Factor XIII-A transglutaminase in neutrophils, neutrophil extracellular trap formation (NETosis) and metabolic health

Fatemeh Soltani

Department of Experimental Medicine
McGill University
Montreal, Canada
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This thesis is dedicated to:

All those I hold dear, with gratitude and affection

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LIST OF ABBREVIATIONS

AMPK - Adenosine Monophosphate (AMP)-Activated Protein Kinase

AP1S1 - Adaptor-Related Protein Complex 1, Sigma 1 Subunit

ASC - Adipose-Derived Stem Cell

AT - Adipose Tissue

ATGL - Adipose Triglyceride Lipase

AT1 - Angiotensin Receptor 1

BAT - Brown Adipose Tissue

BMI - Body Mass Index

C/EBP - CCAAT Enhancer Binding Protein

CD - Control Diet

cFXIII - Cellular FXIII

citH3 - Citrullinated Histone H3

Col - Collagen

CVD - Cardiovascular Disease

DTR - Diphtheria Toxin Receptor

DVT - Deep Vein Thrombosis

ECM - Extracellular Matrix

EMT - Epithelial-Mesenchymal Transition

FDP - Fibrin Degradation Product

FFA - Free Fatty Acid

FXIII-A - Factor XIII-A

G-CSFR - Granulocyte Colony Stimulating Factor Receptor

GFP - Green Fluorescent Protein

Gfi-1 - Growth Factor Independence-1

GLUT4 - Glucose Transporter-4

GWAS - Genome-Wide Association Study

HAT - Histone Acetyltransferase

HFD - High Fat Diet

HIF-1α - Hypoxia-Inducible Factor 1-Alpha

HMGB1 - High Mobility Group Box 1

HSL - Hormone-Sensitive Lipase

hMRP8 - Human Migration Inhibitory Factor-Related Protein 8

IFN-γ - Interferon-Gamma

IL-6 - Interleukin-6

iNOS - Inducible Nitric Oxide Synthase

IRES - Internal Ribosomal Entry Site

ITT - Insulin Tolerance Test

LAM - Lipid-Associated Macrophage

LOX - Lysyl Oxidase

LTBP - Latent TGF-B Binding Protein

MAPK1 - Mitogen-Activated Protein Kinase 1

MCP-1 - Monocyte Chemotactic Protein-1

MGL1 - Macrophage Galactose-Type Lectin-1

MI - Myocardial Infarction

Mincle - Macrophage-Inducible C-Type Lectin

MMP - Matrix Metalloproteinase

MPO - Myeloperoxidase

MHO - Metabolically Healthy Obesity

NE - Neutrophil Elastase

NET - Neutrophil Extracellular Trap

NF-κB - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NOX - Nicotinamide Adenine Dinucleotide Phosphate Oxidase

ORX - Orchidectomy

OVX - Ovariectomy

PAI - Plasminogen Activator Inhibitor

PDK1 - 3-Phosphoinositide-Dependent Protein Kinase-1

PDGFR - Platelet-Derived Growth Factor Receptor

PMA - Phorbol 12-Myristate 13-Acetate

PMN - Polymorphonuclear

PPAR - Peroxisome Proliferator-Activated Receptor

PRP - Platelet-Rich Plasma

PSGL-1 - P-Selectin Glycoprotein Ligand-1

PTM - Posttranslational Modifications

pFN - Plasma Fibronectin

pFXIII - Plasma Factor XIII

PKB - Protein Kinase B

PLA2G16 - Group 16 Phospholipase A2

RAGE - Receptor for Advanced Glycation Endproduct

RBC - Red Blood Cell

ROS - Reactive Oxygen Species

RhoA - Ras Homolog Family Member A

RP S19 - Ribosomal Protein S9

SAT - Subcutaneous Adipose Tissue

SNP - Single Nucleotide Polymorphism

SPD - Spermidine

SPM - Spermine

T2D - Type 2 Diabetes

TAFI - Thrombin-Activatable Fibrinolysis Inhibitor

TF - Tissue Factor

TGF-β - Transforming Growth Factor -Beta

TFPI - Tissue Factor Pathway Inhibitor

TG - Transglutaminase

TLR - Toll-Like Receptor

TIMP - Tissue Inhibitors of Metalloproteinase

TNF - Tumor Necrosis Factor

tPA - Tissue-Type Plasminogen Activator

Trem2 - Triggering Receptor Expressed on Myeloid Cells 2

UCP1 - Uncoupling Protein 1

VAT - Visceral Adipose Tissue

VEGF - Vascular Endothelial Growth Factor

WAT - White Adipose Tissue

ABSTRACT

Factor XIII-A (FXIII-A) transglutaminase (TG) is crucial in the coagulation cascade and stabilizing fibrin. It is also involved in obesity-related pathologies, including thrombosis and fibrosis. Our lab has reported its correlation with metabolic dysfunctions and neutrophil activation. However, its expression and function in neutrophils remain unexplored. My thesis aimed to investigate the FXIII-A production and activity in neutrophils, as well as its role in neutrophil extracellular trap (NET)-fibrin interaction and metabolic disorders in obesity.

Chapter 2 demonstrates FXIII-A production and transglutaminase activity in mouse bone marrow neutrophils, contributing to the NET-fibrin stabilization. The data showed a sex difference in FXIII-A expression by neutrophils; males expressed higher levels than females, suggesting a hormonal effect on this difference. Chapter 3 focuses on generating the *F13a1*$^{-/-\text{MRP8}}$ mouse model and its metabolic health upon a high-fat diet (HFD) challenge. The male knockout mice exhibited resistance to weight gain and improved metabolic parameters compared to wild types on HFD. Given the normal weight gain of global *F13a1* knockout on HFD, such resistance was unexpected, prompting us to include MRP8-Cre as an additional control. Like knockout mice, these mice did not gain weight, suggesting a significant confounding effect from the model, due to an unintended deletion of the *Serpine 1* (PAI-1) gene. As PAI-1 and FXIII-A share metabolic functions, interpreting the results from *F13a1*$^{-/-\text{MRP8}}$ mice was complicated, leading us to terminate further metabolic studies on this model. In conclusion, my thesis identifies neutrophils as a new source of FXIII-A and shows neutrophil-derived FXIII-A role in stabilizing NETs-fibrin networks. However, making a definite conclusion about neutrophil-FXIII-A effect on metabolic health was not possible due to the MRP8-Cre issue, underscoring the unsuitability of this model in adipose tissue and metabolic-related studies.

RÉSUMÉ

La transglutaminase (TG) du facteur XIII-A (FXIII-A) joue un rôle crucial dans la cascade de la coagulation et la stabilisation de la fibrine. Elle est également impliquée dans les pathologies liées à l'obésité, notamment la thrombose et la fibrose. Notre laboratoire a rapporté sa corrélation avec les dysfonctionnements métaboliques et l'activation des neutrophiles. Cependant, son expression et sa fonction dans les neutrophiles restent inexplorées. Ma thèse visait à étudier la production et l'activité du FXIII-A dans les neutrophiles, ainsi que son rôle dans l'interaction entre le piège extracellulaire des neutrophiles (NET) et la fibrine, et dans les troubles métaboliques liés à l'obésité.

Le chapitre 2 démontre la production de FXIII-A et l'activité TG dans les neutrophiles de la moelle osseuse de souris, contribuant à la stabilisation de la fibrine du piège extracellulaire des neutrophiles. Les données montrent une différence entre les sexes dans l'expression du FXIII-A par les neutrophiles; les mâles expriment des niveaux plus élevés que les femelles, ce qui suggère un effet hormonal sur cette différence. Le chapitre 3 se concentre sur la création du modèle de souris F13a1-/-MRP8 et sur sa santé métabolique lors d'un régime alimentaire riche en graisses (HFD). Les souris knock-out mâles ont montré une résistance à la prise de poids et une amélioration des paramètres métaboliques par rapport aux types sauvages soumis à un régime riche en graisses. Etant donné la prise de poids normale des souris knockout F13a1 sur HFD, une telle résistance était inattendue, ce qui nous a incité à inclure MRP8-Cre comme contrôle supplémentaire. Comme les souris knock-out, ces souris n'ont pas pris de poids, ce qui suggère un effet de confusion significatif du modèle, dû à une délétion involontaire du gène de la Serpine 1 (PAI-1). Comme le PAI-1 et le FXIII-A partagent des fonctions métaboliques, l'interprétation des résultats des souris F13a1-/-MRP8 était compliquée, ce qui nous a conduits à mettre fin à d'autres études métaboliques sur ce modèle. En conclusion, ma thèse identifie les neutrophiles comme une

nouvelle source de FXIII-A et montre le rôle du FXIII-A dérivé des neutrophiles dans la stabilisation des réseaux NETs-fibrine. Cependant, il n'a pas été possible de tirer une conclusion définitive sur l'effet des neutrophiles-FXIII-A sur la santé métabolique en raison du problème de MRP8-Cre, ce qui souligne l'inadéquation de ce modèle pour les études sur le tissu adipeux et le métabolisme.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Obesity leads to a whole-body hypercoagulable state that can initiate and aggravate the weight-gain-related metabolic disorders, thrombotic events, and local adipose tissue inflammation. Neutrophils are among the first immune cells to respond to adipose tissue inflammation, where they initiate a series of proinflammatory events through various mechanisms, including NETosis. While their role and interplay with other cells and molecules in immunothrombosis are evident, several gaps in these processes need further exploration.

Factor XIII-A is a transglutaminase enzyme with an essential function in coagulation but also in pathologies such as thrombosis, adipose tissue and metabolic dysfunctions. Several cell types produce it, but main producers are monocytes and macrophages. To date, there are no reports on the endogenous expression of Factor XIII-A by neutrophils and the potential role of neutrophil-derived Factor XIII-A in adipose tissue health. The thesis presented here contributes the following novel findings about neutrophil-derived FXIII-A in thrombotic processes and adipose tissue metabolic health. The new findings are as follows:

1- **Factor XIII-A production by neutrophils:** Neutrophils from male mouse bone marrow, but not female ones, produce FXIII-A at levels comparable to macrophages and monocytes, which have been identified as the main producers of this enzyme.
2- **Participation of Factor XIII-A in NETosis:** Neutrophil's FXIII-A is externalized during NETosis and remains attached to and colocalizes with NET markers (DNA, citH3, and MPO) *in vitro*.
3- **Factor XIII-A contributes to protein crosslinking activity in NETosis:** Neutrophil-Factor XIII-A possesses transglutaminase activity *in vitro* in both resting neutrophils and

during NETosis stimulation stages. This activity is inhibited by the TG inhibitor, NC9, *in vitro*.

4- **Factor XIII-A crosslinks fibrin(ogen) to NETs:** Neutrophil's Factor XIII-A participates in NETosis-mediated fibrinogenesis and promotes NET-fibrin interaction *in vitro.* The fibrin network promotes the entrapment of neutrophils within thrombi *in vitro*.
5- **Sexual dimorphism in Factor XIII-A expression:** *F13a1* expression exhibits sexual dimorphism, where male neutrophils express more *F13a1* than females. The expression is suppressed in male neutrophils by female and male sex hormones.
6- **Limitations of MRP8-Cre mice:** Research done on MRP8-Cre mice, which are designed for neutrophil-specific knockouts, need to use MRP8-Cre as one control in metabolic studies or research that may involve fibrinolytic systems. MRP8-Cre harbour a global deletion of *Serpine 1* (PAI-1, prothrombotic factor) and a partial deletion of *Ap1s1* in the host genome and their adipose tissue, and the mice do not gain weight on a high-fat diet. This observation and original contribution to knowledge was made via crossing MRP8-Cre with *F13a1* flx/flx mouse model to create a neutrophil-specific FXIII-A null created a 'stronger' phenotype than *F13a1*-/- global knockout.

These findings provide new insight into the presence of Factor XIII-A in neutrophils and its transglutaminase, crosslinking activity in stabilizing fibrin-NET interactions. The findings underscore the potential for further studies to uncover the precise role of neutrophil-derived Factor XIII-A in adipose tissue health and to leverage these concepts into therapeutic strategies for managing immunothrombosis and metabolic issues.

CONTRIBUTION OF AUTHORS

Fatemeh Soltani (Fatemeh S) was the lead investigator and first author of the manuscripts in this thesis. Fatemeh S designed experiments in collaboration with Dr. Mari Kaartinen, and troubleshooted and optimized protocols, collected data, and analyzed results. Fatemeh S also drafted the initial manuscripts and created the figures.

Dr. Mari Kaartinen contributed to the design and funding of the projects. She supervised Fatemeh S throughout her research and was extensively involved in the preparation, writing and finalization of the papers.

Chapter 1. A modified segment of my review paper published in the *American Journal of Physiology-Cell Physiology* was used to discuss the role of FXIII-A in fibrosis for the literature review.

Fatemeh Soltani F, Mari Kaartinen, **Transglutaminases in fibrosis-overview and recent advances**. *Am J Physiol Cell Physiol*. 2023; 325: C885-C894.

Chapter 2. Neutrophil Factor XIII-A transglutaminase contributes to Neutrophil Extracellular Trap (NET)-mediated fibrin network formation and crosslinking

Fatemeh Soltani, Mélanie Welman, Sahar Ebrahimi Samani, Alain Pacis, Marie Lordkipanidzé, Mari T. Kaartinen. Thrombosis and Hemostasis journal, *Accepted for publication*.

M.W. Performed Platelet isoltaion and IF staining of plateletes.

S. E. E. Assisted with monocyte, macrophage and osteoclast isolation.

A. P. Performed single cell RNA sequencing data mining.

M. L. Supervised the platelet preparation and related data interpretation.

Chapter 3. Off-target genomic effects in MRP-Cre driver mice complicate its use in weight gain and metabolic studies

Fatemeh Soltani, Cedric Duval, Robert Ariens, Mari T. Kaartinen. *Ready to submit.*

C.D. Developed *F13a1* loxed model and assisted with genotyping primer design.

R.A. Developed *F13a1* loxed model.

1. Chapter 1: Introduction and Literature Review

1.1. Adipose tissue and its role in metabolic health

Adipose tissue or fat tissue is a unique form of loose connective tissue. Different cells within adipose tissue include adipocytes, stem cells, preadipocytes, endothelial cells, immune cells, fibroblasts, etc. Although adipose tissue is an energy reservoir, it possesses several other important functions, such as insulation, control of thermogenesis, mediation of insulin sensitivity, and acting as an endocrine organ that regulates metabolic homeostasis. These functions make adipose tissue a central player in developing and managing metabolic diseases, including obesity, diabetes, and cardiovascular diseases. Gaining a better understanding of adipose tissue provides essential insights into identifying potential therapeutic targets for improving metabolic health and managing metabolic disorders.

1.1.1. Adipose tissue depots

Adipose tissue is divided into two main groups: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the most abundant form of adipose tissue in the human body and is primarily responsible for energy storage. It also acts as an endocrine organ which secretes hormones and adipokines, thereby influencing various functions of the body. There are two types of WATs in the body: subcutaneous (SAT) and visceral (VAT). SAT is located beneath the skin, whereas VAT is distributed within the abdominal cavity. In rodents, SAT is further divided into anterior and posterior groups, corresponding to upper and lower SAT in humans. In humans and rodents, VAT includes depots around the heart (epicardial /pericardial) and surrounding intrabdominal organs (mesenteric, omental, perigonadal, perirenal, and peritoneal). The main subcutaneous depot in

humans is typically located around the hips and thighs, while the predominant VAT is the omental one. In rodent models, SAT and VAT are referred to as inguinal and epididymal or gonadal WAT, respectively [1, 2] (**Figure 1.1**).

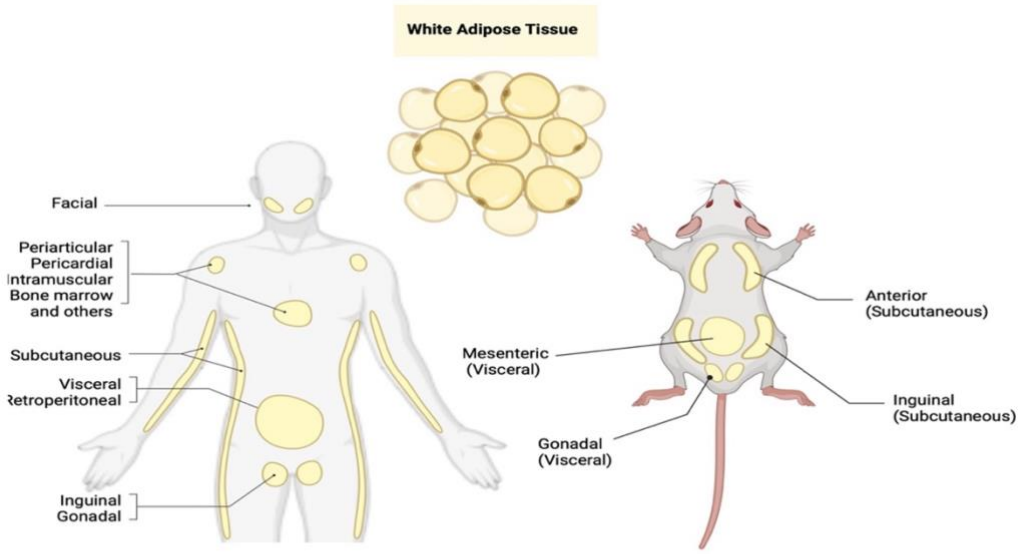

Figure 1.1. Distribution of white adipose tissue (WAT) in humans and mice. Inspired by Ref. [3] and created in Biorender.com.

Brown adipose tissue (BAT) is less abundant in the human body and is critical in non-shivering thermoregulation. It generates heat via the oxidation of lipids and glucose, facilitated by uncoupling protein 1 (UCP1) located in the inner mitochondrial membrane of brown adipocytes. BAT is characterized by multilocular lipid droplets, rich vascularization, and a high mitochondria content [3, 4]. Anatomically, in mice, BAT is mainly found in the interscapular region between the shoulder blades and extends to areas such as the armpit, neck, and around the kidneys. In humans, it is predominantly located in the neck and upper back, with additional depots around the

shoulders, along the spine, and near the kidneys [5]. The activity and distribution of BAT spots can be changed by factors such as aging, specific diets, and environmental conditions [6, 7].

1.1.2. Energy metabolism

Adipose tissue is vital for metabolic regulation and energy homeostasis. It maintains metabolic balance by acting as a reservoir of fats in WAT and as an organ for energy expenditure by thermogenesis in BAT. In conditions of nutrient excess, adipose tissue stores the surplus nutrients as triglyceride, so-called lipogenesis, which is regulated by insulin. After food intake, insulin activates its receptor tyrosine kinase on adipocytes, which leads to a cascade of signalling pathways that involve phosphoinositide 3-kinases (PI3K) and protein kinase B (PKB, Akt) activation. This leads to the translocation of glucose transporter-4 (GLUT4) to the plasma membrane, increasing glucose uptake, necessary for converting free fatty acids into triglycerides [8, 9]. Conversely, during energy scarcity, adipose tissue breaks down triglyceride into free fatty acids and glycerol through lipolysis [10]. Lipolysis is initiated by activating adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). This process is regulated by hormones such as glucagon, epinephrine, cortisol, and growth hormones. Insulin inhibits lipolysis via the mammalian target of rapamycin complex 1(mTORC1) and Akt/PKB pathways[11, 12].

Adipose tissue also plays a crucial role in energy metabolism by releasing various hormones and adipokines. These substances circulate and stimulate signalling pathways in organs such as the muscle, liver, pancreas, and brain, thereby regulating systemic energy metabolism. Adipokines are involved in various physiological and metabolic processes, including glucose uptake, insulin signalling, and fatty acid oxidations. Adipokines, including adiponectin, leptin, and resistin, are associated with obesity-related metabolic disorders. Adiponectin (AdipoQ), the most abundant

adipokines, enhances insulin sensitivity by activating adenosine monophosphate (AMP)-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor- alpha (PPAR-α) in liver and muscle tissues [13-15]. Leptin regulates energy balance, metabolism, and glucose homeostasis by suppressing appetite and increasing energy expenditure. Leptin resistance, during obesity, further complicates obesity-related metabolic issues[16]. Resistin has also been linked to obesity and insulin resistance and activates the toll-like receptor 4 (TLR4) signalling pathway. It also has pro-inflammatory functions, opposing the beneficial effects of adiponectin [17, 18].

1.1.3. Adipogenesis

Adipose tissue expansion occurs through two distinct mechanisms, including hyperplasia (increase in number) and hypertrophy (increase in size) of adipocytes. The process of increasing adipocyte number is known as adipogenesis, a series of differentiation processes that adipose stem cells undergo to transform into mature adipocytes. In the process of adipogenesis, initially, fibroblast-like cells form preadipocytes without significant alteration in their morphology. Subsequently, these cells differentiate further, and their morphology changes significantly. Then they accumulate lipids and gradually shift from a fibroblast shape to a multilocular and ultimately to a spherical unilocular form. This final form, namely mature adipocyte, is specialized for efficient triglyceride storage and is characterized by enhanced insulin responsiveness [19, 20] (**Figure 1.2**). The extracellular matrix (ECM) composition of adipose-derived stem cells (ASCs) is altered during adipogenesis. For instance, the expression of fibrillar collagens (Col I and III) shifts to basement membrane collagens (Col IV, VI, XV and XVIII). These changes are important for cell differentiation and affect the physical properties of the tissue environment [21, 22].

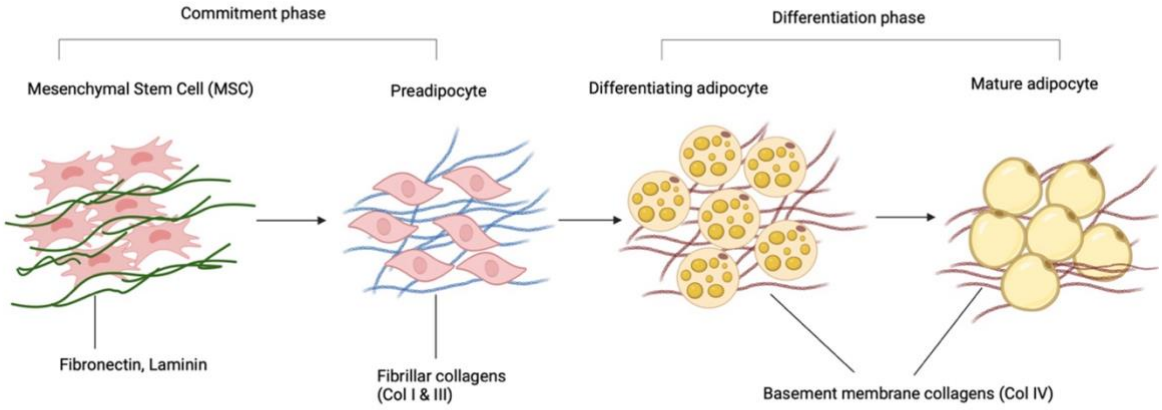

Figure 1.2. The process of adipogenesis and ECM alteration. Created in Biorender.com.

The balance between hypertrophic expansion and adipogenesis is critical for metabolic health. Hypertrophic adipocytes are linked to obesity and are associated with insulin resistance. Smaller adipocytes are beneficial and can help counteract obesity-related metabolic dysfunctions like diabetes. These adipocytes differ in lipolysis rates and cytokine secretion. In adults, adipose tissue growth is primarily driven by adipocyte hypertrophy. However, hyperplasia can occur under certain circumstances and varies across different adipose tissue depots, affecting tissue functions and metabolism [20, 23, 24].

Adipogenesis is regulated by different signalling pathways, including insulin and glucocorticoid signalling [25-27]. The differentiation process involves multiple transcription factors, notably PPARγ and the CCAAT enhancer binding protein (C/EBP) family, which control genes critical for lipid metabolism and insulin sensitivity [28, 29]. In addition, adipogenesis is influenced by inflammatory processes. Inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) can impair adipocyte differentiation [30, 31]. Macrophage infiltration into

adipose tissue in obese individuals releases pro-inflammatory cytokines, which are shown to be correlated with adipocyte hypertrophy. Furthermore, transforming growth factor -beta (TGF-β), a potent anti-adipogenic protein produced by hypertrophic adipocytes and immune cells, inhibits adipogenesis by blocking the PPARγ–C/EBPα pathway[32, 33]. Additionally, some adipocyte precursors may adopt inflammatory roles, leading to tissue fibrosis and inhibiting adipogenesis. Interestingly, while chronic inflammation exacerbates adipose tissue dysfunction by impairing adipocyte formation, transient acute inflammation can stimulate beneficial adipogenic responses [20, 34].

1.1.4. Obesity and adipose tissue dysfunction

Obesity has become one of the most important global health issues, with its prevalence tripling over the past 50 years [35, 36]. Obese people are at high risk for comorbidities such as type 2 diabetes (T2D), hypertension, cardiovascular diseases (CVD), osteoarthritis, certain types of cancers and increased coronavirus disease 2019 (COVID-19) hospitalization risk [37-40].

During weight gain and obesity, adipose tissue undergoes significant changes affecting metabolic health (**Figure 1.3**). Adipose tissue is characterized by its plasticity, which can expand and remodel in response to metabolic stimuli such as diet, obesity, and exercise. However, the response of adipose tissue can vary significantly among individuals and between genders [20, 41].

Impaired expansion of adipose tissue in response to excess calories contributes to insulin resistance and ectopic fat deposition in other organs, including the liver, visceral depots, skeletal muscle, and pancreas [42, 43]. Healthy weight fluctuations maintain metabolic flexibility with balanced adipocyte growth, controlled inflammation and insulin sensitivity [20, 44]. In healthy adipose tissue, expansion mainly occurs through hyperplasia associated with insulin sensitivity. However,

in obesity, expansion predominantly occurs through hypertrophy, leading to adipose tissue dysfunction, characterized by ectopic fat deposition, inadequate vascularization, hypoxia, immune cell infiltration, and fibrosis [23]. In obesity, VAT expansion is more detrimental to metabolic health than SAT, which is correlated with insulin resistance [45]. Excess lipid storage and adipocyte hypertrophy challenge adipose tissue homeostasis and mitochondrial function, generating reactive oxygen species (ROS) and oxidative stress [46].

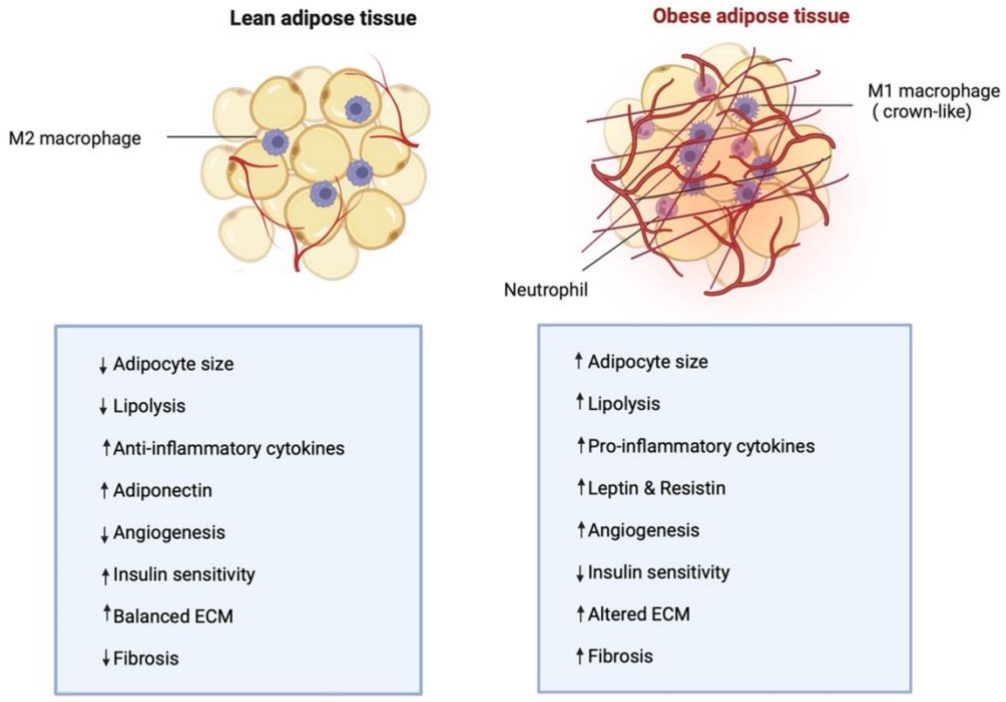

Figure 1.3. Changes of adipose tissue during weight gain and obesity. Template adapted from Biorender.com, with modifications.

Obese adipose tissue also shifts to an inflammatory state due to adipocyte expansion and stress, leading to hypoxia and increased immune cell infiltration. Immune cells play key roles during obesity, among which neutrophils first respond to inflammatory cues and infiltrate adipose tissue

within days. They can be activated by different stimuli, such as free fatty acids (FFA) released from dying adipocytes because of lipolysis [47]. The released FFA bind to receptors such as TLR4 on neutrophils and activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway. Neutrophils contribute to various obesity-related dysfunctions through different mechanisms, including secreting inflammatory cytokines and releasing neutrophils extracellular traps, so-called NETosis. Neutrophil's functions will be discussed in more detail in the context of thromboinflammation on page 57. Activation and infiltration of neutrophils and chemokine-like monocyte chemotactic protein-1 (MCP-1) from hypertrophic adipocytes precede macrophage infiltration to clear dead adipocytes in adipose tissue during the high-fat diet in mice [48, 49]. Their accumulation primarily in VAT forms crown-like structures around dying cells and secretes inflammatory cytokines [50]. Macrophages mainly originate from circulating monocytes and a smaller fraction from resident anti-inflammatory M2 macrophages (expressing CD206, MGL1, ARG1). They undergo polarization to pro-inflammatory M1 macrophages in obesity, characterized by CD11c, TNF-α, and inducible nitric oxide synthase (iNOS) markers. Additionally, lipid-associated macrophages (LAMs) expressing the triggering receptor expressed on myeloid cells 2 (Trem2) receptor, increase in obesity [51]. Deletion of Trem2 exacerbates adipocyte hypertrophy and metabolic dysfunction, including hypercholesterolemia and insulin resistance [52]. All these processes contribute to a chronic, low-grade inflammatory state in adipose tissue, closely linked to metabolic complications such as insulin resistance and non-alcoholic fatty liver disease [44, 53, 54].

Metabolically healthy obesity (MHO) is when some obese individuals do not show metabolic and cardiovascular complications. This difference has been linked to favourable fat distribution, particularly a higher proportion of SAT versus VAT [55, 56]. These individuals often exhibit

smaller adipocytes, lower adipose tissue inflammation, and better adipokine profiles, with higher adiponectin and lower TNF-α and IL-6 levels [57]. Although MHO individuals are still at risk of diabetes and cardiovascular diseases, the risk is lower compared to unhealthy obese ones [58, 59]. MHO is considered a healthy condition, however, its lasting is the subject of debate; MHO may be a transient condition and capable of changing into unhealthy obesity after a couple of years [60]. Identifying factors contributing to MHO and monitoring the transition from MHO into the unhealthy obesity can help prevent obesity-related metabolic complications.

1.1.5. Sex differences in obesity, adipose tissue and metabolism

Sex differences in adipose tissue function and deposition are well-documented and involve differences in both SAT and VAT distribution and related health risks [61, 62]. Men typically have more VAT depots, leading to an android body shape, which has been linked to cardiovascular risks, whereas women accumulate more SAT depots prior to menopause. This feature in women is associated with protection from obesity-related comorbidities such as insulin resistance and metabolic syndrome. The difference in fat distribution is associated with varying levels of lipoprotein lipase, an enzyme responsible for breaking down triglycerides. For instance, lipoprotein lipase activity is higher in gluteal SAT in women, whereas men exhibit higher activity in abdominal VAT. Additionally, testosterone suppresses lipoprotein lipase activity in the femoral SAT of men [63].

Menopause, which involves a decline of ovarian function and a decrease in female hormones, increases the risk of metabolic syndrome by 60% via altering body-fat distribution that leads to central obesity or VAT accumulation. This is directly linked to the decreased levels of estrogen and progesterone [64, 65]. Estrogens decrease food intake, increase energy expenditure, and

enhance adipocyte expandability in SAT while inhibiting in VAT[66]. Estrogens are also necessary in men, and inhibition of the conversion of androgens to estrogens leads to reduced insulin sensitivity and metabolic health. Moreover, in men, decreased or deficient androgens can increase the development of metabolic syndrome, although there is some controversy in this regard [67]. Hormone replacement therapy, either female or male hormones, may have favourable effects on mitigating central obesity, improving glycemic control, lowering circulating lipids and decreasing thrombotic risks [65, 68].

Studies using a mouse model on a HFD have shown similar effects, where gonadectomies, i.e. ovariectomy (OVX) and orchidectomy (ORX), exacerbate weight gain and worsen metabolic dysfunction and atherosclerosis [69-71]. A sexual dimorphic response of immune cells in obesity is also known [62, 72]. For example, male monocytes are more inflammatory than female monocytes, responding more strongly to metabolic stimuli and maturing into inflammatory macrophages. Males have higher levels of M1 macrophages in WAT than females, who present higher levels of M2 macrophages [73]. Furthermore, sex hormones regulate adipocyte differentiation and function, and modulate lipolysis and lipogenesis via their receptors [74, 75].

1.2. Adipose tissue extracellular matrix

1.2.1. Overview of the extracellular matrix

The extracellular matrix (ECM) in adipose tissue is a dynamic network that provides mechanical support scaffolding and crucial information about the extracellular environment, influencing adipocyte functions [76]. Based on the location, the ECM is categorized into two groups: interstitial (e.g. Col I, III, and V) located in the lamina propria, submucosa, and muscular and serosa layers, and basement membrane (e.g. Col IV, laminin, fibronectin, and hyaluronan) matrix

located beneath the epithelial and endothelial cells. The former is produced by mesenchymal cells, including fibroblasts and myofibroblasts, and the latter is synthesized by epithelial cells, endothelial cells, and pericytes [77]. In adipose tissue, the main ECM component is collagen, contributing considerably to the non-cell mass of the adipose tissue. Collagen is primarily produced by adipocytes; however, other cells can produce it as well. The most abundant collagen in adipose tissue is type I, constituting approximately 90% of the total tissue structure, and predominantly is present within SAT. Up to now, twelve types of collagens have been identified in rodent adipose tissue. They provide structural support and affect cell functions and tissue morphogenesis. Col IV is the major component in adipocytes as a basement membrane and, along with laminin networks, provides a structured basement membrane essential for adipocytes. Col VI also interacts with various ECM proteins and affects the elasticity and hypertrophy of adipose tissue [78, 79].

