONTENTS 10

ABSTRACT 17

ABRÉGÉ 19

LIST OF TABLES 21

LIST OF FIGURES 22

AUTHOR CONTRIBUTIONS 23

SUMMARY OF ORIGINAL CONTRIBUTIONS 24

GENERAL INTRODUCTION 26

1. Cholesterol structure and function 26
   1.1 Structure and function of cholesterol 26
   1.2 *De novo* cholesterol synthesis 27
   1.3 Regulation of cholesterol synthesis 28
   1.4 Cholesterol transport – the importance of lipoproteins 29
2. The cholesteryl ester transfer protein 31
   2.1 Function of CETP 31
   2.2 Structure of CETP 32
3. Cholesterol & lipids in disease 33
   3.1 Treatment strategies in cardiovascular disease – statins 35
   3.2 Treatment strategies in cardiovascular disease – PCSK9 inhibitors 35
   3.3 Treatment strategies in cardiovascular disease – CETP inhibition 36
4. Lipoprotein particles in the brain and the blood-brain barrier 38
5. CETP in the central nervous system 40
6. Alzheimer's disease 42
   6.1 Alzheimer's disease pathology 42
   6.2 The amyloid pathology in Alzheimer's disease 43
   6.3 The role of lipids and cholesterol in Alzheimer's disease 45
   6.4 Genetic risk factors for Alzheimer's disease 48
   6.5 Treatment options for Alzheimer's disease 49
7. A role for CETP in Alzheimer's disease 52
8. Aim of the thesis 55

EXPERIMENTAL SECTION 56

FOREWORD MANUSCRIPT 1 57

MANUSCRIPT 1: 58

1. Abstract 59

2. Introduction 59
2.1 Cholesterol and lipoproteins 59
2.2 The cholesteryl ester transfer protein 60
3. Materials & Methods: 61
3.1 Cell culture 62
3.2 Western blot analysis of mouse tissue samples 62
3.3 Quantitative real time PCR (RT-qPCR) 62
3.4 Imaging mass spectrometry (IMS) 64
3.5 Mouse housing 65
3.6 Mouse genotyping 65
3.7 Plasma lipid analysis 66
3.8 CETP activity assay 66
3.9 Astrocyte purification 66
3.10 Flow cytometry 66
3.11 Astrocyte microarray 66
3.12 Statistical analysis 67
4. Results 67
4.1 Dietary cholesterol intake induces CETP expression 67
4.2 CETP promotes TREM2 expression in the liver 71
4.3 CETP activity promotes peripheral inflammation 73
4.4 CETP changes the brain cholesterol composition 76
4.5 Transcriptional changes in CETPtg brain induced by presenilins 78
4.6 CETP activates γ-secretase *in vitro* ***81***
5. Discussion 83
5.1 CETP-mediated increase in brain cholesterol 83
5.2 Several Alzheimer-related changes triggered in CETPtg mice 84
6. Acknowledgements 87
7. Author contribution 87
FOREWORD MANUSCRIPT 2 88
MANUSCRIPT 2 89
1. Abstract 90
2. Introduction 90
2.1 Alzheimer's disease 90
2.2 The role of cholesterol in Alzheimer's disease 91
2.3 Risk factors for sporadic Alzheimer's disease 92
2.4 CETP and Alzheimer's disease pathology 93
3. Materials & methods 94
3.1 HEK293T Cell culture 94
3.2 Aβ quantification from cell culture supernatant 95
3.3 CETP activity assay 95
3.4 Western blot analysis of mouse tissue samples 95
3.5 Mouse housing and breeding 95
3.6 Mouse genotyping 96

3.7 Aβ measurement from mouse cortex 96
3.8 Quantitative real-time PCR (RT-qPCR) 97
3.9 Novel Object recognition (NOR) 97
3.10 Y-Maze spontaneous alteration 98
3.11 Evans blue BBB integrity assay 98
3.12 Statistical analysis 98
4. Results 99
4.1 CETP increases Aβ *in vitro* ***99***
4.2 CETP expression does not increase Aβ in 5xFAD mice 102
4.3 CETP expression increases Aβ in McGill-Thy1-APP mice 104
4.4 Memory impairment in CETP/Thy1 APP McGill mice 108
4.5 BBB is intact in CETP/Thy1 APP McGill mice 109
5. Discussion 111
5.1 Cholesterol metabolism in Alzheimer's disease 111
5.2 Does CETP activity increase Aβ generation? 112
5.3 How could CETP increase Aβ generation 114
5.4 Difference between the 5xFAD and Thy1 APP McGill mouse models 115
6. Acknowledgements 117
GENERAL DISCUSSION 118
1. Alzheimer's disease 118
1.1 Pathological hallmarks of Alzheimer's disease 118
1.2 The amyloid cascade hypothesis 118
1.3 Struggles and pitfalls in current Alzheimer's disease research 119
1.4 Epidemiologic and genetic risk factors 119
2. CETP is linked with Alzheimer's disease 120
2.1 CETP transgenic mice 121
2.2 Does CETP modify brain lipids? 122
2.3 How do these changes relate to CETP studies in humans? 123
3. Where does extra brain-cholesterol come from? 124
3.1 What are the downstream effects of the cholesterol increase? 125
4. CETP in Alzheimer's disease mouse models 126
4.1 What are the differences between our mouse models? 126
5. Targeting cholesterol to treat Alzheimer's disease? 127
6. The need to include a cholesterol phenotype in AD models 128
7. What comes next? 129
CONCLUSION 132
ABBREVIATIONS 133
REFERENCES 136
APPENDIX 185
Embedded in the membrane: How lipids confer activity and specificity to intramembrane proteases 186

ABSTRACT

Alzheimer's disease is a devastating neurodegenerative disease, affecting millions of families every year. Decades of research has widened our understanding of the biochemistry, the pathology, as well as genetic and lifestyle risks factors underlying the disease. Nonetheless, after over 30 years of biochemical research, this knowledge has not transformed into successful therapeutic strategies. Virtually, all drug candidates whether an antibody or small molecule have failed to show clinical efficacy.

A potential explanation for these failures could be that a significant proportion of fundamental Alzheimer's disease research has focussed on animal models harboring mutations found only in very rare and aggressive cases of familial Alzheimer's disease. However, most risk factors for sporadic Alzheimer's disease have been disregarded. The logical next step to improve current disease models is to include cardiovascular risk factors as they include some of the strongest genetic and epidemiological risk factors identified thus far. The most striking difference between the human and murine lipid and cholesterol metabolism is the lack of the cholesteryl ester transfer protein (CETP). As a result, mice have negligible levels of low-density lipoproteins, a strong risk factor for both Alzheimer's disease and cardiovascular disease. CETP is a lipid transfer protein responsible for the exchange of cholesteryl esters and triglycerides between lipoproteins and based on its ability to increase the levels of the low-level lipoprotein, is considered pro-atherogenic. In return, decreased CETP activity is associated with longevity, cardiovascular health, and reduced incidence of Alzheimer's disease. With the goal to generate an Alzheimer's disease mouse model with a humanized lipoprotein profile, we studied mice transgenic for human CETP. While there is abundant literature available investigating CETP and its role in the periphery, its role in the central nervous system is poorly defined.

Our analysis revealed that CETP transgenic mice had up to 31% higher cholesterol levels in the brain. Further, a microarray using astrocyte-derived mRNA showed that this cholester-

ol increase is unlikely the result of increased astrocytic *de novo* synthesis. Additionally, leakiness of the blood-brain barrier was not responsible either. More research effort is required to identify the source of this remarkable cholesterol increase. Nonetheless, we were able to describe downstream effects of the cholesterol increase. Strikingly, both presenilin 1 and presenilin 2 were among the top targets. Presenilins are the catalytic subunits of the γ-secretase complex, a key component in the generation of amyloid-β in the brain. Our data suggest that CETP transgenic mice phenocopy human plasma-cholesterol levels and cholesterol exposure of the brain as a valuable research tool to investigate the impact of the cholesterol metabolism on brain functions in relation to Alzheimer's disease.

To further study the effect of CETP on Alzheimer's disease, we generated a novel mouse model combining a humanized cholesterol metabolism with Alzheimer's disease pathology. Our analysis revealed that CETP activity increases soluble and insoluble levels of amyloid-β in a presenilin-dependent manner. The CETP-induced increase of amyloid production was suppressed in mice expressing familial presenilin mutations, underlining problems with most current animal models for Alzheimer's disease.

ABRÉGÉ

La maladie d'Alzheimer est une maladie neurodégénérative et dévastatrice qui chaque année affecte des millions de familles. Des décennies de recherche ont élargi notre compréhension de la biochimie, de la pathologie, ainsi que des facteurs de risque, soit génétiques soit liés au mode de vie sous-jacents de la maladie. Pourtant, après plus de 30 ans de recherche biochimique, ces connaissances ne se sont pas transformées en stratégies thérapeutiques efficaces. Pratiquement, tous les médicaments candidats, qu'il s'agisse d'un anticorps ou d'une petite molécule, n'ont pas démontré d'efficacité clinique.

Une explication possible de ces échecs pourrait être qu'une grande partie des recherches fondamentales sur la maladie d'Alzheimer est concentrée sur des modèles animaux portant des mutations découvertes dans des cas très rares et très agressifs de la maladie d'Alzheimer familiale. Pourtant, la plupart des facteurs de risque de la maladie d'Alzheimer sporadique ont été ignorés. Un prochain pas logique pour améliorer les modèles de maladie consiste à inclure les facteurs de risque cardiovasculaires, qui demeurent les facteurs de risque génétiques et épidémiologiques les plus puissants identifiés jusqu'à présent. La différence la plus frappante entre le métabolisme humain et murin des lipides et du cholestérol est l'absence de 'cholesterol ester transfer protein' (CETP). En conséquence, les souris ont des niveaux négligeables de 'low density lipoprotein' (LDL), un facteur de risque important pour la maladie d'Alzheimer et les maladies cardiovasculaires. CETP est une protéine de transfert lipidique responsable pour l'échange d'esters de cholestérol et de triglycérides entre lipoprotéines. Elle est considérée pro-athérogène en raison de sa capacité à augmenter les taux de LDL. En contrepartie, une diminution de l'activité du CETP a été associée à la longévité, à la santé cardiovasculaire et à une incidence réduite de la maladie d'Alzheimer. Dans le but de générer un modèle de souris atteints de la maladie d'Alzheimer avec un profil de lipoprotéines humanisé, nous avons étudié des souris transgéniques pour la CETP humaine. Bien que la littérature disponible sur le CETP et son rôle à la périphérie soit abondante, son rôle dans le système

nerveux central est mal défini.

Notre analyse a révélé que les souris transgéniques CETP avaient des niveaux de cholestérol dans le cerveau jusqu'à 30% plus élevés. Fait intéressant, une microarray utilisant des ARNm dérivés d'astrocytes a montré que cette augmentation de cholestérol est peu probable du fait d'une augmentation de la synthèse *de novo* astrocytaire. De plus, la fuite de la barrière hémato-encéphalique n'était pas non plus responsable. Des efforts de recherche supplémentaires sont nécessaires pour identifier la source de cette augmentation spectaculaire du taux de cholestérol. Néanmoins, nous avons pu identifier les effets en aval de l'augmentation du cholestérol. De manière frappante, la préséniline 1 et la préséniline 2 figuraient parmi les principales cibles en aval. Les présénilines sont les sous-unités catalytiques du complexe γ-sécrétase, un composant clé de la génération de β-amyloïde dans le cerveau. Nos données suggèrent que les souris transgéniques CETP phénocopient les taux plasmatiques de cholestérol humain et l'exposition cérébrale au cholestérol comme un outil de recherche précieux pour étudier l'impact du métabolisme du cholestérol sur les fonctions cérébrales et la maladie d'Alzheimer.

Pour étudier plus l'effet du CETP sur la maladie d'Alzheimer, nous avons créé un nouveau modèle murin combinant un métabolisme du cholestérol humanisé à une pathologie de la maladie d'Alzheimer. Notre analyse a révélé que l'activité de la CETP augmente les taux d'amyloïde-β solubles et insolubles de manière dépendante de la préséniline. L'augmentation de la production d'amyloïde induite par la CETP a été supprimée chez les souris exprimant des mutations familiales de la préséniline, soulignant les problèmes rencontrés avec la plupart des modèles animaux actuels de la maladie d'Alzheimer.

LIST OF TABLES

Table 1: Summary of Apolipoproteins, their properties, and roles 27
Table 2: Summary of past and ongoing clinical trials on CETP inhibitors 34
Table 3: Summary genetic Alzheimer's disease risk factors identified by GWAS..... 45
Table 4: Studies linking CETP with Alzheimer's disease 50
Table 5: Primers sequences for RT-qPCR. 181
Table 6: Calculated fatty acid a lipids composition of the animal diets used here. 181

LIST OF FIGURES

Figure 1: Structure of cholesterol 22
Figure 2: Structure and function of CETP..... 29
Figure 3: Cholesterol transport and lipoproteins in the CNS..... 36
Figure 4: Alzheimer's disease pathology 38
Figure 5: APP processing 40
Figure 6: Ongoing clinical trials for Alzheimer's disease therapies..... 47
Figure 7: Dietary cholesterol intake induces CETP expression 65
Figure 8: CETP promotes TREM2 expression in the liver 69
Figure 9: CETP activity promotes peripheral inflammation 72
Figure 10: CETP changes the brain cholesterol composition 74
Figure 11: Transcriptional changes in CETPtg brain induced by presenilins 77
Figure 12: CETP activates γ-secretase 78
Figure 13: CETP activity increases Aβ *in vitro*..... *97*
Figure 14: CETP activity does not increase Aβ in 5xFAD mice..... 99
Figure 15: Behaviour and Alzheimer's disease risk gene expression in 5xFAD/CETP mice 101
Figure 16: CETP activity increases Aβ in Thy1 APP mice 103
Figure 17: CETP activity does not affect the blood-brain barrier 106

AUTHOR CONTRIBUTIONS

As outlined in the McGill Thesis Guideline, this thesis is based on two manuscripts. The first manuscript is currently in revision at the Journal of lipid research (2018). The second manuscript will be submitted in altered form for publication. I am the sole first author of both manuscripts. A detailed summary of the individual contributions of all co-authors is listed below:

Manuscript 1: The Cholesteryl Ester Transfer Protein (CETP) raises Cholesterol Levels in the Brain and affects Presenilin-mediated Gene Regulation.

Felix Oestereich, Elizabeth-Ann Kranjec, Ethan Yang, Pierre Chaurand, Lisa Marie Munter

I designed and performed the majority of experiments, analyzed all experiments, illustrated all figures and wrote the first draft of the manuscript. EY and EAK acquired the MALDI-IMS data and EY analyzed the MALDI-IMS data. I purified, astrocyte mRNA and transcribed it into cDNA. The McGill University and Génome Québec Innovation Centre performed the microarray and I analyzed the data. LMM designed the project. LMM, PC, and EY edited and revised the manuscript. All Authors approved the manuscript for publication.

Manuscript 2: CETP activity increases Aβ production

Felix Oestereich, Helen Wu, Lisa Marie Munter

I designed and performed all experiments presented in manuscript 2. HW analyzed all behavioral experiments. I illustrated all figures and wrote the first draft of the manuscript. LMM designed the project and revised the manuscript. All authors approved the final version of this manuscript.

Appendix: Embedded in the membrane: How lipids confer activity and specificity to intramembrane proteases

Sandra Paschkowsky, Felix Oestereich, Lisa Marie Munter

J Membr. Biol., 2017 Dec 19., doi: 10.1007/s00232-017-0008-5

Additionally, I contributed to a review article published in J Membr. Biol. In 2017. Here, I wrote the section regarding γ-secretase, SP wrote the part concerning rhomboid proteases as well as the abstract and conclusion and LM wrote the introduction and parts about intramembrane proteases and drug discovery.

SUMMARY OF ORIGINAL CONTRIBUTIONS

i. The liver-mRNA-levels of CETP can be induced 8.8-fold using a cholesterol enriched diet.

ii. CETP transgenic mice show elevated levels of liver TREM2 mRNA. This effect is potentiated in animals on a cholesterol diet.

iii. CETP activity in mice leads to a peripheral inflammatory response.

iv. CETP transgenic mice have elevated brain-cholesterol levels.

v. These increased brain-cholesterol-levels are not due to *de novo* synthesis.

vi. I found that the γ-secretase subunits presenilin 1 & 2 are affected in CETP transgenic mice fed with a cholesterol diet.

vii. Additionally, I identified several genes linked to Alzheimer's disease whose expression was altered in CETP transgenic mice fed with a cholesterol diet.

viii. I Found that CETP activity increases brain IL1β mRNA.

ix. Using HEK293T cells, I measured that CETP activity activates γ-secretase.

x. I found that CETP activity increases Aβ438, Aβ40 and Aβ42 in cell culture.

xi. Using various CETP mutants, I found that the Aβ increase in cell culture relies on the CE-transfer activity of CETP and not its lipid transfer activity.

xii. Using the CETP inhibitor evacetrapib, I determined that inhibiting CETP abolished its effect on the generation of Aβ.

xiii. Further, I found that the CETP-dependent Aβ-increase relies on the presence of lipoproteins in the cell-culture medium.

xiv. I found that in 5xFAD mice, CETP transgenicity does not induce a Aβ increase at three or six months of age.

xv. In 5xFAD mice, CETP transgenicity does not affect animal behaviour in the Y-maze test.

xvi. 5xFAD mice show elevated levels of brain TREM2 mRNA.

xvii. In Thy1 APP McGill mice, CETP transgenicity increases the generation of soluble Aβ38, Aβ40 and Aβ42.

xviii. In Thy1 APP McGill mice, CETP transgenicity increases the generation of insoluble Aβ38, Aβ40 and Aβ42.

xix. CETP activity does not affect the blood-brain barrier

GENERAL INTRODUCTION

1. Cholesterol structure and function

1.1 Structure and function of cholesterol

Cholesterol is an amphipathic lipid belonging to the family of sterols. It is composed out of a steroid ring structure with four hydrocarbon rings as hydrophobic core and a hydrophilic hydroxyl group on the first ring (ring A) (**Figure 1**).

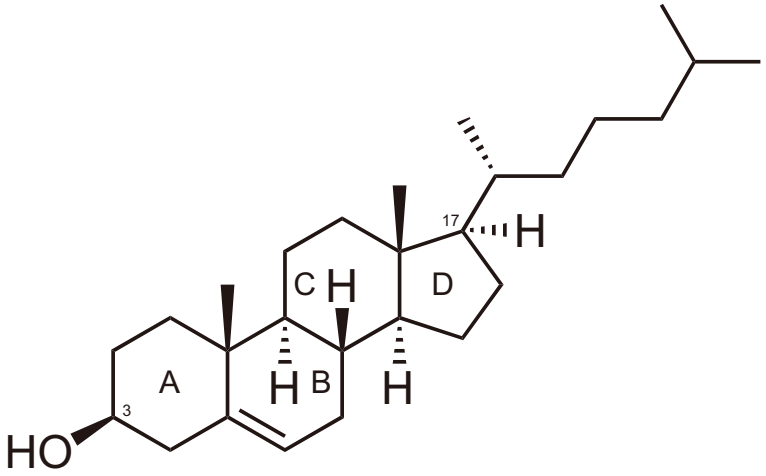


Figure 1: Structure of cholesterol
Cholesterol is a lipid, composed a steroid core consisting of four hydrocarbon rings (A-D), a hydroxyl group (OH) on carbon atom number 3 of ring A and a hydrocarbon tail on carbon atom 17.

Cholesterol was first identified in 1769 by the French doctor François Poulletier de la Salle in the form of cholesterol crystals from gallstones (Dam, 1958). However, the molecule received its name cholesterol only 46 years later when the French chemist Michel Eugène Chevreul named the molecule (Chevreul, 1816).

Cholesterol is a major constituent of all plasma membranes in mammals, a primary component of the myelin sheath covering axons in the nervous system and precursor for various hormones, bile acid as well as vitamin D. In most tissues, the cholesterol concentration is about 2 mg/g tissue, however, it reaches 15-20 mg/g in the tissue of the central nervous system (CNS) (Dietschy and Turley, 2004). Thus, the brain comprises 25% of the total body

cholesterol, implying a particular need of the brain for cholesterol since it is a vital part of the myelin sheet covering mammalian axons (Bjorkhem and Meaney, 2004).

As a vital component of plasma membranes, cholesterol is essential in maintaining various biophysical properties. The hydrophilic hydroxyl group can interact with the head groups of neighboring membrane lipids while the hydrophobic steroid core remains embedded in the center of membranes, contacting the fatty acid side chains of lipids. This interaction of cholesterol with its neighbors is the basis for its role to act as a buffer of membrane fluidity. At lower temperatures, cholesterol increases the membrane fluidity by breaking up the intermolecular interactions of phospholipids. However, at higher temperatures, the phospholipids move freely, and cholesterol can fill the space, decreasing the fluidity of the membrane (Ohvo-Rekila *et al.*, 2002).

1.2 *De novo* cholesterol synthesis

While all cells are able to synthesize cholesterol, most of the body's cholesterol is generated through *de novo* synthesis in either liver or brain, or taken up through the diet and subsequently redistributed. However, only about 20% of the body's cholesterol comes from dietary sources (reviewed in (van der Wulp *et al.*, 2013)). In liver and brain, the *de novo* cholesterol synthesis starts with acetyl-CoA, an intermediate during energy generation from carbohydrates via glycolysis and citric acid cycle, or from fatty acids via β-oxidation. Acetyl-CoA enters the mevalonate or HMG-CoA reductase pathway where first two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA, which is catalyzed by the enzyme thiolase. Subsequently, HMG-CoA synthase catalyzes the addition of another molecule of acetyl-CoA, leading to the formation of HMG-CoA. Finally, HMG-CoA reductase reduces HMG-CoA to mevalonate. This reaction is irreversible and additionally the rate limiting step in the cholesterol biosynthesis. Through several conversion steps, mevalonate is transformed into isopentanyl 5-pyrophosphate and dimethylallyl pyrophosphate. Further, through a series of condensation reactions, squalene is formed. The linear molecule squalene is first cyclised into

lanosterol, catalyzed by the enzyme lanosterol synthetase. In an intricate 20-step remodeling process, lanosterol is remodeled into cholesterol (Zetterstrom, 2009). Since the irreversible reduction of mevalonate by HMG-CoA reductase is the rate-limiting step of the cholesterol synthesis, the enzyme is the target of numerous cholesterol-lowering drugs known as statins (Alberts *et al.*, 1980). Approximately 95% of brain cholesterol comes from *de novo* synthesis in glial cells such as astrocytes. Rapid cholesterol synthesis in the brain is essential to the development of the central nervous system.

1.3 Regulation of cholesterol synthesis

The extent of *de novo* cholesterol synthesis varies by cell type and organ. The liver is responsible for the majority of cholesterol synthesis in mammals.

Within the membrane of the endoplasmic reticulum (ER), the sterol regulatory element-binding protein 2 (SREBP2) together with its binding partners the SREBP cleavage-activating protein (SCAP) and the Insulin-Induced Gene 1 (INSIG-1) sense cellular cholesterol levels and regulate the *de novo* synthesis. The N-terminal domain or SREBP2 is a transcriptional regulator and has to be released from the ER trough two successive cleavages.

SCAP is required for the activation of SREBP2. When ER cholesterol levels are high, SCAP undergoes a conformational change, leading to the binding of INSIG-1 to SCAP and the retention of the SCAP/SREBP complex in the ER. In contrast, when cholesterol levels drop, the SREBP2-SCAP complex can be transported to the to the Golgi apparatus via COP-II coated vesicles (Gong *et al.*, 2006; Nohturfft *et al.*, 2000). Here, SREBP2 is activated through cleavage by the site-1 protease (S1P) that cleaved SREBP2 into two membrane-bound fragments. Next, the N terminus undergoes a second cleavage, catalyzed by the enzyme site-2 protease (S2P). The now activated transcription factor SREBP2 migrates into the nucleus and initiates the transcription of genes under the control of a sterol responsive element (SRE) (Brown *et al.*, 2000). Among the genes controlled by SREs are the HMG-CoA reductase as well as the

low-density lipoprotein receptor (LDLR) (Horton *et al.*, 2002).

1.4 Cholesterol transport – the importance of lipoproteins

Like all hydrophobic lipids, cholesterol cannot be transported in the blood without modifications or the help of lipid-carrying particles (Goldstein and Brown, 2001). In the blood, lipoprotein particles are responsible for the distribution of lipids between different organs. Various classes of lipoproteins have been found, and their distinct functions have been characterized (Feingold and Grunfeld, 2000). Lipoproteins are round or spherical particles consisting of a hydrophobic core that contains non-polar lipids such as cholesteryl esters (CE) and triglycerides, and an outer membrane layer that is made up of free cholesterol, phospholipids and apolipoproteins.

They are classified based on their density, size and apolipoprotein content (Table 1) (Feingold and Grunfeld, 2000). They range from the large and lowest density chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) to high-density lipoproteins (HDL). Chylomicrons, containing dietary lipids are generated in the digestive tract. Tissues such as muscle and adipose tissue can metabolize triglycerides carried by chylomicrons through the release of free fatty acids through the action of lipoprotein lipase (Hussain, 2000). The remaining chylomicron remnants can be taken up by the liver. The liver is the primary source of endogenous lipoprotein generation. Here, the liver generates VLDL with ApoB-100 as the primary apolipoprotein which is found on each particle. Peripheral tissues remove triglycerides from VLDL, generating IDL which is enriched in cholesterol (Feingold and Grunfeld, 2000). Through further removal of triglycerides, LDL is obtained from IDL, thus possessing an even higher cholesterol concentration. Their major apolipoprotein is Apo B-100. LDL particles vary in size and small and dense particles have been associated with obesity, type 2 diabetes as well as inflammation and hypertriglyceridemia.

HDL particles carry ApoA-I, A-II, A-IV, C-I, C-II, C-III and E as mayor lipoproteins

(Feingold and Grunfeld, 2000; Yao and McLeod, 1994). HDL particles are responsible for reverse cholesterol transport from the periphery back to the liver (Glomset, 1968). The initial step in HDL generation involves the synthesis of the main apolipoprotein ApoA-I in liver and intestine. Next, the ATP-binding cassette transporter A1 (ABCA1) lipidates ApoA-I with cholesterol and phospholipids to form mature HDL particles. ABCA1 belongs to the family of ABC transporters, ATPases responsible for the transport of molecules across various biomembranes. Various peripheral tissues such as muscle, adipocytes express ABCA1 and transfer lipids on circulating HDL particles.

To increase the efficiency of cholesterol transport, a large percentage of cholesterol is esterified to cholesteryl esters (CE) by cholesterol acyltransferases. In the small intestine, this catalytic reaction is performed by the acyl-CoA cholesterol acyltransferase (ACAT) while in the liver and on the surface of HDL particles this job is performed by the Lecithin: cholesterol acyltransferase (LCAT). In contrast to cholesterol on the surface of lipoproteins, CE is transferred to the core of HDL, allowing for increased packing of HDL with cholesterol.

Excess cholesterol can be transported back to the liver in what is defined as reverse cholesterol transport. Here, cholesterol rich HDL particles can be taken up by the liver trough the action of the scavenger receptor B1 (SR-B1). Alternatively, it can be converted into bile acids by various sterol hydroxylases. Removal of excess cholesterol has been suggested to play an important role in the prevention of atherosclerosis.

Table 1: Summary of Apolipoproteins, their properties, and roles

Lipoprotein	Density (g/ml)	Size (nm)	Major lipids	Major apoproteins
Chylomicrons	<0.930	75-1200	Triglycerides	ApoB-48, ApoC, ApoE, ApoA-I, A-II, A-IV
Chylomicron Remnants	0.930- 1.006	30-80	Triglycerides, Cholesterol	ApoB-48, ApoE
VLDL	0.930- 1.006	30-80	Triglycerides	ApoB-100, ApoE, ApoC
IDL	1.006- 1.019	25-35	Triglycerides Cholesterol	ApoB-100, ApoE, ApoC
LDL	1.019- 1.063	18- 25	Cholesterol	ApoB-100
HDL	1.063- 1.210	5-12	Cholesterol, Phospholipids	ApoA-I, ApoA-II, ApoC, ApoE
Lp (a)	1.055- 1.085	~30	Cholesterol	ApoB-100, Apo(a)

2. The cholesteryl ester transfer protein

2.1 Function of CETP

In addition to cholesterol and lipid transport between organs, carried out by lipoproteins, another form of lipid transport occurs between individual lipoproteins in circulation. This is facilitated by the cholesteryl ester transfer protein (CETP). CETP is a lipid transfer protein that exchanges cholesteryl esters and neutral lipids between plasma lipoproteins (Nichols and Smith, 1965; Zilversmit *et al.*, 1975). Secreted from the liver, CETP transfers cholesteryl esters from HDL to LDL, VLDL, and chylomicrons. Further, it can transfer triglycerides back from HDL to VLDL and LDL. As its transfer activity is passive and does not require energy, it can be assumed that the transfer directions are determined by the concentration gradient of lipids between the different lipoproteins. Seeing that there are no other proteins with similar activity to our knowledge, CETP is responsible for all cholesteryl ester and triglyceride transfer activity in human plasma (Morton and Zilversmit, 1982).

The net result of this transfer activity is increased cholesterol content in pro-atherogenic LDL and especially VLDL particles and decreased cholesterol levels in anti-atherogenic HDL

particles, driving the substantial interest in CETP (Zhong *et al.*, 1996). Several epidemiological studies in statin-treated patients have highlighted the value of raising HDL in the prevention of cardiovascular events (Vergeer *et al.*, 2010). While most research has focussed on the role of CETP in plasma, there is growing evidence that intracellular CETP may influence the cellular lipid metabolism, perhaps by facilitating the formation of lipid droplets (Huang *et al.*, 2003; Izem and Morton, 2001; Zhang *et al.*, 2001). The expression of CETP can be induced via dietary cholesterol where increased levels of cholesterol lead to increased transcription of the CETP gene (Jiang et al., 1992). Here, both liver X receptors as well as SPREBP can induce transcription of CETP. The CETP gene carries a sterol upregulatory element that can be activated through binding of LXRα and LXRβ as well as SREBP1. (Gauthier et al., 1999; Luo and Tall, 2000).

2.2 Structure of CETP

CETP is a soluble, highly glycosylated protein with a molecular mass of 53 kDa (**Figure 2A**). However, CETP has much higher apparent molecular weight of approximately 70 kDa due to multiple glycosylation sites (Swenson *et al.*, 1987). Structurally, CETP has a 'boomerang' shape with a hydrophobic tunnel that enables the transfer of lipids through an aqueous environment (Qiu *et al.*, 2007). Even though the exact details of the transfer mechanism are not clear, there is good evidence that the N-terminal part of CETP dives into HDL while the C-terminal part binds LDL or VLDL particles at the same time, forming a tunnel between the two particles that enable the transfer (**Figure 2B**). Evidence for this has been gained from electron microscopy as well as molecular dynamics simulations (Lei *et al.*, 2016; Qiu *et al.*, 2007). The concentrations of CE and TG dissolved in the phospholipid surface monolayer influence CETP lipid transfer rates (Morton and Steinbrunner, 1990).

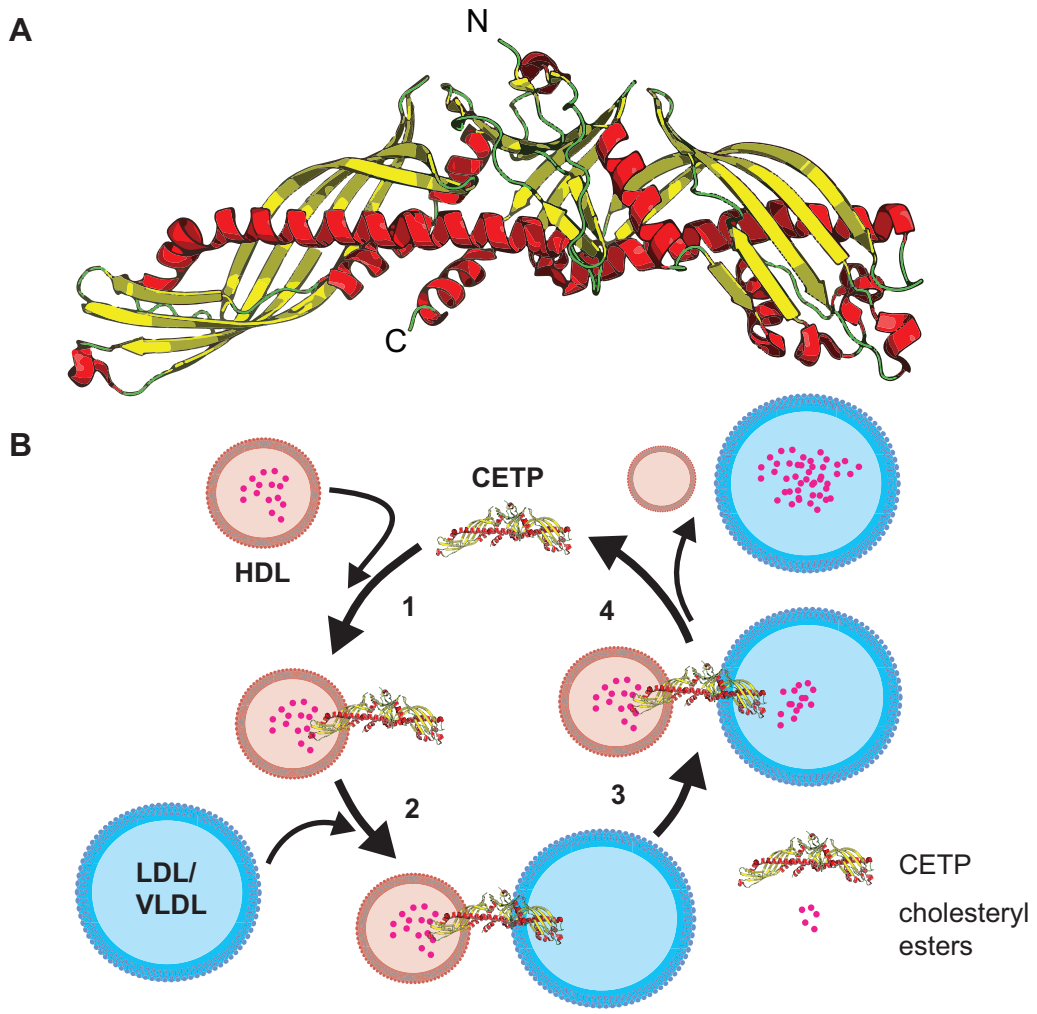


Figure 2: Structure and function of CETP

A: Crystal structure of CETP (Qiu *et al.*, 2007). α-helices are shown in red and β-sheet structures in yellow. The N and C terminus are labeled with N and C, respectively. PDB: 2OBD **B**: Proposed model of CETP mechanism. 1. The N terminus of CETP contacts HDL and dives into the cholesteryl-ester core of the particle. 2. The C-terminal end of CETP can interact with LDL or VLDL particles, and 3. form a continuous tunnel between lipoproteins. 4. Based on the concentration gradient between the particles, cholesteryl-esters are transferred from HDL to LDL or VLDL. Illustration adapted from (Zhang *et al.*, 2012).

3. Cholesterol & lipids in disease

While cholesterol plays a crucial role in various physiological processes, cholesterol deficiency can lead to severe neurological and developmental defects. Throughout evolution, humans have lived mostly as hunters and gatherers with high physical activity and the intestinal

metabolism has evolved on a diet that was largely vegetarian. When dietary fats were available, the human metabolism has evolved to store available fats or use them to supply energy to tissues, using lipoproteins as transporters. Further, in times of limited dietary cholesterol, de novo cholesterol synthesis is sufficient to ensure an adequate cholesterol supply (Babin and Gibbons, 2009). However, in modern times most humans have access to large quantities of animal food products enriched in fats and cholesterol. This increase in the consumption of animal fats and cholesterol has given rise to increases plasma cholesterol and LDL-cholesterol (LDL-C) levels. This increase is responsible an increase in obesity, insulin resistance, and atherosclerosis which can be aggravated by a sedentary lifestyle (Linton et al., 2000).

Atherosclerosis is one of the leading causes of death in Canada, just surpassed by malignant cancer (Stats Canada, 2016). The disease is defined by the build-up of atherosclerotic plaques on the inner walls of arteries, leading to the narrowing of vessels and a restriction in oxygen supply. The formation of atherosclerotic plaques begins with the aggregation of (oxidized) LDL particles on the endothelial cells, potentially in response to inflammatory processes (Li *et al.*, 2016). Early processes include the infiltration of the plaque by monocytes and macrophages and aggregation of platelets. Over time the plaque grows which can lead to stenosis and plaque rupture.

While the individual causes for atherosclerosis are multifaceted, multiple common risk factors have been found over the years. The most common being smoking, type-2 diabetes and high blood pressure (Rafieian-Kopaei *et al.*, 2014). However, they are eclipsed by the effects elevated LDL-C on the risk of atherosclerosis. Based on its role in increasing LDL-C, CETP has been linked tightly with atherosclerosis and cardiovascular disease (reviewed in (de Grooth *et al.*, 2004)).

While atherosclerosis is the most prominent disease associated with excess cholesterol, gallstones, non-alcoholic fatty liver disease as well as dermatological disorders have also been linked with increased cholesterol levels, highlighting the importance of controlling cholesterol levels (Arguello et al., 2015; Shenoy et al., 2015).

3.1 Treatment strategies in cardiovascular disease – statins

There are multiple prevention strategies and treatments in use, ranging from lifestyle changes, healthy diets to anti-inflammatory or blood pressure lowering medications. In the end, all those treatments aim at normalizing the patient's lipid profile. Among the most prescribed drugs are statins, HMG-CoA reductase inhibitors. Reduced liver *de novo* cholesterol synthesis goes hand-in-hand with upregulation of LDL receptors at the cell surface, increasing the reabsorption of cholesterol. Most statins lower circulating LDL-C by about 25-40% and lead to a slight increase in HDL cholesterol.

Lovastatin, the first statin, was identified by Merck in late 1970 from the fungus Aspergillus terreus (Alberts *et al.*, 1980). The success of lovastatin paved the way for the development of multiple drugs targeting cardiovascular disease (CVD), and today there are various statins on the market such as simvastatin (Zocor), atorvastatin (Lipitor), fluvastatin (Lescol), and pravastatin (Lipostat) with similar effects regarding their LDL-lowering capacity but with different biological properties.

3.2 Treatment strategies in cardiovascular disease – PCSK9 inhibitors

More recently, new LDL lowering drugs have been developed, such as inhibitors for proprotein convertase subtilisin/kexin type 9 (PCSK9) and CETP. PCSK9 is an inactive, extracellular proprotein convertase that binds the LDL receptor (LDLR), leading to its internalization and lysosomal degradation. Inhibition of PCSK9 increases cell-surface LDLR levels and consequently increases the hepatic clearance of circulating LDL particles (Benjannet *et al.*, 2004; Lagace *et al.*, 2006). PCSK9 was first implicated in cardiovascular disease when Abifadel *et al.* identified a gain of function mutation in PCSK9 leading to an autosomal dominant form of hypercholesteremia (Abifadel *et al.*, 2003). Later, polymorphisms in PCSK9 have been linked with a lowered incidence of coronary heart disease and lowered circulating LDL levels (Cohen *et al.*, 2005; Cohen *et al.*, 2006). In 2017, the first PCSK9 inhibitor, alirocumab a monoclonal

antibody targeting PCSK9, was approved by the FDA and since then various other PCSK9 inhibitors have been developed (Chaudhary *et al.*, 2017; Robinson *et al.*, 2015; Sheridan, 2013).

3.3 Treatment strategies in cardiovascular disease – CETP inhibition

Similarly, polymorphisms, reducing the transfer activity or transcription levels of CETP have been linked with longevity and a reduced risk of atherosclerosis presumably based its pro-atherogenic effects as it well characterized that these polymorphisms raise HDL and lower LDL levels (Brousseau *et al.*, 2004; Clark *et al.*, 2004; de Grooth *et al.*, 2002; Koizumi *et al.*, 1985; Masson, 2009; Okamoto *et al.*, 2000; Sanders *et al.*, 2010; Vasan *et al.*, 2009). Notably, the I405V polymorphism (rs5882) has drawn interest due to its effect on decreasing plasma CETP mass, LDL levels and reduced risk of coronary heart disease. Here, several studies found a reduced incidence of cardiovascular disease in carriers of the CETP I405V polymorphism. (Bustami *et al.*, 2016; Corbex *et al.*, 2000; Papp *et al.*, 2012).

Given the evidence for CETP activity to raise LDL and VLDL and lower HDL as well as the positive effect of genetic CETP polymorphisms, pharmaceutical companies developed potent CETP inhibitors to treat CVD (Brousseau *et al.*, 2004; Cao *et al.*, 2011; Gutstein *et al.*, 2012; Huang *et al.*, 2002).

Torcetrapib was the first CETP inhibitor to be tested in clinical trials. Despite reducing LDL-C and increasing HDL-cholesterol (HDL-C) levels drastically the drug was eventually abandoned in clinical phase three due to an increased risk of mortality. While the ILLUMINATE study showed that Torcetrapib in combination with atorvastatin was able to increase HDL-C by 72% and additionally lower LDL-C by 25% compared to atorvastatin alone, Barter et al. found an increased risk of cardiac events and mortality from any cause in patients treated with Torcetrapib. However, there was no significant interaction between the treatment group and the cause of death. Death due to cancer and infection were the most prevalent causes of death due to noncardiovascular causes in both study groups (Barter et al., 2007). Dalcetrapib, the next CETP inhibitor to be tested in clinical trials showed fewer ad-

verse side effects and was deemed to be save for clinical use. However, despite raising HDL-C by 30%, clinical trials were abandoned due to a lack of clinical outcomes, defined as time to the first major cardiovascular event such as coronary heart disease, myocardial infarcts or stroke (Schwartz *et al.*, 2012). The next group of inhibitors tested was Evacetrapib (Eli Lilly & Company) and Anacetrapib (Merck). Both showed an efficient reduction of LDL-C. Here, Evacetrapib showed an impressive 37% reduction in LDL-C levels and a 132% increase in HDL-C when compared with the placebo-treated group. Yet, Evacetrapib was eventually abandoned due to a lack of efficacy since the treatment did not yield a lower rate of cardiovascular events when compared to placebo in high-risk patients (Lincoff *et al.*, 2017; Doggrell, 2017). The only CETP inhibitor successfully reaching its clinical goals was Anacetrapib. The REVEAL (Randomized Evaluation of the Effects of Anacetrapib through Lipid modification) trial showed a 104% increase in HDL-C and a 17% reduction of LDL-C. Further, patients receiving o Anacetrapib showed a significantly reduced occurrence of the primary outcome of coronary death, myocardial infarction, or coronary revascularization (Group *et al.*, 2017). It is important to note, however, that Merck has stated that they do not intend to file Anacetrapib with the Food and Drug Administration (FDA) due to the accumulation of the drug in fatty tissues after prolonged dosing and potential associated safety concerns (Merck, 2017). Another very potent CETP inhibitor TA-8995 (Amgen) showed even better efficacy and safety profile than previous inhibitors and was able to reduce LDL-C by 68% while increasing HDL-C by 179% (Hovingh *et al.*, 2015). While Anacetrapib is a very lipophilic molecule with retention of up to two years, TA-8995 (Obicetrapib) is eliminated from the body within two weeks. However, in light of the recent clinical failures of other CETP inhibitors, Amgen halted the development of TA-8995 in 2017 after the completion of phase two clinical trials. As of now, there is only one clinical trial ongoing. In 2016, DalCor started their dalGenE study, that targets explicitly patients with the acute coronary syndrome (ACS) carrying a polymorphism in the adenylate cyclase type 9 ADCY9 gene (Table 2) (Tardif *et al.*, 2015).

The clinical failure of almost all CETP inhibitors trials put the idea of raising HDL and

reducing LDL using CETP inhibition for the treatment of CVD into question. However, it has to be noted that the failures of these drugs were not solely due to the lack of efficacy since most inhibitors were well tolerated and showed impressive LDL-lowering and HDL-raising capabilities. One issue is that all CETP inhibitors were co-administered with statins and the beneficial effects of CETP inhibition on top of statin therapy are limited. Due to the fact that patients enrolled in CETP inhibitor trials had largely very well controlled LDL-C levels means that the total reductions in LDL-C were low and the benefit of CETP inhibition drastically lower than they could have been in patients with higher LDL-C at randomization (Tall and Rader, 2018). However, there could be a market for specific patient populations that do not respond to statin therapy as the encouraging results of the dal-GenE trial are showing.

Table 2: Summary of past and ongoing clinical trials on CETP inhibitors

Drug	Trial	Company	Status	Other drugs[1]	LDL-C	HDL-C	Identifier[2]
Torcetrapib	ILLUMINATE	Pfizer	Terminated in 2006 due to **adverse side effects (high blood pressure, elevated plasma aldosterone levels)**	Statin	-24%	+61%	NCT00134264
Dalcetrapib	dal-OUTCOMES	Proche/ DalCor	Terminated in 2012 due to **lack of clinical outcome (no difference to placebo)**	Statin	-	+31%	NCT00658515
Evacetrapib	ACCELERATE	Eli Lilly	Terminated in 2016 due to **lack of clinical outcome**	Statin	-37%	+130%	NCT01687998
Anacetrapib	REVEAL	Merck	Abandoned in 2017 due to an **unsatisfactory clinical profile**	Statin	-36%	+139%	NCT01252953
TA-8995	TULIP	Xention/ Dezima/ Amgen	**Discontinued** in 2017 in after completion of Phase II trials	Statin	-28-69%	+74 - 177%	NCT01970215
Dalcetrapib	dal-GenE	DalCor	**Phase 3 ongoing,** estimated completion date in 2020	none	n.a.	n.a.	NCT02525939

[1] Other drugs that were co-administered
[2] ClinicalTrials.gov identifier

4. Lipoprotein particles in the brain and the blood-brain barrier

While, the lipids and cholesterol metabolism in the periphery relies on lipoproteins such as VLDL, LDL, and HDL, the majority of these particles are not able to cross the blood-brain barrier (BBB) (Jeske and Dietschy, 1980; Liu *et al.*, 2012). Even though the brain is the most cholesterol-rich organ and contains about 20-25% of the total cholesterol (Bjorkhem and

Meaney, 2004), it cannot rely on dietary cholesterol. Brain cholesterol is almost exclusively derived from *de novo* synthesis during development. Most of the brain cholesterol is found in the myelin sheets of axons as well as the cell membranes (Jeske and Dietschy, 1980). During early development of the CNS, myelination of axons by oligodendrocytes requires extensive *de novo* synthesis of cholesterol.

In the adult brain, astrocytes are primarily involved in lipid synthesis (Dietschy and Turley, 2001, 2004). Further, fully differentiated neurons loose the cholesterol biosynthetic capacity and rely on cholesterol-containing lipoproteins, secreted by glial cells for ongoing needs for maintenance and repair of damaged neurons (Zhang and Liu, 2015). Excess cholesterol in the brain can be stored as cholesteryl esters or is hydroxylated by the enzyme cholesterol 24-hydroxylase to form 24-hydroxycholesterol which shows better solubility and can cross the BBB without the aid of carrier molecules (Russell *et al.*, 2009). Further, the levels of 24-hydroxycholesterol in the brain reflect the number of metabolically active neurons in the brain and it has been reported that the plasma levels of 24-hydroxycholesterol are elevated in neurodegenerative diseases (Leoni and Caccia, 2013).

Within the brain, the cholesterol transport mediated by lipoproteins has long been thought to underlie the proper functioning of the brain and ApoE- and cholesterol-rich lipoproteins ensure the supply of cholesterol required for normal functioning of neurons (Pfrieger, 2003). Indeed, all major types of neuronal cells can bind and internalize lipoproteins present in the extracellular fluid (Beffert *et al.*, 1998). The lipoprotein composition in the brain differs from what is found in the periphery. The primary brain lipoprotein resembles HDL in size and density (Koch *et al.*, 2001). ApoE and ApoJ are the major apolipoproteins in the human brain, with highest concentrations of ApoE (Demeester *et al.*, 2000; Koch *et al.*, 2001; Song *et al.*, 1997). While a small portion of the CNS ApoE is derived from neurons, astrocytic and glial cells are mainly responsible for synthesizing ApoE and generating these HDL-like particles (Knoferle *et al.*, 2014) (**Figure 3**). Nascent ApoE is lipidated by the ABCA1 from astrocytes and the composition can be further modiefied through esteification of cholesterol

by the Lecithin–cholesterol acyltransferase (LCAT) (Liao *et al.*, 2017; Hirsch-Reinshagen *et al.*, 2009). Next, to ApoE, the apolipoprotein J (ApoJ, also known as clusterin) is the second most common apolipoprotein in the brain (Ladu *et al.*, 2000).

Lipoprotein particles are taken up by neurons, astricytes and glia through the family of LDL-receptors. The LDL-receptor related protein 1 (LRP1) shows the highest affinity towards ApoE particles. Yet, the very-low density lipoprotein receptor (VLDLR) and ApoE receptor 2 (ApoER2) can also recognize ApoE and ApoJ (Fernandez-Castaneda *et al.*, 2013; Matsuo *et al.*, 2011). Lastly, there is evidence of lipoproteins crossing the BBB as several reports show that the scavenger receptor B1 (SR-B1) can mediate the transfer of HDL through the BBB, likely via transcytosis. However, it is not clear whether complte HDL particles or simply their protein content are transferred across the BBB (Balazs *et al.*, 2004; Fung *et al.*, 2017; Goti *et al.*, 2001).

