THE ROLE OF REELIN IN HUMAN NEUTROPHIL PEPTIDE-INDUCED ENDOTHELIAL DYSFUNCTION AND PLATELET AGGREGATION

by

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The role of reelin in human neutrophil peptide-induced endothelial dysfunction and platelet aggregation

**Introduction:** Human neutrophil peptides (HNP) may play an important role in the pathogenesis of atherosclerosis. We have shown that HNP-induced platelet aggregation was abolished in the absence of low density lipoprotein-related protein-8 (LRP8), a receptor for the extracellular matrix protein reelin.

**Hypothesis:** The HNP-induced endothelial dysfunction and platelet aggregation is mediated by reelin.

**Methods:** Human coronary artery endothelial cells (HCAEC) were stimulated with HNP to assess reelin protein expression and release. A reelin neutralizing antibody was used to assess endothelial function and platelet aggregation after HNP stimulation.

**Results:** HNP induced reelin protein expression and release in HCAEC. The stimulation of recombinant mouse reelin resulted in oxidative stress and decreased endothelial nitric oxide synthase in HCAEC, and platelet aggregation in human platelets. The HNP-induced endothelial dysfunction and platelet responses were attenuated by using a reelin neutralizing antibody.

**Conclusion:** HNP induce endothelial dysfunction and platelet aggregation mediated by the reelin-LRP8 signal pathway.
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LIST OF ABBREVIATIONS

$\alpha_1$-PI - $\alpha_1$ Protease Inhibitor
ADMA – Asymmetric Dimethyl-Arginine
ADP – Adenosine Diphosphate
ATP – Adenosine Triphosphate
ANOVA – Analysis of Variance
APC – Anaphase Promoting Complex
Apo – Apolipoprotein
Bcl-2 – B-Cell Lymphoma 2
BH$_4$ - (6R)-5,6,7,8-tetrahydrobiopterin
BLAST – Basic Local Alignment Search Tool
BSA – Bovine Serum Albumin
Ca$^{2+}$ - Calcium Ion
CAD – Carotid Artery Disease
CaM – Calmodulin
cAMP – Cyclic Adenosine Monophosphate
CD – Cluster of Differentiation
cGMP – Cyclic Guanosine Monophosphate
CRP – C-Reactive Protein
COX-1 – Cyclooxygenase-1
CXC – A chemokine with the first two amino cysteine residues separated by an amino acid (X)
DTS – Dense Tubular System
EDCF – Endothelial-Derived Constriction Factor
EDHF – Endothelial-Derived Hyperpolarizing Factor
ELISA – Enzyme-Linked Immunosorbent Assay
eNOS – Endothelial Nitric Oxide Synthase (NOS III)
E-Selectin – Endothelial Selectin
ET-1 – Endothelin-1
FAD – Flavin Adenine Dinucleotide
Fe - Iron
GAPDH - Glyceraldehyde 3-Phosphate Dehydrogenase
GC – Guanylyl Cyclase
GDP – Guanosine Diphosphate
GM-CSF – Granulocyte/Macrophage Colony Stimulating Factor
GP – Glycoprotein
GTP – Guanosine Triphosphate
HCAEC – Human Coronary Artery Endothelial Cells
hBD – Human β Defensin
HBSS – Hank’s Balanced Salt Solution
HDL – High-Density Lipoprotein
HNP – Human Neutrophil Peptides
ICAM-1 – Intercellular Adhesion Molecule-1
IgG – Immunoglobulin G
IκBα - Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells Inhibitor α
IL- Interleukin
iNOS – Inducible Nitric Oxide Synthase (NOS II)
IVM – Intravital Microscopy
JAM-3 – Junction Adhesion Molecule-3

JNK - c-Jun N-terminal kinases

K⁺ - Potassium Ion

LDL – Low-Density Lipoprotein

LDLR – Low-Density Lipoprotein Receptor

Lp(a) – Lipoprotein (a)

LRs – LDL Receptor-Related Protein Receptor family members

LRP8 – LDL Receptor-Related Protein 8 (ApoER2; Apolipoprotein E Receptor 2)

MAC-1 – Macrophage Antigen-1

MCP-1 – Monocyte Chemotactic Protein-1

Mg²⁺ - Magnesium Ion

MMP – Matrix metalloproteinase

mRNA – Messenger Ribonucleic Acid

mrRLN – Mouse Recombinant Reelin

Na⁺ - Sodium Ion

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

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

NO – Nitric Oxide

NOHA - N-hydroxyl-L-arginine

NOS – Nitric Oxide Synthase

nNOS – Neuronal Nitric Oxide Synthase (NOS I)

ONOO⁻ - Peroxynitrite (PN)

oxLDL – Oxidized LDL

p38MAPK – p38 Mitogen-Activated Protein Kinase
PAF – Platelet Activating Factor
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PF4 – Platelet Factor 4
PGH₂ – Endoperoxide
PGI₂ – Prostacyclin
PIP₂ – Phosphatidylinositol 4,5 Biphosphate
PKC – Protein Kinase C
PKG – cGMP-Dependent Kinase
PLA₂ – Phospholipase A₂
PMN – Polymorphonuclear Neutrophils
PPP – Platelet Poor Plasma
PRP – Platelet Rich Plasma
P-Selectin – Platelet-Selectin
PSGL-1 - P-selectin glycoprotein ligand-1
RANTES - Regulated on Activation, Normal T Cell Expressed And Secreted
RAP – Receptor Associated Protein
RLN - Reelin
RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species
RT – Room Temperature
SERCA - Sarco/Endoplasmic Reticulum Ca²⁺- ATP Monophosphatase (ATPase)
SNAP - Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein
SNARE - Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein Receptor
SR-A – Scavenger Receptor A
STEMI - ST-Segment Elevation Myocardial Infarction

TNF-α - Tumour Necrosis Factor - α

tPA – Tissue-Type Plasminogen Activator

TxA₂ – Thromboxane A₂

Tyr - Tyrosine

VCAM-1 – Vascular Cell Adhesion Molecule-1

VSMC – Vascular Smooth Muscle Cell

vWF – von Willebrand Factor
Chapter 1: Introduction

1.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the large elastic and muscular arteries, that develops over time to become coronary artery diseases and stroke, diseases which account for 50% of all deaths in westernized societies [1]. The epidemiology of the disease encompasses a coupling of environmental and genetic risk factors, and a complex cascade of cellular events. The elements of atherosclerosis is commonly characterized by sub-endothelial accumulation of cholesterol and lipid-engorged foam cells, leukocyte trafficking from the lumen into the vessel intima, vascular smooth muscle cell (VSMC) migration from the tunica media into the tunica intima, platelet activation and aggregation, T-cell activation, and production of platelet- and leukocyte-derived cytokines and chemokines [1-5]. Unfortunately, these events only unfold silently with time as the disease develops in the inner lining of the arteries. Initially, the buildup of cholesterol-rich lipids in arteries, known as a fatty streak, can be found as early as the first decade of life [1]. These lesions are precursors to the development of atheromas, a more advanced lesion characterized by the presence of lipid-rich necrotic debris perturbed with uncontrolled inflammatory events such as leukocyte recruitment, foam-cell formation, and cytokine and chemokine production. The proliferative environment subsequently stimulates migrated VSMC to produce extracellular matrix molecules, such as interstitial collagen and elastin, and form a fibrous cap to enclose the plaque [1, 6-9]. The growing plaque, that is continuously fuelled by uncontrolled inflammation and apoptosis, can cause clinical complications when 1) it produces a flow-restricting stenosis that causes tissues ischemia; and/or 2) its fibrous cap ruptures, due to enzymatic activity by collagenolytic enzymes and metalloproteinases, to form thrombi that embolize and lodge in distal arteries that causes thrombotic complications [9]. It is upon plaque rupture and
thrombosis that the condition finally manifests itself clinically as acute coronary syndrome, myocardial infarction or stroke.

Despite the medical advances present today, on a global scale, cardiovascular disease accounts for 1 in 3 deaths, and is projected to be the single leading cause of death by 2030 [10]. Owing to its overwhelming burden medically and economically, extensive clinical and laboratory research has been conducted to elucidate the pathophysiology of atherosclerosis for both preventive and precautionary purposes.

1.1.1 Pathophysiology of atherosclerosis

Until the 1970s, atherosclerosis was predominantly viewed as a lipid-retention disease [11]. However, research over the last four decades has increasingly elucidated the significant role of neutrophils, monocytes and leukocytes to the pathogenesis of atherosclerosis. To date, over 10,000 peer-reviewed articles have been published under the US National Library of Medicine that documented the inflammatory events that transpire in the disturbed vascular equilibrium precipitated in atherosclerosis.

1.1.2 Atherosclerosis as an inflammatory disease

The view of atherosclerosis as a chronic inflammatory disease originated from the crucial observation that different subsets of leukocytes were found in atherosclerotic lesions at various stages of atherogenesis [12]. The individual leukocytes, such as monocytes/macrophages, T-cells, mast cells and dendritic cells, and their roles in contributing to atherosclerosis were well reviewed in 2008 by Weber et al published in Nature [5].
Under homeostatic circumstances, the endothelial monolayer resists the firm adhesion and recruitment of circulating leukocytes. However, at certain arterial sites with decreased shear stress and increased turbulence [13], specific adhesion molecules from the endothelium are expressed, and are responsible for the capture and migration of circulating leukocytes such as monocytes and T-cells [3, 5, 13]. These adhesion molecules acts as receptors for the integrins found on the surface membrane of monocytes and T-cells. Once adhered to the endothelial cells, leukocytes enter into the intima by diapedesis between the endothelial junctions [13, 14].

The chemotactic migration of circulating leukocytes may be accounted for by the presence of chemokines such as MCP-1, INF-γ and eotaxin [13]. Once resident in the arterial intima, activated leukocytes amplify receptors that bind to chemoattractant molecules, which further activates the cells to induce cell proliferation, and inflammation at the site of lesion. For instance, monocytes proliferate to become macrophages, which encourage the cell to generate cytokines (such as TNF-α, IL-1 and TGF-β), proteolytic enzymes (such as MMPs), and growth factors (such as PDGF) that could further fuel the inflammatory responses at the lesion site [15]. In addition, activated macrophages are known to express class II histocompatibility antigens that permit them to present antigens to T-cells [13-15].

Since both CD4+ and CD8+ T-cells are present at the lesion site, they too can become activated, which results in the additional generation and secretion of cytokines such as INF-γ, TNF-α and -β [15]. A similar T-cell activation can be elicited by dendritic cells (DC) since DCs are also antigen-presenting cells [15]. In addition to supplying the intima with cytokines and chemokines, leukocytes such as mast cells are equipped with high contents of proteases such as tryptase and chymase [1, 3, 5, 15], which can destabilize the atherosclerotic plaque, subjecting the plaque to increased susceptibility to rupture. Therefore, the cascade of
inflammatory responses collectively further endorses the recruitment of circulating leukocytes, which can ultimately destabilize the atherosclerotic plaque.

1.1.2.1 Foam Cells

Monocytes represent an essential cellular component of the host defense system, particularly in the native and acquired immunity [16]. By recognizing specific molecular patterns and interacting with specific receptors, monocytes can mediate phagocytosis and various protein expressions. Although these functions were evolved to protect the host from microbial infection, however, these mechanisms also inadvertently contribute to the development of atherosclerosis.

Monocyte recruitment begins with the attraction to sites of injury by the presence of pro-inflammatory molecules such as monocyte chemotactic protein (MCP-1), macrophage colony stimulating factor (M-CSF), TNF-α, endothelin-1 (ET-1), granulocyte/macrophage colony stimulating factor (GM-CSF) and transforming growth factor-β (TGF-β) [16-20]. Using cell adhesion molecules expressed on activated endothelial cells, monocytes roll, adhere and migrate across the endothelium barrier under the influence of chemoattractant protein [16]. Once in the subendothelial space, monocytes proliferate and differentiate into macrophages with high expressions of scavenger receptors such as CD36 and CD68 [21, 22]. This class of proteins recognizes polyanionic macromolecules such as phosphatidylserine and oxidized lipoproteins to serve as a mechanism for lipid clearance [16]. Unfortunately, the continual uptake of oxidized LDL leads to the formation of large lipid-engorged macrophages known as foam cells [16]. Consequently, foam cells can 1) release cytokines and chemokines to further fuel the inflammatory responses; and 2) release metalloproteinases that can destabilize the
plaque and cause a rupture, which leads to the clinical complication of myocardial infarction or stroke [16].

1.1.2.2 **Endothelial dysfunction**

1.1.2.2.1 **Endothelial cell in vascular homeostasis**

The endothelium is a major regulator of vascular homeostasis by exerting vasoprotective effects such as vasodilation, inhibition of VSMC migration and proliferation, and suppression of inflammatory events [23-25]. Strategically situated as the interface between the vascular lumen and vessel body, it is optimally placed to respond to both physical and chemical signals that regulate cellular adhesion, thrombogenesis and fibrinolysis, VSMC proliferation and vascular inflammation [25]. The importance of the endothelium was first appreciated through its role in maintaining vascular tone, which is achieved through the production and release of vasoactive molecules that would relax or constrict the vessel [25, 26]. Several endothelial-derived vasodilating molecules have been documented including prostacyclin (PGI$_2$), endothelial-derived hyperpolarizing factor (EDHF), and nitric oxide (NO) [23, 27].