Overall, the ECM regulates various cellular processes such as adipocyte differentiation, proliferation, and apoptosis through interaction with cell surface receptors like integrins. Additionally, ECM is considered a source of growth factors like TGF-β, fibroblast growth factor, and vascular endothelial growth factor (VEGF) [80]. Understanding the composition of ECM and its interaction with adipocytes provides insight into how adipose tissue responds to and manages metabolic demands, especially in conditions like weight gain and obesity.

1.2.2. ECM homeostasis and remodeling in adipose tissue

In various conditions, ECM changes occur to maintain the tissue structure and integrity. It affects cellular functions critical for metabolic health, including cellular senescence, proliferation, migration, and differentiation. In adipose tissue, homeostasis of the ECM involves a balance

between synthesis and degradation of its components [79, 81]. This balance is important for maintaining the integrity of adipose tissue and allows it to adapt to new conditions such as pregnancy, injury, and inflammation. In such conditions, ECM changes and remodels in terms of composition and deposition, which is important for normal cellular functions [82]. For instance, during adipogenesis, fibronectin secretion decreases while laminin production increases. This influences the rigidity of the matrix, thereby facilitating the accumulation of lipid droplets within the cells [22].

The ECM remodelling is controlled by different factors such as matrix metalloproteinases (MMPs), which degrade ECM components, and tissue inhibitors of metalloproteinases (TIMPs), which regulate MMP activity. Several hormones, such as estrogen and TGF-β, also control ECM remodelling [83, 84]. Dysregulation of adipose tissue remodelling and, thereby, excessive production and accumulation of ECM components can lead to the stiffening of adipose tissue and hindering angiogenesis, which is linked to metabolic dysfunction, fibrosis and insulin resistance [79, 85]. Studies showed that obese individuals with insulin resistance exhibit higher ECM deposition in their AT than MHO ones, highlighting the role of ECM remodelling in the pathophysiology of insulin resistance [86]. Intriguingly, interventions like exercise have been shown to reduce ECM expansion and improve metabolic health [87, 88].

1.2.3. ECM remodeling and contribution to inflammation

Extensive ECM remodelling in adipose tissue during obesity increases rigidity and fibrotic events. This rigidity puts mechanical stress on adipocytes, triggering pro-inflammatory responses [89, 90]. Consequently, several signalling pathways, such as Ras homolog family member A (RhoA) and NF-κB, are activated in response to adipocyte hypertrophy and cell stress. RhoA disrupts the

transcriptional activity of PPARγ, inhibiting healthy adipocyte expansion and exacerbating metabolic dysfunction. NF-κB also enhances the inflammatory state and activates monocytes, further affecting metabolic health [91-93]. In addition, the cellular stress caused by ECM remodelling triggers the release of pro-inflammatory cytokines, including plasminogen activator inhibitor-1 (PAI-1) and MCP-1 from adipocytes and macrophages [89, 91]. During obesity, the level of TGF-β in adipose tissue also increases, leading to the stimulation of corresponding signalling pathways related to ECM production. The elevated levels of inflammatory cytokines and TGF-β, further enhance ECM production, promoting fibrosis and aggravation of inflammatory responses [94, 95].

Additionally, the altered ECM promotes adipocyte death, which attracts further inflammatory immune cells to the site, exacerbating ECM stiffness and metabolic complications. The fragmentation of ECM components during remodeling can also activate various inflammatory responses, extending tissue dysfunction and systemic metabolic disorders [96, 97].

1.2.4. Adipose tissue fibrinogenic processes

The inflammatory responses triggered by obesity can initiate repair events, leading to adipose tissue fibrosis. While these events are detrimental to obese individuals, often they are undetected and rarely reversed, thereby exacerbating obesity-related comorbidities [98-100]. Like other fibrotic diseases, altered ECM remodeling in adipose tissue leads to stiffening and fibrosis, through various mechanisms (**Figure 1.4**). During the development of fibrosis, there is an imbalance between ECM production and degradation. Different cells in adipose tissue, including adipocyte progenitors, adipocytes, fibroblasts, and myofibroblasts, are responsible for ECM production [90, 101]. Key ECM proteins, including fibronectin, collagen, proteoglycan, and non-proteoglycans,

are present in adipose tissue, among which fibronectin and collagens are the most abundant in both healthy and fibrotic adipose tissue[21]. Col I, III, V and VI concentrations are increased in obese mice with insulin resistance. Coll VI is specifically correlated to adipose tissue fibrosis, and its absence leads to reduced inflammation and insulin resistance [102].

Hypertrophic adipocytes, due to a high-fat diet, are more prone to hypoxia and cell death upon uncontrolled lipid accumulation. This results in the activation of hypoxia-inducible factor 1- alpha (HIF-1α) and other transcription factor expressions that affect genes involved in various functions, including ECM remodeling and inflammation[21, 103]. For instance, the expression of collagen type I, alpha 1 (Col1A1), Col3A1 and lysyl oxidase (LOX), and MMP14 are increased [104-106]. Persistent local hypoxia leads to necrotic adipocytes, attracting proinflammatory immune cells like M1 macrophages, which form crown-like structures around dying adipocytes and release proinflammatory cytokines (e.g. TNF-α, IL-6). These cytokines promote the infiltration and activation of fibroblasts and further ECM production, mediated by TLR4 activation and macrophage-inducible C-type lectin (Mincle) [107, 108] (**Figure 1.4**). The increase in ECM production and interstitial fibrosis disrupts normal adipocyte expansion and healthy interactions. Moreover, an imbalance between MMPs and TIMPs during excessive weight gain leads to uncontrollable ECM accumulation [104, 109]. Mice lacking MMP14, thereby impaired collagen degradation, show stiffening and reduced lipid accumulation in adipocytes [110]. Also, inhibition of LOX, which contributes to collagen cross-linking and ECM stiffening, has been shown to improve adipose tissue dysfunction in obesity [105]. During a high-fat diet, adipocytes change into a fibroblast-like phenotype, specifically into cells known as myofibroblasts, which exhibit fibrogenic behavior. This transformation is characterized by an increased expression of genes

involved in ECM production [94], making a shift towards a more fibrotic state within the adipose tissue.

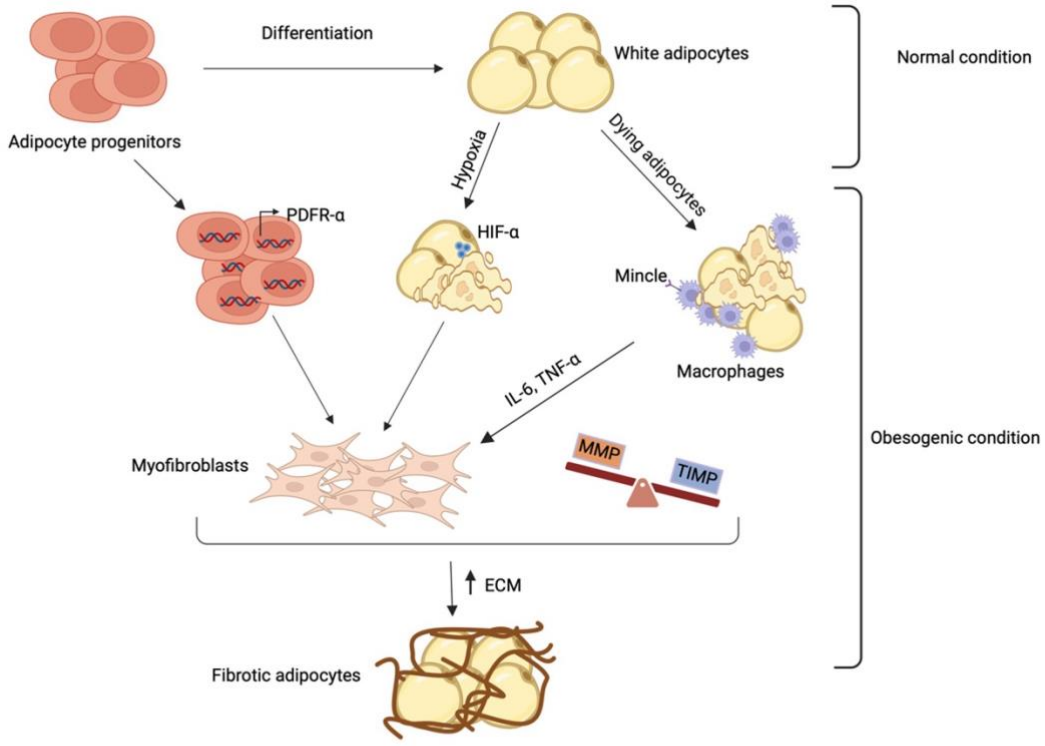

Figure 1.4. Representation of the cellular and molecular events contributing to adipose tissue fibrosis. Created in Biorender.com.

Moreover, a specific subset of adipocyte progenitors, those positive for platelet-derived growth factor receptor-alpha (PDGFR-α), also adopts a fibrogenic phenotype. These cells express a high amount of PDGFR-α, which has a negative correlation with adipogenesis. The overproduction of PDGFR-α promotes the differentiation of these cells into a pro-fibrotic state, thereby driving pathological functions and remodeling within WAT [111]. During adipose tissue expansion, these

progenitors differentiate into white adipocytes; however, in extensive obesogenic conditions they adopt a myofibroblastic phenotype, further exacerbating the fibrotic process.

1.3.Factor XIII-A: Essential roles in hemostasis, wound healing and beyond

1.3.1. FXIII-A - plasma transglutaminase: Structure and activation

Factor XIII-A (*F13A1* gene in humans and *F13a1* gene in mice) is a member of the transglutaminase enzyme (TG) family, which consists of nine members (FXIII-A and TG1-TG7). Of these TG, eight possess enzymatic activity. The most prevalent function of TGs is to create covalent isopeptide bonds between their substrate proteins. The crosslinking reaction mediated by FXIII-A includes attacking Cys314 to the glutamine-carboxamide group of the substrate, resulting in displacing an ammonia molecule and forming a thioester intermediate. This reaction is followed by attacking the intermediate by a lysine-amine group of amine donor protein, and then the Cys314 is displaced, and the ε-(γ-glutamyl)lysine, i.e. isopeptide bond is formed between glutamine and lysine residues of proteins [112]. This crosslinking can occur between one substrate like fibrin or between different proteins, which can alter their biological functions [113]. Transglutaminase activity can also link primary amines and polyamines to glutamine residues in proteins, affecting protein activities. When lysine-residues or primary amines are absent, water reacts with the intermediate, and glutamine is converted into glutamic acid [114, 115] (**Figure 1.5**).

FXIII-A is the only transglutaminase that forms a dimer with itself, i.e., homodimer (FXIII-A2). It plays a pivotal role in blood coagulation and exists as a zymogen that requires enzymatic activation before its function as a transglutamianse. In plasma, FXIII is present as a heterotetrameric form (FXIII-A2B2), consisting of two catalytic A (FXIII-A2) and two carrier B (FXIII-B2) subunits. FXIII-B is synthesized in the liver by hepatocytes and binds to FXIII-A in

plasma. The source of plasma FXIII-A was originally postulated to be platelets, but the normal level of FXIII-A in thrombocytopenic mice failed to support the idea of platelets as a source [116]. Recent cell-specific knockouts using *F13a1*flx/flx mouse identified the main source of FXIII-A to be monocytes and tissue-resident macrophages [117]. Since FXIII-A lacks an endoplasmic reticulum signal sequence, its secretory mechanisms are not clear. The plasma concentration of complex FXIII-A2B2 is approximately 68 nM, with an excess of B homodimers (~50%) also found in circulation. FXIII-A deficiency is an autosomal recessive disorder characterized by bleeding tendency and impaired wound healing [116, 118-120].

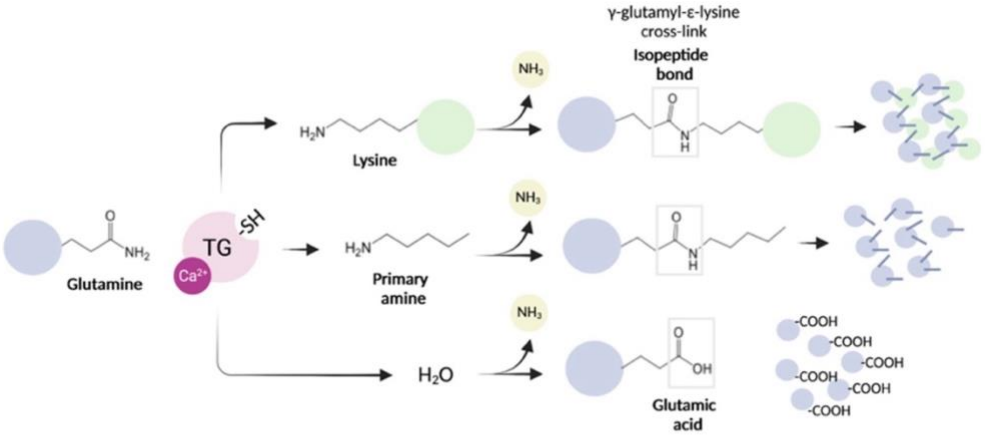

Figure 1.5. Crosslinking reactions mediated by FXIII-A. Adapted from Ref. [116] with permission from the American Physiological Society.

The cellular form of FXIII-A is a homodimer of the A subunits. It can be activated by a slight increase in intracellular Ca^{2+} concentrations [121] and is present in different cells, including bone marrow and mesenchymal lineage, notably platelets, megakaryocytes, monocytes, circulating and tissue macrophages, dendritic cells, chondrocytes, osteoblasts and preadipocytes [114]. FXIII-A is involved in several physiological events like clot formation, wound healing, angiogenesis, bone

metabolism, and pregnancy. Also, it contributes to various diseases such as thrombosis, diabetes, obesity, and cancer [122].

FXIII-A consists of 732 amino acids, and its molecular mass is 83 kDa. There are four principal domains in the structure of FXIII-A, including NH_2-terminal activation peptide (1-37), β-sandwich (38-184), catalytic core (185-515), β-barrel 1 and β-barrel 2 domains (516-731) (**Figure 1.6**).

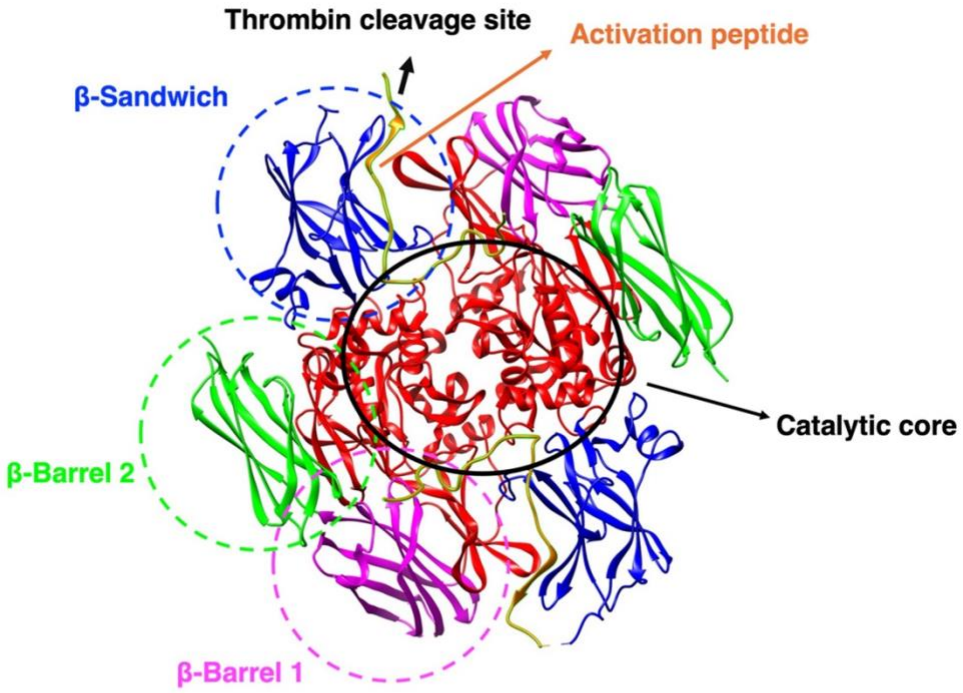

Figure 1.6. Tertiary structure of the FXIII A dimer (FXIII-A2). Image was created using UCSF chimera.

To form FXIII-A2, the catalytic cores align together. The activation peptide covers the active site, preventing interaction with target substrates. Plasma FXIII-A is activated by thrombin, which cleaves the Arg37-Gly38 bond in the activation peptide, leading to destabilization of the complex (**Figure 1.7**). This is followed by the binding of Ca^{+2} to its site, which is critical for

transglutaminase activity and promotes removing FXIII-B2 subunits, activating the transglutaminase enzyme FXIII-A*2. Upon dissociation of the activation peptide, the catalytic triad (Cys314, His373, Asp396) is rearranged and thereby exposed to the substrates. Other serine proteases, including platelet acid protease, calpain, and neutrophil elastase (NE), can also activate FXIII-A [121]. The physiological relevance of the NE-mediated activation is not known; however, it was reported in 2016 that transglutaminase activity can stabilize neutrophil extracellular traps (NETs) via crosslinking polyamines into several NET proteins [123]. FXIII-A can also be activated via a non-proteolytic mechanism, namely by high Ca^{2+} and substrate binding to the enzyme, which is postulated to be the main activation mechanism in the dimeric form, cellular FXIII-A (**Figure 1.7**)

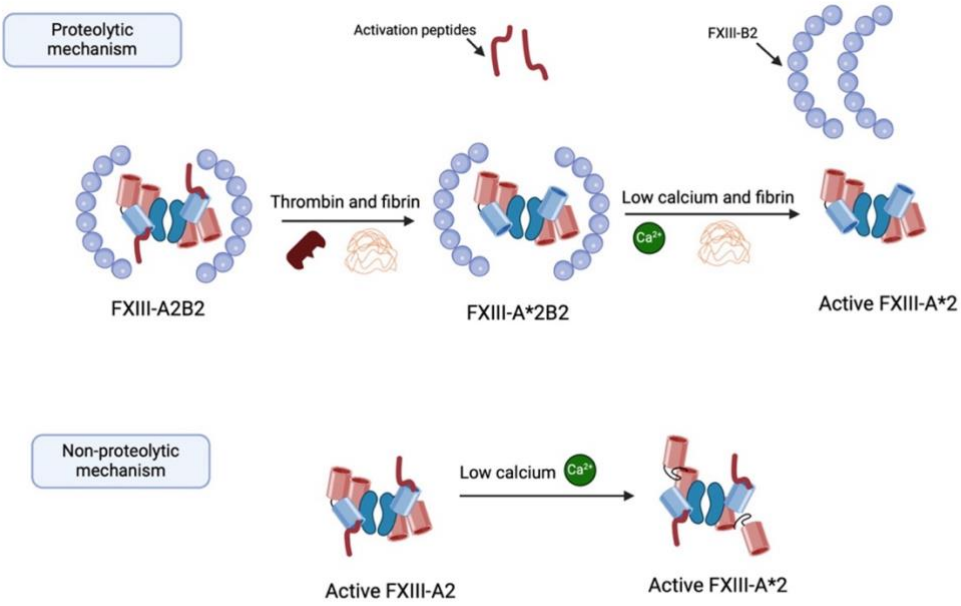

Figure 1.7. Activation of FXIII-A through proteolytic and non-proteolytic mechanisms. Inspired by Ref. [121] and created in Biorender.com.

1.3.2. Role of FXIII-A in hemostasis

The role of FXIII-A in the final stage of the coagulation cascade and hemostasis is established. Maintaining approximately 50% of normal circulating levels of FXIII-A is crucial for efficient hemostasis [120]. During coagulation, thrombin cleaves N-terminal fibrinopeptide from the Aα- and Bβ-chains of fibrinogen (will be discussed in detail in the context of Fibrinogen on page 52), leading to the polymerization of fibrin monomers into fibrin fibres [124]. FXIII-A then catalyzes the formation of isopeptide bonds between glutamine 398/399 and lysine 406 residues in the fibrin γ-chain and between glutamine and lysine residues in the α-chain, enhancing thrombus stability and mechanical strength. This specificity for fibrin is likely influenced by the pre-localization of FXIII-A on fibrinogen in circulation, which effectively reaches FXIII-A to the site of clot formation [125].

To date, 147 substrates have been identified for FXIII-A, with 48 of these substrates incorporated into the clot [126]. Fibrinolytic inhibitors like α2-antiplasmin (α2-AP) are crosslinked into the fibrin matrix, protecting thrombi against fibrinolysis and ensuring the durability of the clot during tissue repair [127]. The incorporation of various ECM proteins, such as vitronectin and thrombospondin, further assists in tissue repair [125]. The crosslinking activity of FXIII-A is crucial as it also reduces the binding of plasminogen and tissue-type plasminogen activator (tPA) to fibrin. Indeed, the denser and thinner fibers are more resistant to fibrinolysis due to reduced solute access and diminished tPA binding [127].

Additionally, FXIII-A within platelets plays a role in stabilizing clots by translocating to the platelet membrane upon stimulation, where it can perform extracellular crosslinking reactions [128]. FXIII-A is synthesized during thrombopoiesis in megakaryocytes and remains in the cytoplasm of platelets, not α-granules. Its activation occurs through an elevation in intracellular

calcium levels. Once activated, FXIII-A translocates to the outer membrane, mainly to the cap area. Despite its significant role within platelets, FXIII-A is not released to the outside of platelets, suggesting that its primary function occurs on the surface and within platelets [129]. Platelet FXIII-A also interacts with integrin βIIbβ3, influencing clot retraction and size. Plasma FXIII-A, not platelet one, contributes to retaining red blood cells (RBCs) in clots and enhancing clot stability [128, 130]. Studies have also demonstrated that monocyte and macrophage-derived FXIII-A is expressed on the surface of these cells and may be involved in hemostasis by promoting fibrin crosslinking and thereby stabilizing thrombi [131, 132]. Hence, FXIII-A is a critical factor in the regulation of hemostasis and thrombosis, regardless of its sources.

1.3.3. FXIII-A in inflammatory cells

The inflammatory function of FXIII-A is closely related to its significant intercellular presence in inflammatory cells such as monocytes, macrophages and dendritic cells, highlighting its role in immune cell functions [117]. The mRNA expression of FXIII-A has been identified in various monocyte-derived macrophages, including, tumor-associated macrophages, dendritic reticulum cells, and alveolar macrophages, indicating its endogenous production [133]. FXIII-A is mainly located in the cytoplasm; however, evidence shows its translocation to the cell membrane upon activation, as observed in U937 cells (a human promonocytic tumor line) treated with phorbol myristate acetate, lipopolysaccharide and interferon-gamma (INF-γ). Additionally, FXIII-A detected in the culture media of dendritic cells, differentiated from monocytes, was correlated to dying cells rather than secretion, owing to the lack of a secretion signal [134]. Moreover, its translocation to the nucleus and crosslinking activity have also been studied [135]. A recent study shows that stimulation of monocytes and THP-1 cells with certain cytokines (e.g. IL-4, IL-10)

promotes the externalization of FXIII-A and stabilization of thrombus against fibrinolytic degradation [131]. Also, its upregulation, alongside other transglutaminases like TG2, in these cells upon differentiation and activation has been reported, though FXIII-A remains the predominant enzyme responsible for the observed transglutaminase activity [136, 137].

Activated polymorphonuclear (PMN) leukocytes, containing enzymes such as human NE, cathepsin G and MMPs, break down FXIII-A within fibrin clots, leading to reduction of the crosslinking activity during coagulation. This degradation is much faster in the presence of α1-antitrypsin (α1-AT), suggesting a regulatory role of these enzymes in modulating FXIII-A function. During inflammation, human NE can activate pFXIII-A and cFXIII-A by cleavage in Val39-Gln40 peptide bond, followed by a gradual inactivation. The inactivation rate ensures stable fibrin clot formation, limiting overactivity with potential deleterious effects [121, 138].

Moreover, FXIII-A contributes to the proliferation, survival and infiltration of PMNs to inflammatory sites [138]. Several glycoproteins on immune cells are substrates of FXIII-A. For example, on the surface of activated platelets, FXIII-A dimerizes ribosomal protein S9 (RP S19) and converts it into MCP, promoting monocyte/inflammatory macrophage migration to the injury site [139, 140]. A similar dimerization mediated by cFXIII-A has also been reported in monocytes, in which angiotensin receptor 1 (AT1) is covalently dimerized, promoting the adhesion of these cells to endothelial cells, crucial in hypertensive conditions and atherosclerosis development [141]. In addition, FXIII-A influences PMNs activities, such as neutrophils' functional responses [142] .It is also implicated in the mechanisms of phagocytosis and motility in monocytes and macrophages, possibly through the crosslinking of cytoskeletal proteins [114, 138, 139, 143].

The expression of FXIII-A in macrophages is both stimuli- and phenotype-dependent. M1 or classically activated macrophages, stimulated by immune mediators, show a downregulation of

FXIII-A [144, 145], while M2 or alternatively activated macrophages in response of IL-4 and IL-13 display upregulation of FXIII-A [145, 146]. This increase is in line with the anti-inflammatory functions of macrophages in matrix remodeling and wound healing. Although, the precise role of monocyte/macrophage-derived FXIII-A in haemostasis needs further research, their involvement in fibrin crosslinking, wound healing, and inflammatory conditions is evident.

1.3.4. FXIII-A in ECM stabilization and wound healing

Wound healing is a complex physiological process that involves the formation of a scaffold composed of a matrix and different cells crucial for tissue regeneration. Research has shown that both FXIII-deficient animal models and individuals exhibit impaired wound healing, highlighting the pivotal role of FXIII-A in this process [147, 148].

The biochemical activities of FXIII-A in ECM stabilization and wound healing extend beyond simple crosslinking; its primary function begins with stabilizing the fibrin clot and the formation of the initial scaffold. FXIII significantly enhances the mechanical strength and stability of key ECM proteins such as fibronectin and collagen. This stabilization is essential for the migration, adhesion, proliferation, and differentiation of fibroblasts, endothelial cells and other cells involved in tissue regeneration. Moreover, it crosslinks fibrin to proteins on the surface of bacteria, thereby trapping invading bacteria and reducing the infection risks at the injured site [120, 149, 150].

Additionally, FXIII-A plays an important role in angiogenesis, a critical process during wound healing. The stabilized ECM serves as a scaffold that supports the proliferation of endothelial cells and the formation of new blood vessels. Crosslinked fibronectin and vitronectin activate VEGFR-2 and $\alpha_v\beta_3$ receptors on vascular endothelial cells. The formation of receptor complexes, mediated by FXIII-A, activates their signaling pathways and promotes angiogenesis [150-153].

The immobilized FXIII-A in the wound enhances platelet adhesion via integrin receptors, contributing to matrix stability. Additionally, FXIII-A crosslinks ECM proteins such as fibronectin and vitronectin, which bind to integrin on inflammatory cells. This interaction facilitates the migration of immune cells, like neutrophils and macrophages, to the injury site, activating integrin-related signaling cascades. Crosslinked vitronectin has been observed to inhibit neutrophil apoptosis, which are first responders to injury [154-156]. FXIII-A also regulates the release rate of growth factors embedded within ECM, controlling the bioavailability of these active molecules. Thus, FXIII-A ensures a regulated environment for effective wound healing through its ability to stabilize the ECM components [114].

1.4.Factor XIII-A in pathological conditions

Increasing evidence revealed that FXIII-A is not only involved in hemostasis, but also it may contribute to the pathogenesis of several diseases such as thrombosis, fibrosis and metabolic disorders [114, 122].

1.4.1. FXIII-A in thrombosis

Thrombosis, or intravascular coagulation, is a significant global health concern, causing considerable morbidity and mortality with varying rates depending on the location and severity [157, 158]. Thrombosis occurs when blood clots form within vessels, obstructing normal blood flow and potentially leading to serious health complications such as myocardial infarction (MI), stroke, pulmonary embolism and deep vein thrombosis (DVT) [159].

Arterial thrombosis typically develops from atherosclerotic plaque within arteries, resulting in the formation of occlusive white thrombi that are rich in platelets as a result of platelet activation and fibrin deposition at the site[160]. Conversely, venous thrombosis, or thromboembolism, occurs in

a state of hypercoagulability and is triggered by the inappropriate expression of cell adhesion molecules. The formed thrombi are rich in RBCs, termed red thrombi, and characterized by a high fibrin content. Despite the differences in triggering stimuli, both arterial and venous thrombosis share the common feature of fibrin formation [161]. Therefore, targeting this process is a sophisticated strategy to treat both types of thrombosis.

FXIII-A activation initiates early during clot formation, and its activity is detectable within thrombi several hours thereafter. FXIII-A is bound to fibrin and may be released into circulation upon fibrinolysis. Under normal physiological conditions, activated FXIII-A is gradually deactivated within the clot over time [162]. Increased crosslinking activity of FXIII-A contribute to the stiffness of fibrin networks, which play a role in thrombus remodeling [163]. The inhibition of FXIII-A is achieved through various enzymes, including thrombin, plasmin and enzymes released from granulocytes [162, 164].

The FXIII-fibrinogen axis is crucial in venous thrombogenesis. Rapid activation of FXIII-A upon thrombus formation contributes to the retention of RBCs in clots and affects the thrombus size. Studies from FXIII-deficient human and mouse models have shown thrombi with less RBC content and smaller size. FXIII-A affects RBC retention through fibrin α-chain crosslinking [165]. Also, mice with the Fibγ390-396A mutation (which will be discussed in detail in the context of Fibrinogen on page 52), which exhibit reduced FXIII-A binding to fibrinogen and delayed fibrin crosslinking, showed decreased RBC retention [130, 166]. Additionally, mice with FXIII V35L polymorphism (which will be discussed in the context of FXIII-A mutation on page 49) that shows faster FXIII activation, revealed reduced RBC retention in a fibrinogen-dependent manner. The activation and activity of FXIII are dependent on its binding to soluble and circulating fibrinogen [167, 168].

A positive correlation between FXIII-A and arterial thrombosis in diabetic mouse models has also been reported [169]. In animal models, it was shown that the administration of FXIII-A inhibitors prior to thrombus formation significantly reduced the thrombus stability more than tPA alone. However, administrating the inhibitors after thrombus formation had no effect, highlighting the critical role of FXIII-A from the onset of thrombus formation [169]. Overall, FXIII-A contributes to multiple stages of thrombus formation and stability.

1.4.2. FXIII-A in fibrosis

The body responds to tissue damage through two phases to repair the injured site. In the regenerative phase, damaged cells are replaced with new ones identical to the originals. However, if regeneration is insufficient, the repair process enters the second phase, called fibroplasia or fibrosis. During this phase, parenchymal tissue is replaced with connective tissue, resulting in scar formation. Although this process is critical for maintaining tissue integrity, prolonged and uncontrolled activation can lead to an excessive deposition of ECM proteins, which ultimately can cause organ damage and fibrosis [170, 171].

The involvement of transglutaminase activity in fibrotic events in various organs, including the kidney, heart, liver, and adipose tissue, has been well-documented (**Figure 1.8**). Several stimuli, such as hypoxia, inflammation, and hyperglycemia can trigger fibrosis. Among transglutaminases, TG2 has been the most studied enzyme in the context of tissue fibrosis which activates epithelial-mesenchymal transitions (EMT), latent TGF-β binding protein (LTBP)-TGF-ß signaling and matrix crosslinking [115].

While FXIII-A is widely recognized for its critical role in hemostasis, its potential role in fibrotic diseases affecting various organs is gaining increasing attention. Its upregulation in pathological

states underscores its involvement in complex fibrotic events. Early studies demonstrated its expression in cells within lung and oral lesions [172, 173]. Furthermore, V34L mutation (which will be discussed in the context of FXIII-A mutation on page 49) in FXIII-A structure has been linked to accelerated progression of liver fibrosis in patients with chronic hepatitis B and C [115, 174].

Research from our laboratory has demonstrated that the complete absence of FXIII-A in knockout mice leads to fewer complications related to diet-induced obesity in adipose tissue. Particularly, there was a significant reduction in Col I and fibronectin accumulation within ECM, indicative of reduced adipose tissue fibrosis. This decrease in fibrosis correlated with reduced macrophage infiltration in adipose tissue and improved insulin sensitivity [175]. A study on renal transplant biopsies and quantitative proteomics associated FXIII-A and three other proteins with interstitial fibrosis. This positions FXIII-A as a significant predictor of fibrosis alongside other markers [176]. Furthermore, another study revealed that FXIII-A global knockout male mice displayed cardiac fibrosis linked to defective hemorrhaging events, with no change in TG2 levels within their heart [177]. Additionally, it was noted that while fibrosis did not occur in apolipoprotein E (ApoE)/FXIII-A double knockout and FXIII-A single knockout mice, increased cardiac fibrosis was observed in a triple knockout involving ApoE/FXIII-A/TG2. This increase was attributed to defects in tissue integrity and a pro-fibrotic stimulus from extravasated blood in the myocardium [178].