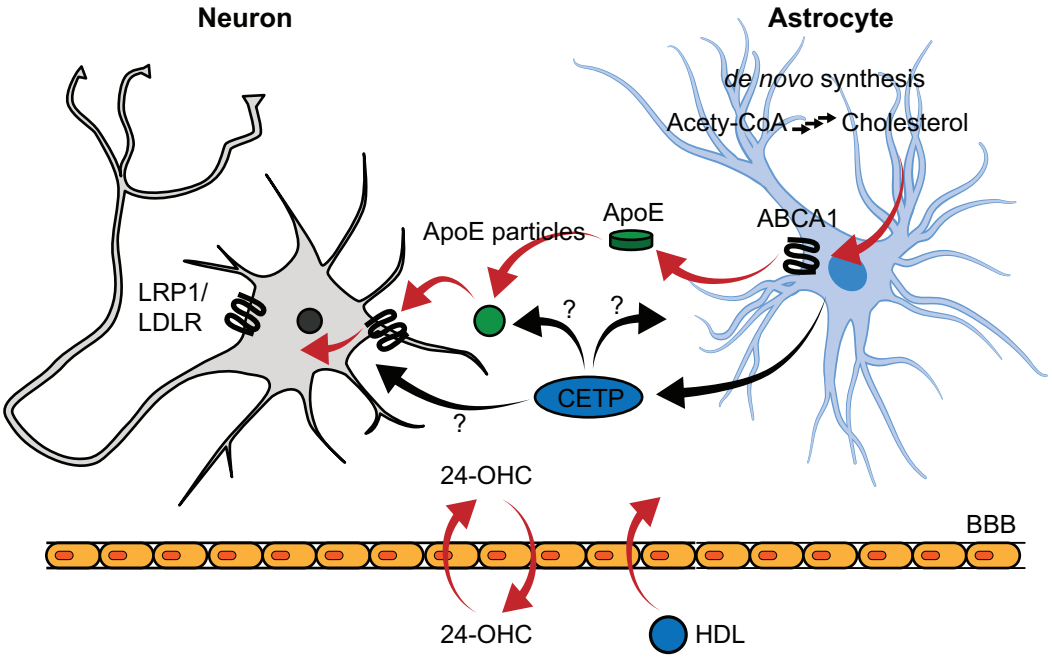


Figure 3: Cholesterol transport and lipoproteins in the CNS

Astrocytes are the primary cell type in the CNS responsible for cholesterol synthesis. LCAT synthesized by astrocytes esterifies cholesterol which is loaded on ApoE by ABCA1. Nascent ApoE particles are modified, potentially through the action of CETP before they deliver CE and lipids to neurons with the help of LDLR and LRP1 (Vitali *et al.*, 2014; Zhang and Liu, 2015). HDL and 24-

OHC can cross the BBB.

5. CETP in the central nervous system

While peripheral CETP has been studied extensively, several recent studies suggest a role for CETP in brain functioning, which may suggest a role for CETP in the brain itself. CETP is expressed in the brain, predominantly in astrocytes (Yamada *et al.*, 1995). Further, CETP is found in cerebral spinal fluid implicating a role in the brain cholesterol distribution (Albers *et al.*, 1992). Recently, several groups studying the effects of CETP polymorphisms on brain-lipid homeostasis found that CETP polymorphisms affecting the expression or activity of CETP had drastic effects on brain lipid and cholesterol distribution. First, Salminen *et al.* found that the CETP I405V polymorphism, linked with lower CETP expression (Blankenberg *et al.*, 2003), was associated with grey matter abnormalities (Salminen *et al.*, 2015). Here, homozygous carriers of the 405I allele, i.e. non-carriers of the polymorphism reducing CETP activity showed a higher risk for grey matter abnormalities in posterior brain regions, independent of an ApoE4 allele. These abnormalities can be interpreted as neurodegeneration and atrophy.

Further, diffusion tensor imaging (DTI) assessing the white matter fiber structure found that the same polymorphism showed association with physiological features such as edema, demyelination, and inflammation (Warstadt *et al.*, 2014; Huppi and Dubois, 2006; Tamnes *et al.*, 2010). These studies suggest that CETP is not only expressed in the CNS but further, that CETP plays a role in brain cholesterol and lipid homeostasis. While the studies mentioned above did not test for cognitive differences between their cohorts, there are multiple studies linking CETP with cognition. First, studies investigating the genetic predisposition of "super-agers" or "centenarians," aged people with well-maintained health and cognitive performance, revealed an association with specific CETP polymorphisms. Here, the same CETP polymorphisms that affect brain structure and CETP activity additionally associate with longevity and improved cognitive performance (Barzilai *et al.*, 2006; Barzilai *et al.*, 2003; Murphy *et al.*, 2012; Sanders *et al.*, 2010).

Despite this evidence supporting a role of CETP in the brain, its exact role remains poorly understood. The brain only contains ApoE-lipoprotein particles, resembling HDL in size and density.

Based on the lack of other lipoproteins, the substrates of CETP in the brain are not clear. Further, the biochemical and molecular effects of CETP in the brain other than the evidence gathered from epidemiological studies are not known. It is tempting to speculate that the function of CETP in the CNS is similar to its role in the periphery and CETP may play a role in lipoprotein metabolism in the brain. However, more research is required to unravel the role of CETP in the brain

6. Alzheimer's disease

6.1 Alzheimer's disease pathology

Alzheimer's disease is the most common form of neurodegenerative diseases and has a prevalence of over 750,000 patients in CAnada alone. Further, it is the 7th leading cause of death in Canada (Alzheimer's disease international, 2015; Selkoe, 2001). It is a progressive neurodegenerative disorder that manifests with a loss of cognition, mood changes, problems with spatial orientation, depression, memory loss, and speech impairment (Becker and Overman, 2002; Forstl and Kurz, 1999; Frank, 1994). Due to our aging population, we expect an exponential increase in Alzheimer's disease incidence (Bertram and Tanzi, 2004). This rise in the incidence will come with an immense financial burden.

On a macroscopic level, the disease is characterized by diffuse brain atrophy, the loss of synapses and especially cholinergic neurons (Holtzman *et al.*, 2011). Further, a pathological hallmark of the disease is the occurrence of amyloid plaques in the extracellular space and neurofibrillary tangles inside neurons (**Figure 4**) (Alzheimer *et al.*, 1995; Dickson *et al.*, 1988; Holtzman *et al.*, 2011).

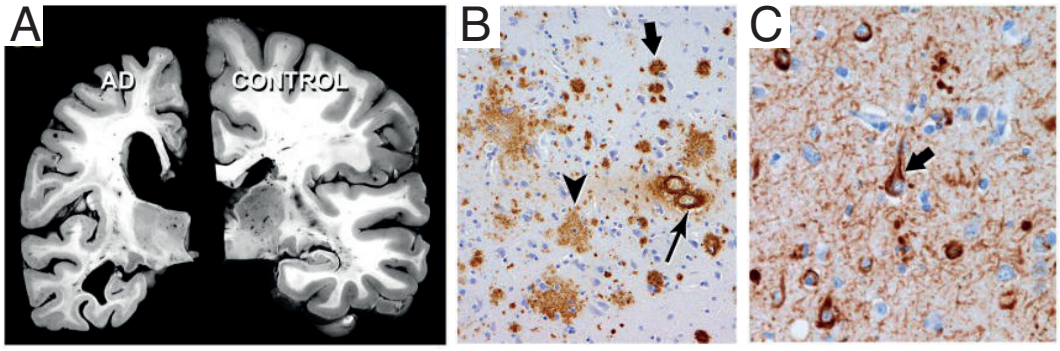


Figure 4: Alzheimer's disease pathology
Pathological hallmarks of AD are **A**: A diffuse brain atrophy. Compare the diseased brain hemisphere or the left to the healthy control in post-mortem brain slices **B:** Amyloid depositions in the form of diffuse Aβ plaques (arrowhead), compact plaque (upper arrow) and cerebral amyloid angiopathy (lower arrow). The staining was obtained using an anti-Aβ antibody on human tissue samples. **C:** Neurofibrillary tangles, stained by an antibody recognizing hyperphosphorylated tau. (From (Holtzman *et al.*, 2011). Reprinted with permission from AAAS).

Those neurofibrillary tangles consist of aggregates of hyperphosphorylated tau protein (Bancher *et al.*, 1989). In Alzheimer's disease, the microtubule-binding protein is hyperphosphorylated and aggregates intraneuronally (Wischik *et al.*, 1988). Together, amyloid and neurofibrillary tangles contribute to neuroinflammation, the next important pillar in the pathology of Alzheimer's disease. Here, activated microglia migrate towards plaques and secrete inflammatory cytokines (Lucin and Wyss-Coray, 2009; Webster *et al.*, 1997). While it is well established that inflammation in AD seems to stem from within the CNS, but it is not clear what mechanisms induce the start of inflammatory processes (Heneka *et al.*, 2015). Many inflammatory mediators such as cytokines have been linked with AD. Several lines of evidence have linked activation of the complement system with AD (Rogers *et al.*, 1992) that can be activated by both amyloid and tau aggregates (Shen *et al.*, 2013). Additionally, the disease has a strong vascular component. Here, Aβ peptides aggregate in cerebral vasculature and cause an illness associated with Alzheimer's, i.e., cerebral amyloid angiopathy (Esiri and Wilcock, 1986; Yamada *et al.*, 1987).

6.2 The amyloid pathology in Alzheimer's disease

The amyloid plaques consist of aggregated amyloid beta peptides (Aβ) that are generated from the amyloid precursor protein (APP) through sequential cleavages by two transmembrane secretases, the β- (BACE) and γ-secretase (Glenner and Wong, 1984; Hussain *et al.*, 1999; Masters and Beyreuther, 1989; Masters *et al.*, 1985). The latter enzyme cleaves the membrane residing C-terminal fragment of APP in several sequential photolytic steps. As a result, a mixture of Aβ peptides with varying lengths is generated whereas longer peptides with 42 amino acids are more prone to aggregation and show higher toxicity (**Figure 5**). The γ-secretase complex consists of the four subunits presenilin (PS), anterior pharynx-defective 1 (Aph1), nicastrin (NCT) and presenilin enhancer 2 (PEN-2). These subunits build an enzymatic complex with 19 transmembrane helices (Edbauer *et al.*, 2003).

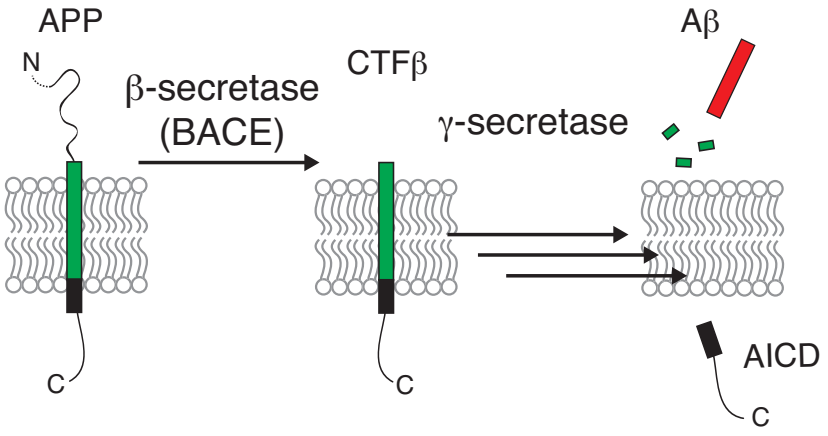


Figure 5: APP processing
During the amyloidogenic processing of APP, the protein is first cleaved by the β-secretase to generate sAPPβ and the β-CTF. The β-CTF is then cleaved by γ-secretase through multiple successive cleavages, releasing AICD and Aβ peptides of various lengths.

Further reinforcing a role for APP in AD, various disease-causing mutations in both APP and PS have been identified. This genetic form of AD (familial AD – FAD) is different from the much more common sporadic, late-onset AD (LOAD) and usually manifest at a much earlier age familiar AD (FAD) (Sherrington *et al.*, 1995; St George-Hyslop *et al.*, 1987; Tanzi,

2012). Based on this evidence, Hardy *et al.* formulated the amyloid cascade hypothesis (Hardy and Higgins, 1992). This hypothesis characterizes Aβ as the major toxic element underlying AD pathology (Glenner and Wong, 1984).

In humans, APP has two homologs, the APP-like proteins 1 and 2 (APLP1 and APLP2). Together they have been implicated in various neuronal processes. All APP family member proteins have a small intracellular and a larger extracellular domain. Several genetic knock-out studies have suggested a role for the APP-family proteins in various neurological processes. While the knock out of a single member of the protein family showed viability with minor defects such as reduced locomotor activity and memory performance as well as axonal deficits (Dawson et al., 1999; Zheng et al., 1995; Magara et al., 1999), double knockouts showed more severe phenotypes, indicating that the members of the APP family may compensate for each other. Here, knock outs of APLP2 together with either APP or APLP1 are lethal (Heber et al., 2000). This would indicate that all three members are having overlapping functions and that while APLP2 is able to compensate for the loss of either APP or APLP1 but both APP and APLP1 are not able to compensate for the loss of both other members. However, it is important to note that there is a high possibility that all members have their own non-overlapping functions that could be tissue specific. In the brain, APP has been linked with functions in in the development of the CNS such as neuronal migration (Young-Pearse et al., 2007). Another suggested role for APP is as support in synapse formation or maintenance as APP is present at synapses (Kamenetz et al., 2003). Another interesting role for APP in the brain lipoprotein metabolism has been suggested. Here, APP can interact with members of the LDL-receptor family proteins such as LRP1, VLDLR or APOER2 (Dumanis et al., 2012; Hoe et al., 2005; Kounnas et al., 1995). Despite this interesting evidence for APP in development, the role for APP in the adult brain and especially the role of Aβ in the brain remains poorly understood.

However, despite dominating the field for the last 20 years, all efforts to develop treatments for AD by targeting Aβ have failed, and it may be time to look past the amyloid hypothesis or at least expand on it (Canevelli *et al.*, 2017; Morris *et al.*, 2014). One of the problems could be

that the it is heavily based on findings in patients with rare FAD. However, the more common LOAD may have more complicated and diverse etiology.

6.3 The role of lipids and cholesterol in Alzheimer's disease

The most significant risk factor for sporadic Alzheimer's disease is the age, and the incidence of the disease increases rapidly after the age of 65 (Bertram and Tanzi, 2004).

The molecular mechanisms underlying the neurodegeneration are not fully understood. However, the importance of cholesterol metabolism as a risk factor for AD has been of particular interest since epidemiological studies cemented the involvement of cholesterol metabolism in Alzheimer's disease. Further, high cholesterol level have been suggested as a key pathological factor contributing to the disease (Ghribi, 2008). Defects in cholesterol homeostasis have long been linked the risk of AD (Di Paolo and Kim, 2011). Additionally, it is well established that Alzheimer's disease and cardiovascular disease share multiple risk factors. Among those, elevated LDL-C levels, hypertension, obesity, and type-II diabetes increase the risk substantially (Camejo *et al.*, 1976; Gordon *et al.*, 1977). The most potent genetic risk factor for sporadic Alzheimer's disease identified thus far is the ε4 allele of the Apolipoprotein E (ApoE ε4). Homozygous carriers of the ε4 allele have a 12-fold higher risk of developing the disease while hemizygous carriers still have a 4-fold higher risk (Saunders *et al.*, 1993). Further, a recent plasma lipidomic analysis found especially long-chain cholesteryl esters to be associated with Alzheimer's disease and members of the LDL receptor superfamily such as LRP1 have been implicated in the clearance of Aβ (Deane *et al.*, 2004; Proitsi *et al.*, 2015). Lastly, a recent meta analysis of statin use and dementia incidence found a reduced risk of dementia in statin users (Chu *et al.*, 2018).

Additionally, cholesterol is a vital part of subdomains of the plasma membrane enriched in cholesterol and glycosphingolipids that are known as lipid rafts. They contain up to five times the concentration of cholesterol when compared to the surrounding plasma membrane (Simons and Ehehalt, 2002; Simons and Sampaio, 2011). Lipid rafts contain sphingolipids and

cholesterol in the outer leaflet and phospholipids as well as cholesterol in the inner leaflet. Due to the saturation of the hydrocarbon side chain of lipids found in rafts, they show tight packing and a high degree of order compared to the bulk part of the plasma membrane. Lipid rafts play a role in membrane trafficking and synaptic transmission. However, they have been implicated in Alzheimer's disease as they promote the interaction of APP and β-secretase, leading to increased levels of Aβ (Hicks *et al.*, 2012; Parkin *et al.*, 1999). Here, it has been postulated that APP is recruited to lipid rafts though the interaction with flotillin-1 (Chen *et al.*, 2006). Further, it has been suggested that LRP1 can additionally promote raft trafficking of APP and the β-secretase (Yoon *et al.*, 2007). Several studies have reported increased levels of Aβ production from rafts localized APP (Ehehalt *et al.*, 2003; Rushworth and Hooper, 2010).

Further, multiple studies have analyzed the intricate relationship between the activity of the γ-secretase complex and its lipid environment. We know that increased levels of cholesterol lead to an increase in the generation of the longer Aβ42 species (Hur *et al.*, 2008; Riddell *et al.*, 2001; Runz *et al.*, 2002; Rushworth and Hooper, 2010; Simons and Ikonen, 1997). Accordingly, depleting cellular membranes of cholesterol using agents such as methyl-beta-cyclodextrin reduces the generation of Aβ (Golde and Eckman, 2001; Simons *et al.*, 1998). Further, multiple studies aimed at elucidating the effects of other lipid species as well as the effects of membrane fluidity and thickness on γ-secretase. They found that sphingolipids such as sphingomyelin or cerebrosides, that form a crucial component of lipid microdomains or lipid rafts increase the generation of Aβ by γ-secretase (Hur *et al.*, 2008; Vetrivel *et al.*, 2004). Additionally, Osenkowski *et al.* used lipid mixtures resembling different organs and organelles and tested the effect of such lipids on the activity of the γ-secretase complex and found the highest activity in brain-derived lipids and a lipid composition that resembles the plasma membrane and especially lipid rafts (Osenkowski *et al.*, 2008). Moreover, both Ayciriex *et al.* and Yang *et al.* found lipids interacting directly with γ-secretase (Ayciriex *et al.*, 2016; Yang *et al.*, 2017). In addition to these direct interaction between lipids and the enzyme, general membrane properties also affect the enzymatic activity. Here, Winkler *et al.*

determined the optimal membrane thickness to be around 26-29 Angström and Holmes *et al.* used a systematic approach, altering fatty acid chain length and saturation and measured the highest activity in membranes with 18-20 C-atom FA-chains to determine the optimal fatty acid chain length for facilitating γ-secretase activity (Holmes *et al.*, 2012; Winkler *et al.*, 2012). Further, monounsaturated FA with trans double bonds increases activity compared to cis double bonds (Holmes *et al.*, 2012). While the above evidence is stemming from *in vitro* studies and studying the effects of lipids on γ-secretase remains challenging *in vivo*, there are several studies noting changes in sphingolipids, cholesterol and phospholipids in the brain and cerebrospinal fluid (CSF) of AD patients (Dietschy, 2009; Fonteh *et al.*, 2013; Haughey *et al.*, 2010; He *et al.*, 2010; Kosicek and Hecimovic, 2013; van Echten-Deckert and Walter, 2012; Wells *et al.*, 1995). Additionally, administering cholesterol-lowering drugs in mouse models for AD leads to a reduction in amyloid pathology, and conversely, a diet enriched in cholesterol aggravates pathology (Levin-Allerhand *et al.*, 2002; Petanceska *et al.*, 2002; Refolo *et al.*, 2001).

Lastly, it has been documented that ApoE is able to bind Aβ, leading to its elimination and clearance out of the brain via a LRP1 mediated transport across the BBB. Here the ε3 allele of ApoE shows higher affinity towards Aβ when compared to ApoE ε4, suggesting that the increased AD risk of ApoE ε4 carriers may be due to insufficient clearance of Aβ from the brain (Kanekiyo *et al.*, 2014). Further, the binding of ApoE ε3 to Aβ has been shown to reduce its aggregation while this was not the case for ApeE ε4 (Petrlova *et al.*, 2011).

6.4 Genetic risk factors for Alzheimer's disease

Over the last decade, large genome-wide association studies (GWAS) have identified a large set of variants associated with AD (**Table 3**). These screens have been able to shed light on the proteins and pathways central to the disease. Unsurprisingly, they reinforced the involvement of the lipid and cholesterol metabolism in the disease (Fenoglio *et al.*, 2007; Harold *et al.*, 2009; Hollingworth *et al.*, 2011). The highest genetic risk factors can a grouped

into four groups according to their known functions. Here it is apparent that a large portion of genetic AD-risk factors is involved in lipid and cholesterol metabolism. As expected, GWAS found the ε4 allele of the Apolipoprotein E (ApoE ε4) (Grupe *et al.*, 2007; Saunders *et al.*, 1993). Further, the Apolipoprotein J (ApoJ or Clusterin) (Lambert et al., 2009), the ATP-binding cassette transporter A (ABCA7), the sortilin-related receptor-1 (SORL1) and the phosphatidylinositol binding clathrin assembly protein (PICALM) also play a role lipid metabolism or trafficking (Bertram and Tanzi, 2004; Harold et al., 2009; Hollingworth et al., 2011; Lambert et al., 2013). In the brain, ABCA7 is predominantly expresses in microglia, yet its exact role in AD pathology remains unknown (Fu et al., 2016). However, based on its involvement in phagocytosis it is suggested that ABCA7 could play a role in the clearance of Aβ (Fu et al., 2016).

#	Gene	Protein	Role	Reference
1.	APOE ε2/3/4	apolipoprotein E (ε2/3/4)	major apoprotein of chylomicrons and HDL; major apolipoprotein in the CNS	Lambert et al. 2009
2.	BIN1	bridging integrator 1	tumor suppressing MYC-interacting protein; may be involved in synaptic vesicle endocytosis	Harold et al. 2009
3.	CLU	clusterin (apolipoprotein J)	secreted chaperone involved in cell death, tumor progression and neurodegenerative disorders	Harold et al. 2009
4.	ABCA7	ATP-binding cassette, sub-family A (ABC1), member 7	suggested a role in lipid homeostasis; role in AD unknown	Hollingworth et al. 2011
5.	CR1	complement component (3b/4b) receptor 1	member of the receptors of complement activation (RCA) family	Harold et al. 2009
6.	PICALM	phosphatidylinositol binding clathrin assembly protein	clathrin assembly protein; involved in AP2-dependent clathrin-mediated endocytosis at the neuromuscular junction	Harold et al. 2009
7.	MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	contributes to a shift in anti-inflammatory cells to damaging pro-inflammatory cells	Hollingworth et al. 2011
8.	CD33	CD33	regulator of innate immunity; slows phagocytosis and Aβ clearance	Hollingworth et al. 2011
9.	MS4A4E	membrane-spanning 4-domains, subfamily A, member 4E	unknown role	Hollingworth et al. 2011
10.	CD2AP	CD2-associated protein	implicated in dynamic actin remodeling and membrane trafficking	Naj et al. 2011

Table 3: Summary genetic Alzheimer's disease risk factors identified by GWAS
The top ten genes are listed in order of their relative AD risk.

Another important contributor to AD are genes involved in inflammation such as the complement receptor 1 (CR1), Siglec-3 (CD33), the membrane-spanning 4A is a family of genes (MS4A) and the ephrin receptor A1 (EPHA1) (Harold *et al.*, 2009; Hollingworth *et al.*, 2011; Lambert *et al.*, 2009; Lambert *et al.*, 2013). Additionally, several genes increasing the risk of LOAD are involved in endocytosis. Among those, ABCA7, BIN1, PICALM, CLU,

and SORL1 carry the highest risk (Harold *et al.*, 2009; Hollingworth *et al.*, 2011; Lambert *et al.*, 2009; Lambert *et al.*, 2013). Together with the epidemiological data available, these observations depict a critical role of the cholesterol metabolism and inflammatory pathways in Alzheimer's disease. Another very interesting variant identified in GWAS is the triggering receptor expressed in myeloid cells 2 (TREM2) where loss of function mutants were associated with an increased risk of Alzheimer's disease (Steinberg et al., 2015). TREM2 is a cell-surface receptor of the immunoglobulin (Ig) superfamily and within the CNS, TREM2 is predominantly expressed in microglia. TREM2 forms a complex with its adapter the DNAX activation protein of 12kDa (DAP12) and TREM2 activation, potentially through lipids, leads to the activation of downstream signalling cascades such as the Phosphoinositide 3-kinase (PI3K) or the protein kinase C (PKC) pathways (Feng et al., 2006). Several studies have shown that TREM2 plays a role in the phagocytosis of apoptotic cells as well as bacteria (Takahashi et al., 2005). The lack of TREM2 in the brain has been linked with increased Aβ deposition in the brain and a reduction in gliosis surrounding Aβ plaques (Ulrich et al., 2016).

6.5 Treatment options for Alzheimer's disease

As of now, there is only symptomatic treatment for AD available. Two classes of drugs aiming at the treatment of memory loss and other cognitive problems like confusion and problems with reasoning have been approved. Cholinesterase inhibitors such as Aricept, Exelon or Razadyne are commonly prescribed for mild AD, and N-methyl D-aspartate (NMDA) antagonist like memantine are prescribed for moderate to severe AD. Antidepressants such as sertraline (Zoloft) or fluoxetine (Prozac) are prescribed to combat mood disturbances and irritability and anxiolytics such as lorazepam (Ativan) or oxazepam (Serax) to treat anxiety. However, the effectiveness of antidepressants for the treatment of AD is controversial, and multiple studies were not able to demonstrate statistically significant improvements when comparing antidepressants with placebos (Orgeta *et al.*, 2017). Furthermore, antipsychotic medications are prescribed in the case of hallucinations, delusions, and aggression. Other symptoms such as sleep disturbances and insomnia can be combatted using sleeping pills

(zolpidem or chloral hydrate) or benzodiazepines such as lorazepam or oxazepam (Deschenes and McCurry, 2009).

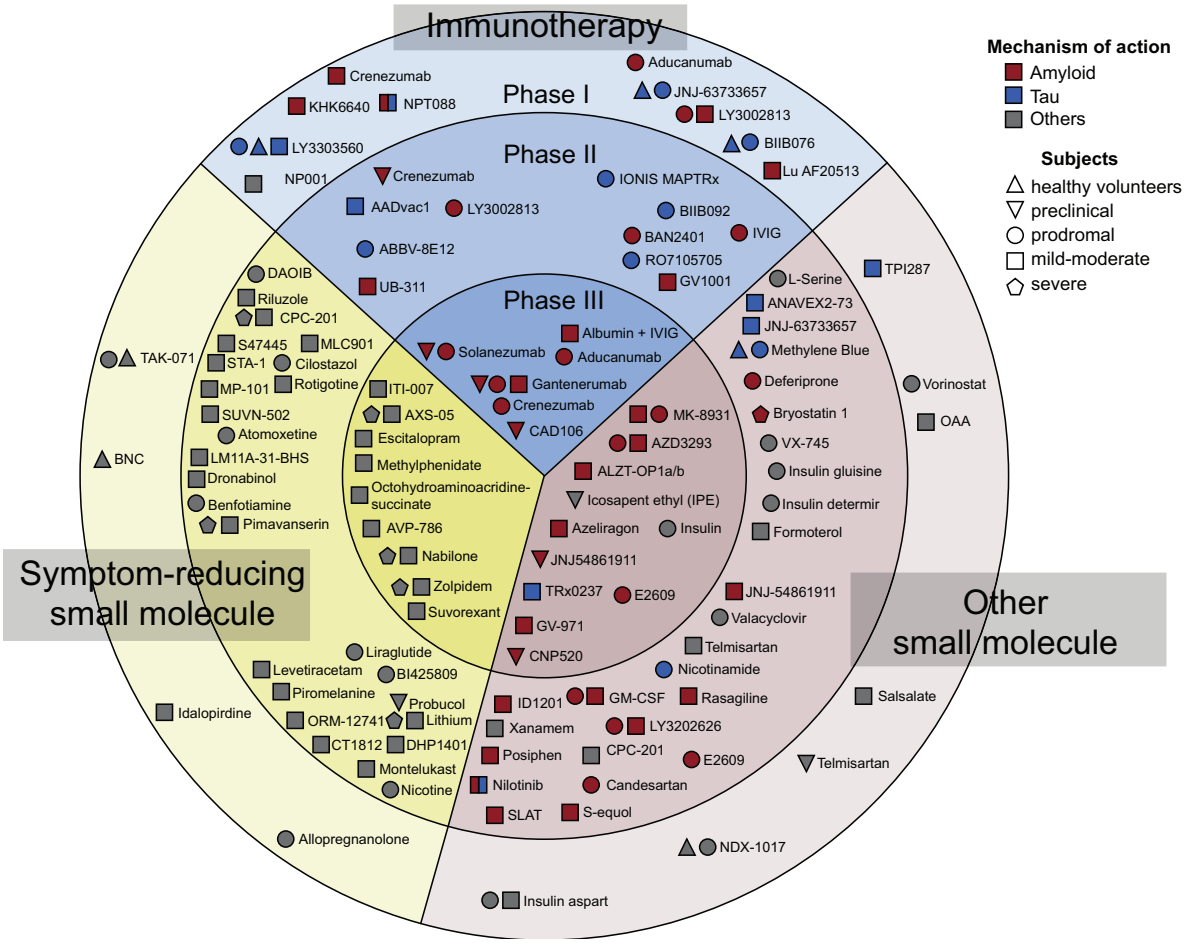


Figure 6: Ongoing clinical trials for Alzheimer's disease therapies
All ongoing trials as found on Clinicaltrials.gov are listed and categorized according to their current clinical phase and mechanism of action. Out of 112 total drugs, 26 are in clinical phase III, 63 in phase II and 23 in phase I. (Figure adapted from Cummings *et al.*, 2018).

Even though all drugs targeting AD failed, there are still several clinical trials ongoing (**Figure 6**). Multiple companies developed drugs targeting the amyloid peptides and removing it or preventing its aggregation or generation. Sargramostim (GM-CSF leucine) and the drug AZD3293 are examples of drugs aiming at reducing the levels of Aβ. Sargramostim is a synthetic granulocyte-macrophage-colony-stimulating factor that can reduce amyloid pa-

thology in transgenic mouse models of AD (Boyd *et al.*, 2010). Another strategy to reduce amyloid pathology is aiming at reducing the generation of Aβ. Most drugs in this category target one of the enzymes involved in the generation of Aβ, BACE or γ-secretase. While γ-secretase inhibitors (GSI) have shown significant reductions in Aβ generation, inhibiting γ-secretase has been proven to be challenging due to the many substrates of the enzyme.

Most importantly, the cleavage of notch1 is governed by γ-secretase, and non-selective GSIs showed target-based toxicity. Notch1 is a transmembrane receptor involved in the regulation of cell fate during development but also synaptic plasticity and neuronal stem cell maintenance (Wong *et al.*, 1997). While most GSIs such as Semagacestat failed in clinical trials, some are still pursued. Examples of notch-sparing GSIs that are still in clinical trials are BMS-708163 (Avagacestat) or GSI-953 (Begacestat).

More recently developed γ-secretase modulators (GSMs) appear to a safer alternative to GSIs as they do not interfere with the regular enzymatic activity but rather interact with an allosteric site on the enzyme and can drastically shift the levels of toxic Aβ42 towards less harmful shorter species such as Aβ38 (Bursavich *et al.*, 2016). Multiple classes of GSMs have been developed and tested in clinical trials such as GSM-1 and NGP-555 (Ebke *et al.*, 2011; Weggen *et al.*, 2001). Similar to GSIs, early GSMs suffered from target-based toxicity which has to be closely monitored during the development of new GSMs. Lastly, there are reports that some GSMs may interact with APP and not γ-secretase (Kukar *et al.*, 2008). In addition to GSMs, BACE inhibitors have been intensely studied. The development of potent BACE inhibitors has been challenging, but the upsides could be immense since BACE has few known substrates and the potential of lower-target based toxicity. Unfortunately, most BACE inhibitors developed eventually failed in clinical studies either due to their side effects (LY2811376) or lack of efficacy (MK8931, Verubecestat) (May *et al.*, 2011).

Based on the failure of drugs targeting Aβ, it has been questioned whether targeting the amyloid is a worthwhile strategy. While several drugs were able to reduce the amyloid burden significantly, no cognitive improvement was reported. Additionally, it became clear that the

molecular changes underlying the amyloid pathology start well before the onset of the clinical symptoms, complicating the design of clinical trials (Jack *et al.*, 2013). Despite these challenges, more than half of AD drugs currently in clinical phase III target amyloid (Cummings *et al.*, 2018).

7. A role for CETP in Alzheimer's disease

As discussed previously, CETP is expressed in astrocytes in the brain, yet its function there remains elusive (Albers *et al.*, 1992). Interestingly, the same polymorphism linked with longevity has been recently associated with a reduced risk of dementia (Barzilai *et al.*, 2003; Chen *et al.*, 2014; Sanders *et al.*, 2010; Thompson *et al.*, 2008; Yu *et al.*, 2012). Subsequently, several studies analyzed if CETP polymorphisms may further decrease the risk for Alzheimer's disease (AD). Using brain imaging data, Salminen *et al.* linked the CETP I405V polymorphism with medial temporal lobe volume loss in an ApoE ε4-dependent fashion (Salminen *et al.*, 2015). Furthermore, some studies reported protective effects of CETP polymorphisms at early AD stages, particularly in carriers of the apolipoprotein E ε4 (ApoEε4). Rodriguez *et al.* found that in APOE ε4 carriers, the homozygosity for the CETP I405V polymorphism led to a three-fold reduced risk of developing AD (Rodriguez *et al.*, 2006). Further, Murphy *et al.* investigated two CETP polymorphisms in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort and found that both polymorphisms correlated with increased cortical thickness and reduced atrophy in ApoE ε4 carriers (Murphy *et al.*, 2012). Lastly, Sundermann *et al.* hypothesized that the CETP polymorphism may buffer the adverse effects on the APOE ε4 allele. Using the Einstein aging cohort of non-demented elderly, they measured the episodic memory and found that the CETP I405 polymorphism indeed buffers the detrimental effect on memory decline (Sundermann *et al.*, 2016).

Those epidemiological findings indicate that CETP activity may impact on cognitive performance. However, the underlying molecular mechanisms remain unclear.

Finally, we believe that the evidence for a role of CETP in AD is staggering. Yet, almost all

development of CETP inhibitors has been abandoned due to the availability of potent drugs that already efficiently lower LDL cholesterol levels (Gu *et al.*, 2014). Still, the picture looks bleak for the case of a treatment for AD. The high potency and tolerability of CETP inhibitors make them, accordingly prime candidates to be tested for potential effects on AD.

While the majority of epidemiological studies found that CETP polymorphisms associated with reduced CETP activity correlate with a lower incidence of AD, Arias-Vasquez *et al.* reported a slightly increased risk of AD in homozygous carriers of the I405V polymorphism (Arias-Vasquez *et al.*, 2007). The discrepancy between the different epidemiological studies could be due to differences in the cohorts that were analyzed. Further, this increase in AD risk was only observed in non-carriers of the ApoE ε4 allele. Most studies that found a reduced risk for AD, reported particular strong effects in ε4 carriers and the effects of CETP on AD could rely strongly on the ApoE isoform present.

Table 4: Studies linking CETP with Alzheimer's disease

Several studies have studied links between CETP polymorphisms and AD and/or dementia.

Finding	Reference
CETP polymorphism modifies AD risk associated with ApoE ε4	Rodriguez *et al.* J Neurol., 2006
Longevity associates with a lower prevalence of hypertension, cardiovascular disease, the metabolic syndrome, and increased homozygosity for the CETP I405V variant.	Barzilai *et. al.* JAMA, 2003
CETP genotype associated with lower CETP levels implicated as a modulator of age-related cognitive function	Barzilai *et. al.* Neurology, 2006
CETP I405V polymorphism increases AD risk in ApoE ε4 non-carriers	Arias-Vásquez *et al.* Neurogenetics, 2007
No association of CETP genotype with cognitive function or age-related cognitive change	Johnson *et. al.* Neurosci Lett., 2007
CETP I405V homozygosity is associated with lower AD risk	Sanders *et al.* JAMA, 2010
CETP I405V associates with lower risk of dementia in ApoE ε4 non-carriers	Murphy *et al.* Brain Imaging Behav., 2012
The CETP I405V polymorphism is associated with an increased risk of Alzheimer's disease	Yu *et al.* Aging Cell, 2012
CETP associates with less intense frontally mediated behaviors	Warstadt *et. al.* Neurobiol. Aging, 2014
Meta-analysis: Suggests that CETP rs5882 polymorphism increases the risk of AD	Chen *et al.* DNA and Cell Biology, 2014
Meta-analysis: No association found between CETP SNPs and the risk of AD, carotid atherosclerosis, longevity, and the efficacy of statin therapy	Li *et. al.* Neurobiol. Aging, 2014
CETP I405V is a predisposing risk factor for gray matter abnormalities in posterior brain regions in healthy older adults, independent of an ApoE4 allele.	Salmienen *et. al.* J Neural Transm., 2015
CETP I405V allele buffers ApoE ε4-associated memory decline in a gene dose-dependent manner	Sunderman *et al.* Neurobiol Aging, 2016
CETP I405V is associated with preserved cognition over time but not with LOAD status.	Lythgoe *et al.* Neurobiol Aging, 2015

8. Aim of the thesis

Alzheimer's disease poses an immense socioeconomic burden on society, yet all therapeutic approaches have failed thus far. Given the overwhelming evidence of the involvement of the cholesterol metabolism in the disease, it is indispensable to expand our understanding of how cholesterol modifies the risk and pathology of the disease.

Unfortunately, mice and rats have significantly different cholesterol and lipoprotein metabolisms compared to humans. Hence, all currently available Alzheimer's disease models are not suitable to study the role of cholesterol in the disease.

Logically, the first aim of the thesis was to generate an Alzheimer's disease model that closely models the human cholesterol metabolism. We chose to use CETP transgenic mice as they are a well-characterized model and have been shown to model the human cholesterol metabolism. However, CETP transgenic mice do not develop cognitive deficits or other markers of Alzheimer's disease pathology and had to be crossed with models for Alzheimer's disease.

Using these models, we investigated the effects of a humanized cholesterol metabolism and CETP activity on the pathological hallmarks of Alzheimer's disease for my second aim.

EXPERIMENTAL SECTION

In order to study a potential role of CETP in AD, we turned to a mouse model transgenic for human CETP since mice completely lack the protein (Jiang *et al.*, 1992). While there are other CETP transgenic models available, they carry other transgenes or gene knockouts like an LDLR knockout, and we wanted to use the simplest model showing a humanized lipoprotein profile (Harada *et al.*, 2006; van den Hoek *et al.*, 2014). CETP transgenic mice have been used extensively to study cardiovascular disease, but data regarding its effects in the brain are sparse. Hence, our initial experiments were carried out in CETP transgenic mice, and the first manuscript addresses some of the effects of CETP in the brain. For the second manuscript, we turned our focus on AD and crossed the CETP transgenic mice with models for AD.

FOREWORD MANUSCRIPT 1

Alzheimer's disease is a devastating disease with an immense burden, both financially and socially. Clinical trials over the last decades have not been able to develop a cure or prevention for the disease and have put the current dogmas in question. However, it is well understood that the cholesterol metabolism is a substantial contributing factor to the disease (Di Paolo and Kim, 2011). It may be time to re-evaluate whether targeting Aβ is a fruitful strategy to treat the disease. Several clinical trials on cardiovascular disease (CVD) hinted at beneficial effects of cholesterol-lowering drugs in AD.

Most animal models studying aspects of AD pathology are based on mice and rats. However, both species show striking differences in their cholesterol metabolism compared to humans and may not be a suitable model system to study the interrelationship of AD pathology and cholesterol metabolism. The most striking difference lies in the complete lack of the cholesteryl ester transfer protein (CETP). CETP loads LDL with cholesteryl esters and is primarily responsible for LDL levels in humans. Luckily, mice transgenic for human CETP have been studied for a long time in CVD research but have not been analyzed beyond their cardiovascular system. However, the role of CETP in the brain is poorly understood. This work aims at studying the effects of CETP on brain cholesterol metabolism, especially in relation to known AD risk factors.

MANUSCRIPT 1:

The Cholesteryl Ester Transfer Protein (CETP) raises Cholesterol Levels in the Brain and affects Presenilin-mediated Gene Regulation.

Felix Oestereich[1, 2], Elizabeth-Ann Kranjec[3], Ethan Yang[3], Pierre Chaurand[3], Lisa Marie Munter[1]

[1]Department of Pharmacology and Therapeutics, Cell Information Systems group, McGill University, 3649 Promenade Sir William Osler, Montreal, QC, Canada H3G 0B1
[2]Integrated Program in Neuroscience, McGill University, Montréal, QC, Canada H3A 2B4
[3]Department of Biochemistry, Université de Montréal, Montréal, QC, Canada H3C 3J7

Correspondence:
Dr. Lisa Munter, lisa.munter@mcgill.ca, McGill University, Department of Pharmacology and Therapeutics, Bellini Life Sciences Complex, 3649 Promenade Sir-William Osler, Montreal, QC, Canada, H3G 0B1

1. Abstract

The cholesteryl ester transfer protein (CETP) is a lipid transfer protein responsible for the exchange of cholesteryl esters and triglycerides between lipoproteins. Decreased CETP activity is associated with longevity, cardiovascular health, and maintenance of good cognitive performance. Interestingly, mice lack CETP and have very low levels of low-density lipoprotein (LDL) particles compared to humans. To understand how CETP activity affects the brain, we utilized CETP transgenic (CETPtg) mice with a normolipidemic, humanized cholesterol profile. We found that the brains of CETPtg mice showed up to 25% higher cholesterol levels. Using a microarray on astrocyte-derived mRNA, we found that this cholesterol increase is likely not due to *de novo* synthesis of cholesterol. However, several genes linked to Alzheimer's disease were affected. Most interestingly, we found activation of the G protein-coupled receptor EP4 and γ-secretase as upstream regulators of the transcriptional changes observed in the CETPtg mice. Further, CETP expression was sufficient to activate γ-secretase activity *in vitro*. The data suggest that CETPtg mice phenocopy human plasma cholesterol levels and cholesterol exposure of the brain as a valuable research tool to investigate the impact of the cholesterol metabolism on brain functions, especially concerning Alzheimer's disease.

2. Introduction

2.1 Cholesterol and lipoproteins

Cholesterol is a major constituent of biomembranes and precursor for various hormones. In most tissues, the cholesterol concentration is about 2 mg/g tissue. However, it reaches 15-20 mg/g in the tissue of the central nervous system (CNS) (Dietschy and Turley, 2004). Consequently, the brain contains 25% of the total body cholesterol, suggesting a particular need of the brain for cholesterol (Bjorkhem and Meaney, 2004). In the blood, dietary cholesterol is transported by very-low-density lipoprotein (VLDL) or low-density lipoprotein (LDL) particles that are secreted by the liver to deliver cholesterol to extrahepatic tissues (Yao

and McLeod, 1994). Reverse cholesterol transport from the periphery back to the liver occurs via high-density lipoprotein (HDL) particles (Glomset, 1968). However, the brain seems to be excluded from those distribution cycles. Neither VLDL or LDL particles cross the blood-brain barrier (Jeske and Dietschy, 1980; Liu *et al.*, 2012).

In the CNS, astrocytes are the cell type primarily involved in lipid synthesis and secrete HDL-like lipoprotein particles that contain predominantly apolipoprotein E (ApoE) as their apolipoprotein (Vance and Hayashi, 2010). Such particles are taken up by neurons through members of the LDL-receptor family that recognize ApoE including the LDL-receptor related protein 1 (LRP1) (Fryer *et al.*, 2005; Liu *et al.*, 2007).

2.2 The cholesteryl ester transfer protein

The cholesteryl ester transfer protein (CETP) is a lipid transfer protein that facilitates the exchange of cholesteryl esters in HDL for triglyceride in VLDL and LDL (Nichols and Smith, 1965; Zilversmit *et al.*, 1975). The net result of this transfer activity is a increased cholesterol content in pro-atherogenic LDL particles and decreased cholesterol levels in anti-atherogenic HDL particles (Zhong *et al.*, 1996). Studies investigating the genetic predisposition of "super-agers" or "centenarians", aged people with well-maintained health and cognitive performance, revealed an association with CETP. Here, polymorphisms that impair CETP's activity associated with longevity, cardiovascular health, and good cognitive performance (Barzilai *et al.*, 2006; Barzilai *et al.*, 2003; Murphy *et al.*, 2012; Sanders *et al.*, 2010). Based on these findings, several studies analyzed whether CETP polymorphisms could decrease the risk of Alzheimer's disease, an aging-associated neurodegenerative disease. Indeed, protective effects of CETP polymorphisms at early Alzheimer's disease stages were reported, particularly in carriers of the strongest genetic risk factor, the ε4 allele of the apolipoprotein E (ApoEε4) (Arias-Vasquez *et al.*, 2007; Murphy *et al.*, 2012; Rodriguez *et al.*, 2006; Sundermann *et al.*, 2016). In contrast to the peripheral lipoproteins, ApoE is the predominant lipoprotein of the

brain (Wang and Eckel, 2014). Those epidemiological findings indicate that CETP activity may impact on cognitive performance and brain function. However, the underlying molecular mechanisms remain unclear.

While CETP is predominantly expressed in the liver and secreted to the blood, it is expressed in astrocytes in the brain (Yamada *et al.*, 1995). Its function in the CNS remains elusive. In light of the effects of CETP on cognition, we hypothesized that CETP might affect the brain's cholesterol levels.

It is important to note that mice lack CETP, resulting in significant differences in the plasma lipoprotein profile between mice and humans. Mice have negligible LDL-levels compared to humans. Here, we used a well-established CETP transgenic mouse model expressing the human CETP gene under its natural promoter (CETPtg) that is frequently used in the cardiovascular research field (Cazita *et al.*, 2003; Cheema and Rashid-Kolvear, 2003; Jiang *et al.*, 1992). The promoter contains a cholesterol responsive element (CRE), and dietary lipids induce CETP gene expression. CETP expression in mice leads to increased LDL levels, mimicking a humanized (normolipidemic) lipoprotein profile (Gauthier *et al.*, 1999). We herein characterized the effects of CETP expression on molecular changes in the brain in CETPtg mice. We observed higher cholesterol levels in the brains of CETPtg as compared to wild-type mice. Transcriptome profiling of astrocytes indicated decreased cholesterol synthesis, regulation of several genes linked to Alzheimer's disease and overall activation of presenilin-mediated signaling.

3. Materials & Methods:

All experiments were conducted following McGill University environmental health and safety regulations (EHS) as well as the Canadian biosafety standards and guidelines.

3.1 Cell culture

HEK293T cells were cultivated in 1:1 Dulbecco´s modified Eagle medium (DMEM) supplemented with 0.584 g/l L-glutamine and 0.11 g/l sodium pyruvate (Wisent), and 10% FCS (Wisent), at 37°C and 5% CO_2. For transient transfections, 1.5 x 10^5 cells per well (12-well plates) were seeded 24 h before transfection. Cells were transiently transfected with 1 µg DNA in total and 2 µl polyethyleneimine (PEI) per well. 36 hours after transfection, cell culture supernatant was collected, and cells were lysed with TNE-lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, and complete protease inhibitors, Roche) and prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.2 Western blot analysis of mouse tissue samples

Fresh frozen liver or brain samples (approximately 100 mg) were lysed in 5x volume of lysis buffer (150 mM NaCl, 10% glycerol, 2 mM EDTA, 0.5% NP-40, 0.1% sodium-deoxycholate, 20 mM HEPES, 1x complete protease-inhibitor cocktail (Roche), pH 7.4) using lysing-matrix D at 6000 rpmin a Roche MagNA Lyser for 40 seconds. The lysates were further diluted 1:5 in lysis buffer. For western-blot analysis, liver samples were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on either 10% or 15% SDS-polyacrylamide gels. The following primary antibodies were used: 22C11 (Millipore), rabbit-anti-GAPDH (14C10, Cell Signaling), TP2 (kind gift of the Ottawa Heart Institute), anti-TREM2 (Mab1729 R&D systems) and anti-ABCA7 (polyclonal, Thermo Fisher). Horseradish peroxidase (HRP)-coupled secondary antibodies directed against mouse or rabbit IgG were purchased from Promega. Chemiluminescence images were acquired using the ImageQuant LAS 500 system (GE Healthcare).

3.3 Quantitative real time PCR (RT-qPCR)

mRNA was isolated from mouse tissue using the Macherey & Nagel mRNA-isolation kit in combination with lysing matrix D. Briefly, 25-50 µg of fresh frozen tissue were lysed in 450 µL RNA preparation buffer (with β-mercaptoethanol) in lysing matrix D tubes using a Roche MagNA Lyser (6000 rpm 2x 30 seconds) according to manufacturer's in-

structions. The RNA concentration was adjusted to 100 pg/mL and 500 ng of RNA were transcribed into cDNA using the high-capacity cDNA reverse-transcription kit (Applied Biosystems) according to manufacturer's instructions. RT-qPCR was performed using the SsoAdvanced SYBR green supermix (Biorad) according to manufacturer's instructions on a Biorad CFX384Touch cycler. All primers were ordered from integrated DNA technologies. Primers used were: CETP forward: CAGATCAGCCACTTGTCCAT, CETP reverse: CAGCTGTGTGTTGATCTGGA, ABCA7 forward: TTCTCAGTCCCTCGTCACCCAT, ABCA7 reverse: GCTCTTGTCTGAGGTTCCTCGT, TNFα forward: GGTGCCTATGTCTCAGCCTCTT, TNFα reverse: GCCATAGAACTGATGAGAGGGAG, IL1β forward: TGGACCTTCCAGGATGAGGACA IL1β reverse: GTTCATCTCGGAGCCTGTAGT, TLR4 forward: AGCTTCTCCAATTTTTCAGAACTTC, TLR4 reverse: TGAGAGGTGGTGTAAGCCATGC, TREM2 forward: ACAGCACCTCCAGGAATCAAG, TREM2 reverse: AACTTGCTCAGGAGAACGCA, IL6 forward: CCTCTGGTCTTCTGGAGTACC, IL6 reverse: ACTCCTTCTGTGACTCCAGC, HES1 forward: p21 forward: GCCTTAGCCCTCACTCTGTG p21 reverse: AGCTGGCCTTAGAGGTGACA, HES1 forward: CGGAATCCCCTGTCTACCTC, HES1 reverse: AATGCCGGGAGCTATCTTTCT. The following primers were used as reference genes: HPRT forward: CCAGTTTCACTAATGACACAAACG, HPRT reverse: CTGGTGAAAAGGACCTCTCGAAG, PSMC4 forward: CCGCTTACACACTTCGAGCTGT, PSMC4 reverse: GTGATGTGCCACAGCCTTTGCT, GAPDH forward: CATCACTGCCACCCAGAAGACTG, GAPDH reverse: ATGCCAGTGAGCTTCCCGTTCAG, Actin-β forward: CATTGCTGACAGGATGCAGAAGG, Actin-β reverse: TGCTGGAAGGTGGACAGTGAGG. Primer efficiency for all primers was determined to be between 90-110%. For normalization of gene expression, the four genes ACT, GAPDH, HPRT and PSMC4 were used as reference genes. RT-qPCR was analysed using the CFX manager software (Biorad).