Briefly, prostacyclin is a major product derived from the activities of vascular cyclooxygenase that is primarily formed in response to shear stress, bradykinin, and hypoxia [24]. This endothelium-derived arachidonic acid metabolite has previously been shown to cause VSMC relaxation by activating adenylate cyclase [28], and inhibit platelet aggregation by diminishing fibrinogen receptor and P-selectin expression [29, 30]. Now, in addition to PGI$_2$, EDHF, a product of the phospholipase A$_2$-dependent arachidonic acid metabolism [31], also plays an important role in inducing endothelium-dependent relaxation. In response to extracellular mediators such as bradykinin and acetylcholine, EDHF mediates VSMC relaxation by activating potassium (K$^+$) channels to allow K$^+$ efflux and membrane hyperpolarization [31, 32]. Finally, due to NO’s pivotal role in governing endothelial-dependent vasodilation, I will
further expand on the molecule’s role and production later in the thesis. Interestingly, under certain conditions, such as the presence of cytokines, thrombin, and serotonin, the endothelium may begin to produce endothelial-derived constriction factors (EDCF) such as endothelin-1 (ET-1), angiotensin II, superoxide anions, endoperoxide (PGH₂) and thromboxane A₂ [24, 33-36]. One of the common pathways in which EDCF induces VSMC constriction is by mobilizing Ca²⁺ influx to increase the intracellular Ca²⁺ content and mediate vascular muscle contraction [37].

1.1.2.2.2 Nitric oxide

1.1.2.2.2.1 Nitric oxide synthase

Now, the chief regulator of vasodilation is nitric oxide (NO). Its vasodilating properties were first demonstrated by experiments conducted by Furchgott and Zawadzki in 1980. In their study, rabbit aorta with intact endothelium was relaxed upon the treatment of acetylcholine, but was found to constrict in aortas with denuded endothelium [38]. The culprit responsible for acetylcholine-mediated relaxation was later found to be nitric oxide [39, 40]. NO is principally derived from L-arginine by the enzymatic action of a family known as nitric oxide synthase (NOS). Currently, there are three known isoforms that can synthesize NO: neuronal NOS (nNOS, also known as NOS I), endothelial NOS (eNOS, also known as NOS II), and inducible NOS (iNOS, also commonly known as NOS II). Numerous studies have shown a similarity in three-dimensional crystallographic structure shared by all three isoforms: a flavin-containing reductase and an iron protoporphyrin IX (heme)-containing oxygenase domain, bridged by a flexible and calmodulin (CaM)-containing protein strand [41-44]. In addition to the dimeric structure, there are tightly-bound cofactors required for the proper functioning of the enzyme: (6R)-5,6,7,8-tetrahydrobiopterin (BH₄), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH) [23, 41]. The generation of NO begins
with the electron donation of NAPDH to the reductase domain of the enzyme and transfer, via FAD redox carrier, to the oxygenase domain. There, the electrons interact with the heme-containing BH₄ to catalyze the reaction of oxygen with L-arginine to generate an intermediate species called \(N\)-hydroxyl-L-arginine (NOHA), prior to the final oxidation to citrulline and NO [41, 45, 46]. However, even though the three isoforms share a protein structure, the proteins also contain distinct differences in expression among each other. Firstly, the three isoforms’ expression varies across different tissues. nNOS is predominately found in neuronal tissues; eNOS is found in vascular endothelial cells; and iNOS is found in a wide range of cells and tissues such as macrophages and endothelial cells [41]. Secondly, the genes governing the expression of the proteins are located on different chromosomes. nNOS is found on 12q24.2-12q24.3 of chromosome 12; eNOS is found on 17cen-q11.2 of chromosome 17; and iNOS is found on 7q35-7q36 of chromosome 7 [41]. Finally, while eNOS and nNOS are \(Ca^{2+}\)-dependent enzymes, iNOS is an inducible \(Ca^{2+}\)-independent enzyme that is often up regulated by the presence of cytokines to generate 100-1000-fold more NO than its constitutive counterparts [46-49]. Paradoxically, high content of NO can begin to lose its anti-proliferative properties, which will be elaborated further later on [50].

1.1.2.2.2 Nitrix oxide as an anti-inflammatory molecule

As a gaseous molecule, NO released from the endothelium is able to diffuse across neighbouring cell’s plasma membrane and bind to its intracellular receptor: soluble guanylyl cyclase (GC). By binding onto the heme prosthetic group on GC \(\beta1\) subunit, NO triggers a conformational change that increases the catalysis of cyclic guanosine monophosphate (cGMP) synthesis by several hundred-folds [51-54]. Depending on the cell stimulated, NO-mediated cGMP production has many implications. For instance, in VSMCs, an increase in
cGMP has previously been shown to activate cGMP-dependent kinases (PKG), which can promote SERCA activity and inhibit L-type Ca\(^{2+}\) channels and Na\(^{+}/Ca^{2+}\) exchange, thus resulting in a decrease in intracellular Ca\(^{2+}\) content and VSMC relaxation \([55-58]\). Similarly, in platelets, a rise in cGMP potently inhibited the activation of thromboxane A\(_2\) receptor by phosphorylating the carboxyl terminus of the receptor, rendering it inactive for platelet activation and aggregation \([59]\). Additionally, NO has previously been shown to stimulate other pathways that resulted in an inhibition of inflammatory responses. For instance, NO was previously shown to inhibit VSMC proliferation by increasing the ubiquitination and degradation of UbcH10, an enzyme known to interact with the anaphase promoting complex (APC) \([60]\). In a study conducted by Spiecker \textit{et al}, it was demonstrated that NO was able to inhibit TNF-\(\alpha\)-induced NF-\(\kappa\)B activation in endothelial cells by increasing the expression and nuclear translocation of the NF-\(\kappa\)B inhibitor, \(I\kappaB\alpha\). Interestingly, this was also correlated with a reduction in vascular cell adhesion molecule-1 (VCAM-1) expression \([61]\), a crucial molecule involved in capturing and recruiting leukocytes for migration into the subendothelial compartment \([3, 62]\). Congruently, a study by De Caterina \textit{et al} showed similar results when IL-1\(\alpha\)-induced VCAM-1 expression in endothelial cells was significantly inhibited in the presence of NO. Not only did this resulted in a reduction in monocyte adhesion to the endothelial monolayer, but also in a diminished production of cytokine levels as assessed by IL-6 and IL-8 concentrations \([63]\). Collectively, it becomes evident that NO plays a pivotal role in regulating vascular homeostasis, and that the impairment of production or activity of NO leads to a loss of endothelium-dependent vasodilation, and a gain in pro-inflammatory, proliferative and pro-coagulatory environment conditioned for the development of atherosclerosis \([64]\). Such environment is the hallmark feature of endothelial activation, which
eventually leads to endothelial dysfunction [65]. Now, it is here that I would like to address the difference between endothelial activation and endothelial dysfunction.

### 1.1.2.2.3 Endothelial dysfunction

The most recent definition of endothelial activation was illustrated in the *Journal of Clinical Investigation*, where it defined the phenomenon as “the endothelial expression of cell-surface adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin...induced by proinflammatory cytokines... (that) facilitates the recruitment and attachment of circulating leukocytes to the vessel wall” [66]. Therefore, endothelial activation is the transformation of endothelial cells from a quiescent phenotype to one that involves in a host defense response [25]. In contrast, endothelial dysfunction is characterized by the reduction of NO bioavailability [65, 66]. Endothelial activation can lead to endothelial dysfunction, however, it is not uncommon for some papers use the two terminologies interchangeably. In order to avoid confusion, this thesis will regard the two events separately.

Interestingly, over the past 30 years, a growing body of evidence has suggested that atherosclerosis begins with the disruption of vascular endothelium homeostasis, with endothelial dysfunction as the pioneer event [25]. It has been suggested that it is the imbalance between vessel dilation and constriction by NO that compels the pathophysiology in atherosclerosis, including endothelial permeability, leukocyte recruitment, platelet aggregation and cytokine production [67]. Remarkably, endothelial dysfunction has recently been used clinically as a diagnostic marker of early atherosclerosis.

### 1.1.2.2.4 Endothelial dysfunction assessment
A common and non-invasive way of assessing endothelial function is by quantifying the diameter of coronary arteries before and after infusion of acetylcholine by angiography [68, 69]. As healthy coronary arteries are expected to respond by vasodilation, patients with endothelial dysfunction would exhibit vasoconstriction [23]. Similarly, venous occlusion plethysmography can also been used to measure vascular responses after infusion of acetylcholine into the brachial artery [70]. Alternatively, endothelial dysfunction can also be measured by high-resolution ultrasound to assess the brachial arterial diameter in response to reactive hyperemia [70]. Lastly, due to its inverse correlation to endothelial function, serum C-reactive protein (CRP) has also been used to indirectly assess endothelial impairment [71].

1.1.2.2.5 Nitric oxide bioactivity vs. bioavailability.

The primary driving force for endothelial dysfunction is the net reduction of NO bioavailability [23, 65, 66, 72]. The mechanisms of diminished NO bioavailability is variable: from a reduction in eNOS protein expression due to oscillatory shear stress [73, 74], or a reduction in the eNOS cofactor BH₄ [75], to an elevation in eNOS inhibitors such as asymmetric dimethylarginine (ADMA) [76]. However, this is not to be confused with NO bioactivity. In a study conducted by Minor et al, it was found that rabbits fed on a high cholesterol diet for 6 months exhibited an increase in NO, but an impairment of endothelial-dependent relaxation [77].

This counter-protective model of NO was first proposed by Stuehr et al when the group discovered two different catalytic pathways of iNOS [78, 79]. Prior to being surrendered from the oxygenase domain of iNOS, NO is bound to the Fe³⁺-containing heme, a composite known as the Fe³⁺-NO complex [50]. The Fe³⁺-NO complex could either: a) release the NO, or b) act as a nucleophile to attack oxygen and generate the reactive nitrogen species (RNS), peroxynitrite (PN or ONOO⁻) [50]. Peroxynitrite is a potent cytotoxic and pro-inflammatory
molecule works to nitrosylate the aromatic ring of tyrosine residues on proteins to form 3-nitrotyrosine, which modifies protein functional activities, and drives reactive oxygen species production [4, 80-84]. Previous studies have observed that nitrosylation of residue 385 (Tyr385) on the catalytic site of cyclooxygenase (COX) in mice and human atherosclerotic lesions resulted in an interference of the enzymatic reaction of arachidonic acid to prostaglandins [85-87]. The underlying NOS that governed the NO-derived PN was later found to be iNOS [85]. This notion was further supported by in vivo studies conducted by Upmacis et al, showing attenuation of protein nitrosylation of the heart, lungs and liver in diet-induced atherosclerotic mice lacking iNOS expression [88]. As a consequence of the accumulation of RNS, the LDL in the environment undergoes accelerated oxidation to form oxidative LDL (oxLDL), a modified LDL that promotes foam cell formation and plaque destabilization [89]. Therefore, it is crucial to appreciate the importance of diminished bioavailability and bioactivity of NO in contributing to endothelial dysfunction, which then initiates the inflammatory cascade of events observed in atherosclerosis.

1.1.2.3 Platelets and platelet aggregation

1.1.2.3.1 Platelets

As early as 1874, microscopist Dr. William Osler made a pioneering discovery of a disk-shaped unit, which he coined the term “the third corpuscle” or “blood plates”, that forms “irregular masses of colorless globules” upon bleeding [90]. Today, we recognize this discoid unit as the anuclear fragments shed from bone-marrow derived megakaryotypes [91]. During the terminal differentiation phase of thrombopoiesis, megakaryocytes form proplatelet extensions that are eventually shed as platelets [92]. Constituting a dimension of 2.0-µm in length, and 0.5-µm in thickness, platelets are one of the smallest circulating cell-types in the
blood [93]. Although lacking a nucleus, platelets are equipped with protein-synthesizing machineries, including messenger RNA (mRNA) and translational proteins [94]. Their short lifespan of 8-10 days is governed by an “internal clock” based on the activities of anti- and pro-apoptotic Bcl-2 family proteins [95]. With a normal count of 150-400 x 10^9 platelets/L in humans [96], they play a vital role in the integrity and homeostasis of vascular thrombosis. Upon an appropriate signal, platelets’ prime directive is to rapidly form platelet-rich thrombi to stop vascular bleeding [97, 98]. Nonetheless, beyond the apparent role in hemostasis and thrombosis, platelets are also considered to play a significant role in facilitating inflammatory responses by recruiting a subset of leukocytes (monocytes, neutrophils, dendritic cells, and T-cells) to the lesion site, and releasing inflammatory mediators into the lesion milieu [99]. It is their inherent inflammatory nature that recognizes them as atherosclerosis promoters.