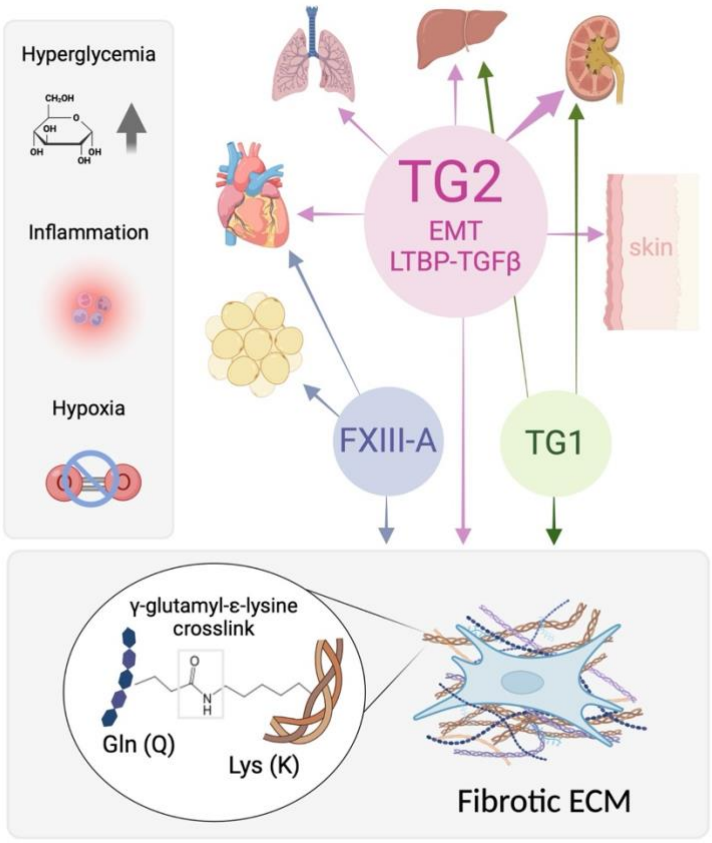

Figure 1.8. Overview of the contributions of TG1, TG2 and FXIII-A to organ fibrosis. Adapted from Ref. [116] with permission from the American Physiological Society.

1.4.3. FXIII-A in human obesity and metabolic diseases

F13A1 was identified as a significant gene associated with obesity through a genome-wide association study (GWAS) involving a rare cohort of weight-discordant monozygotic twins (lean-heavy twin pairs). The study also included data from the large European ENGAGE consortium study, which involved over 21,000 unrelated individuals and showed seven single nucleotide

polymorphisms (SNPs) in *F13A1* gene associated with body mass index (BMI), suggesting the potential regulatory role of FXIII-A in adipose tissue biology [179].

Our laboratory has conducted pioneering research on the role of FXIII-A in connective tissues, particularly focusing on its expression in osteoblasts and adipocytes and its involvement in the fibronectin and Col I matrix [180-188]. Our group has demonstrated that FXIII-A acts as a regulatory switch in insulin signaling of 3T3-L1 cells, affecting transitioning from a pro-proliferative to a pro-differentiating state. The mechanism involved FXIII-A promoting the presence of plasma fibronectin ECM in the adipocyte cultures [185]. Other studies in our laboratory demonstrated that in *F13a1* knockout mice (*F13a1*$^{-/-}$) on a HFD, there were significant changes in the cellularity of adipose tissue, a reduction in macrophage infiltration, and a marked improvement in insulin sensitivity [175]. This suggests that the absence of FXIII-A may be beneficial to metabolic health in mice. Building on this foundation, our laboratory further explored the expression of *F13A1* in weight-discordant monozygotic twins, where *F13A1* was originally identified as an obesity-associated gene [189, 190]. It was shown that *F13A1* expression is increased in in heavier twin and associates negatively to adiponectin levels in a manner where a decrease in adiponectin correlates with an increase in *F13A1* level. Our group's research further indicates that *F13A1* is positively linked to hypertrophic adipocytes, cell stress, inflammation, and neutrophils, in human adipose tissue. These findings support the role of FXIII-A in the expansion of adipose tissue and the inflammation linked to obesity. Interestingly, the data also showed a positive correlation between FXIII-A expression and group 16 phospholipase A2 (PLA2G16) and mitogen-activated protein kinase 1 (MAPK1) genes, both of which are involved in lipolysis process [189, 190].

1.4.4. FXIII-A mutations and diseases

FXIII-A deficiency is a rare bleeding disorder, occurring in approximately 1 in 2-5 million individuals, characterized by impaired wound healing. SNPs in the FXIII-A gene is relatively common [114, 191] and can influence the expression, stability or activity of FXIII-A. One of the most studied SNPs in the *F13A1* gene involves a G>T transition in the second exon, causing the substitution of valine (V) with leucine (L) at position 34 in human's FXIII-A (V34L) [114, 192]. This variant, V34L, which corresponds to V35L in mice, is located just three amino acids upstream from the thrombin cleavage site on the FXIII-A protein. This results in about 2.5 times faster activation of FXIII-A by thrombin, thereby enhancing the rate of fibrin crosslinking, which is essential for clot stability. The V34L variant also influences the plasma levels of FXIII-A, leading to faster elimination and consequently lower enzyme levels in the blood stream [167, 192]. Research indicates that this polymorphism also affects the fibrin clot structure in a fibrinogen level-dependent manner. At low or normal fibrinogen levels, this mutation leads to the formation of thinner fibrin fibers and results in clots with low permeability. Conversely, a high concentrations of fibrinogen result in thicker clots, which exhibit increased permeability and a higher susceptibility to fibrinolysis. Since the high concentration of fibrinogen is associated with the risk of cardiovascular diseases, this mutation can be beneficial [167, 193, 194]. The V34L mutation, associated with alterations in clot structure, has been linked to a decreased risk of arterial and venous thrombosis, CVD and MI in humans[192]. These protective effects are particularly noticeable in populations at higher risk for CVD, such as obese and menopausal women [195, 196]. Notably, in women who carry at least two copies of the V34L-FXIII-A alleles and who use estrogen, the risk of MI is further reduced compared to those who do not use estrogen. While this mutation affects cardiovascular health, it does not have an association with BMI [197]. *F13A1*

SNP rs7766109 has been associated with BMI and insulin resistance in individuals with polycystic ovary syndrome [198]. It is not known how the V34L mutation affects adipose tissue health and development of insulin resistance in obesity, which needs further studies.

1.4.5. FXIII-A and PAI-1: Interplay in fibrinolysis and metabolic diseases

Fibrinolysis is critical for maintaining vascular health by breaking down fibrin clots after bleeding stops. A normal balance between coagulation and fibrinolysis is essential to prevent pathological conditions such as excessive bleeding, thrombosis, stroke, and heart attacks. The key step in fibrinolysis is the conversion of plasminogen to plasmin, which is facilitated by t-PA and u-PA. t-PA is particularly effective in the presence of fibrin, forming a complex that enhances its activity and specifically targets fibrin degradation [199, 200].

FXIII-A significantly impacts the fibrinolysis process by crosslinking fibrin chains, thereby enhancing their resistance to fibrinolysis (**Figure 1.9**). It interacts with other proteins involved in modulating fibrinolysis, such as α2-AP, PAI-2 and thrombin-activatable fibrinolysis inhibitor (TAFI), crosslinking them directly to fibrin and significantly enhancing their antifibrinolytic activities. Changes in the levels of these inhibitors can profoundly affect clot formation and dissolution, leading to cardiovascular diseases and thrombotic disorders [201, 202].

While FXIII-A does not interact directly with PAI-1, a major inhibitor of plasminogen activators. Indeed, they play complementary and synergistic roles in inhibiting fibrinolysis and stabilizing blood clots. High levels of PAI-1 and FXIII-A are associated with dense clot formations, typically observed in patients with metabolic syndrome [114]. PAI-1 regulates the fibrinolytic system by controlling the formation of plasmin [127, 203, 204]. PAI-1 is produced in various tissues, including adipose tissue, liver and spleen, and its levels can vary in response to physiological and

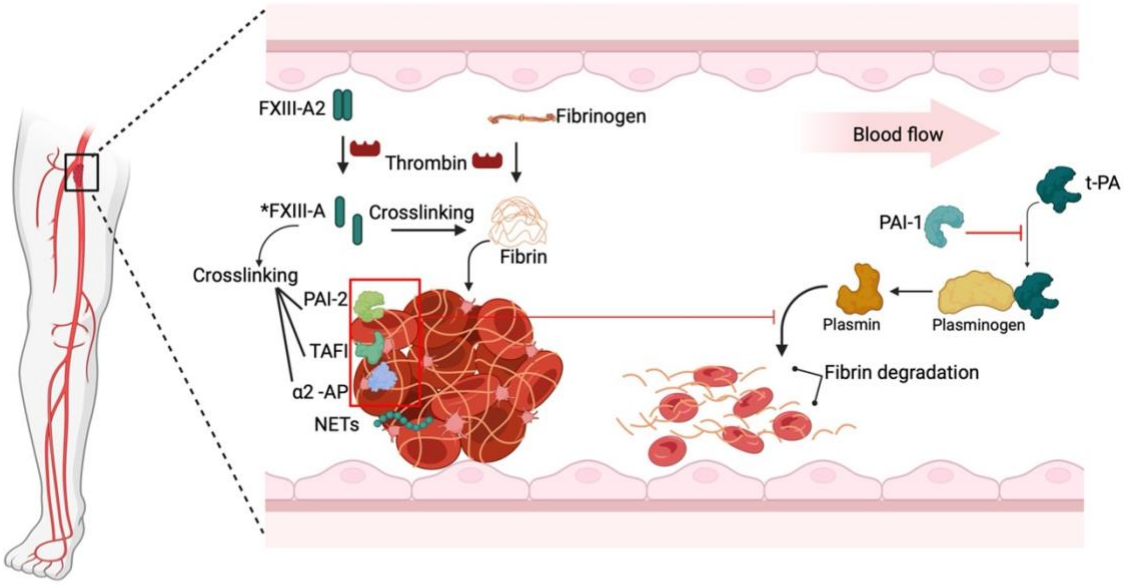

Figure 1.9. The role of FXIII-A and PAI-1 in fibrinolysis. Created in Biorender.com.

pathological stimuli such as inflammation or metabolic conditions. Notably, adipocytes, particularly those within VAT, are the major sources of circulating PAI-1 in obese individuals. PAI-1 influences adipocyte differentiation and regulates immune cell infiltration into adipose tissue during obesity [205, 206]. Besides its role in fibrinolysis system, PAI-1 also contributes to multiple physiological processes including degradation of ECM, tissue remodeling, angiogenesis and inflammation. Although the absence of PAI-1 does not impair hemostasis, it is linked to a hypofibrinolytic state and resistance to thrombosis [207].

Furthermore, elevated levels of PAI-1 contribute to the progression of thrombosis, fibrosis, and obesity- related complications such as insulin resistance [208-211]. Studies have shown that PAI-1 knockout mice are protected against obesity and metabolic dysfunction [212]. Inhibition of PAI-1 has been shown to reduce macrophage infiltration, while PAI-1 knockout models exhibit an increased number of anti-inflammatory macrophages in adipose tissue [213, 214]. The role of PAI-

1 in body weight regulation has been related to hypothalamic leptin resistance, particularly during a high-fat diet. Unlike PAI-1, FXIII-A does not directly affect body weight regulation [175, 215].

1.5. Fibrinogen and its relation to metabolic health

1.5.1. Fibrinogen: Structure

Fibrinogen, a 340-kDa hexameric glycoprotein, is one of the key plasma proteins, and one of the most abundant proteins in the bloodstream with concentration ranging from 2–4 mg/mL. It is also a component of natural ECM. At physiological conditions, its expression rate is constant, however in certain circumstances like inflammatory its production can be increased by two to three times [216-218].

Fibrinogen plays a crucial role during hemostatic processes by forming the insoluble fibrin matrix. It possesses a cell-signaling domain composed of protease degradation and cell adhesion motifs. The protein is encoded by three genes, including *FGA*, *FGB*, and *FGG*, located on chromosome 4, and includes three pairs of polypeptide chains: Aα, Bβ, and γ (**Figure 1.10**). These chains are synthesized primarily in hepatocytes and then undergo various posttranslational modifications, which are important for its proper function in the coagulation cascade. The six chains are arranged symmetrically around a central E region, supporting the interactions with the D domains at each end of the molecule. Fibrinogen has two D domains containing the Bβ and γ chain C-termini and a central E domain with the N-termini of all chains, essential for initiating fibrin polymerization by binding thrombin [219, 220]. Several alternatively spliced forms of fibrinogen are present in plasma. For example, there is another form of the γ chain, γ′, in which the last four amino acids in γ chain are replaced with 20 amino acids resulting in a more negative charge residue. A relation between the presence of γA/γ′ -fibrinogen in plasma and the incidence of various diseases like

cardiovascular diseases has been found [221]. Also, clots having this altered structure are more resistant to fibrinolysis [222]. Moreover, disulfide bond formation and glycosylation occur upon fibrinogen synthesis. The variations in PTMs may lead to different biological and pathological conditions, depending on the final fibrin structure [223]. Therefore, the fibrinogen structural type and arrangement is crucial for transforming fibrinogen into fibrin during blood clotting as well as interaction with various cell types. This mediates processes like cell adhesion, migration, and inflammation, which are related to specific motifs within the fibrinogen chains. These motifs bind to cellular receptors, such as integrins and stimulate signaling pathways critical for cellular responses during tissue repair and inflammatory reactions[224-227].

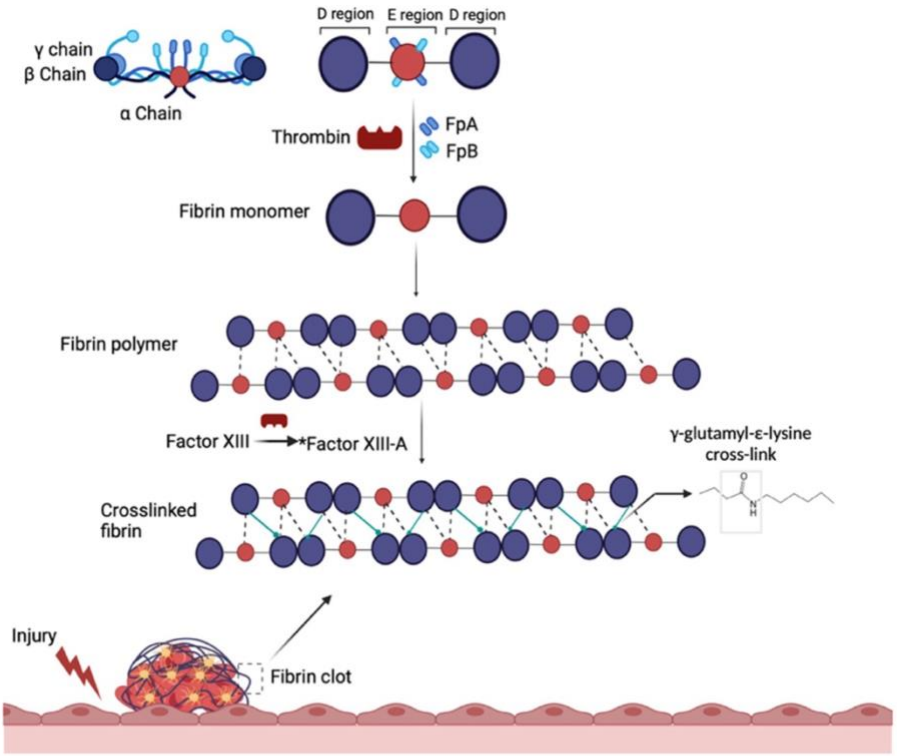

Figure 1.10. Schematic representation of fibrinogen structure and fibrin formation. Created in Biorender.com.

1.5.2. Fibrinogen and hemostasis

Fibrinogen is a crucial protein in the blood coagulation, playing a key role in clot formation and maintaining hemostasis. Upon vascular injury, the coagulation cascade is triggered, leading to thrombin activation. Thrombin cleaves fibrinopeptides from the Aα chains of fibrinogen, releasing Bβ chains, which leads to the formation of fibrin monomers. These monomers interact with other fibrin molecules, facilitating the formation of fibrin oligomers and protofibrils. These structures then laterally aggregate and branch to form a stable, three-dimensional fibrin network, namely clot [228, 229] (**Figure 1.10**).

The characteristics of the clot are significantly influenced by thrombin concentrations. Lower thrombin levels lead thick, loose networks that are more susceptible to fibrinolysis, while higher levels create denser networks with thinner fibers, which are more resistant to fibrinolytic degradation. Different variants of fibrinogen affect clot properties and its interaction with other cells. For instance, the γA/γ′ isoform, which is prevalent in plasma, can also participate in fibrin networks, resulting in thinner fibers more resistant to fibrinolysis. This isoform has a higher affinity for thrombin and can influence thrombotic processes depending on its concentration and interactions [221, 230].

As earlier mentioned, FXIII-A plays a crucial role in fibrin formation, by crosslinking fibrin fibers, enhancing their mechanical strength and stability. Additionally, FXIII-A mediates the crosslinking of other molecules like α2-AP and vWF into the fibrin structure, contributing to the biophysical characteristics of fibrin, including resistance to fibrinolysis [231]. Therefore, the formation of fibrin initiated by thrombin and its subsequent stabilization by FXIII-A are essential for adequate hemostasis and wound healing. Moreover, the presence of other isoforms of fibrinogen introduces additional complexity to the coagulation process outcome.

1.5.3. Fibrinogen role in inflammation and obesity

Fibrinogen is not only well-recognized for its role in coagulation but also plays an important role in inflammatory responses and leukocyte activities. Elevated fibrinogen levels have been linked to various inflammatory conditions, including rheumatoid arthritis and cardiovascular diseases, indicating its involvement in both acute and chronic inflammation [217].

Moreover, increased concentrations of fibrin degradation products (FDPs), such as d-dimer, are used as a biomarker for inflammation and thrombotic events [232]. Fibrinogen facilitates the migration of leukocytes from blood vessels into injured tissues, activating their effector functions. Its proinflammatory effects are related to interactions with immune cells, leading to the release of reactive oxygen species, which serves as a localized signal within damaged areas. Fibrinogen contributes to inflammation through acting as a ligand for various cell surface receptors. Its binding to αMβ2 integrin on immune cells including neutrophils, macrophages, Kupffer cells and microglial cells activates signalling cascades, such as NF-κB, which trigger the release of pro-inflammatory cytokines, including TNF-α or IL-1β, making fibrinogen a potent driver of local inflammation and tissue damage [233]. Additionally, the conversion of fibrinogen into a fibrin mesh supports further cellular migration and angiogenesis, enhancing tissue remodelling and healing [234].

Studies utilizing the Fib$\gamma^{390\text{-}396A}$ mouse models, which carry a mutation in the gamma chain of fibrinogen, preventing its interaction with αMβ2 integrin have demonstrated reduced inflammatory responses compared to the wild-type mice. These mice were protected in models of inflammatory diseases such as colitis, arthritis and neuroinflammatory diseases. It is noteworthy that Fib$\gamma^{390\text{-}396A}$ transgenic mice maintain normal levels of fibrinogen and fibrin formation. Engagement of

extravascular fibrin deposits by αMβ2-expressing immune cells like neutrophils and macrophages contributes to local inflammation and tissue damage [235-237].

Fibrinogen deposits have also been shown to impact regenerative processes; for example, injection of wild-type fibrinogen into the central nervous system induces severe inflammatory demyelination compared to Fib$\gamma^{390\text{-}396A}$ variant [238]. Fibrinogen may be involved in obesity pathologies and metabolic syndrome [239]. The obese individuals with type 2- diabetes show increased fibrinogen levels [240, 241]. Also, fibrinogen from diabetic patients is known to form denser, more fibrinolysis-resistant clots, potentially contributing to the thrombotic state. Treatment with insulin can affect the glycation of fibrinogen and thereby changing the architecture of formed clot into a more permeable thrombus [230]. A study involving Fib$\gamma^{390\text{-}396A}$ mouse models on a high-fat diet, showed that the mutation protects mice from obesity and its complications, such as hypercholesterolemia and diabetes. These mice exhibited less weight gain and reduced adipose tissue inflammation. Conversely, mice with reduced or no expression of fibrinogen (Fib- and siFga-treated mice) developed obesity-associated pathologies similar to wild-type mice [238]. Moreover, a study using homozygous thrombomodulin-mutant ThbdPro mice showed that elevated thrombin activity leads to increased weight gain and worsen fatty liver conditions. Inhibition of fibrin formation using dabigatran, a thrombin inhibitor, protected against obesity and reduced the obesity-related complications, underscoring the profound effect of fibrin networks on the inflammatory response and weight modulation [242].

Mice expressing fibrinogen locked in its monomeric form (FibAEK) and mice deficient in FXIII-A displayed an intermediate phenotype, showing partial protection from some obesity-associated pathologies. This supports the concept that fibrin polymerization and crosslinking are crucial for fibrin-driven inflammatory responses, which refers to the inflammatory processes that are initiated

or exacerbated by the presence of fibrin. These protective effects underscore the significant role of fibrinogen- αMβ2 interactions in inflammation [242, 243].

Given the role of FXIII-A in fibrin stabilization, it seems that FXIII-A contributes to inflammation through both fibrin-dependent and independent events. The stiff and stable fibrin networks mediated by FXIII-A activity enhance inflammatory responses by facilitating cell adhesion, migration and inflammatory cytokine production[226].

1.6.Thromboinflammation and neutrophils

Thrombosis and inflammation are intertwined and contribute to various biological processes. Thrombosis often occurs following an inflammatory response to prevent the spread of invading pathogens. This process, known as immunothrombosis and first described in 2013[244], appears to be beneficial and typically does not lead to pathological symptoms. Conversely, platelets and the coagulation system can influence inflammatory responses by interacting with immune cells. This interaction can exacerbate the inflammation resulting from thrombotic events, a detrimental process referred to as thromboinflammation. Therefore, these two processes are in a “cause and effect” relationship with each other, necessitating a balanced interplay between immunothrombosis and thromboinflamation[245, 246]. Neutrophils are one of the predominant players in acute thromboinflammatory responses, such as those seen in ischemic stroke, through various mechanisms. Their interactions with platelets involve different adhesion molecules including P-selectin, GPIb, aIIbb3 (on platelets), as well as PSGL-1, and Mac-1(on neutrophils) [247]. Consequently, targeting neutrophils represents a sophisticated strategy to combat related pathological disorders.

1.6.1. NETosis: Neutrophil-mediated immune responses

Neutrophils are the most abundant leukocytes in circulation. They are produced in bone marrow and form a crucial arm of the innate immune system. Neutrophils are often the first immune cells to arrive at sites of injury or infection and play a pivotal role in internal immunity. Upon recruiting to the relevant sites, neutrophils employ a variety of strategies to combat infections, including phagocytosis, degranulation, ROS production, neutrophil extracellular trap formation (NETosis), cytokine, and chemokine production [248, 249].

The concept of NETosis, the process by which neutrophils release their cellular contents, has advanced the understanding of neutrophil immune response. In 1996, Takei et al. [250] described a distinct form of neutrophil morphological changes after treatment with phorbol 12-myristate 13-acetate (PMA), later named neutrophil extracellular traps (NETs) [251]. NETosis has been recognized as a key defense mechanism against infection. However, its contribution to other pathological conditions including cancer, diabetes, atherosclerosis, and thrombosis has been also reported [252-254]. NETosis, unlike apoptosis and necrosis, is a form of programmed cell death that happens upon neutrophil activation. It is initiated by several events, including actin disassembly, plasma microvesicle formation, and chromatin decondensation, which leads to the permeation of nuclear and plasma membranes. Following the rupture of these membranes, the chromatin is released into the extracellular space [255].

NETs are composed of a DNA backbone, histones, and granular proteins such as myeloperoxidase (MPO), and NE, which can entrap and neutralize pathogens. NETosis can be activated by different stimuli, such as activated platelets, PMA, lipopolysaccharide (LPS), and pathogen contact [256, 257]. It was shown that PMA and LPS induce NETosis through nicotinamide adenine dinucleotide phosphate oxidase (NOX)-dependent ROS production, whereas some other stimuli like

ionomycin, derived from bacteria act via a NOX-independent pathway involving calcium influx [258].

NETosis occurs in two distinct forms including, vital and suicidal (**Figure 1.11**). Vital NETosis is a rapid process that can be activated by microorganisms, complements and platelets. During this type of NETosis, the intracellular calcium is increased, and the nuclear materials are released. Also, the cells remain alive and retain their chemotactic and phagocytic functions. In contrast, suicidal or lytic NETosis is a slower process and depends on the mitogen-activated protein kinase (MEK)-ERK pathway and NADPH oxidase activation, ultimately leading to cell death [259].

During both types, DNA is decondensed and released into the extracellular space. However, histone citrullination, mediated by peptidyl arginine deiminase 4 (PAD4), happens only in the suicidal form. The citrullination involves the deimination of arginine in histones H2A, H3, and H4 within the DNA binding site. PAD4 is found in the nucleus of neutrophils, granulocytes, and macrophages and plays a role in diseases such as thrombosis [260, 261]. Once the chromatin is released, it serves as a matrix for other active components, including histones, MPO, NE, cathepsin, and antibacterial proteins, which collectively contribute to inflammation, antimicrobial activity and thrombosis. Therefore, this process is not only about combating infection but also contributes to the pathogenesis of various diseases [262].

1.6.2. Posttranslational modifications of NETs

Posttranslational modifications (PTMs) of neutrophil proteins are crucial in regulating neutrophil physiological and pathophysiological functions [263]. PTMs such as histone acetylation, methylation and citrullination influence processes like differentiation, migration, phagocytosis, and neutrophil survival [264, 265]. Specifically, PTMs of NETs are implicated in the pathogenesis

of various diseases, including thrombosis, sepsis and lung diseases [266, 267]. For instance, citrullinated histone H3 (citH3) has been associated to acute lung injury, pulmonary fibrosis and venous thrombosis [268, 269].

Besides histone modifications, other protein modifications occur on NET proteins. These modifications are mediated by enzymes such as peptidyl arginine deiminase, methyltransferase, and histone acetyltransferase. Transglutaminases catalyze covalent bond formation between proteins by crosslinking glutamine residues to primary amines, typically lysine residues. This results in forming ε-(γ-glutamyl) lysine isopeptide bonds, which stabilize the proteins [270-273]. The presence of bis(γ-glutamyl) polyamine and ε-(γ-glutamyl) lysine bonds in NET protein fractions suggest a transglutaminase-mediated crosslinking process during NETosis. Proteomic analysis has revealed that during NETosis, transglutaminase activity in neutrophils leads to the crosslinking of polyamines through chlorinated polyamines, ε-(γ-glutamyl) lysine, and bis-γ-glutamyl polyamine bonds into NET proteins such as MPO, NE, histones, and lactoferrin. This mechanism is essential for the stabilization and formation of functional NETs. Additionally, endogenous polyamines like spermine (SPM) and spermidine (SPD), as endogenous polyamines, have been detected as covalently bound to NETs, dependent on MPO activity. This confirms the critical role of crosslinking in maintaining neutrophil activity[274].

The NET-specific crosslinks as PTMs not only contribute to protein stabilization but also help prevent the spread of harmful neutrophil contents into the circulation. However, these crosslinks can also enhance binding of coagulatory protein to the NETs, leading to complications such as fibrosis and thrombosis.

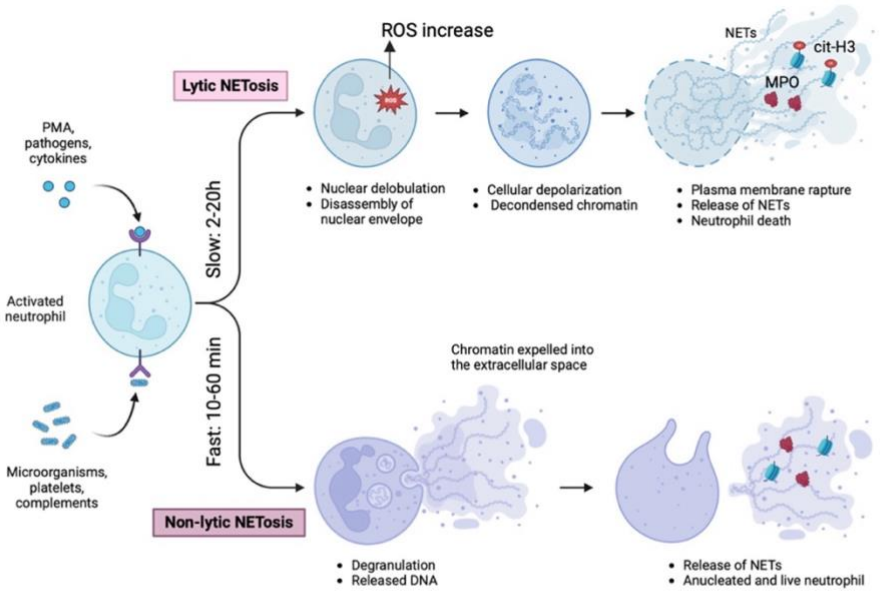

Figure 1.11. Two different types of NETosis and their characteristics. Template adapted from Biorender.com with modifications.

1.6.3. Neutrophils and NETs in blood coagulation

While the role of monocytes and macrophages in coagulation is well-documented, recent studies have highlighted the involvement of neutrophils in the pro-coagulant process [275, 276]. Emerging evidence has shown their interaction with injured endothelium, which facilitates a coagulative response [277-280]. Exposed tissue factor on the dying neutrophils and released microparticles can initiate a coagulant event [281]. Additionally, neutrophils release proteases such as cathepsin G and elastase, which activate platelets and other key proteins in the coagulation process [282, 283].

Neutrophils suppress the tissue factor pathway inhibitor (TFPI) through their content of cathepsin G, elastase, and MMPs, thereby promoting coagulation. Neutrophil elastase also plays a role in the

degradation of antithrombin III, further affecting the coagulation cascade [284, 285]. Moreover, neutrophils contribute to the immune response and coagulation through the generation of NETs [279, 286]. When NETs are installed in a flow chamber perfused with blood, they can simulate coagulation, leading to the formation of fibrin networks. These networks interact with proteins such as fibronectin and vWF, providing a scaffold that supports the adhesion of RBCs, leukocytes, and platelets, facilitating fibrin deposition and clot formation [287].

Partially activated neutrophils and NETs can induce clot formation in plasma, independent of triggers [279]. Components of NETs, including DNA and histones. also exhibit a coagulative effect [288, 289]. DNA activates the contact system and the intrinsic pathway due to its highly negative charge, leading to the activation of Factor XII and Factor XI, and subsequent thrombin generation. It also affects fibrinolysis by inhibiting the plasmin-mediated fibrin degradation [287]. *In vivo* studies have shown that histones, particularly H3 and H4 promote coagulation and enhance fibrin deposition by affecting platelets and their αIIbβ3 integrin receptors [290]. Histones trigger vWF release from endothelial cells and promote thrombin generation. Moreover, they induce macrophage lysis and expose phosphatidyl serine, which activates tissue factor and triggers systemic coagulation [291].

Furthermore, NETs stabilize plasma proteins like fibronectin and vWF during coagulation and enhance the coagulation cascade through interaction with TF and Factor XII [277]. Although there is substantial evidence linking neutrophils and NETs to coagulation, the precise mechanisms of how NETosis affect coagulation remain an actively researched area. Many studies suggest that NETs promote coagulation by interacting with blood clotting factors and platelets, but more detailed investigations are needed to uncover these interactions. Overall, NETs and fibrin networks

are intertwined, and the colocalization of NETs and fibrin networks might have functional consequences on both inflammation and coagulation.

1.6.4. Neutrophils and NETs in thrombosis

NETs, while necessary for host defense, can lead to adverse effects, including tissue damage, and thrombosis, when excessively activated. Increasing evidence shows the involvement of NETs in thromboinflammation and immunothrombosis processes that link the host defense to thrombotic events [244, 269, 292-294]. NETs also serve as biomarkers for monitoring disease progression [295]. Citrullinated histones have been identified in various thrombi in both human and animal models [296-298]. Infusion of histones in mice has been shown to increase vWF release, leading to microthrombosis in mouse models [299].

The mechanisms by which NETs contribute to thrombosis are complex and not fully understood. It is believed that NETs, in conjunction with fibrin, play a role in the immune response that may lead to thromboinflammation and immunothrombosis [244]. For instance, the intraperitoneal administration of Alum adjutant (aluminum potassium sulfate) results in the formation of nodules comprised of extracellular DNA and fibrin [300].

NETs likely influence coagulation through interactions with blood cells, plasma proteins, and the endothelial vessel wall, affecting blood flow (**Figure 1.12**). Upon endothelial damage, these cells release cytokines, including IL-1b and IL-8. The interaction between neutrophils and platelets at injury sites, mediated by P-selectin/ P-selectin glycoprotein ligand-1 (PSGL-1) and high mobility group box 1(HMGB1)/ receptor for advanced glycation end products (RAGE), triggering NETosis. The adhered NETs can obstruct blood flow and potentially cause endothelial damage through the enzymatic activity of neutrophils. Additionally, the electrostatic interactions between NET DNA

and vWF facilitate the retention of NETs on endothelial cells. [301, 302]. NETs provide a scaffold for cells and coagulation factors, contributing to the development of thrombosis and inflammation at the site of injury [303-306],

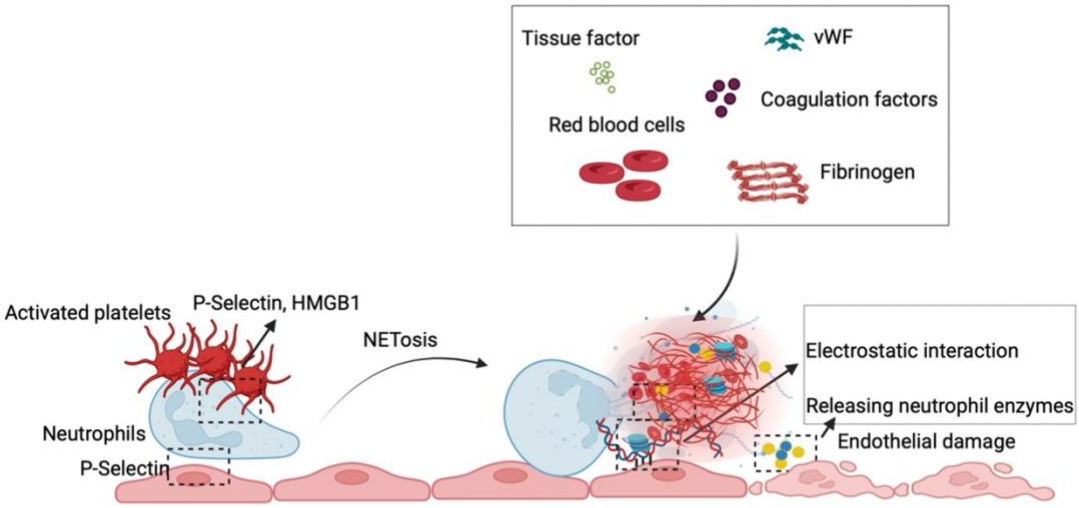

Figure 1.12. The interaction between NETs, cells and proteins leading to thrombosis. Created in Biorender.com.

thus, prevention of neutrophil adhesion or NET formation can be one of the therapeutic interventions to protect from thrombosis.