3.4 Imaging mass spectrometry (IMS)

Sample preparation: The fresh frozen brain samples were sectioned sagittally at 14 μm thickness and the frozen brain homogenates at 20 μm thickness with a Leica CM3050 cryostat at -20°C (Leica Microsystems GmbH, Wentzler, Germany). All brain specimens were cut at approximately the same Bregma in order to clearly delineate the hippocampus. Brain homogenates were prepared according to published protocols (Groseclose and Castellino, 2013) and were used to normalise data across experiments. For each technical replicate, one tissue section of each condition was thaw-mounted in a 2 x 2 pattern on a 25 x 75 mm indium-tin-oxide (ITO) coated microscope slide (Delta Technologies, Loveland, CO), along with two sections of frozen brain homogenate on the left and right of the grid. After desiccation in a vacuum pump desiccator for ≤ 1 hour, a 23 ± 2 nm silver layer was deposited onto the sections using a Cressington 308R sputter coater (Ted Pella Inc, Redding, CA) as per the protocol detailed in Dufresne et al 2013 (Dufresne *et al.*, 2013). The argon partial pressure was set at 0.02 mbar and the current at 80 mA. Data acquisition: IMS data were acquired at 50 μm spatial resolution and 100 shots per raster position with a "small" laser setting using a "matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF) ultrafleXtreme mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a SmartBeam-II Nd:YAG/355-nm laser operating at a repetition rate of 1 kHz using flexImaging 4.1 software (Bruker Daltonics, Billerica, MA). All instrumental parameters (source voltages, laser energy, delayed extraction parameters, etc.) were optimised for maximum signal-to-noise ratio within the 100-1100 m/z range in the reflectron geometry, with the acceleration voltage set to 25 kV. Two 400-pixel squares were also acquired from each brain tissue homogenate section at the same spatial resolution. Data Analysis: Raw IMS data were first internally calibrated with the silver isotopic peaks using the flexAnalysis Batch Process software (Bruker Daltonics, Billerica, CA) to obtain a ~5 ppm mass accuracy. Next, IMS data from the hippocampal and whole brain regions of interests (ROIs) were exported into the common imzML format using flexImaging 4.1 (Schramm *et al.*, 2012). Using an in-house code based on the Cardinal package (x1.6.0) in R (x3.2.5), the mean area and standard deviation of the two cholesterol signals

(m/z 493.26 and m/z 495.26, corresponding to the [M+107Ag]+ and [M+109Ag]+ molecular ions, respectively) were calculated for the ROIs after independent TIC normalization (Bemis *et al.*, 2015). The same code was used to obtain the mean of the summed areas of the ten most abundant signals in the homogenate squares. This value acted as the correction factor to correct for variations in signal intensity across all experiments. The final cholesterol intensity reported is the mean across the three technical triplicates for one group normalised against the correction factor. Unless otherwise noted, all solvent and material were purchased from Thermo Fisher Scientific (Ottawa, ON). The silver target 3N5 (99.95% purity) used for tissue sputter-coating was purchased from ESPI Metals (Ashland, OR).

3.5 Mouse housing

The CETP transgenic mouse strain B6.CBA-Tg(CETP)5203Tall/J (Jackson strain no.: 003904) (Jiang *et al.*, 1992) were housed according to the McGill University standard operating procedure mouse breeding colony management #608. Mice were bred heterozygous and non-transgenic littermates were used as controls. All mouse diets were purchased from Envigo. The diets used in this study were: low fat control diet (TD.08485), low fat diet enriched with 1% cholesterol (TD.140215) and a diet containing 21% fatty acids (FA) and 1% cholesterol (TD.95286). The FA composition was 65% saturated FA (SFA), 31% monounsaturated FA (MUFA), and 4% polyunsaturated FA (PUFA). Animals were assigned randomly to treatment groups. All procedures were approved by McGill's Animal Care Committee and are in accordance with the McGill policy on the study and care of animals. The reporting of all mouse data in accordance with the ARRIVE guidelines (Animal Research: Reporting *in Vivo* Experiments).

3.6 Mouse genotyping

Genotyping was performed by Transnetyx genotyping using real-time PCR from ear punch tissue. Ear punches were lysed in at 56 °C overnight. Primers used for the transgene were: forward: GAATGTCTCAGAGGACCTCCC, reverse: CTTGAACTCGTCTCCCATCAG.

Primers for internal controls were: Forward: CTAGGCCACAGAATTGAAAGATCT, reverse: GTAGTGGAAATTCTAGCATCATCC.

3.7 Plasma lipid analysis

The lipid analysis of mouse plasma samples was performed using the COBAS Integra 400 Plus analyzer (ROCHE) and the following kits: COBAS INTEGRA CHOL 2, COBAS INTEGRA HDL-C gen3, and COBAS Integra TRIG GPO 250, respectively. The levels of LDL-C were calculated using the Friedewald formula: [total cholesterol] – [HDL-C] – [TG/2.2].

3.8 CETP activity assay

CETP activity was measured using the Roar biomedical Inc. fluorescent CETP activity assay. Here, 5 µL of cell culture supernatant was incubated with 0.3 µL donor and 0.3 µL acceptor molecules in 30 µL reaction volume. The reaction mix was incubated for 3 hours at 37 °C in a water bath and the fluorescence (λ_{ex} 465/λ_{em} 535) was measured.

3.9 Astrocyte purification

Astrocytes were purified using the Anti-GLAST (ACSA-1) MicroBead Kit (Miltenyi biotec). Briefly, whole mouse brains were dissociated using a miltenyi gentleMACS Octo dissociator with Heaters, and GLAST positive astrocytes were isolated using anti-GLAST (ACSA-1) antibody magnetic beads according to manufacturer's instructions.

3.10 Flow cytometry

Purified astrocytes were EtOH fixed and stained with a Cy3 labelled anti-GFAP antibody (1:1000, Sigma). The samples were run on a BD LSRFortessa flow cytometer and the GFAP-Cy3 emission was detected using a 561 nm laser for excitation. The detector channel used was 586/15 nm. BD FACSDIVA 8.0.1. was used for analysis.

3.11 Astrocyte microarray

RNA from GLAST-positive astrocytes was isolated using the Macherey & Nagel mRNA isolation kit. The Affymetrix clariom-S nano microarray was performed at the Genomecenter

Quebec according to manufacturer's instructions. The initial microarray dataset was analysed using Transcriptome Analysis Software (Affymetrix). Upstream regulator and pathway analyses were performed using Ingenuity Pathway Analysis (IPA). GEO accession number: GSE111242.

3.12 Statistical analysis

Statistical analysis was performed using the Graphpad Prism 7 software.

4. Results

4.1 Dietary cholesterol intake induces CETP expression

CETP transgenic animals have been widely used in cardiovascular research. However, it remained unclear whether fatty acids could induce CETP expression in addition to dietary cholesterol (Cazita *et al.*, 2003; Cheema and Rashid-Kolvear, 2003; Jiang *et al.*, 1992). Hence, we compared a diet enriched with 1% (w/w) cholesterol to a diet containing 1% cholesterol plus 21% (w/w) fatty acids for their effects on CETP expression in CETPtg mice. Here we fed wild type and CETPtg with our lipid diets for one month starting at the age of two months (**Figure 7A**). As expected, CETPtg, but not wild type mice showed CETP activity, confirming that mice do not express CETP or a protein compensating for the lack of CETP. To understand the effect of dietary fatty acids of cholesterol, we compared CETP transgenic mice on a control diet with mice fed a diet enriched in cholesterol or cholesterol and fatty acids. CETP activity was increased 2-fold when animals were fed with a diet enriched in cholesterol or cholesterol and fatty acids as compared to mice on a standard diet (**Figure 7B**). Likewise, the protein levels of circulating CETP, as determined by western blotting, were increased 2-fold in mouse plasma with either diet (**Figure 7D, E**).

Further, we quantified CETP mRNA levels from the liver by RT-qPCR and found that the diet supplemented only with cholesterol showed the most robust increase (8.8-fold) as compared to the high cholesterol/fat diet (7-fold) (**Figure 7C**). Additional fatty acids did not

affect CETP activity or expression levels (**Figure 7B-D**)

When assessing the lipid profile in plasma, were interested in poteintial diet-dependent aas well as CETP-dependent effects. Here, the 1% cholesterol diet in itself had no effect on HLD levels. However, we found that CETPtg mice showed lower HDL levels on the standard and cholesterol diets, an effect that vanished in mice receiving the cholesterol/fat diet (**Figure 7F**).

The only group with significantly elevated LDL cholesterol levels were CETPtg animals fed with a cholesterol-enriched diet. These mice shoed 2-3-fold higher LDL-C compared to CET tg mice on a standard mice as well as wild type mice. However, this was not observed in animals fed with a cholesterol and fat diet although both diets led to a similar increase in CETP activity and protein levels (**Figure 7G**). This could potentially be explained by increased cholesterol secretion in the form of bile acid. Cappel *et al.* described a protective effect of CETP in a obesity model where CETP was able to ameliorate insulin resistance induced by obesity (Cappel *et al.*, 2013). However, to our knowledge this is the only report showing protective effects of CETP on a diet enriched in lipids and cholesterol.

Note that LDL levels of approximately 1.2 mmol/L are still relatively low considering that human LDL levels < 3 mmol/L are still considered healthy levels. Free cholesterol was not significantly affected by the diets (**Figure 7H**). However, we found a trend towards decreased levels of triglycerides in animals fed with the cholesterol diet, independent of the genotype (**Figure 7I**). Finally, we analyzed the net weight gain of mice during the 4-week feeding period. In contrast to the cholesterol/fatty acid diet, mice on the cholesterol diet did not show an additional weight gain as compared to a standard diet (**Figure 7J**). Together, high CETP expression and activity is achieved with both diets enriched in either cholesterol alone or cholesterol and fatty acids. However, the blood lipoprotein profile only changed towards a more human-like profile. Mice on regular chow diet show only negible LDL levels and rely predominantly on HDL particles.

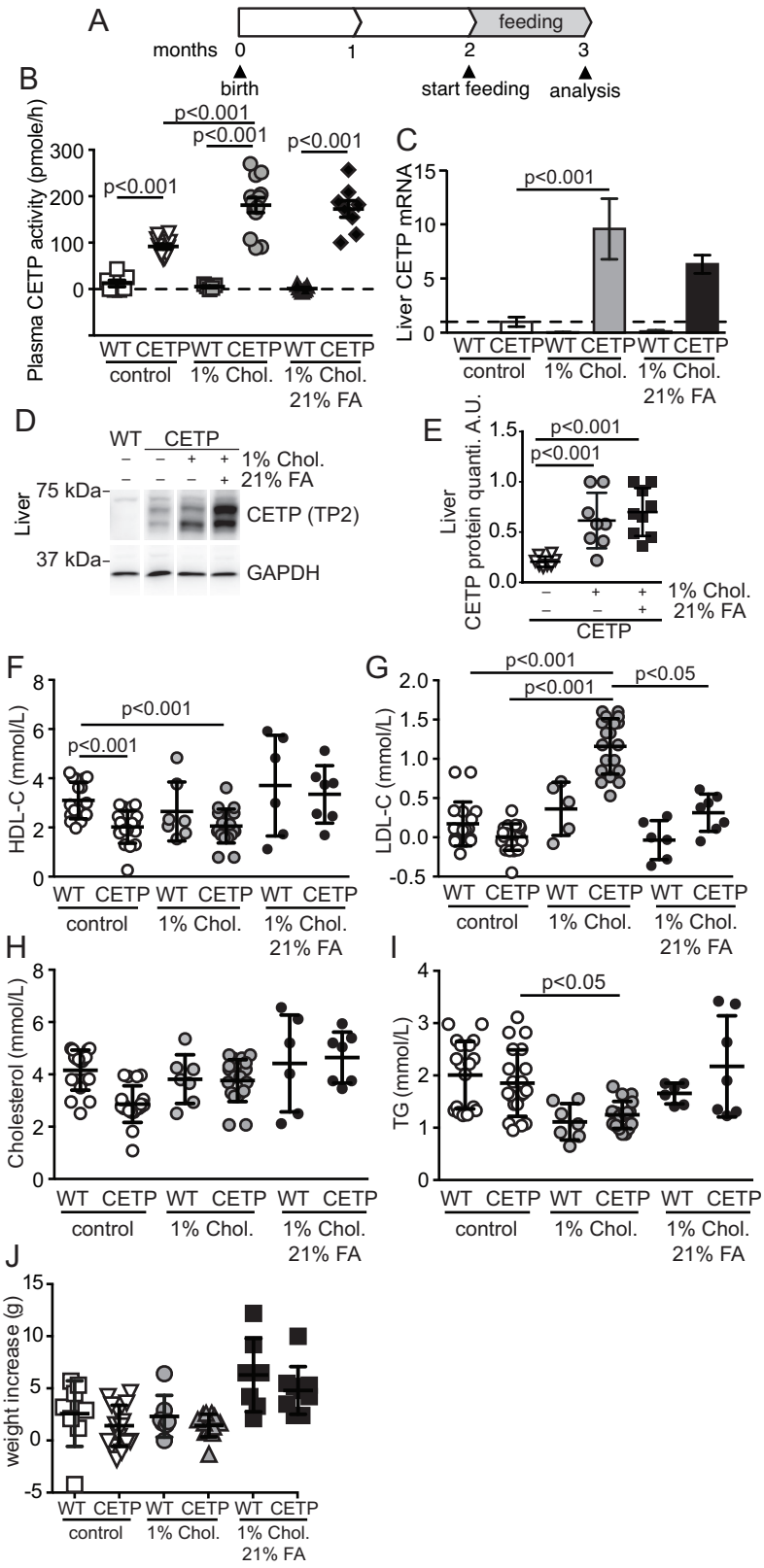


Figure 7: Dietary cholesterol intake induces CETP expression

A: Feeding schedule & study design. Wild type and CETP transgenic animals were fed for 1 month starting at the age of 2 months. Biochemical analyses were performed after 3 months of age. **B**: CETP activity: CETP activity of CETP transgenic or wild type animals was measured from 1 µL plasma using the fluorescence-based CETP activity assay (Roar biomedical). n=6-14, mean ± SEM; 2-way ANOVA, Tukey's multiple comparison. **C**: Relative normalized CETP expression. RT-qPCR of liver samples at the age of 5 months. n=5-8, mean ± SEM; 2-way ANOVA, Tukey's multiple comparison. **D**: CETP western blot from liver lysates. Liver lysates were separated on 10% SDS-PA gels. CETP was detected using the TP2 monoclonal antibody. **E**: Quantification of CETP western blots as shown in **D**: n=8, mean ± SEM; Students T-test. **F-I**: Plasma lipoprotein analysis: **F**: HDL-C, **G**: LDL-C, **H**: free cholesterol **I**: and triglycerides from mouse plasma samples. Plasma samples were analysed on a COBAS Integra 400 Plus (ROCHE) analyser using the following kits: COBAS INTEGRA CHOL 2, COBAS INTEGRA HDL-C gen3, and COBAS Integra TRIG GPO 250, respectively. The levels of LDL-C were calculated using the Friedewald formula: [total cholesterol] – [HDL-C] – [TG/2.2]. n=6-14. mean ± SEM; 2-way ANOVA, Tukey's multiple comparison. **J**: Mouse weight increase: Net weight increase of wild type and CETP transgenic animals during the feeding period.

Using CETPtg mice fed a 1% cholesterol diet led to increased LDL levels similar to a human lipoprotein profile. In addition, since cholesterol-enriched food did not impact on the weight of mice, this diet has the advantage that potentially confounding factors such as obesity can be excluded. Thus, we continued to use the 1% cholesterol diet for further experiments.

Note that LDL levels of approximately 1.2 mmol/L are still relatively low considering that human LDL levels < 3 mmol/L are still considered healthy levels. Free cholesterol was not significantly affected by the diets (**Figure 7H**). However, we found a trend towards decreased levels of triglycerides in animals fed with the cholesterol diet, independent of the genotype (**Figure 7I**). Finally, we analyzed the net weight gain of mice during the 4-week feeding period. In contrast to the cholesterol/fatty acid diet, mice on the cholesterol diet did not show an additional weight gain as compared to a standard diet (**Figure 7J**). Together, high CETP expression and activity is achieved with both diets enriched in either cholesterol alone or cholesterol and fatty acids. However, the blood lipoprotein profile only changed towards a more human-like profile. Mice on regular chow diet show only negible LDL levels and rely predominantly on HDL particles. Using CETPtg mice fed a 1% cholesterol diet led to increased LDL

levels similar to a human lipoprotein profile. In addition, since cholesterol-enriched food did not impact on the weight of mice, this diet has the advantage that potentially confounding factors such as obesity can be excluded. Thus, we continued to use the 1% cholesterol diet for further experiments.

4.2 CETP promotes TREM2 expression in the liver

To ultimately study changes in the brain, we expanded the diet period to 3 months to enhance the effects of CETP. First, the effect of CETP on plasma LDL levels should lead to transcriptional changes in the liver. Here, we analysed diet-dependent effecs by comparing wild type mice on standard diet with wild type mice on a diet containing 1% cholesterol as well as CETP-dependent effects by comparing mice with highest CETP expression (CETP mice on 1% cholesterol diet) with wild type mice with on cholesterol diet. Here, we found both dietary, CETP-dependent effects and in some cases a combination of both. Dietary cholesterol is known to decrease cholesterol synthesis and gene transcription regulated by the sterol-regulatory binding protein-2 (SREBP-2)(Brown and Goldstein, 1997). Indeed, the mRNA levels for HMGCR decreased on high cholesterol diet in wild type and CETPtg mice as compared to wild type mice on a standard diet at the age of 5 months (**Figure 8B**) (Berger *et al.*, 2015; Engelking *et al.*, 2004; Llorente-Cortes *et al.*, 2006). We found that the expression of LDLR and was repressed in mice fed a 1% cholesterol diet or CETP tg mice (**Figure 8C**). Moreover, the mRNA levels of LRP1 were reduced in a strictly CETP-dependent fashion (**Figure 8D).**

We were further interested in two Alzheimer's risk genes, the ATP-binding cassette transporter A7 (ABCA7) that is also regulated by SREBP-2, and triggering receptor expressed in myeloid cells 2 (TREM2) (Guerreiro *et al.*, 2013; Hollingworth *et al.*, 2011; Iwamoto *et al.*, 2006; Wang *et al.*, 2015). While the mRNA levels were statistically unchanged, we found a CETP-dependent increase in ABCA7 protein levels (**Figure 8E-G**). TREM2 gene transcription was increased by diet as well as CETP leading to an 8-fold increase of transcript levels comparing the two extremes, wild type mice on a standard diet with CETPtg mice on cho-

lesterol diet (**Figure 8H**). However, this increase could not be replicated at the protein level, which could be attributed to overall low signal intensities (**Figure 8E, I**) (Daws *et al.*, 2001; Gelissen *et al.*, 1998; Tanaka *et al.*, 2011; Ulyanova *et al.*, 1999; Zhu *et al.*, 2004). Further, transcript levels of ABCA7 and TREM2 were not affected in total cortical mRNA (data not shown).

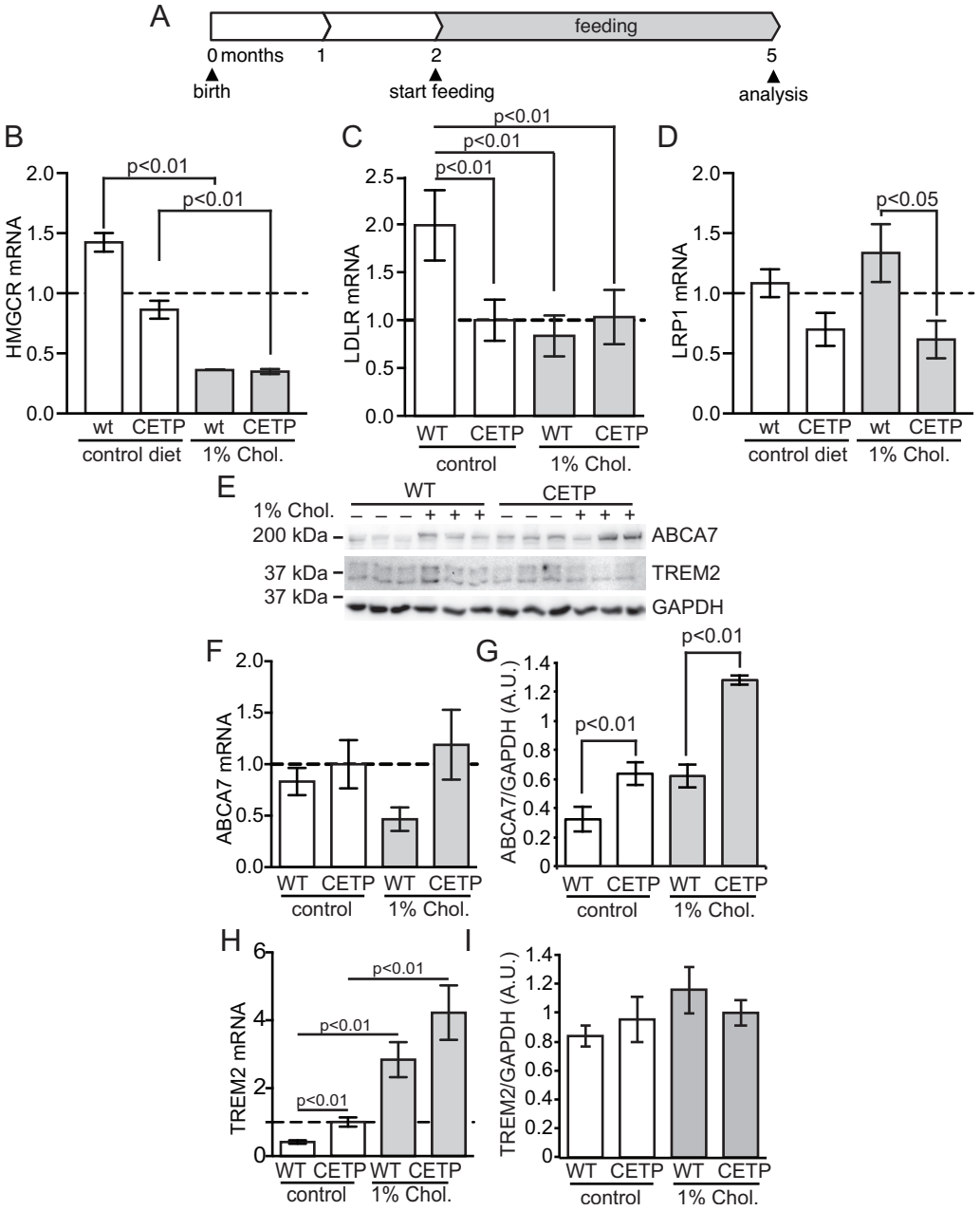


Figure 8: CETP promotes TREM2 expression in the liver

A: Feeding schedule & study design. Wild type and CETP transgenic animals were fed for 3 months starting at the age of 2 months. Biochemical analyses were performed at the age of 5 months. **B-D**: RT-qPCR from mouse liver tissue. **B**: Normalised HMGCR, **C**: LDLR and **D**: LRP1 expression, n=6-14, mean ± SEM; 2-way ANOVA, Tukey's multiple comparison. **E**: Western blot analysis of ABCA7 and TREM2 from 5-month old liver samples. Antibodies used: rabbit-anti-GAPDH (14C10, Cell Signaling), anti-TREM2 (Mab1729 R&D systems) and anti-ABCA7 (polyclonal, Thermo Fisher), n=6; mean ± SEM, Students T-test. **F-I**: Expression analysis of ABCA7 and TREM2 from liver samples of 5-month old mice. **F**: mRNA levels of liver ABCA7 and **H**: TREM2. n=6-14, mean ± SEM; 2-way ANOVA, Tukey's multiple comparison. **G**: Western-blot quantification of ABCA7 and **I**: TREM2. n=6; mean ± SEM, Students T-test.

4.3 CETP activity promotes peripheral inflammation

It has been previously shown that cholesterol-enriched diets induce inflammation (Wouters *et al.*, 2008). Therefore, we quantified the inflammatory cytokines IL1β and TNFα in mouse plasma samples using multiplex ELISA after three months of feeding a 1% cholesterol or control diet. To distinguish diet-mediated effects from CETP-dependent effects, we compared wild type mice on standard diet with wild type mice on a diet containing 1% cholesterol to obrain a baseline for dietary-mediated effects and compared mice with highest CETP expression (CETP mice on 1% cholesterol diet) with wild type mice with on cholesterol diet to obtain the magnitude of CETP-dependent increase in cytokines.

For circulating TNFα and IL1β levels, we found a strong CETP-dependent increase, comparing CETPtg to wild type mice on cholesterol diet. However, it should be noted that out of the 10 plasma samples analyzed, 6 samples had very low TNFα levels comparable to the control diets, and only four mice showed elevated TNFα levels (**Figure 9A, B**). . This could be explaind by the increase in LDL levels in said animals as elevated LDL has been linked with inflammatory responses in the liver (Seo *et al.*, 2013).

Since the liver mainly secretes CETP, we determined mRNA expression of such cytokines in the liver by qRT-PCR. As expected, the same mice with elevated plasma cytokine levels also had elevated TNFα and IL1β mRNA levels in the liver (**Figure 9C, D**). Yet, in addition to

CETP-dependent effects, the liver-mRNA levels also showed a clear diet-dependent increase as evident when comparing animals on a standard diet with their respective counterpart on a cholesterol diet. Furthermore, we performed a D'Agostino & Pearson test to test for normal distribution which showed that we have a population of mice with elevated cytokine expression and one that showed no change in cytokine mRNA. This was the case for IL1β, TNFα and IL6 liver mRNA levels but not for TLR4.

Furthermore, mRNA expression of the toll-like receptor 4 (TLR4) as an upstream regulator of TNFα, and IL1β was also increased in mice with the highest cytokine levels (**Figure 9E**). Similarly, transcript levels of IL6, an interleukin that was reported to induce the expression of lipid-regulating proteins were high in 3 out of 12 mice (**Figure 9F**) (Muller *et al.*, 2015). Overall, the mRNA levels of all cytokines measured in this study showed strongest increase when comparing CETP tg mice on standard diet with CETP tg mice on a cholesterol diet, suggesting a combined effect of diet and genotype.

To analyze whether inflammatory cytokine production was extended to the central nervous system, transcript levels were determined from cortical samples. While we were able to demonstrate that CETP is expressed in the cortex of CETPtg mice, its expression levels were not affected by dietary cholesterol intake, which may relate to the fact that LDL and HDL particles do not cross the blood-brain barrier (**Figure 9G**). Importantly, cytokine levels were not significantly increased in the brain at that age, except for IL1β levels which showed a modest, yet significant increase in a CETP-dependent fashion (**Figure 9H-J**). In summary, CETP expression and a cholesterol diet induced inflammatory responses in the periphery, with attenuated effects in the brain.

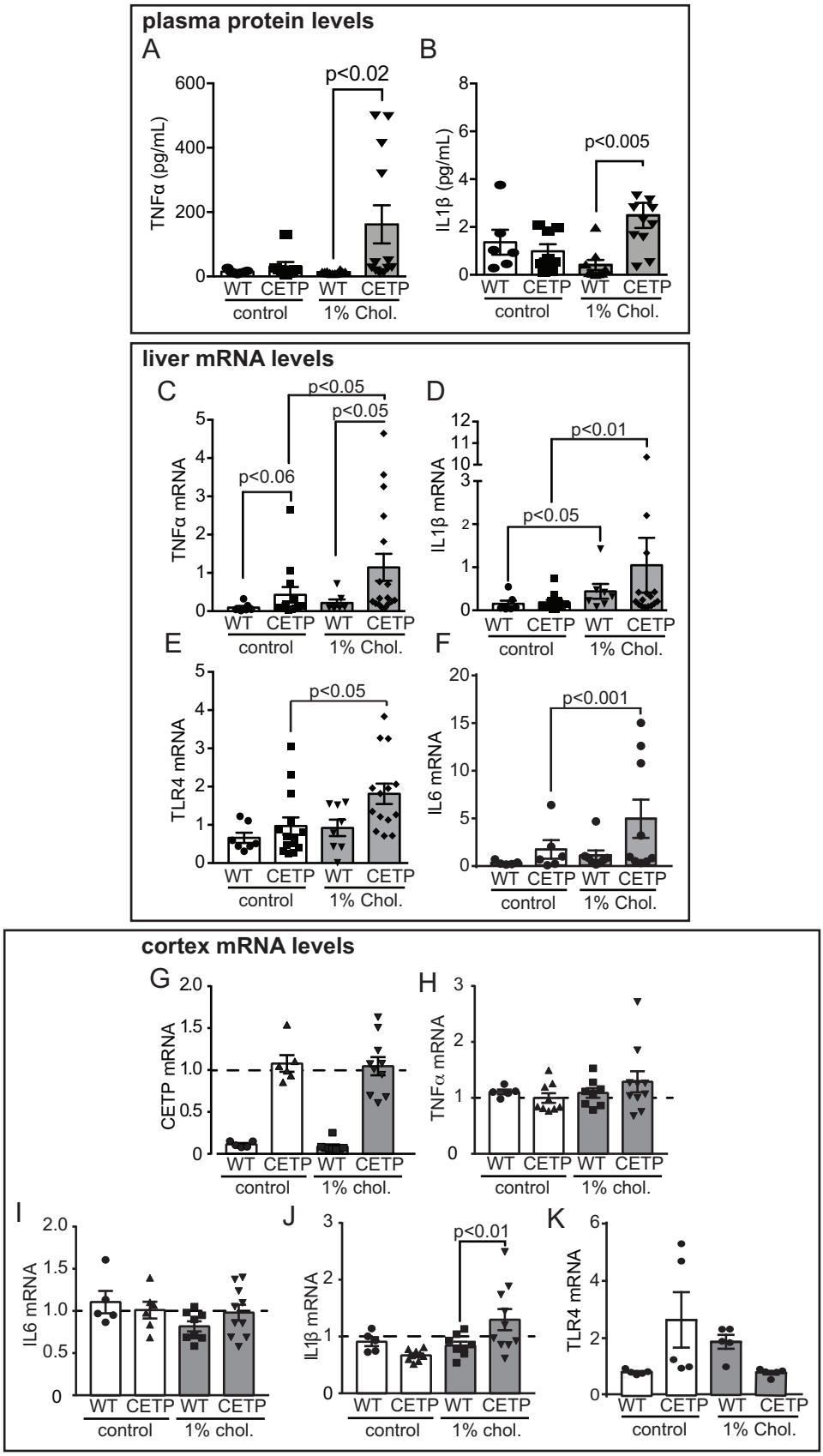

plasma protein levels
A
TNFα (pg/mL)
p<0.02
B
IL1β (pg/mL)
p<0.005
WT CETP WT CETP
control 1% Chol.
liver mRNA levels
C
TNFα mRNA
p<0.05
p<0.05
p<0.06
D
IL1β mRNA
p<0.01
p<0.05
E
TLR4 mRNA
p<0.05
F
IL6 mRNA
p<0.001
cortex mRNA levels
G
CETP mRNA
H
TNFα mRNA
I
IL6 mRNA
J
IL1β mRNA
p<0.01
K
TLR4 mRNA
control 1% chol.

Figure 9: CETP activity promotes peripheral inflammation
A, B: Plasma cytokine levels. **A**: TNFα and **B**: IL1β measured in 25 µL EDTA plasma using a multiplex ELISA (mesoscale discoveries). n=6-11. mean ± SEM; 2-way ANOVA, Tukey's multiple comparison, D'Agostino & Pearson normality test. **C-F**: RT-qPCR of liver samples from 5-month old mice. Normalised expression of **C**: TNFα, **D**: IL1β, **E**: TLR4 and **F**: IL6 expression. n=6-14, mean ± SEM; 2-way ANOVA, Tukey's multiple comparison. **G-K**: Cytokine mRNA expression in brain samples: **G**: CETP, **H**: TNF α, **I**: IL6, **J**: IL1β and **K**: TLR4 expression. n=6-10. mean ± SEM; 2-way ANOVA, Tukey's multiple comparison.

4.4 CETP changes the brain cholesterol composition

To study the potential effects of CETP activity on the composition and distribution of lipids in the brain, we employed imaging mass spectrometry (IMS) to obtain the spatial distribution of cholesterol in the brain. While several studies have looked at the distribution of lipids in the brain by IMS using 1,5-Diaminonaphthalene or other organic matrices (Caughlin *et al.*, 2017; Thomas *et al.*, 2012), the visualization of cholesterol using IMS remained challenging. Here, we deposit a fine homogeneous silver layer over the tissue sections to promote the laser desorption/ionization (LDI) and allow the imaging of cholesterol and olefin-containing fatty acids with high specificity and sensitivity (Dufresne *et al.*, 2013). The heat map images depict the distribution of cholesterol in sagittal mouse brain sections detected at m/z = 493 ([M+107Ag]+, silver adduct molecular ion) (**Figure 10A**). Cholesterol is found at the highest concentrations in the myelin-rich fiber tracts, whereas lower levels are observed in cortex, hippocampus, and cerebellum (**Figure 10A**, wild type on a standard diet). To understand whether either a 1% cholesterol diet of CETP could affect brain cholesterol levels, we compared wild type mice with CETP tg mice as well as either group on standard diet with their counterpart on a cholesterol diet. Most interestingly, CETPtg mice showed overall higher cholesterol levels in the brain than wild type mice with a 23±4% increase between wild type and CETPtg mice on a standard diet and a 31±4% increase between wild type and CETPtg mice on cholesterol diet over the whole brain (**Figure 10C, D**). The hippocampal region showed similar trends, albeit without statistically significant changes (**Figure 10E**). Since pe-

ripheral cytokine levels, as well as brain IL1β mRNA levels, were elevated, we further analyzed levels of the fatty acid arachidonic acid as a precursor of eicosanoids and prostaglandins. Signals for arachidonic acid were comparable between genotypes and diets (while there may be a trend towards higher levels in CETPtg mice on cholesterol diet) suggesting an overall low abundance of neuroinflammation in CETPtg mice (**Figure 10B, F**).

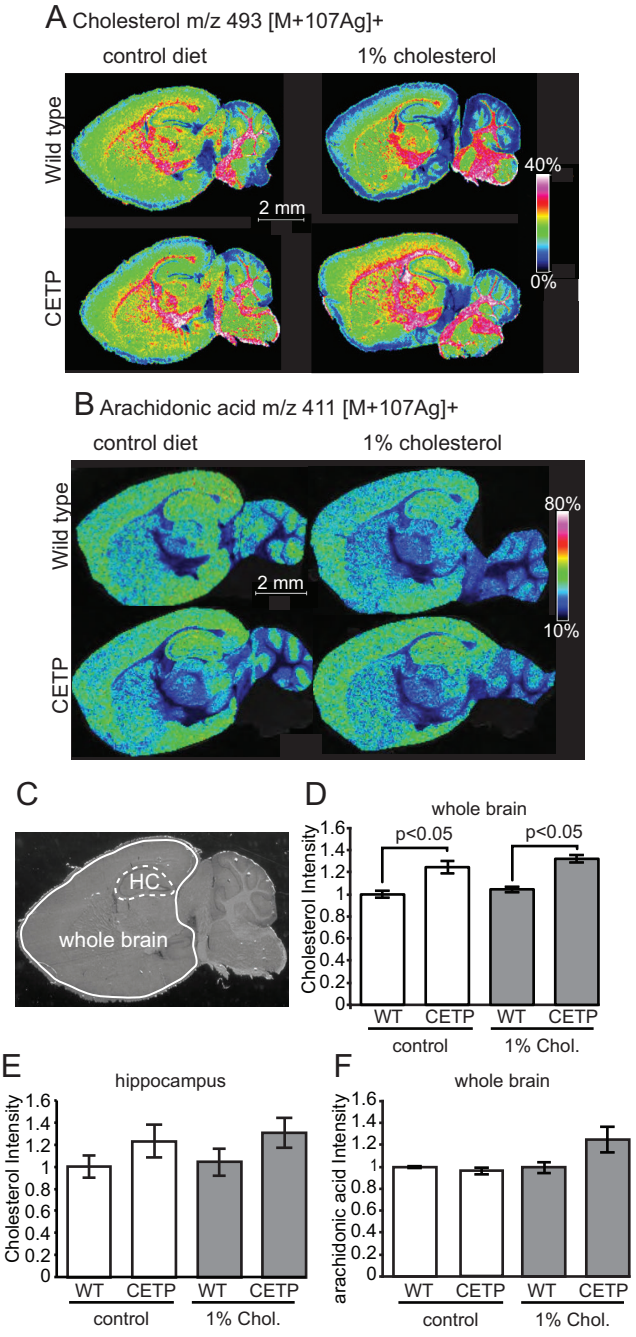


Figure 10: CETP changes the brain cholesterol composition

A, B: MALDI-IMS of 5-month-old wild type and CETPtg sagittal brain slices. **A**: Heatmap representation of peak intensities corresponding to cholesterol (m/z 493 [M+107Ag]+) and (**B**) arachidonic acid (m/z 411 [M+107Ag]+). **C**: representation of whole brain sagittal section and illustration of regions of interest selected for whole brain or hippocampal quantification. **D, E**: Quantification of peak intensities corresponding to cholesterol from whole brain (**D**) and hippocampus (**E**). **F**: Quantification of peak intensities corresponding to arachidonic acid from whole brain. n=5. mean ± SEM; One way ANOVA.

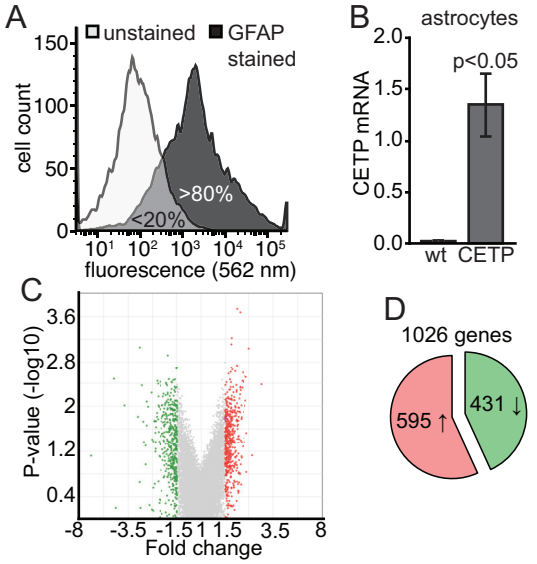
4.5 Transcriptional changes in CETPtg brain induced by presenilins

To investigate whether changes in brain cholesterol composition were a result of changes in the transcription of genes that induce cholesterol synthesis, we performed a microarray from purified astrocyte RNA (**Figure 11**). The two extreme conditions of lowest and highest cholesterol content in the brain were chosen, i.e., wild type animals on a control diet compared to CETPtg mice on cholesterol diet (yielding a 31% cholesterol increase, **Figure 10D**). Cells positive for the glutamate-aspartate transporter (GLAST) from freshly dissected and dissociated whole brains were enriched using the ACSA-1 MicroBead Kit (Sharma *et al.*, 2015). GLAST is expressed explicitly by astrocytes (Perego *et al.*, 2000). To verify the enrichment of astrocytes, approximately $8x10^5$ cells were stained for the glial fibrillary acidic protein (GFAP) and analyzed by flow cytometry, revealing a purity of more than 80% across all samples (**Figure 11A**). Of note, there may be a basal expression of GLAST in some neurons (Rothstein *et al.*, 1994). Total mRNA was purified. First, CETP expression was validated in the astrocyte mRNA by qPCR (**Figure 11B**). Second, astrocyte transcripts were analyzed on a Clariom S microarray (Affymetrix). 595 genes were significantly up and 431 genes significantly down-regulated (**Figure 11C, D**). Interestingly, genes involved in cholesterol or lipid synthesis were not among the strongest differentially regulated genes (**Figure 11E**). In fact, genes encoding for proteins involved in cholesterol synthesis and homeostasis were downregulated, among those the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) (1.57-fold down). The sterol regulatory element-binding proteins 1 and 2

(SREBF1, SREBF2) were downregulated 1.71- and 1.84-fold, respectively, and the mevalonate kinase (MVK), was reduced 1.42-fold. Also, mRNA levels of two LDL receptors (LDLR and LRP1) were reduced (**Figure 11E**). Overall, this data implies that it is unlikely that increased *de novo* cholesterol synthesis is responsible for the elevated cholesterol levels in the brains of CETPtg mice.

Since we were interested if mice with humanized cholesterol metabolism show changes in the brain that may be of interest for Alzheimer's disease, we analyzed if genes linked to Alzheimer's were affected (**Figure 11F**). Seven genes were identified. Upregulated genes included *I*) the prime Alzheimer's risk gene apolipoprotein E (ApoE), involved in lipid transport and multiple epidemiological studies already suggested an interaction between CETP and ApoE in the context of Alzheimer's disease ((Murphy *et al.*, 2012; Rodriguez *et al.*, 2006; Sundermann *et al.*, 2016); *II*) The angiotensin-converting enzyme (ACE) producing the vasoconstrictor angiotensin II, which is upregulated and implicated in hypoperfusion in Alzheimer's disease (Love and Miners, 2016); *III*) Caspase 8 is a part of the apoptotic machinery and polymorphisms have been associated with Alzheimer's disease (Rehker *et al.*, 2017; Rohn *et al.*, 2001); *IV*) IL1β (though just below the threshold of 1.5), an inflammatory cytokine that is elevated in Alzheimer's disease brains (Griffin *et al.*, 1989). Downregulated genes included *V*) the insulin-degrading enzyme (IDE), which has been implicated in the degradation of Aβ peptides and was associated with sporadic Alzheimer's disease (Bertram and Tanzi, 2004; Qiu *et al.*, 1998); *VI*) TREM2, which has been genetically linked to Alzheimer's disease and recently been intensively studied in activated microglia (Guerreiro *et al.*, 2013; Jonsson and Stefansson, 2013; Perugorria *et al.*, 2018; Suarez-Calvet *et al.*, 2016). *VII*) Sortilin-related receptor 1 (SORL1, though again just below threshold), which was described to shuttle APP away from subcellular locations of Aβ production (Andersen *et al.*, 2005; Yin *et al.*, 2015). Together, all these changes are in line with pathological changes in Alzheimer's disease and imply that due to the presence of CETP several different molecular changes co-occur. Next, we performed an upstream-regulator analysis, identifying common regulators that may ac-

count for the overall changes in mRNA expression in the dataset. The top upstream regulator was the prostaglandin E receptor 4 (PTGER4) as 24 downstream targets of PTGER4 were differentially regulated (**Figure 11G**). PTGER4 is a G protein-coupled receptor that binds prostaglandin E2 (PGE2) and has been associated with neurotoxicity and neuroinflammation (Higgins and Lees, 1984; Samuelsson, 1991; Zhang and Rivest, 2000).



E

Function	Gene	Expr. Fold Change
cholesterol biosynthesis	HMGCR	-1.63
	SREBF1	-1.71
	SREBF2	-1.84
	MVK	-1.42
lipoprotein receptors	LRP1	-1.41
	LDLR	-1.48

F

Function	Gene	Expr. Fold Change
Alzheimer's disease	IDE	-1.51
	TREM2	-2.34
	IL1b	1.46
	CASP8	1.53
	ACE	2.03
	APOE	1.57
	SORL1	-1.42

G

Top upstream regulators	p-value	Affected genes	Expr. Fold Change
PTGER4	1.09E-06		
PSEN1	1.26E-05	C1QA	3.48
PSEN2	1.91E-05	CD74	3.24
		CTSS	2.86
		C1QC	2.1
		CTSZ	1.83
		Erdr1	1.69
		SELPLG	1.63
		C3AR1	1.58
		C1QB	1.55
		CD9	1.48
		KIF5B	1.48
		ENPP2	1.44
		SLC38A2	1.41
		HLA-E	1.4
		WARS	-1.42
		FOXO3	-1.44
		RELN	-1.44
		BDNF	-1.53
		FMN2	-1.7
		CUEDC1	-1.8
		GDF11	-1.84

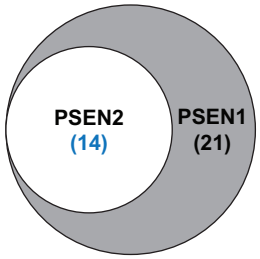


Figure 11: Transcriptional changes in CETPtg brain induced by presenilins

A: Flow cytometry analysis of astrocyte purification from mouse brains. GLAST-positive astrocytes were stained with GFAP. All purifications showed 80% or more of the purified cells were positive for GFAP. **B**: CETP RT-qPCR of astrocyte RNA, n=2-3. mean ± SEM; 2-way ANOVA. **C**: Volcano plot of the mouse microarray results. Each dot represents an individual gene. The P-value of plotted against the gene regulation fold change of the corresponding gene. P-values cut-off for significance was set to <0.05. **D**: Overall, 595 genes were found to be significantly up-regulated and 431 genes were found to be significantly down-regulated in our data set. **E**: genes involved in the *de novo* synthesis of cholesterol, generation of arachidonic acid and lipoprotein receptors. **F**: Alzheimer's disease risk genes regulated in our data set. **G**: Pathways analysis of upstream regulators. Analyzing the fold changes in the dataset, PTGER4, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) are the top 3 predicted upstream regulators. A total of 21 genes that have been reported to be regulated via PS1 and have been found in our dataset. 14 of these genes have also been reported to be regulated via PS2 (highlighted in blue). Upstream regulator analysis was performed using ingenious pathway analysis.

NCBI gene numbers: HMGCR: 15357; SREBF1: 20787; SREBF2: 20788; MVK: 17855; LRP1: 16971; LDLR: 16835; IDE: 15925; TREM2: 83433; IL1B: 16176; CASP8: 12370; ACE: 11421; APOE: 11816; SORL1: 20660; C1QA: 20660; CD74: 16149; CTSS: 13040; C1QC: 12262; CTSZ: 64138; Erdr1: 170942; SELPLG: 20345; C3AR1: 12267; C1QB: 12260; CD9: 12527; KIF5B: 16573; ENPP2: 18606; SLC38A2: 67760; HLA-E: 15040; WARS: 22375; FOXO3: 56484; RELN: 19699; BDNF: 12064; FMN2: 54418; CUEDC1: 103841; GDF11: 14561

Most interestingly, the second and third hit of upstream regulators are presenilin-1 and -2 (PSEN1 and PSEN2), the catalytic subunits of γ-secretase, a critical protease in the etiology of Alzheimer's disease generating Aβ peptides.

Presenilin-1 and -2 were identified by 21 and 14 known downstream target genes, respectively (**Figure 11G**). γ-Secretase cleaves multiple substrates and is at the center of many signaling pathways. However, it is remarkable that the presence of CETP and the subsequent humanized cholesterol metabolism activates presenilin signaling in the mouse brain (Bai *et al.*, 2011; Haapasalo and Kovacs, 2011).

4.6 CETP activates γ-secretase *in vitro*

Given the changes in brain cholesterol composition and its potential stimulation of γ-secretase-mediated signaling, we investigated if CETP activity stimulates γ-secretase signaling *in vitro*. To this end, we took advantage of the well-known γ-secretase substrate, notch. After the notch intracellular domain has been released by γ-secretase, it activates transcription of notch target genes, i.e., HES1 (Hes Family BHLH transcription factor 1) and p21 (cyclin-dependent kinase inhibitor 1A) (Balaganapathy *et al.*, 2017; Guo *et al.*, 2009; Iso *et al.*, 2003; Jarriault *et al.*, 1995; Zhang *et al.*, 2014). CETP or an inactive CETP mutant (L457/M459W (Qiu *et al.*, 2007)) were expressed in HEK293T cells (**Figure 12A**). mRNA levels were determined by RT-qPCR and revealed that active CETP indeed increased HES1 and p21 levels, whereas the inactive CETP mutant had no effect (**Figure 12B**). The data shows that CETP activity causes cellular changes that stimulate γ-secretase activity *in vitro*.

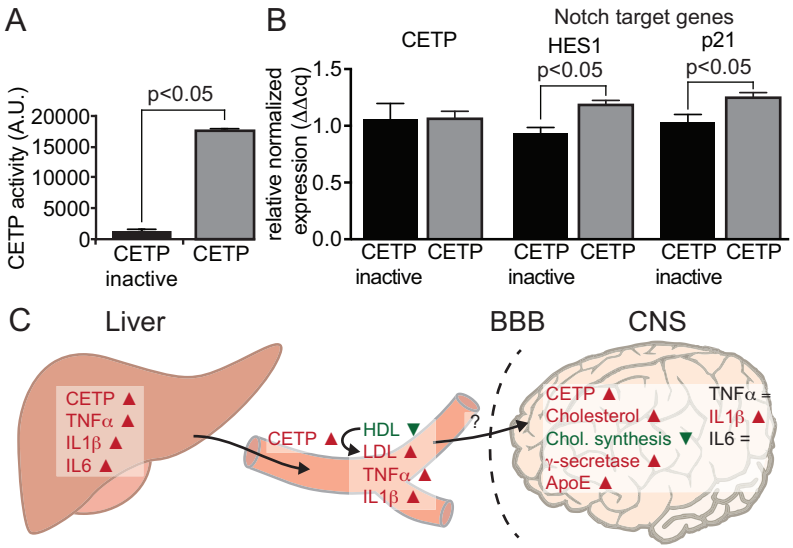


Figure 12: CETP activates γ-secretase

A: CETP activity assay of HEK293T cells transfected with wild type CETP or an inactive mutant (CETP M457/L459W). N=3, mean ± SEM, students-T test **B**: Normalised relative expression of CETP, HES1 and p21. Expression levels are normalised to GAPDH and. N=3, mean ± SEM, students T-test. **C**: Schematic representation of changes observed in liver, plasma and brain of CETPtg animals.

5. Discussion

5.1 CETP-mediated increase in brain cholesterol

In this study, we aimed to understand the effects of CETP on brain lipid composition and gene regulation. Based on our analysis, CETPtg mice show a humanized lipoprotein profile in the blood and importantly, a 23% - 31% increase in brain cholesterol levels when compared to wild type mice. To our knowledge, this is the first report of a transgenic mouse model showing elevated brain cholesterol levels to this extent. CETPtg mice on the cholesterol diet showed peripheral inflammation, but no elevated cytokine levels in total cortical mRNA, with the exception of IL-1β, which was elevated. The enhanced inflammatory response in liver and plasma could be attributed to higher cholesterol levels in immune cells where it was already demonstrated that cholesterol augments, for instance, TLR receptor signaling, and modulates immune cells surrounding tumors (Tall and Yvan-Charvet, 2015; Yang *et al.*, 2016).