1.1.2.3.2 Platelet activation and aggregation

Under normal conditions, platelets circulate in a quiescent state, characterized by the lack of platelet-selectin (P-selectin) expression, an integral membrane glycoprotein responsible for leukocyte recruitment [99]. Upon exposure to a stimulus, platelets undergo three distinct phases: 1) platelet tethering, activation and adhesion, 2) platelet aggregation and recruitment, and 3) thrombus stabilization [100]. Platelet tethering is first achieved by the expression and binding of glycoprotein (GP) Ibα to the collagen-immobilized protein, von Willebrand factor (vWF) [101]. The security of the adherence is then further supported by the expression of integrin receptors αIIbβ3 (GP IIb/IIIa) and α2β1 (GP Ib/IIa) upon platelet GPVI interaction with collagen [102-105]. Once firmly attached, platelets undergo rapid morphology changes from a disk-like shape to a sphere with pseudopodia extensions [106]. Subsequently, platelets release their granular contents composed of platelet agonists, such as ADP and TXA2, that
act in a paracrine and positive feedback loop that amplifies platelet activation, and assist with platelet aggregation by promoting the conformational change of $\alpha_{\text{IIB}}\beta_3$ integrin into its active form [107]. Active $\alpha_{\text{IIB}}\beta_3$ is essential for the bridging of activated platelets with fibrinogen, vWF and fibronectin in order to form platelet aggregates [108]. These platelet aggregates are then stabilized by the presence of fibrin converted from fibrinogen by thrombin to form a stable thrombus [109].

1.1.2.3.3 Platelet granules

Platelets are highly secretory cells, with over 300 distinct molecules organized and packaged into three main organelles: dense granules, $\alpha$-granules and lysosomes [107, 110]. Firstly, dense granules are small granules, with a diameter of 150-nm, containing pro-aggregating factors including nucleotides (ATP, ADP, GTP, GDP), amines (serotonin 5-HT, histamine) and $\text{Ca}^{2+}$ [107]. Their opaque dense cores are surrounded by a single membrane that is easily recognizable under an electron microscope [111]. Secondly, $\alpha$-granules are large spherical or ovoid organelles, with a diameter of 200-400-nm, that houses a pool of platelet coagulant factors, fibrinolysis mediators, adhesive glycoproteins, protease inhibitors, and other immunological proteins [107]. They represent the majority of granule population in both size and number in platelets [107]. Finally, the third category of granules concerns the acidic compartment, lysosomes. Measuring in between the dense and $\alpha$-granules in size, at 175-250-nm, the lysosome carries a variety of proteases, cathepsins, and glycohydrolases with bactericidal activity [107]. Degranulation of these compartments occurs once a platelet agonist, such as thrombin, collagen and thromboxane $A_2$ (TXA$_2$), binds to its corresponding receptor and initiate a signal transduction for platelet activation [110]. Following activation, $\text{Ca}^{2+}$ is moved from the platelet dense tubular system (DTS) into the cytosol, which results in
an increase in intracellular \( \text{Ca}^{2+} \) from an undetectable concentration of 100-nM to 2-10-\( \mu \text{M} \).

This results in: 1) the movement of granules confined within a bundle of microtubules to the membranes, and 2) dock and fusion of granular membrane with platelet plasma membrane to unload its contents by a SNARE- and SNAP- dependent exocytosis [112].

### 1.1.2.3.4 Platelets in Atherosclerosis

As early as the 1960s, studies have shown the presence of platelets at sites of inflammation and endothelial injury [113]. A growing body of evidence now suggests their crucial role in the development of atherosclerotic lesions. Fundamentally, platelets are able to adhere to intact endothelial cells both in vitro and in vivo, even in the absence of endothelial lesion [113-115], thereby resulting in platelet activation and release of platelet-derived mediators. Consequently, the platelet-derived mediators have the potential of recruiting leukocytes by eliciting chemokine production and adhesion molecule expression. For instance, among the platelet-derived mediators released is interleukin-1\( \beta \), a potent inflammatory cytokine known to activate endothelial cells to induce intracellular adhesion molecule-1 (ICAM-1) expression and cytokine production, particularly IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF) [116]. These cytokines are known to facilitate neutrophil and monocyte recruitment [116]. In addition, another very potent mediator released by platelets is RANTES, a chemokine known to attract monocytes and promote monocyte-endothelial interactions [117]. Finally, platelet factor 4 (PF4) is a CXC chemokine found in the \( \alpha \)-granules of platelets at high concentrations of 1500-\( \mu \text{g/mL} \) [118]. Upon binding to low-density lipoprotein related protein (LRP) on endothelial cells, PF4 has previously been shown to induce endothelial NF-\( \kappa \text{B} \) activity, which resulted in an increase in expression of endothelial (E)-selectin, an endothelial-leukocyte adhesion molecule involved in leukocyte recruitment [118]. Moreover, the same
PF4 has also been demonstrated to promote the binding of oxidized low-density lipoprotein (oxLDL) to endothelial cells and macrophages, which further endorses the development of a fatty streak in atherosclerosis [119]. Similarly, in a study conducted by Daub et al, it was shown that platelets were able to bind to LDL to form an LDL-platelet complex that could be phagocytosed by macrophages to promote the formation of lipid-engorged foam cells [120]. Interestingly, secondary capture by platelets have also been observed to facilitate leukocyte recruitment to the endothelial vessel wall. Activated and endothelium-adhered platelets express a wide array of leukocyte-binding receptors including P-selectin, GPIbα and JAM-3, which can bind to their receptor counterparts PSGL-1 and MAC-1 expressed in leukocytes [113]. By capturing the circulating leukocytes, platelets are inherently contributing to leukocyte recruitment that can augment the inflammatory responses found in atherosclerosis. Collectively, these data compels us to appreciate the significant role of platelets in atherogenesis.

1.1.3 Neutrophils in atherosclerosis

It is evident that atherosclerosis is a complex inflammatory disease of the vessel, with established and defined roles of monocytes/macrophages, T-cells, and platelets. However, only recently has the role of polymorphonuclear neutrophils (PMN) in atherosclerosis been evolved, since under a classical inflammatory response, they are the first line of defense that responds to invading microbials and tissue injury [121-123]. In 2002, using coronary artery segments from patients with acute myocardial infarction, Naurko et al have observed distinct neutrophil infiltration in patients with ruptured plaques, suggesting that neutrophil infiltration is actively associated with acute coronary events [122]. This was in congruent with findings from Haumer et al, showing patients with higher neutrophil counts were at higher risk for major adverse cardiovascular events [124]. In addition, similar findings were also shown by Grau et...
al whereby it was found that high neutrophil counts were independent predictors of recurrent ischemic events, better than monocyte and lymphocyte counts [125]. In a study conducted by Rotzius et al in 2010, the group utilized neutrophil- or monocyte- depleted ApoE$^{-/-}$ mice to quantify the population of leukocytes interacting with the endothelium at the lesion site [126]. Interestingly, the results showed that neutrophils accounted for majority of the leukocyte infiltration [126]. This first wave of neutrophil extravasation into the lesion site would thus provide the setting to attract monocytes. In 2008, Soehnlein et al demonstrated that by depleting neutrophils in C57BL/6 mice, extravasation of inflammatory monocytes were markedly reduced compared to mice with intact neutrophil count [123]. It was later found that it was the components of neutrophil secretion that attracted and activated the monocytes. Collectively, these evidences strongly suggest the pioneer and crucial role of neutrophils in the development atherosclerosis. Moreover, the mechanism by which neutrophils can trigger atherosclerosis may lie directly through a neutrophil-derived molecule that contributes to the development of the disease.

1.2 Defensins

1.2.1 Three families of defensins

Antimicrobial peptides are small cationic polypeptides composed of less than 100 amino acids with significant roles in host defense. The first study of antimicrobial peptides originated 1960, when Zeya and Spitznagel isolated an arginine-rich cationic peptide from rabbit and guinea-pig leukocyte lysates that displayed a broad spectrum of antimicrobial activity [127]. Humans, especially leukocytes and epithelial cells, express two main families of antimicrobial peptides: defensin and cathelicidin [128]. Although structurally and evolutionarily similar to one another, attention is directed more to defensins due to their extensive gene pool, various isoforms, and ubiquitous participation in infection [128].
Defensins are a family of antimicrobial peptides with 30-40 amino acids folded into a protein of molecular weight 3-5kDa [128, 129]. The members of the family share a characteristic triple stranded β-sheet fold with three cysteine-disulfide bonds [128]. There are three subfamilies of defensins: α-, β- and θ-defensins, with α- and β-defensin playing a more prominent role in human host defense due to the presence of a pre-mature stop codon in θ-defensins [130]. Therefore, due to the deficiency of θ-defensin expression in human, we will not elaborate θ-defensins in this thesis. Moreover, although β-defensins play a role in human host defense, however, unlike α-defensins, they are not expressed in immune cells, but in epithelial cells [128, 131]. Due to the different avenues of α- and β-defensins in mediating host defense, our project will only focus on the role of α-defensins in atherosclerosis.

1.2.1.1 α-defensin

Human α-defensins were studied as early as 1985 by Dr. Thomas Ganz [132], and has now been expanded to a breadth of knowledge with research. α-defensins contain 6 members: 1-4 designated as human neutrophil peptides (HNP-1, HNP-2, HNP3- and HNP-4) due to their high expression in neutrophils, and human α-defensin 5- and 6 (HD-5 and HD-6) which are chiefly expressed in intestinal paneth cells [129, 130, 133-135]. HD-5 and HD-6, together, are termed the enteric defensins due to their nature as an innate defense molecule for the gastrointestinal surface [136]. As such, since HD-5 and -6 are not involved in neutrophil-mediated immune response, our project will not focus on these two α-defensins.

Differing only in their first amino acid, HNP-1, HNP-2, and HNP-3 constitute more than 50% of the azurophilic granule content [137], with HNP-1 and HNP-2 collectively accounting for 90%
of the defensin composition [138]. In contrast, HNP-4 contains an amino acid sequence distinct from HNP1-3, and only accounting for less than 2% of the defensin content [139]. Due to the prominent concentration of HNP1-3 in neutrophils, herein forth, HNP will be referred to as a collection of HNP1-3.

In addition to neutrophils, HNPs are expressed by a wide range of immune cells including monocytes, macrophages, natural killer cells, B cells, dendritic cells and γδ T cells [129, 140-142]. HNP expressions within the various cells are also observed across different species. HNP has been isolated from rats, guinea pigs, hamsters, rhesus macaques and paneth cells from mice, suggesting an evolutionary conservation of HNP [128-130, 143-148].

Synthesis of HNP begins as a 90-100 amino acid prepropeptide with three main components: a 19 amino acid N-terminus, a 45 amino acid anionic propiece, and a 30 amino acid cationic C-terminus [149, 150]. Extensive processing and folding occurs in the neutrophil precursor cells known as promyelocyte, with mature HNPs packaged in the azurophillic (or primary) granules of neutrophils [129, 137, 151-153]. Once mature neutrophils circulate in the blood, they no longer synthesize the HNP peptides, nor their mRNAs [129]. Degranulation of HNP is regulated by the presence of microbial, cytokine or neuroendocrine signals [129]. Consequently, plasma concentrations of HNP can range from an undetectable level of 50-100-ng/mL in healthy humans to a significant range of 900-170,000-ng/mL in septic patients [154]. Thus, given its high concentration, HNP can play a substantial role in inflammation outside of its antimicrobial activities, including atherosclerosis.

1.2.2 HNP neutralization
α₁-protease inhibitor or anti-trypsin (α₁-PI or α₁-AT) is a member of the serpins superfamily, expressed by hepatocytes, monocytes, macrophages and neutrophils [155]. Under a steady-state condition, plasma α₁-PI concentration ranges between 15-30-μM, however, low α₁-PI concentrations (at plasma levels below 11-μM) have previously been shown to have important clinical implications in atherosclerosis [155, 156]. Clinically, patients with low serum levels of α₁-PI during an acute phase of myocardial infarction are associated with a higher risk of cardiogenic shock and mortality [157]. In addition, in patients undergoing coronary angiography, the severity of coronary atherosclerosis has been associated with low plasma levels of α₁-PI [156]. Therefore, low levels of α₁-PI have become an important indicator of the development of atherosclerosis. Interestingly, α₁-PI is an endogenous neutralizer of HNP [158-160].

In 1995, a study conducted by Panyutich et al showed that HNP would bind to α₁-PI to form a complex that would inactivate the two molecules. Using human lung carcinoma cell line A549, it was found that cytotoxicity induced by HNP was inhibited by the presence of α₁-PI [161]. Indeed, using A549 and small airway epithelial cells (SAEC) co-cultured with CD4⁺ lymphocytes, it was previously demonstrated that HNP-induced IL-8 production was significantly inhibited by the treatment of α₁-PI [162]. Taken together, this may potentially explain the poor prognosis of patients with low α₁-PI, as they become incapable of neutralizing the burden resulted from high concentrations of HNP during inflammation.

1.2.3 Properties of HNP

1.2.3.1 HNP and its anti-microbial role
HNP exhibit host defense activities against a wide array of microbes including gram-positive and –negative bacteria, fungus, and viruses [163-165]. Its activity can be observed at concentrations as low as 1-10-µg/mL [128]. However, its activity can be impeded by the presence of metabolic inhibitors, nutrient deprivation, hyperosmotic conditions, divalent cations and certain plasma proteins [166].