DNA and histones within NETs also trigger thrombin generation through various mechanisms. Administration of DNase has been shown to reduce thrombus formation in mice, indicating the potential therapeutic targets for DVT and other thrombotic disease [269]. DNA and histones increase thrombin production via the FXII- or FXI-dependent pathways [287], and histones activate platelets through TLR2 and TLR4 receptors [307]. Histones also interact with αIIbβ3

integrins on platelets and induce fibrinogen -mediated platelet aggregation [290]. Moreover, other NET components, such as neutrophil elastase and cathepsin G, promote coagulation in a tissue factor- and factor XII- dependent manner [308].

Despite substantial evidence supporting the procoagulant effects of NETs, some studies present conflicting results. For instance, it was reported that isolated NETs possess a weaker procoagulant effect compared to DNA or histones alone [308, 309]. Also, using NETosis mouse model, it was shown that depleting platelets and inhibiting thrombin did not prevent NET-induced mortality in DNase-deficient mice, suggesting that vascular obstruction by NETs might occur independently of coagulation and fibrin formation [310].

Additionally, complexing isolated DNA with histones in the structure of chromatin abolished the DNA-induced coagulation, however, DNA itself were able to increase thrombin generation and coagulation, indicating the properties of DNA change when part of chromatin structure in NETs [288]. These variations might be due to the experimental conditions which each group adopted, highlighting the need for careful experimental design and interpretation in studies investigating the procoagulant effects of NETs and their components.

1.6.5. Neutrophils and NETs in obesity

As earlier explained, chronic, systemic low-grade inflammation frequently accompanies obesity. Neutrophils, as one of the first responders, infiltrate adipose tissue within a week after the initiation of HFD, preceding the infiltration of macrophages [311, 312]. The inflammation in adipose tissue, exacerbated by factors such as FFAs released from dying adipocytes, attracts neutrophils [47]. The inflammatory state during weight gain is associated with an expansion of neutrophil populations [311, 313, 314]. Indeed, the number of circulating neutrophils is elevated in individuals with

obesity and metabolic syndrome, correlating with higher BMI [315-318]. The increased levels of pro-inflammatory cytokines observed in obesity stimulate granulopoiesis in the bone marrow, releasing of high numbers of neutrophils and other immune cells into the circulation [319, 320]. Additionally, leptin, produced by adipose tissue, affects the production and activation of these cells [321, 322]. In obesity, neutrophils exhibit an activated phenotype, marked by increased expression of the degranulation marker, CD66, as well as MPO and NE[323]. Activating signaling pathways such as NF-κB in obesity further highlights their enhanced activation state [324-326].

NETosis has recently garnered more attention in the context of obesity and its pathological consequences. Studies indicate elevated levels of plasma DNA-MPO complexes in obese individuals compared to their lean counterparts, with NET markers correlating with BMI [327]. Nonetheless, the specific role of NETosis in obesity is still under debate, with studies presenting both supportive and contradictory findings. For example, higher levels of NETosis marker, cathelicidin-related antimicrobial peptide (CRAMP), have been observed in the mesenteric arterial walls of mice on a HFD. This increase was mitigated by treatment with Cl-amidine, a PAD4 inhibitor, or DNase, suggesting that NETs may impair endothelial function through MPO activity and free radical production [328]. Additionally, granule proteins such as NE released during NETosis have been linked to the induction of insulin resistance, probably by degrading insulin receptor substrate 1 and enhancing leukocyte infiltration through TLR4 signaling [329].

Conversely, other studies report lower levels of NETosis in obese mice compared to non-obese ones despite signs of neutrophil activation [330, 331]. Intravital microscopy in mice on a HFD revealed reduced NET production in the liver vasculature compared to lean mice [331]. Furthermore, while inhibiting NETosis in the early stages of obesity does not appear to be beneficial in terms of leukocyte infiltration, activation, and insulin resistance, it may improve

metabolic parameters in more advanced stages of the disease [332]. These discrepancies between studies may stem from differences in the methods used to assess NETosis.

1.7. Mouse models and tools for the *in vivo* study of neutrophils

1.7.1. Traditional models: Neutrophil depletion

Various mouse models have been developed to study neutrophil biology by either depleting neutrophils or knocking out the key proteins [333] (**Table 1.1**). In order to deplete neutrophils and study their role *in vivo*, inducible neutrophil depletion models using either pharmacological agents or specific antibodies have been developed [334-336]. This strategy offers a convenient and cost-effective approach which is applicable across different mouse strains. For example, cyclophosphamide [335], a chemotherapeutic agent, is injected intraperitoneally or subcutaneously into the mice to trigger the death of hematopoietic stem cells and, consequently, rapid depletion of neutrophils. However, its lack of selectivity, potential side effects and affecting other cell types such as monocytes, B cells, and T cells may complicate result interpretations and necessitate further validation with other methods [335, 337, 338].

Antibodies targeting neutrophils, such as anti-Gr-1 and anti-Ly6G, are also used for depletion. The Gr-1 antibody targets both Ly6G and Ly6C proteins; Ly6G is more specific to neutrophils, whereas Ly6C is expressed in a broader range of cells, including monocytes, macrophages and T-cells. Neutrophil death via these antibodies occurs primarily via complement-dependent opsonization. Despite their effectiveness, anti-Gr-1 antibodies have several drawbacks, including non-specificity and severe side effects like microvascular obstruction and respiratory defects in certain conditions [339]. Anti-Ly6G is more specific and does not bind to Ly6C but requires higher doses for effective neutrophil depletion, and their effects are transient [340]. These antibodies can be used

for neutrophil depletion in pure form; however, when conjugated to fluorophores, they can be used to detect neutrophils. Moreover, the age and strain of mice as well as the type of fluorophores can affect the consistency of results. These antibodies require macrophages for neutrophil depletion. Therefore, the role of macrophages in the phagocytosis of antibody-bound neutrophils should be considered when studying macrophage function alongside neutrophils [341-343].

1.7.2. Genetic models: Studying neutrophil function

1.7.2.1. Global knockout models

Various genetic models have been developed to induce constitutive neutropenia. Granulocyte colony-stimulating factor receptor (G-CSFR$^{-/-}$) knockout mice, where the G-CSFR gene is deleted, displayed reduced neutrophil counts in both blood and bone marrow. However, their application is limited due to the involvement of G-CSFR in other critical physiological processes, which can lead to abnormalities. and confound experimental results [344, 345]. Cxcr2$^{-/-}$ mice are other neutropenia models which exhibit mild reduction in circulating neutrophils. Cxcr2 is one of the key chemokine receptors expressed by neutrophils and contributes to the migration of neutrophils from bone marrow to the circulation. Knockout of this protein leads to the accumulation of mature neutrophils in bone marrow [346, 347]. The primary limitation of this model is its lack of specificity, in which this receptor is expressed on other cells, including monocytes and macrophages [348]. Gfi-1-deficient mice lack the growth factor independence-1 (Gfi-1) gene, which is necessary for the differentiation of hematopoietic cells and immune regulation [349-351]. The Gfi expression is not limited to neutrophils, and other cells, including granulocytes and LPS-activated macrophages, also produce Gfi. So, the deletion results in a reduction of neutrophils in bone marrow, blood and spleen, profound neutropenia, but the

emergence of certain features including delayed growth and atypical cells complicate the interpretation of experimental outcomes [349-351]. Genista mice carry a point mutation in the Gfi-1 gene, in which the interaction between Gfi-1 and DNA is impaired. They show severe neutropenia and less phenotypic abnormalities compared to Gfi-$1^{-/-}$ mice. Nevertheless, they still present several disadvantages, including impaired responses of NK cells and anomalies in certain myeloid cells [352, 353].

Foxo3a$^{-/-}$ mice lack Foxo3a expression, which is a transcription factor involved in regulating apoptosis, proliferation, and oxidative stress in lymphoid tissues [354-356]. They can be used to study neutrophil functions during inflammation. These mice show a 50% reduction in circulating neutrophils due to increased apoptosis. They exhibit T-cell hyperactivity and enhanced inflammatory responses. Although these characteristics make them valuable for studying the inflammatory functions of neutrophils, the high number of apoptotic neutrophils and inflammatory state are the major limitations [357].

1.7.2.2.Cre-driver mouse models for neutrophil-specific knockout

Besides the above-mentioned methods for neutrophil depletion, there are other methods using transgenic mouse models which can be used to specifically manipulate neutrophils. The Cre/lox system is the most used approach in this regard. In this system, a gene of interest flanked by two lox sites (Floxed) is deleted or inverted by Cre recombination in a specific target. Generally, the knockout model is generated by mating Cre-driver mice with Floxed mice [358]. Up to now, over 1300 Cre-driver mice have been generated, and they show tissue or stage-specific expression of Cre recombinase (https://www.findmice.org/).

The promoter of lysozyme M is expressed by myeloid cells and has been used to direct Cre recombinase expression in these cells [359]. Mcl-1flx/flx mice model employs the LysM-Cre system to delete the Mcl-1gene in neutrophils and macrophages. Mcl-1 is essential for delaying apoptosis in neutrophils, particularly during activation. Thus, its targeted deletion leads to inducing apoptosis in neutrophils. While this model is a valuable tool for studying neutrophil survival and immune function, incomplete deletion of Mcl-1 in some neutrophils renders complete neutrophil depletion. Also, the deletion affects all cells expressing LysM markers [360].

Expression of Cre recombinase under the control of human migration inhibitory factor-related protein 8 (hMRP8) or neutrophil-associated S100 calcium binding protein A8 (S100A8) promoter has also been used to target neutrophils [361]. For instance, in the *hMRP8cre; ROSA-iDTRKI* mouse model, the introduction of the human diphtheria toxin receptor (DTR) via the hMRP-8 Cre promoter allows for neutrophil depletion upon diphtheria toxin administration [362]. However, the prolonged DT administrating stimulates the immune responses, which affect the depletion efficiency [363]. hMRP8-ATTAC mice express high levels of Apoptosis Through Targeted Activation of Caspase 8 (ATTAC) in neutrophils, administration of Dimerizer leads to neutrophil removal; however, in conditions with higher pressure on bone marrow, like in cancer, the neutrophil depletion needs optimization [364]. MRP8-Cre Mcl-1flx/flx mice model also uses MRP8-Cre. Although this model is more specific to neutrophils, it shows higher mortality and breeding productivity [361].

MRP8-Cre-ires/EGFP transgenic mouse is another variant of MRP8-Cre models widely used in immunological studies due to its ability to express Cre recombinase and an EGFP reporter specifically in neutrophils, enabling detecting neutrophils by flow cytometry. MRP8-Cre-ires/GFP transgene includes the Cre recombinase gene, internal ribosomal entry site (IRES), and a green

fluorescent protein (GFP) gene under the control of the MRP8 promoter. It has been found that other cells, including monocytes and macrophages, may also be affected [365]. Additionally, the integration of the MRP8-Cre transgene into the host genome resulted in a 44 kb deletion of the locus on chromosome 5 (5qG2). This locus includes the serine (or cysteine) peptidase inhibitor, clade E, member 1 (*Serpine1*) gene and a part of the adaptor protein complex AP-1, sigma 1 (*Ap1s1*) gene [366].

Table 1.1. Different mouse models for neutrophil study

Category	Type	Model/Tool	Description	Pros	Cons	Ref.
Traditional models	Drug -based depletion	Cyclophosphamide	Chemotherapeutic agent injected to deplete neutrophils	Convenient, cost-effective, applicable in any mouse strains	Non-selective, needs repetitive injections	[337, 339]
		Vinblastine	Chemotherapeutic agent injected to deplete neutrophils	Convenient, cost-effective, applicable in any mouse strains	Non-selective, needs repetitive injections	[340]
	Antibody -based depletion	Anti-Gr1	Targets Ly6G and Ly6C proteins on neutrophils, leading to neutrophil depletion	Effective, rapid, applicable in any mouse strains	Non-specific, severe side effects, needs repetitive injections	[341]
		Anti-Ly6G	Specifically targets Ly6G protein on neutrophils, leading to neutrophil depletion	More specific than anti-Gr-1, applicable in any mouse strains	Less efficient than anti-Gr-1, needs repetitive injections	[342]
Genetic models	Global knockout	G-CSFR$^{-/-}$	Deletion of G-CSFR gene reduces neutrophil counts	Provides insight into neutropenia	Abnormalities and involvement in other physiological processes	[346, 347]
		Cxcr2$^{-/-}$	Deletion of Cxcr2 reduces migration of neutrophils from bone marrow to circulation	Useful for studying the role of Cxcr2 in neutrophil migration and recruitment	Lack of specificity	[348-350]
		Gfi-1$^{-/-}$	Deletion of Gfi-1 causes profound neutropenia	Valuable for understanding the role of Gfi-1 in hematopoiesis and immune regulation	Delayed growth, high mortality rate, atypical cells, affects other cell types	[351-353]
		Genista	Point mutation in Gfi-1 gene, severe neutropenia	Less phenotypic abnormalities compared to Gfi-1$^{-/-}$	Impaired NK cell responses, anomalies in certain myeloid cells	[354, 355]
		Foxo3a$^{-/-}$	Deletion of Foxo3a affects apoptosis, proliferation, and oxidative stress regulation	Valuable for studying inflammatory functions of neutrophils	High number of apoptotic neutrophils, T-cells hyperactivity	[356-359]

Table 1.1. Different mouse models for neutrophil study (continued)

Category	Type	Model/Tool	Description	Pros	Cons	Ref.
Genetic models	Conditional knockout	LysM-Cre Mcl-1flx/flx	Deletion of Mcl-1 gene in neutrophils and macrophages using LysM-Cre system	Valuable for studying neutrophil survival and immune function	Incomplete deletion in some neutrophils, affects all cells expressing LysM markers	[362]
		hMRP8cre; ROSA-iDTRKI	Introduction of DTR via hMRP-8 Cre promoter allows for neutrophil depletion upon diphtheria toxin administration	Conditional targeting of neutrophils	Prolonged DT treatment stimulates immune responses, need repetitive injection	[365]
		hMRP8-ATTAC	Administration of dimerizer leads to neutrophil removal	High specificity for neutrophils	Neutrophil depletion needs optimization in conditions with higher bone marrow pressure (e.g., cancer)	[366]
		MRP8-Cre Mcl-1flx/flx	MRP8-Cre model with specific targeting of neutrophils	Higher specificity	Higher mortality, breeding productivity issues	[363]
		MRP8-Cre-ires/EGFP	Expression of Cre recombinase and EGFP reporter specifically in neutrophils	Allows detection of neutrophils by flow cytometry	Off-target effects, not useful for metabolic studies	[367-368]

1.8.Rationale and Objective of Research

Weight gain and obesity induce significant alterations in adipose tissue to accommodate for the increased need for energy storage. In excess, these demands induce adipocyte stress and death which in turn induces immune cell infiltration, and inflammation of the tissue. The excessive repair and remodeling can lead to the accumulation of ECM, adipose tissue fibrosis, which contribute to the failure of the tissue to meet its metabolic role. Despite extensive research, gaps remain in our understanding the links between adipose tissue failure and obesity-related comorbidities.

FXIII-A transglutaminase was identified as a potentially causative obesity gene in humans. Studies from our group have demonstrated that FXIII-A regulates insulin signaling in mice, and its absence results in decreased macrophage infiltration, reduced adipose tissue inflammation, and increased insulin sensitivity. Furthermore, FXIII-A's role in obesity has been linked to ECM accumulation in adipose tissue. FXIII-A is produced by multiple cell types, including monocytes, macrophages, fibroblasts, megakaryocytes, osteoblasts, dendritic cells, and preadipocytes. Recent transcriptome-wide association studies conducted by our group showed a correlation between human *F13A1* gene expression in obese adipose tissue and neutrophil activation, suggesting a role in neutrophils. Thus, the overall aim of this project was to explore the role of neutrophil-derived FXIII-A in obesity-related metabolic disorders. I hypothesized that FXIII-A is produced by neutrophils, and is active as a transglutaminase, and a component of NETs where it plays a role in forming fibrin-NET thrombi. Furthermore, I aimed to investigate the function of neutrophil FXIII-A in emergency matrix formation, that may be a factor contributing to thromboinflammation and tissue fibrosis.

To two main objectives were:

1. To examine the production and activity of FXIII-A in neutrophils during NETosis and its role in crosslinking NETs with the ECM, particularly fibrin(ogen): This objective will explore if neutrophil FXIII-A influences the NET-fibrin network.
2. To study the immunometabolic phenotype of neutrophil-specific, FXIII-A knockout mice (*F13a1*$^{-/-MRP8}$) on high fat diet. This objective aims to study whether the absence of neutrophil-FXIII-A is beneficial in terms of metabolic and inflammatory responses in knockout mice on HFD.

By addressing these objectives, I aimed to uncover a possible function for FXIII-A in neutrophils and the potential implications of this new role to obesity-related inflammation and metabolic diseases. This research introduces a novel role for FXIII-A in neutrophils and opens the possibility to create enzymatic inhibitors for it for manage adipose tissue inflammation in obesity.

2. Chapter 2: Factor XIII-A transglutaminase contributes to Neutrophil Extracellular Trap (NET)-mediated fibrin(ogen) network formation and crosslinking

Fatemeh Soltani[1], Mélanie Welman[5], Sahar Ebrahimi Samani[1], Alain Pacis[2,3], Marie Lordkipanidzé[4,5], Mari T. Kaartinen[1,6]

[1]Division of Experimental Medicine, Department of Medicine, Faculty of Medicine and Health Sciences, McGill University, Montreal, Canada
[2]Victor Phillip Dahdaleh Institute of Genomic Medicine at McGill University, Montreal, Quebec, Canada
[3]Canadian Centre for Computational Genomics, McGill University, Montreal, Quebec, Canada
[4]Faculté de pharmacie, Université de Montréal, Montreal, Quebec, Canada.
[5]Research Center, Montreal Heart Institute, Montreal, Quebec, Canada.
[6]Faculty of Dental Medicine and Oral Health Sciences, McGill University, Montreal, Canada

Mari T. Kaartinen ORCID ID: 0000-003-2999-6502
Fatemeh Soltani ORCID ID: 0009-0005-9280-3058
Sahar Ebrahimi Samani ORCID ID: 0009-0004-5656-9252
Marie Lordkipanidzé ORCID ID: 0000-0001-7418-6766

Corresponding author:
Mari T. Kaartinen, Ph.D.
Faculty of Dental Medicine and Oral Health Sciences,
McGill University,
3640 University Street, Rm. 72
Montreal, QC, Canada, H3A 0C7
Tel: +1-514-3961499
E-mail: mari.kaartinen@mcgill.ca

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2.1. Abstract

Background: Neutrophil Extracellular Traps can contribute to thrombosis via stabilization fibrin network, which is normally conducted by plasma transglutaminase, Factor XIII-A as part of coagulation cascade. The possible presence and activity of FXIII-A in neutrophils or during NETosis is unknown. Here, we investigated potential presence of FXIII-A in neutrophils and participation in NET-fibrin interaction.

Methods: Data mining of human and mouse *F13A1/F13a1* mRNA expression in whole-body scRNA sequencing Atlases was conducted. *F13a1* mRNA and protein expression was assessed in isolated mouse bone marrow neutrophils. NETosis was induced using 12-phorbol 13-myristate acetate (PMA), and the transglutaminase activity was assessed with 5-(biotinamido) pentylamine incorporation to plasma fibronectin and a fluorescence-fibrin-based activity assay using ATTO488-Cadaverine. Externalization of FXIII-A and its interaction with neutrophil extracellular trap (NET) markers; decondensed DNA, CitH3, MPO was examined with immunofluorescence microscopy. NET-fibrin(ogen) interaction was investigated with and without serum and/or transglutaminase inhibitor, NC9. Effect of soluble fibrinogen and fibrin network on NETosis was also assessed.

Results: Data mining of RNAseq Atlases, showed *F13A1/F13a1* expression in adipose tissue, blood and bone marrow neutrophils. mRNA expression and protein production were confirmed in isolated neutrophils where expression was comparable to that of macrophages and monocytes. FXIII-A was externalized and active as a transglutaminase and colocalized with NET markers during NETosis. FXIII-A transglutaminase activity promoted NET-fibrin(ogen) interaction and entrapment of neutrophils within fibrin(ogen) matrix network. Soluble fibrinogen or fibrin network did not induce NETosis.

Conclusion: This study identifies neutrophils as a new source of FXIII-A and suggests it role in stabilizing NET-fibrin(ogen) structures.

2.2. Introduction

Factor XIII-A (FXIII-A) - plasma transglutaminase (TG)- introduces ε-(γ-glutamyl)lysine (isopeptide) bonds within protein networks and acts as a fibrin-stabilizer in the last step of the coagulation cascade[1, 2] . The crosslinking of fibrin fibrils as well as covalent integration of plasmin inhibitors, such as alpha-2-antiplasmin within the thrombi enhances the stability and resistance of the clot to plasmin-mediated fibrinolysis. The FXIII-A-mediated increase in clot resistance is essential for clot function, and its activity allows for subsequent steps towards wound healing via plasma fibronectin matrix stabilization. FXIII-A also contributes to thrombotic events like deep vein thrombosis (DVT) and myocardial infarction (MI) [3, 4]. Circulating FXIII-A is complexed to liver-derived FXIII-B inhibitory subunits which together form the circulating tetrameric FXIII (FXIIIA2B2). The production of the circulating FXIII-A is attributed to hematopoietic lineage cells of bone marrow where monocytes and macrophages show high expression levels. Accumulating evidence has demonstrated that several other cell types can produce FXIII-A in tissues including fibroblasts, megakaryocytes, and dendritic cells [5-7]. Our laboratory has identified FXIII-A expression in mesenchymal stem cells, embryonic fibroblasts, pre- and mature osteoblasts and adipocyte lineage cells, and suggested functions to FXIII-A outside the classic clotting cascade in matrix stabilization with functions in cell proliferation and differentiation [5, 8]. Our recent transcriptome-wide association studies (TWAS) involving adipose tissue and adipocytes of weight-discordant monozygotic twins linked the *F13A1* gene expression to hypertrophic adipocytes and to adipose tissue neutrophils with possible involvement

in neutrophil activation, degranulation, neutrophil-mediated immunity, as well as links to hemostasis genes controlling fibrinolysis [9-11]. Neutrophils play a crucial role in initiation and resolution of inflammation via various mechanisms including phagocytosis, degranulation, and formation of Neutrophil Extracellular Trap (NETs) which capture and neutralize invading pathogens [12]. NET formation, i.e., NETosis is a process where neutrophils release decondensed DNA, citrullinated histones, chromatin as a form of network that also contains myeloperoxidase (MPO), antimicrobial proteins, proteolytic enzymes such as neutrophil elastase (NE) [13, 14]. Neutrophils were demonstrated to have TG activity during NETosis, and FXIII-A mRNA was detected in neutrophils jointly with TG1 mRNA. However, FXIII-A was not investigated in detail, and its presence in neutrophils or its activity during NETosis was suggested to arise from phagocytosed of platelets [15]. NET presence and their defective clearance have been linked to number of inflammatory diseases, sepsis and thrombosis where NETs promote fibrin fibrillogenesis[16-19]. Also, the interaction between αMβ2-expressing immune cells including neutrophils with extracellular fibrin deposits contributes to inflammatory response and tissue damage, highlighting the role of fibrin in exacerbating disease conditions [20, 21]. Furthermore, FXIII-A was recently demonstrated to crosslink circulating histones to fibrin *in vitro* [22]. In this study, we sought to investigate the potential expression and activity of FXIII-A in neutrophils and NETs. We report that FXIII-A is produced by neutrophils and is a component of NETs where its activity can stabilize NET-fibrinogen interactions. Our findings add to our understanding of mechanisms of NET-mediated thrombosis and NET stability, and identify neutrophils as a novel cellular source of FXIII-A.

2.3. Materials and Methods

2.3.1. Animals

Male and female mice C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and maintained under standard housing conditions (23 ± 2°C in a 12 h light/12 h dark cycle). All animal related experiments were conducted according to the Guidelines for Animal Experimentation and were approved by the Animal Care Committee of McGill University.

2.3.2. Reagents and antibodies

Dulbecco's Modified Eagle Medium (DMEM, 10569010) and penicillin-streptomycin were obtained from Gibco (Burlington, ON, Canada). Fetal bovine serum (FBS) was purchased from Hyclone (Waltham, MA, USA). Antibodies used for flow cytometry and FACS sorting including anti-CD11b, M1/70 SB436 (62-0112-82), anti-Ly6G 1A8LY6G PE (12-9668-80) and anti-CD45 30-F11 APC-EF780 (47-0451-80) were obtained from Invitrogen (Thermo Fischer Scientific, Waltham, MA, USA). Sheep-anti-Factor XIII-A (SAFXIII-IG) antibody was purchased from Affinity Biologicals (Ancaster, ON, Canada). Mouse-anti- biotin (BN-34) and rabbit-anti-beta actin (SAB4301137) antibodies were obtained from Sigma-Aldrich (Oakville, ON, Canada). Rabbit-anti-citrullinated H3 (citH3, ab5103) and rabbit-anti-myeloperoxidase (MPO, ab208670) were provided by Abcam (Cambridge, UK). The mouse-anti-CD41 antibody (MAB7616) was purchased from R&D Systems, Inc. (Minneapolis, MN, US). The rat-anti-Ly6G (127601) for immunofluorescence staining was obtained from BioLegend, San Diego, CA, USA). The HRP-linked anti-mouse IgG secondary antibody was obtained from GE Healthcare (Mississauga, ON, Canada). The HRP-linked anti-rabbit IgG was purchased from Cell Signaling (Whitby, ON,

Canada). The HRP-conjugated anti-sheep IgG was obtained from Abcam (Cambridge, Cambridgeshire, UK). AlexaFluor 594 donkey anti-sheep (A11016), AlexaFluor 568 goat ant-rabbit (A11011), AlexaFluor 488 goat anti-rabbit (A11008) antibodies were purchased from ThermoFischer Scientific (Waltham, MA, USA). LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (L34965), SytoxGreen (S7020), AlexaFluor 568 NHS Ester (A20003), and 5-(Biotinamido)pentylamine (bPA) were all purchased from ThermoFischer Scientific (Waltham, MA, USA). Phorbol-12-myristate-13-acetate (PMA, 524400), Poly-L-Lysine (PLL, P4707), and human fibrinogen (341576-M) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Thrombin activated recombinant human FXIII-A (T070) and ATTO488-Cadaverine (ATTO488-Cad, A106) were purchased from Zedira GmbH (Darmstadt, Germany). Human M-CSF was purchased from PeproTech (Rocky Hill, NJ, USA). All other reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Fisher Scientific (Hampton, NH, USA).

2.3.3. scRNA-sequence Atlas data mining

Human and mouse pre-processed single-cell datasets from all tissues were obtained from the Tabula Sapiens [23] and Tabula Muris [24] databases, respectively. UMAP visualization, and gene expression data mining were performed using Seurat v5 R package [25] with focus on cell types in adipose tissue, blood, and bone marrow (human) as well as adipose tissue and bone marrow (mouse). Gene expression barplots were constructed using the anno_barplot function from the R package ComplexHeatmap [26].

2.3.4. Bone marrow macrophage, monocyte, and neutrophil isolation

Monocytes and macrophages were isolated from bone marrows of C57Bl/6, at the age 8-10 weeks, as described elsewhere [27]. In brief, bone marrow cells were cultured overnight with 25 ng/mL M-CSF, followed by collecting non-adherent cells after 24 h. This fraction was used as monocytes. The cells were further cultured at 5×10^4 cells/cm^2 for 48 h in the presence of M-CSF. The adherent cells including M-CSF-dependent bone marrow-derived macrophages (BMMs) were used as our macrophage cells. Osteoclasts were differentiated from BMMs with M-CSF and RANKL as described before [27]. Neutrophils were also isolated from the bone marrow cells using the EasySep Mouse Neutrophil Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. The isolated neutrophils were resuspended in DMEM containing 10% FBS. The viability of neutrophils was measured using Trypan blue exclusion [28]. As part of the assessing the purity of the neutrophil preparations, cells were fixed and stained with SytoxGreen to visualize nuclear morphology (multilobed nuclei that distinguishes neutrophils from monocytes or myeloid precursors which exhibiting less segmented nuclei) under the Zeiss Axioscope 5 fluorescence microscope at 20x magnification.

2.3.5. Flow cytometry and fluorescence-activated cell sorting (FACS)

The purity of isolated neutrophils (CD11b+Ly6G+) was determined using a BD LSR Fortessa flow cytometer (BD Biosciences, CA, USA) at the Flow Cytometry Core Facility at McGill University. In brief, cells were stained with a Live-dead dye to identify viable cells. The dye was added to the neutrophils in phosphate buffered saline (PBS), followed by a 30-minutes incubation in darkness. After this, the cells were washed and then resuspended in a staining buffer containing PBS, 1% FBS and 1mM EDTA. To prevent non-specific binding, the neutrophils were then blocked with bovine serum albumin (BSA) prior to incubation with fluorochrome-conjugated antibodies

including anti-CD11b (M1/70 SB436), anti-Ly6G (1A8LY6G PE) and anti-CD45 (30-F11 APC-EF780) for 45 min on ice. Cells were then washed with staining buffer and centrifuged at 300g for 5 min at 4 °C to remove any unbound antibodies, followed by fixation with 1% paraformaldehyde. The presence of CD45, CD11b, and Ly6G positive cells was detected by flow cytometry, capturing a minimum of 10,000 events. Data was analyzed using FlowJo software (Tree Star Inc, BD, USA) and gated based on a forward and side scatter gate. For fluorescence-activated cell sorting (FACS), the cell suspension was kept on ice without fixation to maintain their viability. FACS was performed using a BD FACSAria III (BD Biosciences, CA, USA) equipped with 85 μm nozzle and sheath fluid under low pressure. Single cells were distinguished from clusters or doublets by plotting forward scatter area (FSC-A) versus forward scatter height (FSC-H) to isolate singlets. Neutrophils were specifically sorted based on the positive expression of CD11b, Ly6G, and CD45, a common leukocyte marker. Dead cells were excluded using a live-dead stain, and a minimum of 10,000 events were captured for each sample.

2.3.6. Platelet isolation

Blood was collected through cardiac puncture from C57BL/6 mice, followed by immediate mixing with acid citrate dextrose solution (ACD-A) at ratio of 1:4 volume of blood to ACD. This mixture was further diluted with Tyrode's buffer (137mM NaCl, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2.7 mM KCl, 1.1 mM $MgCl_2$, 5.6 mM glucose, pH of 7.4) at 1:4 volume ratio of ACD/Tyrode buffer. The diluted blood was then centrifuged at 200g for 10 min at room temperature, without a brake, to facilitate the separation of platelet-rich plasma (PRP). The PRP was transferred to a new tube, and 1μM prostaglandin E1 was added to prevent platelet activation during processing. After that, the PRP was centrifuged at 1000g for 10 min at room temperature, without braking. The

supernatant plasma was discarded, and platelets were resuspended in Tyrode's buffer at a final platelet concentration of 250 × 10^8 platelets/ml. The isolated platelets were then seeded onto LabTek 8-well chamber slides, pre-coated with poly-L-lysine, at a density of 5 × 10^5 cells per well (Thermo Fisher Scientific, Waltham, USA) and incubated overnight at 4°C to allow attachment, with the subsequent day dedicated to the labeling of platelets. Neutrophil and platelet marker counts (MPO and CD41) were determined using Image J 1.8.0 software (National Institutes of Health, USA).

2.3.7. Neutrophil cultures and induction of NETosis

Neutrophils were seeded into eight-well chamber slides pre-coated with poly-L-lysine, at a density of 5 × 10^5 cells per well and incubated at 37 °C with 5% CO_2 for 30 min to allow for cell attachment. After that, the cells were treated with 20nM PMA for 4 h to induce NETosis. The cells were then fixed using 4% paraformaldehyde solution for 15 min, after which they were stored at 4 °C until staining for immunofluorescence microscopy.

2.3.8. Quantification of NETosis

Neutrophils were seeded at 2 × 10^5 cells/ml in a 96-well plate in RPMI1640 minus phenol red. After 30 min incubation at 37°C, the cells were treated with four different conditions: 100nM PMA, 100nM PMA plus 40 μM NC9, 2mg/mL soluble fibrinogen or pre-formed fibrin and incubated for an additional 3 h at 37°C. Afterward, 5μM SytoxGreen was added to each well, and after 30 min incubation at 37°C, the fluorescence intensity was measured at an excitation wavelength of 488nm using an Odyssey image analyzer (LI-COR Biosciences, Lincoln, USA).

2.3.9. Qualitative and quantitative Real Time-PCR

Total RNA was extracted from neutrophils using the RNAeasy mini plus kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The concentration of RNA was measured using a spectrophotometer, followed by cDNA synthesis from 1000 ng total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). For qualitative RT-PCR, the used forward and reverse primers have sequence of CAGTTCGAAGACGGCATCCT and AACAAGATCACTGTTGACCTCT, respectively. Quantitative RT-PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems) using TaqMan® Fast Advanced Master Mix. The relative expression levels of *F13a1*(Mm00472334_m1)*, Tgm1* (Mm00498375_m1) and *Tgm2* (Mm00436979_m1) genes were determined using the comparative Ct method and normalized to beta-actin (Mm00486707_m1) in triplicate. RNA from isolated monocytes, macrophages, and osteoclasts [27, 29] was used for comparison.