We investigated if the increase of brain cholesterol arises from *de novo* synthesis. However we were unable to confirm this using our transcriptome analysis. Yet, one of the following alternative pathways may explain the elevated cholesterol levels. While most lipoprotein particles cannot cross the blood-brain barrier, some lipid exchange between the brain and the blood can occur (Bjorkhem *et al.*, 1998; Bjorkhem and Meaney, 2004; Zlokovic, 2008). It is well established that dietary ω3-fatty acids enter the brain (Nguyen *et al.*, 2014; Ouellet *et al.*, 2009). Also, 24S- and 27-hydroxysterols efficiently cross the blood-brain barrier, and polymorphisms in 24S-hydroxylase were associated with Alzheimer's Disease (Bjorkhem, 2006; Bjorkhem and Meaney, 2004). Lastly, HDL particles were described to be capable of transporting cholesterol into the brain via scavenger-receptor mediated transport or transcytosis (Balazs *et al.*, 2004; Stukas *et al.*, 2014a). However, the function of CETP in the brain remains unclear. While CETP shuttles cholesterol between HDL and VLDL in the blood, those lipoprotein particles do not exist in the brain (Fagan *et al.*, 1999; Stukas *et al.*, 2014b; Vance, 2012; Zhang *et al.*, 2012). In the brain, ApoE is the predominant lipoprotein, and most lipoprotein particles are HDL-like in size and decorated with ApoE or ApoJ (Fagan *et al.*, 1999; Xu *et al.*, 2000). While a role for CETP in the brain is not clear, it is likely that it is active as a lipid

transporter. However, the interaction partners may differ from those in the periphery, and it is a possibility that CETP is involved in cholesterol redistribution between cells or acts as an intracellular shuttle between organelles. CETP may be involved in the storage of lipids in microglia and astrocytes. Along these lines, the Morton laboratory reported a role of CETP in lipid droplet formation (Izem and Morton, 2001, 2007). Consequently, it is possible that lifetime exposure to CETP activity in the brain may cause overall retention of cholesterol in the brain, leading to increased cholesterol levels observed in CETPtg mice on either diet. It will be most interesting to reveal if blood-derived CETP, centrally expressed CETP, or both are responsible for the molecular changes of the brain described herein.

In the liver of CETPtg mice, we observed an upregulation of ABCA7 and TREM2 as compared to wild type mice. TREM2 mutations associated with Alzheimer's disease, and it was thus far discussed as an immune receptor in the brain (Li and Zhang, 2018). However, two recent manuscripts linked ABCA7 and TREM2 to bile acid formation in the liver (Mahmoudiandehkordi *et al.*, 2018; Nho *et al.*, 2018). Thus, the elevated ABCA7 and TREM2 levels in CETPtg mice on cholesterol diet in the liver may reflect an increase in bile acid formation. It is tempting to speculate that ABCA7 and TREM2 may be involved in cholesterol transport or redistribution in the brain (Linsel-Nitschke *et al.*, 2005; Tanaka *et al.*, 2010; Tanaka *et al.*, 2011; Wang *et al.*, 2015).

5.2 Several Alzheimer-related changes triggered in CETPtg mice

Alzheimer's disease is the most common form of dementia and defined by the occurrence of amyloid plaques composed of Aβ peptides. Aβ peptides are generated from the amyloid precursor protein (APP) through two subsequent proteolytic cleavages. First, the ectodomain of APP is removed by β-secretase, and then the membrane-bound C-terminal fragment is cleaved by γ-secretase (Hussain *et al.*, 1999; Sinha *et al.*, 1999; Vassar *et al.*, 1999; Zhao *et al.*, 2007). It is well established that higher cellular levels of cholesterol stimulate β- and γ-secretase activity (Miller and Chacko, 2004; Osenkowski *et al.*, 2008; Puglielli *et al.*, 2003;

Wolozin, 2001). To date, the physiological function of APP, as well as the triggers that lead to Aβ production, remain unclear. However, it is evident that cellular pathways that stimulate Aβ production could qualify as the underlying mechanism leading to Alzheimer's disease. We analyzed herein a mouse model with humanized cholesterol metabolism as a result of the expression of a protein that mice lack, i.e., CETP. The cerebral changes that we observed (high cholesterol levels, transcriptional changes, γ-secretase activity) resemble changes that have previously been described in Alzheimer's disease.

We performed an upstream regulator analysis identifying proteins that could explain the observed changes in gene expression in the microarray dataset. We identified the prostaglandin E2 receptor EP4 (PTGER4) as the most significant upstream regulator (**Figure 11G**). In the brain, the EP4 receptor binds prostaglandin E2 (PGE2) a key inflammatory mediator in response to circulating IL1β (Fujikawa *et al.*, 2017; Fujikawa *et al.*, 2016; Zhang and Rivest, 2000). However, its role in mediating an inflammatory response is not completely clear as PGE2 can have both pro- and anti-inflammatory effects (Echeverria *et al.*, 2005; Shi *et al.*, 2010; Woodling and Andreasson, 2016; Woodling *et al.*, 2014). Mice lacking an inducible Prostaglandin E synthase and therfore lacking PGE_2, showshow a reduction in inflammatory responses (Trebino *et al.*, 2003). Yet, in model sfor athrosclerosis, PGE2 has been shown to have pro-inflammatory properties in a mouse model for hyperlipedemia (Wang *et al.*, 2006). Yet, multiple studies have linked activation of EP4 with an increase in Aβ peptides and memory loss in the context of Alzheimer's disease (Hoshino *et al.*, 2007; Hoshino *et al.*, 2012; Hoshino *et al.*, 2009; Li *et al.*, 2015). Such effects are explained by an increase in γ-secretase activity and not through the activation of conventional G protein-coupled receptor signaling involving adenylate cyclase and cAMP. Hoshino *et al.* showed that upon stimulation with PGE2, EP4 is co-internalised with γ-secretase to endosomal and lysosomal compartments where γ-secretase activity is elevated (Grbovic *et al.*, 2003; He *et al.*, 2007; Takahashi *et al.*, 2004). These detrimental effects were abolished in EP4 knockout animals or through pharmacological inhibition of EP4 (Hoshino *et al.*, 2007; Hoshino *et al.*, 2012). In line with this

mechanism, activation of γ-secretase activity was indeed observed as second and third top upstream regulators identified in CETPtg mice (**Figure 11G**). γ-Secretase activity is stimulated by membrane cholesterol and co-internalization with EP4 (Hartmann *et al.*, 2007; Jung *et al.*, 2015; Marquer *et al.*, 2011). It is important to note that while it has been well established that EP4 internalization occurs upon PGE2 binding, the receptor also carries a cholesterol consensus motif and directly senses changes in cellular cholesterol levels (Hanson *et al.*, 2008). Interestingly, expression of CETP in cell culture models is already sufficient to increase γ-secretase activity. Given this evidence, it is likely that the effects on presenilins/γ-secretase are downstream effects of elevated cholesterol levels in the brain. The altered cholesterol metabolism, at least in the CETPtg model presented here, drives multiple molecular changes that recapitulate changes already described in Alzheimer's disease (**Figure 12C**).

Lipidomic studies have found that abnormal plasma lipid profiles, and consequently abnormal lipid biomarker panels, yield specific markers of Alzheimer's disease (Alzheimer *et al.*, 1995; Hartmann *et al.*, 2007; Kosicek and Hecimovic, 2013; Mapstone *et al.*, 2014; Solomon *et al.*, 2014; Zarrouk *et al.*, 2018). However, most animal models focus on overexpression of mutated forms of human APP (Arendash and King, 2002; Chishti *et al.*, 2001; Mucke *et al.*, 2000), presenilin (Jankowsky *et al.*, 2004; Kimura and Ohno, 2009) or tau, involved in another hallmark pathology of Alzheimer's disease (Andorfer *et al.*, 2003; Billings *et al.*, 2005; Taylor *et al.*, 2002). All these mouse models have low levels of circulating LDL due to the lack of CETP and therefore do not report on the impact of the lipid metabolism on Alzheimer's pathology, which may have been underestimated thus far (Steenbergen *et al.*, 2010). Here, we report that mice expressing human CETP present a humanized cholesterol metabolism and exhibit elevated levels of cholesterol in the brain. In the absence of APP or presenilin overexpression, CETPtg mice show a transcriptional profile that reflects a multitude of changes previously described in the Alzheimer's diseased brain. Taken together, our data suggest that a mouse model expressing CETP and APP will be a valuable tool to unravel the molecular mechanisms between the peripheral and central cholesterol metabolism, ApoE and Alzheimer's disease.

6. Acknowledgements

We thank Dr. Bernard Robaire for software support to evaluate the microarray data and Dr. Sandra Paschkowsky and Sasen Efrem for valuable feedback on the manuscript.

7. Author contribution

FO performed and analysed all experiments presented here with the exception of the MALDI-IMS data that were acquired by EY and EAK and analysed by EY. LMM designed the project. FO wrote draft, LMM, PC, and EY edited and revised the manuscript. All Authors approved the manuscript for publication.

FOREWORD MANUSCRIPT 2

In my first manuscript, I was able to show that CETP transgenic animals show elevated cholesterol levels in their brains. Further, these mice show effects on the γ-secretase complex. Together, this poised us to study whether CETP would accelerate AD pathology in AD model systems. I started using fundamental cell culture *in vitro* system and moved on to two different mouse models of AD.

MANUSCRIPT 2

CETP activity increases Aβ production

Felix Oestereich[1, 2], Helen Wu[1], Lisa Marie Munter[1]

[1] Department of Pharmacology and Therapeutics, Cell Information Systems group, McGill University, 3649 Promenade Sir William Osler, Montreal, QC, Canada H3G 0B1

[2] Integrated Program in Neuroscience, McGill University, Montréal, QC, Canada H3A 2B4

Correspondence:
Dr. Lisa Munter, lisa.munter@mcgill.ca, McGill University, Department of Pharmacology and Therapeutics, Bellini Life Sciences Complex, 3649 Promenade Sir-William Osler, Montreal, QC, Canada, H3G 0B1

1. Abstract

The cholesterol metabolism is tightly linked with Alzheimer's disease (AD). However, the human cholesterol homeostasis is significantly different compared to mice. Hence, all current mouse models lack a crucial aspect of the underlying pathology. We generated a novel AD-mouse model combining a humanized cholesterol metabolism with AD pathology. We crossed mice transgenic for the human cholesteryl ester transfer protein (CETP) with models for AD. Analyzing our novel models, we found that CETP activity increases soluble and insoluble levels of Aβ in mice expressing the amyloid precursor protein (APP) containing familial mutations. When CETP was combined with an AD model expressing familial AD mutations in presenilin, the Aβ increase was abandoned. Presenilin is the catalytic subunit of γ-secretase, and its lipid environment tightly regulates its activity.

Together with previous work, we hypothesize that CETP modulated brain cholesterol levels which in return affects γ-secretase, leading to increased Aβ levels.

2. Introduction

2.1 Alzheimer's disease

Alzheimer's disease is the most common neurodegenerative disease and affects around 50 million people worldwide with an estimated global economic burden of around one billion US dollars (Alzheimer's disease international, 2015). This neurodegenerative disorder is characterized by diffuse brain atrophy, the loss of synapses, aggregation of amyloid beta (Aβ) in extracellular plaques and neurofibrillary tangles inside neurons, consisting of hyperphosphorylated tau (Holtzman *et al.*, 2011; Wischik *et al.*, 1988).

Aβ is a small peptide generated from the amyloid precursor protein (APP) through sequential cleavages by two transmembrane secretases, the β- and γ-secretase (Glenner and Wong, 1984; Hussain *et al.*, 1999; Masters and Beyreuther, 1989; Masters *et al.*, 1985). The Aβ peptides generated by γ-secretase are heterogeneous in length, and longer peptides with

42 amino acids (Aβ42) are more prone to aggregation and show higher toxicity (Chen and Glabe, 2006; Cummings *et al.*, 1996; Kuperstein *et al.*, 2010). Due to its central role in the AD pathology, Aβ is and has been a major target in the development of therapeutics to prevent the disease. Therapeutic strategies, ranging from inhibiting or modulating β- or γ-secretase, removing Aβ aggregates from the brain, or reducing its neurotoxic effects have been studied in clinical trials (Frozza *et al.*, 2018).

2.2 The role of cholesterol in Alzheimer's disease

In addition to the enzymes directly involved in the proteolytic cascade of APP, the levels of cholesterol have been shown to play a profound role in the generation of Aβ on multiple levels. First, it is known that cholesterol-rich diets, as well as hypercholesteremia, stimulate the generation of Aβ (Di Paolo and Kim, 2011). Further, Osenkowski *et al.* showed that the levels of cellular and membrane cholesterol levels influence the activity and processivity of the γ-secretase complex (Osenkowski *et al.*, 2008). Here, the levels of membrane cholesterol positively correlate with the amount of Aβ42 generation by γ-secretase (Golde and Eckman, 2001; Hur *et al.*, 2008; Rushworth and Hooper, 2010; Simons *et al.*, 1998). However, it is not clear whether cholesterol directly affects the enzyme, the substrate or whether these effects are due to changes in the physical properties of the membrane such as thickness or fluidity (Ayciriex *et al.*, 2016; Holmes *et al.*, 2012; Hur *et al.*, 2008; Paschkowsky *et al.*, 2018; Vetrivel *et al.*, 2004; Winkler *et al.*, 2012; Yang *et al.*, 2017). However, there is sufficient evidence that the γ-secretase is regulated by its lipid environment (Grimm *et al.*, 2005). These *in vitro* observations are supported by animal studies showing that pharmacologically lowering cholesterol levels reduces amyloid pathology while raising cholesterol levels using dietary cholesterol aggravates pathology (Levin-Allerhand *et al.*, 2002; Petanceska *et al.*, 2002; Refolo *et al.*, 2001). Further, several epidemiological studies note changes in sphingolipids, cholesterol and phospholipids in the brains and/or cerebrospinal fluid of AD patients (Dietschy, 2009; Fonteh *et al.*, 2013; Haughey *et al.*, 2010; He *et al.*, 2010; Kosicek and Hecimovic, 2013; van

Echten-Deckert and Walter, 2012; Wells *et al.*, 1995).

2.3 Risk factors for sporadic Alzheimer's disease

Apart from age, the most significant risk factors for sporadic Alzheimer's disease are heavily entangled with the lipid and cholesterol metabolism, and we understand that AD patients show elevated cholesterol and cholesteryl-ester (CE) levels (Bertram and Tanzi, 2004; Stefani and Liguri, 2009). Hence, it is not surprising that AD and cardiovascular disease (CVD) share multiple risk factors like elevated levels of low-density lipoprotein (LDL) cholesterol levels, hypertension, and type-II diabetes, as well as the most potent genetic risk factor known, the ε4 allele of the Apolipoprotein E (ApoE ε4). Homozygous carriers of the ε4 allele have a 12-fold higher risk of developing the disease while hemizygous carriers still have a 4-fold higher risk (Camejo *et al.*, 1976; Gordon *et al.*, 1977; Saunders *et al.*, 1993). Additionally, despite some controversies and limitations, there is staggering evidence that statin treatment, especially in mid-life could slow the onset of AD. While the HPS and PROSPER clinical trials, testing pravastatin and simvastatin, showed no effect on preventing dementia, multiple studies found an association of statin use and dementia (Heart Protection Study Collaborative, 2002; Houx *et al.*, 2002). Initially, two studies reported a reduced risk of AD in statin users (Jick *et al.*, 2000; Wolozin *et al.*, 2000). These reports were later followed up by further studies, and collectively the evidence supports a role for statins in the reduction of dementia and AD (Chatterjee *et al.*, 2015; Li *et al.*, 2010; Rockwood *et al.*, 2002; Shah *et al.*, 2015; Wong *et al.*, 2013). Is important to note, that there may be a window of opportunity for statins before the onset of symptoms of dementia (Li *et al.*, 2010) and the effects of statins on AD may be more pronounced in homozygous ApoE ε4 carriers (Geifman *et al.*, 2017). Additionally, elevated midlife cholesterol levels are a risk factor for the development of AD later in life (Anstey *et al.*, 2017). Further, there is evidence that other cholesterol-lowering drug may show similar effects to statins (Sparks *et al.*, 2008).

2.4 CETP and Alzheimer's disease pathology

An exciting drug candidate for the treatment of AD is the cholesteryl ester transfer protein (CETP). CETP is a lipid transfer protein that facilitates the exchange of cholesteryl esters and neutral lipids between plasma lipoproteins, and the activity of CETP led to increased LDL cholesterol and decreased HDL cholesterol (Morton and Zilversmit, 1982; Nichols and Smith, 1965; Zhong *et al.*, 1996; Zilversmit *et al.*, 1975). Extensive research for the prevention of cardiovascular disease has been devoted to CETP as soon as single nucleotide polymorphisms (SNPs) in CETP had been linked with longevity and a reduced risk of atherosclerosis (Brousseau *et al.*, 2004; Clark *et al.*, 2004; de Grooth *et al.*, 2002; Koizumi *et al.*, 1985; Masson, 2009; Okamoto *et al.*, 2000; Sanders *et al.*, 2010; Vasan *et al.*, 2009). More recently, SNPs in CETP have been linked with a reduced risk of dementia (Arias-Vasquez *et al.*, 2007; Barzilai *et al.*, 2003; Chen *et al.*, 2014; Sanders *et al.*, 2010; Thompson *et al.*, 2008; Yu *et al.*, 2012). Subsequently, several laboratories further analyzed the involvement of CETP SNPs in AD and found a possible correlation of CETP SNPs with the ε4 allele of ApoE. In homozygous carriers of the ε4 allele the CETP I405V polymorphism buffers the detrimental effects of the ε4 genotype (Murphy *et al.*, 2012; Rodriguez *et al.*, 2006; Sundermann *et al.*, 2016). Additionally, imaging studies showed that CETP SNPs could modify brain lipids and structure (Murphy *et al.*, 2012; Salminen *et al.*, 2015).

Together, these studies make a compelling case that the cholesterol metabolism and especially CETP may be a modifying factor in AD. However, while mice and rats are the most common animal models for AD, neither mice or rats express CETP, and their cholesterol metabolism differs from humans as they only have very low levels of LDL particles (Gauthier *et al.*, 1999; Gordon *et al.*, 2015). Hence, all current animal models for AD are not able to test the effects of CETP on the pathology of AD in an animal model.

The scope of this study was to generate a novel mouse model for AD that incorporates a humanized cholesterol metabolism. To achieve this, we used a CETP transgenic mouse model that expresses human CETP under its natural promoter (Jiang *et al.*, 1992). CETP expression

and LDL levels can be induced using a diet containing 1% cholesterol leading to a humanized lipoprotein profile (Cazita *et al.*, 2003; Cheema and Rashid-Kolvear, 2003; Jiang *et al.*, 1992). In a previous study using the same CETP transgenic animals, we were able to show that CETP expression has a profound role on the brain cholesterol distribution (Oestereich *et al.*, 2018, in revision at JLR). Here, transgenic animals fed a diet supplemented with 1% cholesterol showed a significant 30% increase in brain cholesterol. Most interestingly, this increase was not due to the elevated *de novo* synthesis of cholesterol. Furthermore, we found that these increased cholesterol levels were accompanied by changes in presenilin 1 and 2, the catalytic subunits of the γ-secretase complex, indicating a connection between brain cholesterol levels and presenilin activity.

To further analyze the connection of CETP and AD, we crossed CETP transgenic mice with mouse models for AD to analyze the effects of CETP on the generation of Aβ.

3. Materials & methods

All experiments were conducted following McGill University environmental health and safety regulations (EHS).

3.1 HEK293T Cell culture

HEK293T cells were cultivated in 1:1 Dulbecco´s modified Eagle medium (DMEM) supplemented with 0.584 g/l L-glutamine and 0.11 g/l sodium pyruvate (Wisent), and 10% fetal calf serum (FCS, Wisent), at 37°C and 5% CO_2. For transient transfections, 1.5 x 10^5 cells per well (12-well plates) were seeded 18 h before transfection. Cells were transiently transfected with 1 µg DNA in total and 2 µl polyethyleneimine (PEI) per well. The cell culture supernatant was changed 18 h after transfection. For experiments containing lipoprotein deprived serum (LPDS) FCS was replaced by LPDS (Sigma). A further 24 h later, cell culture supernatant was collected, and cells were lysed with TNE-lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, and complete protease inhibitors, Roche) and prepared for

SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.2 Aβ quantification from cell culture supernatant

Aβ38, Aβ40, and Aβ42 were measured from 25 µL cell-culture supernatant using the V-PLEX Plus Aβ Peptide Panel 1 (6E10) Kit (Mesoscale discovery) according to manufacturer's instructions.

3.3 CETP activity assay

CETP activity was measured using a fluorescent CETP activity assay (Sigma-Aldrich). Here, 1 µL of serum or 3 µL cell culture supernatant was incubated with 0.3 µL donor and 0.3 µL acceptor molecules in 30 µL reaction volume. The reaction mix was incubated for 3 hours at 37 °C in a water bath, and the fluorescence (λ_{ex} 465/λ_{em} 535) was measured.

3.4 Western blot analysis of mouse tissue samples

Fresh frozen liver or brain samples (approximately 100 mg) were lysed in 5x volume of lysis buffer (150 mM NaCl, 10% glycerol, 2 mM EDTA, 0.5% NP-40, 0.1% sodium deoxycholate, 20 mM HEPES, 1x complete protease-inhibitor cocktail (Roche), pH 7.4) using lysing-matrix D at 6000 rpm for 40 seconds. The lysates were further diluted 1:5 in lysis buffer. For western-blot analysis, liver samples were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on either 10% or 15% SDS-polyacrylamide gels. The following antibodies were used as primary antibodies: 22C11 (Millipore) to detect APP, the mouse monoclonal TP2 (kind gift of the Ottawa Heart Institute) to detect CETP, rabbit-anti-GAPDH (14C10, Cell Signaling) and mouse-anti β-tubulin (Abcam) were used. Horseradish peroxidase (HRP)-coupled secondary antibodies directed against mouse or rabbit IgG were purchased from Promega. Chemiluminescence images were acquired using the ImageQuant LAS 500 system (GE Healthcare).

3.5 Mouse housing and breeding

The CETP transgenic mouse strain B6.CBA-Tg(CETP)5203Tall/J (JAX MMRRC Stock# 003904), the 5xFAD mice (APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I

(London), PSEN1 M146L, PSEN1 L286V) (JAX MMRRC Stock# 034840) and McGill-Thy1-APP Tg mice (hAPP KM670/671NL (Swedish) and V717F (Indiana)) were housed according to the McGill University standard operating procedure mouse breeding colony management #608 (Ferretti *et al.*, 2011; Jiang *et al.*, 1992; Oakley *et al.*, 2006). Double transgenic mice were bred heterozygous and non-transgenic littermates were used as controls. All mouse diets were purchased from Envigo. The diets used in this study were: low-fat control diet (TD.08485) and a low-fat diet enriched with 1% (w/w) cholesterol (TD.140215). Animals were assigned randomly to treatment groups. All mouse procedures were approved by McGill's Animal Care Committee and are following the McGill policy on the study and care of animals. The reporting of all mouse data under the ARRIVE guidelines (Animal Research: Reporting *in Vivo* Experiments).

3.6 Mouse genotyping

Genotyping was performed by Transnetyx (Transnetyx, Cordova, TN) genotyping using real-time PCR from ear punch tissue.

3.7 Aβ measurement from mouse cortex

Aβ from mouse brains was measured using the V-PLEX Plus Aβ Peptide Panel 1 (6E10) Kit (Mesoscale discovery) according to manufacturer's instructions. In brief, 40-80 mg of cortex tissue was mechanically homogenized in 10x vol. of cold tissue homogenization buffer (2 mM Tris (pH 7.4), 250 mM Sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 1x protease inhibitor cocktail). 250 µL homogenate was mixed thoroughly with 250 µL DEA buffer (0.4% DEA, 0.1 M NaCl) and centrifuged at 120000 x g for 1h at 4 °C. 425 µL supernatant was neutralized with 42 µL 0.5 M Tris-HCl (pH 6.8) buffer, the soluble DEA extract. The pellet was dissociated in 125 µL cold formic for 30 mins on ice. The homogenate was centrifuged at 120000 x g for 1 h at 4 °C. 105 µL supernatant was neutralized with 1.895 mL formic-acid neutralization buffer (1M Tris base, 0.5 M Na_3HPO_4, 0.05% NaN3), the insoluble formic acid extract. 25 µL of soluble DEA extract and formic acid extract were used in the ELISA.

3.8 Quantitative real-time PCR (RT-qPCR)

mRNA was isolated from mouse tissue using the Macherey & Nagel mRNA-isolation kit in combination with lysing matrix D. Briefly, 40-80 µg of fresh frozen tissue were lysed in 450 µL RNA preparation buffer (with β-mercaptoethanol) in lysing matrix D tubes using a Magna lyser (6000 rpm 2x 30 seconds) according to manufacturer's instructions. The RNA concentration was adjusted to 100 pg/mL, and 500 ng of RNA was transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. RT-qPCR was performed using the SsoAdvanced SYBR-green supermix (Biorad) according to manufacturer's instructions on a Biorad CFX384Touch cycler. All primers were ordered from Integrated DNA technologies. Primers used were: CETP forward: CAGATCAGCCACTTGTCCAT, CETP reverse: CAGCTGTGTGTTGATCTGGA, hAPP forward: CCTTCTCGTTCCTGACAAGTGC, hAPP reverse: GGCAGCAACATGCCGTAGTCAT, TNFα forward: GGTGCCTATGTCTCAGCCTCTT, TNFα reverse: GCCATAGAACTGATGAGAGGGAG, IL1β forward: TGGACCTTCCAGGATGAGGACA IL1β reverse: GTTCATCTCGGAGCCTGTAGT, TREM2 forward: ACAGCACCTCCAGGAATCAAG, TREM2 reverse: AACTTGCTCAGGAGAACGCA, The following primers were used as reference genes: HPRT forward: CCAGTTTCACTAATGACACAAACG, HPRT reverse: CTGGTGAAAAGGACCTCTCGAAG, GAPDH forward: CATCACTGCCACCCAGAAGACTG, GAPDH reverse: ATGCCAGTGAGCTTCCCGTTCAG, Actin-β forward: CATTGCTGACAGGATGCAGAAGG, Actin-β reverse: TGCTGGAAGGTGGACAGTGAGG. Primer efficiency for all primers was determined to be between 90-110%. For normalization of gene expression, the three genes ACT, GAPDH and HPRT were used as reference genes. RT-qPCR was analyzed using the CFX maestro software (Biorad).

3.9 Novel Object recognition (NOR)

6-months-old mice animals were first acclimated to an empty open field box in a dark

room. Twenty-four hours after the habituation session, mice were exposed to two identical, non-toxic 'familiar' objects. After a retention interval of 2 h or 24 h, the animals were returned to the arena in which one of the two objects was exchanged with a novel object. Each session lasted 10 min, during which all the mice were allowed to interact with the objects freely and the amount of time exploring each object was recorded using an infrared camera. The two objects were placed in two opposing corners in the box. All Objects were randomized between animals.

3.10 Y-Maze spontaneous alteration

6-month-old animals were placed in a Y-Maze with the dimensions of 40cm x 8cm x 10 cm with the arms at an angle of 120°. The animal was allowed to explore the maze freely for 5 minutes while being recorded. An arm entry was recorded when all four limbs of the animal wholly entered the arm. Over multiple entries, cognitive intact animals show a preference to enter a less recently visited arm. The experiments were performed in a dark room with only red light and recorded using an infrared camera.

3.11 Evans blue BBB integrity assay

Mice were anesthetized using isoflurane, and 5 μL/g body weight of a 2% Evans blue solution in PBS was injected into the caudal vein. The mouse was returned to its cage for 30 minutes. Finally, the animal was perfused with PBS and sacrificed under isoflurane anesthesia. The brain was extracted and documented.

3.12 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 7 software.

4. Results

4.1 CETP increases Aβ *in vitro*

Based on our hypothesis that CETP could modulate the processing of APP by γ-secretase via the CETP-mediated cholesterol modulation, we first sought out to test whether CETP could alter γ-secretase *in vitro* and affect Aβ generation. Here, we used a HEK293T cell-culture system and transiently co-transfected human APP and CETP (**Figure 13A**). To test our hypothesis, we compared the effects of wild-type CETP with an inactive CETP mutant ($CETP_{inac.}$, CETP L457W M459W) and the CETP inhibitor evacetrapib (Cao *et al.*, 2011; Qiu *et al.*, 2007). We found the APP expression levels to be consistent between different transfections (**Figure 13B**). However, our western-blot analysis revealed that our CETP antibody only shows a low affinity towards the inactive CETP mutant, presumably due to the interference of the mutations with the antibody epitope. We confirmed the results of our inactive mutant by generating another mutant with impaired activity (data not shown).

Additionally, we verified the CETP mutants as well as the inhibitor treatments with a CETP-activity assay. Here, the inactive mutant, the triglyceride-transfer (TG) deficient CETP V198W, and cells treated with the 100 nM evacetrapib showed reduced activity. As the assay only detects TG transfer, the CE-transfer deficient mutant CETP Q199A showed no impairment (**Figure 13C**). Next, we used a multiplex ELISA to simultaneously detect Aβ38, Aβ40, and Aβ42 from the cell culture supernatant. When comparing cells transfected with APP and CETP with cells transfected with APP alone, we found that the former showed a 30% increase in Aβ40 and 23% increase in Aβ42 (**Figure 13F, H**). Changes in Aβ38 were less pronounced (**Figure 13D**). We were able to confirm that this increase in Aβ is indeed dependent on CETP using the inactive mutant $CETP_{inac.}$ as well as CETP inhibition with 100 nM evacetrapib, as both showed Aβ levels identical to the APP transfection alone (**Figure 13D-I**).

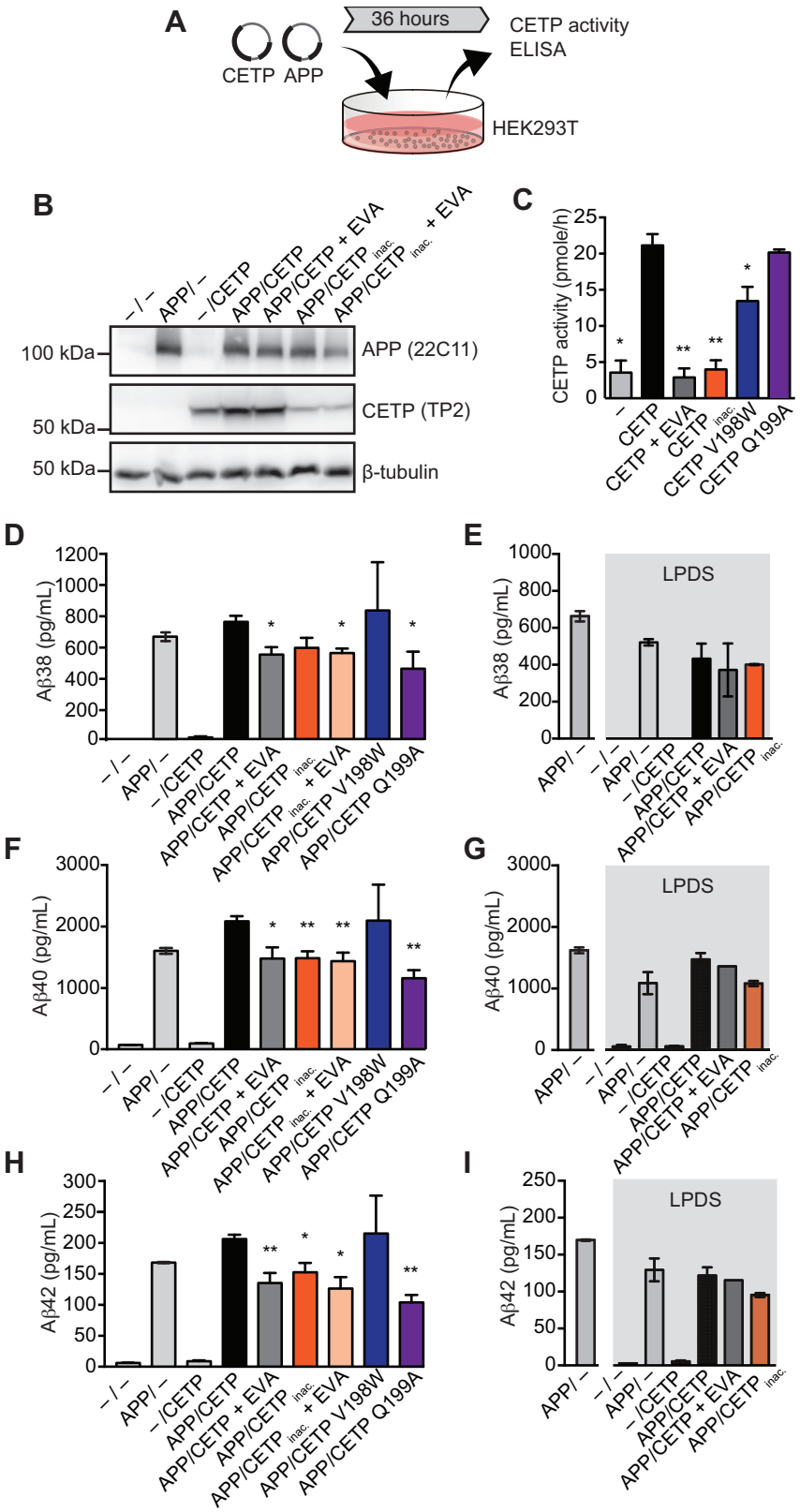

A
36 hours
CETP activity
ELISA
CETP APP
HEK293T
B
– / –
APP/ –
– /CETP
APP/CETP
APP/CETP + EVA
APP/CETP inac.
APP/CETP inac. + EVA
100 kDa
APP (22C11)
CETP (TP2)
50 kDa
50 kDa
β-tubulin
C
CETP activity (pmole/h)
CETP
CETP + EVA
CETP inac.
CETP V198W
CETP Q199A
D
Aβ38 (pg/mL)
APP/CETP V198W
APP/CETP Q199A
E
LPDS
Aβ38 (pg/mL)
F
Aβ40 (pg/mL)
G
LPDS
Aβ40 (pg/mL)
H
Aβ42 (pg/mL)
I
LPDS
Aβ42 (pg/mL)

Figure 13: CETP activity increases Aβ *in vitro*

A: Experimental setup: CETP and APP were co-transfected in HEK293T cells. After 36h the Aβ levels were measured from the cell culture supernatant using a multiplex ELISA. **B**: Western-blot control to test equal expression levels of CETP and APP using the TP2 and 22C11 antibodies, respectively. Note that the TP2 antibody shows a reduced affinity towards the $CETP_{inac.}$ mutant. **C**: CETP-activity assay of 2 µL cell culture supernatant. 100 nM Evacetrapib were added to the cell culture supernatant for CETP inhibition. **D-I**: Multiplex ELISA to measure Aβ levels in the cell culture supernatant of cells transfected with APP and CETP and cultured in medium containing either FCS or LPDS. **D, E**: Aβ38 levels. **F, G**: Aβ40 levels. **H, I**: Aβ42 levels. n=3-4, mean ± SEM; 2-way ANOVA, Tukey's multiple comparisons. * $p<0.05$, ** $p<0.001$ compared to APP/CETP.

Considering that CETP can transfer both CE as well as triglycerides, we were interested in testing whether either the cholesteryl ester or lipid-transfer activity is responsible for the Aβ increase. Here, we generated two additional CETP mutants lacking either the CE or lipid transfer while leaving the other mostly unaffected. CETP V198W shows impaired TG-transfer, and CETP Q199A lacks the CE transfer (Qiu *et al.*, 2007). While CETP V198W showed similar Aβ levels as wild-type CETP, the Q199A mutant resembled inactive $CETP_{inac}$. This shows that is the CE transfer activity of CETP is responsible for the effects on APP processing and Aβ generation (**Figure 13D, F, H**). These results are in line with our previous work demonstrating that CETP activity can activate γ-secretase *in vitro* and increase brain cholesterol levels *in vivo* (Oestereich *et al.*, 2018, in revision at JLR).

Seeing that CETP transfers lipids between lipoproteins and it is unlikely that it directly interacts with the γ-secretase complex, we hypothesized that removing lipoproteins from the cell culture medium should show similar effects as CETP inhibition. Hence, we replaced the fetal bovine serum component of the cell culture medium with lipoprotein-deprived serum (LPDS) and found no significant differences between any of the treatment groups in the absence of lipoproteins. In this case, the levels of Aβ were similar to the levels measured in the presence of lipoproteins but with either inactive or inhibited CETP (**Figure 13E, G, I**).

Together, these results suggest that at least in cell culture, CETP activity is necessary for the redistribution of cholesterol and lipids which results in elevated levels of Aβ generated.

4.2 CETP expression does not increase Aβ in 5xFAD mice

We had previously been able to show that CETP activity alters brain cholesterol levels *in vivo* and can increase Aβ secretion most likely via an LDL-dependent mechanism *in vitro*. To test whether CETP activity could affect Aβ in a mouse model for AD, we crossed CETP transgenic mice with the 5xFAD mouse model for AD (Jiang *et al.*, 1992; Oakley *et al.*, 2006). The 5xFAD model shows signs of amyloid deposition at the age of 1.5 months and cognitive impairment measured in the Y-maze at 4-5 months of age (Oakley *et al.*, 2006). We measured the soluble and insoluble Aβ38, Aβ40 and Aβ42 from brain homogenates at the age of 3 (**Figure 14A-C**) and 6 months (**Figure 14D-J**). In 3-month old animals, we found no difference in Aβ38, Aβ40 or Aβ42 between 5xFAD and double transgenic animals. However, we found a robust diet-dependent increase in all measured Aβ species (**Figure 14B, C**).

At the age of 6 months (**Figure 14D**), we found no differences in the levels of soluble Aβ40 or Aβ42 between 5xFAD and double transgenic mice on either diet (**Figure 14E-J**). However, 5xFAD/CETP transgenic mice had significantly reduced levels of insoluble Aβ40 while insoluble Aβ42 remained unchanged on either diet (**Figure 14H**). While not statistically significant, we found increased Aβ42/40 ratios in 5xFAD/CETP mice on a high cholesterol diet (**Figure 14J**).

Next, we asked whether the memory of 5xFAD/CETP would be affected through CETP. To measure cognitive performance, we performed the Y-maze behavioral test to assess spatial memory. However, at the age of 6 months, we were not able to show any significant differences in spatial memory between any of the experimental groups (**Figure 15A**). Surprisingly, neither 5xFAD nor 5xFAD/CETP mice showed significant differences compared to our control groups. Apart from the wild-type control animals, all groups showed signs of cognitive impairment, albeit not statistically significant.

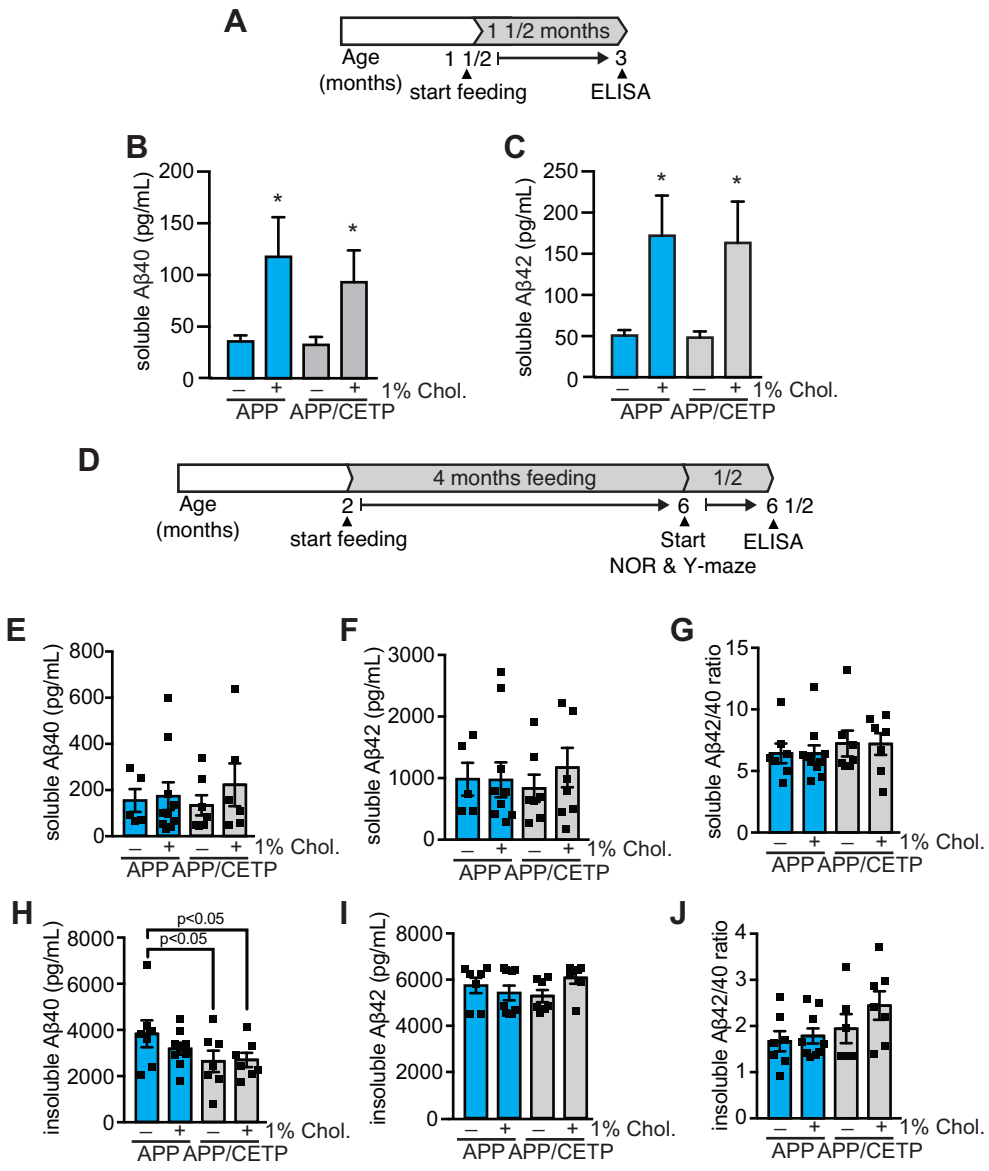


Figure 14: CETP activity does not increase Aβ in 5xFAD mice

A: Breeding scheme of the 3-month trial: Mice were put on a control diet or a diet containing 1% (w/w) cholesterol at the age of 6 weeks. Soluble Aβ levels in the brain were measured at the age of 3 months. **B, C**: Soluble Aβ40 (**B**) and Aβ42 (**C**) levels. **D**: Breeding scheme of the 6-month trial: Mice were put on a control diet or a diet containing 1% (w/w) cholesterol at the age of 2 months. At the age of 6 months, the NOR and Y-maze behavioral tests were performed, and the animals were sacrificed afterward to measure brain-Aβ levels. **E-G**: Levels of soluble Aβ40 and Aβ42 levels as well as the Aβ42/40 ratio. **H-J**: Levels of insoluble Aβ40 and Aβ42 levels and the Aβ42/40 ratio. n=5-8, mean ± SEM; 2-way ANOVA, Tukey's multiple comparisons. * $p<0.05$.

To exclude that differences in APP expression between 5xFAD and 5xFAD/CETP animals is responsible for our negative results, we purified mRNA from cortex samples and performed an RT-qPCR analysis. Here, we found no differences in the mRNA levels of APP between 5xFAD and 5xFAD/CETP animals (**Figure 15B**). Additionally, there was no detectable difference in CETP mRNA between CETP and double transgenic mice (**Figure 15C**). In line with previous experiments in CETP transgenic animals, we observed a trend towards increased levels of the inflammatory cytokines TNFα and IL1β both in a diet and genotype-dependent manner (**Figure 15D, E**). Animals fed with a diet containing 1% cholesterol a trend towards higher cytokine mRNA levels when compared to their diet counterpart. However, this was not statistically significant.

Additionally, 5xFAD and 5xFAD/CETP had increased cytokine levels when compared to the wild-type of CETP controls. Preliminary experiments hinted at changes in the triggering receptor expressed on myeloid cell 2 (TREM2) in our transgenic mice. Consequently, we analyzed the brain-expression levels of TREM2 and found a 6-8-fold increase in TREM2 mRNA in 5xFAD and 5xFAD/CETP mice (**Figure 15F**). Lastly, we detected APP-protein levels using western blotting. While we detected great individual differences in APP between different animals, we could not detect statistically significant differences between groups (**Figure 15G**).

4.3 CETP expression increases Aβ in McGill-Thy1-APP mice

Seeing that the γ-secretase complex is a central component in the generation of Aβ and is modulated by surrounding cholesterol and lipid levels, but we were not able to show changes in Aβ in 5xFAD/CETP mice, we hypothesized that familiar presenilin mutations might not be susceptible to changes in membrane cholesterol and thus represent a poor model for sporadic AD. Several studies have published that γ-secretase incorporating familial presenilin mutations cleave APP in a different mechanism (Qi *et al.*, 2003). Hence, we used a different model for AD to cross with the CETP transgenic mice.

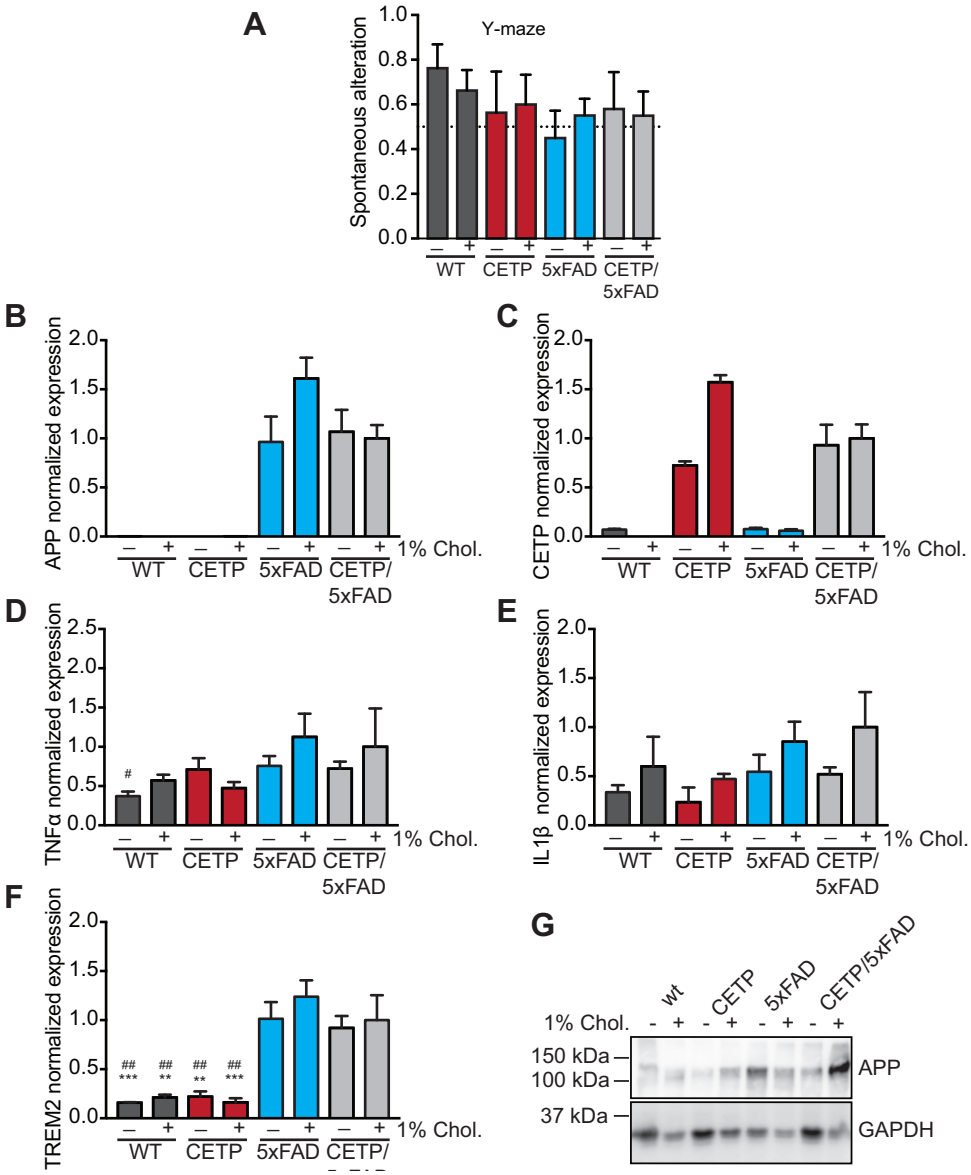


Figure 15: Behaviour and Alzheimer's disease risk gene expression in 5xFAD/CETP mice
A: Y-maze behavioral test. **B-F**: Brain-mRNA levels of (**B**) APP, (**C**) CETP, (**D**) TNFα, (**E**) IL1β and (**F**) TREM2. n=7-8, mean ± SEM; 2-way ANOVA, Tukey's multiple comparisons.* p<0.05 compared to APP/CETP 1%C. diet, ** p<0.01 compared to APP/CETP 1%C. diet, *** p<0.001 compared to APP/CETP 1%C. diet, # p<0.05 compared to APP/CETP standard diet, ## p<0.01 compared to APP/CETP standard diet, ### p<0.001 compared to APP/CETP standard diet. **G**: Western blot analysis of human APP expression.

We used a model that does not have any presenilin FAD mutations and crossed the Thy1 APP McGill mice with the CETP transgenic mice (Ferretti *et al.*, 2011; Jiang *et al.*, 1992).

The Thy1 APP McGill mice carry the Swedish (K670N & M671L) and Indiana V717F APP mutations and show cognitive deficits as early as three months and early onset of amyloid pathology (Ferretti *et al.*, 2011).

Akin to our initial mouse study, we analyzed the double transgenic Thy1 APP McGill/CETP mice together with wild-type, CETP and Thy1 APP McGill as controls at the age of 3 months and 6 months.

Unlike the 5xFAD model, the Thy1 APP McGill/CETP mice had significantly higher levels of Aβ40 and Aβ42 at the age of 3 months, while Aβ38 was below the detection limit for most animals (data not shown) (**Figure 16A-C**). Based on these initial experiments, we bred a larger cohort and analyzed the animals at the age of 6 months (**Figure 16D**). First, we measured the CETP activity in plasma samples from all groups. As expected, wild-type and Thy1 APP McGill mice showed no CETP activity. CETP as well double transgenic mice showed CETP on both control and 1% cholesterol diet(**Figure 16E**).