The mechanism of HNP’s antimicrobial and cytotoxic activity involves two crucial steps: 1) the binding, and 2) permeabilization of target membranes. Firstly, as a positively charged molecule, HNP is able to bind to negatively charged molecules by electrostatic interactions. Specifically, HNP binds to the negatively charged components found on microbial membranes such as lipopolysaccharides (LPS) in gram-negative bacteria, (lipo)teichoic acids in gram-positive bacteria polysaccharides, phosphatidyl glycerol and cardiolipin [167-169]. The importance of such electrostatic interaction supports the diminished activity observed when HNP is cultured with high salt conditions. Due to HNPs’ higher affinity for negatively charged microbial surface molecules than the native divalent cations (Ca^{2+} or Mg^{2+}), HNP can competitively displace these ions and disrupt the normal outer membrane to create a transient opening [170]. Using their hydrophobic surfaces, HNPs can coalesce into a hexamer of dimers to form channels with a diameter of 25 Å [128, 165, 170-172]. The electrically inserted channels subsequently allows for an increase in membrane permeability that ultimately inhibits RNA, DNA and protein synthesis, thereby decreasing microbial viability [173].

1.2.3.2 HNP in inflammation

1.2.3.2.1 HNP as chemoattractant

HNP contains an intrinsic chemotactic property for T-cells, mast cells, monocytes and immature dendritic cells [174, 175], a property that labels HNP as an immunological effector.
Indeed, in a study by Yang et al, HNP was demonstrated to induce the chemotactic migration of T-cells and dendritic cells, a response that was dependent on a G\textsubscript{i\alpha}-protein coupled receptor [175]. Likewise, using human monocyte- or murine bone marrow-derived macrophages and macrophage cell line J774A1, Grigat et al, showed that upon treatment of HNP, the cells’ chemotactic migration was induced [176].

Intriguingly, aside from HNP’s direct role in chemoattraction, HNP can also induce the production of secondary mediators to further support leukocyte migration. Our group has previously demonstrated that intratracheal instillation of HNP over 5 h induced an increase in production of chemoattractants TNF-\(\alpha\), and MCP-1 in the bronchial alveolar lavage fluid [177]. Tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is a potent chemokine known to be chemotactic for neutrophil even at nanomolar concentrations [178]. Similarly, monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine for monocytes, memory T-cells and basophils [179]. Its importance in recruiting monocytes in atherosclerosis was confirmed when mice deficient in MCP-1 exhibited a decrease in severity of diet-induced atherosclerosis due to the attenuation of monocyte accumulation at the site of lesion [180, 181].

1.2.3.2.2 HNP and P2Y\textsubscript{6}

Purinergic receptors are a class of receptors that bind to extracellular nucleotides, and have been previously implicated to mediate systemic inflammatory responses [182]. Of particular interest is the P2Y\textsubscript{6} receptor, a seven-transmembrane G-protein-coupled receptor that conventionally binds to UDP [182]. In a study conducted by Riegel et al, upon treatment of TNF-\(\alpha\) for 24 h in human microvascular endothelial cells (HMEC-1), there was a selective induction of P2Y\textsubscript{6} expression, a response that was not observed in a pool of 14 other
purinergic receptors [182]. The role of P2Y$_6$ in mediating vascular inflammation was further supported in P2Y$_6^{-/-}$ mice, whereby treatment of lipopolysaccharide (LPS) had an attenuated production of VCAM and neutrophil-activating protein 3 (KC) [182].

To evaluate the potential role of P2Y$_6$ in acting as an HNP receptor to induce inflammation, our lab sought to determine the pathway of HNP-mediated IL-8. IL-8 is a pivotal neutrophil chemoattractant expressed by a variety of cells including monocytes, lymphocytes, granulocytes, endothelial cells, epithelial cells, and fibroblasts [183]. In additional to neutrophils, IL-8 can also attract basophils, and eosinophils [184], immune cells that have well documented roles in atherosclerosis [3]. Therefore, the ability of HNP to induce IL-8 production would have significant impact in recruiting leukocytes and consequently, atherogenesis.

Indeed, using A549 and SAEC, our lab has previously found that stimulation with HNP would induce IL-8 production that was mediated through the purinergic P2 receptor, P2Y$_6$ [185]. Interestingly, stimulation of HCAEC and monocytes with HNP has also been shown to increase IL-8 mRNA and protein expression [162, 174, 185-190]. The resultant interaction between HNP and P2Y$_6$ receptor instigated activities of the PI3K/Akt and ERK1/2 pathway in lung epithelial cells [186] that were responsible in the IL-8 production. Finally, the observed HNP-mediated responses were shown to be significantly masked in the presence of P2Y$_6$ antisense [185]. Now, although it is possible that HNP can promote atherosclerosis through the P2Y$_6$ signaling pathway, however, our lab has shown in vitro and in vivo that another receptor may also play a prominent role.

1.2.4 HNP and atherosclerotic events
1.2.4.1 Plasma HNP levels in inflammatory disease

Under normal physiological conditions, plasma HNP concentration ranges from 0.2554±0.007- to 42±53-ng/mL [154, 191]. However, in patients with bacterial and non-bacterial infections, HNP concentration can significantly increase up to 1,075±101.8-ng/mL and 816.7±164.5-ng/mL, respectively [191]. In addition to plasma, the enhanced HNP concentrations can be found in other bodily fluids of patients with infection such as pleural, bronchoalveolar lavage, urine and cerebrospinal [191]. Interestingly, elevated HNP concentrations were also clinically observed in patients with bacterial septicemia, whereby their concentrations can reach as high as 170,000-ng/mL [154]. The detection of elevated HNP suggests a clinical significance of HNP in mediating inflammatory responses, perhaps even in the inflammatory events found in atherosclerosis.

1.2.4.2 HNP in atherosclerosis

Recently, a study conducted by Zhao et al compared the systemic concentration of HNP in patients with and without CAD, and acute ST-segment elevation myocardial infarction (STEMI). Unlike other antimicrobial peptides found in PMN such as LL-37, HNP levels were found to be significantly higher in patients with CAD and STEMI, in comparison to patients without CAD [192]. Clinically, this translated to three times the risk of cardiovascular mortality compared to patients with lower plasma HNP concentration [193]. These results suggest that circulating HNP, released from activated PMN during vascular inflammation, can complicate and exacerbate the events observed in atherosclerosis.

In general, HNP can be detected in three areas: 1) leukocyte and epithelial granules; 2) systemic circulation; 3) localized tissues. Indeed, previous studies have also detected HNP
deposition in the intima of atherosclerotic vessels [194]. Most prominently, HNP was found to be co-localized with the intimal smooth muscle cells and endothelium [194, 195], suggesting its potential in activating the endothelial and VSMC. In addition, increases in HNP deposition on the skin overlaying the femoral artery was significantly correlated with the severity of CAD, as assessed by coronary angiography [196].

To evaluate the role of HNP in the development of atherosclerosis, our lab designed a double transgenic mice that expresses HNP in the neutrophils and was prone to development of atherosclerosis called ApoE<sup>−/−</sup>/HNP<sup>+</sup>. Since mice do not express α-defensins in their neutrophils [128, 131], our double transgenic mice would express HNP regulated by a neutrophil elastase promoter, thereby making the mouse a more representative model of the human physiology. After feeding the mice and their littermate controls (ApoE<sup>−/−</sup>/HNP<sup>−/−</sup>) on chow or high fat for 10 weeks, their aorta was extracted to assess for lipid deposition. Remarkably, mice expressing HNP showed a positive trend, although not significant, of more lipid deposition in their aorta when fed on high fat diet (Figure 1) [197]. On an inflammation perspective, mice expressing HNP also showed a significant increase in leukocyte adhesion in the carotid artery (Figure 2) [197]. This was perhaps due to an increase in cytokine and chemokine production. Indeed, HNP-expressing mice plasma was found to have a significant increase in MCP-1, granulocyte macrophage colony stimulating factor (GM-CSF) and neutrophil-activating protein-3 (KC) in comparison to the littermate controls (Figure 3). Although not significant, regulated on activation, normal T cell expressed and secreted (RANTES) showed a positive trend in HNP-expressing mice. Collectively, these results provide evidence for HNP’s role in amplifying atherosclerotic lesion, leukocyte recruitment, and chemokine production.
Figure 1. HNP enhances lipid deposition in aorta of ApoE<sup>−/−</sup>/HNP<sup>(+)</sup> mice fed on high fat diet. A and B. Total and descending aortic lesion was stained by Oil-Red-O (ORO) in ApoE<sup>−/−</sup>/HNP<sup>(−)</sup> and ApoE<sup>−/−</sup>/HNP<sup>(+)</sup> mice fed on chow and high fat. Aortic lesion was quantified as percentage of total or descending aortic area. C. Images are representative images of at least 6 experiments.
Figure 2. HNP recruits leukocyte to carotid artery in vivo. **A.** Plasma HNP was measured in male ApoE<sup>-/-</sup>/HNP<sup>(-)</sup> and ApoE<sup>-/-</sup>/HNP<sup>(+)</sup> mice fed on chow or high fat diet for 10 weeks (n=6). **B.** Mice were injected with 150-µL of rhodamine 6G to non-specifically label leukocytes. Mean values of leukocyte rolling was monitored by in vitro microscopy (IVM). **C.** Adherent leukocytes were quantified by ImageJ. **D.** Representative images of leukocyte adherence under fluorescent microscopy. *p<0.05 vs ApoE<sup>-/-</sup>/HNP<sup>(+)</sup> chow; §p<0.05 vs ApoE<sup>-/-</sup>/HNP<sup>(+)</sup> high fat.
Figure 3. HNP increases cytokine and chemokine production in ApoE−/−/HNP+(+) mice. Plasma from ApoE−/−/HNP+(+) and ApoE−/−/HNP(−) mice were collected after 10 weeks of chow or high fat diet for analysis of monocyte-chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), Regulated on Activation Normal T cellExpressed and Secreted (RANTES), and neutrophil activating protein 3 (NAP-3 or KC). *p<0.05 vs ApoE−/−/HNP(−) chow or high fat. §p<0.05 vs ApoE−/−/HNP+(−) on chow and high fat.
1.2.4.3  HNP-induced leukocyte adhesion and migration

Leukocyte infiltration in atherosclerotic lesions plays an adverse role in the pathophysiology of atherosclerosis. Previously, our laboratory has demonstrated surface expression of CD80, CD86 and ICAM-1 in primary human small airway epithelial cells and A549 alveolar type II cells, as well as their counterpart ligands CD28, CD152 and CD11a/CD18 in CD4\(^+\) T-lymphocytes were increased in response to HNP stimulation [162]. Correspondingly, an increase in leukocyte-epithelial interaction was observed [162]. Likewise, HNP treatment was also shown to be able to enhance monocyte adhesion and migration across primary human coronary artery endothelial cells by increasing endothelial ICAM-1 and monocytic CD11b expression [174]. Functionally, this translated into an increase in leukocyte adherence and rolling found in mice given a bolus of HNP, as captured by intravital microscopy [174]. By enhancing the expression of proteins involved in leukocyte recruitment, HNP plays a valuable role in capturing circulating leukocytes and promoting their transmigration and accumulation to the site of atherosclerotic lesions, therefore endorsing the inflammatory cascade.

In addition, our group has previously demonstrated that HNP was able to induce leukocyte rolling in the carotid artery of wild-type mice. Moreover, this response was dependent on a lipoprotein receptor known as LRP8, since leukocyte rolling induced by HNP was significantly abolished in LRP8\(^{-/-}\) mice in comparison to wild-type mice. Interestingly, in mice deficient of all LDLR and LRP family members (LDLR\(^{-/-}\)/LRP\(^{-/-}\) mice), there was no significant difference in leukocyte rolling in comparison to LRP8\(^{-/-}\) mice after receiving a bolus of HNP, thereby suggesting that HNP-induced leukocyte recruitment was chiefly due to the LRP8 signaling pathway [174], and not only through a P2Y\(_{6}\)-dependent signaling pathway.
1.2.4.4 HNP-mediated oxidative stress

Oxidative stress is one of the hallmark characteristics of endothelial dysfunction and a prominent event in atherosclerosis [198-200]. A study conducted by Kougias et al demonstrated the influential effect of HNP in reducing endothelial-dependent vasorelaxation that was associated with an increase in superoxide radical production and a decrease in eNOS mRNA expression in porcine coronary arteries [201]. This was consistent with findings in our laboratory where HNP was able to stimulate hydrogen peroxide (H$_2$O$_2$) productions in murine lung explants [202], and nitrotyrosine in human acute monocytic leukemia cell line (THP-1) derived macrophage [174]. The buildup of these reactive oxygen and nitrogen species can perturb atherosclerosis by oxidizing lipids into oxLDL, which activates the endothelium [4, 203-205], promotes foam cell formation [206-209] and attracts circulating leukocytes [206].