2.3.10. Protein extraction and Western blotting

The proteins were extracted from neutrophils using RIPA buffer (50 mM Tris (pH 7.5), 0.5 M NaCl, 2% Igepal, 1% protease and phosphatase inhibitor cocktails) followed by BCA protein quantification. Equal amounts of proteins of monocytes, macrophages and osteoclasts and 5-fold of that of neutrophils were electrophoresed in 10% SDS-polyacrylamide gels and then electroblotted onto a PVDF membrane. The membrane was blocked with 5% skim milk, and then incubated with primary antibodies against FXIII-A and beta- actin overnight at 4°C. Then the membranes were incubated for 1 h with HRP-conjugated anti -sheep Ig G and anti-rabbit IgG secondary antibodies. After this, the membranes were washed with TBS-T and developed using

an ECL Western blotting detection reagent (GE Healthcare, Chicago, USA). This was followed by capturing the signals using an Odyssey image analyzer.

2.3.11. Fibrinogen labelling and *in vitro* fibrin scaffolds

Human fibrinogen (hFBN) was labelled with AlexaFluor 568 NHS Ester according to manufacturer's instruction. In brief, the DMSO-dissolved dye was mixed with the hFBN solution at 8-fold molar excess and incubated for one hour at room temperature with constant and gentle shaking, ensuring protection from light. Subsequently, the solution was dialyzed twice at 4°C against 100 mM $NaHCO_3$ and 500mM NaCl, at pH 8.4. Following dialysis, the protein concentration was measured using the BCA protein assay. The degree of labelling (DOL) was calculated based on the absorbance of the labelled protein at 280nm and 568nm, applying the Lambert-Beer law equation. Additionally, the success of the labeling process was verified through SDS-PAGE analysis and observing using an Odyssey image analyzer. AF568-fibrin scaffold was formed within 8-well chamber slides by incubating AF568-FBN (0.1mg/ml) with unlabelled FBN (2mg/ml) along with 0.2U/ml thrombin and 5mM $CaCl_2$ for 1.5 h at 37 °C. After incubation, the scaffold was gently rinsed with PBS. The scaffolds were used to assess TG activity (below) and to investigate the potential influence of fibrin(ogen) on NETosis, where the cells were cultured on a pre-formed fibrin scaffold for 4 h at 37 °C. Following these, the cells were gently washed with PBS and then fixed with 4% paraformaldehyde solution for 15 min, after which they were stored at 4 °C until staining for immunofluorescence microscopy.

2.3.12. *In vitro* and *in situ* transglutaminase activity assays

For *in vitro* TG activity assay, neutrophil lysate was incubated for 2 h at 37 °C with 2mM 5-(biotinamido) pentylamine (bPA) with or without 1 µg bovine plasma fibronectin (pFN), in a reaction buffer containing 1mM dithiothreitol (DTT), 3mM $CaCl_2$, 10mM Tris-HCl (pH 8.0). After incubation, the reaction was stopped by addition of sample buffer followed by western blotting and detection using anti- biotin antibody to visualize covalent bPA incorporation into pFN or other potential substrates. Neutrophil TG activity was also assessed using the formed AF568-fibrin scaffold. For this, either neutrophil extract or recombinant FXIII-A was added together with ATTO488-Cad as a fluorescence amine donor, in the presence of reaction buffer. The slides were then incubated for 2h at 37 °C. Following this period, the scaffold was washed with PBS, and image analysis was performed using confocal microscopy, capturing images from three distinct fields. The integration of ATTO488-Cad into the AF568-fibrin network serves as an indicator of TG activity. To assess TG activity during NETosis, the cells were treated with or without ATTO488-Cad along with PMA in the presence or absence of TG inhibitor, NC9. To assess the impact of NETosis on fibrin formation, the cells were treated with soluble AF568-FBN for 10 min followed by addition of PMA in DMEM with or without 10% FBS. Also, to evaluate the role of FXIII-A, NC9 was used as a TG inhibitor.

2.3.13. Immunofluorescence microscopy

The fixed cells were washed with PBS and then permeabilized with 0.25% Triton-x for 10 min. After washing with PBS, the cells were blocked using 2% BSA for 30 min. For the detection of specific protein markers, the cells were incubated with respective primary antibodies including anti-citH3, anti- MPO, anti-Ly6G, anti-FXIII-A, and anti-CD41 for 2 h at room temperature. Then,

the cells were washed three times with PBS and incubated with fluorescence secondary antibodies for 1 h. Finally, to stain the nuclei DAPI was added and incubated for 5 min at room temperature. SytoxGreen was also used to visualize NETosis. The slides were observed, and images were captured under the Zeiss Axioscope 5 fluorescence microscope, and Zeiss LSM 900 confocal microscope. Three distinct visual fields were randomly selected, and the images were analyzed by Image J 1.8.0 software (National Institutes of Health, USA).

2.3.14. Statistical Analysis

Data analysis was performed using GraphPad Prism software (version 9), and results are presented as the mean ± SEM (standard error of the mean). Each experiment was performed with cells isolated from 3-5 mice, and each experiment was done in triplicate. For qRT-PCR and WB, tissues from each mouse were processed separately, and each sample was analyzed in duplicate. Statistical significance was assessed using one-way and two-way ANOVA, with subsequent Tukey multiple comparison post hoc testing or Kruskal-Walis for non-parametric tests. Statistical significance was assigned at p-values < 0.05, denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; 'ns' indicates a non-significant difference.

2.4. Results

2.4.1. scRNA-seq data analysis revealed the expression of *F13a1* by neutrophils

To investigate FXIII-A mRNA production by neutrophils, we first conducted data mining on Tabula Sapiens [23] (human) and Tabula Muris [24] (mouse) whole body scRNA sequence (scRNAseq) atlases for *F13a1/F13A1* expressing cells with focus on bone marrow (to confirm expression in known-expressors monocytes and macrophages), blood and adipose tissue

(subcutaneous). **Figure 2. 1A** shows *F13A1/F13a1* expressing tissues with high specificity to those harboring immune cells. The blood scRNAseq data was only available for the human atlas. In humans, *F13a1/F13A1* mRNA was confirmed in all tissues investigated with strong expression by adipose tissue macrophages, bone marrow and blood monocytes. Expression in bone marrow granulocytes (which included neutrophils but also eosinophils and basophils) was also seen. Human neutrophils showed positive expression of *F13A1* albeit to lesser levels compared to other cells. In mice, adipose tissue showed high levels of *F13a1* in granulocytes, macrophages, and neutrophils as well as bone marrow monocytes and neutrophils as low, but detectable levels (**Figure 2. 1B**).

2.4.2. Neutrophils express *F13a1* gene and FXIII-A protein

To investigate *F13a1* (FXIII-A) gene and protein expression in neutrophils, we isolated murine neutrophils from C57BL/6 mouse bone marrow using a commercial kit. The nuclei staining of neutrophils with SytoxGreen confirmed the morphological characteristics of neutrophils, having multilobed nuclei (**Supplementary Figure 2**. **2**). Flow cytometry method also verified that the isolated neutrophils were 73% pure, consisting of CD11b+, Ly6G+ cells. Following FACS-based sorting (gating with CD11b+, Ly6G+, CD45), we assessed *F13a1* expression qualitatively using RT-PCR. Both the unsorted (73% purity) and sorted populations (100% purity) showed clear *F13a1* mRNA, as shown in **Figure 2. 2A**. Comparative qRT-PCR and Western blot analysis also revealed that neutrophils express FXIII-A mRNA and protein; using bone marrow-derived monocytes as a gold standard for *F13a1* expression (100%), neutrophils showed 57% of the monocyte *F13a1* mRNA expression levels, whereas bone marrow macrophages showed less, 35% of the monocyte expression levels (**Figure 2. 2B, C**). Osteoclasts, as a low expressor control [27],

showed no detectable *F13a1* mRNA. This pattern was also reflected in the Western blot analysis (**Figure 2. 2B**). Comparing the *F13a1* expression levels to the other transglutaminases, namely *Tgm1* and *Tgm2* showed no expression for neither at significant levels - *Tgm1* mRNA expression was less than 1% of *F13a1* mRNA and *Tgm2 mRNA* was undetectable (**Figure 2. 2D***).* The production of FXIII-A protein in neutrophils was confirmed by immunofluorescence staining, where it was detected intracellularly colocalizing with neutrophil markers, Ly6G and MPO (**Figure 2. 2E**). *F13a1* expression analysis from male and female neutrophils demonstrated that female neutrophils express significantly lower level of *F13a1* (**Supplementary Figure 2. 1**), thus, our further studies were conducted with neutrophils isolated from male mice. To assess if the detection could arise from co-purifying platelets, the neutrophil preparations (obtained from the commercial kit), were stained for the platelet marker, CD41. Negligible CD41 expression was observed; after calculating cell numbers, 1.8% of neutrophils showed interacting platelets (**Supplementary Figure 2. 3**).

2.4.3. Neutrophil transglutaminase activity

We next assessed if neutrophils exhibit transglutaminase (TG) and fibrin or plasma fibronectin (pFN) crosslinking activity. For this, we first used a classic primary amine incorporation assay. **Figure 2. 3A** illustrates covalent bPA incorporation into the pFN in the presence of neutrophil extract, confirming the TG activity. Given the negligible expression of TG1 and TG2, it is plausible to attribute the detected TG activity to FXIII-A. We also devised a fibrin-based TG activity assay using AF568-fibrin network scaffold (formed via thrombin and calcium) and fluorescence - primary amine incorporation (ATTO488-cadaverine) (**Figure 2. 3B**). The TG activity in neutrophil extracts was assessed in the presence of calcium and DTT using ATTO488-Cad. Negative controls,

AF568-FBN alone and AF568-FBN together with ATTO488-Cad exhibited no labeling of the fibrin network. Human recombinant FXIII-A (thrombin activated) served as a positive control to validate the assay. The results with added neutrophil extract show clear incorporation of ATTO488-Cad into the AF568-fibrin network scaffold and similar labeling was seen with FXIII-A positive control (**Figure 2. 3C**).

2.4.4. Neutrophil FXIII-A is active during NETosis

We next examined whether FXIII-A is active as a TG in NETosis. To induce NETosis, neutrophils were treated with PMA for 4 hours and cells were subsequently stained for FXIII-A and NET components using immunofluorescence microscopy. The results, presented in **Figure 2. 4,** demonstrate that PMA treatment induced neutrophils to release their nuclear contents which involve degranulation and expulsion of decondensed chromatin and proteins in the form of NETs. FXIII-A is present in neutrophils and co-localized with NET components; decondensed DNA (SytoxGreen), CitH3 and MPO which all take slightly different shapes in NETs. To assess the TG activity and FXIII-A together during NETosis, NETosing cells were also incubated with ATTO488-Cad. The results, shown in **Figure 2. 5**, indicate significant incorporation of the TG-activity probe into the NETs, and colocalization of the probe with FXIII-A. The probe incorporation was decreased by TG inhibitor NC9, confirming the active FXIII-A in the NETs (**Figure 2. 5**).

2.4.5. Neutrophil FXIII-A promotes fibrin(ogen)-NET interaction

We next assessed if FXIII-A contributes to NET-fibrin(ogen) network formation. For this, the NETosis was induced in the presence of soluble AF568-FBN (**Figure 2. 6A**) but in the absence of

serum to specifically focus on the potential interaction of NETs and fibrin(ogen) and to eliminate any confounding factors. A clear colocalization and interaction between NETs and fibrin(ogen) was observed in NETosing cells upon PMA induction, which was blocked by addition of NC9, TG inhibitor (**Figure 2. 6B**). We next investigated the effect of NETosis on fibrin(ogen) network formation in the presence of serum. **Figure 2. 6C** shows that upon induction of NETosis, soluble AF568-FBN is dramatically assembled into AF568-fibrin(ogen) matrix fibrillar network. This assembly was specific to NETosis event as resting neutrophils did not promote assembly and fibrinogen showed only short fibrils. In this serum-containing experimental setup, addition of NC9, had no effect on fibrin(ogen) network formation (as expected) but appeared to cause a network that did not envelope neutrophils (**Figure 2. 6C**).

2.4.6. Soluble or fibrillar fibrin(ogen) does not induce NETosis

To explore the influence of fibrinogen or fibrin on stimulation of NETosis, we plated neutrophils onto wells either harboring the fibrin scaffold or in the presence of soluble FBN (**Figure 2. 7A**). PMA was used as a positive control. NETosis assay, involving SytoxGreen quantification (**Figure 2. 7B**), showed that neither fibrin nor fibrinogen induced significant NETosis. NC9 did not alter the extent of NETosis induced by PMA, suggesting that FXIII-A does not play any significant role in the actual NETosis process (**Figure 2. 7B**). The IF results under same conditions confirmed the result, i.e., no visible NETs (**Figure 2. 7C**).

2.5. Discussion

Factor XIII-A plays a crucial role in hemostasis as a clot stabilization factor, however, its involvement in thrombotic events is increasingly acknowledged. In thrombotic events, such as

DVT, almost half of FXIII-A has been demonstrated to arise from the circulating platelets, where FXIII-A is exposed on the activated platelet membrane and exerts its antifibrinolytic activity [4, 30, 31]. In thromboinflammatory pathologies that include sepsis, ischemia-reperfusion injury, atherosclerosis, ischemic stroke and DVT, FXIII-A activity has been attributed to the stabilization of fibrin clots, modulation of inflammation, and mediating wound healing process and its activity likely arises from the immune cells which are abundant expressors of FXIII-A [32-35]. The abundant FXIII-A mRNA expression in immune cells was also demonstrated in our whole body scRNAseq atlas data mining in humans and mice. In our study, we bring new evidence on the production of FXIII-A by neutrophils and the participation of FXIII-A in NET-fibrin(ogen) interactions. Out work adds neutrophils to the cell pool that can produce FXIII-A and a mechanism how FXIII-A may contribute to thrombotic events via NETs. Our work also suggests sexual dimorphism in *F13a1* expression as male neutrophils show higher *F13a1* expression compared to female neutrophils.

Previous reports showed the presence of mRNA of TG1 and FXIII-A in circulating human neutrophils, however, the high amount of *F13A1* mRNA in neutrophils was suggested to arise from phagocytosed platelets which serve as a storage for FXIII-A enzyme [15]. In our study, we assessed the possible presence of platelets in our preparations done using a commercial kit [36]. Our data showed negligible presence of CD41 positive platelets attached to less than 2% of the purified neutrophils. Thus, platelets are a marginal source of FXIII-A in our experiments. Moreover, FXIII-A colocalization with neutrophil markers, namely Ly6G and MPO, as well as the clear intracellular presence of FXIII-A is indicative of FXIII-A production by neutrophils. We also investigated if TG1 or TG2 could contribute to TG activity we observed; their low expression (1% and 0%, respectively) compared to *F13a1* supports the identification of FXIII-A as the main

transglutaminase in mouse bone marrow neutrophils. Previous work showing TG1 presence in neutrophils was conducted with human neutrophils, and TG2 expression was demonstrated in neutrophil-like cells derived from promyelocytic leukemia lineage. In the study by Balajthy's group, differences in neutrophil activity between TG2 knockout and wild type mice, particularly after yeast injection, was linked to TG2 activity [37]. Thus, the discrepancy between our findings and these studies can be attributed to different cells and methodologies used. Our TG activity assay was performed on fibrin scaffold demonstrated that both neutrophil extract and Factor XIII-A (as a positive control) were able to incorporate the ATTO488-Cad into the fibrin network. Negative controls did not show any labeling which shows that that the minor amount of FXIII-A present in the preparation is washed out during the scaffold preparation and does not contribute to the detectable activity.

The presence of neutrophils within thrombi has been observed in both mouse models and clots from patients with myocardial infarction, which highlights their significant role in coagulation, and beyond their established immune functions. NETosis is a key mechanism by which neutrophils contribute to innate immune responses and thrombosis. Different stimuli such as bacteria, activated platelets and PMA (*in vitro*) activate neutrophils leading to releasing NETs [38, 39]. The details of NET-induced thrombosis mechanism are not fully understood but NETs recruit, interact and activate soluble clotting factors such as von Willebrand Factor (vWF), fibronectin, fibrinogen as well as platelets and leukocytes [16, 40, 41]. Our *in situ* TG activity assay performed on PMA-stimulated neutrophils, using ATTO488-Cad shows clear incorporation of the probe into the NETs and the inhibition of the process by TG inhibitor, NC9. Also, FXIII-A mediated *in vitro* crosslinking of histones into the fibrin has been demonstrated [22]. In addition to histones, several other NET components including neutrophil elastase, lactoferrin, MPO and cathepsin G were

identified as TG substrates (TG1) by Csomos et al. via classical primary amine pull down experiment [15]. Interestingly, it was demonstrated that human neutrophil elastase cleavage results in a truncated, but active form of FXIII-A which can effectively crosslink fibrin chains and contribute to clot formation and stabilization, albeit with a slightly lower activity compared to thrombin-activated FXIII-A [42].

The clotting function of neutrophils is further supported by a recent report showing that neutrophils, in the presence of plasma, facilitate clotting primarily through Factor XI, however, the clot formation induced by isolated NETs occurs independently of this factor [43]. According to the authors, in a purified fibrinogen system and in the absence of thrombin, neither neutrophils nor isolated NETs induce clot formation [43]. The authors related the procoagulant effect of neutrophils, in the presence of plasma, to releasing the proteins and enzymes like S100-A8 and MPO during the isolation process. Besides, NETs enhanced the density of clot, but the clot formed by partially activated neutrophils during isolation was less stable [43]. Our data also showed that the incubation of resting neutrophils with fibrinogen did not result in fibrin(ogen) matrix formation, whereas the fibrin(ogen) network formation occurred dramatically upon NETosis induction. Interestingly, our data demonstrates a tight interaction and 'wrapping' of NETosing neutrophils by fibrin(ogen) matrix. It also seems that the formation of fibrin(ogen) network during NETosis restrict the full expansion and spreading of NETs as the morphology of the PMA-treated neutrophils in the presence of fibrin(ogen) network was dramatically different from those without fibrin(ogen) matrix. The wrapping or enveloping of NETosing neutrophils by fibrin(ogen) matrix is likely mediated by integrin receptor αMβ2 (Mac-1), which is known to be upregulated upon neutrophil activation and in an activated conformation in NETosing cells [44]. The results from the NC9 treatment group showed that inhibiting the TG activity resulted in altered distribution of

neutrophils within the fibrin(ogen) network reflecting likely decreased interaction between cells and fibrin(ogen). The crosslinking of fibrin(ogen) network might affect the exposure of binding sites on fibrinogen [45]. In another study it was reported that stimulation of neutrophils by PMA enhances thrombin generation in platelet-poor plasma [46] which was associated to DNA acting as a pre-coagulatory role via intrinsic pathway [46]. Also, it was reported that fibrinogen binds to both neutrophils and NETs, which is critical for thrombus formation [47]. The interaction between fibrin and neutrophils in mucosal barrier has been correlated to αMβ2 integrin receptor which leads to the production of reactive oxygen species and NETosis [48].

In summary, our work highlights the role of neutrophil FXIII-A in bridging innate immune responses with coagulation processes, where it likely influences structural integrity of NET-induced thrombi as well as NET clearance and neutrophil-fibrin(ogen) interactions. Furthermore, the observed significant difference in *F13a1* expression between male and female neutrophils merits further investigations as thrombotic events, such as DVT, myocardial infraction, and pulmonary embolism, are more prevalent in men [49-51] and higher basal FXIII-A levels in neutrophils could affect the NET-related thrombotic outcomes.

2.6. Conclusion

This study provides new evidence on neutrophils as a cellular source of FXIII-A and suggests a role for neutrophil FXIII-A in fibrin(ogen)-NET stabilization where its role has not been previously accounted for. This activity likely affects fibrinolysis-NET clearance and emphasizes the importance of FXIII-A inhibition as a therapeutic target in immune-thrombotic states. Further studies are required to examine the implications of FXIII-A inhibition on neutrophil function, particularly in the development of FXIII-A based therapeutics.

2.7. References

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2.8. Figures

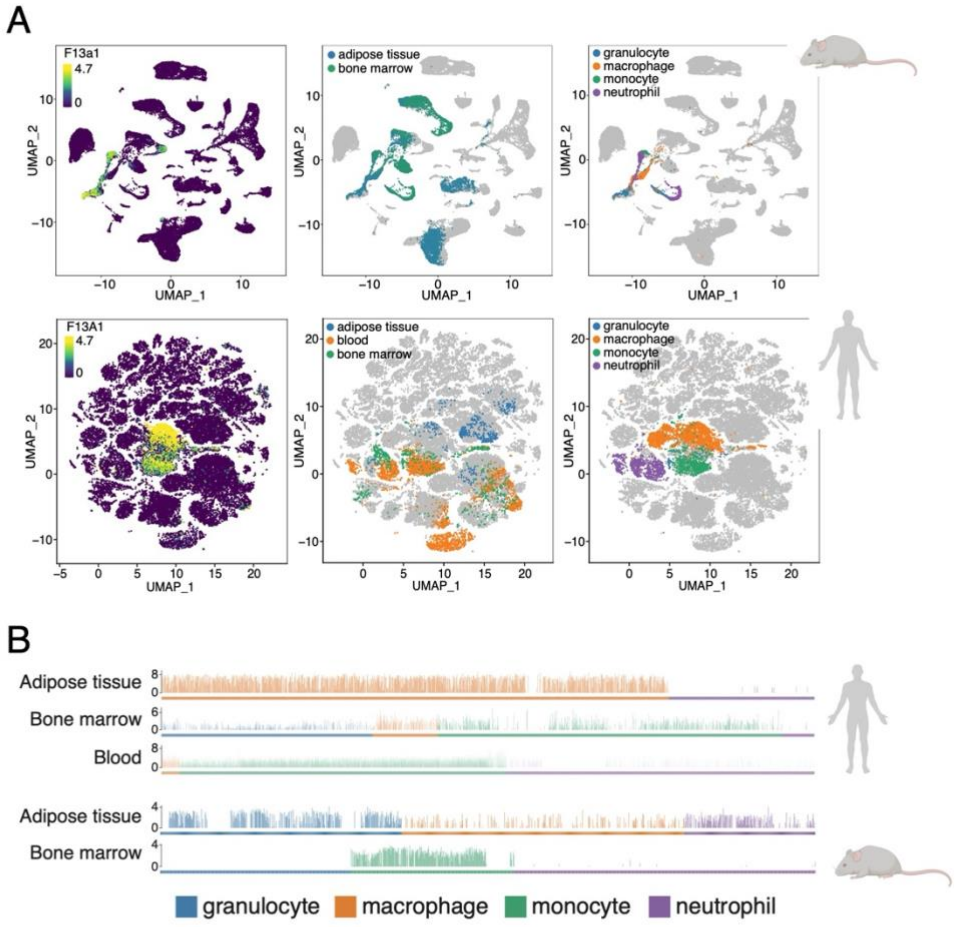


Figure 2.1. Data mining of Tabula Sapiens and Tabula Muris scRNA sequence atlases for *F13a1*/F13A1 expression. A. UMAP plot of cells, color-coded *F13A1* expression, tissue and cell-type annotation, in mouse (top) and human (bottom). **B.** Barplots showing the *F13A1/F13a1* expression profiles in adipose tissue, bone marrow and blood identifying four cell-types; granulocytes, macrophages, monocytes and neutrophils as expressors in these tissues. Other cell types were not identified. Each bar represents an expressor cell.

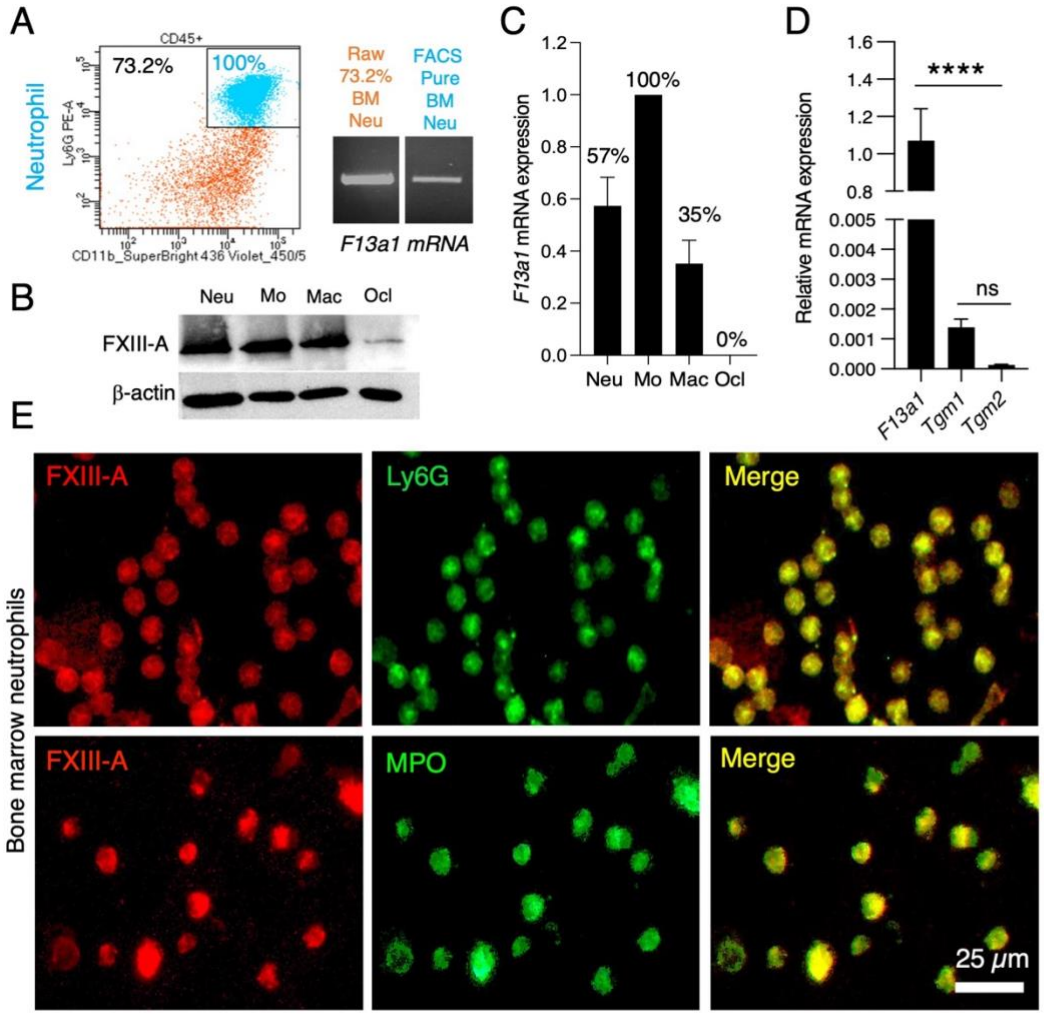


Figure 2.2. Neutrophils express and produce FXIII-A. A. Purified neutrophils from C57BL/6 mouse bone marrow were isolated using neutrophil enrichment method (purity: 73.2%), and FACS sorting (purity: 100%). Neutrophils show *F13a1* mRNA expression. **B, C.** The protein (Western blot) and mRNA (qRT-PCR) levels of *F13a1* in neutrophils are comparable to those in monocytes (Mo) and macrophages (Mac), all of which produce FXIII-A protein. Osteoclasts (Ocl), which express negligible amount of *F13a1,* were used as a negative control. **D.** *Tgm1* and *Tgm2* transglutaminase mRNA expression is not detected in mouse neutrophils. **E.** FXIII-A shows intracellular localization and colocalization with the neutrophil markers; Ly6G and MPO.

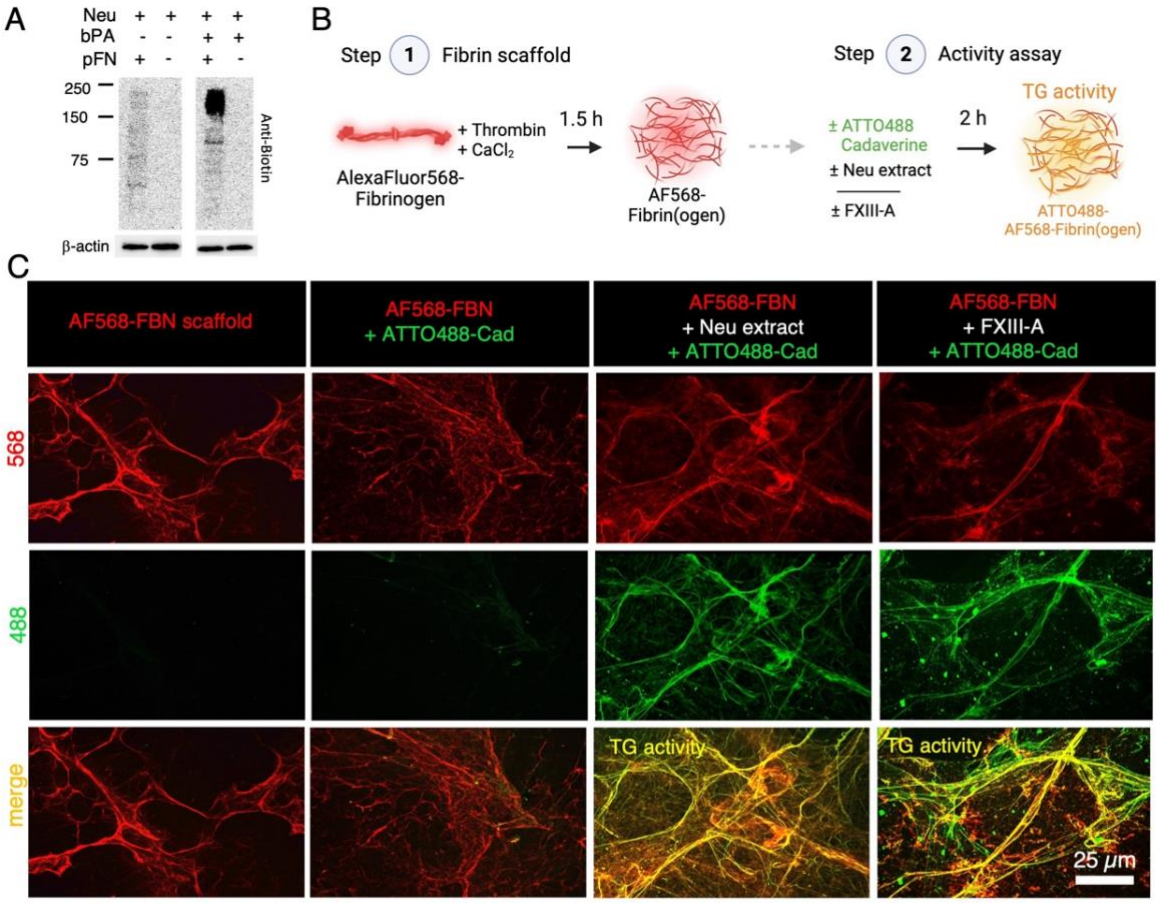


Figure 2.3. Neutrophils extracts show transglutaminase (TG) activity. A. *In vitro* transglutaminase activity was conducted via visualizing covalent incorporation of primary amine, 5-biotin-pentylamine (bPA) to endogenous substrates in neutrophils (Neu) and to plasma fibronectin (pFN). Neutrophil extract showed TG activity with and without pFN addition to the assay. **B.** Schematic representation of the fibrin-based TG activity assay involving formation of a AF568-fibrin scaffold followed addition of ATTO488-Cadaverine (Cad) (primary amine) as a fluorescence activity probe. Scaffold was incubated either with or without neutrophil (Neu) extract or FXIII-A enzyme as positive control. **C.** Addition of neutrophil extract to AF568-fibrin scaffold resulted in crosslinking of ATTO488-Cad into the fibrin network (merge, yellow) at similar levels as FXIII-A enzyme indicating presence of TG activity and FXIII-A in neutrophils. Negative controls showed no activity.

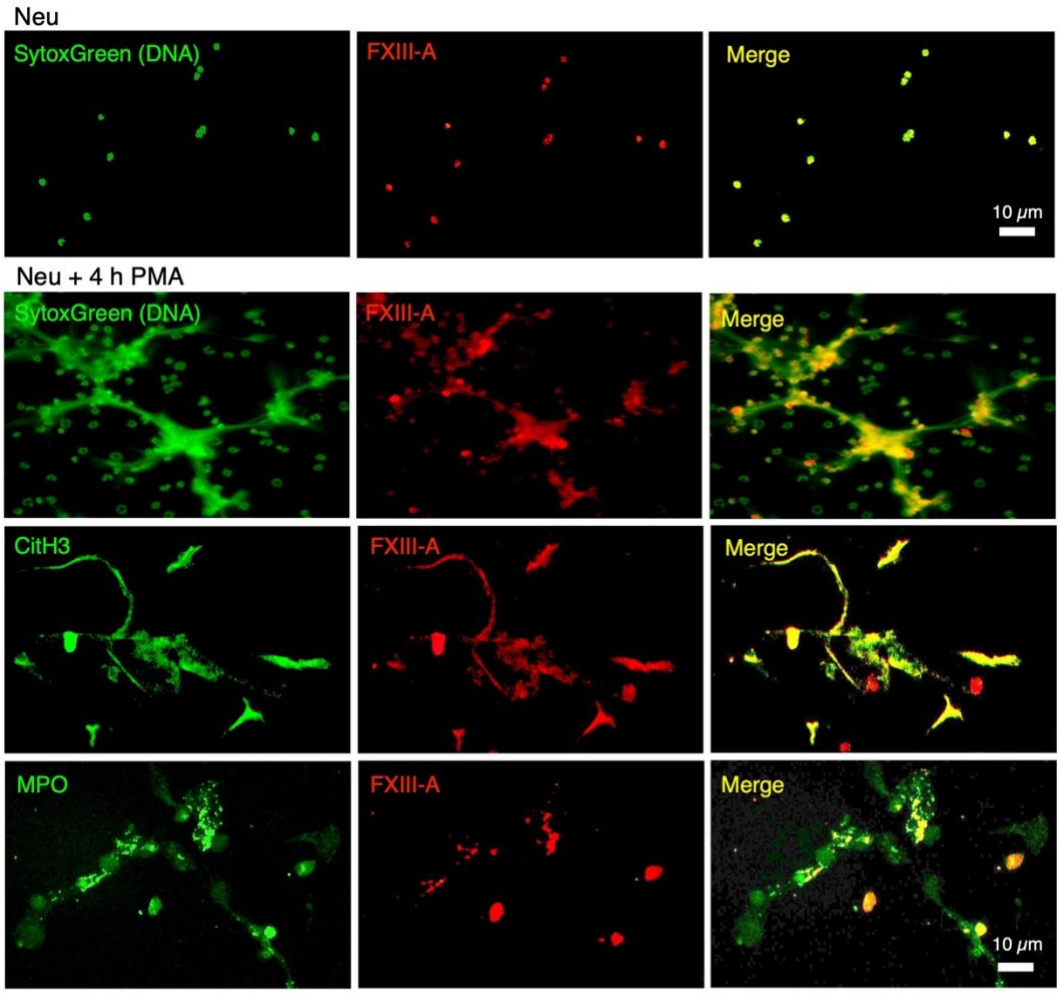


Figure 2.4. Neutrophil FXIII-A is a component of Neutrophil Extracellular Traps (NETs). Immunofluorescence staining of FXIII-A in resting neutrophils (Neu) and neutrophils that were treated with phorbol-12-myristate 13-acetate (PMA) for 4h. NETs were visualized using SytoxGreen (decondenced DNA), and NET markers; citrullinated histone 3 (CitH3) and myeloperoxidase (MPO). FXIII-A colocalized with all NET markers (merge, yellow).