Further, we analyzed the expression levels of CETP and APP in the mouse brain using RT-qPCR and western blotting. The expression levels of the human APP were indistinguishable between Thy1 APP McGill and double transgenic mice (**Figure 16H**). Additionally, the CETP expression was identical between CETP and double transgenic mice. However, the levels of CETP in the brain as detected by western blot were very low (**Figure 16F**).

Subsequently, we measured the levels of soluble and insoluble Aβ38, Aβ40 and Aβ42 in Thy1 APP McGill and Thy1 APP McGill/CETP mice at the age of 6 months (**Figure 16I-P**). In line with our 3-month cohort, the double transgenic mice had increased levels of Aβ compared to the Thy1 APP McGill mice. Analyzing the amount of TBS-soluble Aβ, we found that both the genotype and diet had a profound effect on all Aβ species (**Figure 16I-L**). Most strikingly, we were able to measure a 3-fold increase in Aβ42 comparing Thy1 APP McGill with double transgenic mice on a 1% cholesterol diet (**Figure 16K**). We observed very similar effects for the insoluble Aβ42 fractions (**Figure 16O**). For Aβ38, we found that double transgenic animals had higher Aβ levels as compared to their Thy1 APP McGill counterpart with

the highest values measured in double transgenic mice fed with a diet supplemented with 1% cholesterol (**Figure 16M**).

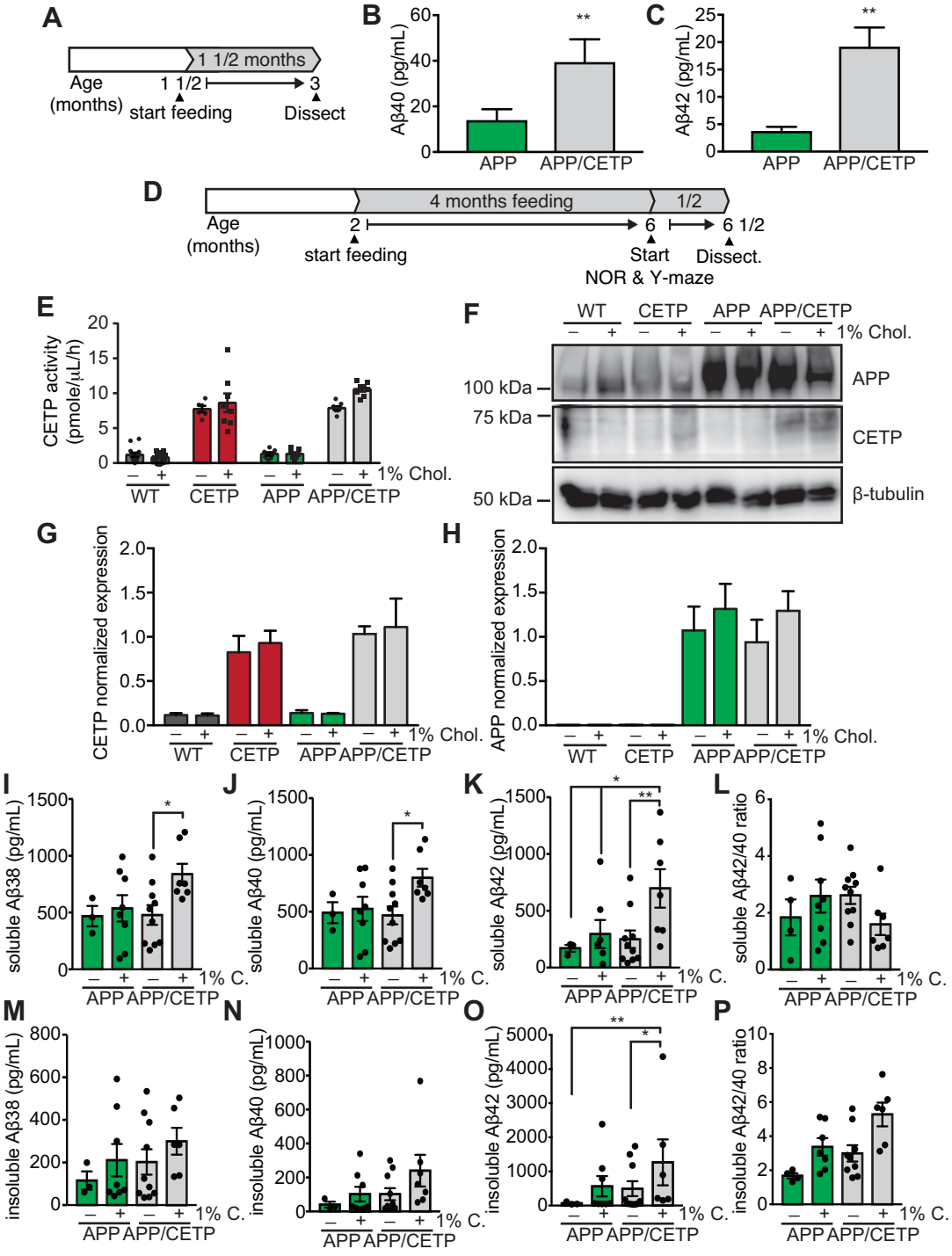


Figure 16: CETP activity increases Aβ in Thy1 APP mice

A: Breeding scheme of the 3-month trial: Mice were put on a control diet or a diet containing 1% (w/w) cholesterol at 6 weeks. Soluble Aβ levels in the brain were measured after 3 months. **B,**

C: Soluble Aβ40 (**B**) and Aβ42 (**C**) levels. n=5-6, mean ± SEM; 2-way ANOVA, Tukey's multiple comparisons. **D**: Mice were put on a control diet or a diet containing 1% (w/w) cholesterol at the age of 2 months. At the age of 6 months, behavioral (NOR & Y-maze) and biochemical analyses (RT-qPCR, western blot & ELISA) were performed. **E**: CETP-activity assay of 1 µL mouse serum. **F**: Western blot analysis of APP and CETP expression. **G**: Gene expression of CETP as measured from cortical mRNA using RT-qPCR. **H**: Gene expression of APP as measured from cortical mRNA using RT-qPCR. **I-L**: Levels of soluble Aβ40 and Aβ42 levels as the Aβ42/40 ratio. **M-P**: Levels of insoluble Aβ40 and Aβ42 levels as the Aβ42/40 ratio. n=4-8, mean ± SEM; 2-way ANOVA, Tukey's multiple comparisons. * $p<0.05$, ** $p<0.001$.

For Aβ40 we saw strong diet-dependent effects with 30% and 50% increases in Aβ40 when comparing Thy1 APP McGill and Thy1 APP McGill/CETP mice on control with a 1% cholesterol diet, respectively (**Figure 16N**). Again, double transgenic mice on a diet containing 1% cholesterol showed the highest values. In a similar fashion to the soluble Aβ42, we found the most potent effects, both in a diet and genotype-dependent fashion for Aβ42 (**Figure 16O**). While Thy1 APP McGill mice had about 2-fold higher Aβ42-levels on a 1% cholesterol diet, double transgenic mice had almost 6-fold more Aβ42. Additionally, double transgenic mice had almost 4-fold higher Aβ42 values compared to Thy1 APP McGill mice on a 1% cholesterol.

4.4 Memory impairment in CETP/Thy1 APP McGill mice

Next, we measured spatial memory using the Y-maze test. Here, both Thy1 APP McGill and Thy1 APP McGill/CETP mice showed impaired spatial memory when compared to wild-type or CETP transgenic mice. However, there were no detectable differences between the diets or Thy1 APP McGill and Thy1 APP McGill/CETP mice (**Figure 17A**). We further analyzed the behavior of our novel transgenic mice using the novel object recognition (NOR) test (Baxter, 2010; Ennaceur and Delacour, 1988). As expected, both wild-type and CETP transgenic mice showed no signs of cognitive impairment on either diet. We were not able to measure significant cognitive deficits of the double transgenic or Thy1 APP McGill mice

compared to controls using the novel object recognition test. However, we observed a trend towards memory impairment in both strains (**Figure 17B**).

4.5 BBB is intact in CETP/Thy1 APP McGill mice

Lastly, we analyzed whether the combination of CETP activity and APP transgenicity could lead to disruptions of the blood-brain barrier (BBB). CETP is a known pro-atherogenic factor and in combination with Aβ deposition could accelerate lesions in the BBB which could be involved in the increase in brain-Aβ levels. To test this, we injected the BBB impermeable dye Evans blue into the tail vein, let the dye circulate for 30 minutes and dissected the animals (**Figure 17C, D**). While the dye was able to penetrate every organ, we could not detect Evans blue diffusion into the brain in any of our experimental treatment groups. As a positive control for BBB leakage, we used a much older animal that had spontaneously developed BBB leaks (**Figure 17E**). These results indicate that the increasing levels of Aβ stem from changes within the brain.

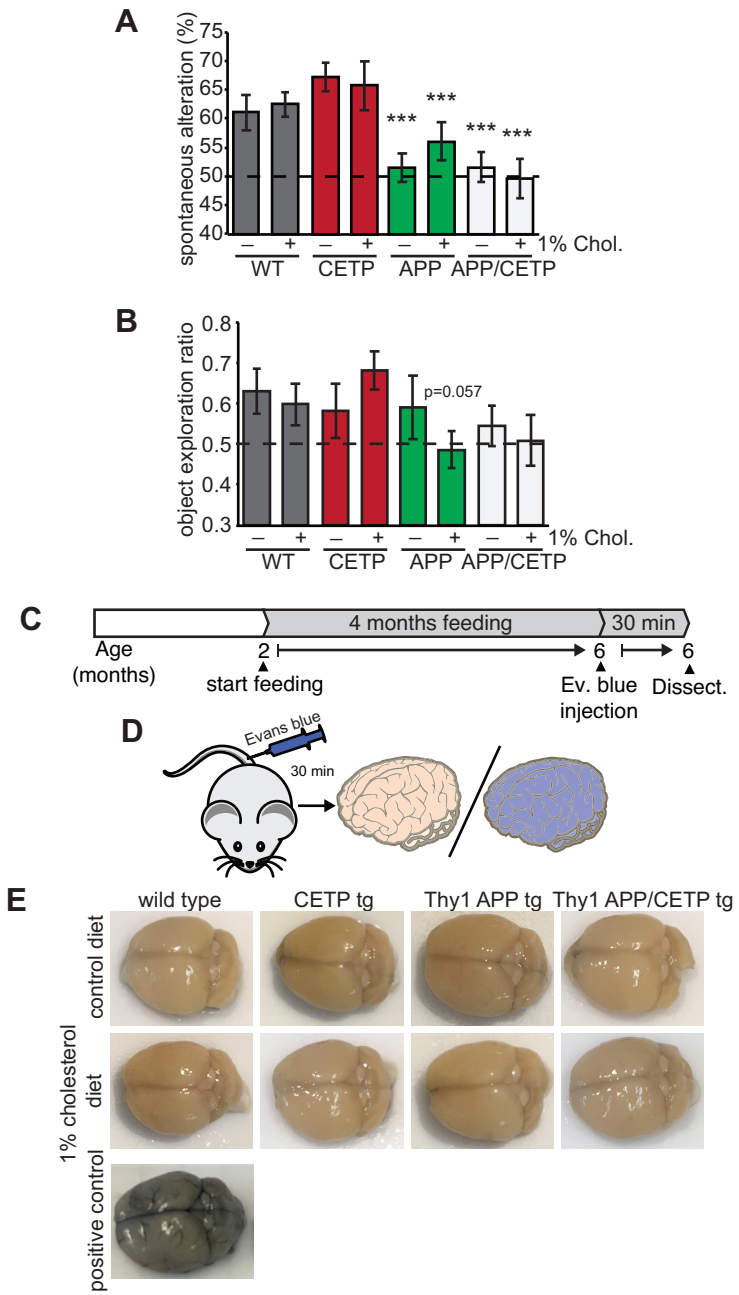


Figure 17: CETP activity does not affect the blood-brain barrier

A: Y-maze analysis. n=9-16, mean ± SEM; 2-way ANOVA, Tukey's multiple comparisons. **B**: NOR analysis. n=9-16, mean ± SEM; 2-way ANOVA, Tukey's multiple comparisons. **C**: Breeding scheme for Evans blue injections. Mice were put on a control diet or a diet containing 1% (w/w) cholesterol at the age of 2 months. At the age of 6 months, the Evans blue injections were performed, and BBB integrity was determined. **D**: Schematic representation of Evans blue injections. **E**: Extracted brains from mice injected with Evans blue. A much older animal with BBB impairment was used as a reference. n=4. *** $p<0.001$.

5. Discussion

5.1 Cholesterol metabolism in Alzheimer's disease

Since the beginning of AD research, the cholesterol metabolism has been tightly linked to AD (Fassbender *et al.*, 2001; Runz *et al.*, 2002). Epidemiologically, it is well understood that elevated cholesterol and LDL levels, as well as hyperlipidemia, are strong risk factors for AD. Further, multiple of the strongest genetic risk factors for AD, such as the ε4 allele of ApoE, polymorphisms in clusterin (ApoJ) or the ABCA7 are critical players in lipid and cholesterol homeostasis (Di Paolo and Kim, 2011). Additionally, Marquer *et al.* noted that an early increase in cholesterol levels mimics most the early phenotype of AD in a mouse model (Marquer *et al.*, 2014). Today, a large proportion of AD research uses mouse or rat model systems to unravel the pathological mechanisms underlying AD.

However, both mice and rats have essential differences in their cholesterol homeostasis compared to humans. While both humans and mice/rats share the same lipoprotein particles, most apolipoproteins and most other proteins associated with lipoproteins, mice completely lack CETP (Guyard-Dangremont *et al.*, 1998). Seeing that CETP transports cholesteryl esters from HDL to ApoB-containing LDL, it is of no surprise that mice and rats have negligible levels of LDL and ApoB (Gauthier *et al.*, 1999; Gordon *et al.*, 2015). In light of these differences, we were interested to use mice transgenic for the human CETP to study AD. Compared to wild-type mice, CETP transgenic animals show a humanized lipoprotein profile with elevated LDL levels (Agellon *et al.*, 1991; Grass *et al.*, 1995; Oestereich *et al.*, 2018, in revision at JLR). Previous work in our group demonstrated that CETP expression leads to increased levels of cholesterol in the brain which is accompanied by increased plasma and brain cytokine levels. Furthermore, a microarray analysis of astrocytes revealed that the γ-secretase complex might be one of the mayor targets downstream of this cholesterol increase (Oestereich *et al.*, 2018, in revision at JLR).

However, we were not able to study whether this would affect the generation of Aβ pep-

tides by γ-secretase. This was attributed to the fact that CETP transgenic animals show only low levels of murine Aβ, which additionally shows different biophysical and biochemical properties compared to human Aβ. Several studies were able to demonstrate that murine Aβ does not aggregate or show toxicity to the same extent as human Aβ (Lv *et al.*, 2013). As of now, there were no animal models available incorporating both CETP and amyloid pathology to study AD. Based on our data using CETP transgenic mice, we hypothesized that the CETP induced changes in brain cholesterol would affect the processing of APP by the γ-secretase complex the levels of Aβ in the brain (Wahrle *et al.*, 2002; Wrigley *et al.*, 2005).

5.2 Does CETP activity increase Aβ generation?

To test this hypothesis, we transfected APP and CETP in HEK293T cells and measured the levels of Aβ in the cell-culture supernatant. Here, were able to show that CETP activity stimulates the generation of the Aβ species Aβ38, Aβ40 and Aβ42 *in vitro* (**Figure 13D-I**). Further, we were able to demonstrate that the CE transfer activity of CETP is required for this effect (**Figure 13D-I**).

To study whether this effect would translate into an animal model, we crossed mice transgenic for the human CETP (B6.CBA-Tg(CETP)5203Tall/J) with the 5xFAD mouse model for AD (Jiang *et al.*, 1992; Oakley *et al.*, 2006). The 5xFAD mouse is a very well-established mouse model of amyloidosis in the context of AD. It carries the APP-familiar mutations KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London) and the familiar presenilin mutations PSEN1 M146L and PSEN1 L286V (Oakley *et al.*, 2006).

First, we studied our novel 5xFAD/CETP mice and analyzed the levels of soluble and insoluble Aβ at 3 months as well as Aβ and memory at the age of 6 months. Since the expression of CETP can be induced via dietary cholesterol intake and transgenic mice on a standard chow diet show low CETP expression, we decided to feed the mice with a chow supplement with 1% cholesterol. We found that the 5xFAD, as well as the 5xFAD/CETP mice, showed a swift and early onset of amyloid pathology. However, we were not able to show that CETP activity

increases the levels of soluble or insoluble Aβ. At the age of 3 months, we found that animals fed with a diet containing 1% cholesterol had 2-3-fold higher Aβ levels. Yet, there was no difference between 5xFAD and double transgenic mice (**Figure 14B, C**). Similarly, there was no increase in Aβ levels in 6-month old mice (**Figure 14E-J**). In line with this, no additional cognitive impairment was detected (**Figure 15A**).

Seeing that our hypothesized mechanism for the CETP induced increase in Aβ is based on the effects of cholesterol on the activity of γ-secretase, we wondered whether familial mutations in presenilin, the catalytic subunit of γ-secretase, would confound our experiments. While this has not been extensively studied, there is evidence, that the cleavage mechanism of FAD mutations in presenilin may differ from wild-type presenilin (Qi *et al.*, 2003). Further, there is evidence showing that the lack of PS activity, as seen in PS FAD, could increase CE production and decrease cellular lipoprotein uptake (Area-Gomez *et al.*, 2012; Tamboli *et al.*, 2008). This PS FAD-dependent increase in CE could mask the effects of CETP or potentially prevent cellular uptake of CE.

To circumvent these drawbacks of a model carrying PS FAD-mutations, we decided to cross the CETP transgenic mice with the Thy1 APP McGill model for AD (Ferretti *et al.*, 2011). This model only carries the APP KM670/671NL (Swedish) and V717F (Indiana) mutations. Herein, we analyzed the Thy1 APP McGill/CETP mice in a similar way to our previous double transgenic model. Strikingly, we found that in a model without PS-FAD mutations, CETP activity led to an increase in both soluble and insoluble Aβ (**Figure 16I-P**). While all measured Aβ species (Aβ38, Aβ40, and Aβ42) were increased both at 3, and 6 months of age, the most striking increase was detected for both soluble and insoluble Aβ42 in 6-month old mice (**Figure 16K, O**).

Next, we asked whether these changes would prompt cognitive deficits. We measured cognitive performance using the Y-maze and NOR test. Here, both tests revealed cognitive deficits of the Thy1 APP McGill and Thy1 APP McGill/CETP mice when compared to either wild-type or CETP transgenic animals (**Figure 16A, B**). However, both groups performed

equally poorly and explored the Y-maze as well as the novel object in the NOR test randomly by chance. It is possible that CETP activity would accelerate the onset of cognitive decline, but at the age of 6 months, both groups already showed big cognitive deficits. Future research assessing cognition at earlier ages could provide insight into whether CETP can accelerate cognitive deficits in our mouse model.

5.3 How could CETP increase Aβ generation

As for the mechanism behind the CETP dependent increase in Aβ levels, we hypothesized that CETP activity in the brain would redistribute lipids and especially cholesterol and thus affect γ-secretase and Aβ generation. This is in line with *in vitro* data by Holmes *et al.* reporting that changes in γ-secretase lipid environment have effects on Aβ similar in magnitude to PS and APP FAD mutations (Holmes *et al.*, 2012).

Here, we were able to show that the CE-transfer activity and not the TG transfer of CETP is responsible for the Aβ increase *in vitro* (**Figure 13D, F, H**). Our findings are in line with our hypothesis that CETP-induced changes in the brain cholesterol homeostasis would cause increased Aβ levels.

However, while CETP expression is found in the brain, especially in astrocytes, and could be responsible for the increase in Aβ, several other possibilities may account for the effects observed in our mouse model. First, we cannot rule out that peripheral and not CNS-derived CETP activity could be responsible for the increase in Aβ (Albers *et al.*, 1992). To exclude the hypothesis that peripheral Aβ, lipids or CETP the changes in the brain, we tested the integrity of the blood-brain barrier using Evans blue injections. We found that peripherally injected Evans blue did not cross the BBB, indicating that a damaged BBB is not responsible for the neural Aβ increase (**Figure 17E**).

5.4 Difference between the 5xFAD and Thy1 APP McGill mouse models

Seeing that we measured striking diferences in Aβ generation between both mouse models, we wonderend what differences could underlie the observed effects. To minimize differences in the genetic background of our two animal models, we bred both strains homozygous with the same C57BL/6J wild-type strain. Additionally, both AD models share the same C57BL/6J background, and lastly, we used the same C57BL/6J CETP mice to generate our double transgenic models.

However, a difference between the models is the onset and rate of amyloid pathology. Due to its five FAD mutations, the 5xFAD strain shows a very aggressive pathology which may precede the slower changes induced by CETP activity. CETP expression in the brain is significantly lower compared to the periphery, and the detrimental effects of CETP may only manifest at a later time point. The Thy1 APP McGill mice on the other hand only carry three FAD mutations, and none in PSEN1 or PSEN2. Their rate of amyloid deposition could be slow enough to be affected by changes in the cholesterol content in the brain and CETP activity. This point can be supported by the fact that 5xFAD mice have 4-fold higher levels of soluble and 20-fold higher levels of insoluble Aβ42 at the age of 6 months compared to Thy1 APP McGill mice (**Figure 14E-J, 16I-P**).

Additionally, the FAD mutations may directly influence the effect of cholesterol and membrane lipids on the activity of γ-secretase or the enzyme could be insensitive to any changes in membrane cholesterol. The two PSEN1 mutations in 5xFAD (PSEN1 M146L and PSEN1 L286V) lead to an increase in the Aβ42/40 ratio (Sherrington *et al.*, 1995; Weggen and Beher, 2012).

While there may be further differences between the 5xFAD and Thy1 APP McGill mice that are not related to the presenilin FAD mutations, it is the most striking difference between the models. Additionally, the familiar APP mutations I716V (Florida) and V717I (London) are located right at the γ-secretase cleavage site and lead to elevated levels of Aβ42 and Aβ43 (Florida) or an increase in the ratio of Aβ42/40 (London) (Eckman *et al.*, 1997; Goate *et*

al., 1991). However, the Thy1 APP McGill mice also carry the FAD mutation APP V717F (Indiana) at the γ-secretase cleavage site (Murrell *et al.*, 1991). This mutation shows similar effects as the London mutation as it increases the Aβ42/40 ratio by selectively increasing longer Aβ species such as Aβ42 and Aβ43 while leaving shorter species unaffected (Suzuki *et al.*, 1994; Tamaoka *et al.*, 1994). Nonetheless, carrying four mutations directly associated with alterations of γ-secretase cleavage could render the enzyme-substrate pair unresponsive to its lipid environment or overwhelm any effects of the lipid environment.

Additionally, Woodruff *et al.* found that PS1 FAD shows defective transcytosis and lipoprotein uptake which would be a cornerstone in our hypothesized mechanism (Woodruff *et al.*, 2016). Unfortunately, there is not enough data available to establish whether the PS FAD mutations lack regulations through their lipid environment. However, given the similarities regarding the background strain between our mouse models we believe that the different mutations in APP and especially PSEN1 are responsible for the lack of CETP-dependent increase in Aβ in the 5xFAD mice.

Altogether, our data is in line with several laboratories that have been reporting changes in brain lipids in AD (Barbash *et al.*, 2017; Chan *et al.*, 2012; Foley, 2010; Mendis *et al.*, 2016). In a similar fashion, several studies found changes in brain lipids on carriers of CETP single nucleotide polymorphisms (SNPs) (Huppi and Dubois, 2006; Salminen *et al.*, 2015; Tamnes *et al.*, 2010; Warstadt *et al.*, 2014). It is not clear that these changes observed in humans are solely due to CETP activity, but it is evident that a CETP transgenic mouse model could be a valuable tool to incorporate these changes into a mouse model.

As of now all Alzheimer's disease drugs targeting Aβ eventually failed in clinical trials (May *et al.*, 2011) Hence, it has been questioned whether targeting amyloid is the best strategy (Castello *et al.*, 2014; Hardy and De Strooper, 2017). While several drugs were able to reduce the amyloid burden significantly, no cognitive improvement was reported. Further, it became clear that the molecular changes underlying the disease start well before the onset of the clinical symptoms, complicating the design of clinical trials (Mullane and Williams, 2018). Other

therapeutic strategies need to be pursued and targeting CETP could be a successful strategy especially since several epidemiological studies have linked SNPs in CETP with a lower risk of AD. Several studied reported protective effects of the CETP polymorphism I405V in carriers of the AD risk allele APOE ε4 (Murphy *et al.*, 2012; Rodriguez *et al.*, 2006; Sundermann *et al.*, 2016). Altogether, we believe that our novel mouse model with a humanized lipoprotein metabolism could shed light on new therapeutic avenues (Mullane and Williams, 2018).

6. Acknowledgements

We would like to thank Dr. Cuello for generously gifting us his Thy1 APP McGill mouse strain, Dr. Whitman and Anna Toma at the Ottawa heart institute for providing us with the TP2 antibody and Sasen Efrem, Sherilyn Recinto and Sandra Paschkowski for their help with dissecting the mice.

GENERAL DISCUSSION

In this manuscript-based Ph.D. thesis, I am presenting two manuscripts that collectively aim at describing the role of the cholesteryl ester transfer protein in the progression of Alzheimer's disease. During the first chapter, we used a mouse model transgenic for the human CETP to analyze its effects on brain lipids. In the second chapter, we generated a novel mouse model by crossing CETP transgenic mice with common models for AD.

1. Alzheimer's disease

1.1 Pathological hallmarks of Alzheimer's disease

Alzheimer's disease is the most common form of dementia and inflicts an immense socioeconomic burden worldwide (Alzheimer's disease international, 2015). The main characteristic of the disease is a progressive loss of cognition and memory. Additionally, mood changes, depression and speech impairment accompany the memory dysfunctions (Becker and Overman, 2002; Forstl and Kurz, 1999; Frank, 1994). Pathologically, the main features underlying the disease are synapse loss, a diffuse brain atrophy, and protein aggregates in the form of Aβ and tau deposits (Holtzman *et al.*, 2011; Wischik *et al.*, 1988). In 1984, Glenner and Wong were the first to postulate that APP is the source of the amyloid deposits in the AD brain (Glenner and Wong, 1984). Yet, it took another three years until multiple groups purified and sequenced Aβ from plaques and mapped it to APP on chromosome 21 (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Tanzi *et al.*, 1987; Tanzi *et al.*, 1988).

1.2 The amyloid cascade hypothesis

In order to generate amyloidogenic Aβ peptides, APP has to be cleaved by multiple transmembrane proteases. First, the β-secretase cleaves APP, generating a soluble sAPPβ and the β-C-terminal fragment (β-CTF). Subsequently, the γ-secretase complex cleaves the β-CTF to release the APP intracellular domain (AICD), and Aβ peptides of various lengths (Glenner

and Wong, 1984; Hussain *et al.*, 1999; Masters and Beyreuther, 1989; Masters *et al.*, 1985).

Not long after these central discoveries, several mutations in APP as well as presenilin, the catalytic subunit of the γ-secretase complex, have been identified and linked to early-onset AD (Sherrington *et al.*, 1995; St George-Hyslop *et al.*, 1987). This purely genetic and rare form has been termed familial AD (FAD) due to its Mendelian inheritance in contrast to the late onset, sporadic form of the disease (late-onset AD, LOAD) (Tanzi, 2012). Based on these critical findings the amyloid cascade hypothesis, depicting Aβ as the main culprit in AD, became the most wildly accepted hypothesis aiming to explain the molecular changes underlying AD (Hardy and Higgins, 1992).

1.3 Struggles and pitfalls in current Alzheimer's disease research

Despite decades of biochemical research and clinical trials, there are only symptomatic treatment options approved today (Yiannopoulou and Papageorgiou, 2013). Based on the overwhelming data depicting Aβ as the principal culprit of the disease, the majority of clinical trials have focussed on reducing the amyloid burden. Here, strategies to reduce the levels of either soluble Aβ, amyloid plaques or soluble oligomeric Aβ included passive immunization approaches, therapeutic antibody-based strategies as well as inhibition or modulation of the Aβ-generating enzymes β- and γ-secretase secretase. While several studies showed efficacious removal of Aβ, this did not lead to significant improvements in clinical outcomes (Lobello et al., 2012). In light of this bleak perspective, this would mean that for the majority of patients, not carrying FAD mutations, targeting Aβ may not achieve clinical efficacy (Lane *et al.*, 2012). Further, we understand now that most of the underlying biochemical changes (Aβ and tau deposition, synapse loss, and brain atrophy) occur years before the first occurrence of clinical symptoms (Jack *et al.*, 2013).

1.4 Epidemiologic and genetic risk factors

It is imperative to expand our list of potential drug targets or treatment approaches to advance treatments for AD. The most logical steps in finding suitable targets are to test the most significant AD risk factors that have emerged from epidemiological studies and large-scale genomic screens on AD patients. Based on our current understanding of the disease, the prime candidate to demand our attention is the cholesterol metabolism. Multiple epidemiologic and genome-wide association studies (GWAS) established the cholesterol metabolism among the strongest genetic risk factors (Bertram and Tanzi, 2004; Cuyvers and Sleegers, 2016; Di Paolo and Kim, 2011; Stefani and Liguri, 2009. Additionally, several epidemiological studies on cholesterol-lowering drugs have suggested that controlling cholesterol levels may not only be beneficial for CVD but also reduce the risk of AD (Chu *et al.*, 2018). However, as of now, we do not entirely understand the molecular mechanisms determining how the lipid and cholesterol metabolism modifies the risk and the pathology of AD. More research is necessary to unravel this intricate relationship. Yet, in order to progress in this endeavour, we have to rethink our choices of model systems. Today, most AD research relies on mouse or rat models. However, both species show significant differences in their cholesterol metabolism when compared to humans. Most importantly, they lack CETP and as a result, show negligible levels of LDL-C and cannot mirror a human lipoprotein profile.

2. CETP is linked with Alzheimer's disease

In my thesis work, we focussed on generating a novel model incorporating human CETP in mouse models of AD. While there are more differences than just the lack of CETP between human and murine cholesterol handling, it is of particular interest because it had directly been previously linked with AD and represents a significant step towards humanizing the murine lipoprotein profile. In epidemiological studies, SNPs in CETP have been linked with a reduced risk of AD. Most interestingly, the lack of CETP activity showed greatest effects

in carriers of the ε4 allele of ApoE (Arias-Vasquez *et al.*, 2007; Barzilai *et al.*, 2006; Chen *et al.*, 2014; Sanders *et al.*, 2010; Yu *et al.*, 2012). Prompted by these promising epidemiological studies and seeing that minimal research on CETP in the brain had been done, we set out to focus on the effects of CETP on the pathology of AD.

2.1 CETP transgenic mice

We started our endeavor using a well-characterized transgenic mouse expressing human CETP with expression patterns very similar to humans (Jiang *et al.*, 1992). The B6.CBA-Tg(CETP)5203Tall/J mouse strain was initially developed in the lab of Dr. Allan Tall in 1992 to study CVD. Here, Jiang *et al.* generated the transgenic strain by microinjection of a mini-gene consisting of all native CETP introns and its natural flanking regions into the male pronucleus of a fertilized mouse egg (Jiang *et al.*, 1992). Similar to humans, CETP expression in this model can be stimulated via dietary cholesterol as the promoter contains a cholesterol responsive element (CRE). During all our trials we made use of this by inducing CETP expression through feeding of a diet enriched with 1% cholesterol, resulting in an 8-9-fold increase in liver-mRNA levels of CETP. In this context, it is important to note that a standard mouse diet is devoid of cholesterol (**Table 6**) and does not accurately reflect an average human nutritional profile. Using this dietary approach, we were able to humanize the murine lipoprotein profile, meaning increased LDL-C and decreased HDL-C.

While CETP transgenic mice alone cannot be considered a model for AD, we wanted to study whether it would affect known risk factors for AD. Both cardiovascular disease and AD show an upregulation of inflammatory cytokines as a response to the injury, and we were curious to study whether CETP would inflict a similar inflammatory response (Heneka *et al.*, 2015; Ruparelia *et al.*, 2017). We measured the plasma levels of the inflammatory cytokines TNFα and IL1β in the circulation, as well as the corresponding mRNA levels in both liver and brain. We focussed on liver-mRNA levels since the liver is the primary organ secreting CETP and a key player in cholesterol homeostasis. Our analysis showed that in animals with highest

CETP expression, i.e., CETP transgenic mice fed with our 1% cholesterol diet have elevated levels of circulating TNFα and IL1β, which was matched by increased liver expression. These results were reassuring but not unexpected as it is well documented that dietary cholesterol and elevated LDL-C induces inflammation (Tall and Yvan-Charvet, 2015; Wouters *et al.*, 2008). While most of the research studying the role of inflammation in Alzheimer's disease had been focussed on the brain, there are several reports that show that peripheral inflammation, especially in the liver is able to induce disturbances in the brain, likeliy through humoral and/or neural pathways (Garcia-Martinez and Cordoba, 2012). Further, Non-alcoholic fatty liver disease (NAFLD) has been shown to accelerate neuroinflammation and promote Alzheimer's disease in mouse models fed a high fat diet (Kim *et al.*, 2016).

2.2 Does CETP modify brain lipids?

Since our overall goal was to study the effect of CETP in the brain, we turned our focus to the central nervous system. Opposed to the increase in peripheral inflammation, we only detected a very modest increase in brain-mRNA levels of IL1β while other cytokines remained unchanged. Next, we were able to confirm that the brain expression of CETP in our mice was similar to what has been published for humans with the highest expression in astrocytes (Yamada *et al.*, 1995). While being a central player in CVD research, potential roles of CNS-derived CETP remain elusive. Taken the effects of CETP activity in the periphery, we wondered if CETP transgenic mice would show abnormalities in their brain-lipid levels. Imaging of brain lipids is challenging due to the unspecific nature of most dyes available and the lack of spatial information in classic mass-spectrometry approaches. This poised us to collaborate with the laboratory of Dr. Pierre Chaurand, an expert in imaging mass spectrometry (IMS) of lipids.

Using MALDI-IMS, we found that CETP transgenic mice have up to 30% increased brain cholesterol levels. The highest concentrations were found in the myelin-rich fiber tracts. Additionally, we found a similar trend towards increased levels of cholesterol on the hip-

pocampus of CETP transgenic mice, albeit this was not statistically significant. The nature of MALDI-IMS allows for the quantification of other lipid species. Due to its involvement in inflammatory processes, and as a precursor for eicosanoids and prostaglandins, we analyzed the levels of arachidonic acid (Samuelsson, 1991). However, we were not able to detect significant differences between genotypes or diets. Seeing that MALDI-IMS of cholesterol is still a very recent technique and is not broadly used, few studies have been able to study brain cholesterol levels to a similar degree as presented here.

Nonetheless, Xu *et al.* used mass spectrometry on plated fibroblasts to measure cholesterol levels from Smith-Lemli-Opitz syndrome (SLOS) patients that accumulate 7-dehydrocholesterol due to a mutation in the gene for the enzyme 7-dehydrocholesterol reductase. They successfully imaged cholesterol and its precursors but were limited to a cell monolayer in culture (Xu *et al.*, 2015). Several other studies have analyzed cholesterol level in mouse models for atherosclerosis. However, just like the study carried out by Hutchins *et al.* most often they resort to analyzing the composition of atherosclerotic plaques or other pathological features and not the whole brain (Hutchins *et al.*, 2011). Further analysis and especially advances in the quantification of MALDI-IMS datasets will be very valuable in expanding our knowledge on the effects of CETP on the brain lipid and cholesterol homeostasis. One way of investigating whether the effects observed could be facilitated by peripheral CETP would be to use mice that lack expression of CETP in the CNS. One possible way of accomplishing this would be using a viral expression system to allow CETP expression only in liver cells. Here, promoters such as the thyroxine binding globulin (TBG) promoter allow for specific expression in hepatic cells (Yan *et al.*, 2012).

2.3 How do these changes relate to CETP studies in humans?

While we were first to demonstrate that CETP transgenic mice show elevated brain cholesterol levels, several studies have hinted at similar changes in humans. Multiple publications studying carriers of CETP polymorphisms report changes in brain structure and lipids.

Several laboratories using Diffusion Tensor Imaging investigated the CETP I405V polymorphism (rs5882) that is associated with reduced CETP activity, and found significant abnormalities in grey matter microstructure (Blankenberg *et al.*, 2003; Boekholdt and Thompson, 2003; Oliveira and de Faria, 2011; Salminen *et al.*, 2015). These abnormalities in white matter microstructure can be interpreted as neurodegeneration and atrophy leading to reduced fiber-tract integrity (Warstadt *et al.*, 2014; Huppi and Dubois, 2006; Tamnes *et al.*, 2010). Similar changes have been reported for AD patients, and it has been hypothesized that white-matter microstructure may be a valuable marker for cognitive capacity (Teipel *et al.*, 2009). Further, several groups have employed DTI to study the brains of AD patients and found significant reductions in fractional anisotropy in brains of patients with AD when compared to healthy controls (Bozzali and Cherubini, 2007; Liu *et al.*, 2011). Increased fractional anisotropy in DTI is considered a marker for atrophy in fiber tracts (Whitwell *et al.*, 2010).

3. Where does extra brain cholesterol come from?

Despite the evidence that CETP has a role in the CNS, the biochemical effects of CETP activity in the brain remain elusive. Considering that the majority of CNS cholesterol is derived from *de novo* synthesis, we wanted to test whether this could account for the increased cholesterol levels in the brain of CETP transgenic mice. Here, we purified astrocytes from CETP transgenic mouse brains fed with a diet enriched with 1% cholesterol, extracted mRNA, transcribed it into a cDNA library and performed a *RNA microarray*. Interestingly, our analysis revealed that increased *de novo* synthesis is not responsible for the increase in cholesterol.

Our next hypothesis was that CETP could induce increased cholesterol transport into the brain. Yet, an intact BBB does not allow for direct cholesterol transport into the brain. The brain cholesterol metabolism is separated from the peripheral cholesterol homeostasis (Zhang and Liu, 2015). However, BBB integrity can be impaired in several pathological conditions. CETP has been associated with atherosclerosis which is known to damage the BBB and hence could allow for cholesterol influx into the brain (Inazu *et al.*, 1990). Lastly, it has been

reported such damage could lead to similar white matter lesion as discussed above (Claus *et al.*, 1996; de Leeuw *et al.*, 2000). To test this possibility, we tested the BBB integrity using Evans blue injections but were not able to show any signs of permeability.

Nonetheless, further possible scenarios could explain the CNS-cholesterol increase. While cholesterol is not able to cross the BBB, some hydroxycholesterol species such as 24-hydroxycholesterol can (Russell *et al.*, 2009). Further, smaller lipoprotein particles can enter the brain via SR-B1-mediated transcytosis (Balazs *et al.*, 2004; Fung *et al.*, 2017; Goti *et al.*, 2001).

Unfortunately, as of now, we were not able to measure 24-hydroxycholesterol or other hydroxycholesterols, but further MALDI-IMS analyses could shed light on the distribution and concentrations of various hydroxycholesterols.

Another possible source for the increase in cholesterol could be the release via de-esterification of cholesteryl esters. CETP has been linked with lipids droplet formation, and CETP activity could affect retention of cholesterol or cholesteryl esters in lipid droplets and their release in the form of cholesterol.

3.1 What are the downstream effects of the cholesterol increase?

While our microarray analysis was not able to determine the source of the cholesterol increase in the brain, it was able to reveal some of its downstream effects. Looking at changes in gene expression, we found that both PSEN1 and PSEN2 among the top upstream targets. PSEN1 and PSEN2 are the genes coding for presenilins, the catalytic subunits of the γ-secretase complex (De Strooper *et al.*, 1998). We further studied this effect *in vitro* where we were able to reproduce the effects of CETP on γ-secretase and showed that CETP activity could activate γ-secretase. Further cementing a role for CETP in γ-secretase processing, CETP activity leads to increased generation of Aβ in cell culture. Using *in vitro* cell culture allowed us to explore the mechanism behind this effect. We found that the increase in Aβ relies on the presence of lipoproteins in the cell culture supernatant and the cholesteryl-ester transfer activity of CETP. Removing lipoproteins from the cell-culture medium as well as transfection of

CE-transfer deficient CETP mutant did not increase Aβ generation. Based on our results, we propose that CETP increases the cholesterol content of LDL and potentially brain-lipoprotein particles that are subsequently taken via LDL-receptors. This increase in cellular cholesterol is affecting the lipid environment of γ-secretase, leading to increased levels of Aβ.

4. CETP in Alzheimer's disease mouse models

So far, we had been able to demonstrate that CETP increases the brain cholesterol levels *in vivo* and affects γ-secretase, as evident by increased Aβ generation *in vitro*. To study the effects of CETP on Aβ generation *in vivo*, we generated two novel mouse models by crossing CETP transgenic mice with the 5xFAD and Thy1 APP McGill models for AD. Both models are mouse models for amyloidosis and rely on the overexpression of human APP, carrying several FAD mutations. Surprisingly, while we were able to measure a CETP-dependent Aβ increase Thy1 APP McGill mice, this was not the case in our 5xFAD model.

4.1 What are the differences between our mouse models?

This glaring inconsistency is puzzling, but there are several potential reasons for the discrepancy between the different models. While both are a model for amyloidosis, the severity and rate of Aβ generation are different between the models. As the name suggests, the 5xFAD model carries five familiar AD mutations (APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L, PSEN1 L286V) and shows one of the fastest onsets of amyloid pathology when compared to other mouse models for AD (Oakley *et al.*, 2006). The Thy1 APP McGill model, on the other hand, shows a much slower progression of amyloid deposition (Ferretti *et al.*, 2011). At the age of 6 months, the 5xFAD/CETP mice have 4-fold higher levels of soluble and 20-fold higher levels of insoluble Aβ42 compared to Thy1 APP McGill/CETP mice. The fast amyloid generation could precede effects of CETP on the generation of Aβ.

Further, we hypothesize that the effect of CETP on Aβ is mediated through CETP's ability to affect the lipid environment of γ-secretase. The 5xFAD mouse model carries the PSEN1 mutations M146L and PSEN1 L286V. While most PS FAD mutations have been described as a lack of function, the M146L and PSEN1 L286V mutations lead to an increase in the Aβ42/40 ratio but also the overall amounts of Aβ42 generated (Sherrington *et al.*, 1995; Sun *et al.*, 2017; Weggen and Beher, 2012). It could very well be that this gain of function regarding the ability to generate Aβ42 could render the enzyme insensitive to its lipid environment.

Despite the difficulties and limitations of models aiming at incorporating cardiovascular risk factors in AD models, our results demonstrate that it could be a valuable tool to study the role of cholesterol in AD.

Only very few groups have employed similar approaches. One example would be a study on a model transgenic for human ApoB-100. Bjelik at al. used this transgenic mouse in combination with a diet enriched with cholesterol. Similar to our approach, using a pro-atherogenic factor they found effects on the APP metabolism in the brain (Pitas *et al.*, 1987b). While promising, it is important to note that ApoB-100 not expressed in the brain and unlikely to cross the BBB. Further, their analysis was limited and did not include a measurement of Aβ in the brain (Bjelik *et al.*, 2006). However, it does underline that pro-atherogenic factors even when expressed in the periphery play an essential role in the APP metabolism in the CNS. Multiple researchers have acknowledged that most current models are very artificial and may not represent sporadic AD (Keene *et al.*, 2016; Onos *et al.*, 2016). Some even blame the lack of appropriate models of sporadic, late-onset AD for some of the failures in recent clinical trials (Cummings, 2018; Drummond and Wisniewski, 2017). Given the knowledge about AD risk factors, it is surprising that most current animal models still heavily rely on the overexpression of FAD mutations at the expense of other risk factors.

5. Targeting cholesterol to treat Alzheimer's disease?

While the final verdict is still out, several epidemiological studies on cholesterol-lowering

drugs and their effects on Alzheimer's disease provide hope that they may be able to slow the onset of AD.

Several recent studies showed that statin use was associated with a reduced risk of dementia, especially in ApoE ε4 carriers (Chatterjee *et al.*, 2015; Chu *et al.*, 2018; Geifman *et al.*, 2017; Li *et al.*, 2010; Rockwood *et al.*, 2002; Shah *et al.*, 2015; Wong *et al.*, 2013). Additionally, Zimetti *et al.* recently reported a pathophysiological link between PCSK9 and AD. Here, they found higher CSF PCSK9 levels in AD patients compared to controls (Zimetti *et al.*, 2017).

We believe the studies as mentioned earlier together with our data present a strong case for the use of cholesterol-lowering drugs in the prevention of Alzheimer's disease. However, cholesterol-lowering drugs have been reported to slow inflammatory processes promoting to Alzheimer's disease or by affecting the levels of Aβ either through reducing the generation or by promoting its clearance. Both, the inflammatory component as well as Aβ pathology have been shown to occur much earlier than the onset of clinical symptoms (Jack *et al.*, 2013).

Hence, it is likely that cholesterol-lowering drugs such as statins, PCSK9 inhibitors or CETP inhibitors would have to be administered 15-20 years before the onset of clinical symptoms (Li *et al.*, 2010). Unfortunately, this very long treatment timeline, the lack of early diagnostic and the cost associated with long-term clinical trials make it incredibly challenging to prove the validity of cholesterol-lowering drugs as a treatment for AD.

6. The need to include a cholesterol phenotype in AD models

Altogether, there is a significant need for innovation in developing novel models that can model late-onset AD. There is a concerted effort in generating models that include multiple risk factors and pathological features. A good example would be mouse models combining both amyloid and tau pathology such as the ADLPAPT mouse model (Kim *et al.*, 2018). Additionally, the laboratory of Dr. De Strooper has recently developed a novel mouse model that is built around humanizing an AD mouse model by injecting human PSCs into mouse brains. These mice show additional pathological hallmarks such as neurite dystrophy, neu-

rodegeneration, and tau that are not present to the same extend previous models (Espuny-Camacho *et al.*, 2017).

One of the most interesting new models is the hAPP/APOE4/Trem2*R47H mouse as developed by Mike Sasner at the Jackson Laboratory for the IU/JAX MODEL-AD consortium. This mouse model has humanized APP and ApoE ε4, and carries the TREM2 R47H mutation. The ε4 allele and TREM2 R47H polymorphism are among the strongest risk factors for late-onset AD and make this a very promising model. Not only is this model lacking APP and presenilin FAD, it also incorporates risk factors involved in the cholesterol homeostasis. This model is currently in a phenotyping pipeline that includes a histological analysis including white matter, NeuN, tau staining as well as imaging of neuroinflammation and vascular health.

Further, *in vivo* imaging studies including amyloid, tau, blood flow, and glucose imaging will be performed. Lastly, biochemical analyses include Aβ and tau measurements as well as the quantification of soluble TREM2. While human ApoE ε4 on its own will not lead to a humanized lipoprotein metabolism, this latest approach takes a step forward towards generating a mouse modeling multiple pathologies of human AD pathology closely.

Apart from various efforts in generating better mouse models, recent advances in the generations of so called mini brains promise to provide insight into the pathology of sporadic AD. For example, Lin *et al.* generated human iPSC-derived organoids from APOE4 iPSCs. Comparing APOE4 with APOE3 organoids, they were able to demonstrate that Aβ clearance, as well as aggregation, depends on the ApoE isoform (Lin *et al.*, 2018).

7. What comes next?

We believe that our Thy1 APP McGill/CETP model is a very valuable tool to expand our knowledge in the interrelationship between CVD and AD. One big question we were not yet able to definitively answer is whether CETP in the CNS or the periphery is responsible for the Aβ increase. As previously discussed, animals transgenic for human ApoB-100 show effects on brain Aβ levels, while ApoB-100 is most likely restricted to the periphery and not

able to cross the BBB. Yet, it shows effects on brain APP levels. Hence, it is not impossible that the effects we observed are due to the action of CETP in the periphery. However, in contrast to ApoB-100, CETP is expressed in the brain as we and others have shown. However, restricting CETP expression to either the liver or CNS should give us answers to that question. Furthermore, using a different model for hypercholesteremia, such as LDLR knock out mice of ApoB-100 overexpressing mice could shed light whether peripheral hypercholesteremia of neural CETP expression is responsible for the effects observed in our animal models. Additionally, Fast protein liquid chromatography (FPLC) of brain lipoproteins of CETP and wild type animals could show if CETP affects the generation of composition of brain lipoprotein particles. To further understand the precise role of CETP in the brain, LCAT inhibitors could be used to prevent the generation of cholesteryl esters, the substrate of CETP and should yield similar effects as CETP inhibition, which would help understand whether the cholesteryl ester transfer activity of CETP or a different, yet unknown, role of CETP plays a part in the effects of CETP in the brain.

Based on our microarray data, the increased brain cholesterol levels are not due to increased *de novo* cholesterol synthesis in astrocytes. However, we cannot exclude that other cell types such as neurons of glia cells. One way of further studying this would be the use of radioactively labelled precursors such as acetyl-CoA to measure the incorporation rate in cholesterol. Further, the increased levels of cholesterol could be due to reduced transport of 24-hydroxyholesterol out of the brain or 27-hydroxycholesterol into the brain. MALDI-IMS studies, analysing those hydroxycholesterols in periphery and the brain could shed light on whether this could be a potential mechanism for the cholesterol increase in the brain.

Based on our *in vitro* results, pharmacologically inhibiting CETP abolished the detrimental effects of CETP on Aβ. It is tempting to speculate that one of the recently developed CETP inhibitors may show beneficial effects on the progression of AD *in vivo*. Most of the CETP inhibitors have been discontinued for the treatment of CVD mostly due to their lack of efficacy (Barter *et al.*, 2007; Doggrell, 2017; Schwartz *et al.*, 2012; Tall and Rader, 2018). However,

the latest generation of CETP inhibitors are very well tolerated and could be useful in a combination therapy targeting AD (Duivenvoorden and Fayad, 2012; Hendrix *et al.*, 2016). Nonetheless, the brain penetration of current CETP inhibitors is poorly tested and based on their chemical properties (molecular weight>500 g/mole, logP>5) their BBB penetration may be limited. However, Hartmann *et al.* were able to detect anacetrapib in the brain, albeit at low levels (Hartmann *et al.*, 2016). Nevertheless, current research depicts a vital role for CETP in the brain, likely in conjunction with ApoE and we believe that is indispensable to explore potential benefits of CETP inhibition on AD.

CONCLUSION

For my Ph.D. thesis, I aimed at generating and analyzing a novel mouse model for AD that includes one of the strongest risk factors for the disease that had thus far mostly been ignored. Including a humanized lipoprotein profile was able to replicate multiple pathologies that most current AD-mouse models lack. Unlike most other models, we observed an increase in the brain cholesterol levels. Additionally, we were able to show that CETP leads to increased Aβ generation *in vitro* as well as *in vivo*, at least in our novel model without presenilin FAD mutations.