1.2.4.5 HNP and foam cells

On a cellular level, deposited HNP have been shown to have multiple implications. Firstly, our lab has previously found that treatment of HCAEC with HNP was able to induce MCP-1 secretion [174], a phenomenon that would promote the recruitment of monocytes to the lesion site and increase the chances of foam cell formation. Secondly, our lab has also demonstrated that the same HNP was able to accelerate foam cell formation in human acute monocytic leukemia cell line (THP-1)-derived macrophages treated with oxLDL [174]. It was later found that this was due to an increase in expression of macrophage scavenger receptor responsible for lipid uptake: CD36 and CD68 [174]. Consequently, the HNP-stimulated macrophages was able to elicit nitrotyrosine production, suggestive of HNP’s role in mediating nitrosative stress that can further perturb atherosclerosis [174].
1.2.4.6  HNP and platelet aggregation

Platelet activation and aggregation plays an integral role in atherosclerosis. Our lab has previously shown that stimulation of human platelets with HNP can induce platelet activation, a response assessed by measuring P-selectin (CD62P) expression [174], the surface adhesion marker responsible for platelet-endothelial interaction [210]. Moreover, when human platelets were primed with HNP, platelets were able to sustain their aggregation response once stimulated with ADP [174]. The physiological relevance of these results was demonstrated in vivo. In mice injected with a bolus of HNP intravenously, platelet aggregation was significantly increased upon application of ferric chloride in comparison to mice that received vehicle control. Likewise, the amount of time to occlude the vessel was also significantly reduced in mice that received HNP [174]. Interestingly, the observed platelet responses were also mediated by the LRP8-signaling pathway [174]. Therefore, additional research is warranted to provide a better understanding of the role in LRP8 in mediating the pathogenesis of atherosclerosis.

1.3  Low-density lipoprotein related protein-8 (LRP8)

1.3.1  LDLR and LRP family

The lipoprotein receptor (LR) superfamily is a class of eleven structurally related transmembrane surface proteins: 1) LRP1, also known as α-2-macroglobulin; 2) LRP1b, also known as LR32; 3) megalin/LRP2, previously known as gp320; 4) LDL receptor (LDLR); 5) very low-density lipoprotein receptor (VLDL receptor); 6) MEG7/LRP4; 7) LRP8/apolipoprotein E receptor 2; 8-11) LR 3, 5, 6 and 11 [211, 212]. The structural domains found common to majority of the receptors are: 1) ligand binding domain, consisting of seven cysteine-rich type A repeats enriched with negatively charged clusters of Ser-Asp-Glu residues; 2) epidermal growth factor (EGF), a 400 amino acid residue that facilitates acid-dependent ligand
dissociation; 3) O-linked sugar domain, a 58 amino acid residue enriched with 18 serines and threonines containing O-linked carbohydrate chains; 4) transmembrane domain, a single 20 amino acid domain that serves to anchor the receptor to the plasma membrane; and 4) cytoplasmic tail domain, a 50 amino acid residue the receptor internalization sequence Asn-Pro-Val-Tyr in the coated pit [212]. Members of the superfamily participate in a wide range of homeostatic responses including lipid metabolism, neurodevelopment, signal transduction, nutrient and vitamin trafficking, and ligand endocytosis [211]. The expression of the individual proteins, their biological function, and corresponding ligands vary across different tissues (Table 1).

In light of LRs' role in lipid homeostasis and signal transduction, several studies have been dedicated to elucidate their role in atherogenesis. Firstly, LRP family members are highly expressed in the vascular wall and is associated with macrophages and vascular smooth muscle cells [213], suggesting its role in maintaining vascular integrity. Secondly, many of the LRs' ligands are mediators of atherosclerosis such as thrombospondin, ApoE, coagulant factor Xi, thrombin, tPA, uPA, lipoprotein lipase, hepatic lipase, protease nexin-1 and -2, and protein C [214, 215], which warrants the assumption that LRs may be responsible in the signaling transduction of atherosclerosis development. Moreover, in a study using genomewide linkage scan of 428 families, it was found that genetic variants of LRP8 contribute to the development of premature myocardial infarction and familial CAD [216]. Congruently, mutation in the LDLR gene has previously been found to cause familial hypercholesterolemia [211], due to cellular loss-in-function of LDL uptake. Together, it suggests that LRs play an important role in shaping atherosclerosis development.
<table>
<thead>
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<th>Expression</th>
<th>Ligands</th>
<th>Biological Function</th>
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<td>ApoE, ApoB, LDL</td>
<td>Cholesterol homeostasis</td>
</tr>
<tr>
<td>LRP1</td>
<td>Hepatocytes, neurons, VSMC, macrophage, trophoblast, embryonic tissues</td>
<td>ApoE, chylomicron remnants, a2-macroglobulin, amyloid percursor protein, tPA, protease inhibitor complexes, lipoprotein lipase, PDGF, TGFβ</td>
<td>Endocytosis, chylomicron remnant receptor, phagocytosis of apoptotic cells, embryonic development, PDGF receptor regulator, Ca²⁺ current regulator</td>
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<tr>
<td>VLDLR</td>
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<td>ApoE, Reelin, lipoprotein lipase, tissue factor pathway inhibitor</td>
<td>Neuronal migration, synaptic transmission</td>
</tr>
<tr>
<td>LRP8</td>
<td>Brain, testis, platelet, endothelial cells, hepatocytes, VSMC, placenta, ovaries</td>
<td>ApoE, Reelin, thrombospondin-1, coagulant factor XI, antiphospholipids</td>
<td>Neuronal migration, synaptic transmission, male fertility, platelet activation and aggregation</td>
</tr>
<tr>
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</tr>
<tr>
<td>MEGF7</td>
<td>Central nervous system</td>
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Table 1. Ligands and functions of LDL receptor family members. The table highlights only a selection of LDL receptor family members. (Adaptation from May²¹ with updated information from recent publications).
1.3.2 LRP8 and atherosclerosis

1.3.2.1 LRP8

Until 1996, lipid metabolism in the central nervous system was poorly understood, even though it was widely known that lipids played a crucial role in myelin formation [217]. Although most of the lipids found in the brain are synthesized by the neuronal cells such as astrocytes, however, constitutive cholesterol, particularly ApoE, and fatty acid uptake from the systemic circulation also contributes significantly to the lipid content in the central nervous system (CNS) [218, 219]. It wasn’t until the study conducted by Kim et al that the receptor-mediated ApoE endocytosis was found to be due to the actions of LRP8 [217]. However, despite LRP8’s ability to bind and internalize apoE in neurons, transgenic mice deficient in LRP8 do not exhibit hypercholesterolemia [220]. Indeed, in a study conducted by Li et al, they found LRP8 had the lowest endocytosis rate when compared to LRP1, LRP4, LRP2 and LDLR [221]. This suggests vascular LRP8 plays a more prominent role in signal transduction, not lipid internalization.

1.3.2.2 LRP8 in platelet

Although initially characterized as a receptor responsible for lipid metabolism in the central nervous system, LRP8 has recently gained more attention to its role in atherosclerosis. Originally believed to be chiefly expressed in the brain, placenta, testes and ovaries, expression of LRP8 has now been expanded to include heart, endothelial cells, vascular smooth muscle cells and platelets [222]. Interestingly, of all the LRP family members, only LRP8 is found in platelets [223], therefore, many studies have first focused on platelet LRP8 to provide insight on the role of LRP8 in atherogenesis.
To date, different splice variants of LRP8 on platelets have been discovered. Using a homology cloning approach and degenerate PCR primers to amplify the binding domain of LDL-R, Riddell et al found that platelet LRP8 lacked repeating units 4-6 (LRP8Δ4-6) [223]. Later using immune-blotting, Pennings et al have documented three more LRP8 splice variants: 1) shApoER2Δ5, lacking LDL binding domains 4, 5, and 6; 2) shApoER2Δ4-5, lacking LDL binding domains 3, 4, 5, and 6; and 3) shApoER2Δ3-4-5, lacking LDL binding domains 3, 4, 5, 6 and 7 [224]. Although it remains unclear why platelets contain multiple LRP8 variants, however, it could suggest the diversity of ligands LRP8 can bind to.

The functional role of LRP8 in platelets was first described by Desai et al in 1989 when they discovered that high density lipoprotein E inhibited ADP-mediated platelet aggregation through a surface receptor they called HDL₂ [225]. It was later confirmed by Riddell that the receptor was the LDL receptor family member, LRP8 [223]. Moreover, it was elucidated that ApoE-induced LRP8 activation mediated an intracellular up regulation of eNOS activity, which generated a large quantity of NO that augmented guanylate cyclase activity and cGMP content [226]. Later in 2004, Korporaal et al expanded Riddell’s work to include the downstream pathway of LRP8 activation. By sensitizing isolated human platelets with an LDL constituent, apolipoprotein B100, the group found that it induced a rapid phosphorylation of p38 mitogen-activated protein kinase (p38MAPK), a response that was significantly attenuated by the presence of the LDL receptor family inhibitor: receptor-associated protein (RAP) [227]. The response was later confirmed ex vivo using platelets isolated from LRP8-deficient mice, whereby LDL was also unable to induce p38MAPK activation [227]. By inhibiting p38MAPK, it can attenuate cytosolic phospholipase A₂ (cPLA₂) activity, which results in a decrease in cytosolic Ca²⁺ load, and TxA₂ production and release [228]. This suggests a critical role of LRP8 in
mediating intracellular signaling for platelet aggregation. Indeed, in 2009, Robertson et al examined the role of LRP8 in platelet aggregation in vivo. The group demonstrated that in LRP8-deficient (LRP8^{-/-}) and LRP8 heterozygous (LRP8^{-/+}) mice, platelets treated with ADP or thrombin exhibited a significant attenuation in platelet activation and aggregation, as quantified by $\alpha_{IIb}\beta_3$ activation and aggregometer, respectively [229]. Correspondingly, in comparison to the littermate controls, LRP8^{-/-} and LRP8^{-/+} mice exhibited an increase in carotid artery occlusion time post topical injection of FeCl$_3$ [229]. It thus becomes evident that LRP8 can play a critical role in mediating platelet aggregation and shape the course of atherosclerosis.

1.3.2.3 LRP8 in endothelial cells

With a growing body of evidence for the role of LRP8 in platelet aggregation, studies have shifted to investigating the role of LRP8 in endothelial cell behaviour. Interestingly, in 2011, Ramesh et al established the role of LRP8 in mediating leukocyte rolling. The group showed that activation of LRP8’s first LDL-binding domain in vascular endothelial cells, by anti-phospholipid antibodies (aPL), was able to induce an increase in phosphatase PP2A activity [230]. The resultant PP2A activation induced a decline in eNOS activity, and hence NO bioavailability [230]. Using intravital microscopy, the group also found that in LRP8-expressing mice, stimulation with aPL mediated an increase in leukocyte adhesion and rolling, a phenomenon that was absence in LRP8-knock out mice [230].

1.3.3 LRP8 Ligands

Outside of ApoE, several other extracellular ligands of LRP8 have recently been identified that can induce LRP8 signal transduction: 1) reelin, a 400kDa extracellular matrix protein that binds to the first ligand binding domain of LRP8; 2) thrombospondin, a trimeric or pentameric
protein involved in cell-cell communication; 3) selenoprotein P, a transporter for selenium that is required for spermatogenesis; and 4) coagulation factor XI, a 160-kDa glycoprotein that participates in the intrinsic blood coagulation pathway [215, 231-233]. Consequently, the activation of LRP8 can induce binding of multiple scaffold proteins onto the intracellular domains of LRP8 such as: 1) disabled-1 and -2, a cytoplasmic docking protein that is essential for Src, Fyn and MAPK kinase activity; 2) JIP-1 and -2, molecular scaffolds involved in the C-jun N-terminal kinase (JNK)-signaling pathway; and 3) PSD-95, an adaptor protein responsible for synaptic and dendritic spine formation [234-236] [Table 2]. Interestingly, previous studies have linked MAPK activity to the increased production and release of platelet agonist thromboxane A2 (TxA2) [228], and cholesterol ester accumulation in macrophages to promote foam cell formation [237]. Likewise, numerous studies have established the key role of JNK in atherogenesis. Studies have shown that atherosclerotic lesions exhibit an elevation of JNK-activity, and that inhibition of the JNK pathway attenuated CD-36 and SR-A induced LDL uptake, foam cell formation and atherogenesis in mice [238-240]. Collectively, this suggests that activation of LRP8 can induce downstream pathways that promote atherosclerosis.