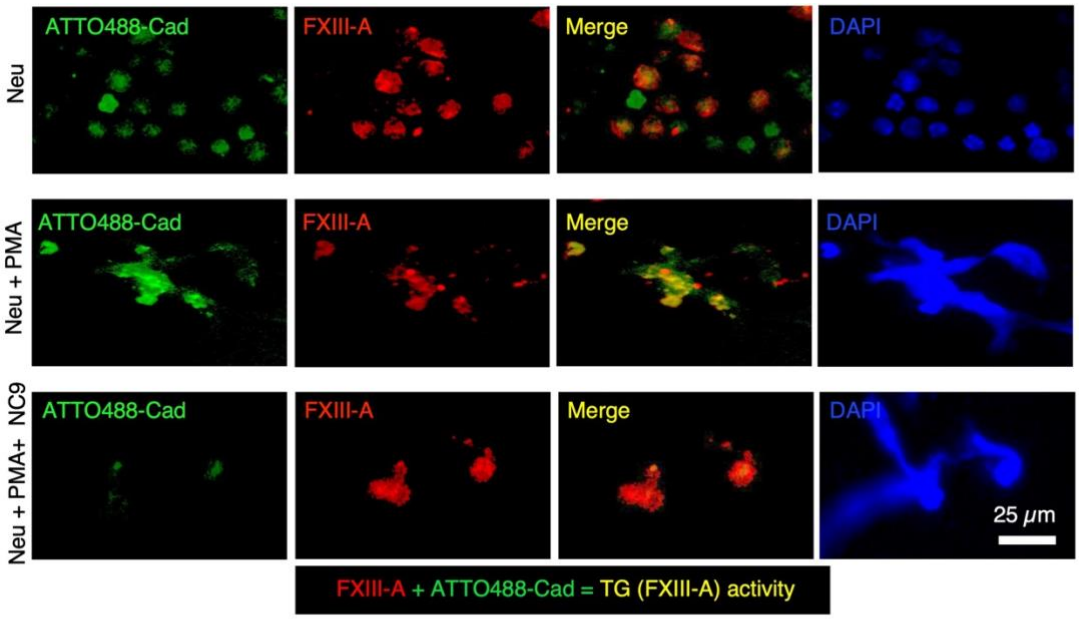


Figure 2.5. Induction of NETosis reveals TG activity of FXIII-A. Neutrophils were treated with phorbol-12-myristate 13-acetate (PMA) and TG activity probe - primary amine, ATTO488-cadaverine (Cad) for 4 h. FXIII-A transglutaminase activity was visualized as a colocalization between ATTO488-Cad and NET-associated FXIII-A (merge, yellow). ATTO488-Cad incorporation to NETs and colocalization with FXIII-A was inhibited by transglutaminase inhibitor, NC9.

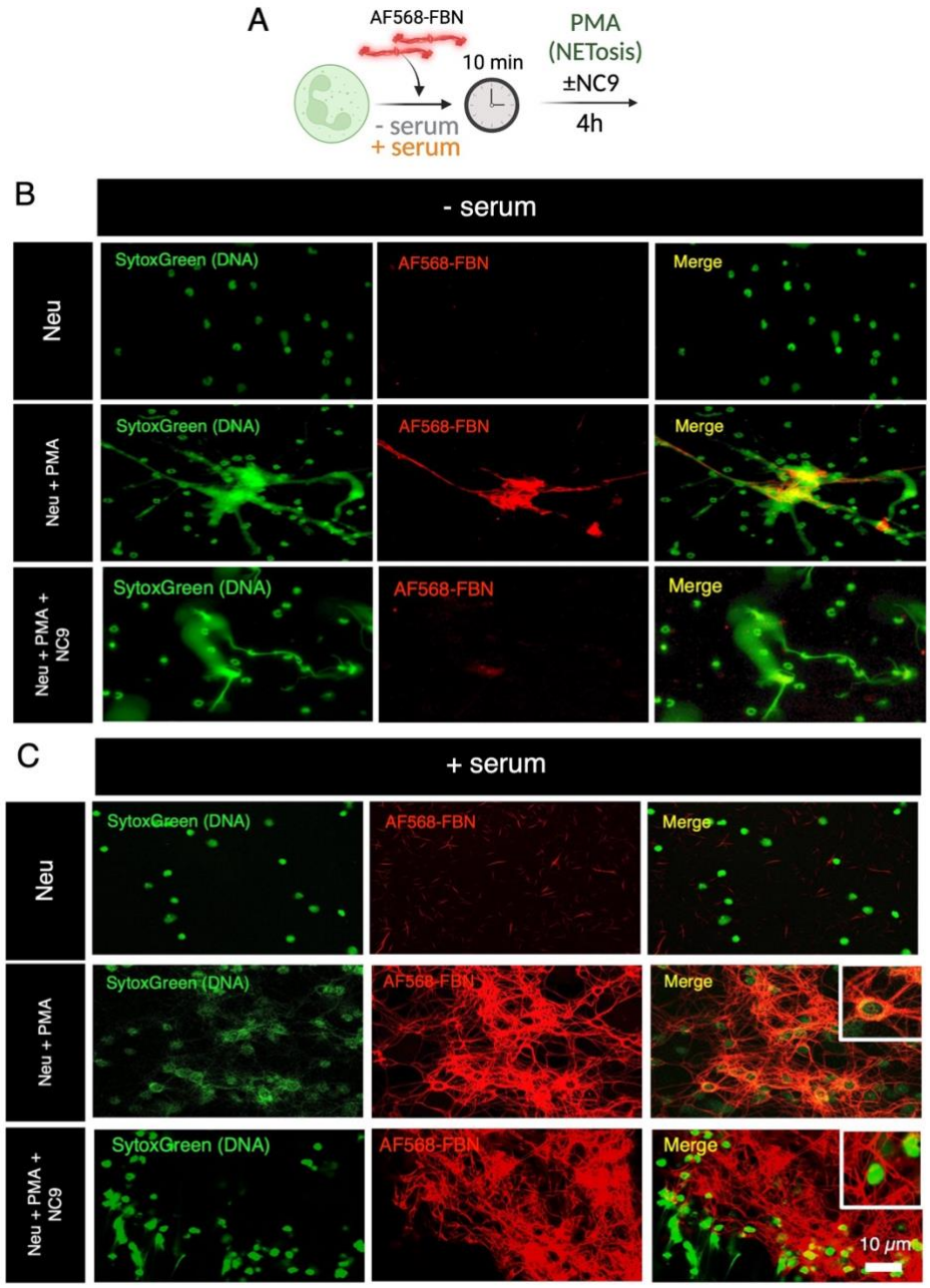


Figure 2.6. Transglutaminase activity promotes NET-fibrin(ogen) interaction. **A**. Schematic illustration of the experimental set up; AF568-fibrin(ogen) (AF568-FBN) was given to plated neutrophils (Neu) in the presence or absence of serum and allowed to stabilize for 10 min which was followed by addition of PMA to induce NETosis for 4h. **B**. Induction of NETosis in the absence of serum promotes association and interaction of AF568-FBN with NETs, which is inhibited by TG inhibitor NC9 suggesting involvement of crosslinking and presence of TG activity. **C**. Induction of NETosis in the presence of AF568-FBN and serum promotes fibrin network formation which show entrapment of Neu (inset) but not typical NET decondensed DNA morphology. TG inhibitor, NC9, influences Neu-fibrin interaction.

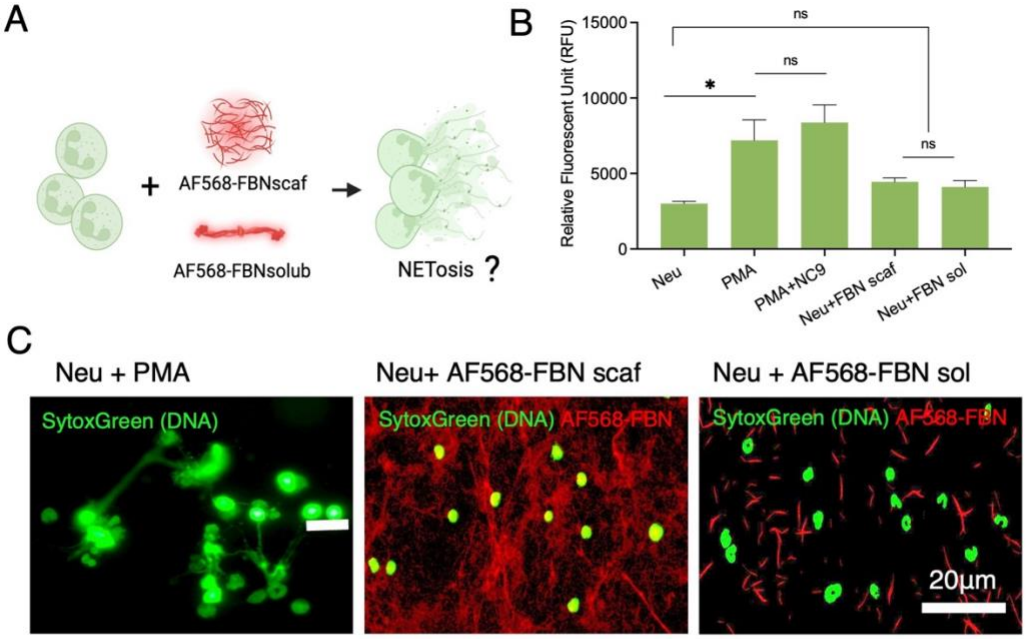
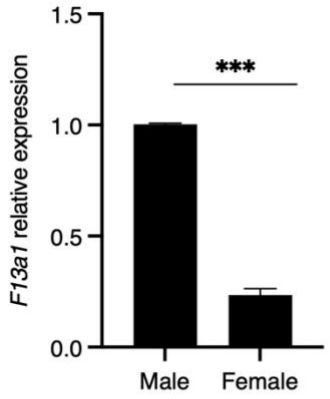
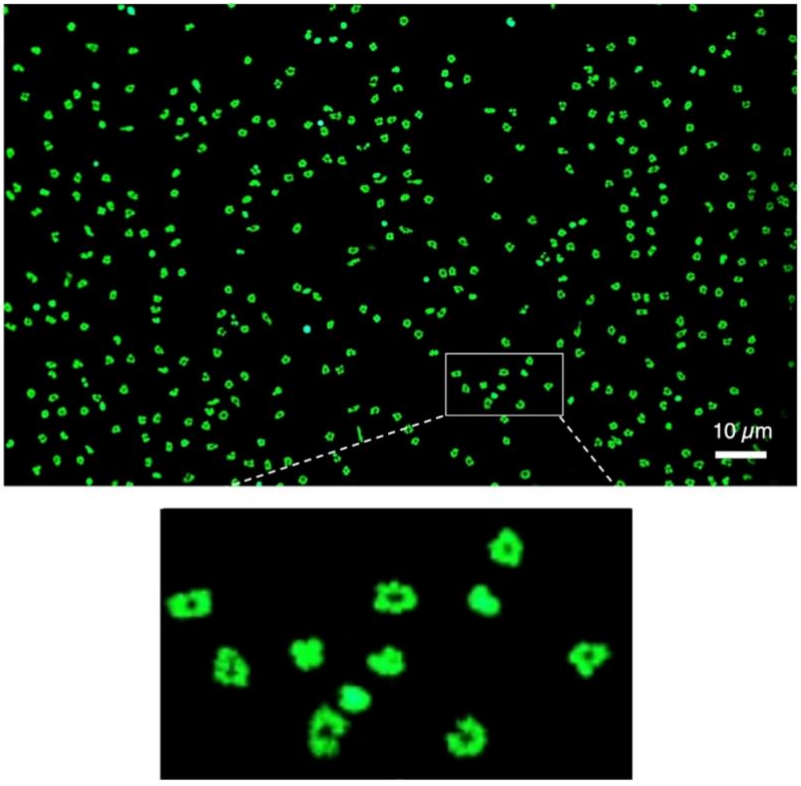


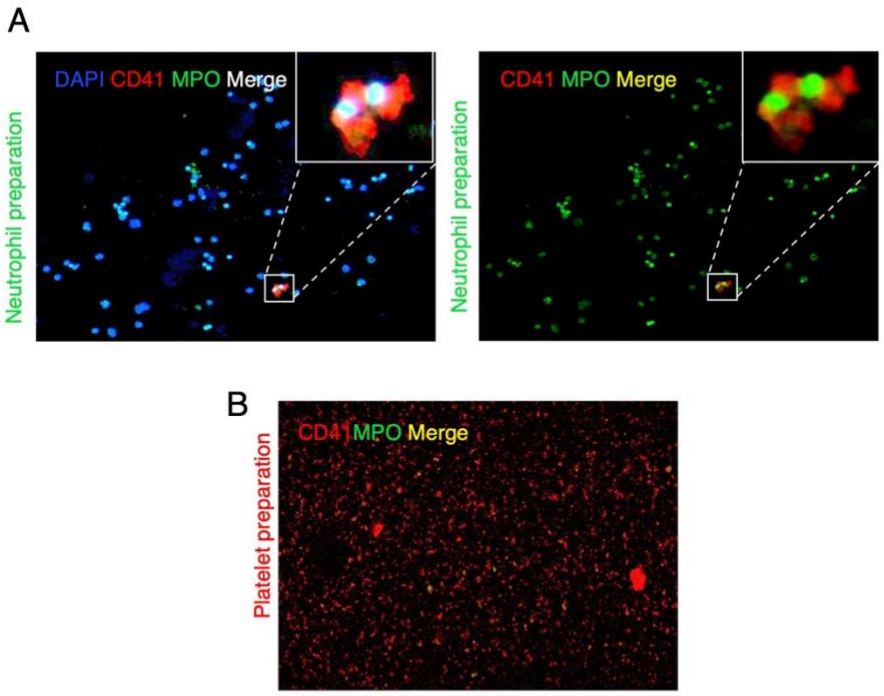
Figure 2.7. Soluble fibrinogen or fibrin scaffold do not promote NETosis of neutrophils. A. Schematic representation of the experiment assessing induction of NETosis by either fibrin scaffold or soluble fibrinogen (FBN). **B.** Quantification of NETosis using SytoxGreen assay shows no NETosis induction. **C.** Immunofluorescence imaging of resting neutrophils cultured on AF568-fibrin scaffold and with soluble AF568-fibrinogen indicates no significant NETosis, i.e., presence of decondensed extracellular DNA.



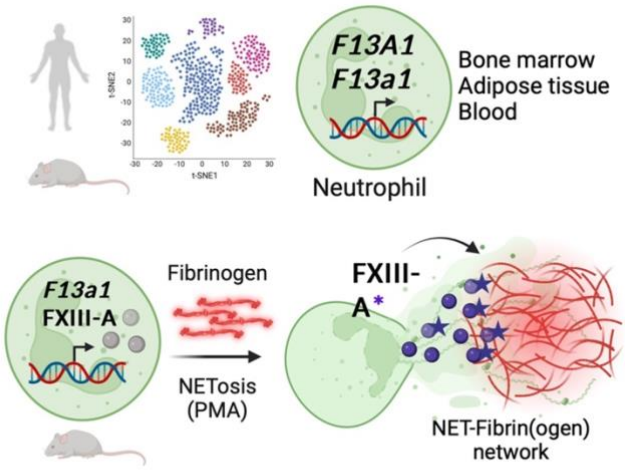
Supplementary Figure 2.1. *F13a1* expression in mouse bone marrow neutrophils is sex-dependent. mRNA gene expression analysis of *F13a1* shows significantly lower (-80%) level of *F13a1* in female compared to male neutrophils.



Supplementary Figure 2.2. SytoxGreen staining of isolated neutrophils shows multilobed nuclei, a typical characteristic of neutrophils. Isolated neutrophils were fixed and stained with SytoxGreen to visualize nuclear morphology under fluorescence microscopy at 20x magnification, to distinguish neutrophils from monocytes or myeloid precursors which have less segmented nuclei. The multilobed morphology is observed throughout the preparation.



Supplementary Figure 2.3. Bone marrow neutrophils show negligible platelet co-purification. A. Immunofluorescence staining of bone marrow neutrophils and platelets with MPO and CD41, as neutrophil and platelet markers, respectively, show that only ~1.8% of neutrophils had bound platelets. **B.** Mouse platelet preparation validates CD41 antibody detection.



Visual summary of Chapter 2

Connecting statement: Chapter 2 and 3

In Chapter 2, we explored the production of FXIII-A by neutrophils and its transglutamianse activity, establishing neutrophils as a novel source for this enzyme. Neutrophil-FXIII-A participation in NET-fibrin interactions and network formation provides a foundational understanding of neutrophil activity during fibrinogenesis.

The promising results obtained *in vitro* motivated us to investigate the potential function of neutrophil-derived FXIII-A in metabolic health *in vivo.* To advance our understanding, we bridge towards generating a neutrophil-specific *F13a1* knockout model (*F13a1*$^{-/-MRP8}$) to further elucidate neutrophil behaviour under obesity-simulated conditions and in metabolic disorders. However, due to the emergence of an unexpected confounding factor in our Cre model, i.e. the absence of *Serpine 1*, which influenced our results, this chapter will discuss the unsuitability of the MRP8-Cre model in the context of adipose tissue and metabolic research, highlighting the critical need for appropriate models and controls in such studies.

3. Chapter 3: Off-target genomic effects in MRP-Cre driver mice complicate its use in weight gain and metabolic studies

Fatemeh Soltani[1], Cedric Duval[2], Robert Ariens[2], Mari T. Kaartinen[1,3]

[1]Faculty of Medicine and Health Sciences (Division of Experimental Medicine), McGill University, Montreal, QC, Canada
[2]Leeds Thrombosis Collective, Discovery & Translational Science Department, Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds LS2 9NL, United Kingdom
[3]Faculty of Dental Medicine and Oral Health Sciences (Biomedical Sciences), McGill University, Montreal, QC, Canada

Mari T. Kaartinen ORCID ID: 0000-003-2999-6502
Fatemeh Soltani ORCID ID: 0009-0005-9280-3058
Cedric Duval ORCID ID: 0000-0002-4870-6542
Robert Ariens ORCID ID: 0000-0002-6310-5745

Corresponding author:
Mari T. Kaartinen, Ph.D.
Faculty of Dental Medicine and Oral Health Sciences,
McGill University,
3640 University Street, Rm. 72
Montreal, QC, Canada, H3A 0C7
Tel: +1-514-3961499
E-mail: mari.kaartinen@mcgill.ca

Key words: Obesity, Immune system, Thrombosis, Factor XIII-A, Cre Model

3.1. Abstract

Thromboinflammation is a major driver of metabolic dysfunctions. This process involves the accumulation of pro-fibrinogenic factors in adipose tissue in obesity, which promotes immune cell infiltration and affects weight gain. Neutrophils contribute to adipose tissue inflammation, and thrombosis, and their roles and component contribution to metabolism have been investigated using MRP8-Cre driver mouse to generate neutrophil-specific knockouts. In this study, we demonstrate the limitation of using the MRP8-Cre mouse to study adipose tissue, weight-related dysfunctions and/or metabolic disorders. MRP8-Cre mouse was recently demonstrated to have off-target deletions in *Serpine1* and *Ap1s1* genes. *Serpine1* encodes for plasminogen activator inhibitor-1, a major anti-fibrinolytic factor known to induce thromboinflammation. MRP8-Cre has no *Serpine1* expression in subcutaneous and visceral adipose tissue, and when fed a high-fat diet, does not gain weight. The resistance to weight gain translates to a neutrophil-specific knockout model, $F13a1^{-/-MRP8}$, which was not expected to show resistance to weight gain as its global knockout does not exhibit this phenotype. Our work underscores the critical need for careful selection of controls for genetic models.

3.2. Introduction

Obesity is a major risk factor for cancers and several metabolic and thrombotic diseases [1-3]. Many of the comorbidities have underlining contributions from the chronic, systemic low-grade inflammation originating from adipose tissue (AT) dysfunction during weight gain [4-6]. Adipose tissue inflammation also contributes to further weight gain, and it arises from several factors, including adipocyte stress and death, mechanical stress on cells from the surrounding fibrotic extracellular matrix (ECM), and dysregulation of fatty acid metabolism [7-9]. This creates a dysfunctional AT with hampered adipogenesis and lipogenesis which in turn causes ectopic lipid accumulation in the metabolic organs, such as the liver and skeletal muscle [10, 11].

AT inflammation is initially driven by neutrophils whose activation generally precedes the pro-inflammatory macrophage infiltration which is promoted by the presence of fibrin deposits and pro-coagulatory and anti-fibrinolytic molecules such as thrombin, Factor XIII-A transglutaminase, and plasminogen activator inhibitor-1 (PAI-1) in AT [12-14]. The process, referred to as thromboinflammation, is crucial in obesity-related metabolic dysfunction. Knocking out PAI-1, a key anti-fibrinolytic factor leads to resistance to weight gain and protection from AT inflammation and obesity-related metabolic dysfunctions in mice [15-19]. Similarly, thrombin inhibitors administered during a high-fat diet (HFD) decrease weight gain [12]. Transgenic mice with the mutation in fibrinogen that eliminates leukocyte integrin binding site exhibit decreased AT inflammation and resistance to weight gain [12]. The global knockout of fibrin crosslinking enzyme, Factor XIII-A (FXIII-A) on an HFD displays normal weight gain but improved insulin sensitivity, decreased ECM in AT, and reduced macrophage infiltration [13].

Neutrophils play a significant role in obesity and thrombotic disorders [20-23]. Upon weight gain, they infiltrate adipose tissue within days, promoting pro-inflammatory macrophage recruitment and thereby worsening adipose tissue inflammation [24]. Neutrophils contribute to this through

mechanisms such as secreting inflammatory cytokines and releasing neutrophil extracellular traps (NETs), leading to a pro-thrombotic state and thromboinflammation [25, 26]. As first responders to injury sites, they also contribute to hemostasis and thrombus formation [27-30].

Research models and methods to study the effects of neutrophils on whole-body biology include pharmacological depletion of neutrophils [31] and neutrophil-specific knockouts generated using LysM-Cre and MRP8-Cre-ires/GFP (MRP8-Cre), Cre-driver mice, former not being specific to neutrophils, making MRP8-Cre model more used in this specific context [31-33]. MRP8-Cre-model expresses Cre recombinase via MRP8 (S100A8) promoter [34-36]. A recent study reported off-target effects in MRP8-Cre [31] - authors reported that integration of a single copy of MRP8-Cre-ires/GFP transgene into chromosome 5 leads to the complete deletion of *Serpine1 (*PAI-1*)* gene and partial deletion of *Ap1s1* (Adaptor Related Protein Complex 1 Subunit Sigma 1) genes in the host genome [37].

In this report, we present evidence that MRP8-Cre may not be suitable for studies related to AT or weight-related metabolic conditions. We show that MRP8-Cre subcutaneous and visceral AT do not express *Serpine 1* mRNA and the mice phenocopy resistance to the published lack of weight gain of the global PAI-1 knockout on HFD. We further support this claim by showing that the *F13a1*$^{-/-MRP8}$ model created with MRP8-Cre mice also exhibits resistance to weight gain upon HFD, which is a phenotype not found in published study on global *F13a1* null mice [13]. Our finding suggests the careful evaluation of this Cre-model in weight gain and AT studies and including it as an important control to any studies where PAI-1 and AP1S1 may have functions.

3.3. Materials and methods

3.3.1. Animals and MRP8-Cre

All animal housing and procedures were conducted according to the guidelines for animal experimentation and were approved by the Animal Care Committee of McGill University. Animals were maintained in a pathogen-free environment and under standard housing conditions (23±2°C in a 12 h light/12 h dark cycle) with ad libitum access to food and water. MRP8-Cre-ires/GFP (MRP8-Cre) transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA; JAX, stock #021614). These mice express Cre recombinase under the control of the MRP8 promoter, which is active in myeloid cells.

3.3.2. Neutrophil-deletion of *F13a1* using MRP8-Cre driver mouse

For the development of neutrophil-specific *F13a1* knockouts (*F13a1*$^{-/-MRP8}$), MRP8-Cre mice were bred to *F13a1* flx/flx mice, which carry loxP sites inserted into exon 8 of *F13a1* gene, to delete *F13a1* gene in neutrophils. The knockout mice were selected based on the genotyping of pups using Selleck Direct PCR Kit and primers specific to LoxP (Forward: tctgggccaaaccaagtacctgg, Reverse: caagaccagactgtgcaaaggg) and Cre (Forward: gcggtctggcagtaaaaactatc, Reverse: gtgaaacagcattgctgtcactt) alleles. Qualitative RT-PCR using *F13a1* specific primers (Forward: cagttcgaagacggcatcct, Reverse: aacaagatcactgttgacctct) was also conducted to further confirm the knockout process.

3.3.3. Dietary intervention and weekly wight monitoring

All mice MRP8-Cre, *F13a1* flx/flx, and *F13a1*$^{-/-MRP8}$ were placed on specific diets at the age of 4 weeks for 16 weeks. The diets, including the control diet (CD)(TD.08806) with 10% calory from fat and high-fat diet (HFD)(TD.06414) containing 60% calory from fat, were purchased from

Envigo, headquartered in Indianapolis, Indiana, USA. Mice were weighed on a weekly basis to monitor the effect of a specific diet on each group's weight. *F13a1* flx/flx acted here as positive control with expected normal weight gain on HFD. The weight gain data was recorded and analyzed using GraphPad Prism v9. For better visualization, graphs were generated to represent weekly weight gain over 16 weeks on diets. The area under the curve (AUC) values were calculated using GraphPad Prism v9. The higher AUC represents the higher weight gain.

3.3.4. Tissue collection, RNA extraction and cDNA synthesis

After 16 weeks on a diet, the mice were euthanized by isoflurane followed by CO_2 asphyxiation. The nose-to-tail of mice was measured as a parameter for growth. The white adipose tissue (WAT) depots, including VAT (epididymal) and SAT (inguinal), were dissected, snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted from snap-frozen tissues using the RNeasy Mini Kit (Qiagen, Germany), following the manufacturer's protocol. Briefly, tissues were homogenized in QIAzol buffer, and RNA was purified using silica-membrane spin columns. The quantity and quality of RNA were assessed using a spectrophotometer, and the complementary DNA (cDNA) was synthesized from 1000ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The reaction setup included the following components: 10x RT buffer (2.0 µL), 25x dNTP mix (100 mM, 0.8 µL), 10x RT random primers (2.0 µL), MultiScribe Reverse Transcriptase (1.0 µL), and nuclease-free water (4.2 µL). The reaction conditions were 25°C for 10 minutes, and 37°C for 120 minutes, followed by a termination step at 85°C for 5 minutes.

3.3.5. Quantitative Real-Time PCR (qRT-PCR)

Each 20 µL reaction contained 9 µL (50 ng) synthesized cDNA, 10 µL TaqMan Fast Advanced Master Mix, and 1 µL of each TaqMan Gene Expression Assay. Relative gene expression of *Serpine 1* (Mm01204470_m1), *Ap1s1* (Mm00475917_m1) and *Adgre1*(Mm00802529_m1) was calculated using the $2^{\wedge(-\Delta\Delta Ct)}$ method with TATA-box binding protein (*TBP*) (Mm01277044_m1) as the internal control for normalization.

3.3.6. Statistical analysis

Data were analyzed using GraphPad Prism software (version 9). Results are expressed as the mean ± SEM (standard error of the mean). Each experiment was performed in triplicate, with n representing the number of mice (n= 7). For comparisons involving more than two groups, one-way or two-way ANOVA was performed, as appropriate, with a Tukey post hoc test for multiple comparisons. The area under the curve (AUC) for weight gain was calculated with a baseline 4-week weight set at zero. Differences were considered statistically significant at p-values < 0.05. Plots of the data were generated to visually assess trends and variability.

3.4. Results

3.4.1. MRP8-Cre and *F13a1*$^{-/-\ MRP8}$ are resistant to weight gain on HFD

Diet-induced obesity models were created from MRP8-Cre mice, *F13a1* flx/flx, and *F13a1*$^{-/-MRP8}$ models. *F13a1* flx/flx was used as a positive control with expected normal weight gain on HFD. The mice at the age of 4 weeks, the starting point for the diet challenge, showed similar weight (**Supplemental Figure 3.1**). Weekly weight monitoring showed a significant difference in weight gain on HFD onwards of 8 weeks resistance in both MRP8-Cre and *F13a1*$^{-/-MRP8}$ mice compared to the *F13a1* flx/flx control mice (**Figure 3.2A, B**). This difference persisted until the endpoint at

16 weeks, when the mice appeared slimmer and smaller (**Figure 3.2C, D**). A similar pattern was observed in female groups, with differences showing after 10 weeks on HFD (data not shown). To determine if the weight difference was due to altered general growth and body size, nose-to-tail length was measured. The data indicated no significant differences in these parameters, suggesting normal general growth in all mouse models (**Figure 3.2E**).

3.4.2. WAT from MRP8-Cre mice show negligible *Serpine 1, as well as reduced Ap1s1* and *Adgre1* expression compared to *F13a1* flx/flx mice

To investigate whether the reported off-target genomic deletions of *Serpine1* and *Ap1s1* have also resulted in deletions in AT depots, possibly affecting the weight gain, gene expression analysis of SAT and VAT was performed from the CD and HFD-fed mouse tissues. The analysis revealed that the expression of *Serpine1* in both AT depots and after both diets was, indeed, dramatically down in MRP8-Cre and *F13a1*$^{-/-MRP}$ mice but increased in the control model on HFD. A similar pattern was also observed for the expression of *Ap1s1* in VAT and SAT (**Figure 3.3A, B**). The MRP8-Cre background also generated SAT and VAT with significantly decreased macrophage infiltration (*Adgre1* -F8/40), suggesting protection from inflammation (**Figure 3.3A, B**).

3.5. Discussion

PAI-1 inhibits fibrin lysis as part of the hemostatic process to sustain blood clots. Recent advances have linked its function also to metabolic processes, AT inflammation, adipogenesis, development of insulin resistance, and glucose metabolism disturbances [15-17, 38, 39]. Emerging evidence shows the positive impact of PAI-1 inhibition or knockout on adipose tissue health and metabolic functions [14, 18, 40]. For instance, pharmacological inhibition of PAI-1 using PAI-039 or complete deletion of the PAI-1 gene led to a significant decrease in macrophage infiltration into

AT and improved metabolic status in HFD-induced models [14]. In addition, targeting PAI-1 with chemical inhibitors ameliorates AT inflammation, atherosclerosis and associated metabolic dysfunctions [41]. Mechanistically, PAI-1's role in weight gain in mice was linked to enhanced hypothalamic leptin sensitivity, but roles directly in AT are also likely based on recent advances in our understanding of the essential roles of fibrinolytic system on AT health and inflammation [18]

[12]. Factor XIII-A, a transglutaminase enzyme that stabilizes fibrin clots, was originally linked to obesity as a putative causative gene in a GWAS study on obesity-discordant monozygotic twins [42]. However, global deletion of FXIII-A in mice does not contribute to weight gain albeit the mice have significantly improved insulin sensitivity on HFD compared to controls [13]. Our recent transcriptome-wide association studies (TWAS) on adipocytes and adipose tissues from monozygotic twins linked the *F13A1* gene to neutrophil activation justifying the question of this FXIII-A in neutrophils is relevant to AT health [43-45]. Generation of neutrophil-specific *F13a1* knockout model using MRP8-Cre mice revealed that MRP8-Cre background creates resistance to weight gain, a finding confirmed in this paper using the MRP8-Cre alone. Likely, the recently reported off-target effects in the MRP8-Cre [37] are the underlying cause of the weight phenotype. Our gene expression analysis verified the complete deletion of *Serpine 1* in WAT and a significant reduction in *Ap1s1* expression. AP1S1, an adaptor protein complex family member of the, contributes to cargo transport between intracellular organelles [46-48]. To date, no data is available on the role of protein's contribution to weight modulation and metabolic health.

So far, MRP8-cre mice have been used to generate several neutrophil-specific knockouts to analyze of metabolic effects. These involve PAD4$^{-/-\ MRP8}$, CXCR2$^{-/-\ MRP8}$, CXCR4$^{-/-\ MRP8}$, and α9$^{-/-\ MRP8}$ [49-52]. The MRP8-Cre model was not used as a control. PAD4$^{-/-\ MRP8}$ study showed a

reduction in weight gain from 7 weeks onwards of HFD, and showed no increase in cardiac collagen, and exhibited a decreased thrombus formation after 10 weeks of metabolic challenge [49]. Additionally, the obese $\alpha 9^{-/-\ MRP8}$ model showed improved stroke outcomes and reduced thrombosis susceptibility [52]. A study on the effects of neutrophil aging on metabolic profiles in $CXCR2^{-/-\ MRP8}$ mice, challenging with a 20-week HFD, revealed protection from metabolic alterations and reduced proinflammatory macrophage recruitment in AT. These mice also exhibited resistance to weight gain [51]. While our report is based on simple weight gain observations, it is important to emphasize that reduced weight gain on HFD will naturally improve many metabolic outcomes, including insulin sensitivity, glucose clearance and AT inflammation. Furthermore, the global elimination of PAI-1 in the MRP8-Cre may cause other phenotypes, particularly those related to thrombosis and thromboinflammation. We thus encourage re-investigating some of the discovered effects using the MRP8-Cre as a control. Our report underscores the critical need for thorough characterizing genetically engineered models such as Cre and LoxP and using them as controls in studies.