Further, our data indicate that the effect of CETP on Aβ relies on changes in brain cholesterol and subsequently γ-secretase, in line with various studies. This signifies a significant step forward to better models to study AD. Nonetheless, it is indispensable to improve current models further. Finally, this study is only the beginning of our studies on AD model with a humanized lipoprotein metabolism.

ABBREVIATIONS

ABCA7	ATP Binding Cassette Subfamily A Member 7
Aβ	Amyloid beta
ACAT	Acyl-CoA cholesterol acyl transferase
AD	Alzheimer's disease
ADNI	Alzheimer's Disease Neuroimaging Initiative
Aph1	Anterior pharynx-defective 1
ApoE	Apolipoprotein A
ApoER2	ApoE receptor 2
ApoJ	Apolipoprotein J (also known as clusterin)
APP	Amyloid precursor protein
ARRIVE	Aspirin to Reduce Risk of Initial Vascular Events
β-CTF	β-C-terminal fragment
BBB	Blood-brain barrier
cDNA	Complementary deoxyribonucleic acid
CE	Cholesteryl ester
CETP	Cholesteryl-ester transfer protein
CoA	Coenzyme A
CNS	Central-nervous system
CRE	Cholesterol-response element
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
CVD	Cardiovascular disease
DEA	Diethanolamine

DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTI	Diffusion Tensor Imaging
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EHS	Environmental health and safety regulations
ER	Endoplasmic reticulum
FAD	Familiar Alzheimer's disease
FCS	Fetal calf serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome-wide association studies
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDL	High-density lipoprotein
HMG-CoA	3-Hydroxy-3-Methylglutaryl-CoA Reductase
HPS	Heart Protection Study
HRP	Horseradish peroxidase
IDL	Intermediate-density lipoprotein
IMS	Imaging-mass spectrometry
INSIG1	Insulin Induced Gene 1
LBP	Lipopolysaccharide binding protein
LCAT	Lecithin: cholesterol acyltransferase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LOAD	Late-onset Alzheimer's disease
Lp(a)	Lipoprotein (a)
LPDS	Lipoprotein-deprived serum

LRP1	Low-density lipoprotein receptor related protein 1
NMDA	N-methyl-D-aspartate
NOR	Novel-object recognition
NCT	Nicastrin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEN-2	Presenilin enhancer 2
PICALM	Phosphatidylinositol Binding Clathrin Assembly Protein
PLTP	Phospholipid-transfer protein
PSC	Pluripotent stem cell
PS1	Presenilin 1
PS2	Presenilin 2
REVEAL	Randomized Evaluation of the Effects of anacetrapib through Lipid modification
RNA	Ribonucleic acid
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
SCAP	SREBP cleavage-activating protein
SDS	Sodium-dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
S1P	Site-1 protease
SLOS	Smith–Lemli–Opitz syndrome
SNP	Single-nucleotide polymorphism
SRE	Sterol-regulatory element
SREBP	Sterol regulatory element-binding proteins
TG	Triglyceride
TNE	Tris-SDS-EDTA
TREM2	Triggering receptor expressed on myeloid cells 2

VLDL	Very-low density lipoprotein
VLDLR	Very-low density lipoprotein receptor

REFERENCES

Abifadel, M., Varret, M., Rabes, J.P., Allard, D., Ouguerram, K., Devillers, M., Cruaud, C., Benjannet, S., Wickham, L., Erlich, D., *et al.* (2003). Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 34, 154-156.

Agellon, L.B., Walsh, A., Hayek, T., Moulin, P., Jiang, X.C., Shelanski, S.A., Breslow, J.L., and Tall, A.R. (1991). Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. The Journal of biological chemistry 266, 10796-10801.

Albers, J.J., Tollefson, J.H., Wolfbauer, G., and Albright, R.E., Jr. (1992). Cholesteryl ester transfer protein in human brain. Int J Clin Lab Res 21, 264-266.

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., *et al.* (1980). Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proceedings of the National Academy of Sciences of the United States of America 77, 3957-3961.

Alzheimer, A., Stelzmann, R.A., Schnitzlein, H.N., and Murtagh, F.R. (1995). An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". Clin Anat 8, 429-431.

Andersen, O.M., Reiche, J., Schmidt, V., Gotthardt, M., Spoelgen, R., Behlke, J., von Arnim, C.A., Breiderhoff, T., Jansen, P., Wu, X., *et al.* (2005). Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. Proceedings of the National Academy of Sciences of the United States of America 102, 13461-13466.

Andorfer, C., Kress, Y., Espinoza, M., de Silva, R., Tucker, K.L., Barde, Y.A., Duff, K., and Davies, P. (2003). Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. Journal of neurochemistry 86, 582-590.

Anstey, K.J., Ashby-Mitchell, K., and Peters, R. (2017). Updating the Evidence on the Association between Serum Cholesterol and Risk of Late-Life Dementia: Review and Meta-Analysis. J Alzheimers Dis 56, 215-228.

Area-Gomez, E., Del Carmen Lara Castillo, M., Tambini, M.D., Guardia-Laguarta, C., de Groof, A.J., Madra, M., Ikenouchi, J., Umeda, M., Bird, T.D., Sturley, S.L., *et al.* (2012). Upregulated function of mitochondria-associated ER membranes in Alzheimer disease. EMBO J 31, 4106-4123.

Arendash, G.W., and King, D.L. (2002). Intra- and intertask relationships in a behavioral test battery given to Tg2576 transgenic mice and controls. Physiol Behav 75, 643-652.

Arguello, G., Balboa, E., Arrese, M., and Zanlungo, S. (2015). Recent insights on the role of cholesterol in non-alcoholic fatty liver disease. Biochimica et biophysica acta 1852, 1765-1778.

Arias-Vasquez, A., Isaacs, A., Aulchenko, Y.S., Hofman, A., Oostra, B.A., Breteler, M., and van Duijn, C.M. (2007). The cholesteryl ester transfer protein (CETP) gene and the risk of Alzheimer's disease. Neurogenetics 8, 189-193.

Ayciriex, S., Gerber, H., Osuna, G.M., Chami, M., Stahlberg, H., Shevchenko, A., and Fraering, P.C. (2016). The lipidome associated with the gamma-secretase complex is required for its integrity and activity. Biochem J 473, 321-334.

Babin, P.J., and Gibbons, G.F. (2009). The evolution of plasma cholesterol: direct utility or a "spandrel" of hepatic lipid metabolism? Prog Lipid Res 48, 73-91.

Bai, G., Chivatakarn, O., Bonanomi, D., Lettieri, K., Franco, L., Xia, C., Stein, E., Ma, L., Lewcock, J.W., and Pfaff, S.L. (2011). Presenilin-dependent receptor processing is required for axon guidance. Cell 144, 106-118.

Balaganapathy, P., Baik, S.H., Mallilankaraman, K., Sobey, C.G., Jo, D.G., and Arumugam, T.V. (2017). Interplay between Notch and p53 promotes neuronal cell death in ischemic stroke. J Cereb Blood Flow Metab, 271678X17715956.

Balazs, Z., Panzenboeck, U., Hammer, A., Sovic, A., Quehenberger, O., Malle, E., and Sattler, W. (2004). Uptake and transport of high-density lipoprotein (HDL) and HDL-associated alpha-tocopherol by an *in vitro* blood-brain barrier model. Journal of neurochemistry 89, 939-950.

Bancher, C., Brunner, C., Lassmann, H., Budka, H., Jellinger, K., Wiche, G., Seitelberger, F., Grundke-Iqbal, I., Iqbal, K., and Wisniewski, H.M. (1989). Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. Brain Res 477, 90-99.

Barbash, S., Garfinkel, B.P., Maoz, R., Simchovitz, A., Nadorp, B., Guffanti, A., Bennett, E.R., Nadeau, C., Turk, A., Paul, L., *et al.* (2017). Alzheimer's brains show inter-related changes in RNA and lipid metabolism. Neurobiol Dis 106, 1-13.

Barter, P.J., Caulfield, M., Eriksson, M., Grundy, S.M., Kastelein, J.J., Komajda, M., Lopez-Sendon, J., Mosca, L., Tardif, J.C., Waters, D.D., *et al.* (2007). Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med 357, 2109-2122.

Barzilai, N., Atzmon, G., Derby, C.A., Bauman, J.M., and Lipton, R.B. (2006). A genotype of exceptional longevity is associated with preservation of cognitive function. Neurology 67, 2170-2175.

Barzilai, N., Atzmon, G., Schechter, C., Schaefer, E.J., Cupples, A.L., Lipton, R., Cheng, S., and Shuldiner, A.R. (2003). Unique lipoprotein phenotype and genotype associated with exceptional longevity. JAMA : the journal of the American Medical Association 290, 2030-2040.

Baxter, M.G. (2010). "I've seen it all before": explaining age-related impairments in object recognition. Theoretical comment on Burke *et al.* (2010). Behav Neurosci 124, 706-709.

Becker, J.T., and Overman, A.A. (2002). [The semantic memory deficit in Alzheimer's disease]. Revista de neurologia 35, 777-783.

Beffert, U., Danik, M., Krzywkowski, P., Ramassamy, C., Berrada, F., and Poirier, J. (1998). The neurobiology of apolipoproteins and their receptors in the CNS and Alzheimer's disease. Brain research Brain research reviews 27, 119-142.

Bemis, K.D., Harry, A., Eberlin, L.S., Ferreira, C., van de Ven, S.M., Mallick, P., Stolowitz, M., and Vitek, O. (2015). Cardinal: an R package for statistical analysis of mass spectrometry-based imaging experiments. Bioinformatics 31, 2418-2420.

Benjannet, S., Rhainds, D., Essalmani, R., Mayne, J., Wickham, L., Jin, W., Asselin, M.C., Hamelin, J., Varret, M., Allard, D., *et al.* (2004). NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. The Journal of biological chemistry 279, 48865-48875.

Berger, S., Raman, G., Vishwanathan, R., Jacques, P.F., and Johnson, E.J. (2015). Dietary cholesterol and cardiovascular disease: a systematic review and meta-analysis. Am J Clin Nutr 102, 276-294.

Bertram, L., and Tanzi, R.E. (2004). Alzheimer's disease: one disorder, too many genes? Human molecular genetics 13 Spec No 1, R135-141.

Billings, L.M., Oddo, S., Green, K.N., McGaugh, J.L., and LaFerla, F.M. (2005). Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. Neuron 45, 675-688.

Bjorkhem, I. (2006). Crossing the barrier: oxysterols as cholesterol transporters and metabolic modulators in the brain. J Intern Med 260, 493-508.

Bjorkhem, I., Lutjohann, D., Diczfalusy, U., Stahle, L., Ahlborg, G., and Wahren, J. (1998). Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation. Journal of lipid research 39, 1594-1600.

Bjorkhem, I., and Meaney, S. (2004). Brain cholesterol: long secret life behind a barrier. Arterioscler Thromb Vasc Biol 24, 806-815.

Blankenberg, S., Rupprecht, H.J., Bickel, C., Jiang, X.C., Poirier, O., Lackner, K.J., Meyer, J., Cambien, F., Tiret, L., and AtheroGene, I. (2003). Common genetic variation of the cholesteryl ester transfer protein gene strongly predicts future cardiovascular death in patients with coronary artery disease. J Am Coll Cardiol 41, 1983-1989.

Boyd, T.D., Bennett, S.P., Mori, T., Governatori, N., Runfeldt, M., Norden, M., Padmanabhan, J., Neame, P., Wefes, I., Sanchez-Ramos, J., *et al.* (2010). GM-CSF upregulated in rheumatoid arthritis reverses cognitive impairment and amyloidosis in Alzheimer mice. J Alzheimers Dis 21, 507-518.

Brousseau, M.E., Schaefer, E.J., Wolfe, M.L., Bloedon, L.T., Digenio, A.G., Clark, R.W., Mancuso, J.P., and Rader, D.J. (2004). Effects of an inhibitor of cholesteryl ester transfer protein on HDL cholesterol. N Engl J Med 350, 1505-1515.

Brown, M.S., and Goldstein, J.L. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89, 331-340.

Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell 100, 391-398.

Bursavich, M.G., Harrison, B.A., and Blain, J.F. (2016). Gamma Secretase Modulators: New Alzheimer's Drugs on the Horizon? J Med Chem 59, 7389-7409.

Bustami, J., Sukiasyan, A., Kupcinskas, J., Skieceviciene, J., Iakoubov, L., Szwed, M., Kleinle, C., Schumann, R.R., Puzianowska-Kuznicka, M., and Hamann, L. (2016). Cholesteryl ester transfer protein (CETP) I405V polymorphism and cardiovascular disease in eastern European Caucasians - a cross-sectional study. BMC Geriatr 16, 144.

Camejo, G., Waich, S., Quintero, G., Berrizbeitia, M.L., and Lalaguna, F. (1976). The affinity of low density lipoproteins for an arterial macromolecular complex. A study in ischemic heart disease and controls. Atherosclerosis 24, 341-354.

Canada, A.S. (2014 - 2015). Alzheimer Society Canada annual report

Canevelli, M., Bruno, G., and Cesari, M. (2017). The sterile controversy on the amyloid cascade hypothesis. Neurosci Biobehav Rev 83, 472-473.

Cao, G., Beyer, T.P., Zhang, Y., Schmidt, R.J., Chen, Y.Q., Cockerham, S.L., Zimmerman, K.M., Karathanasis, S.K., Cannady, E.A., Fields, T., *et al.* (2011). Evacetrapib is a novel, potent, and selective inhibitor of cholesteryl ester transfer protein that elevates HDL cholesterol without inducing aldosterone or increasing blood pressure. Journal of lipid research 52, 2169-2176.

Cappel, D.A., Palmisano, B.T., Emfinger, C.H., Martinez, M.N., McGuinness, O.P., and Stafford, J.M. (2013). Cholesteryl ester transfer protein protects against insulin resistance in obese female mice. Mol Metab 2, 457-467.

Castello, M.A., Jeppson, J.D., and Soriano, S. (2014). Moving beyond anti-amyloid therapy for the prevention and treatment of Alzheimer's disease. BMC Neurol 14, 169.

Caughlin, S., Park, D.H., Yeung, K.K., Cechetto, D.F., and Whitehead, S.N. (2017). Sublimation of DAN Matrix for the Detection and Visualization of Gangliosides in Rat Brain Tissue for MALDI Imaging Mass Spectrometry. J Vis Exp.

Cazita, P.M., Berti, J.A., Aoki, C., Gidlund, M., Harada, L.M., Nunes, V.S., Quintao, E.C., and Oliveira, H.C. (2003). Cholesteryl ester transfer protein expression attenuates atherosclerosis in ovariectomized mice. Journal of lipid research 44, 33-40.

Chan, R.B., Oliveira, T.G., Cortes, E.P., Honig, L.S., Duff, K.E., Small, S.A., Wenk, M.R., Shui, G., and Di Paolo, G. (2012). Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. The Journal of biological chemistry 287, 2678-2688.

Chatterjee, S., Krishnamoorthy, P., Ranjan, P., Roy, A., Chakraborty, A., Sabharwal, M.S., Ro, R., Agarwal, V., Sardar, P., Danik, J., *et al.* (2015). Statins and cognitive function: an updated review. Curr Cardiol Rep 17, 4.

Chaudhary, R., Garg, J., Shah, N., and Sumner, A. (2017). PCSK9 inhibitors: A new era of lipid lowering therapy. World J Cardiol 9, 76-91.

Cheema, S.K., and Rashid-Kolvear, F. (2003). Streptozotocin-induced increase in cholesterol ester transfer protein (CETP) and its reversal by insulin in transgenic mice expressing human CETP. Can J Physiol Pharmacol 81, 997-1004.

Chen, J.J., Li, Y.M., Zou, W.Y., and Fu, J.L. (2014). Relationships between CETP genetic polymorphisms and Alzheimer's disease risk: a meta-analysis. DNA Cell Biol 33, 807-815.

Chen, Y.R., and Glabe, C.G. (2006). Distinct early folding and aggregation properties of Alzheimer amyloid-beta peptides Abeta40 and Abeta42: stable trimer or tetramer formation by Abeta42. The Journal of biological chemistry 281, 24414-24422.

Chevreul, M.E. (1816). Recherches chimiques sur les corps gras, et particulièrement sur leurs combinaisons avec les alcalis. Sixième mémoire. Examen des graisses d'homme, de mouton, de boeuf, de jaguar et d'oie. In Annales de Chimie et de Physique, pp. 339–372.

Chishti, M.A., Yang, D.S., Janus, C., Phinney, A.L., Horne, P., Pearson, J., Strome, R., Zuker, N., Loukides, J., French, J., *et al.* (2001). Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. The Journal of biological chemistry 276, 21562-21570.

Chu, C.S., Tseng, P.T., Stubbs, B., Chen, T.Y., Tang, C.H., Li, D.J., Yang, W.C., Chen, Y.W., Wu, C.K., Veronese, N., *et al.* (2018). Use of statins and the risk of dementia and mild cognitive impairment: A systematic review and meta-analysis. Sci Rep 8, 5804.

Clark, R.W., Sutfin, T.A., Ruggeri, R.B., Willauer, A.T., Sugarman, E.D., Magnus-Aryitey, G., Cosgrove, P.G., Sand, T.M., Wester, R.T., Williams, J.A., *et al.* (2004). Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: an initial multidose study of torcetrapib. Arterioscler Thromb Vasc Biol 24, 490-497.

Cohen, J., Pertsemlidis, A., Kotowski, I.K., Graham, R., Garcia, C.K., and Hobbs, H.H. (2005). Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. Nat Genet 37, 161-165.

Cohen, J.C., Boerwinkle, E., Mosley, T.H., Jr., and Hobbs, H.H. (2006). Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 354, 1264-1272.

Corbex, M., Poirier, O., Fumeron, F., Betoulle, D., Evans, A., Ruidavets, J.B., Arveiler, D., Luc, G., Tiret, L., and Cambien, F. (2000). Extensive association analysis between the CETP gene and coronary heart disease phenotypes reveals several putative functional polymorphisms and gene-environment interaction. Genet Epidemiol 19, 64-80.

Cummings, B.J., Pike, C.J., Shankle, R., and Cotman, C.W. (1996). Beta-amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer's disease. Neurobiology of aging 17, 921-933.

Cummings, J., Lee, G., Ritter, A., and Zhong, K. (2018). Alzheimer's disease drug development pipeline: 2018. Alzheimers Dement (N Y) 4, 195-214.

Dam, H. (1958). Historical introduction to cholesterol. In Chemistry, Biochemistry and Pathology (New York, NY.: Academic Press), pp. 1-14.

Daws, M.R., Lanier, L.L., Seaman, W.E., and Ryan, J.C. (2001). Cloning and characterization of a novel mouse myeloid DAP12-associated receptor family. Eur J Immunol 31, 783-791.

Dawson, G.R., Seabrook, G.R., Zheng, H., Smith, D.W., Graham, S., O'Dowd, G., Bowery, B.J., Boyce, S., Trumbauer, M.E., Chen, H.Y., et al. (1999). Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. Neuroscience 90, 1-13.

Dumanis, S.B., Chamberlain, K.A., Jin Sohn, Y., Jin Lee, Y., Guenette, S.Y., Suzuki, T., Mathews, P.M., Pak, D., Rebeck, G.W., Suh, Y.H., et al. (2012). FE65 as a link between VLDLR and APP to regulate their trafficking and processing. Mol Neurodegener 7, 9.

de Grooth, G.J., Klerkx, A.H., Stroes, E.S., Stalenhoef, A.F., Kastelein, J.J., and Kuivenhoven, J.A. (2004). A review of CETP and its relation to atherosclerosis. Journal of lipid research 45, 1967-1974.

de Grooth, G.J., Kuivenhoven, J.A., Stalenhoef, A.F., de Graaf, J., Zwinderman, A.H., Posma, J.L., van Tol, A., and Kastelein, J.J. (2002). Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose-response study. Circulation 105, 2159-2165.

Deane, R., Wu, Z., Sagare, A., Davis, J., Du Yan, S., Hamm, K., Xu, F., Parisi, M., LaRue, B., Hu, H.W., *et al.* (2004). LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. Neuron 43, 333-344.

Demeester, N., Castro, G., Desrumaux, C., De Geitere, C., Fruchart, J.C., Santens, P., Mulleners, E., Engelborghs, S., De Deyn, P.P., Vandekerckhove, J., *et al.* (2000). Characterization and functional studies of lipoproteins, lipid transfer proteins, and lecithin:cholesterol acyltransferase in CSF of normal individuals and patients with Alzheimer's disease. Journal of lipid research 41, 963-974.

Deschenes, C.L., and McCurry, S.M. (2009). Current treatments for sleep disturbances in individuals with dementia. Curr Psychiatry Rep 11, 20-26.

Di Paolo, G., and Kim, T.W. (2011). Linking lipids to Alzheimer's disease: cholesterol and beyond. Nat Rev Neurosci 12, 284-296.

Dickson, D.W., Farlo, J., Davies, P., Crystal, H., Fuld, P., and Yen, S.H. (1988). Alzheimer's disease. A double-labeling immunohistochemical study of senile plaques. Am J Pathol 132, 86-101.

Dietschy, J.M. (2009). Central nervous system: cholesterol turnover, brain development and neurodegeneration. Biol Chem 390, 287-293.

Dietschy, J.M., and Turley, S.D. (2001). Cholesterol metabolism in the brain. Curr Opin Lipidol 12, 105-112.

Dietschy, J.M., and Turley, S.D. (2004). Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. Journal of lipid research 45, 1375-1397.

Doggrell, S.A. (2017). No cardiovascular benefit with evacetrapib - is this the end of the road for the 'cetrapibs'? Expert Opin Pharmacother 18, 1439-1442.

Dufresne, M., Thomas, A., Breault-Turcot, J., Masson, J.F., and Chaurand, P. (2013). Silver-assisted laser desorption ionization for high spatial resolution imaging mass spectrometry of olefins from thin tissue sections. Anal Chem 85, 3318-3324.

Ebke, A., Luebbers, T., Fukumori, A., Shirotani, K., Haass, C., Baumann, K., and Steiner, H. (2011). Novel gamma-secretase enzyme modulators directly target presenilin protein. The Journal of biological chemistry 286, 37181-37186.

Echeverria, V., Clerman, A., and Dore, S. (2005). Stimulation of PGE receptors EP2 and EP4 protects cultured neurons against oxidative stress and cell death following beta-amyloid exposure. Eur J Neurosci 22, 2199-2206.

Eckman, C.B., Mehta, N.D., Crook, R., Perez-tur, J., Prihar, G., Pfeiffer, E., Graff-Radford, N., Hinder, P., Yager, D., Zenk, B., *et al.* (1997). A new pathogenic mutation in the APP gene (I716V) increases the relative proportion of A beta 42(43). Human molecular genetics 6, 2087-2089.

Edbauer, D., Winkler, E., Regula, J.T., Pesold, B., Steiner, H., and Haass, C. (2003). Reconstitution of gamma-secretase activity. Nat Cell Biol 5, 486-488.

Engelking, L.J., Kuriyama, H., Hammer, R.E., Horton, J.D., Brown, M.S., Goldstein, J.L., and Liang, G. (2004). Overexpression of Insig-1 in the livers of transgenic mice inhibits SREBP processing and reduces insulin-stimulated lipogenesis. The Journal of clinical investigation 113, 1168-1175.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 31, 47-59.

Esiri, M.M., and Wilcock, G.K. (1986). Cerebral amyloid angiopathy in dementia and old age. J Neurol Neurosurg Psychiatry 49, 1221-1226.

Fagan, A.M., Holtzman, D.M., Munson, G., Mathur, T., Schneider, D., Chang, L.K., Getz, G.S., Reardon, C.A., Lukens, J., Shah, J.A., *et al.* (1999). Unique lipoproteins secreted by primary astrocytes from wild type, apoE (-/-), and human apoE transgenic mice. The Journal of biological chemistry 274, 30001-30007.

Fassbender, K., Simons, M., Bergmann, C., Stroick, M., Lutjohann, D., Keller, P., Runz, H., Kuhl, S., Bertsch, T., von Bergmann, K., *et al.* (2001). Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 *in vitro* and *in vivo*. Proceedings of the National Academy of Sciences of the United States of America 98, 5856-5861.

Feingold, K.R., and Grunfeld, C. (2000). Introduction to Lipids and Lipoproteins. In Endotext, L.J. De Groot, G. Chrousos, K. Dungan, K.R. Feingold, A. Grossman, J.M. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, *et al.*, eds. (South Dartmouth (MA)).

Feng, J., Call, M.E., and Wucherpfennig, K.W. (2006). The assembly of diverse immune receptors is focused on a polar membrane-embedded interaction site. PLoS Biol 4, e142.

Fenoglio, C., Galimberti, D., Piccio, L., Scalabrini, D., Panina, P., Buonsanti, C., Venturelli, E., Lovati, C., Forloni, G., Mariani, C., *et al.* (2007). Absence of TREM2 polymorphisms in patients with Alzheimer's disease and Frontotemporal Lobar Degeneration. Neurosci Lett 411, 133-137.

Fernandez-Castaneda, A., Arandjelovic, S., Stiles, T.L., Schlobach, R.K., Mowen, K.A., Gonias, S.L., and Gaultier, A. (2013). Identification of the low density lipoprotein (LDL) receptor-related protein-1 interactome in central nervous system myelin suggests a role in the clearance of necrotic cell debris. The Journal of biological chemistry 288, 4538-4548.

Ferretti, M.T., Partridge, V., Leon, W.C., Canneva, F., Allard, S., Arvanitis, D.N., Vercauteren, F., Houle, D., Ducatenzeiler, A., Klein, W.L., *et al.* (2011). Transgenic mice as a model of pre-clinical Alzheimer's disease. Curr Alzheimer Res 8, 4-23.

Foley, P. (2010). Lipids in Alzheimer's disease: A century-old story. Biochimica et biophysica acta 1801, 750-753.

Fonteh, A.N., Chiang, J., Cipolla, M., Hale, J., Diallo, F., Chirino, A., Arakaki, X., and Harrington, M.G. (2013). Alterations in cerebrospinal fluid glycerophospholipids and phospholipase A2 activity in Alzheimer's disease. Journal of lipid research 54, 2884-2897.

Forstl, H., and Kurz, A. (1999). Clinical features of Alzheimer's disease. European archives of psychiatry and clinical neuroscience 249, 288-290.

Frank, E.M. (1994). Effect of Alzheimer's disease on communication function. Journal of the South Carolina Medical Association 90, 417-423.

Frozza, R.L., Lourenco, M.V., and De Felice, F.G. (2018). Challenges for Alzheimer's Disease Therapy: Insights from Novel Mechanisms Beyond Memory Defects. Front Neurosci 12, 37.

Fryer, J.D., Demattos, R.B., McCormick, L.M., O'Dell, M.A., Spinner, M.L., Bales, K.R., Paul, S.M., Sullivan, P.M., Parsadanian, M., Bu, G., *et al.* (2005). The low density lipoprotein receptor regulates the level of central nervous system human and murine apolipoprotein E but does not modify amyloid plaque pathology in PDAPP mice. The Journal of biological chemistry 280, 25754-25759.

Fu, Y., Hsiao, J.H., Paxinos, G., Halliday, G.M., and Kim, W.S. (2016). ABCA7 Mediates Phagocytic Clearance of Amyloid-beta in the Brain. J Alzheimers Dis 54, 569-584.

Fujikawa, R., Higuchi, S., Nakatsuji, M., Yasui, M., Ikedo, T., Nagata, M., Hayashi, K., Yokode, M., and Minami, M. (2017). Deficiency in EP4 Receptor-Associated Protein Ameliorates Abnormal Anxiety-Like Behavior and Brain Inflammation in a Mouse Model of Alzheimer Disease. Am J Pathol 187, 1848-1854.

Fujikawa, R., Higuchi, S., Nakatsuji, M., Yasui, M., Ikedo, T., Nagata, M., Yokode, M., and Minami, M. (2016). EP4 Receptor-Associated Protein in Microglia Promotes Inflammation in the Brain. Am J Pathol 186, 1982-1988.

Fung, K.Y., Wang, C., Nyegaard, S., Heit, B., Fairn, G.D., and Lee, W.L. (2017). SR-BI Mediated Transcytosis of HDL in Brain Microvascular Endothelial Cells Is Independent of Caveolin, Clathrin, and PDZK1. Front Physiol 8, 841.

Gauthier, B., Robb, M., Gaudet, F., Ginsburg, G.S., and McPherson, R. (1999). Characterization of a cholesterol response element (CRE) in the promoter of the cholesteryl ester transfer protein gene: functional role of the transcription factors SREBP-1a, -2, and YY1. Journal of lipid research 40, 1284-1293.

Geifman, N., Brinton, R.D., Kennedy, R.E., Schneider, L.S., and Butte, A.J. (2017). Evidence for benefit of statins to modify cognitive decline and risk in Alzheimer's disease. Alzheimers Res Ther 9, 10.

Gelissen, I.C., Hochgrebe, T., Wilson, M.R., Easterbrook-Smith, S.B., Jessup, W., Dean, R.T., and Brown, A.J. (1998). Apolipoprotein J (clusterin) induces cholesterol export from macrophage-foam cells: a potential anti-atherogenic function? Biochem J 331 (Pt 1), 231-237.

Ghribi, O. (2008). Potential mechanisms linking cholesterol to Alzheimer's disease-like pathology in rabbit brain, hippocampal organotypic slices, and skeletal muscle. J Alzheimers Dis 15, 673-684.

Glenner, G.G., and Wong, C.W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochemical and biophysical research communications 120, 885-890.

Glomset, J.A. (1968). The plasma lecithins:cholesterol acyltransferase reaction. Journal of lipid research 9, 155-167.

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., *et al.* (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349, 704-706.

Golde, T.E., and Eckman, C.B. (2001). Cholesterol modulation as an emerging strategy for the treatment of Alzheimer's disease. Drug Discov Today 6, 1049-1055.

Goldstein, J.L., and Brown, M.S. (2001). Molecular medicine. The cholesterol quartet. Science 292, 1310-1312.

Gong, Y., Lee, J.N., Lee, P.C., Goldstein, J.L., Brown, M.S., and Ye, J. (2006). Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. Cell Metab 3, 15-24.

Gonzalez de San Roman, E., Manuel, I., Giralt, M.T., Ferrer, I., and Rodriguez-Puertas, R. (2017). Imaging mass spectrometry (IMS) of cortical lipids from preclinical to severe stages of Alzheimer's disease. Biochim Biophys Acta Biomembr 1859, 1604-1614.

Gordon, S.M., Li, H., Zhu, X., Shah, A.S., Lu, L.J., and Davidson, W.S. (2015). A comparison of the mouse and human lipoproteome: suitability of the mouse model for studies of human lipoproteins. J Proteome Res 14, 2686-2695.

Gordon, T., Castelli, W.P., Hjortland, M.C., Kannel, W.B., and Dawber, T.R. (1977). High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. The American journal of medicine 62, 707-714.

Goti, D., Hrzenjak, A., Levak-Frank, S., Frank, S., van der Westhuyzen, D.R., Malle, E., and Sattler, W. (2001). Scavenger receptor class B, type I is expressed in porcine brain capillary endothelial cells and contributes to selective uptake of HDL-associated vitamin E. Journal of neurochemistry 76, 498-508.

Grass, D.S., Saini, U., Felkner, R.H., Wallace, R.E., Lago, W.J., Young, S.G., and Swanson, M.E. (1995). Transgenic mice expressing both human apolipoprotein B and human CETP have a lipoprotein cholesterol distribution similar to that of normolipidemic humans. Journal of lipid research 36, 1082-1091.

Grbovic, O.M., Mathews, P.M., Jiang, Y., Schmidt, S.D., Dinakar, R., Summers-Terio, N.B., Ceresa, B.P., Nixon, R.A., and Cataldo, A.M. (2003). Rab5-stimulated up-regulation of the endocytic pathway increases intracellular beta-cleaved amyloid precursor protein carboxyl-terminal fragment levels and Abeta production. The Journal of biological chemistry 278, 31261-31268.

Griffin, W.S., Stanley, L.C., Ling, C., White, L., MacLeod, V., Perrot, L.J., White, C.L., 3rd, and Araoz, C. (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America 86, 7611-7615.

Grimm, M.O., Grimm, H.S., Patzold, A.J., Zinser, E.G., Halonen, R., Duering, M., Tschape, J.A., De Strooper, B., Muller, U., Shen, J., *et al.* (2005). Regulation of cholesterol and sphingomyelin metabolism by amyloid-beta and presenilin. Nat Cell Biol 7, 1118-1123.

Groseclose, M.R., and Castellino, S. (2013). A mimetic tissue model for the quantification of drug distributions by MALDI imaging mass spectrometry. Anal Chem 85, 10099-10106.

Group, H.T.R.C., Bowman, L., Hopewell, J.C., Chen, F., Wallendszus, K., Stevens, W., Collins, R., Wiviott, S.D., Cannon, C.P., Braunwald, E., *et al.* (2017). Effects of Anacetrapib in Patients with Atherosclerotic Vascular Disease. N Engl J Med 377, 1217-1227.

Grupe, A., Abraham, R., Li, Y., Rowland, C., Hollingworth, P., Morgan, A., Jehu, L., Segurado, R., Stone, D., Schadt, E., *et al.* (2007). Evidence for novel susceptibility genes for late-onset Alzheimer's disease from a genome-wide association study of putative functional variants. Human molecular genetics 16, 865-873.

Gu, Q., Paulose-Ram, R., Burt, V.L., and Kit, B.K. (2014). Prescription cholesterol-lowering medication use in adults aged 40 and over: United States, 2003-2012. NCHS Data Brief, 1-8.

Guerreiro, R., Wojtas, A., Bras, J., Carrasquillo, M., Rogaeva, E., Majounie, E., Cruchaga, C., Sassi, C., Kauwe, J.S., Younkin, S., *et al.* (2013). TREM2 variants in Alzheimer's disease. N Engl J Med 368, 117-127.

Guo, D., Ye, J., Dai, J., Li, L., Chen, F., Ma, D., and Ji, C. (2009). Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. Leuk Res 33, 678-685.

Gutstein, D.E., Krishna, R., Johns, D., Surks, H.K., Dansky, H.M., Shah, S., Mitchel, Y.B., Arena, J., and Wagner, J.A. (2012). Anacetrapib, a novel CETP inhibitor: pursuing a new approach to cardiovascular risk reduction. Clinical pharmacology and therapeutics 91, 109-122.

Guyard-Dangremont, V., Desrumaux, C., Gambert, P., Lallemant, C., and Lagrost, L. (1998). Phospholipid and cholesteryl ester transfer activities in plasma from 14 vertebrate species. Relation to atherogenesis susceptibility. Comp Biochem Physiol B Biochem Mol Biol 120, 517-525.

Haapasalo, A., and Kovacs, D.M. (2011). The many substrates of presenilin/gamma-secretase. J Alzheimers Dis 25, 3-28.

Hanson, M.A., Cherezov, V., Griffith, M.T., Roth, C.B., Jaakola, V.P., Chien, E.Y., Velasquez, J., Kuhn, P., and Stevens, R.C. (2008). A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor. Structure 16, 897-905.

Harada, L.M., Carrilho, A.J., Oliveira, H.C., Nakandakare, E.R., and Quintao, E.C. (2006). Regulation of hepatic cholesterol metabolism in CETP/LDLr mice by cholesterol feeding and by drugs (cholestyramine and lovastatin) that lower plasma cholesterol. Clin Exp Pharmacol Physiol 33, 1209-1215.

Hardy, J., and De Strooper, B. (2017). Alzheimer's disease: where next for anti-amyloid therapies? Brain 140, 853-855.

Hardy, J.A., and Higgins, G.A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. Science 256, 184-185.

Harold, D., Abraham, R., Hollingworth, P., Sims, R., Gerrish, A., Hamshere, M.L., Pahwa, J.S., Moskvina, V., Dowzell, K., Williams, A., *et al.* (2009). Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet 41, 1088-1093.

Hartmann, T., Kuchenbecker, J., and Grimm, M.O. (2007). Alzheimer's disease: the lipid connection. Journal of neurochemistry 103 Suppl 1, 159-170.

Haughey, N.J., Bandaru, V.V., Bae, M., and Mattson, M.P. (2010). Roles for dysfunctional sphingolipid metabolism in Alzheimer's disease neuropathogenesis. Biochimica et biophysica acta 1801, 878-886.

He, X., Cooley, K., Chung, C.H., Dashti, N., and Tang, J. (2007). Apolipoprotein receptor 2 and X11 alpha/beta mediate apolipoprotein E-induced endocytosis of amyloid-beta precursor protein and beta-secretase, leading to amyloid-beta production. J Neurosci 27, 4052-4060.

He, X., Huang, Y., Li, B., Gong, C.X., and Schuchman, E.H. (2010). Deregulation of sphingolipid metabolism in Alzheimer's disease. Neurobiology of aging 31, 398-408.

Heart Protection Study Collaborative, G. (2002). MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 360, 7-22.

Heneka, M.T., Carson, M.J., El Khoury, J., Landreth, G.E., Brosseron, F., Feinstein, D.L., Jacobs, A.H., Wyss-Coray, T., Vitorica, J., Ransohoff, R.M., *et al.* (2015). Neuroinflammation in Alzheimer's disease. Lancet Neurol 14, 388-405.

Heber, S., Herms, J., Gajic, V., Hainfellner, J., Aguzzi, A., Rulicke, T., von Kretzschmar, H., von Koch, C., Sisodia, S., Tremml, P., et al. (2000). Mice with combined gene knockouts reveal essential and partially redundant functions of amyloid precursor protein family members. J Neurosci 20, 7951-7963.

Hoe, H.S., Wessner, D., Beffert, U., Becker, A.G., Matsuoka, Y., and Rebeck, G.W. (2005). F-spondin interaction with the apolipoprotein E receptor ApoEr2 affects processing of amyloid precursor protein. Mol Cell Biol 25, 9259-9268.

Higgins, A.J., and Lees, P. (1984). The acute inflammatory process, arachidonic acid metabolism and the mode of action of anti-inflammatory drugs. Equine Vet J 16, 163-175.

Hirsch-Reinshagen, V., Donkin, J., Stukas, S., Chan, J., Wilkinson, A., Fan, J., Parks, J.S., Kuivenhoven, J.A., Lutjohann, D., Pritchard, H., *et al.* (2009). LCAT synthesized by primary astrocytes esterifies cholesterol on glia-derived lipoproteins. Journal of lipid research 50, 885-893.

Hollingworth, P., Harold, D., Sims, R., Gerrish, A., Lambert, J.C., Carrasquillo, M.M., Abraham, R., Hamshere, M.L., Pahwa, J.S., Moskvina, V., *et al.* (2011). Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. Nat Genet 43, 429-435.

Holmes, O., Paturi, S., Ye, W., Wolfe, M.S., and Selkoe, D.J. (2012). Effects of membrane lipids on the activity and processivity of purified gamma-secretase. Biochemistry 51, 3565-3575.

Holtzman, D.M., Morris, J.C., and Goate, A.M. (2011). Alzheimer's disease: the challenge of the second century. Science translational medicine 3, 77sr71.

Horton, J.D., Goldstein, J.L., and Brown, M.S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. The Journal of clinical investigation 109, 1125-1131.

Hoshino, T., Nakaya, T., Homan, T., Tanaka, K., Sugimoto, Y., Araki, W., Narita, M., Narumiya, S., Suzuki, T., and Mizushima, T. (2007). Involvement of prostaglandin E2 in production of amyloid-beta peptides both *in vitro* and *in vivo*. The Journal of biological chemistry 282, 32676-32688.

Hoshino, T., Namba, T., Takehara, M., Murao, N., Matsushima, T., Sugimoto, Y., Narumiya, S., Suzuki, T., and Mizushima, T. (2012). Improvement of cognitive function in Alzheimer's disease model mice by genetic and pharmacological inhibition of the EP(4) receptor. Journal of neurochemistry 120, 795-805.

Hoshino, T., Namba, T., Takehara, M., Nakaya, T., Sugimoto, Y., Araki, W., Narumiya, S., Suzuki, T., and Mizushima, T. (2009). Prostaglandin E2 stimulates the production of amyloid-beta peptides through internalization of the EP4 receptor. The Journal of biological chemistry 284, 18493-18502.

Houx, P.J., Shepherd, J., Blauw, G.J., Murphy, M.B., Ford, I., Bollen, E.L., Buckley, B., Stott, D.J., Jukema, W., Hyland, M., *et al.* (2002). Testing cognitive function in elderly populations: the PROSPER study. PROspective Study of Pravastatin in the Elderly at Risk. J Neurol Neurosurg Psychiatry 73, 385-389.

Hovingh, G.K., Kastelein, J.J., van Deventer, S.J., Round, P., Ford, J., Saleheen, D., Rader, D.J., Brewer, H.B., and Barter, P.J. (2015). Cholesterol ester transfer protein inhibition by TA-8995 in patients with mild dyslipidaemia (TULIP): a randomised, double-blind, placebo-controlled phase 2 trial. Lancet 386, 452-460.

Huang, X.G., Yee, B.K., Nag, S., Chan, S.T., and Tang, F. (2003). Behavioral and neurochemical characterization of transgenic mice carrying the human presenilin-1 gene with or without the leucine-to-proline mutation at codon 235. Experimental neurology 183, 673-681.

Huang, Z., Inazu, A., Nohara, A., Higashikata, T., and Mabuchi, H. (2002). Cholesteryl ester transfer protein inhibitor (JTT-705) and the development of atherosclerosis in rabbits with severe hypercholesterolaemia. Clinical science 103, 587-594.

Huppi, P.S., and Dubois, J. (2006). Diffusion tensor imaging of brain development. Semin Fetal Neonatal Med 11, 489-497.

Hur, J.Y., Welander, H., Behbahani, H., Aoki, M., Franberg, J., Winblad, B., Frykman, S., and Tjernberg, L.O. (2008). Active gamma-secretase is localized to detergent-resistant membranes in human brain. FEBS J 275, 1174-1187.

Hussain, I., Powell, D., Howlett, D.R., Tew, D.G., Meek, T.D., Chapman, C., Gloger, I.S., Murphy, K.E., Southan, C.D., Ryan, D.M., *et al.* (1999). Identification of a novel aspartic protease (Asp 2) as beta-secretase. Molecular and cellular neurosciences 14, 419-427.

Hussain, M.M. (2000). A proposed model for the assembly of chylomicrons. Atherosclerosis 148, 1-15.

Alzheimer's disease International, Prince, M., Anders Wimo, A., Guerchet, M., Ali, GC., Wu, YT., Prina, M. (2015). World Alzheimer Report 2015.

Iso, T., Kedes, L., and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 194, 237-255.

Iwamoto, N., Abe-Dohmae, S., Sato, R., and Yokoyama, S. (2006). ABCA7 expression is regulated by cellular cholesterol through the SREBP2 pathway and associated with phagocytosis. Journal of lipid research 47, 1915-1927.

Izem, L., and Morton, R.E. (2001). Cholesteryl ester transfer protein biosynthesis and cellular cholesterol homeostasis are tightly interconnected. The Journal of biological chemistry 276, 26534-26541.

Izem, L., and Morton, R.E. (2007). Possible role for intracellular cholesteryl ester transfer protein in adipocyte lipid metabolism and storage. The Journal of biological chemistry 282, 21856-21865.

Jack, C.R., Jr., Knopman, D.S., Jagust, W.J., Petersen, R.C., Weiner, M.W., Aisen, P.S., Shaw, L.M., Vemuri, P., Wiste, H.J., Weigand, S.D., *et al.* (2013). Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. Lancet Neurol 12, 207-216.

Jankowsky, J.L., Fadale, D.J., Anderson, J., Xu, G.M., Gonzales, V., Jenkins, N.A., Copeland, N.G., Lee, M.K., Younkin, L.H., Wagner, S.L., *et al.* (2004). Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide *in vivo*: evidence for augmentation of a 42-specific gamma secretase. Human molecular genetics 13, 159-170.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R., and Israel, A. (1995). Signalling downstream of activated mammalian Notch. Nature 377, 355-358.

Jeske, D.J., and Dietschy, J.M. (1980). Regulation of rates of cholesterol synthesis *in vivo* in the liver and carcass of the rat measured using [3H]water. Journal of lipid research 21, 364-376.

Jiang, X.C., Agellon, L.B., Walsh, A., Breslow, J.L., and Tall, A. (1992). Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. The Journal of clinical investigation 90, 1290-1295.

Jick, H., Zornberg, G.L., Jick, S.S., Seshadri, S., and Drachman, D.A. (2000). Statins and the risk of dementia. Lancet 356, 1627-1631.

Jonsson, T., and Stefansson, K. (2013). TREM2 and neurodegenerative disease. N Engl J Med 369, 1568-1569.

Jung, J.I., Price, A.R., Ladd, T.B., Ran, Y., Park, H.J., Ceballos-Diaz, C., Smithson, L.A., Hochhaus, G., Tang, Y., Akula, R., *et al.* (2015). Cholestenoic acid, an endogenous cholesterol metabolite, is a potent gamma-secretase modulator. Mol Neurodegener 10, 29.

K., Lapointe, J.M., Saha, S., Roach, M.L., *et al.* (2003). Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. Proceedings of the National Academy of Sciences of the United States of America 100, 9044-9049.

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., Sisodia, S., and Malinow, R. (2003). APP processing and synaptic function. Neuron 37, 925-937.

Kanekiyo, T., Xu, H., and Bu, G. (2014). ApoE and Abeta in Alzheimer's disease: accidental encounters or partners? Neuron 81, 740-754.

Kimura, R., and Ohno, M. (2009). Impairments in remote memory stabilization precede hippocampal synaptic and cognitive failures in 5XFAD Alzheimer mouse model. Neurobiol Dis 33, 229-235.

Knoferle, J., Yoon, S.Y., Walker, D., Leung, L., Gillespie, A.K., Tong, L.M., Bien-Ly, N., and Huang, Y. (2014). Apolipoprotein E4 produced in GABAergic interneurons causes learning and memory deficits in mice. J Neurosci 34, 14069-14078.

Koch, S., Donarski, N., Goetze, K., Kreckel, M., Stuerenburg, H.J., Buhmann, C., and Beisiegel, U. (2001). Characterization of four lipoprotein classes in human cerebrospinal fluid. Journal of lipid research 42, 1143-1151.

Koizumi, J., Mabuchi, H., Yoshimura, A., Michishita, I., Takeda, M., Itoh, H., Sakai, Y., Sakai, T., Ueda, K., and Takeda, R. (1985). Deficiency of serum cholesteryl-ester transfer activity in patients with familial hyperalphalipoproteinaemia. Atherosclerosis 58, 175-186.

Kosicek, M., and Hecimovic, S. (2013). Phospholipids and Alzheimer's disease: alterations, mechanisms and potential biomarkers. Int J Mol Sci 14, 1310-1322.

Kounnas, M.Z., Moir, R.D., Rebeck, G.W., Bush, A.I., Argraves, W.S., Tanzi, R.E., Hyman, B.T., and Strickland, D.K. (1995). LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. Cell 82, 331-340.

Kukar, T.L., Ladd, T.B., Bann, M.A., Fraering, P.C., Narlawar, R., Maharvi, G.M., Healy, B., Chapman, R., Welzel, A.T., Price, R.W., *et al.* (2008). Substrate-targeting gamma-secretase modulators. Nature 453, 925-929.

Kuperstein, I., Broersen, K., Benilova, I., Rozenski, J., Jonckheere, W., Debulpaep, M., Vandersteen, A., Segers-Nolten, I., Van Der Werf, K., Subramaniam, V., *et al.* (2010). Neurotoxicity of Alzheimer's disease Abeta peptides is induced by small changes in the Abeta42 to Abeta40 ratio. EMBO J 29, 3408-3420.

Ladu, M.J., Reardon, C., Van Eldik, L., Fagan, A.M., Bu, G., Holtzman, D., and Getz, G.S. (2000). Lipoproteins in the central nervous system. Ann N Y Acad Sci 903, 167-175.

Lagace, T.A., Curtis, D.E., Garuti, R., McNutt, M.C., Park, S.W., Prather, H.B., Anderson, N.N., Ho, Y.K., Hammer, R.E., and Horton, J.D. (2006). Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. The Journal of clinical investigation 116, 2995-3005.

Lambert, J.C., Heath, S., Even, G., Campion, D., Sleegers, K., Hiltunen, M., Combarros, O., Zelenika, D., Bullido, M.J., Tavernier, B., *et al.* (2009). Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nat Genet 41, 1094-1099.

Lambert, J.C., Ibrahim-Verbaas, C.A., Harold, D., Naj, A.C., Sims, R., Bellenguez, C., DeStafano, A.L., Bis, J.C., Beecham, G.W., Grenier-Boley, B., *et al.* (2013). Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. Nat Genet 45, 1452-1458.

Lei, D., Rames, M., Zhang, X., Zhang, L., Zhang, S., and Ren, G. (2016). Insights into the tunnel mechanism of cholesteryl ester transfer protein using all-atom molecular dynamics simulations. The Journal of biological chemistry.

Leoni, V., and Caccia, C. (2013). 24S-hydroxycholesterol in plasma: a marker of cholesterol turnover in neurodegenerative diseases. Biochimie 95, 595-612.

Levin-Allerhand, J.A., Lominska, C.E., and Smith, J.D. (2002). Increased amyloid- levels in APPSWE transgenic mice treated chronically with a physiological high-fat high-cholesterol diet. J Nutr Health Aging 6, 315-319.

Li, G., Shofer, J.B., Rhew, I.C., Kukull, W.A., Peskind, E.R., McCormick, W., Bowen, J.D., Schellenberg, G.D., Crane, P.K., Breitner, J.C., *et al.* (2010). Age-varying association between statin use and incident Alzheimer's disease. J Am Geriatr Soc 58, 1311-1317.

Li, J.T., and Zhang, Y. (2018). TREM2 regulates innate immunity in Alzheimer's disease. J Neuroinflammation 15, 107.

Li, X., Fang, P., Li, Y., Kuo, Y.M., Andrews, A.J., Nanayakkara, G., Johnson, C., Fu, H., Shan, H., Du, F., *et al.* (2016). Mitochondrial Reactive Oxygen Species Mediate Lysophosphatidylcholine-Induced Endothelial Cell Activation. Arterioscler Thromb Vasc Biol 36, 1090-1100.