1.3.3.1 Reelin

1.3.3.1.1 Reelin molecule

Molecularly, reelin is a 3461 amino acid long protein with a molecular mass of 387,497 Da. The protein contains a 27 amino acid signaling peptide, a 28-190 amino acid F-spondin-like (SP) segment, a 191-500 residue H region, and a long chain of 8 repetitive segments, each approximately 350 amino acid long separated by an epidermal growth factor (EGF) [241]. Finally, the protein terminates with a 33 amino acid C-terminus enriched in basic residues [242]. Although reelin is first expressed as a full-length protein, however, other fragments of
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<th>Ligand</th>
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<td>LDL</td>
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<td>Platelet</td>
<td>$\alpha_{IIb}\beta_3$ and P-selectin expression</td>
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<td>Antiphospholipids</td>
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<td>Platelet</td>
<td>$TxB_2$ expression</td>
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<td>ApoE</td>
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<td>$\downarrow$ Platelet aggregation</td>
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<tr>
<td>$\beta_2$GPI</td>
<td>Platelet</td>
<td>$\uparrow$ $TxA_2$ expression</td>
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<tr>
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<td>Lymphatic</td>
<td>$\uparrow$ MCP-1 expression</td>
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<td>Sperm</td>
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<td>Coagulation facto XI</td>
<td>Platelet</td>
<td>Platelet aggregation</td>
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Table 2. Ligands and functions of LRP8. The table highlights the various LRP8 ligands and their corresponding function in the cells.
reelin have been found due to reelin processing by metalloproteinase [243]. By comparing partial recombinant reelin construct, reelin has been shown to be cleaved at 2 locations: 1) between repeats 2 and 3; and 2) between repeats 6 and 7, to yield reelin fragments with molecular weight 100-, 120-, 180-, 220- and 300-kDa [244]. Reelin fragment 100-kDa contains the repeating units 7 to C-terminus (R78C); 120-kDa contains the repeating units 3 to 6 (R36); 180-kDa contains the N-terminus to repeating unit 2 (NR2); 220-kDa contains the repeating units 3 to C-terminus (R38C); and 300-kDa contains the N-terminus to repeating unit 7 (NR6) [244-246] [Figure 4]. Interestingly, not all reelin fragments bind to their receptor, LRP8, or at equal affinity.

1.3.3.1.2 Native vs. active reelin

The ligand-receptor relationship between reelin and LRP8 was first observed when LRP8-deficient mice exhibited reeler-like phenotype [220]. Later, in 1999, D’Arcangelo et al made the revolutionary discovery that reelin binds directly to LRP8 to elicit their response [247]. By constructing recombinant reelin proteins of the various fragments, the binding capacity of other reelin fragments to LRP8 was assessed in vitro [245, 246]. In addition and of equal importance, by quantifying Dab1 tyrosine phosphorylation on LRP8, the group was also able to address the ability of various reelin fragments to induce LRP8 activation [245, 246]. It was found that recombinant reelin proteins containing the N-terminus, the first four repeating units and part of the fifth repeating unit showed no significant binding to LRP8 [246, 248, 249]. However, recombinant reelin proteins containing repeating units 3-8 or 3-6, which coincidently was the central segment of reelin, was able to bind to LRP8 at similar affinities to full-length reelin [246, 249, 250]. Moreover, recombinant reelin containing the central segment was also able to stimulate Dab1 phosphorylation on LRP8 [245], thereby suggesting the importance of reelin’s central fragment in binding to and activating LRP8. Interestingly, although the N-
Figure 4. Reelin fragments. Proteolytic processing of reelin protein and the corresponding molecular weights of the fragment. Figure adapted from D'Arcangelo\textsuperscript{257}.
terminal moiety of reelin was not responsible in binding to LRP8, it does play a crucial role in reelin functioning. Studies have shown that reelin interacts electrostatically with each other on the N-terminus to form a large homopolymer, which is important for reelin’s functional activity [251, 252]. By using an N-terminus blocking antibody, CR50, reelin was unable to form an aggregate and induce LRP8 Dab1 phosphorylation [252]. Indeed, a study by Nakajima et al showed that intraventricular injection of CR-50 at the embryonic stage of mice was able to disrupt the organized development of the hippocampus, an event that was in conjunction to the reeler pattern [253]. Collectively, this suggests that reelin fragment 300-kDa and full-length reelin are the prime activators of LRP8.

1.3.3.1.3 Reelin in inflammation

Reelin was originally believed to be solely expressed by the central nervous system, however, traces of reelin have been detected in mammalian blood, liver, pituitary pars intermedia, adrenal chromaffin cells, hepatic stellate cells, lymphatic endothelial cells and platelets [254-256]. Interestingly, an up regulation of reelin expression was found in patients with liver cirrhosis [257] and ocular injury [258], suggesting a mediatory role of reelin during inflammation. Indeed, recently, a study published by Lutter et al found that in the presence of SMCs, lymphatic endothelial cells release and proteolytically process the endothelium-derived reelin. Through a paracine mechanism, the released reelin correspondingly acts on neighbouring endothelial cells to induce an up-regulation of MCP-1 [255]. This suggests that reelin is capable in mediating leukocyte recruitment. Interestingly, once the monocytes and macrophages have been recruited, reelin can also act on their LRP8 receptor to regulate leukocyte cholesterol efflux. In a study conducted by Chen et al, the group observed that stimulation of mouse macrophages, RAW264.7 cells, with reelin can induce an up-regulation
of the ATP binding cassette transporter A1 (ABCA1), a transporter that regulates cholesterol efflux [259]. Therefore, reelin may play a crucial role in regulating foam cell formation.

To further expand the role of reelin in inflammation, in 2010, Tseng et al sought to determine the effect of reelin on platelet activation since LRP8 was the only LRP family member expressed on platelets [223, 254]. Remarkably, stimulation of reelin in platelets induced platelet spreading, a phenomenon that is characteristic of platelet activation [254]. This proposes the notion that circulatory reelin is able to induce platelet activation, a primary and necessary event preceding platelet aggregation. In summary, the observed association between reelin and inflammation emphasizes the molecule’s potential role in mediating the events found in atherosclerosis.

1.4 Summary

The role of neutrophils in the development of atherosclerosis as recently gained wide attention [5, 126]. As the first line of defense, neutrophils have previously been shown to infiltrate atherosclerotic lesions prior to the recruitment of monocytes and macrophages [126]. However, due to their short life spans, neutrophils do not reside in the lesion over the entire duration of the disease. Therefore, attention has been shifted to focus on neutrophil proteins in mediating the neutrophil-provoked inflammation, specifically HNP.

It is evident that HNP released from activated neutrophils play an immune-modulating role, however, it is also increasingly becoming apparent that HNP may also serve as an atherosclerotic-modulating peptide. Although our lab has previously shown that the LRP8 signaling pathway plays a significant role in mediating HNP responses, nevertheless, the link between HNP and LRP8 was still unclear. Here, we present for the first time the relationship
between HNP and LRP8, whereby the linkage between the two is mediated through the reelin molecule.
Chapter 2: Rationale and Objectives

2.1 Rationale and Novelty

Elevation in neutrophil counts and infiltration to the lesions has been previously observed to correlate with the severity and progression of atherosclerotic lesions \[122, 124\]. Human neutrophil peptides (HNP) are cationic proteins that account for more than 50% of the azurophilic granule contents in neutrophils \[137\] and are released into the extracellular milieu upon PMN activation. This leaves a “foot-print” of the neutrophil’s existence at the site of lesion \[260\]. In epithelial cells, HNP is known to bind to P2Y\(_6\) and elicit IL-8 production \[185\], however, the biological consequence of HNP on P2Y\(_6\) in endothelial cells is not known. Moreover, although primarily known for its antimicrobial properties, recently, HNP has been found to also mediate inflammatory events found in atherosclerosis including the induction of leukocyte chemoattraction, adhesion, and migration across the endothelium, and platelet aggregation \[174\]. Interestingly, these responses involved the communication between HNP and the LRP8-signaling pathway. However, HNP does not directly bind to LRP8 in human microvascular endothelial cells (HMEC-1) \[Figure 5\]. In platelets, LRP8 is the only known LRP family member expressed, suggesting that a traditional LRP8 ligand participates in the crosstalk between HNP and LRP8 to induce platelet aggregation. We believe that HNP is able to stimulate the production and release of an LRP8-ligand, which acts in a paracrine fashion to instigate the activation of the LRP8-signaling pathway. Due to its prominent expression during inflammation and its ability induce inflammatory mediators, reelin may be a candidate ligand that facilitates the HNP-LRP8 crosstalk.
Here, we present for the first time the molecular mechanism of HNP activating the LRP8-signaling pathway through the expression and release of reelin [Figure 6] in endothelial cells and platelet.

2.2 Hypothesis

Human neutrophil peptides induce endothelial activation and platelet aggregation mediated by reelin.

2.3 Objectives

2.3.1 Objective #1: To examine the role of HNP-induced reelin release.

Specific Aim I: To examine the protein expression of reelin in HNP-treated endothelial cells.

Specific Aim II: To evaluate the effect of P2Y6 blocker on HNP-mediated reelin expression.

2.3.2 Objective #2: To determine the specific effect of reelin in endothelial function and platelet aggregation.

Specific Aim I: To examine the direct effects of reelin on nitric oxide synthase (eNOS and iNOS) in human coronary endothelial cells (HCAEC).

Specific Aim II: To determine the direct effects of reelin on measure oxidative stress markers in HCAEC.

Specific Aim III: To evaluate the direct effect of reelin on platelet aggregation.

Specific Aim IV: To assess the effect of reelin neutralizing antibody on endothelial dysfunction and platelet aggregation.
### GST-Pull Down Assay:

<table>
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<th>WCL</th>
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<td>+</td>
<td>+</td>
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<tr>
<td>-</td>
<td>GST</td>
<td>GST-HNP</td>
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**Figure 5. HNP does not bind directly to LRP8.** HCAEC protein lysates were incubated with GST protein, GST-fused HNP, or beads alone overnight at 4°C on a rotator. Direct protein-protein binding was determined by western blot after probing for LRP8 and HNP.
Figure 6. Potential mechanism of HNP in promoting atherogenesis. Activated PMN release HNP to act on endothelial P2Y$_6$ signaling pathway and induce the expression and release of reelin. Reelin acts on platelet LRP8, the only LDL receptor found on platelets, to mediate platelet aggregation and thrombosis. Finally, reelin acts on neighbouring endothelial cells to induce peroxynitrite production and endothelial dysfunction.
Chapter 3: Methods and Materials

3.1 Reagents

Rabbit α-human LRP8 mAb was purchased from Epitomics (Burlingame, CA, USA). Mouse α-human reelin mAb was from EMD Millipore (Billerica, MA, USA). Mouse α-GAPDH and -His mAb were purchased from Biolegend (San Diego, CA, USA). Rabbit α-human eNOS mAb was from Cell Signaling (Beverly, MA, USA). Rabbit α-human iNOS and lamin pAb were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), goat α-rabbit and mouse IgG-HRP secondary antibody were from Jackson ImmunoResearch Laboratories Inc. (WestGrove, PA, USA) and enhanced-chemiluminescence solution was from ThermoScientific (Waltham, MA, USA). Dithiothritol (DTT) was purchased from BioShip (Burlington, ON, Canada). Mouse recombinant reelin was purchased from R&D Systems (Minneapolis, MN, USA), and reelin neutralizing antibody (CR-50) was from MBL (Nagoya, Japan). Adenosine diphosphate (ADP) and 1,4-Di[3-(-isothiocyanatophenyl)thioureido]butane (MRS 2578) were from Sigma (St. Louis, MO, USA).

Purified HNP was a mixture of HNP-1, -2, and -3 isolated from the sputum of cystic fibrosis patients, as previously described [202]. Briefly, 20 cystic fibrosis patient sputums were pooled prior to purification to minimize variability from one patient to another. Sputum was mixed with 5% acetic acid in a 5-mL volume, homogenized and stirred for 24 h at 4°C in a polypropylene conical tube. The solution was centrifuged for 30 min at 27,000 G and supernatant was collected. Ten percent dithiothritol (DTT) was added to the sputum supernatant and loaded onto a polyacrylamide el permeation column of Bio-Gel P-10 (Bio-Rad Laboratories, Hercules, CA, USA) and eluted with 5% acetic acid. Fractions were collected, and HNP-contained eluents were pooled, lyophilized and reconstituted in PBS. Endotoxin detection assay was
performed by using Limulus amebocyte lysate ( Associates of Cape Cod Inc, Falmouth, MA, USA), and bacterial killing was performed to test HNP activity. Protein concentrations were measured by spectrophotometry at an absorbance of 280-nm.

3.2 Cell Culture

3.2.1 Primary human coronary endothelial cells (HCAEC)

Primary human coronary artery endothelial cells (HCAEC, Cell Applications, San Diego, CA) were grown in T75 flasks ( Sarstedt Inc, Newton, NC) and kept at a density of 0.1-1x10^6 cells/mL in MesoEndo Cell Growth Medium ( Cell Applications, San Diego, CA). Cells within the first fifteen doublings of growth were used for experiments. Upon 80% confluency, cells were washed twice with HBSS, trypsinized and seeded at a density of 1x10^5 cells/mL in 12-well plates for 16 h at 37°C in MesoEndo Cell Growth Medium to ensure adherence. Prior to stimulation, cells were washed with HBSS and medium was replaced with Endothelial Serum-Free defined medium with 1% fetal bovine serum (Sigma, St. Louis, MO) to prevent HNP inactivation [261].