3.6. Conclusion

In conclusion, our study underscores the confounding effects of MRP8-Cre mice when investigating weight gain and metabolic parameters and emphasizes the importance of including appropriate controls for any new genetic models.

3.7. References

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3.8. Figures

Figure 1

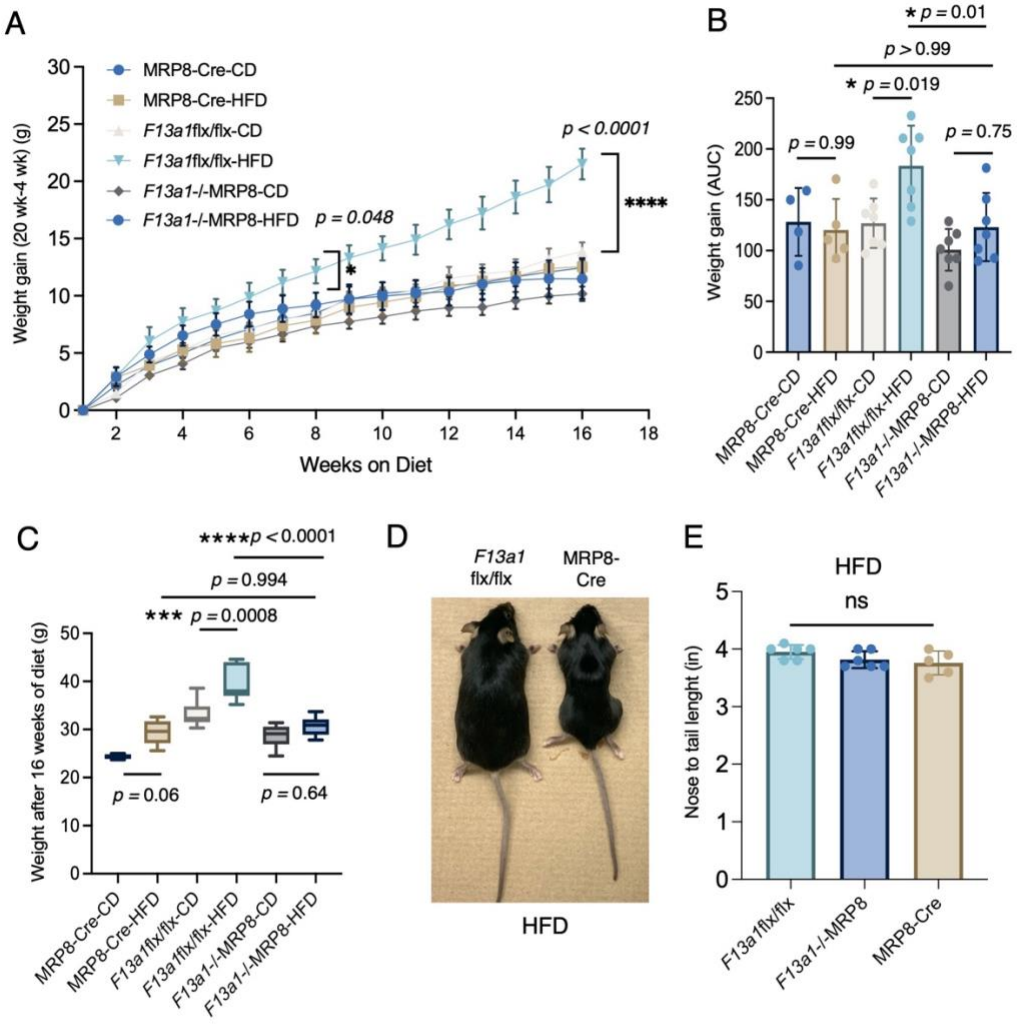

Figure 3.1. MRP8-Cre driver mouse does not gain weight on a high-fat diet (HFD). A. B. Weight monitoring of MRP8-Cre driver mouse on HFD in comparison to *F13a1*flx/flx and *F13a1*-/-MRP8 (conditional knockout generated using MRP8-Cre model). Weekly weight gain and AUC of the weight gain curve are presented. The significant differences in weight gain begun to emerge after 8 weeks on HFD. **C.** Weights at the end point after 16-weeks on diet. MRP8-Cre sustained same weight on HFD as on CD whereas *F13a1*flx/flx (representing WT control genotype) gained significant weight on HFD. *F13a1*-/-MRP8 showed same resistance to weight gain on HFD as MRP-Cre on HFD. **D.** Photographic images of *F13a1*flx/flx and MRP8-Cre after HFD feeding. MRP8-Cre appears slimmer. **E.** Nose-to-tail length measurements of *F13a1*flx/flx (control), *F13a1*-/-MRP8 and MRP8-Cre show no difference in general size of the mice. Data are presented as mean ± SD and significance was analyzed with two-way ANOVA. AUC data was normalized to weight at 4 weeks. Significance is presented as *p*-values and values <0.05 are considered significant (p< 0.05, *; p<0.001;**, p<0.001, *** and p<0.0001, ****).

Figure 2

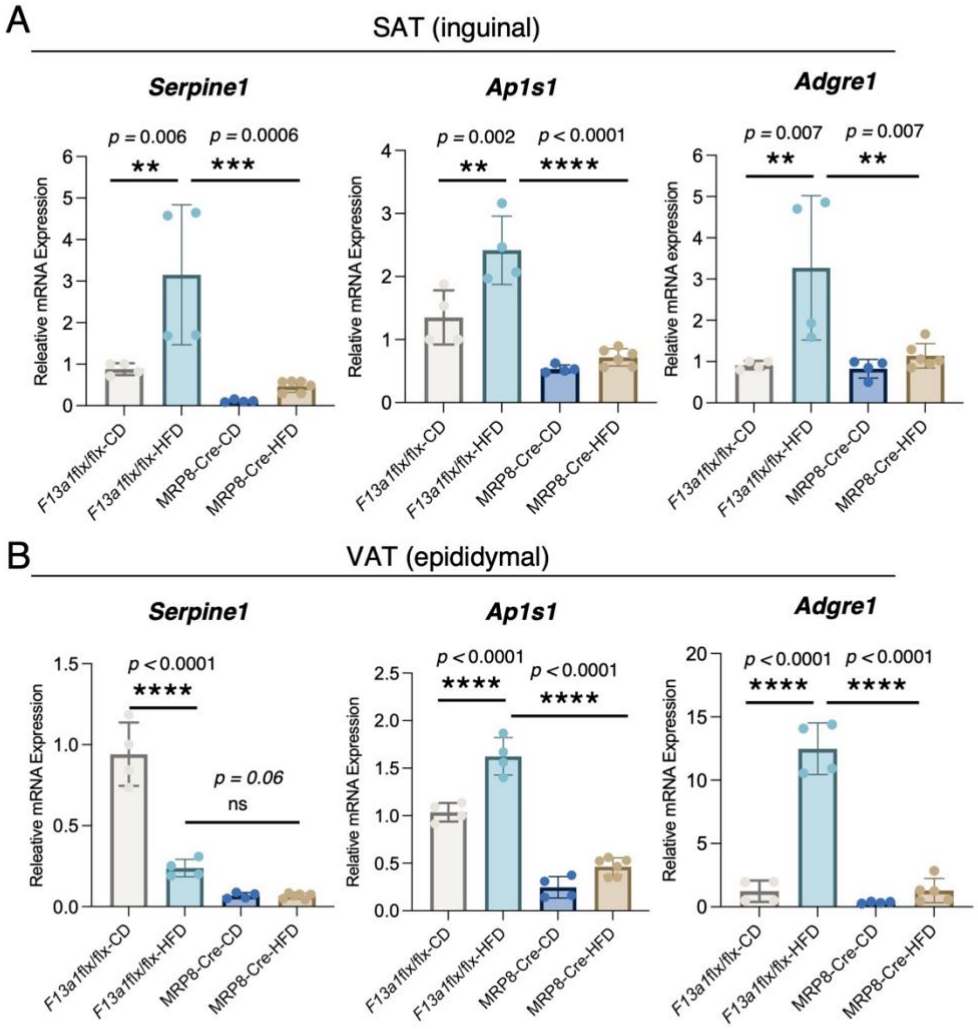

Figure 3.2. White adipose tissue depots of MRP8-Cre driver mouse are knockout for *Serpine1* and *Ap1s* and show protection to macrophage infiltration on a high-fat diet (HFD). Gene expression analysis of *Serpine1* (PAI-1) *and Ap1s1* (AP1S1) and *Adgre1* (F4/80) in subcutaneous (inguinal) **(A)** and visceral (epididymal) **(B)** adipose tissue of MRP8-Cre mice (male) after 16 weeks on control diet (CD) and high-fat diet (HFD), latter which is expected to induce macrophage infiltration as observed in *F13a1* flx/flx on HFD (corresponding to a WT mouse genotype and positive control for diet-induced obesity model.

Supplementary Figure 1

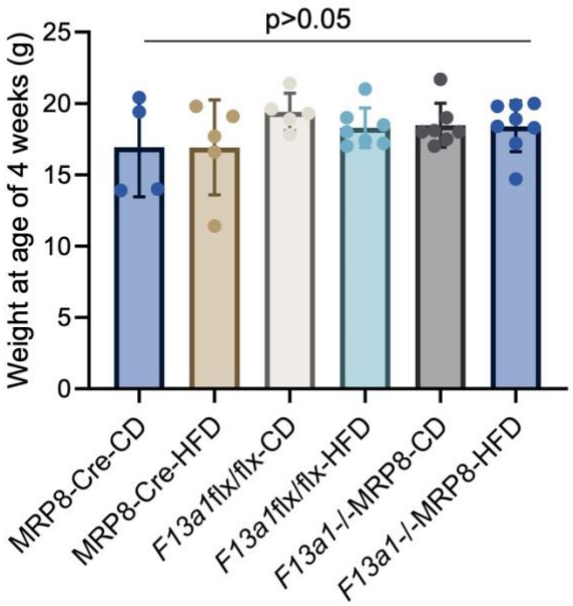

Figure S3.1. Mouse weights at 4-week age prior to starting special diets. No statistical differences were detected.

4. Chapter 4: General Discussion

4.1. Significance of study

Thromboinflammation and fibrosis are crucial concerns during the progression of obesity, leading to metabolic dysfunctions such as insulin resistance. The pathological alterations in adipose tissue during weight gain substantially contribute to metabolic disorders. During obesity, immune cells, particularly neutrophils and macrophages, infiltrate adipose tissue and play pivotal roles in the clearance of dead adipocytes. Understanding the initiation of the infiltration and how this leads to pathological sequela can open an avenue to develop therapeutic strategies to manage adipose tissue inflammation.

4.2. Key findings

The goal of this thesis research was to explore the possible expression of FXIII-A transglutaminase in neutrophils, and its role in NET-fibrin stabilization *in vitro,* and its effects on metabolic parameters during obesity *in vivo* in mice. Chapter 2 used bone marrow neutrophils from C57Bl/6 mice, and in Chapter 3, I developed a neutrophil *F13a1* knockout mice (*F13a1*$^{-/-MRP8}$) using Cre-loxP strategy involving *F13a1*flx/flx and MRP8-Cre models. The results in Chapter 2 confirmed our scRNA sequencing data mining and showed that murine male neutrophils exclusively produce FXIII-A as the sole transglutaminase enzyme. This expression level was not observed in female neutrophils, suggesting a regulatory role for sex hormones. The data also showed a transglutaminase activity of neutrophil FXIII-A during both resting and activation (NETosis) stages. Moreover, the findings revealed the active involvement of neutrophil's FXIII-A in stabilizing NET-fibrin interaction, which was inhibited by a general transglutaminase inhibitor, i.e. NC9. Chapter 3 demonstrates that the metabolic phenotyping of *F13a1*$^{-/-MRP8}$, including weight gain and metabolic parameters, is affected by a critical confounder, which is the deletion of *Serpine 1* (PAI-1) in MRP8-Cre genome.

The weight gain monitoring confirmed that the observed resistance to weight gain might be due to the absence of PAI-1 in adipose tissue rather than FXIII-A in neutrophils, given the normal weight gain observed in previously reported global FXIII-A knockout mice. Therefore, while metabolic parameters were improved in the neutrophil-specific FXIII-A knockout mice (data will be discussed in the Overall discussion part), the overlapping roles of FXIII-A and PAI-1, as well as the dominant role of PAI-1 in metabolic functions in mice, made it challenging to distinguish the precise role of neutrophil-derived FXIII-A in adipose tissue health from that of PAI-1.

Moreover, sexual dimorphism in *F13a1* expression and metabolic functions was observed. Male neutrophils expressed higher levels of *F13a1* compared to females, and after one week on HFD, *F13a1* expression increased in male WAT but not in female WAT. Overall, this dissertation has established the contribution of neutrophil's FXIII-A to the formation of NET-fibrin networks within inflammatory and thrombotic environments and showed that the MRP8-Cre model is not appropriate for studying metabolic functions due to the deletion of PAI-1.

4.2. Overall discussion

Obesity is a growing global problem with a serious impact on public health. It is a complex and multifaceted condition associated with several comorbidities, such as type 2 diabetes, cardiovascular disease, and certain types of cancer [36, 39, 40, 367]. Sex and many genetic factors play important roles in the susceptivity of individuals to obesity, particularly in obesogenic environments [368, 369]. Men are prone to accumulate more visceral fat and certain metabolic disorders compared to women [369]. Several genes, including leptin, the leptin receptor, and the melanocortin 4 receptor, have been linked to the development of obesity [370, 371]. As mentioned in the introduction of this thesis, the gene *F13A1*, which encodes FXIII-A coagulation factor and transglutaminase, has also been recognized as potentially contributing to obesity and its

comorbidities including insulin resistance and thrombotic events [197] [189]. FXIII-A functions as a fibrin-stabilizing enzyme, crosslinking critical substrates such as fibronectin and fibrinogen. This transglutaminase activity may lead to inflammatory responses and dysfunction in metabolic tissues, notably adipose tissue. Furthermore, FXIII-A is produced intracellularly by various cells, including macrophages, which underscores its potential role in the pathogenesis of diseases, particularly those that alter the activity or numbers of these cells, such as obesity [175].

The activity of neutrophils, as primary responders in the immune system, has increasingly garnered attention in the context of fibrinogenesis and thrombosis during obesity [372-374]. Given the diverse sources and functions of FXIII-A and our scRNA sequencing data showing *F13A1/F13a1* mRNA expression in immune cells in adipose tissue, my thesis aimed to investigate whether neutrophils produce FXIII-A endogenously. I also aimed to explore its potential active roles in the context of fibrotic events and obesity-related comorbidities. **Figure 4.1** illustrates a schematic representation of my hypothesis and the overall goal of this thesis.

My *in vitro* experiments showed endogenous production of FXIII-A in neutrophils as an intercellular transglutaminase. FXIII-A lacks a signal peptide to direct it to the outside of the cells, and other non-classical mechanisms might be involved in its externalization. The observed intracellular distribution of FXIII-A aligns with previous studies demonstrating cytoplasmic localization of FXIII-A in certain immune cells, with a known translocation to the cell membrane upon cell activation. For instance, FXIII-A is initially cytoplasmic in platelets and monocytes but can relocate to the cell membrane to participate in coagulation processes and crosslinking

extracellular substrates [131, 375]. Future investigations employing techniques such as subcellular fractionation are necessary to accurately determine the localization of FXIII-A within neutrophils.

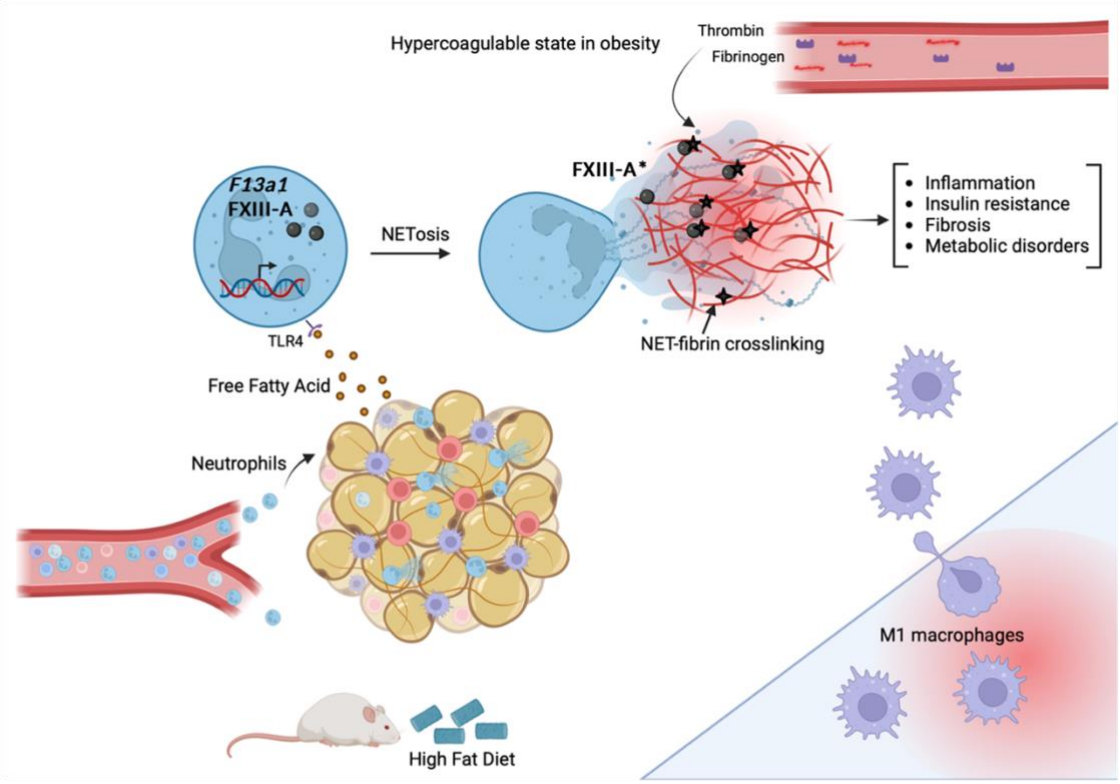

Figure 4. 1. Schematic representation of the hypothesized role of neutrophil FXIII-A in enhancing NET-fibrin stabilization and adipose tissue health during obesity, as proposed in this thesis. Created in Biorender. Com.

Although previous studies have identified TG1 and TG2 in human neutrophils [274, 376], my results indicated that FXIII-A is the sole transglutaminase in mouse bone marrow neutrophils. This inconsistency may be related to the varying sources of neutrophils used in those studies. Furthermore, the expression levels and types of TGs can differ significantly between species and

cell types. For example, human platelets contain FXIII-A as the only transglutaminase, whereas mouse platelets exhibit a range of active transglutaminases [114, 377, 378]. In addition, one of the studies [274], despite showing higher levels of *F13A1* mRNA compared to *Tgm1*, suggested that this abundant *F13A1* mRNA derives primarily from phagocytosed platelets, which are rich in FXIII-A protein, rather than from endogenous production by human neutrophils [274]. Platelet FXIII-A is synthesized in megakaryocyte's precursor during thrombopoiesis and stored as a protein (not mRNA) in platelets during pro-platelet production. It regulates platelet functions and contributes to clot retraction and stability [128]. Platelet activation leads to morphological changes and the upregulation of adhesion molecules such as P-selectin and ß2 and ß3 integrins (CD11b/CD18, CD41/CD61). These molecules enhance interactions between platelets and various innate and adaptive immune system cells, including neutrophils [379, 380]. During the isolation process, the activation of platelets is likely and can lead to their co-purification with neutrophils through these interactions.

Moreover, the internalization of microvesicles from platelets into the neutrophils and thereby transferring their materials into the other cells has been related to the lipid mediator of inflammation [381]. Given the higher abundance and complete maturation of platelets in circulating blood compared to bone marrow, it seems that the possibility of co-purifying platelets is higher when isolating neutrophils from blood rather than from bone marrow. The immunofluorescence imaging confirmed a negligible presence of platelets in my neutrophil preparations, with less than 2% CD41-positive cells. Therefore, it is highly likely platelets minimally contribute to the FXIII-A observed in our experiments.

Upon identifying FXIII-A in bone marrow neutrophils, I next aimed to investigate its potential role in the context of NET-fibrin(ogen) stabilization. Neutrophils participate in coagulation and

thrombosis through various mechanisms, including releasing NETs [244, 269, 292-294]. The administration of DNase or knockout of PAD4 reduces intravascular coagulation, highlighting the significant role of NETs in this process [382]. While purified NETs can induce coagulation [383, 384], conflicting reports exist, such as one indicating that isolated NETs, unlike DNA and histones, do not initiate thrombus formation [288], suggesting the complex underlying interactions. Physical interactions between NETs and neutrophil microparticles are linked to the activation of intrinsic pathways [383]. Furthermore, electrostatic interactions between NET components, such as histone H4, and platelets activate the contact pathway of coagulation [382, 385]. Covalent interactions between NET components and various clotting factors, including vWF, fibronectin, factor XII, and fibrin/fibrinogen, contribute to coagulation and subsequent thrombosis [287]. These interactions enhance clot stability and resistance to fibrinolysis. Nevertheless, the precise mechanisms of these interactions and NET-induced thrombosis remain poorly understood.

My data showing the colocalization of FXIII-A with NET proteins including citH3 and MPO during NETosis suggests active exposure of FXIII-A to other NET and coagulation components, which likely promotes consequent events. Previous investigations have shown that NET components, such as histones, elastase, lactoferrin, MPO and cathepsin G, are substrates for transglutaminases [274]. Therefore, it is plausible that these proteins are crosslinked to coagulatory proteins through transglutaminase activity, contributing to the coagulation process. The *in vitro* crosslinking of histones to fibrin, likely facilitated by FXIII-A activity, contributes to the post-translational modification of proteins through transamidation reactions [386]. The observed transglutamianse activity during NETosis could mediate interactions between NETs and clot proteins. Indeed, in this study, fibrinogen was crosslinked to NETs, particularly in areas of released DNA, in the absence of serum, and this interaction was inhibited by NC9. Previous reports have

documented the critical role of interactions between neutrophils, NETs, and fibrinogen in thrombus formation [387]. Such interactions are likely mediated by the integrin receptor αMβ2 (Mac-1), which is upregulated upon neutrophil activation [388-390]. Also, the crosslinking within the fibrin network may alter the exposure of binding sites on fibrinogen [391]. My thesis did not include mechanistic investigations to further elucidate the interactions between individual NET proteins and fibrin, which necessitates future research. Given that FXIII-A was found to be the sole transglutaminase in murine bone marrow neutrophils, NC9 was used, as a general transglutaminase inhibitor. However, to more accurately determine the role of FXIII-A, especially in the presence of other transglutaminases, future studies should employ a more specific FXIII-A inhibitor such as Tridegin [392]. Also, in future, using neutrophils from *F13a1* global knockout mice would provide a more direct assessment of neutrophil FXIII-A in these processes.

According to my data, fibrin(ogen) matrix formation during NETosis was serum-dependent; that is, NETosis in the presence of serum led to strong fibrin(gen) network formation. Intriguingly, the NETosing cells were tightly enveloped and embedded within the fibrin(ogen) matrix fibrils. This observation aligns with another study [393] showing that a fibrin network can prevent the expansion of sheer-induced NETosis. Such an effect may act as a regulatory factor that limits excessive NET formation and mitigates its pathological effects. While NETs can trap invading pathogens [394, 395], NETs can also be harmful in sterile inflammation and thrombosis [396, 397]. For example, in systemic lupus erythematosus and rheumatoid arthritis, NETs contribute to the pathology of these autoimmune diseases. NET components, including enzymes, can damage tissues. In addition, double-strand DNA and other protein contents in NET can serve as a source for autoantigens, leading to excessive immune responses [398]. Moreover, as discussed earlier, in

thrombotic diseases like DVT, NETs provide a scaffold for the aggreagation of platelets and the activation of coagulation factors, resulting in clot formation [399].

In this study, the findings regarding the fibrin(ogen) matrix formation in serum, was unexpected as serum typically lacks active clotting factors. This phenomenon may be correlated to the complex composition of serum or potentially residual proteins and active components [400], which might influence neutrophil behaviour during NETosis. To further validate and understand these findings, additional experiments using plasma, which contains active clotting factors, are required.

The findings presented in Chapter 2 suggest that neutrophil FXIII-A plays a significant role in stabilizing fibrin(ogen) network formation during NETosis. In obesity, the thrombin/fibrin(ogen) axis contributes to metabolic inflammation, and fibrin crosslinking and polymerization further drive the comorbidities observed in obesity [242, 243].

Building on these findings, we aimed to study the role of neutrophil FXIII-A in metabolic health during obesity. To this end, I generated neutrophil *F13a1* knockout mice (*F13a1*$^{-/-MRP8}$) using MRP8-Cre mice. Previous studies report a high level of Cre recombinase expression, ∼90% in blood neutrophils and ∼ 80% in spleen and bone marrow neutrophils [362, 401].

Upon HFD, the knockout mice exhibited resistance to weight gain, which was unexpected. The weight gain discrepancies between *F13a1*$^{-/-}$ and *F13a1*$^{-/-MRP8}$ mice led us to question whether the observed phenotype in our study might stem from another factor. The weight monitoring of MRP8-Cre mice as an additional control revealed that these mice present a critical confounding factor. Despite the valuable role of this model in neutrophil biology studies, it appears to have off-target effects; the integration of the Cre cassette into chromosome 5 of the host resulted in a sequence deletion within the genome [366]. This deletion leads to a partial deletion of *Ap1s1* and a complete deletion of the *Serpine 1* (PAI-1) gene [366]. Gene deletions and side effects raised

from Cre models have also been reported in other genetic models, underscoring the importance of investigating unexpected recombination and using the Cre-driver as a control [402, 403]. My preliminary analysis of adipocyte size in VAT further confirmed this weight-related phenotype, showing a distribution of smaller adipocytes within the tissue as expected if mice do not gain weight (**Figure 4.2**).

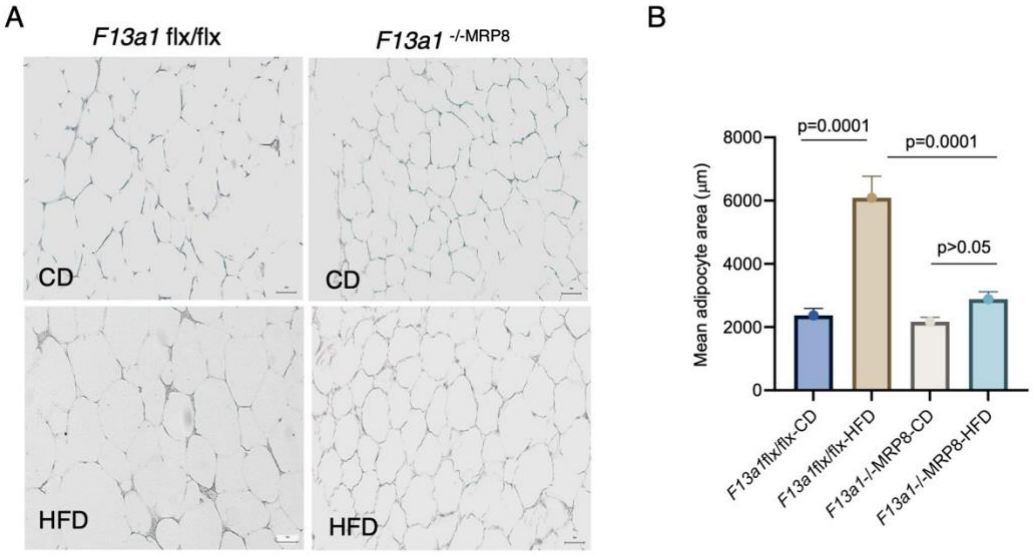

Figure 4.2. Comparison of adipocyte size in visceral adipose tissue (VAT) from *F13a1*$^{-/-MRP8}$ and *F13a1* flx/flx mice on a high-fat diet (HFD). A: Immunohistochemistry (IHC) analysis indicates smaller adipocytes in knockout mice compared to wild-type controls. **B:** Quantitative measurement of adipocyte size using ImageJ software confirmed a smaller size distribution in knockout mice relative to wild-type on HFD, with no significant size differences observed under a control diet (CD). Analyses were conducted on three different repeats, each consisting of three different fields. Each data point represents the mean ± SEM, and p value <0.05 denotes statistical significance.

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AP1S1 is a member of the adaptor protein (AP) complex family, involved in clathrin coat assembly and cargo transport between intracellular organelles, such as endosomes and the plasma membrane. Mutations or deficiencies in AP1S1 are associated with various diseases, including

intestinal epithelial barrier defects, congenital diarrhea, MEDNIK syndrome, and cancer [404-406]. *Ap1s1* has been shown to negatively correlate with immune cell infiltration into breast cancer tissue, indicating its role in modulating cellular immunity [406]. Furthermore, in breast tumors, *Ap1s1* expression positively correlates with estrogen receptor expression and metastasis [406]. To date, there are no reports linking AP1S1 absence with metabolic syndrome or weight modulation. On the other hand, the contribution of PAI-1 (function reviewed in this thesis on page 50) in various processes, including fibrinolysis, inflammation, adipogenesis, insulin resistance, and glucose metabolism, has been widely reported [208, 209, 407-409]. Studies employing the PAI-1 global knockout model have reported the significant role of PAI-1 in obesity-related comorbidities. These mice showed metabolic phenotypes such as resistance to weight gain, reduced inflammatory macrophage infiltration into the adipose tissue, and improved glucose tolerance and insulin sensitivity [214, 215, 410], consistent with my *in vivo* results (which will be discussed in the following).

In addition to weight gain, I explored the phenotype of *F13a1*$^{-/-MRP8}$ mice by conducting additional metabolic experiments. MRP8-Cre mice were not included in these experiments, and further analysis was stopped due to the weight phenotype of this control. The results from *F13a1*$^{-/-MRP8}$ mice indicated an improved glucose tolerance and insulin sensitivity in male knockout mice on HFD (**Figures 4.3 and 4.4**).

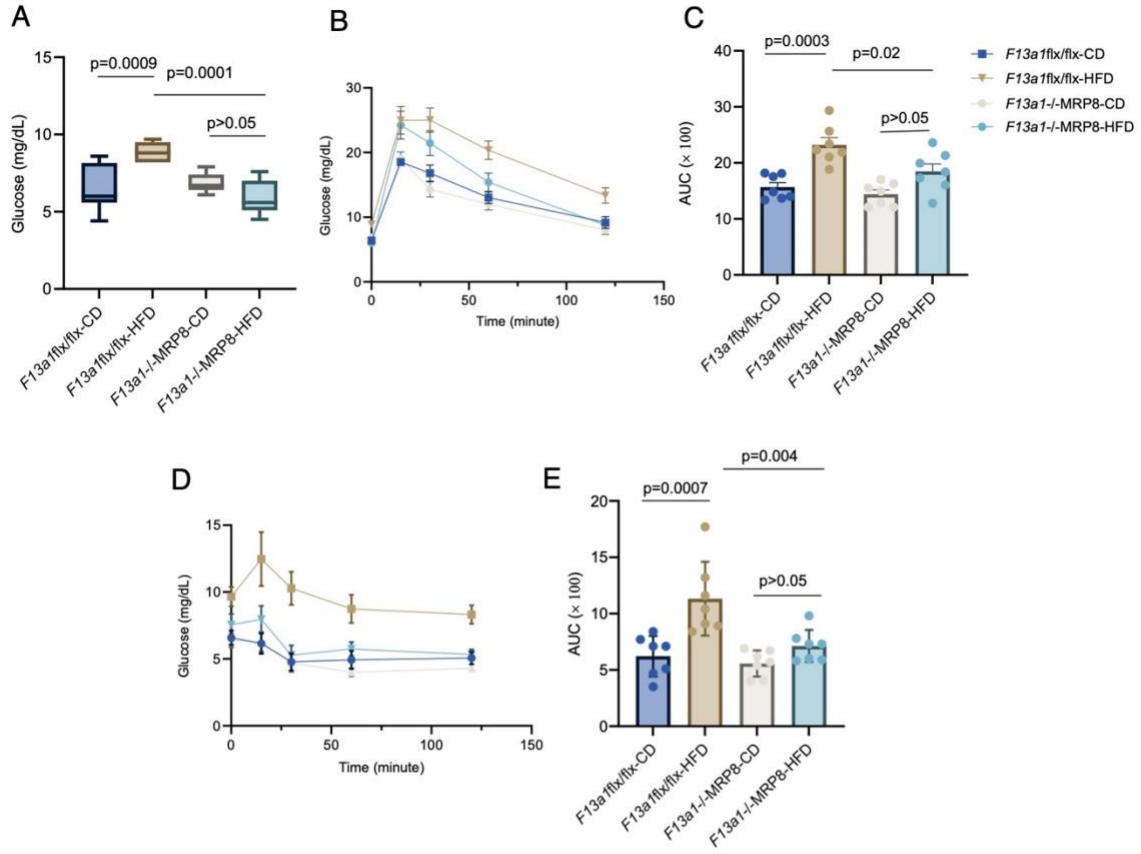

Figure 4.3. Improved glucose tolerance and insulin sensitivity in male *F13a1*$^{-/-\ MRP8}$ mice compared to *F13a1* flx/flx controls on a high-fat diet (HFD). **A:** Baseline glucose levels were significantly lower in knockout mice (n=7), indicating improved glucose regulation. **B** and **D:** Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT) involved measuring blood glucose levels at 0, 15-, 30-, 60-, and 120-minutes post-administration of glucose (2mg/g body weight) and insulin (0.5U/g body weight), respectively. **C** and **E:** Calculation of the area under the curve (AUC) for each test highlighted better metabolic functions in *F13a1*$^{-/-\ MRP8}$ mice. Data are presented as mean ± SEM, with $p < 0.05$ indicating statistical significance.