Li, X., Montine, K.S., Keene, C.D., and Montine, T.J. (2015). Different mechanisms of apolipoprotein E isoform-dependent modulation of prostaglandin E2 production and triggering receptor expressed on myeloid cells 2 (TREM2) expression after innate immune activation of microglia. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 29, 1754-1762.

Liao, F., Yoon, H., and Kim, J. (2017). Apolipoprotein E metabolism and functions in brain and its role in Alzheimer's disease. Curr Opin Lipidol 28, 60-67.

Lincoff, A.M., Nicholls, S.J., Riesmeyer, J.S., Barter, P.J., Brewer, H.B., Fox, K.A.A., Gibson, C.M., Granger, C., Menon, V., Montalescot, G., *et al.* (2017). Evacetrapib and Cardiovascular Outcomes in High-Risk Vascular Disease. N Engl J Med 376, 1933-1942.

Linton, M.R.F., Yancey, P.G., Davies, S.S., Jerome, W.G., Linton, E.F., Song, W.L., Doran, A.C., and Vickers, K.C. (2000). The Role of Lipids and Lipoproteins in Atherosclerosis. In Endotext, K.R. Feingold, B. Anawalt, A. Boyce, G. Chrousos, K. Dungan, A. Grossman, J.M. Hershman, G. Kaltsas, C. Koch, P. Kopp, et al., eds. (South Dartmouth (MA)).

Liu, M., Kuhel, D.G., Shen, L., Hui, D.Y., and Woods, S.C. (2012). Apolipoprotein E does not cross the blood-cerebrospinal fluid barrier, as revealed by an improved technique for sampling CSF from mice. Am J Physiol Regul Integr Comp Physiol 303, R903-908.

Liu, Q., Zerbinatti, C.V., Zhang, J., Hoe, H.S., Wang, B., Cole, S.L., Herz, J., Muglia, L., and Bu, G. (2007). Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. Neuron 56, 66-78.

Llorente-Cortes, V., Costales, P., Bernues, J., Camino-Lopez, S., and Badimon, L. (2006). Sterol regulatory element-binding protein-2 negatively regulates low density lipoprotein receptor-related protein transcription. J Mol Biol 359, 950-960.

Lobello, K., Ryan, J.M., Liu, E., Rippon, G., and Black, R. (2012). Targeting Beta amyloid: a clinical review of immunotherapeutic approaches in Alzheimer's disease. Int J Alzheimers Dis 2012, 628070.

Love, S., and Miners, J.S. (2016). Cerebral Hypoperfusion and the Energy Deficit in Alzheimer's Disease. Brain Pathol 26, 607-617.

Lucin, K.M., and Wyss-Coray, T. (2009). Immune activation in brain aging and neurodegeneration: too much or too little? Neuron 64, 110-122.

Luo, Y., and Tall, A.R. (2000). Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. The Journal of clinical investigation 105, 513-520.

Lv, X., Li, W., Luo, Y., Wang, D., Zhu, C., Huang, Z.X., and Tan, X. (2013). Exploring the differences between mouse mAbeta(1-42) and human hAbeta(1-42) for Alzheimer's disease related properties and neuronal cytotoxicity. Chem Commun (Camb) 49, 5865-5867.

Lythgoe, C., Perkes, A., Peterson, M., Schmutz, C., Leary, M., Ebbert, M.T., Ridge, P.G., Norton, M.C., Tschanz, J.T., Munger, R.G., et al. (2015). Population-based analysis of cholesteryl ester transfer protein identifies association between I405V and cognitive decline: the Cache County Study. Neurobiology of aging 36, 547 e541-543.

Magara, F., Muller, U., Li, Z.W., Lipp, H.P., Weissmann, C., Stagljar, M., and Wolfer, D.P. (1999). Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the beta-amyloid-precursor protein. Proceedings of the National Academy of Sciences of the United States of America 96, 4656-4661.

Mahmoudiandehkordi, S., Arnold, M., Nho, K., Ahmad, S., Jia, W., Xia, G., Louie, G., Kueider, A., Moseley, M.A., Thompson, J.W., *et al.* (2018). Altered Bile Acid Profile Associates with Cognitive Impairment in Alzheimer's Disease: An Emerging Role for Gut Microbiome. bioRxiv.

Mapstone, M., Cheema, A.K., Fiandaca, M.S., Zhong, X., Mhyre, T.R., MacArthur, L.H., Hall, W.J., Fisher, S.G., Peterson, D.R., Haley, J.M., *et al.* (2014). Plasma phospholipids identify antecedent memory impairment in older adults. Nat Med 20, 415-418.

Marquer, C., Devauges, V., Cossec, J.C., Liot, G., Lecart, S., Saudou, F., Duyckaerts, C., Leveque-Fort, S., and Potier, M.C. (2011). Local cholesterol increase triggers amyloid precursor protein-Bace1 clustering in lipid rafts and rapid endocytosis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 25, 1295-1305.

Marquer, C., Laine, J., Dauphinot, L., Hanbouch, L., Lemercier-Neuillet, C., Pierrot, N., Bossers, K., Le, M., Corlier, F., Benstaali, C., *et al.* (2014). Increasing membrane cholesterol of neurons in culture recapitulates Alzheimer's disease early phenotypes. Mol Neurodegener 9, 60.

Masson, D. (2009). Anacetrapib, a cholesterol ester transfer protein (CETP) inhibitor for the treatment of atherosclerosis. Curr Opin Investig Drugs 10, 980-987.

Masters, C.L., and Beyreuther, K.T. (1989). The pathology of the amyloid A4 precursor of Alzheimer's disease. Annals of medicine 21, 89-90.

Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L., and Beyreuther, K. (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proceedings of the National Academy of Sciences of the United States of America 82, 4245-4249.

Matsuo, M., Campenot, R.B., Vance, D.E., Ueda, K., and Vance, J.E. (2011). Involvement of low-density lipoprotein receptor-related protein and ABCG1 in stimulation of axonal extension by apoE-containing lipoproteins. Biochimica et biophysica acta 1811, 31-38.

May, P.C., Dean, R.A., Lowe, S.L., Martenyi, F., Sheehan, S.M., Boggs, L.N., Monk, S.A., Mathes, B.M., Mergott, D.J., Watson, B.M., *et al.* (2011). Robust central reduction of amyloid-beta in humans with an orally available, non-peptidic beta-secretase inhibitor. J Neurosci 31, 16507-16516.

Mendis, L.H., Grey, A.C., Faull, R.L., and Curtis, M.A. (2016). Hippocampal lipid differences in Alzheimer's disease: a human brain study using matrix-assisted laser desorption/ionization-imaging mass spectrometry. Brain Behav 6, e00517.

Merck (2017). Merck Provides Update on Anacetrapib Development Program.

Miller, L.J., and Chacko, R. (2004). The role of cholesterol and statins in Alzheimer's disease. Ann Pharmacother 38, 91-98.

Morris, G.P., Clark, I.A., and Vissel, B. (2014). Inconsistencies and controversies surrounding the amyloid hypothesis of Alzheimer's disease. Acta Neuropathol Commun 2, 135.

Morton, R.E., and Steinbrunner, J.V. (1990). Concentration of neutral lipids in the phospholipid surface of substrate particles determines lipid transfer protein activity. Journal of lipid research 31, 1559-1567.

Morton, R.E., and Zilversmit, D.B. (1982). Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. Journal of lipid research 23, 1058-1067.

Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., and McConlogue, L. (2000). High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J Neurosci 20, 4050-4058.

Mullane, K., and Williams, M. (2018). Alzheimer's Disease (AD) therapeutics - 1: Repeated clinical failures continue to question the amyloid hypothesis of AD and the current understanding of AD causality. Biochem Pharmacol.

Muller, N., Schulte, D.M., Turk, K., Freitag-Wolf, S., Hampe, J., Zeuner, R., Schroder, J.O., Gouni-Berthold, I., Berthold, H.K., Krone, W., *et al.* (2015). IL-6 blockade by monoclonal antibodies inhibits apolipoprotein (a) expression and lipoprotein (a) synthesis in humans. Journal of lipid research 56, 1034-1042.

Murphy, E.A., Roddey, J.C., McEvoy, L.K., Holland, D., Hagler, D.J., Jr., Dale, A.M., Brewer, J.B., and Alzheimer's Disease Neuroimaging, I. (2012). CETP polymorphisms associate with brain structure, atrophy rate, and Alzheimer's disease risk in an APOE-dependent manner. Brain Imaging Behav 6, 16-26.

Murrell, J., Farlow, M., Ghetti, B., and Benson, M.D. (1991). A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science 254, 97-99.

Nguyen, L.N., Ma, D., Shui, G., Wong, P., Cazenave-Gassiot, A., Zhang, X., Wenk, M.R., Goh, E.L., and Silver, D.L. (2014). Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. Nature 509, 503-506.

Nho, K., Kueider-Paisley, A., MahmoudianDehkord, S., Arnold, M., Risacher, S.L., Louie, G., Blach, C., Baillie, R., Han, X., Kastenmueller, G., *et al.* (2018). Altered Bile Acid Profile in Mild Cognitive Impairment and Alzheimer's Disease: Relationship to Neuroimaging and CSF Biomarkers. bioRxiv.

Nichols, A.V., and Smith, L. (1965). Effect of Very Low-Density Lipoproteins on Lipid Transfer in Incubated Serum. Journal of lipid research 6, 206-210.

Nohturfft, A., Yabe, D., Goldstein, J.L., Brown, M.S., and Espenshade, P.J. (2000). Regulated step in cholesterol feedback localized to budding of SCAP from ER membranes. Cell 102, 315-323.

Oakley, H., Cole, S.L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongaarts, A., Ohno, M., Disterhoft, J., Van Eldik, L., *et al.* (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci 26, 10129-10140.

Ohvo-Rekila, H., Ramstedt, B., Leppimaki, P., and Slotte, J.P. (2002). Cholesterol interactions with phospholipids in membranes. Prog Lipid Res 41, 66-97.

Okamoto, H., Yonemori, F., Wakitani, K., Minowa, T., Maeda, K., and Shinkai, H. (2000). A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. Nature 406, 203-207.

Orgeta, V., Tabet, N., Nilforooshan, R., and Howard, R. (2017). Efficacy of Antidepressants for Depression in Alzheimer's Disease: Systematic Review and Meta-Analysis. J Alzheimers Dis 58, 725-733.

Osenkowski, P., Ye, W., Wang, R., Wolfe, M.S., and Selkoe, D.J. (2008). Direct and potent regulation of gamma-secretase by its lipid microenvironment. The Journal of biological chemistry 283, 22529-22540.

Ouellet, M., Emond, V., Chen, C.T., Julien, C., Bourasset, F., Oddo, S., LaFerla, F., Bazinet, R.P., and Calon, F. (2009). Diffusion of docosahexaenoic and eicosapentaenoic acids through the blood-brain barrier: An in situ cerebral perfusion study. Neurochem Int 55, 476-482.

Papp, A.C., Pinsonneault, J.K., Wang, D., Newman, L.C., Gong, Y., Johnson, J.A., Pepine, C.J., Kumari, M., Hingorani, A.D., Talmud, P.J., *et al.* (2012). Cholesteryl Ester Transfer Protein (CETP) polymorphisms affect mRNA splicing, HDL levels, and sex-dependent cardiovascular risk. PLoS One 7, e31930.

Paschkowsky, S., Oestereich, F., and Munter, L.M. (2018). Embedded in the Membrane: How Lipids Confer Activity and Specificity to Intramembrane Proteases. J Membr Biol 251, 369-378.

Perego, C., Vanoni, C., Bossi, M., Massari, S., Basudev, H., Longhi, R., and Pietrini, G. (2000). The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal cocultures. Journal of neurochemistry 75, 1076-1084.

Perugorria, M.J., Esparza-Baquer, A., Oakley, F., Labiano, I., Korosec, A., Jais, A., Mann, J., Tiniakos, D., Santos-Laso, A., Arbelaiz, A., *et al.* (2018). Non-parenchymal TREM-2 protects the liver from immune-mediated hepatocellular damage. Gut.

Petanceska, S.S., DeRosa, S., Olm, V., Diaz, N., Sharma, A., Thomas-Bryant, T., Duff, K., Pappolla, M., and Refolo, L.M. (2002). Statin therapy for Alzheimer's disease: will it work? J Mol Neurosci 19, 155-161.

Petrlova, J., Hong, H.S., Bricarello, D.A., Harishchandra, G., Lorigan, G.A., Jin, L.W., and Voss, J.C. (2011). A differential association of Apolipoprotein E isoforms with the amyloid-beta oligomer in solution. Proteins 79, 402-416.

Pfrieger, F.W. (2003). Outsourcing in the brain: do neurons depend on cholesterol delivery by astrocytes? BioEssays : news and reviews in molecular, cellular and developmental biology 25, 72-78.

Pitas, R.E., Boyles, J.K., Lee, S.H., Foss, D., and Mahley, R.W. (1987). Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. Biochimica et biophysica acta 917, 148-161.

Proitsi, P., Kim, M., Whiley, L., Pritchard, M., Leung, R., Soininen, H., Kloszewska, I., Mecocci, P., Tsolaki, M., Vellas, B., *et al.* (2015). Plasma lipidomics analysis finds long chain cholesteryl esters to be associated with Alzheimer's disease. Transl Psychiatry 5, e494.

Puglielli, L., Tanzi, R.E., and Kovacs, D.M. (2003). Alzheimer's disease: the cholesterol connection. Nat Neurosci 6, 345-351.

Qi, Y., Morishima-Kawashima, M., Sato, T., Mitsumori, R., and Ihara, Y. (2003). Distinct mechanisms by mutant presenilin 1 and 2 leading to increased intracellular levels of amyloid beta-protein 42 in Chinese hamster ovary cells. Biochemistry 42, 1042-1052.

Qiu, W.Q., Walsh, D.M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M.B., Rosner, M.R., Safavi, A., Hersh, L.B., and Selkoe, D.J. (1998). Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. The Journal of biological chemistry 273, 32730-32738.

Qiu, X., Mistry, A., Ammirati, M.J., Chrunyk, B.A., Clark, R.W., Cong, Y., Culp, J.S., Danley, D.E., Freeman, T.B., Geoghegan, K.F., *et al.* (2007). Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. Nature structural & molecular biology 14, 106-113.

Rafieian-Kopaei, M., Setorki, M., Doudi, M., Baradaran, A., and Nasri, H. (2014). Atherosclerosis: process, indicators, risk factors and new hopes. Int J Prev Med 5, 927-946.

Refolo, L.M., Pappolla, M.A., LaFrancois, J., Malester, B., Schmidt, S.D., Thomas-Bryant, T., Tint, G.S., Wang, R., Mercken, M., Petanceska, S.S., *et al.* (2001). A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of Alzheimer's disease. Neurobiol Dis 8, 890-899.

Rehker, J., Rodhe, J., Nesbitt, R.R., Boyle, E.A., Martin, B.K., Lord, J., Karaca, I., Naj, A., Jessen, F., Helisalmi, S., *et al.* (2017). Caspase-8, association with Alzheimer's Disease and functional analysis of rare variants. PLoS One 12, e0185777.

Riddell, D.R., Christie, G., Hussain, I., and Dingwall, C. (2001). Compartmentalization of beta-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. Curr Biol 11, 1288-1293.

Robinson, J.G., Farnier, M., Krempf, M., Bergeron, J., Luc, G., Averna, M., Stroes, E.S., Langslet, G., Raal, F.J., El Shahawy, M., *et al.* (2015). Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. N Engl J Med 372, 1489-1499.

Rockwood, K., Kirkland, S., Hogan, D.B., MacKnight, C., Merry, H., Verreault, R., Wolfson, C., and McDowell, I. (2002). Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. Arch Neurol 59, 223-227.

Rodriguez, E., Mateo, I., Infante, J., Llorca, J., Berciano, J., and Combarros, O. (2006). Cholesteryl ester transfer protein (CETP) polymorphism modifies the Alzheimer's disease risk associated with APOE epsilon4 allele. J Neurol 253, 181-185.

Rogers, J., Cooper, N.R., Webster, S., Schultz, J., McGeer, P.L., Styren, S.D., Civin, W.H., Brachova, L., Bradt, B., Ward, P., *et al.* (1992). Complement activation by beta-amyloid in Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America 89, 10016-10020.

Rohn, T.T., Head, E., Nesse, W.H., Cotman, C.W., and Cribbs, D.H. (2001). Activation of caspase-8 in the Alzheimer's disease brain. Neurobiol Dis 8, 1006-1016.

Rothstein, J.D., Martin, L., Levey, A.I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., and Kuncl, R.W. (1994). Localization of neuronal and glial glutamate transporters. Neuron 13, 713-725.

Runz, H., Rietdorf, J., Tomic, I., de Bernard, M., Beyreuther, K., Pepperkok, R., and Hartmann, T. (2002). Inhibition of intracellular cholesterol transport alters presenilin localization and amyloid precursor protein processing in neuronal cells. J Neurosci 22, 1679-1689.

Rushworth, J.V., and Hooper, N.M. (2010). Lipid Rafts: Linking Alzheimer's Amyloid-beta Production, Aggregation, and Toxicity at Neuronal Membranes. Int J Alzheimers Dis 2011, 603052.

Russell, D.W., Halford, R.W., Ramirez, D.M., Shah, R., and Kotti, T. (2009). Cholesterol 24-hydroxylase: an enzyme of cholesterol turnover in the brain. Annu Rev Biochem 78, 1017-1040.

Salminen, L.E., Schofield, P.R., Pierce, K.D., Luo, X., Zhao, Y., Laidlaw, D.H., Cabeen, R.P., Conturo, T.E., Lane, E.M., Heaps, J.M., *et al.* (2015). Genetic markers of cholesterol transport and gray matter diffusion: a preliminary study of the CETP I405V polymorphism. J Neural Transm (Vienna) 122, 1581-1592.

Samuelsson, B. (1991). Arachidonic acid metabolism: role in inflammation. Z Rheumatol 50 Suppl 1, 3-6.

Sanders, A.E., Wang, C., Katz, M., Derby, C.A., Barzilai, N., Ozelius, L., and Lipton, R.B. (2010). Association of a functional polymorphism in the cholesteryl ester transfer protein (CETP) gene with memory decline and incidence of dementia. JAMA : the journal of the American Medical Association 303, 150-158.

Saunders, A.M., Strittmatter, W.J., Schmechel, D., George-Hyslop, P.H., Pericak-Vance, M.A., Joo, S.H., Rosi, B.L., Gusella, J.F., Crapper-MacLachlan, D.R., Alberts, M.J., *et al.* (1993). Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. Neurology 43, 1467-1472.

Schramm, T., Hester, A., Klinkert, I., Both, J.P., Heeren, R.M., Brunelle, A., Laprevote, O., Desbenoit, N., Robbe, M.F., Stoeckli, M., *et al.* (2012). imzML--a common data format for the flexible exchange and processing of mass spectrometry imaging data. J Proteomics 75, 5106-5110.

Schwartz, G.G., Olsson, A.G., Abt, M., Ballantyne, C.M., Barter, P.J., Brumm, J., Chaitman, B.R., Holme, I.M., Kallend, D., Leiter, L.A., *et al.* (2012). Effects of dalcetrapib in patients with a recent acute coronary syndrome. N Engl J Med 367, 2089-2099.

Seo, Y.Y., Cho, Y.K., Bae, J.C., Seo, M.H., Park, S.E., Rhee, E.J., Park, C.Y., Oh, K.W., Park, S.W., and Lee, W.Y. (2013). Tumor Necrosis Factor-alpha as a Predictor for the Development of Nonalcoholic Fatty Liver Disease: A 4-Year Follow-Up Study. Endocrinol Metab (Seoul) 28, 41-45.

Selkoe, D.J. (2001). Alzheimer's disease: genes, proteins, and therapy. Physiological reviews 81, 741-766.

Shah, N.P., Swiger, K.J., and Martin, S.S. (2015). Impact on cognitive function-are all statins the same? Curr Atheroscler Rep 17, 466.

Sharma, K., Schmitt, S., Bergner, C.G., Tyanova, S., Kannaiyan, N., Manrique-Hoyos, N., Kongi, K., Cantuti, L., Hanisch, U.K., Philips, M.A., *et al.* (2015). Cell type- and brain region-resolved mouse brain proteome. Nat Neurosci 18, 1819-1831.

Shen, Y., Yang, L., and Li, R. (2013). What does complement do in Alzheimer's disease? Old molecules with new insights. Transl Neurodegener 2, 21.

Shenoy, C., Shenoy, M.M., and Rao, G.K. (2015). Dyslipidemia in Dermatological Disorders. N Am J Med Sci 7, 421-428.

Sheridan, C. (2013). Phase 3 data for PCSK9 inhibitor wows. Nat Biotechnol 31, 1057-1058.

Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., *et al.* (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375, 754-760.

Shi, J., Johansson, J., Woodling, N.S., Wang, Q., Montine, T.J., and Andreasson, K. (2010). The prostaglandin E2 E-prostanoid 4 receptor exerts anti-inflammatory effects in brain innate immunity. J Immunol 184, 7207-7218.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature 387, 569-572.

Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C.G., and Simons, K. (1998). Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proceedings of the National Academy of Sciences of the United States of America 95, 6460-6464.

Sinha, S., Anderson, J.P., Barbour, R., Basi, G.S., Caccavello, R., Davis, D., Doan, M., Dovey, H.F., Frigon, N., Hong, J., *et al.* (1999). Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature 402, 537-540.

Solomon, A., Mangialasche, F., Richard, E., Andrieu, S., Bennett, D.A., Breteler, M., Fratiglioni, L., Hooshmand, B., Khachaturian, A.S., Schneider, L.S., *et al.* (2014). Advances in the prevention of Alzheimer's disease and dementia. J Intern Med 275, 229-250.

Song, H., Saito, K., Seishima, M., Noma, A., Urakami, K., and Nakashima, K. (1997). Cerebrospinal fluid apo E and apo A-I concentrations in early- and late-onset Alzheimer's disease. Neurosci Lett 231, 175-178.

Sparks, D.L., Kryscio, R.J., Sabbagh, M.N., Connor, D.J., Sparks, L.M., and Liebsack, C. (2008). Reduced risk of incident AD with elective statin use in a clinical trial cohort. Curr Alzheimer Res 5, 416-421.

St George-Hyslop, P.H., Tanzi, R.E., Polinsky, R.J., Haines, J.L., Nee, L., Watkins, P.C., Myers, R.H., Feldman, R.G., Pollen, D., Drachman, D., *et al.* (1987). The genetic defect causing familial Alzheimer's disease maps on chromosome 21. Science 235, 885-890.

Steenbergen, R.H., Joyce, M.A., Lund, G., Lewis, J., Chen, R., Barsby, N., Douglas, D., Zhu, L.F., Tyrrell, D.L., and Kneteman, N.M. (2010). Lipoprotein profiles in SCID/uPA mice transplanted with human hepatocytes become human-like and correlate with HCV infection success. Am J Physiol Gastrointest Liver Physiol 299, G844-854.

Stefani, M., and Liguri, G. (2009). Cholesterol in Alzheimer's disease: unresolved questions. Curr Alzheimer Res 6, 15-29.

Steinberg, S., Stefansson, H., Jonsson, T., Johannsdottir, H., Ingason, A., Helgason, H., Sulem, P., Magnusson, O.T., Gudjonsson, S.A., Unnsteinsdottir, U., *et al.* (2015). Loss-of-function variants in ABCA7 confer risk of Alzheimer's disease. Nat Genet 47, 445-447.

Stukas, S., Robert, J., Lee, M., Kulic, I., Carr, M., Tourigny, K., Fan, J., Namjoshi, D., Lemke, K., DeValle, N., *et al.* (2014a). Intravenously injected human apolipoprotein A-I rapidly enters the central nervous system via the choroid plexus. J Am Heart Assoc 3, e001156.

Stukas, S., Robert, J., and Wellington, C.L. (2014b). High-density lipoproteins and cerebrovascular integrity in Alzheimer's disease. Cell Metab 19, 574-591.

Suarez-Calvet, M., Araque Caballero, M.A., Kleinberger, G., Bateman, R.J., Fagan, A.M., Morris, J.C., Levin, J., Danek, A., Ewers, M., Haass, C., *et al.* (2016). Early changes in CSF sTREM2 in dominantly inherited Alzheimer's disease occur after amyloid deposition and neuronal injury. Science translational medicine 8, 369ra178.

Sundermann, E.E., Wang, C., Katz, M., Zimmerman, M.E., Derby, C.A., Hall, C.B., Ozelius, L.J., and Lipton, R.B. (2016). Cholesteryl ester transfer protein genotype modifies the effect of apolipoprotein epsilon4 on memory decline in older adults. Neurobiology of aging 41, 200 e207-200 e212.

Suzuki, N., Cheung, T.T., Cai, X.D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T.E., and Younkin, S.G. (1994). An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264, 1336-1340.

Swenson, T.L., Simmons, J.S., Hesler, C.B., Bisgaier, C., and Tall, A.R. (1987). Cholesteryl ester transfer protein is secreted by Hep G2 cells and contains asparagine-linked carbohydrate and sialic acid. The Journal of biological chemistry 262, 16271-16274.

Takahashi, R.H., Almeida, C.G., Kearney, P.F., Yu, F., Lin, M.T., Milner, T.A., and Gouras, G.K. (2004). Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain. J Neurosci 24, 3592-3599.

Takahashi, K., Rochford, C.D., and Neumann, H. (2005). Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med 201, 647-657.

Tall, A.R., and Yvan-Charvet, L. (2015). Cholesterol, inflammation and innate immunity. Nat Rev Immunol 15, 104-116.

Tamaoka, A., Odaka, A., Ishibashi, Y., Usami, M., Sahara, N., Suzuki, N., Nukina, N., Mizusawa, H., Shoji, S., Kanazawa, I., *et al.* (1994). APP717 missense mutation affects the ratio of amyloid beta protein species (A beta 1-42/43 and a beta 1-40) in familial Alzheimer's disease brain. The Journal of biological chemistry 269, 32721-32724.

Tamboli, I.Y., Prager, K., Thal, D.R., Thelen, K.M., Dewachter, I., Pietrzik, C.U., St George-Hyslop, P., Sisodia, S.S., De Strooper, B., Heneka, M.T., *et al.* (2008). Loss of gamma-secretase function impairs endocytosis of lipoprotein particles and membrane cholesterol homeostasis. J Neurosci 28, 12097-12106.

Tamnes, C.K., Ostby, Y., Fjell, A.M., Westlye, L.T., Due-Tonnessen, P., and Walhovd, K.B. (2010). Brain maturation in adolescence and young adulthood: regional age-related changes in cortical thickness and white matter volume and microstructure. Cereb Cortex 20, 534-548.

Tanaka, N., Abe-Dohmae, S., Iwamoto, N., Fitzgerald, M.L., and Yokoyama, S. (2010). Helical apolipoproteins of high-density lipoprotein enhance phagocytosis by stabilizing ATP-binding cassette transporter A7. Journal of lipid research 51, 2591-2599.

Tanaka, N., Abe-Dohmae, S., Iwamoto, N., and Yokoyama, S. (2011). Roles of ATP-binding cassette transporter A7 in cholesterol homeostasis and host defense system. J Atheroscler Thromb 18, 274-281.

Tanzi, R.E. (2012). The genetics of Alzheimer disease. Cold Spring Harb Perspect Med 2.

Tardif, J.C., Rheaume, E., Lemieux Perreault, L.P., Gregoire, J.C., Feroz Zada, Y., Asselin, G., Provost, S., Barhdadi, A., Rhainds, D., L'Allier, P.L., *et al.* (2015). Pharmacogenomic determinants of the cardiovascular effects of dalcetrapib. Circ Cardiovasc Genet 8, 372-382.

Taylor, J.P., Hardy, J., and Fischbeck, K.H. (2002). Toxic proteins in neurodegenerative disease. Science 296, 1991-1995.

Thomas, A., Charbonneau, J.L., Fournaise, E., and Chaurand, P. (2012). Sublimation of new matrix candidates for high spatial resolution imaging mass spectrometry of lipids: enhanced information in both positive and negative polarities after 1,5-diaminonapthalene deposition. Anal Chem 84, 2048-2054.

Thompson, A., Di Angelantonio, E., Sarwar, N., Erqou, S., Saleheen, D., Dullaart, R.P., Keavney, B., Ye, Z., and Danesh, J. (2008). Association of cholesteryl ester transfer protein genotypes with CETP mass and activity, lipid levels, and coronary risk. JAMA : the journal of the American Medical Association 299, 2777-2788.

Ulyanova, T., Blasioli, J., Woodford-Thomas, T.A., and Thomas, M.L. (1999). The sialoadhesin CD33 is a myeloid-specific inhibitory receptor. Eur J Immunol 29, 3440-3449.

van den Hoek, A.M., van der Hoorn, J.W., Maas, A.C., van den Hoogen, R.M., van Nieuwkoop, A., Droog, S., Offerman, E.H., Pieterman, E.J., Havekes, L.M., and Princen, H.M. (2014). APOE*3Leiden.CETP transgenic mice as model for pharmaceutical treatment of the metabolic syndrome. Diabetes Obes Metab 16, 537-544.

van der Wulp, M.Y., Verkade, H.J., and Groen, A.K. (2013). Regulation of cholesterol homeostasis. Mol Cell Endocrinol 368, 1-16.

van Echten-Deckert, G., and Walter, J. (2012). Sphingolipids: critical players in Alzheimer's disease. Prog Lipid Res 51, 378-393.

Vance, J.E. (2012). Dysregulation of cholesterol balance in the brain: contribution to neurodegenerative diseases. Dis Model Mech 5, 746-755.

Vance, J.E., and Hayashi, H. (2010). Formation and function of apolipoprotein E-containing lipoproteins in the nervous system. Biochimica et biophysica acta 1801, 806-818.

Vasan, R.S., Pencina, M.J., Robins, S.J., Zachariah, J.P., Kaur, G., D'Agostino, R.B., and Ordovas, J.M. (2009). Association of circulating cholesteryl ester transfer protein activity with incidence of cardiovascular disease in the community. Circulation 120, 2414-2420.

Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., *et al.* (1999). Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286, 735-741.

Vergeer, M., Holleboom, A.G., Kastelein, J.J., and Kuivenhoven, J.A. (2010). The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis? Journal of lipid research 51, 2058-2073.

Vetrivel, K.S., Cheng, H., Lin, W., Sakurai, T., Li, T., Nukina, N., Wong, P.C., Xu, H., and Thinakaran, G. (2004). Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. The Journal of biological chemistry 279, 44945-44954.

Vitali, C., Wellington, C.L., and Calabresi, L. (2014). HDL and cholesterol handling in the brain. Cardiovasc Res 103, 405-413.

Wahrle, S., Das, P., Nyborg, A.C., McLendon, C., Shoji, M., Kawarabayashi, T., Younkin, L.H., Younkin, S.G., and Golde, T.E. (2002). Cholesterol-dependent gamma-secretase activity in buoyant cholesterol-rich membrane microdomains. Neurobiol Dis 9, 11-23.

Wang, H., and Eckel, R.H. (2014). What are lipoproteins doing in the brain? Trends Endocrinol Metab 25, 8-14.

Wang, M., Zukas, A.M., Hui, Y., Ricciotti, E., Pure, E., and FitzGerald, G.A. (2006). Deletion of microsomal prostaglandin E synthase-1 augments prostacyclin and retards atherogenesis. Proceedings of the National Academy of Sciences of the United States of America 103, 14507-14512.

Wang, Y., Cella, M., Mallinson, K., Ulrich, J.D., Young, K.L., Robinette, M.L., Gilfillan, S., Krishnan, G.M., Sudhakar, S., Zinselmeyer, B.H., *et al.* (2015). TREM2 lipid sensing sustains the microglial response in an Alzheimer's disease model. Cell 160, 1061-1071.

Warstadt, N.M., Dennis, E.L., Jahanshad, N., Kohannim, O., Nir, T.M., McMahon, K.L., de Zubicaray, G.I., Montgomery, G.W., Henders, A.K., Martin, N.G., *et al.* (2014). Serum cholesterol and variant in cholesterol-related gene CETP predict white matter microstructure. Neurobiology of aging 35, 2504-2513.

Webster, S., Lue, L.F., Brachova, L., Tenner, A.J., McGeer, P.L., Terai, K., Walker, D.G., Bradt, B., Cooper, N.R., and Rogers, J. (1997). Molecular and cellular characterization of the membrane attack complex, C5b-9, in Alzheimer's disease. Neurobiology of aging 18, 415-421.

Weggen, S., and Beher, D. (2012). Molecular consequences of amyloid precursor protein and presenilin mutations causing autosomal-dominant Alzheimer's disease. Alzheimers Res Ther 4, 9.

Weggen, S., Eriksen, J.L., Das, P., Sagi, S.A., Wang, R., Pietrzik, C.U., Findlay, K.A., Smith, T.E., Murphy, M.P., Bulter, T., *et al.* (2001). A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature 414, 212-216.

Wells, K., Farooqui, A.A., Liss, L., and Horrocks, L.A. (1995). Neural membrane phospholipids in Alzheimer disease. Neurochem Res 20, 1329-1333.

Winkler, E., Kamp, F., Scheuring, J., Ebke, A., Fukumori, A., and Steiner, H. (2012). Generation of Alzheimer disease-associated amyloid beta42/43 peptide by gamma-secretase can be inhibited directly by modulation of membrane thickness. The Journal of biological chemistry 287, 21326-21334.

Wischik, C.M., Novak, M., Edwards, P.C., Klug, A., Tichelaar, W., and Crowther, R.A. (1988). Structural characterization of the core of the paired helical filament of Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America 85, 4884-4888.

Wolozin, B. (2001). A fluid connection: cholesterol and Abeta. Proceedings of the National Academy of Sciences of the United States of America 98, 5371-5373.

Wolozin, B., Kellman, W., Ruosseau, P., Celesia, G.G., and Siegel, G. (2000). Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. Arch Neurol 57, 1439-1443.

Wong, P.C., Zheng, H., Chen, H., Becher, M.W., Sirinathsinghji, D.J., Trumbauer, M.E., Chen, H.Y., Price, D.L., Van der Ploeg, L.H., and Sisodia, S.S. (1997). Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. Nature 387, 288-292.

Wong, W.B., Lin, V.W., Boudreau, D., and Devine, E.B. (2013). Statins in the prevention of dementia and Alzheimer's disease: a meta-analysis of observational studies and an assessment of confounding. Pharmacoepidemiol Drug Saf 22, 345-358.

Woodling, N.S., and Andreasson, K.I. (2016). Untangling the Web: Toxic and Protective Effects of Neuroinflammation and PGE2 Signaling in Alzheimer's Disease. ACS Chem Neurosci 7, 454-463.

Woodling, N.S., Wang, Q., Priyam, P.G., Larkin, P., Shi, J., Johansson, J.U., Zagol-Ikapitte, I., Boutaud, O., and Andreasson, K.I. (2014). Suppression of Alzheimer-associated inflammation by microglial prostaglandin-E2 EP4 receptor signaling. J Neurosci 34, 5882-5894.

Woodruff, G., Reyna, S.M., Dunlap, M., Van Der Kant, R., Callender, J.A., Young, J.E., Roberts, E.A., and Goldstein, L.S. (2016). Defective Transcytosis of APP and Lipoproteins in Human iPSC-Derived Neurons with Familial Alzheimer's Disease Mutations. Cell Rep 17, 759-773.

Wouters, K., van Gorp, P.J., Bieghs, V., Gijbels, M.J., Duimel, H., Lutjohann, D., Kerksiek, A., van Kruchten, R., Maeda, N., Staels, B., *et al.* (2008). Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology 48, 474-486.

Wrigley, J.D., Schurov, I., Nunn, E.J., Martin, A.C., Clarke, E.E., Ellis, S., Bonnert, T.P., Shearman, M.S., and Beher, D. (2005). Functional overexpression of gamma-secretase reveals protease-independent trafficking functions and a critical role of lipids for protease activity. The Journal of biological chemistry 280, 12523-12535.

Xu, Q., Li, Y., Cyras, C., Sanan, D.A., and Cordell, B. (2000). Isolation and characterization of apolipoproteins from murine microglia. Identification of a low density lipoprotein-like apolipoprotein J-rich but E-poor spherical particle. The Journal of biological chemistry 275, 31770-31777.

Yamada, M., Tsukagoshi, H., Otomo, E., and Hayakawa, M. (1987). Cerebral amyloid angiopathy in the aged. J Neurol 234, 371-376.

Yamada, T., Kawata, M., Arai, H., Fukasawa, M., Inoue, K., and Sato, T. (1995). Astroglial localization of cholesteryl ester transfer protein in normal and Alzheimer's disease brain tissues. Acta Neuropathol 90, 633-636.

Yan, Z., Yan, H., and Ou, H. (2012). Human thyroxine binding globulin (TBG) promoter directs efficient and sustaining transgene expression in liver-specific pattern. Gene 506, 289-294.

Yang, G., Zhou, R., and Shi, Y. (2017). Cryo-EM structures of human gamma-secretase. Curr Opin Struct Biol 46, 55-64.

Yang, W., Bai, Y., Xiong, Y., Zhang, J., Chen, S., Zheng, X., Meng, X., Li, L., Wang, J., Xu, C., *et al.* (2016). Potentiating the antitumour response of CD8(+) T cells by modulating cholesterol metabolism. Nature 531, 651-655.

Yao, Z., and McLeod, R.S. (1994). Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. Biochimica et biophysica acta 1212, 152-166.

Young-Pearse, T.L., Bai, J., Chang, R., Zheng, J.B., LoTurco, J.J., and Selkoe, D.J. (2007). A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. J Neurosci 27, 14459-14469.

Yin, R.H., Yu, J.T., and Tan, L. (2015). The Role of SORL1 in Alzheimer's Disease. Mol Neurobiol 51, 909-918.

Yu, L., Shulman, J.M., Chibnik, L., Leurgans, S., Schneider, J.A., De Jager, P.L., and Bennett, D.A. (2012). The CETP I405V polymorphism is associated with an increased risk of Alzheimer's disease. Aging Cell 11, 228-233.

Zarrouk, A., Debbabi, M., Bezine, M., Karym, E.M., Badreddine, A., Rouaud, O., Moreau, T., Cherkaoui-Malki, M., El Ayeb, M., Nasser, B., *et al.* (2018). Lipid Biomarkers in Alzheimer's Disease. Curr Alzheimer Res 15, 303-312.

Zetterstrom, R. (2009). The 1964 Nobel Prize for the discovery of the biosynthesis of cholesterol. Acta Paediatr 98, 1223-1227.

Zhang, J., and Liu, Q. (2015). Cholesterol metabolism and homeostasis in the brain. Protein Cell 6, 254-264.

Zhang, J., and Rivest, S. (2000). A functional analysis of EP4 receptor-expressing neurons in mediating the action of prostaglandin E2 within specific nuclei of the brain in response to circulating interleukin-1beta. Journal of neurochemistry 74, 2134-2145.

Zhang, L., Yan, F., Zhang, S., Lei, D., Charles, M.A., Cavigiolio, G., Oda, M., Krauss, R.M., Weisgraber, K.H., Rye, K.A., *et al.* (2012). Structural basis of transfer between lipoproteins by cholesteryl ester transfer protein. Nat Chem Biol 8, 342-349.

Zhang, Z., Yamashita, S., Hirano, K., Nakagawa-Toyama, Y., Matsuyama, A., Nishida, M., Sakai, N., Fukasawa, M., Arai, H., Miyagawa, J., *et al.* (2001). Expression of cholesteryl ester transfer protein in human atherosclerotic lesions and its implication in reverse cholesterol transport. Atherosclerosis 159, 67-75.

Zhang, Z., Yan, R., Zhang, Q., Li, J., Kang, X., Wang, H., Huan, L., Zhang, L., Li, F., Yang, S., *et al.* (2014). Hes1, a Notch signaling downstream target, regulates adult hippocampal neurogenesis following traumatic brain injury. Brain Res 1583, 65-78.

Zhao, G., Tan, J., Mao, G., Cui, M.Z., and Xu, X. (2007). The same gamma-secretase accounts for the multiple intramembrane cleavages of APP. Journal of neurochemistry 100, 1234-1246.

Zheng, H., Jiang, M., Trumbauer, M.E., Sirinathsinghji, D.J., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W., et al. (1995). beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81, 525-531.

Zhong, S., Sharp, D.S., Grove, J.S., Bruce, C., Yano, K., Curb, J.D., and Tall, A.R. (1996). Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. The Journal of clinical investigation 97, 2917-2923.

Zhu, Y., Bujo, H., Yamazaki, H., Ohwaki, K., Jiang, M., Hirayama, S., Kanaki, T., Shibasaki, M., Takahashi, K., Schneider, W.J., *et al.* (2004). LR11, an LDL receptor gene family member, is a novel regulator of smooth muscle cell migration. Circ Res 94, 752-758.

Zilversmit, D.B., Hughes, L.B., and Balmer, J. (1975). Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. Biochimica et biophysica acta 409, 393-398.

Zimetti, F., Caffarra, P., Ronda, N., Favari, E., Adorni, M.P., Zanotti, I., Bernini, F., Barocco, F., Spallazzi, M., Galimberti, D., *et al.* (2017). Increased PCSK9 Cerebrospinal Fluid Concentrations in Alzheimer's Disease. J Alzheimers Dis 55, 315-320.

Zlokovic, B.V. (2008). The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron 57, 178-201.

APPENDIX

Table 5: Primers sequences for RT-qPCR.

Target	Forward primer	Reverse primer
hCETP	CAGATCAGCCACTTGTCCAT	CAGCTGTGTGTTGATCTGGA
mABCA7	TTCTCAGTCCCTCGTCACCCAT	GCTCTTGTCTGAGGTTCCTCGT
mTNFα	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
mIL1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGT
mTLR4	AGCTTCTCCAATTTTTCAGAACTTC	TGAGAGGTGGTGTAAGCCATGC
mTREM2	ACAGCACCTCCAGGAATCAAG	AACTTGCTCAGGAGAACGCA
mIL6	CCTCTGGTCTTCTGGAGTACC	ACTCCTTCTGTGACTCCAGC
hHES1	CGGAATCCCCTGTCTACCTC	AATGCCGGGAGCTATCTTTCT
hP21	GCCTTAGCCCTCACTCTGTG	AGCTGGCCTTAGAGGTGACA
mHPRT	CCAGTTTCACTAATGACACAAACG	CTGGTGAAAAGGACCTCTCGAAG
mPSMC4	CCGCTTACACACTTCGAGCTGT	GTGATGTGCCACAGCCTTTGCT
mGAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
mβACT	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG
hAPP	CCTTCTCGTTCCTGACAAGTGC	GGCAGCAACATGCCGTAGTCAT

Table 6: Calculated fatty acid a lipids composition of the animal diets used here.

Calculated fatty acids (g/kg)	Diet			
	control diet	1%Chol.	21% FA	21% FA & 1%Chol.
SFA	26.1	26.1	136.7	136.7
MUFA	14.6	14.6	65.7	65.7
PUFA	9.1	9.1	7.4	7.4
4:0	1.4	1.4	8.0	8.0
6:0	0.9	0.9	4.8	4.8
8:0	0.4	0.4	2.3	2.3
10:0	0.7	0.7	4.2	4.2
11:0	0.0	0.0	0.2	0.2
12:0	1.2	1.2	6.5	6.5
14:0	4.4	4.4	24.6	24.6
14:1	0.3	0.3	1.7	1.7
15:0	0.6	0.6	3.4	3.4
16:0	11.2	11.2	55.0	55.0
16:1	0.7	0.7	4.0	4.0
17:0	0.3	0.3	1.5	1.5
17:1	0.1	0.1	0.4	0.4
18:0	5.2	5.2	26.3	26.3
18:1	13.5	13.5	59.2	59.2
18:2 linolenic acid	7.9	7.9	6.1	6.1
18:3 α-linolenic acid	1.2	1.2	1.1	1.1
20:1	0.1	0.1	0.4	0.4
20:4 arachidonic acid	0.0	0.0	0.2	0.2
n-6	7.9	7.9	6.3	6.3
n-3	1.2	1.2	1.1	1.1
Cholesterol	0.1	10.1	0.6	10.6
n-6:n-3 ratio	6.6	6.6	6.0	6.0
n-3:n-6 ratio	0.2	0.2	0.2	0.2

Embedded in the membrane: How lipids confer activity and specificity to intramembrane proteases

Sandra Paschkowsky, Felix Oestereich, Lisa Marie Munter

J Membr. Biol., 2017 Dec 19., doi: 10.1007/s00232-017-0008-5

The Journal of Membrane Biology
https://doi.org/10.1007/s00232-017-0008-5

Embedded in the Membrane: How Lipids Confer Activity and Specificity to Intramembrane Proteases

Sandra Paschkowsky[1] · Felix Oestereich[1] · Lisa Marie Munter[1]

Received: 16 November 2017 / Accepted: 12 December 2017

Abstract
Proteases, sharp yet unforgivable tools of every cell, require tight regulation to ensure specific non-aberrant cleavages. The relatively recent discovered class of intramembrane proteases has gained increasing interest due to their involvement in important signaling pathways linking them to diseases including Alzheimer's disease and cancer. Despite tremendous efforts, their regulatory mechanisms have only started to unravel. There is evidence that the membrane composition itself can regulate intramembrane protease activity and specificity. In this review, we highlight the work on γ-secretase and rhomboid proteases and summarize several studies as to how different lipids impact on enzymatic activity.

Keywords Membrane lipids in protease regulation · Drug targets · Pathophysiology · Rhomboid protease · Protein-lipid interaction · Gamma-secretase

Intramembrane Proteases: An Emerging Field of Research

Intramembrane proteases, also formerly known as intramembrane cleaving proteases (i-CLips), constitute a relatively new class of proteases with the site-2 protease discovered in 1997 (Rawson 2013), then followed by γ-secretase (Ray et al. 1999; Wolfe et al. 1999a, b; Haass 2000) and rhomboid proteases in 2001 (Urban et al. 2001). In contrast to soluble proteases, intramembrane proteases are multi-pass transmembrane proteins that form a catalytically active center within the plane of the membrane allowing such proteases to cleave peptide bonds within the hydrophobic environment of the lipid bilayer (Wang et al. 2006; Ben-Shem et al. 2007; Lemieux et al. 2007; Langosch et al. 2015). Consequently, cleavages by intramembrane proteases liberate substrate fragments on both sides of the membrane emphasizing the very distinguished roles of intramembrane proteases in receiving and transmitting signals across membranes both within and between cells (McCarthy et al. 2017). Since intramembrane proteases reside in different subcellular membranes and cell types (Urban 2016), an obvious assumption is that the membrane composition may influence their activity. However, the investigation of how lipids may regulate protease activity is a daunting task as our current understanding of lipid–protein interactions is relatively limited and most biophysical and biochemical methods are not well suited to study such interactions. Nevertheless, with improving technology, novel tools are being used to better address the question of how lipids regulate intramembrane proteolysis. Here, we will summarize several findings showcasing the recent advances in the field.

The Four Families of Intramembrane Proteases

To date, four families of intramembrane proteases are distinguished based on their catalytic mechanisms (Verhelst 2017). Site-2 proteases are zinc metalloproteases, γ-secretase and the related signal peptide peptidases (SPPs) are aspartic acid proteases, and rhomboid proteases are serine proteases. Recently, a novel founding member of glutamatergic intramembrane proteases, Rce1, was identified from the archaebacteria *Methanococcus maripaludis* (Manolaridis et al. 2013), reviewed in Verhelst (2017). The following pathways highlight the involvement of intramembrane proteases in diverse biological contexts and the consequent necessity for their tight regulation. The complex mechanism

✉ Lisa Marie Munter
lisa.munter@mcgill.ca

[1] Department of Pharmacology and Therapeutics, McGill University, Montreal, QC H3G 0B1, Canada

Published online: 19 December 2017

of sensing low cholesterol levels in the endoplasmic reticulum (ER) and subsequent trafficking of the sterol regulatory element-binding protein (SREBP) to the Golgi apparatus for its activation was awarded with the Nobel prize in Physiology and Medicine 1985 (Motulsky 1986). Here, the site-2 protease cleaves SREBPs in the Golgi and releases a nuclear transcription factor to induce the transcription of genes involved in lipid and cholesterol synthesis (Brown et al. 2000). In a similar fashion, site-2 protease also deliberates the activating transcription factor-6 (ATF-6) inducing the transcription of genes required for protein folding and biogenesis in the ER upon activation of the unfolded protein response (Sha et al. 2011).

SPPs were named based on their initially described function to degrade signal peptides that were removed from nascent proteins in the ER (Paetzel et al. 2002). However, SPP also generates antigen peptides that are presented by major histocompatibility complexes (MHC) (Oliveira et al. 2013). Similarly, the currently best-characterized SPP-like protease, SPPL2a, cleaves CD74, a component of MHCII, and is required for B-cell maturation (Beisner et al. 2013; Bergmann et al. 2013; Schneppenheim et al. 2013; Mentrup et al. 2017b). Furthermore, the protease SPPL3 was shown to process glycan-modifying enzymes and influence the *N*-glycosylation of cellular proteins (Voss et al. 2014).

Like SPPs, γ-secretase belongs to the family of aspartic acid proteases, but occurs as a complex composed of four subunits, i.e., presenilin (PS1 or PS2 isoforms) carrying the catalytic active residues, anterior pharynx-defective (Aph1αL, Aph1αS, or Aph1β isoforms), nicastrin, and presenilin-enhancer-2 (pen-2) (Kaether et al. 2006). Interestingly, γ-secretase and SPP possess opposite topologies: while PSs are oriented with their N termini in the cytosol, the SPPs have their N termini in the ER lumen. As a consequence, γ-secretase almost exclusively cleaves type-I oriented substrates, whereas SPPs predominantly process single-pass substrates with type-II orientation (Haass and Steiner 2002). γ-Secretase, known as "proteasome of the membrane," processes around 100 substrates (Kopan and Ilagan 2004). Central signaling pathways, such as the Notch signaling pathway regulating embryonic development and cell differentiation, are critically dependent on γ-secretase activity (Bai and Pfaff 2011). Aph and PS come in different isoforms generating six different complexes in human that may regulate processing of different subsets of substrates (Shirotani et al. 2004a, b; Serneels et al. 2005; Acx et al. 2014). γ-Secretase activity and cleavage mechanism was intensively studied in the Alzheimer's disease research field due to its processing of the amyloid precursor protein (APP), the probably best-characterized γ-secretase substrate to date, see below.