3.3 Western blotting

3.3.1 Whole cell lysate preparation

Cells were stimulated according to specific experimental protocols. Culture medium was collected, aliquoted and stored in -80°C freezer. Cells were washed with HBSS and subsequently lysed with 100-μL of cold lysis buffer (50mM Tris-HCl, 2mM EDTA, 2mM EGTA, 150mM NaCl, 1% Triton X-100, pH 7.54). Cells were scraped on ice, centrifuged at 10,000 G for 5 min at 4°C. Supernatants containing the proteins were transferred into a fresh eppendorf tube and store at -80°C.
3.3.2 Protein concentration quantification

Protein concentration was quantified using the Bradford Assay in a 96-well plate. Bovine serum albumin (BSA, Sigma, St. Louis, MO) standard (0-1.0 \( \mu \text{g/\muL} \)) was diluted in 1xPBS and 5-\( \mu \text{L} \) of each standard was added into duplicate wells. Similarly, 5-\( \mu \text{L} \) of samples were added to duplicate wells, followed by the addition of 195-\( \mu \text{L} \) of 1:5 diluted protein assay dye (BioRad, San Diego, CA). The samples were incubated for 10 min at room temperature to allow for colour development. Absorbance was measured at 595-nm with a spectrophotometer (SpectraMax 340, Molecular Devices) and protein concentration was determined with the constructed standard curve.

3.3.3 SDS polyacrylamide gel electrophoresis and Transfer

Samples were ran on a BioRad Mini-PROTEAN gel electrophoresis system. Five-\( \mu \text{g} \) of protein were prepared to ensure equal loading of protein. Into each sample, 6X loading buffer (375mM Tris-HCl, 6% SDS, 48% glycercol, 9% MeSH, 0.03% \( \beta \)-mercaptoethanol, pH 6.8) was added to yield a final concentration of 1X loading buffer in the sample, and boiled for 5 min. Samples and protein standard ladder were loaded onto their corresponding SDS polyacrylamide gels and electrophoresed at 100 V for 1.5 h in 1x running buffer (25mM Tris, 0.1% SDS (w/v), 192 mM glycine). Following electrophoresis, proteins were transferred onto a 0.4-\( \mu \text{m} \) pore nitrocellulose membrane using a wet transfer system (BioRad, San Diego, CA). Electroblotting was conducted for 2.5 h or 16 h at constant 400mA or 15V, respectively, depending on the specific experimental protocols.

3.3.4 Immunoblotting

Following electroblotting, nitrocellulose membranes were blocked with 5% (w/v) skim milk
powder in 0.1% TBS-T (0.5M Tris, 1.5M NaCl, pH 7.4, 0.1% Tween-20) for 1 h at room
temperature on a rotating shaker. Membranes were then rinsed once with 0.1% TBS-T and
incubated with specific primary antibodies in 5% BSA-0.01% TBS-T overnight at 4°C on
rotating shaker. Protein membranes were washed 3 times, 10 min/wash, in 0.01% TBS-T,
followed by the incubation with the respective goat α-rabbit or α-mouse HRP-conjugated IgG
antibody for 1 h at room temperature with gentle shaking. Nitrocellulose membranes were
washed 3 times, 20 min/wash, in 0.01% TBS-T. After the removal of 0.01% TBS-T, 1-mL of
enhanced-chemiluminescence solution (ThermoScientific, Waltham, MA) was added to the
membrane for 1 min. Protein expression were exposed onto a film and developed. Protein
density was determined by GS-800 Calibrated Densitometer (BioRad, San Diego, CA). All
protein band intensities were normalized by the house keeping protein, GAPDH or lamin,
developed from the same membrane.

3.4 Protein-protein interaction

3.4.1 GST-pull down assay

Four hundred-µg of HCAEC protein were incubated with 2-µg mouse recombinant reelin
(mrRLN, R&D Systems, Minneapolis, MN) and 2-µg α-His antibody (Biolegend, San Diego,
CA) for 16 hours at 4°C on a rotator. Subsequently, 30-µL of glutathione-agarose beads
(ThermoScientific, Waltham, MA) were added to the lysates and incubated for 2 hours at
room temperature on a rotator. Sample-bead solution was centrifuged at 8000 rpm
(Centrifuge 5415D) for 1 min at room temperature, and washed twice with high salt solution
(500mM NaCl, 200mM HEPES, 10% glycerol, 0.1% Triton-X 100), followed by 3 times wash
with low salt solution (150mM NaCl, 20mM HEPES, 10% glycerol, 0.1% Triton-X 100). After
the final wash and centrifuge, the supernatant was decanted, and the pellet was prepared for
western blot. Briefly, the sample-bead solution was incubated with 50-µL of 1X loading buffer and boiled for 5 min. The samples were centrifuged at 8000 rpm for 1 min at room temperature, and the supernatant was collected to run on a 6% polyacrylamide gel. Following the electrophoresis, proteins were transferred onto a nitrocellulose membrane for 2.5 h at constant 400mA. The membrane was blocked and incubated with LRP8 antibody overnight at 4°C. The membrane was developed the following day with protocols as previously stated.

3.5 Reelin protein expression

3.5.1 HCAEC stimulation

Seeded HCAEC were stimulated with PBS, HNP 1- or 10-µg/mL for 6 h. Culture medium and protein were collected as previously mentioned. Experiment was repeated to reach a sample size n = 5.

3.5.2 P2Y6 blocked-HCAEC

To examine the specificity of HNP-induced reelin protein expression, in a separate experiment, HCAEC were pre-treated with 0.5- or 1-µM MRS2578 (Sigma, St. Louis, MO), or DMSO control (Sigma, St. Louis, MO) for 30 min in 37°C, followed by the 6 h stimulation of HNP-10 µg/mL or vehicle control. Culture medium and protein were collected as previously mentioned. Experiment was repeated to reach a sample size n = 3.

3.5.3 Measurement of reelin from culture medium and western blot

3.5.3.1 Reelin ELISA

In order to explain the mechanism by which HNP induce endothelial dysfunction, it was important to determine the amount of reelin released from HNP-induced HCAEC. To address
this, the presence of full-length reelin in the culture medium was determined according to a commercially available reelin ELISA kit (USCN, China).

3.5.3.2 Reelin western blot

Reelin is a 400kDa glycoprotein that can undergo protein modification and cleavage, thereby yielding smaller fragments of reelin [244, 246, 262]. Previous studies have determined the LRP8 binding site of reelin to be localized in central fragment, R3-6 [262], which are found in the full-length and 300kDa reelin fragment. Therefore, it is also crucial to determine the expression of reelin fragments expressed in the cells and released into the culture medium from HNP-stimulated HCAEC.

To our knowledge, there is currently no commercially available ELISA kit that can detect specifically other fragments of reelin, therefore, to determine the specific fragments of reelin released in response to HNP, we lyophilized the HNP-induced HCAEC culture medium and used western blot technique to address this matter.

Reelin detected from lyophilized culture medium was prepared as follows. Two hundred and fifty-µL of HNP-stimulated HCAEC culture medium were lyophilized (LabConco, Freeze-Dry System 4.5) continuously at -40°C into powder form for 4 h. The powder was then reconstituted in 100-µL of 1XPBS and prepared for western blotting. Finally, 10-µL of 6X loading buffer was added to 50-µL of lyophilized culture medium. In experiments using cell lysates to detect reelin, 5-µg of HNP-stimulated HCAEC protein were prepared for western blot as previously mentioned. All samples were boiled for 3 min prior to electrophoreses.
Once prepared, samples and protein standard ladder were loaded onto a 5% SDS polyacrylamide gel and electrophoresed at 100V for 1.5 h. Proteins were transferred onto a nitrocellulose membrane at 15V, 4°C for 16 h. Subsequently, the membrane was blocked for 1 h and incubated with reelin antibody overnight at 4°C on a shaker. The following day, the membrane was washed and incubated with goat α-mouse IgG1 HRP for 1 h at room temperature, and the protein expressions were developed onto a film as previously stated.

3.6 Endothelial dysfunction

3.6.1 HCAEC stimulation

Seeded HCAEC were stimulated with PBS, mrRLN 10- or 20-µg/mL, HNP 1- or 10-µg/mL with/without reelin neutralizing antibody, CR-50 (MBL, Nagoya, Japan), or mouse IgG at 10- or 20-µg/mL for 6 h. Culture medium and protein were collected as previously mentioned. Experiment was repeated to reach a sample size n = 5. Experiment with CR-50 treatment was repeated to reach a sample size n = 3.

3.6.2 Measurement of endothelial dysfunction

Endothelial and inducible nitric oxide synthase (eNOS and iNOS, respectively) are nitric-oxide generating enzymes, and imbalances of the two enzymes are classical markers of endothelial activation [23, 263-267]. To determine endothelial activation, eNOS and iNOS protein expression were examined by western blot. Briefly, 5-µg of mrRLN-stimulated HCAEC protein and protein standard ladder were loaded onto 8% SDS polyacrylamide gel and electrophoresed at 100V for 1.5 h. Proteins were transferred onto a nitrocellulose membrane at 400mA for 2.5 h. Subsequent stages of western blotting were conducted as outlined in section 3.2.4.
3.6.3 Measurement of nitric oxide

Nitric oxide is a potent vasodilator and marker of endothelial function. Due to its gaseous properties, nitric oxide is commonly determined indirectly by measuring the nitrate/nitrite concentration by the Griess Assay (Cayman Chemicals, Ann Arbor, MI). Culture medium from HNP-stimulated HCAEC were used to determine the nitrate/nitrite concentration according to the instructions from the commercially available colorimeter assay kit.

3.6.3.1 Measurement of reactive nitrogen species

In order to assess the consequence of nitric oxide synthase expressions, it was important to demonstrate how NOS can influence endothelial function. To address this, the presence of RNS was determined from HNP- and RLN-treated HCAEC protein lysates by a nitrotyrosine ELISA (Oxis International, Foster City, CA). Nitrotyrosine concentration was then normalized by the samples corresponding protein concentrations.

3.7 Platelet aggregation

3.7.1 Platelet-rich plasma (PRP) isolation

The following protocol was approved by the Institutional Research Ethics Research Board. Healthy, non-smoking, non-medicated 20- to 40-year-old male volunteers were recruited for this study. Venous blood was collected with a 21¾-gauge butterfly needle into a 3.8% sodium citrate (1/9 v/v) vacutainer. Blood was centrifuged at 300 G for 7 min at room temperature to obtain the PRP, followed by a centrifugation of 1000 G for 15 min at room temperature to obtain the platelet-poor plasma (PPP). PRP was counted and diluted with PPP to a final concentration of 2x10^6 platelets/mL.
While previous studies have used syringes to draw blood, we used sodium-citrate coated vacutainers to collect blood. However, due to the shear stress from the vacutainers, platelets may have been slightly activated during the collection. Therefore, a lower dosage of ADP was used to induce the ‘first-wave’ of platelet aggregation.

3.7.2 Aggregation protocol

Adenosine diphosphate (ADP) is a physiological agonist of platelet aggregation, known to induce minimal granule release at low concentrations [268], thereby inducing the ‘first-wave’ of platelet aggregation. Remarkably, HNP was has previously been shown to sustain ADP-stimulated platelet aggregation by inducing a ‘second-wave’ of platelet aggregation mediated by the LRP8-signaling pathway [174]. In order to differentiate and observe the first and second wave of aggregation, we continued to utilize ADP as our platelet agonist.

Human PRP (250 µL, 2x10^8 platelets/mL) were pre-treated with 0.5 µM ADP (Sigma, St. Louis, MO) for 1 min, followed by a stimulation of PBS, 10-µg/mL mouse recombinant reelin (R&D Systems, Minneapolis, MN), or 10-µg/mL HNP with or without 10-µg/mL CR-50. Aggregation was evaluated by a computerized Chrono-log Aggregometer (Chrono-Log Corportation, Havertown, PA; generously lent by Dr. Heyu Ni, St. Michael’s Hospital). Experiment was repeated to reach a sample size n = 5. Aggregation was determined by the amount of light passing through the cuvette containing the platelets. As platelets aggregate, more area is created for light to pass through, which directly correlates to the percentage of aggregation. The results are plotted as time elapsed vs. % light transmission on a computerized system. A small magnetic stir bar, adjusted to 1000 rpm, was present to allow continued mixing of platelets and agonist while aggregation was recorded.
3.8 Statistical Analysis

The collected data are presented as mean±standard error of n experiments unless noted otherwise. Statistical significance was determined using one-way ANOVA, followed by a Tukey’s Multiple Comparison Test. A P-value less than 0.05 was considered statistically significant. Analyses were conducted using GraphPad Prism version 5.00c (GraphPad Software Inc., San Diego, CA).
Chapter 4: Results

4.1 Objective #1: To examine the role of HNP-induced reelin release and reelin-LRP8 interaction.

4.1.1 Specific Aim #1: HNP induced reelin protein expression and release in HCAEC

4.1.1.1 Reelin expression by western blot

4.1.1.1.1 Reelin expression from cell lysate

A significant increase in reelin 300-kDa protein expression was observed in HCAEC stimulated with HNP at 1- and 10-µg/mL, as determined by the band intensities from western blots, and normalized by the corresponding loading control: lamin. HNP increased reelin 300-kDa expression from 0.244±0.046 in vehicle control to 0.708±0.24 at 1-µg/mL and 0.846±0.24 at 10-µg/mL HNP (Figure 7A). No significant difference was observed between HNP 1- and 10-µg/mL treatment. Interestingly, endothelial stimulation with 10-µg/mL HNP for 6 h yielded a significant decrease in full-length reelin protein expression (0.428±0.027 fold-decrease; p<0.05 vs control). While stimulation with 1-µg/mL HNP had no significant effect, however, a downward trend was observed (0.504±0.163 fold-decrease vs control) (Figure 7B).