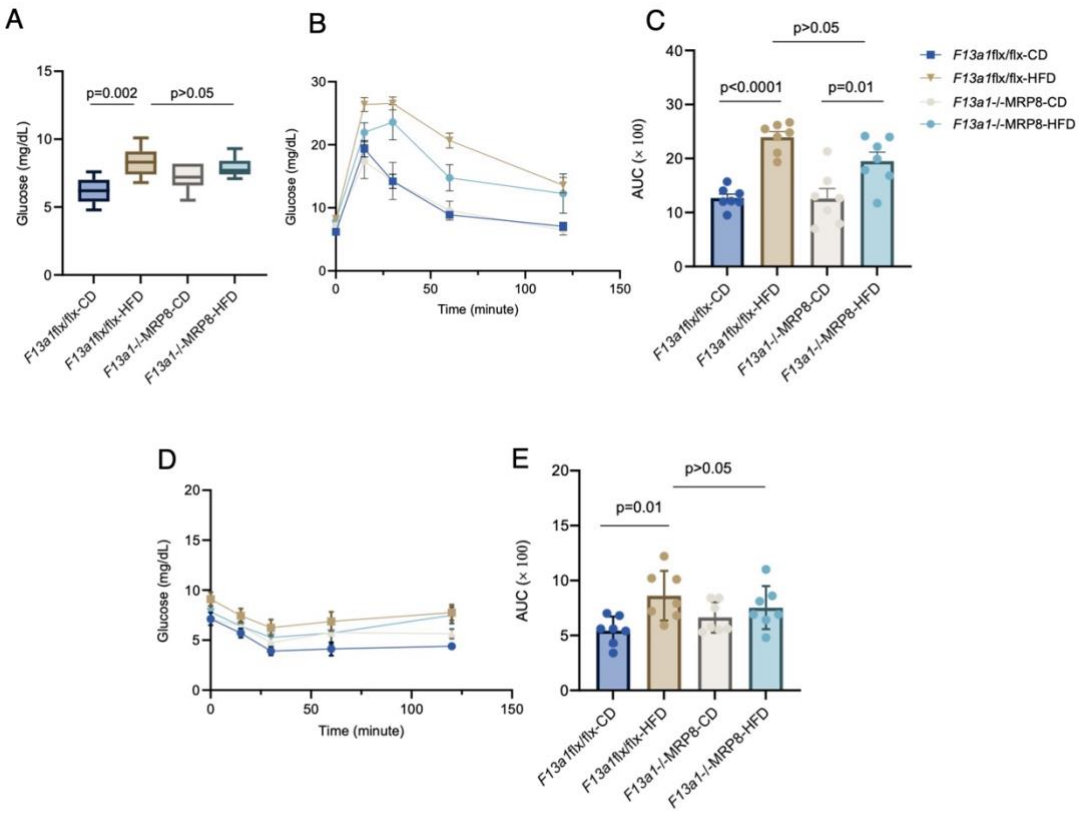

Figure 4.4. Unchanged glucose tolerance and insulin sensitivity in female *F13a1*$^{-/-\ MRP8}$ mice compared to *F13a1* flx/flx controls on a high-fat diet (HFD). **A**: Initial glucose measurements at time zero revealed no significant differences between knockout and wild-type mice (n=7) on HFD. B and D: Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT) involved measuring blood glucose levels at 0, 15-, 30-, 60-, and 120-minutes post-administration of glucose (2mg/g body weight) and insulin (0.5U/g body weight), respectively. C and E: The area under the curve (AUC) for each test was calculated, showing similar glucose metabolism between the groups. Data are presented as mean ± SEM, with $p < 0.05$ indicating statistical significance.

Additionally, my initial findings showed that the *F13a1*$^{-/-MRP8}$ mice have healthier adipose tissue profiles with reduced expression of macrophage, inflammation and fibrosis markers in VAT and SAT (**Figures 4.5 and 4.6**).

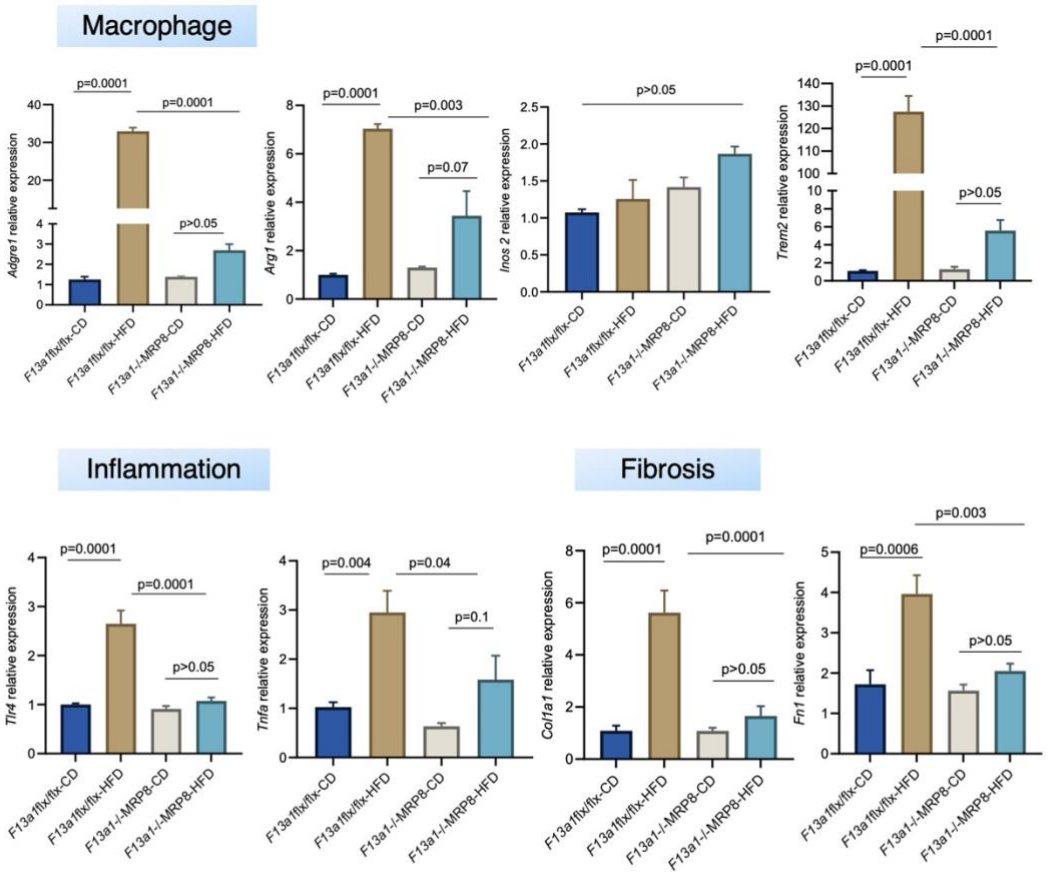

Figure 4.5. Reduced macrophage, inflammation, and fibrosis markers in visceral adipose tissue (VAT) of *F13a1*$^{-/-MRP8}$ mice compared to *F13a1* flx/flx controls. Analysis of VAT from three independent experiments demonstrates significantly lower levels of these markers in male knockout mice versus wild-type mice on a high-fat diet (HFD). Each data point represents mean ± SEM, and p value <0.05 denotes statistical significance.

These effects may also arise from the *F13a1* knockout, as we documented these phenotypes in the global knockout [175]. Expanding the range of markers and assessing the other organs in future studies could provide deeper insights into the underlying molecular mechanisms and enhance our

understanding of overall metabolic health. My preliminary immunohistochemistry (IHC) analysis also confirmed a lower level of fibrin deposits and macrophages in the VAT of *F13a1*$^{-/-MRP8}$ mice compared to the *F13a1* flx/flx on HFD (**Figure 4.7**).

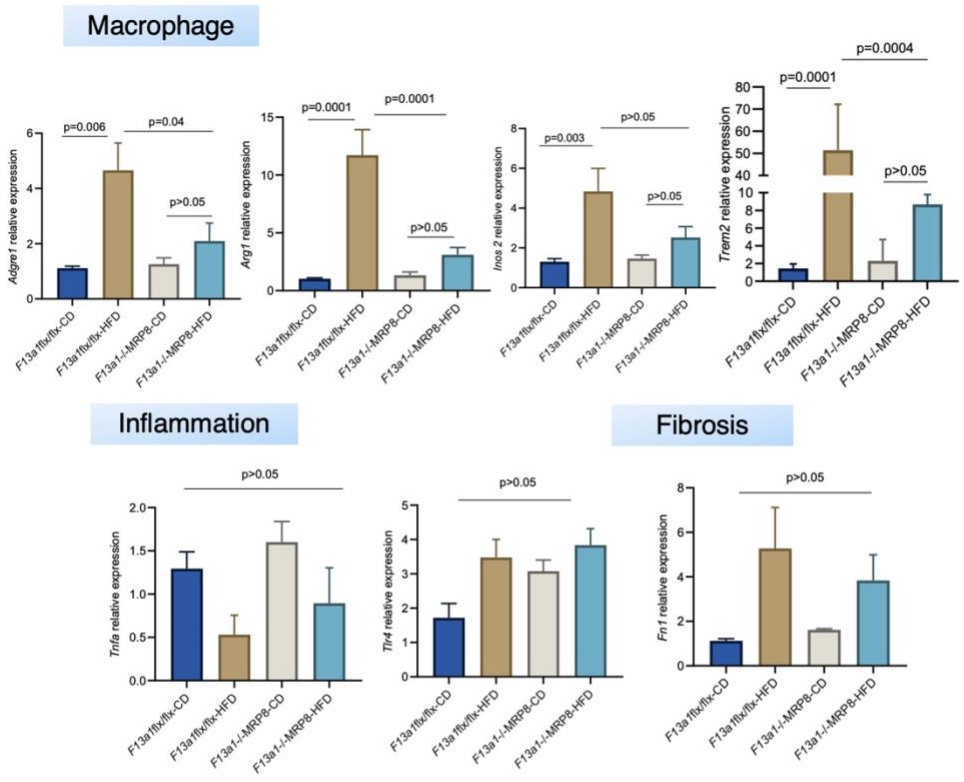

Figure 4.6. Decreased macrophage markers in subcutaneous adipose tissue (SAT) of *F13a1*$^{-/-MRP8}$ mice compared to *F13a1* flx/flx controls. Analysis of SAT from three independent experiments demonstrates significantly lower levels of macrophage markers in knockout mice versus wild-type mice on a high-fat diet (HFD). No significant differences were observed between the groups in inflammation and fibrosis markers. Each data point represents mean ± SEM, and p value <0.05 denotes statistical significance.

Given that both PAI-1 and FXIII-A impact metabolic regulations, distinguishing their individual contributions to obtained results from *F13a1*$^{-/-\ MRP8}$ are not possible that warrants the cost and effort for the work. It is important to note that the *Serpine 1* deletion in MRP8-Cre mice is likely to affect several prior metabolic studies using this model, where PAI-1 may act as a confounding factor.

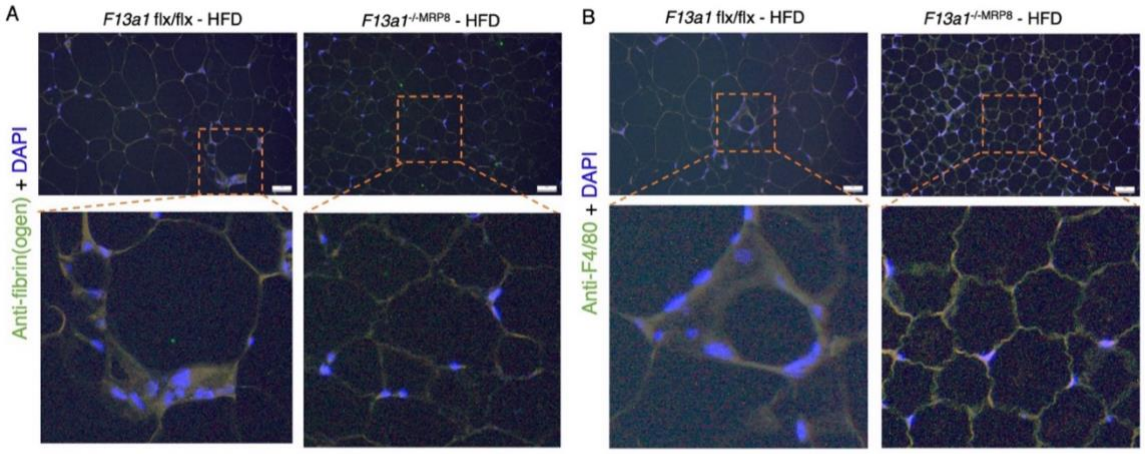

Figure 4.7. Reduced fibrin(ogen) deposition and macrophage accumulation in visceral adipose tissue (VAT) of *F13a1*$^{-/-\ MRP8}$ mice compared to *F13a1* flx/flx controls. A. Immunohistochemistry (IHC) analysis shows lower fibrin(ogen) deposition and B. reduced macrophage accumulation in VAT of knockout compared to that in wild-type mice on HFD. Analyses were conducted on three different groups. Each data point represents mean ± SEM, and p value <0.05 denotes statistical significance.

For example, in studies using PAD4$^{-/-\ MRP8}$ mice on HFD [411], the observed reduced weight gain and thrombosis were associated with the absence of PAD4. However, the concurrent absence of PAI-1 could also influence these findings. The other studies that used the MRP8-Cre model to delete genes such as Nlrp3, ATG5, CXCR2, CXCR4, and α9 integrin in neutrophils also showed a lower weight gain in their developed knockout models, except for CXCR4, and α9 integrin knockouts [411-416]. Without having MRP8-Cre control, it is difficult to determine whether

observed effects were due to the specific gene deletion or from unintended effects of the Cre recombination.

In my project, I observed a clearly different metabolic response between male and female mice; *F13a1*$^{-/-MRP8}$ male mice on HFD exhibited better metabolic profiles, including improved glucose tolerance and insulin sensitivity, whereas female mice did not show this phenotype. The sexual dimorphism in adipose tissue biology, obesity, and metabolic syndrome is well-documented, with men more prone to central obesity, insulin resistance, and cardiovascular diseases [61, 62]. The sexual dimorphism was also reflected in my other *in vivo* preliminary data from C57Bl/6 mice. In mice, 7 days on HFD, a significant increase in *F13a1, Ly6G and Adgre1* gene expressions was observed in male mice (SAT and VAT) but not females (**Figure 4.8**).

The expression of various genes may differ between male and female tissues, despite having identical genomic contents and sequences [417]. Earlier studies have attributed many of these dimorphic genes to sex chromosomes, particularly the X chromosome [418, 419]. In contrast,. others have linked them to autosomal chromosomes, demonstrating that such dimorphisms can be tissue-specific [420]. In addition, sex hormones play a crucial role in mediating gene expression differences, through direct and indirect mechanisms [421, 422]. Given that the *F13a1* gene locus is located on an autosomal chromosome, its observed sexual dimorphism may be influenced by hormonal regulation. Sex hormones exert their effects by binding to specific receptors, including estrogen (ER), androgen (AR) and progesterone (PGR). The distribution of these receptors can vary among different cells, such as immune cells [423]. Upon binding their ligands, these receptors are activated and function as transcription factors. They bind to specific DNA motifs within hormone-responsive regions and influence the transcription of various genes [424]. To explore the role of sex hormones on *F13a1* expression, I treated neutrophils with different concentrations of

estrogen and testosterone. Preliminary results indicated that both hormones reduced *F13a1* expression in male neutrophils but did not alter expression in female neutrophils (**Figure 4.9**). These observations require further investigations to elucidate the signaling pathways and underlying mechanisms, which will help identify potential targets responsible for these observed differences.

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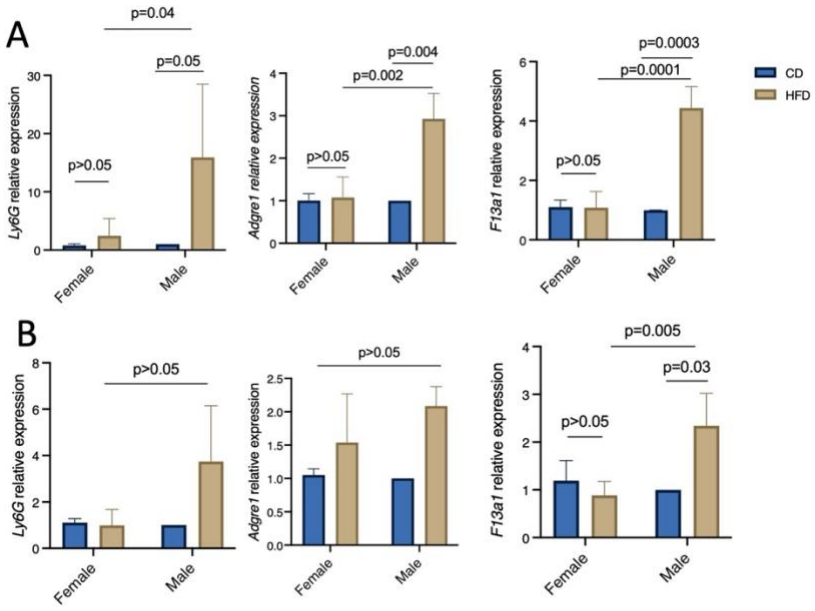

Figure 4.8. Increased expression of neutrophil, macrophage, and F13a1 markers in VAT of male mice after 7 days on a high-fat diet (HFD). A: Gene expression analysis of neutrophil (Ly6G), macrophage (Adgre1), and *F13a1* markers in visceral adipose tissue (VAT) and **B:** subcutaneous adipose tissue (SAT) of male and female C57bl/6 mice on diets. Analysis from three independent repeats shows that the levels of these markers are significantly higher in male mice compared to females on HFD. Each data point represents mean ± SEM, and p value <0.05 denotes statistical significance.

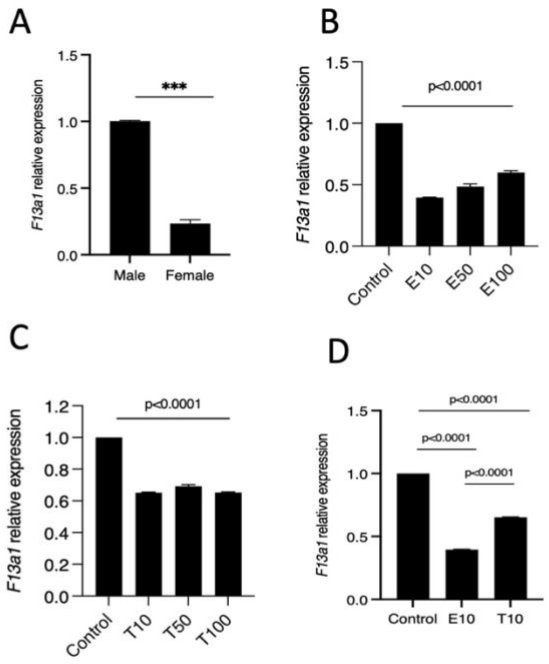

Figure 4.9. Sex-dependent expression of *F13a1* in mouse bone marrow neutrophils. A: mRNA gene expression analysis reveals a significantly higher level of *F13a1* expression in male neutrophils compared to females, showing an 80% reduction in females. **B-D:** Analysis of the effects of sex hormones on *F13a1* expression from three independent experiments indicates that both estrogen and testosterone reduce *F13a1* levels, with estrogen showing a greater reduction than testosterone. Each data point represents mean ± SEM, and p value <0.05 denotes statistical significance.

4.3. Unanswered questions, limitations and future directions

4.3.1. *In vitro* neutrophil study

In my thesis work, neutrophils were initially isolated from mouse bone marrow using a negative selection method. Although this method is effective for many applications, it may not offer the highest purity, which could potentially affect the outcome of my study. Therefore, an additional purification step was conducted using FACS method to enhance the data validity. While this approach addresses the purity concern to some extent, it has its own challenges. It is time-consuming and may affect the cell viability during the processing. Also, the Ly6G and CD11b markers used for gating are not entirely exclusive to neutrophils. Considering these factors,

employing advanced isolation methods like positive selection may improve both the purity and specificity of the neutrophil populations while minimizing cellular stress.

Moreover, I used neutrophils isolated from a mouse model due to their accessibility and similarity in many functional behaviours with human neutrophils. Despite these similarities, there are critical biological differences between mouse and human neutrophils in terms of morphology, protein markers and their frequency in circulation. For instance, Ly6G is expressed exclusively on mouse neutrophils, not on human ones. Also, mouse neutrophils lack defensin antimicrobial proteins [425, 426]. These distinctions could influence the outcomes of studies and limit the applicability of findings to human conditions. Therefore, the need for further studies using peripheral human neutrophils and neutrophils from adipose tissue is not just a suggestion, but a crucial step to address this limitation and validate such observations. Additionally, this can help to bridge the gap between my experimental findings and potential clinical applications.

4.3.2. Genetic mouse model

I aimed to explore the role of neutrophil-derived FXIII-A in metabolic health by utilizing the MRP8-Cre mouse model to selectively delete the *F13a1* gene in neutrophils. However, due to the significant confounding effects associated with this model, I could not conclude the impact of neutrophil FXIII-A on metabolic parameters. This significant limitation highlights the need to carefully consider off-target effects while using the genetic models. Future research should focus on developing more appropriate models for studying neutrophils without having potential confounding effects. In addition, to determine the individual contributions of FXIII-A and PAI-1 to metabolic phenotypes, including *F13a1*$^{-/-}$ and *Serpine1*$^{-/-}$ global knockout mice in such metabolic studies would be useful. Also, the involvement of a complete set of controls, including

both Cre-driver and Floxed mice, in the experimental framework is crucial to validate the outcomes.

4.3.3. Metabolic phenotyping

In my thesis, I utilized various experimental approaches, including weight monitoring, GTT, ITT, and the measurement of inflammatory and fibrosis markers in adipose tissue to assess the metabolic effects of HFD on mice with specific gene deletion. Future studies with specific deletion of *F13a1* in neutrophils should include metabolic cages, which allow for precise measurements of different parameters, including food intake, energy expenditure, and respiratory exchange ratios during HFD challenge. While GTT and ITT are valuable methods to assess acute glucose regulation, they do not represent whole-body insulin sensitivity. Other approaches, like the ELISA method are necessary to determine insulin concentrations in response to glucose injections. Additionally, analysis of insulin signalling pathways in all metabolic organs could provide deeper insight into the systemic effects of *F13a1* deletion in neutrophils on insulin sensitivity. Considering these, we would have a more definitive conclusion on the overall metabolic impacts of neutrophil-derived FXIII-A.

4.3.4. Sexual dimorphism

My preliminary data revealed a sexual dimorphism in *F13a1* expression and associated metabolic responses. Our investigation in this regard is still in its early stages, and further research should consider models with gonadectomies to determine the direct impact of sex hormones on *F13a1* expression and metabolic functions *in vivo*. Moreover, studying mechanistical signaling pathways

using specific agonists and antagonists would provide a deeper understanding of the mechanisms behind these observed sex differences.

4.3.5. V35L mutation

During my thesis work, I successfully established colonies of V35L mutant mice in homozygous and heterozygous forms and confirmed the mutation by genotyping. Preliminary data from these mice shows reduced FXIII-A activity and lower levels of macrophage infiltration (**Figure 4. 10**). This finding justifies numerous follow-up studies focused on metabolic health. Understanding the impact of this polymorphism, which is prevalent in humans (described on page 49), on overall metabolism could enhance our understanding of the variations in metabolic responses that cause serious metabolic dysfunctions or confer protection, such as in metabolically healthy obesity.

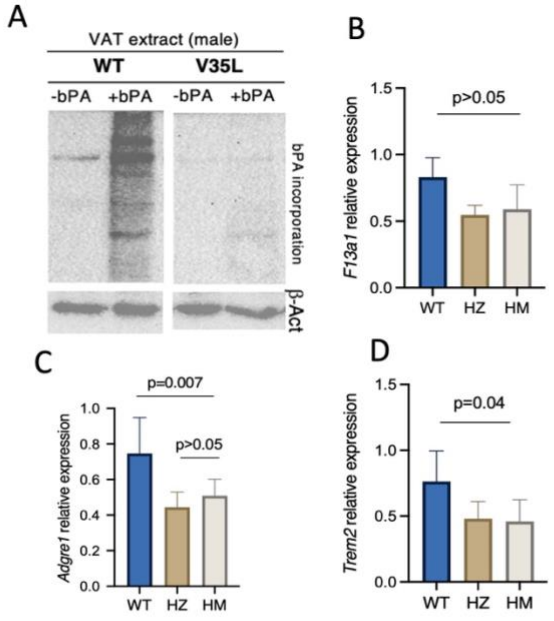

Figure 4.10. Reduced transglutaminase activity and macrophage markers in visceral adipose tissue (VAT) from V35L mutants compared to wild-type mice. A. *In vitro* TG activity assay of VAT extract, in V35L mutants, using biotin-pentylamine (bPA), shows decreased activity relative to wild-type. **B-D.** Gene expression analysis for *F13a1* and macrophage markers shows similar *F13a1* levels and reduced macrophage markers in mutants on a one- week high fat diet (HFD). Analyses were performed on samples from three independent experiments, each data point represents mean ± SEM, and p value <0.05 denotes statistical significance.

The presence of V34L mutation in one allele may also contribute to improved adipose tissue health. In addition, in the context of thrombosis, it is valuable to investigate the effects of neutrophil-derived FXIII-A from these mutants on the structure of fibrin networks. There is some evidence suggesting that the FXIII-A-subunit V34L variant influences thrombus crosslinking in murine thrombosis model [427]. Therefore, examining the impact of neutrophil-derived FXIII-A from these mutants on fibrin network structure is essential for a deeper understanding of its role in thrombosis.

4.3. Summary and Concluding remarks

In conclusion, my thesis project identified neutrophils as a novel source of FXIII-A and their involvement in mediating-fibrin interaction, which suggests its potential impact on metabolic health and thrombotic events. Understanding the various biological aspects of FXIII-A from different cellular sources helps us to develop targeted pharmacological therapeutics for managing obesity and its related complications without affecting FXIII-A's normal role in coagulation.

While my findings from animal model showed the effects of certain genes, *F13a1* or *Serpine 1* on metabolic regulation, the unexpected role of confounding factors, such as the deletion of *Serpine 1* in the MRP8-Cre model, underscores urgent need for selecting sophisticated controls and transgenic models. Overall, this project connects one of the main immune system's arms, i.e. neutrophils, to immunothrombotic events. Further research is required to uncover the definite effects of neutrophil FXIII-A in the context of obesity, metabolic disorders and thrombotic events.

1. Introduction and Discussion References

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2. APPENDIX

Table A3.1. The primers and PCR conditions used for genotyping of *F13a1*$^{-/-\ MRP8}$ mice

	Primer sequences	PCR conditions	Expected band sizes
Flox	F: TCTGGGCCAAACCAAGTACCTGG R: CAAGACCAGACTGTGCAAAGGG	94°C x 2 min, 94°C x 15 sec, 63°C x 20 sec, 72°C x 45 sec, back to step 2 x 29 times, 72°C x 5 minutes, 4°C hold	FLOX: 177 bp Wild type: 126 bp
MRP8-Cre	F: GCGGTCTGGCAGTAA AAACTATC(Cre) R: GTGAAACAGCATTGCTGTCACTT(Cre) F: CTAGGCCACAGA ATTGAA AGATCT (int) R: GTAGGTGGA AATTCTAGCATCATCC (int)	94°C x 2 min, 94°C x 20 sec, 65°C x 15 sec, 68°C x 10 sec, back to step 2 x 10 times, 94°C x 15 sec, 60 °C x 15 sec, 72 °C x 15 sec, back to step 6 x 28, 72°C x 2 minutes, 4°C hold	Knockout: 100 bp Internal positive control: 324 bp
F13a1	F: CAGTTCGAAGACGGCATCCT R: AACAAGATCACTGTTGACCTCT	94°C x 2 min, 94°C x 15 sec, 63°C x 20 sec, 72°C x 45 sec, back to step 2 x 29 times, 72°C x 5 minutes, 4°C hold	Knockout: 498 bp Wild type: 636 bp

2.1. Methods

2.1.1. Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

To determine the effect of diets on the glucose metabolism and insulin sensitivity, GTT and ITT were performed after 16 weeks on each diet. For GTT, mice were fasted for 6 h and then a glucose solution (2 g/kg body weight), prepared in sterile normal saline was administered intraperitoneally. The blood glucose levels were measured at 0, 15-, 30-, 60-, and 120-minutes post-injection using the FreeStyle glucometer (Abbott Laboratories, Chicago, IL, USA). Data were graphed to visually compare the glucose responses between groups, and the area under the curve (AUC) for glucose levels over the 120-minute period was calculated using GraphPad prism. The higher AUC represents the lower clearance of the administrated glucose.

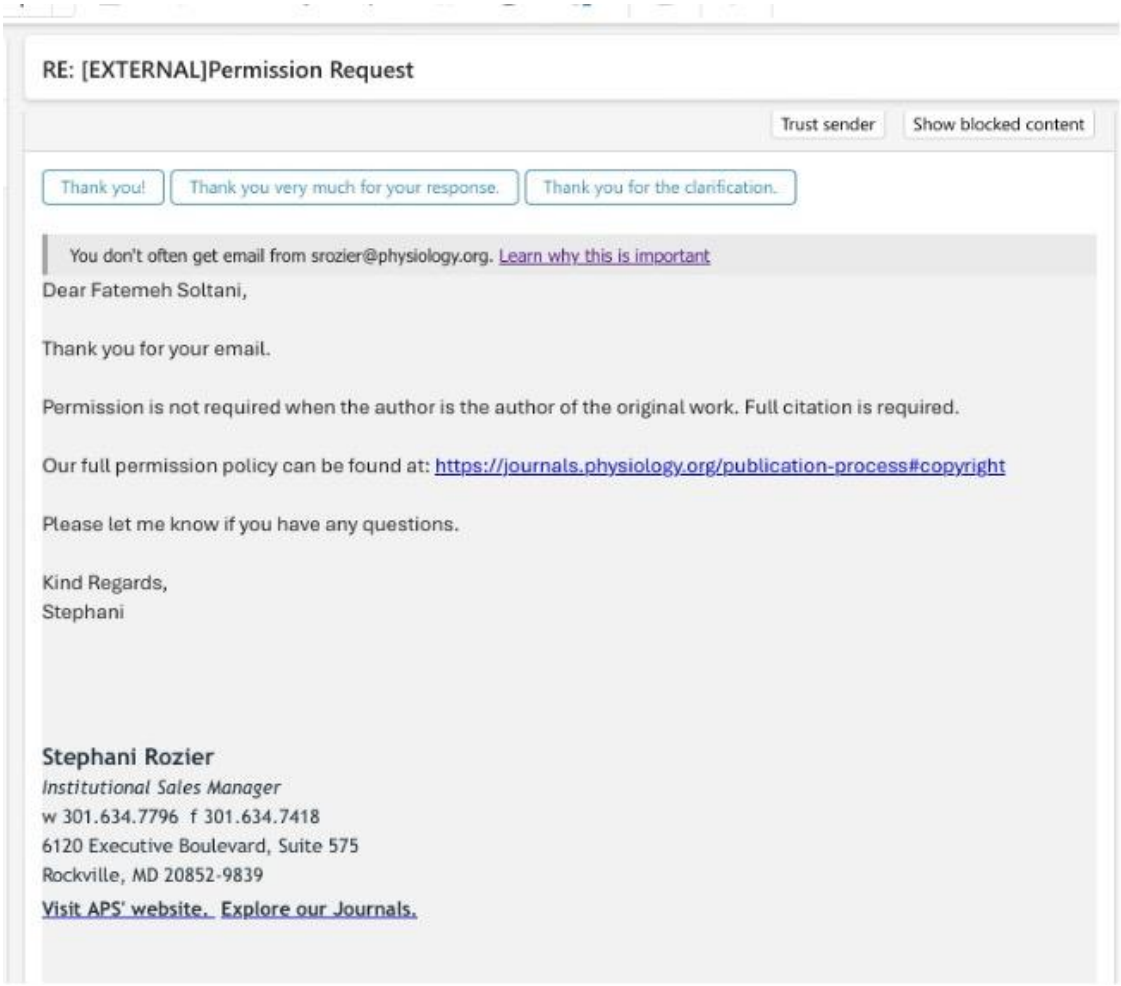
One week after conducting GTT, an ITT was conducted to determine the level of insulin resistance. For this, mice were fasted for 6 hours followed by receiving an intraperitoneal injection of insulin

(0.5 U/kg body weight). Insulin was diluted in sterile saline solution and was prepared fresh. Blood glucose levels were determined at the same time points as in the GTT, following the same procedure. Data were graphed for comparison and the AUC for glucose levels over the 120-minute period was calculated using GraphPad prism. The higher AUC represents the lower insulin sensitivity.

2.1.2. Immunohistochemistry (IHC)

Sectioned formalin-fixed paraffin-embedded tissues were studied using a standard IHC protocol. Briefly, paraffin sections were baked at 65 ºC for one hour, followed by deparaffinization with xylene, a descending ethanol dilution series, and then distilled water. Antigen retrieval was performed using Protease XXIV at concentration of 10ug/mL for 5 min at room temperature. After washing with TBST, blocking was conducted with 2% BSA in TBS for 1 hour at room temperature. Specific primary antibodies targeting fibrin(ogen) and F4/80, as markers of fibrosis and macrophage, respectively, were applied and incubated overnight at 4 ºC in a humidity chamber. This was followed by incubation with fluorescent- labelled secondary antibodies for 1 hour at room temperature. Nuclei were stained with DAPI at 1:7000 dilution for 5 min followed by mounting with Vectashield (Vector labs). Slides were visualized under the Zeiss Axioscope 5 fluorescence microscope at 20X. Cell sizing was determined using ImageJ 1.8.0 software (National Institutes of Health, USA).

2.2. Copyright approval



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