The protease 'Ras and a-factor-converting enzyme 1' (Rce1) is a prenyl-endopeptidase that cleaves farnesylated (and geranylgeranylated) substrates at the CAAX motif, and regulates signaling through the Ras superfamily of small GTPases and subsequent mitogen-activated protein kinase (MAPK) signaling pathways (Wang and Casey 2016). It is possible that the lipid anchor enters the catalytic site of the protease together with the peptide substrate, raising the interesting possibility that lipid modifications may constitute co-factors for substrate recognition or catalysis (Manolaridis et al. 2013). Finally, rhomboid proteases are found in all kingdoms of life and are conserved probably down to the least common ancestor (Lemberg and Freeman 2007). They were discovered in *Drosophila melanogaster* as regulators of signaling through the endothelial growth factor (EGF) during development (Wasserman et al. 2000). In mammals, the inactive pseudo-rhomboid iRhom2 is required for TNF-α release in response to bacterial infections (Adrain et al. 2012). Further, the mitochondrial rhomboid protease PARL has been linked to mitophagy and Parkinson's disease (Jin et al. 2010; McQuibban and Bulman 2011; Meissner et al. 2011; Greene et al. 2012; Shi and McQuibban 2017), and our lab recently found that the rhomboid protease RHBDL4 processes APP linking rhomboid proteases to Alzheimer's disease (Paschkowsky et al. 2016).

Membrane Embedded Proteases: Boon or Bane for Drug Discovery?

As this brief overview hopefully conveyed, intramembrane proteases are involved in many central biological pathways, with implications in multiple diseases certainly rendering them appealing drug targets (Dusterhoft et al. 2017). Some intramembrane proteases are known to exhibit restricted expression patterns and are only found in certain cell types or organs, suggesting that potential drugs may have the benefit of minimal side and/or adverse effects. Drug development excelled for γ-secretase over the last two decades aiming to inhibit the production of toxic Aβ peptides linked to Alzheimer's disease. However, γ-secretase inhibitors had severe side effects through the inhibition of the Notch pathway, so that 2nd generation inhibitors were developed with substrate-selective properties, meaning that they inhibited APP processing, but to a lesser extent Notch processing. In parallel, γ-secretase modulators were developed to alter the cleavage pattern and processivity of this protease (De Strooper and Chavez Gutierrez 2015). However, none of these inhibitors or modulators succeeded in clinical trials to prevent or treat Alzheimer's disease. The compounds are currently being repurposed and tested for the treatment of certain cancers (Kumar et al. 2016). Similarly, inhibitors have been identified for other intramembrane proteases, although most show only low affinity (Wolfe 2010; Pierrat et al. 2011; Strisovsky 2016; Verhelst 2017). Those molecules may represent good

lead structures for further development through medicinal chemistry (Verhelst 2017).

One essential and often misguided aspect of drug design is a solid knowledge of the physiological regulation of intramembrane proteases: which cellular events activate intramembrane proteases and trigger the cleavage of their substrates? And are these triggers substrate specific? Since proteolysis is an irreversible process, it is likely that proteases are not constitutively active, but rather tightly regulated. Several mechanisms have been identified or suggested for the regulation of intramembrane proteases. (A) With the exception of rhomboid proteases, SPPL3 and Rce1, intramembrane proteases usually require an initiating cleavage event mediated by sheddases including the family of 'a disintegrin and metalloproteases' (ADAMs), β-secretase, and others. Such pathways are called 'Regulated intramembrane proteolysis' (RIP) and the regulation seems mediated by sheddases in those cases (Fig. 1). The subsequent intramembrane protease recognizes and further cleaves the remaining stubs (Lichtenthaler et al. 2011). Of note, exceptions for certain substrates have been reported for γ-secretase and SPP (Boname et al. 2014; Hsu et al. 2015; Laurent et al. 2015). (B) Compartmentalization of enzyme and substrate. This is exemplified by the SREBPs that localize in the ER until a change in membrane composition occurs, i.e., a drop in ER cholesterol levels below 5% (Das et al. 2014) triggers the trafficking of SREBPs to the Golgi apparatus where the site-2 proteases localizes, allowing SREBP cleavage exclusively in the Golgi. (C) Transmembrane helix instability, specifically for rhomboid proteases and SPPs. Proposed substrates often contain amino acid residues that may destabilize the transmembrane α-helix or cause a kink (Strisovsky et al. 2009; Fluhrer et al. 2012; Huttl et al. 2016). However, it should be noted that for rhomboid proteases only few substrates have been validated in vivo. (D) Lastly, different lipid compositions of the membrane may affect the protease activity and/or specificity of some intramembrane proteases (Urban and Wolfe 2005). This idea goes hand in hand with the fact that different cellular compartments exhibit different lipid compositions (van Meer et al. 2008). Furthermore, membranes are intrinsically heterogeneous and form microdomains with specialized functions and compositions (Simons and Sampaio 2011). Intramembrane proteases are found in all subcellular compartments thereby raising the intriguing possibility that enzyme activity and specificity is at least in parts driven by lipid composition. In this regard, Table 1 summarizes known membrane lipid compositions

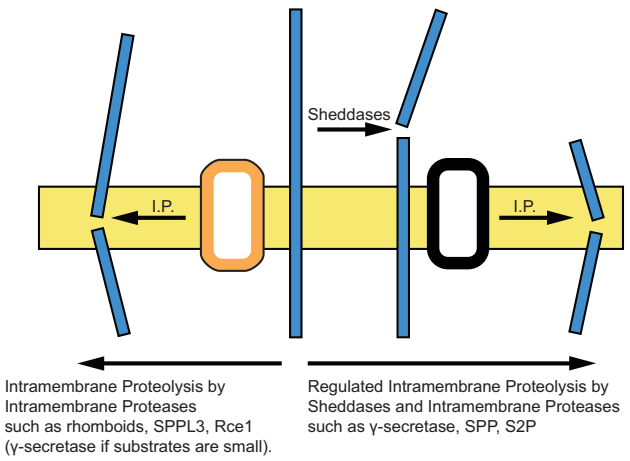


Fig. 1 Substrates are either directly recognized by intramembrane proteases (I.P.) and cleaved (pathway to the left), or they undergo a first ectodomain shedding event before intramembrane proteases recognize the remaining stubs for processing (pathway to the right) resulting in "regulated intramembrane proteolysis" (RIP). Different mechanisms for regulation of proteolysis are implicit

Table 1 Chart comparing the lipid composition of subcellular membranes and the localization of intramembrane proteases

Compartment/organelle	Membrane composition approximations	Intramembrane protease
ER	PC: 50–55% PE: 30% PI: 15% PS: 5% Ratio Chol/PL: 0.15 R: up to 5%	SPP SPPL2c RHBDL4
Golgi	PC: 45–50% PE: 15% PI: 10% PS: 5% SM: 10–15% Ratio Chol/PL: 0.2 R: up to 10%	S2P RHBDL1 SPPL3
Plasma membrane	PC: 35–40% PE: 20–25% PI: 5% PS: 10% SM: 20–25% Ratio Chol/PL: 1 R: up to 5%	SPPL2b RHBDL2 γ-Secretase Rce1
Endosome/lysosome	PC: 45–50% PE: 20–25% PI: 5% PS: 2–3% SM: 10% BMP: 15% Chol/PL: 0.5	SPPL2a RHBDL3 γ-Secretase
Mitochondria	PC: 45% PE: 30% PI: 10% PS: 1% CL: 10% Chol/PL: 0.1 R: up to 5%	PARL

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SM* sphingomyelin, *Chol/PL* cholesterol-to-phospholipid ratio, *R* remaining lipids

as described by van Meer et al. and the respective subcellular localization of mammalian proteases (McQuibban et al. 2003; van Meer et al. 2008; Fleig et al. 2012; Bergbold and Lemberg 2013; Manolaridis et al. 2013; Rawson 2013; Mentrup et al. 2017a).

Most work on lipid-mediated regulation of intramembrane proteases has been done on γ-secretase and rhomboid proteases, and thus we will summarize our knowledge with a focus on these two protease families in the following sections.

γ-Secretase and Its Interplay with Membrane Lipids

The four subunits of γ-secretase form a multi-pass transmembrane protein complex with a total of 19 transmembrane sequences allowing for manifold interactions with the surrounding lipids (Edbauer et al. 2003). In fact, γ-secretase localizes to and is active in lipid rafts, membrane microdomains enriched for cholesterol (Wada et al. 2003; Hur et al. 2008). A better understanding of the complex interplay between γ-secretase and membrane lipids will be helpful to develop strategies selectively targeting γ-secretase complexes or specific substrates.

Most studies on the interaction between γ-secretase and lipids used the substrate APP and its processed fragments as a read-out due to the availability of excellent antibodies and research tools for APP. Therefore, we will first introduce the RIP pathway of APP: APP's ectodomain is first shed off by α- or β-secretase, and then the remaining membrane-bound C-terminal stubs become substrates for γ-secretase. To generate Aβ peptides that are suggested to be causative for Alzheimer's disease, APP has to undergo processing by β- and γ-secretase. One of the puzzling questions for many years was how the different Aβ peptides species differing in only a few amino acids in length are generated. Using sophisticated mass spectrometry analysis, it was revealed that γ-secretase has a sequential substrate processing mechanism in which the substrate (the APP C-terminal stub) is cleaved multiple times starting from the cytoplasmic side of the transmembrane sequence towards the middle leading to different Aβ product lines (Sato et al. 2003; Takami et al. 2009; Olsson et al. 2014).

The interaction of γ-secretase and cholesterol has received particular attention since it had been noted that elevated cholesterol levels increase the production of Aβ peptides (Wolozin 2001; Puglielli et al. 2003; Miller and Chacko 2004). In fact, a large proportion of Aβ is generated in lipid rafts, where γ-secretase localizes (Simons and Ikonen 1997; Riddell et al. 2001; Runz et al. 2002; Hur et al. 2008; Rushworth and Hooper 2010). Vice versa, depleting membrane cholesterol decreased γ-secretase activity (Simons et al. 1998; Golde and Eckman 2001) which may be due to its reduced raft association (Urano et al. 2005; Wang et al. 2013). These observations, however, are not sufficient to conclude that cholesterol is directly interacting with γ-secretase. Recently, Jung et al. (2015) identified the acidic cholesterol metabolite 3b-hydroxy-5-cholestenoic acid (CA) as a potential γ-secretase modulator with an EC_{50} of approximately 250 nM, indicating that sterol metabolites directly bind to γ-secretase. CA enhanced the processivity of γ-secretase leading to the generation of less toxic Aβ42 and more non-toxic Aβ38. The authors confirmed the physiological relevance of this interaction by deleting the enzyme responsible for CA production, Cyp27a1, or CA catabolism, Cyp7b1, in mice, and found the same γ-secretase modulating effects (Jung et al. 2015). Previously, the authors identified also other steroid metabolites to modulate γ-secretase activity (Jung et al. 2013).

Changes in membrane lipid composition other than cholesterol have also been shown to affect the proteolytic processing of γ-secretase (Osenkowski et al. 2008; Grimm et al. 2011; Lemkul and Bevan 2011; Grimm et al. 2012). In a very elegant in vitro experiment using detergent free multi-lamellar liposomes, Osenkowski et al. (2008) analyzed the effects of the lipid species PC, PE, PS, PI, and PA on γ-secretase activity as determined by the amount of Aβ peptides produced. In the presence of PC, the species PE, PS, and PA increased γ-secretase activity, while PI reduced the activity. Additionally, they found that PC liposomes enriched with 5–25% sphingolipids (SM, cerebrosides, and gangliosides), a major component of lipid microdomains or lipid rafts (Vetrivel et al. 2004; Hur et al. 2008), increased Aβ production. Furthermore, they used lipid mixtures resembling those of different organelles and found the highest γ-secretase activity in lipid compositions resembling the plasma membrane, especially lipid rafts. In a similar fashion, they found that brain-derived lipid mixtures yielded the highest γ-secretase activity when compared to the lipid mixtures of other organs (Osenkowski et al. 2008). Again, although very informative, these experiments do not report on a direct interaction of phospholipids with γ-secretase. More striking evidence for a direct interaction of phospholipids was provided in form of the γ-secretase cryo-EM structure that showed phosphatidylcholines bound to the complex (Yang et al. 2017). In a recent unbiased approach by Ayciriex et al. (2016), the authors purified the γ-secretase complex, reconstituted it in proteoliposomes, and analyzed the lipids that interacted and were co-purified with the complex using shotgun lipidomics. They found a 40- to 50-fold molar excess of lipids to the γ-secretase complex. Among the most abundant lipids, they measured a lipid-to-protein ratio of 3:1 for cholesterol and 20:1 for phosphatidylcholines, indicating that those lipids are tightly bound and likely form a specific lipid shell around γ-secretase. Moreover,

mostly saturated and monounsaturated forms of fatty acid side chains associated with the γ-secretase microenvironment. Most interestingly, among the most represented lipids, PI was the only phospholipid found to reduce the activity of the complex (Osenkowski et al. 2008). Pharmacological modifications of the lipid environment also drastically altered its activity. Locally degrading lipids using Phospholipase A2 (PLA2) can alter the interactions between the transmembrane helices, leading to the disruption of the structure of the complex and abolished activity (Ayciriex et al. 2016).

However, a direct interaction of lipids with γ-secretase may not be the only regulatory effect that the local lipid composition confers. It is likely that membrane properties such as thickness, curvature, and fluidity will affect the activity, assembly, or trafficking of the complex as well. In this regard, Winkler et al. focused on the membrane thickness and lipid chain order (Winkler et al. 2012). The authors found that the optimal membrane thickness for the highest γ-secretase activity lies between 26 and 29 Angström. Further, polyunsaturated fatty acids were not beneficial for γ-secretase activity; however, these effects were not due to changes in membrane fluidity. Additionally, activity is abolished when γ-secretase is embedded in a membrane consisting of only saturated lipids. In a similar approach, Holmes et al. systematically altered both the fatty acids' chain length and degree of saturation, as well as the polar lipid head group, and found that monounsaturated fatty acids chaining from a cis to trans double bond led to increased γ-secretase activity (Holmes et al. 2012). When analyzing the fatty acids' chain length, they found that while very short (14 C-atoms) or long (> 22 C-atoms) led to decreased activity, the highest activity was observed with 18–20 C-atom fatty acids' chains.

However, it is important to emphasize that there may be a potential substrate bias in the published studies about membrane alterations and γ-secretase activity to date. Most of these studies focused only on APP as a substrate due to the availability of excellent research tools for APP. Therefore, we must consider that some of the observed lipid-modulatory effects could potentially be mediated through the substrate considering that APP itself was shown to bind cholesterol directly (Beel et al. 2008; Barrett et al. 2012).

Lastly, it has been proven challenging to assess the effect of the lipid environment on γ-secretase in vivo. However, changes in sphingolipids, cholesterol, as well as the phospholipid composition were noticed in Alzheimer's disease patient brains (Wells et al. 1995; Dietschy 2009; Haughey et al. 2010; He et al. 2010; van Echten-Deckert and Walter 2012; Fonteh et al. 2013; Kosicek and Hecimovic 2013). More recently, several studies confirmed and elaborated on altered lipid compositions in brain and CSF of AD patients (Dietschy 2009; He et al. 2010; van Echten-Deckert and Walter 2012). It remains to be determined if those lipid changes are causative or consequential for disease on-set and progression. Furthermore, cholesterol-enriched diets accelerate the amyloid pathology in animal models of AD in contrast to cholesterol lowering drugs that lead to decreased amyloid pathology (Refolo et al. 2001; Levin-Allerhand et al. 2002; Petanceska et al. 2002). Although the mechanisms in vivo may differ, some effects of lipid changes on γ-secretase activity have been reported in vivo (Xiong et al. 2008).

Altogether, there is substantial evidence for a regulatory role of the lipid environment on γ-secretase activity.

Rhomboid Proteases and Membrane Lipids: More Than Just a One-Way Street?

Rhomboid proteases are a rather novel research area that has gained increasing interest recently due to various publications linking them with diseases including Parkinson's disease, Diabetes, or Alzheimer's disease (Walder et al. 2005; Shi et al. 2011; Paschkowsky et al. 2016; Wust et al. 2016). In fact, they are the best structurally characterized class of intramembrane proteases based on the availability of multiple protein crystal structures (Wang et al. 2006; Wu et al. 2006; Lemieux et al. 2007; Lazareno-Saez et al. 2013; Zoll et al. 2014). Intriguingly, soluble proteases were shown to have dramatically higher substrate affinity than rhomboid proteases prompting that proteolysis of rhomboid proteases is driven by the kinetics of the reaction and not by the affinity of the substrate towards rhomboid (Dickey et al. 2013). Consequently, the membrane environment may somehow confer selectivity, specificity, or enzyme activity. In the following paragraph, we will summarize how lipids interact with rhomboid proteases and how they contribute to a hydrolytic reaction within the hydrophobic environment of the lipid bilayer. Note that so far only bacterial homologues have been crystallized (Wang et al. 2006) and therefore most information about the interplay of proteases and membrane environment arises from structural comparisons with mammalian homologues. However, considering the high degree of evolutionary conservation, one may suggest that there are similar mechanisms of regulation (Lemberg and Freeman 2007). Interestingly, in two rhomboid crystal structures, a phospholipid molecule was bound and co-crystallized with the rhomboid protease; however, no functional role of these lipids has been identified, yet (Ben-Shem et al. 2007; Lemieux et al. 2007).

To analyze the ability of membrane lipids to alter the activity of rhomboid proteases, in vitro activity assays of five rhomboid proteases were performed showing a remarkable dependence of rhomboid activity on membrane composition, and each protease preferred different lipids (Urban and Wolfe 2005). The lipid interaction may be mediated

through a highly conserved WR motif in the loop region 1 (L1) connecting transmembrane sequences 1 and 2 of rhomboid proteases (Urban and Baker 2008). Experimental data were complemented by molecular dynamics (MD) simulations analyzing the interaction of rhomboid proteases with lipids (Bondar et al. 2009). Bondar et al. identified several hydrogen-bond interactions between lipid headgroups and the protease backbone. The authors also confirmed the surprising, but intuitive finding that the protein–lipid interaction actually leads to a rearrangement of lipids that induces a non-uniform membrane thinning [described before by Wang et al. (2006)]. The biggest membrane distortion occurs close to the L1 loop of the rhomboid protease GlpG. Strikingly, positioning of the catalytically active serine residue is also dynamically dependent on the membrane composition, which supports the idea of membrane-composition-dependent rhomboid activity. The authors also highlighted the importance of charged amino acids in the L1 loop to interact with lipid headgroups that could further determine differences in specificity for rhomboid proteases in different membrane environments. These results corroborate the idea that the L1 region of rhomboids may act as sensors for the membrane environment (Bondar et al. 2009). Thinning of the membrane in vicinity of the protease was later confirmed, while additional stabilizing interactions were found between helix 5 and lipids that may stabilize the protease in an open substrate gate conformation (Zhou et al. 2012).

We would like to emphasize two key papers, published by Urban and Moin from 2012 and 2014, providing striking mechanistic insights into the regulatory role of membranes in rhomboid protease activity and specificity. The authors showed that rhomboid proteases are highly specific and cleave at one exact site; however, this is not an intrinsic ability of the enzyme, but is rather determined by the membrane environment. By performing very elegant electron paramagnetic resonance (EPR) spectroscopy experiments, the authors concluded that the membrane confers site specificity by restricting the gating dynamics of rhomboids and imposing constraints (Moin and Urban 2012). Consequently, Urban et al. were able to transform non-substrates into substrates simply by altering the membrane composition using membrane perturbing agents like MβCD or lysoPC in cell cultures. The authors further provided evidence for the ability of different non-steroidal anti-inflammatory drugs (NSAIDs), such as flurbiprofen and sulindac sulfide, to change membrane physics inducing non-specific cleavages of rhomboid proteases (Urban and Moin 2014). Previously, NSAIDs were described to affect γ-secretase processivity and initiated the development of γ-secretase modulators (Zettl et al. 2010). In a broader context, these findings raise the question if potential off-target effects of γ-secretase modulators that are in clinical trials for Alzheimer's disease occur (Urban and Moin 2014).

The notion of membrane composition being able to alter rhomboid protease activity is further supporter by Foo et al. (2015). The authors investigated the effects of hydrophobic mismatch due to membrane thinning and how it affects enzymatic activity. Using a water soluble substrate and altering the length of alkyl chains of detergents and lipids, the authors observed a maximal activity with a defined alkyl length and an inhibition for longer chains. This raises the intriguing possibility that longer and more ordered lipid bilayers might provide an inhibitory environment for various rhomboid proteases and raises the question if lipid rafts might provide compartmentalized activity for specific mammalian rhomboids (Foo et al. 2015).

So far, we have focused on the impact of membrane lipids on protease activity and specificity. However, intramembrane proteases are also directly linked to lipid metabolism, with S2P being the prime example via processing of SREBP [see above, (Brown et al. 2000)]. With regard to rhomboid proteases, recent findings suggest a second layer of complexity indicating that rhomboid proteases may be involved in the regulation of lipid metabolism, too. Cortesio et al. (2015) described a significant role for the budding yeast rhomboid homologue Rbd2 in lipid organization, specifically in compartments necessary for endocytosis. Surprisingly, this regulation does not require protease activity, but solely requires the C-terminus of the protease. This rather unusual finding suggests additional roles for rhomboid proteases on top of their ability to proteolytically process substrates. This notion is widely supported by the existence of inactive pseudorhomboid proteases that gain increasing interest among different research fields due to their ability to chaperone and guide transmembrane proteins, such as TNF-α converting enzyme (TACE) (Adrain et al. 2012; McIlwain et al. 2012; Issuree et al. 2013; Siggs et al. 2014). Another rather unexpected finding came from the yeast research field, where the rhomboid protease Rbd2 was originally discovered in a genetic screen as an important factor for survival under hypoxic conditions (Kim et al. 2015). Kim et al. identified Rbd2 as the potential protease for cleaving Sre1, the yeast homolog of the human SREBP, leading to the activation of transcriptional programs essential for survival under low-oxygen conditions. Hwang et al. (2016) further corroborated these results by providing a more detailed pathway for Sre1 activation and identified Rbd2 as a key switch between SREBP activation and degradation. In parallel, a similar mechanism was also described for *Aspergillus fumigatus*, where survival upon hypoxic conditions is critical for fungal virulence: The *A. fumigatus* rhomboid homolog RbdB/RbdA was found to cleave the SREBP homolog SrbA and to induce downstream gene transcription (Dhingra et al. 2016). Independently, Vaknin et al. (2016) came to the same conclusion (note that the nomenclature RbdA and B refers to the same protein). These findings may close the circle of lipids

regulating rhomboid activity, while rhomboid proteases may regulate the membrane composition, thus raising the intriguing question whether mammalian rhomboid proteases may be involved in lipid or cholesterol metabolism pathways and be potentially part of a feed-forward mechanism.

Conclusion

As laid out in this review, there is increasing evidence that lipids are not only important for embedding intramembrane proteases in the membrane, but rather convey activity and specificity. These exciting findings do not only shed light on the functionality of different lipids, but also provide mechanistic insight into how the membrane environment modulates protein activity. While proteomic as well as lipidomic data have been on the rise given the technological advances, little attention has been paid to the interactions between proteins and lipids. We think that there is surmounting evidence that this area might be of global interest, especially regarding drug development strategies and drug targeting. Finally, considering the high degree of evolutionary conservation within the protease families, we speculate that lipid–protein interactions may have been a very early way of modulating protein activities. We hope that this review may spark new interest in understanding the complex biology of membrane lipids and intramembrane proteases.

Acknowledgements We thank Sherilyn Junelle Recinto for eloquent suggestions for the manuscript.

Funding This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant RGPIN-2015-04645, the Canada Foundation of Innovation Leaders Opportunity Fund Grant 32565, Alzheimer Society of Canada Research Grant 17-02, Fonds d'innovation Pfizer-Fonds de recherche Santé Québec (FRQS) sur la maladie d'Alzheimer et les maladies apparentées 31288, and the Scottish Rite Charitable Foundation 16112.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Acx H, Chavez-Gutierrez L, Serneels L, Lismont S, Benurwar M, Elad N, De Strooper B (2014) Signature amyloid beta profiles are produced by different gamma-secretase complexes. J Biol Chem 289(7):4346–4355

Adrain C, Zettl M, Christova Y, Taylor N, Freeman M (2012) Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. Science 335(6065):225–228

Aychiriex S, Gerber H, Osuna GM, Chami M, Stahlberg H, Shevchenko A, Fraering PC (2016) The lipidome associated with the gamma-secretase complex is required for its integrity and activity. Biochem J 473(3):321–334

Bai G, Pfaff SL (2011) Protease regulation: the Yin and Yang of neural development and disease. Neuron 72(1):9–21

Barrett PJ, Song Y, Van Horn WD, Hustedt EJ, Schafer JM, Hadziselimovic A, Beel AJ, Sanders CR (2012) The amyloid precursor protein has a flexible transmembrane domain and binds cholesterol. Science 336(6085):1168–1171

Beel AJ, Mobley CK, Kim HJ, Tian F, Hadziselimovic A, Jap B, Prestegard JH, Sanders CR (2008) Structural studies of the transmembrane C-terminal domain of the amyloid precursor protein (APP): does APP function as a cholesterol sensor? Biochemistry 47(36):9428–9446

Beisner DR, Langerak P, Parker AE, Dahlberg C, Otero FJ, Sutton SE, Poirot L, Barnes W, Young MA, Niessen S, Wiltshire T, Bodendorf U, Martoglio B, Cravatt B, Cooke MP (2013) The intramembrane protease Sppl2a is required for B cell and DC development and survival via cleavage of the invariant chain. J Exp Med 210(1):23–30

Ben-Shem A, Fass D, Bibi E (2007) Structural basis for intramembrane proteolysis by rhomboid serine proteases. Proc Natl Acad Sci USA 104(2):462–466

Bergbold N, Lemberg MK (2013) Emerging role of rhomboid family proteins in mammalian biology and disease. Biochim Biophys Acta 1828(12):2840–2848

Bergmann H, Yabas M, Short A, Miosge L, Barthel N, Teh CE, Roots CM, Bull KR, Jeelall Y, Horikawa K, Whittle B, Balakishnan B, Sjollema G, Bertram EM, Mackay F, Rimmer AJ, Cornall RJ, Field MA, Andrews TD, Goodnow CC, Enders A (2013) B cell survival, surface BCR and BAFFR expression, CD74 metabolism, and CD8-dendritic cells require the intramembrane endopeptidase SPPL2A. J Exp Med 210(1):31–40

Boname JM, Bloor S, Wandel MP, Nathan JA, Antrobus R, Dingwell KS, Thurston TL, Smith DL, Smith JC, Randow F, Lehner PJ (2014) Cleavage by signal peptide peptidase is required for the degradation of selected tail-anchored proteins. J Cell Biol 205(6):847–862

Bondar AN, Val C, White SH (2009) Rhomboid protease dynamics and lipid interactions. Structure 17(3):395–405

Brown MS, Ye J, Rawson RB, Goldstein JL (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell 100(4):391–398

Cortesio CL, Lewellyn EB, Drubin DG (2015) Control of lipid organization and actin assembly during clathrin-mediated endocytosis by the cytoplasmic tail of the rhomboid protein Rbd2. Mol Biol Cell 26(8):1509–1522

Das A, Brown MS, Anderson DD, Goldstein JL, Radhakrishnan A (2014). Three pools of plasma membrane cholesterol and their relation to cholesterol homeostasis. Elife 3:e02882

De Strooper B, Chavez Gutierrez L (2015) Learning by failing: ideas and concepts to tackle gamma-secretases in Alzheimer's disease and beyond. Annu Rev Pharmacol Toxicol 55:419–437

Dhingra S, Kowlaski CH, Thammahong A, Beattie SR, Bultman KM, Cramer RA (2016). RbdB, a rhomboid protease critical for SREBP Activation and Virulence in *Aspergillus fumigatus*. mSphere 1(2)

Dickey S, Baker R, Cho S, Urban S (2013) Proteolysis inside the membrane is a rate-governed reaction not driven by substrate affinity. Cell 155(6):1270–1281

Dietschy JM (2009) Central nervous system: cholesterol turnover, brain development and neurodegeneration. Biol Chem 390(4):287–293

Dusterhoft S, Kunzel U, Freeman M (2017) Rhomboid proteases in human disease: mechanisms and future prospects. Biochim Biophys Acta 1864(11 Pt B):2200–2209

Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C (2003) Reconstitution of gamma-secretase activity. Nat Cell Biol 5(5):486–488

Fleig L, Bergbold N, Sahasrabudhe P, Geiger B, Kaltak L, Lemberg MK (2012) Ubiquitin-dependent intramembrane rhomboid protease promotes ERAD of membrane proteins. Mol Cell 47(4):558–569

Fluhrer R, Martin L, Klier B, Haug-Kroper M, Grammer G, Nuscher B, Haass C (2012) The alpha-helical content of the transmembrane domain of the British dementia protein-2 (Bri2) determines its processing by signal peptide peptidase-like 2b (SPPL2b). J Biol Chem 287(7):5156–5163

Fonteh AN, Chiang J, Cipolla M, Hale J, Diallo F, Chirino A, Arakaki X, Harrington MG (2013) Alterations in cerebrospinal fluid glycerophospholipids and phospholipase A2 activity in Alzheimer's disease. J Lipid Res 54(10):2884–2897

Foo ACY, Harvey BGR, Metz JJ, Goto NK (2015) Influence of hydrophobic mismatch on the catalytic activity of Escherichia coli GlpG rhomboid protease. Protein Sci 24(4):464–473

Golde TE, Eckman CB (2001) Cholesterol modulation as an emerging strategy for the treatment of Alzheimer's disease. Drug Discov Today 6(20):1049–1055

Greene A, Grenier K, Aguileta M, Muise S, Farazifard R, Haque M, McBride H, Park D, Fon E (2012) Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. EMBO Rep 13(4):378–385

Grimm MO, Kuchenbecker J, Grosgen S, Burg VK, Hundsdorfer B, Rothhaar TL, Friess P, de Wilde MC, Broersen LM, Penke B, Peter M, Vigh L, Grimm HS, Hartmann T (2011) Docosahexaenoic acid reduces amyloid beta production via multiple pleiotropic mechanisms. J Biol Chem 286(16):14028–14039

Grimm MO, Rothhaar TL, Grosgen S, Burg VK, Hundsdorfer B, Haupenthal VJ, Friess P, Kins S, Grimm HS, Hartmann T (2012) Trans fatty acids enhance amyloidogenic processing of the Alzheimer amyloid precursor protein (APP). J Nutr Biochem 23(10):1214–1223

Haass C (2000). Presenilin proteins and their function during embryonic development and Alzheimer's disease. Ernst Schering Res Found Workshop 29:57–64

Haass C, Steiner H (2002) Alzheimer disease gamma-secretase: a complex story of GxGD-type presenilin proteases. Trends Cell Biol 12(12):556–562

Haughey NJ, Bandaru VV, Bae M, Mattson MP (2010) Roles for dysfunctional sphingolipid metabolism in Alzheimer's disease neuropathogenesis. Biochim Biophys Acta 1801(8):878–886

He X, Huang Y, Li B, Gong CX, Schuchman EH (2010) Deregulation of sphingolipid metabolism in Alzheimer's disease. Neurobiol Aging 31(3):398–408

Holmes O, Paturi S, Ye W, Wolfe MS, Selkoe DJ (2012) Effects of membrane lipids on the activity and processivity of purified gamma-secretase. Biochemistry 51(17):3565–3575

Hsu FF, Yeh CT, Sun YJ, Chiang MT, Lan WM, Li FA, Lee WH, Chau LY (2015) Signal peptide peptidase-mediated nuclear localization of heme oxygenase-1 promotes cancer cell proliferation and invasion independent of its enzymatic activity. Oncogene 34(18):2360–2370

Hur JY, Welander H, Behbahani H, Aoki M, Franberg J, Winblad B, Frykman S, Tjernberg LO (2008) Active gamma-secretase is localized to detergent-resistant membranes in human brain. FEBS J 275(6):1174–1187

Huttl S, Helfrich F, Mentrup T, Held S, Fukumori A, Steiner H, Saftig P, Fluhrer R, Schroder B (2016) Substrate determinants of signal peptide peptidase-like 2a (SPPL2a)-mediated intramembrane proteolysis of the invariant chain CD74. Biochem J 473(10):1405–1422

Hwang J, Ribbens D, Raychaudhuri S, Cairns L, Gu H, Frost A, Urban S, Espenshade PJ (2016) A Golgi rhomboid protease Rbd2 recruits Cdc48 to cleave yeast SREBP. EMBO J 35(21):2332–2349

Issuree PD, Maretzky T, McIlwain DR, Monette S, Qing X, Lang PA, Swendeman SL, Park-Min KH, Binder N, Kalliolias GD, Yarilina A, Horiuchi K, Ivashkiv LB, Mak TW, Salmon JE, Blobel CP (2013) iRHOM2 is a critical pathogenic mediator of inflammatory arthritis. J Clin Invest 123(2):928–932

Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ (2010) Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J Cell Biol 191(5):933–942

Jung JI, Ladd TB, Kukar T, Price AR, Moore BD, Koo EH, Golde TE, Felsenstein KM (2013) Steroids as gamma-secretase modulators. FASEB J 27(9):3775–3785

Jung JI, Price AR, Ladd TB, Ran Y, Park HJ, Ceballos-Diaz C, Smithson LA, Hochhaus G, Tang Y, Akula R, Ba S, Koo EH, Shapiro G, Felsenstein KM, Golde TE (2015) Cholestenoic acid, an endogenous cholesterol metabolite, is a potent gamma-secretase modulator. Mol Neurodegener 10:29

Kaether C, Haass C, Steiner H (2006) Assembly, trafficking and function of gamma-secretase. Neurodegener Dis 3(4–5):275–283

Kim J, Ha HJ, Kim S, Choi AR, Lee SJ, Hoe KL, Kim DU (2015) Identification of Rbd2 as a candidate protease for sterol regulatory element binding protein (SREBP) cleavage in fission yeast. Biochem Biophys Res Commun 468(4):606–610

Kopan R, Ilagan M (2004) Gamma-secretase: proteasome of the membrane? Nat Rev Mol Cell Biol 5(6):499–504

Kosicek M, Hecimovic S (2013) Phospholipids and Alzheimer's disease: alterations, mechanisms and potential biomarkers. Int J Mol Sci 14(1):1310–1322

Kumar R, Juillerat-Jeanneret L, Golshayan D (2016) Notch antagonists: potential modulators of cancer and inflammatory diseases. J Med Chem 59(17):7719–7737

Langosch D, Scharnagl C, Steiner H, Lemberg MK (2015) Understanding intramembrane proteolysis: from protein dynamics to reaction kinetics. Trends Biochem Sci 40(6):318–327

Laurent SA, Hoffmann FS, Kuhn PH, Cheng Q, Chu Y, Schmidt-Supprian M, Hauck SM, Schuh E, Krumbholz M, Rubsamen H, Wanngren J, Khademi M, Olsson T, Alexander T, Hiepe F, Pfister HW, Weber F, Jenne D, Wekerle H, Hohlfeld R, Lichtenthaler SF, Meinl E (2015) gamma-Secretase directly sheds the survival receptor BCMA from plasma cells. Nat Commun 6:7333

Lazareno-Saez C, Arutyunova E, Coquelle N, Lemieux MJ (2013) Domain swapping in the cytoplasmic domain of the *Escherichia coli* rhomboid protease. J Mol Biol 425(7):1127–1142

Lemberg MK, Freeman M (2007) Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. Genome Res 17(11):1634–1646

Lemieux MJ, Fischer SJ, Cherney MM, Bateman KS, James MN (2007) The crystal structure of the rhomboid peptidase from Haemophilus influenzae provides insight into intramembrane proteolysis. Proc Natl Acad Sci USA 104(3):750–754

Lemkul JA, Bevan DR (2011) Lipid composition influences the release of Alzheimer's amyloid beta-peptide from membranes. Protein Sci 20(9):1530–1545

Levin-Allerhand JA, Lominska CE, Smith JD (2002) Increased amyloid- levels in APPSWE transgenic mice treated chronically with a physiological high-fat high-cholesterol diet. J Nutr Health Aging 6(5):315–319

Lichtenthaler SF, Haass C, Steiner H (2011) Regulated intramembrane proteolysis–lessons from amyloid precursor protein processing. J Neurochem 117(5):779–796

Manolaridis I, Kulkarni K, Dodd RB, Ogasawara S, Zhang Z, Bineva G, Reilly NO, Hanrahan SJ, Thompson AJ, Cronin N, Iwata S, Barford D (2013) Mechanism of farnesylated CAAX protein processing by the intramembrane protease Rce1. Nature 504(7479):301–305

McCarthy AJ, Coleman-Vaughan C, McCarthy JV (2017) Regulated intramembrane proteolysis: emergent role in cell signalling pathways. Biochem Soc Trans 27:BST20170002

McIlwain DR, Lang PA, Maretzky T, Hamada K, Ohishi K, Maney SK, Berger T, Murthy A, Duncan G, Xu HC, Lang KS, Haussinger D, Wakeham A, Itie-Youten A, Khokha R, Ohashi PS, Blobel CP, Mak TW (2012) iRhom2 regulation of TACE controls TNF-mediated protection against Listeria and responses to LPS. Science 335(6065):229–232

McQuibban G, Bulman D (2011) The PARLance of Parkinson disease. Autophagy 7(7):790–792

McQuibban GA, Saurya S, Freeman M (2003) Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. Nature 423(6939):537–541

Meissner C, Lorenz H, Weihofen A, Selkoe DJ, Lemberg MK (2011) The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. J Neurochem 117(5):856–867

Mentrup T, Fluhrer R, Schroder B (2017a) Latest emerging functions of SPP/SPPL intramembrane proteases. Eur J Cell Biol 96(5):372–382

Mentrup T, Loock AC, Fluhrer R, Schroder B (2017b) Signal peptide peptidase and SPP-like proteases—possible therapeutic targets? Biochim Biophys Acta 1864(11 Pt B):2169–2182

Miller LJ, Chacko R (2004) The role of cholesterol and statins in Alzheimer's disease. Ann Pharmacother 38(1):91–98

Moin SM, Urban S (2012) Membrane immersion allows rhomboid proteases to achieve specificity by reading transmembrane segment dynamics. Elife 1:e00173

Motulsky AG (1986) The 1985 nobel prize in physiology or medicine. Science 231(4734):126–129

Oliveira CC, Querido B, Sluijter M, de Groot AF, van der Zee R, Rabelink MJ, Hoeben RC, Ossendorp F, van der Burg SH, van Hall T (2013) New role of signal peptide peptidase to liberate C-terminal peptides for MHC class I presentation. J Immunol 191(8):4020–4028

Olsson F, Schmidt S, Althoff V, Munter LM, Jin S, Rosqvist S, Lendahl U, Multhaup G, Lundkvist J (2014) Characterization of intermediate steps in amyloid beta (Abeta) production under near-native conditions. J Biol Chem 289(3):1540–1550

Osenkowski P, Ye W, Wang R, Wolfe MS, Selkoe DJ (2008) Direct and potent regulation of gamma-secretase by its lipid microenvironment. J Biol Chem 283(33):22529–22540

Paetzel M, Karla A, Strynadka NCJ, Dalbey RE (2002) Signal peptidases. Chem Rev 102(12):4549–4579

Paschkowsky S, Hamze M, Oestereich F, Munter LM (2016) Processing of the amyloid precursor protein family by rhomboid protease RHBDL4. J Biol Chem 291(42):21903–21912

Petanceska SS, DeRosa S, Olm V, Diaz N, Sharma A, Thomas-Bryant T, Duff K, Pappolla M, Refolo LM (2002) Statin therapy for Alzheimer's disease: will it work? J Mol Neurosci 19(1–2):155–161

Pierrat OA, Strisovsky K, Christova Y, Large J, Ansell K, Bouloc N, Smiljanic E, Freeman M (2011) Monocyclic beta-lactams are selective, mechanism-based inhibitors of rhomboid intramembrane proteases. ACS Chem Biol 6(4):325–335

Puglielli L, Tanzi RE, Kovacs DM (2003) Alzheimer's disease: the cholesterol connection. Nat Neurosci 6(4):345–351

Rawson RB (2013) The site-2 protease. Biochim Biophys Acta 1828(12):2801–2807

Ray WJ, Yao M, Mumm J, Schroeter EH, Saftig P, Wolfe M, Selkoe DJ, Kopan R, Goate AM (1999) Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. J Biol Chem 274(51):36801–36807

Refolo LM, Pappolla MA, LaFrancois J, Malester B, Schmidt SD, Thomas-Bryant T, Tint GS, Wang R, Mercken M, Petanceska SS, Duff KE (2001) A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of Alzheimer's disease. Neurobiol Dis 8(5):890–899

Riddell DR, Christie G, Hussain I, Dingwall C (2001) Compartmentalization of beta-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. Curr Biol 11(16):1288–1293

Runz H, Rietdorf J, Tomic I, de Bernard M, Beyreuther K, Pepperkok R, Hartmann T (2002) of intracellular cholesterol transport alters presenilin localization and amyloid precursor protein processing in neuronal cells. J Neurosci 22(5):1679–1689

Rushworth JV, Hooper NM (2010) Lipid rafts: linking Alzheimer's amyloid-beta production, aggregation, and toxicity at neuronal membranes. Int J Alzheimers Dis 2011:603052

Sato T, Dohmae N, Qi Y, Kakuda N, Misonou H, Mitsumori R, Maruyama H, Koo EH, Haass C, Takio K, Morishima-Kawashima M, Ishiura S, Ihara Y (2003) Potential link between amyloid beta-protein 42 and C-terminal fragment gamma 49–99 of beta-amyloid precursor protein. J Biol Chem 278(27):24294–24301

Schneppenheim J, Dressel R, Huttl S, Lullmann-Rauch R, Engelke M, Dittmann K, Wienands J, Eskelinen EL, Hermans-Borgmeyer I, Fluhrer R, Saftig P, Schroder B (2013) The intramembrane protease SPPL2a promotes B cell development and controls endosomal traffic by cleavage of the invariant chain. J Exp Med 210(1):41–58

Serneels L, Dejaegere T, Craessaerts K, Horre K, Jorissen E, Tousseyn T, Hebert S, Coolen M, Martens G, Zwijsen A, Annaert W, Hartmann D, De Strooper B (2005) Differential contribution of the three Aph1 genes to gamma-secretase activity in vivo. Proc Natl Acad Sci USA 102(5):1719–1724

Sha HB, He Y, Yang L, Qi L (2011) Stressed out about obesity: IRE1 alpha-XBP1 in metabolic disorders. Trends Endocrinol Metab 22(9):374–381

Shi G, Lee JR, Grimes DA, Racacho L, Ye D, Yang H, Ross OA, Farrer M, McQuibban GA, Bulman DE (2011) Functional alteration of PARL contributes to mitochondrial dysregulation in Parkinson's disease. Hum Mol Genet 20(10):1966–1974

Shi G, McQuibban GA (2017) The mitochondrial rhomboid protease PARL is regulated by PDK2 to integrate mitochondrial quality control and metabolism. Cell Rep 18(6):1458–1472

Shirotani K, Edbauer D, Kostka M, Steiner H, Haass C (2004a) Immature nicastrin stabilizes APH-1 independent of PEN-2 and presenilin: identification of nicastrin mutants that selectively interact with APH-1. J Neurochem 89(6):1520–1527

Shirotani K, Edbauer D, Prokop S, Haass C, Steiner H (2004b) Identification of distinct gamma-secretase complexes with different APH-1 variants. J Biol Chem 279(40):41340–41345

Siggs OM, Grieve A, Xu H, Bambrough P, Christova Y, Freeman M (2014) Genetic interaction implicates iRhom2 in the regulation of EGF receptor signalling in mice. Biol Open 3(12):1151–1157

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387(6633):569–572

Simons K, Sampaio JL (2011) Membrane organization and lipid rafts. Cold Spring Harb Perspect Biol 3(10):a004697

Simons M, Keller P, De Strooper B, Beyreuther K, Dotti CG, Simons K (1998) Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc Natl Acad Sci USA 95(11):6460–6464

Strisovsky K (2016) Rhomboid protease inhibitors: emerging tools and future therapeutics. Semin Cell Dev Biol 60:52–62

Strisovsky K, Sharpe HJ, Freeman M (2009) Sequence-specific intramembrane proteolysis: identification of a recognition motif in rhomboid substrates. Mol Cell 36(6):1048–1059

Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, Funamoto S, Ihara Y (2009) gamma-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. J Neurosci 29(41):13042–13052

Urano Y, Hayashi I, Isoo N, Reid PC, Shibasaki Y, Noguchi N, Tomita T, Iwatsubo T, Hamakubo T, Kodama T (2005) Association of active gamma-secretase complex with lipid rafts. J Lipid Res 46(5):904–912

Urban S (2016) SnapShot: cartography of Intramembrane Proteolysis. Cell 167(7):1898–1898

Urban S, Baker RP (2008) In vivo analysis reveals substrate-gating mutants of a rhomboid intramembrane protease display increased activity in living cells. Biol Chem 389(8):1107–1115

Urban S, Lee J, Freeman M (2001) Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. Cell 107(2):173–182

Urban S, Moin SM (2014) A subset of membrane-altering agents and gamma-secretase modulators provoke nonsubstrate cleavage by rhomboid proteases. Cell Rep 8(5):1241–1247

Urban S, Wolfe MS (2005) Reconstitution of intramembrane proteolysis in vitro reveals that pure rhomboid is sufficient for catalysis and specificity. Proc Natl Acad Sci USA 102(6):1883–1888

Vaknin Y, Hillmann F, Iannitti R, Ben Baruch N, Sandovsky-Losica H, Shadkchan Y, Romani L, Brakhage A, Kniemeyer O, Osherov N (2016) Identification and characterization of a novel aspergillus fumigatus rhomboid family putative protease, RbdA, Involved in hypoxia sensing and virulence. Infect Immun 84(6):1866–1878

van Echten-Deckert G, Walter J (2012) Sphingolipids: critical players in Alzheimer's disease. Prog Lipid Res 51(4):378–393

van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9(2):112–124

Verhelst SHL (2017) Intramembrane proteases as drug targets. FEBS J 284(10):1489–1502

Vetrivel KS, Cheng H, Lin W, Sakurai T, Li T, Nukina N, Wong PC, Xu H, Thinakaran G (2004) Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J Biol Chem 279(43):44945–44954

Voss M, Kunzel U, Higel F, Kuhn PH, Colombo A, Fukumori A, Haug-Kroper M, Klier B, Grammer G, Seidl A, Schroder B, Obst R, Steiner H, Lichtenthaler SF, Haass C, Fluhrer R (2014) Shedding of glycan-modifying enzymes by signal peptide peptidase-like 3 (SPPL3) regulates cellular *N*-glycosylation. EMBO J 33(24):2890–2905

Wada S, Morishima-Kawashima M, Qi Y, Misono H, Shimada Y, Ohno-Iwashita Y, Ihara Y (2003) Gamma-secretase activity is present in rafts but is not cholesterol-dependent. Biochemistry 42(47):13977–13986

Walder K, Kerr-Bayles L, Civitarese A, Jowett J, Curran J, Elliott K, Trevaskis J, Bishara N, Zimmet P, Mandarino L, Ravussin E, Blangero J, Kissebah A, Collier GR (2005) The mitochondrial rhomboid protease PSARL is a new candidate gene for type 2 diabetes. Diabetologia 48(3):459–468

Wang J, Wu F, Shi C (2013) Substitution of membrane cholesterol with beta-sitosterol promotes nonamyloidogenic cleavage of endogenous amyloid precursor protein. Neuroscience 247:227–233

Wang M, Casey PJ (2016) Protein prenylation: unique fats make their mark on biology. Nat Rev Mol Cell Biol 17(2):110–122

Wang Y, Zhang Y, Ha Y (2006) Crystal structure of a rhomboid family intramembrane protease. Nature 444(7116):179–180

Wasserman JD, Urban S, Freeman M (2000) A family of rhomboid-like genes: *Drosophila* rhomboid-1 and roughoid/rhomboid-3 cooperate to activate EGF receptor signaling. Genes Dev 14(13):1651–1663

Wells K, Farooqui AA, Liss L, Horrocks LA (1995) Neural membrane phospholipids in Alzheimer disease. Neurochem Res 20(11):1329–1333

Winkler E, Kamp F, Scheuring J, Ebke A, Fukumori A, Steiner H (2012) Generation of Alzheimer disease-associated amyloid beta 42/43 peptide by gamma-secretase can be inhibited directly by modulation of membrane thickness. J Biol Chem 287(25):21326–21334

Wolfe MS (2010) Structure, mechanism and inhibition of gamma-secretase and presenilin-like proteases. Biol Chem 391(8):839–847

Wolfe MS, Xia W, Moore CL, Leatherwood DD, Ostaszewski B, Rahmati T, Donkor IO, Selkoe DJ (1999a) Peptidomimetic probes and molecular modeling suggest that Alzheimer's gamma-secretase is an intramembrane-cleaving aspartyl protease. Biochemistry 38(15):4720–4727

Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999b) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 398(6727):513–517

Wolozin B (2001) A fluid connection: cholesterol and Abeta. Proc Natl Acad Sci USA 98(10):5371–5373

Wu Z, Yan N, Feng L, Oberstein A, Yan H, Baker RP, Gu L, Jeffrey PD, Urban S, Shi Y (2006) Structural analysis of a rhomboid family intramembrane protease reveals a gating mechanism for substrate entry. Nat Struct Mol Biol 13(12):1084–1091

Wust R, Maurer B, Hauser K, Woitalla D, Sharma M, Kruger R (2016) Mutation analyses and association studies to assess the role of the presenilin-associated rhomboid-like gene in Parkinson's disease. Neurobiol Aging 39:217e213

Xiong H, Callaghan D, Jones A, Walker DG, Lue LF, Beach TG, Sue LI, Woulfe J, Xu H, Stanimirovic DB, Zhang W (2008) Cholesterol retention in Alzheimer's brain is responsible for high beta- and gamma-secretase activities and Abeta production. Neurobiol Dis 29(3):422–437

Yang G, Zhou R, Shi Y (2017) Cryo-EM structures of human gamma-secretase. Curr Opin Struct Biol 46:55–64

Zettl H, Weggen S, Schneider P, Schneider G (2010) Exploring the chemical space of gamma-secretase modulators. Trends Pharmacol Sci 31(9):402–410

Zhou Y, Moin SM, Urban S, Zhang Y (2012) An internal water-retention site in the rhomboid intramembrane protease GlpG ensures catalytic efficiency. Structure 20(7):1255–1263

Zoll S, Stanchev S, Began J, Skerle J, Lepsik M, Peclinovska L, Majer P, Strisovsky K (2014) Substrate binding and specificity of rhomboid intramembrane protease revealed by substrate-peptide complex structures. EMBO J 33(20):2408–2421