4.1.1.1.2 Reelin expression from lyophilized culture medium

When quantifying the amount of reelin from culture medium of HNP 1- and 10-µg/mL stimulated HCAEC, we observed a negative trend, but not significant, in full-length reelin release (0.22±0.146 and 0.062±0.044, respectively, compared to control at 0.882±0.516, p=0.22) (Figure 8A). Conversely, HNP treatment at 1- and 10-µg/mL only showed a moderate increase in 300-kDa reelin compared to control (1.598±0.076 and 1.840±0.23, respectively, compared to control at 1.33±0.19, p=0.266) (Figure 8A). Although not
Figure 7. HNP induce reelin 300kDa protein expression. HCAEC were stimulated for 6 h with 1- or 10-µg/mL HNP, or vehicle control in 1% serum medium. Protein lysates were collected to determine reelin protein expression by western blotting. *p<0.05 vs control.
Figure 8. HNP decrease reelin full-length secretion. HCAEC were treated with HNP 1- or 10-µg/mL, or vehicle control for 6 h. A. Specific fragments of reelin released were determined by western blotting of lyophilized culture medium. B. Culture medium was collected for analysis of reelin release by ELISA. *p<0.05 vs control.
statistically significant, the pattern of the reelin fragments released treads in parallel with the expression found inside the cell. It is possible that the insignificance may be due to the degradation of reelin protein during the lyophilization procedure, which is a technical limitation to our study. Future studies may look into the availability of specific reelin fragment antibodies to construct a homemade ELISA kit. The specific reelin fragment antibodies could be used to coat the ELISA plate, and non-lyophilized culture medium would be used in the experiment. This would avoid the potential degradation of the reelin fragments during the sample preparation phase.

4.1.1.2 Reelin expression by ELISA

To confirm the potential paracrine property of reelin, we first sought to investigate the effect of HNP on reelin release. When HCAEC were treated with HNP, a decrease in full-length reelin concentration in the culture medium was observed (Figure 8B). Stimulation of HNP 1- and 10-µg/mL yielded a reelin full-length concentration of 0.189±0.029- and 0.074±0.025-ng/mL respectively. Only stimulation of HNP-10-µg/mL yielded a significant decrease in reelin concentration when compared to vehicle control at 0.219±0.055-ng/mL (p<0.05).

4.1.2 Specific Aim #2: HNP-induced reelin protein expression and release was mediated by the P2Y6-signaling pathway

To confirm that reelin was indeed induced by HNP stimulation, we examined reelin’s expression in HCAEC blocked of P2Y6 but stimulated with HNP. P2Y6-blockade was performed by pre-treating HCAEC with an irreversible P2Y6 antagonist, MRS2578, for 30 min prior to the stimulation of HNP for 6 h. Like previously, the presence of 10-µg/mL HNP in HCAEC significantly increased the expression of reelin 300-kDa compared to PBS conditions.
(0.641±0.45 and 0.135±0.112, respectively; p<0.05). The observed response was significantly attenuated when HCAEC were pre-treated with MRS 2578 at 0.5-µM and 1-µM (0.038±0.035 and 0.174±0.098, respectively; p<0.05), even in the presence of HNP 10-µg/mL (Figure 9A). MRS 0.5- and 1-µM stimulation alone had no significant effect in inducing reelin 300kDa protein expression (0.307±0.134 and 0.330±0.097, respectively, vs control at 0.098±0.057). We then evaluated if pre-treatment of MRS 1-µM was able to recover the diminished full-length reelin release upon HNP stimulation. Indeed, the pre-treatment of MRS 1-µM demonstrated a significant increase in full-length reelin compared to stimulation of HNP alone (1.66±1.39 vs 0.311±0.11, respectively; p<0.05) (Figure 9B). These results suggest that P2Y₆ mediated HNP-induced reelin expression in HCAEC. Future studies would need to confirm the role of P2Y₆ in mediating the release of reelin by determining the reelin content in the culture medium of HNP-treated HCAEC.

4.2 Objective #2: To determine the specific effect of reelin in endothelial function and platelet aggregation.

Endothelial dysfunction and platelet aggregation are paradigm events in atherogenesis. Given the notion that HNP was able to induce reelin protein expression and release, we sought to explain the functional role of reelin in mediating endothelial dysfunction and platelet aggregation. Endothelial activation and dysfunction was assessed by measuring the protein expression of nitric-oxide (NO)-producing enzymes, endothelial and inducible nitric oxide synthase (eNOS and iNOS, respectively), and NO content [23-25, 65, 264, 269, 270]. PRP was used in platelet aggregation experiment in order to characterize a more physiological condition, whereby plasma proteins are present to also participate in the platelet aggregation.
Figure 9. HNP-induced reelin expression is mediated by P2Y6-signaling pathway. HCAEC seeded at 1x10^5 cells/mL were pre-treated in 1% FBS medium with MRS2578 at 0.5- or 1-µM, or DMSO control 30 min prior to the addition of HNP 10-µg/mL or PBS control for 6 h. A. Reelin 300kDa was quantified by western blot from protein lysate. B. Culture medium were collected for analysis of full-length reelin by ELISA. *p< 0.05 vs control; §<0.05 vs HNP 10-µg/mL.
4.2.1 Specific Aim #1: Reelin attenuated eNOS and increased iNOS protein expression

4.2.1.1 HNP-induced endothelial activation (proof-of-concept)

Before we could measure the impact of reelin in modulating NOS expression, we had to validate the role of HNP in modulating NOS protein expression. Application of 10-µg/mL HNP was found to significantly decrease eNOS protein expression in HCAEC following a 6 h stimulation (0.045±0.01 vs 0.172±0.055 in control; p<0.05). HNP 1-µg/mL showed a downward trend in eNOS protein expression, however, it was not significant compared to control (0.062±0.0173 vs 0.172±0.055; p=0.16) (Figure 10A). Conversely, endothelial stimulation with 10-µg/mL HNP demonstrated a significant increase in iNOS expression compared to control cells (0.169±0.034 vs 0.066±0.016; p<0.05) (Figure 10B). Similar to the eNOS response, HCAEC stimulated with HNP 1-µg/mL for 6 h did not yield a significant increase in iNOS expression (0.078±0.041 vs 0.066±0.016; p=0.75).

4.2.1.2 Reelin-induced endothelial activation

We evaluated the effect of reelin on NOS protein expressions. Stimulation of HCAEC with mouse recombinant reelin (mrRLN) at 1- and 5-µg/mL for 6 h resulted in a significant decrease in eNOS expression (0.098±0.031 and 0.13±0.037, respectively, vs control at 0.376±0.096; p<0.05) (Figure 11A). Such response due to reelin was comparable to HNP 10-µg/mL stimulation at 0.099±0.036. Correspondingly, parallel to HNP 10-µg/mL stimulation, treatment of mrRLN 5-µg/mL in HCAEC revealed a significant increase in iNOS expression from 0.041±0.008 in control cells to 0.097±0.024 (p<0.05) (Figure 11B). Stimulation with mrRLN at 1-µg/mL had no effect on iNOS expression (0.04±0.003 vs control). The
Figure 10. HNP attenuates eNOS, while augmenting iNOS, protein expression. HCAEC seeded at 1x10^5 cells/mL were stimulated in 1% serum medium with vehicle control, or HNP at 1- or 10-µg/mL for 6 h prior to the collection of protein lysates. Protein expression of eNOS (A) and iNOS (B) were measured by western blots and analyzed by BioRad GS-800 Densitometer. *p<0.05 vs control.
Figure 11. Reelin reduces eNOS, and enhances iNOS protein expression in HCAEC. HCAEC were stimulated in 1% serum medium with vehicle control, or mouse recombinant reelin at 5- or 10-µg/mL for 6 h prior to the collection of protein lysates. Protein expression of eNOS (A) and iNOS (B) were quantified by western blots, and band intensities were analyzed by the BioRad GS-800 Densitometer. *p<0.05 vs control.
comparable responses of both HNP and mrRLN serve to suggest that RLN mediates HNP-induced NOS protein expression. However, the specificity of reelin must still be proven.

The variance observed in eNOS response after stimulation of HNP 10-µg/mL in the proof-of-concept experiment vs reelin experiment may be accountable by technicalities of the experiment including different lot numbers of eNOS antibody used in the western blot and passages of endothelial cells used.

4.2.2 Specific Aim #2: Reelin induced reactive nitrogen species production

In order to address the consequence of RLN-induced eNOS decrease and iNOS increase, we measured the effects of RLN on peroxynitrite formation by measuring nitrotyrosine concentration.

4.2.2.1 HNP-induced nitrosative stress (proof-of-concept)

Like previously, we first validated the nitrogen species concentration in HNP-induced HCAEC. A significant increase in nitrate/nitrite concentration was observed in HCAEC stimulated with 10-µg/mL HNP vs control (14.2±1.68-µM vs 2.05±0.17-µM; p<0.05), but not with 1-µg/mL HNP (3.18±0.17-µM) (Figure 12A). Compatibly, nitrotyrosine formation also revealed a similar trend, with a significant increase from 71.33±8.83-nmol/g protein in control to 174.9±23.8- and 183±34.2-nmol/g protein in HNP 1- and 10-µg/mL, respectively (Figure 12B).
Figure 12. HNP induces production of nitrogenous species. 1x10^5 cell/mL HCAEC were incubated with 1- or 10-µg/mL HNP, or vehicle control for 6 h. A. Culture medium were analyzed for production of nitrate/nitrite, reflecting the presence of nitric oxide. B. Cell lysates were collected for analysis of nitrotyrosine, indicating the presence of ONOO^- *p<0.05 vs control.
4.2.2.2 Reelin-induced nitrosative stress

We quantified the amount of nitrotyrosine concentration in RLN-stimulated HCAEC. RLN stimulation revealed a significant increase in nitrotyrosine production from 63.5±3.64-nmol/g protein in vehicle to 158.5±8.7- and 191.2±21.1-nmol/g protein with RLN at 1- and 5-µg/mL, respectively (Figure 13).

4.2.3 Specific Aim #3: Reelin enhanced human platelet aggregation in response to ADP

To preserve the physiological condition of platelet aggregation, platelet-rich plasma (PRP) was used to perform the aggregation experiments. After incubating human PRP with 0.5-µM ADP for 1 min, PRP elicited the ‘first-wave’ of platelet aggregation that was not sustained over the 12 min of recording time (Figure 14). In addition, no difference in final platelet aggregation was observed between incubation of PRP with ADP alone and ADP with vehicle control (4.207±2.43% vs 4.817±1.69%; p=0.804) (Figure 14). Interestingly, after 1 min of ADP priming, HNP 10-µg/mL generated a second wave of platelet aggregation and significantly prolonged the aggregation response of ADP, with an end aggregation of 50.871±14.38% (Figure 14). Subsequently, we evaluated the effect of reelin on platelet aggregation. Comparable to HNP, reelin at 5-µg/mL also prolonged the transient aggregation response of human platelets to ADP with an end aggregation of 37.683±15.52% (Figure 14).

Finally, the ADP concentration chosen was based on our dose-dependent ADP curve for human PRP (data not shown). We have found that 0.5-µM ADP to be the minimal possible concentration to provoke the ‘first-wave’, but not ‘second-wave’, of platelet aggregation in human PRP. Although previous studies have used higher doses of ADP to generate the ‘first-
Figure 13. **Reelin induce nitrotyrosine production.** Following the treatment of HCAEC with HNP 10-µg/mL, reelin 1- or 5-µg/mL, or vehicle control for 6 h, cell lysates were analyzed for the production of nitrotyrosine by ELISA. *p<0.05 vs control
Platelet aggregation is prolonged in platelets stimulated with ADP and reelin in comparison to stimulation with ADP alone. A. Human PRP were primed with 0.5-µM ADP for 1 min, followed by the addition of HNP 10-µg/mL with or without reelin neutralizing antibody (CR 50) 10-µg/mL, or recombinant reelin at 5-µg/mL. Platelet aggregation was recorded for 12 min with the Chronolog Aggregometer. B. After 12 min of recording, the final aggregation of platelets were compared across groups. *p<0.05 vs control; §p<0.05 vs HNP 10-µg/mL.
wave’ of platelet aggregation [174], however, this may be explained by the differences in PRP isolation protocols.

In summary, like HNP, reelin was able to prolong the platelet aggregation response ADP, that is not present in ADP stimulation alone.

4.2.4 Specific Aim #4: Neutralized reelin (CR50) alleviated endothelial dysfunction and platelet aggregation.

To confirm the specificity of reelin in HNP-induced endothelial dysfunction and platelet aggregation, an inhibition experiment was performed whereby reelin neutralizing antibody (CR50) was co-treated with HNP 10-µg/mL. CR50 antibody inhibits reelin function by interrupting the crucial formation of the reelin-reelin homopolymer complex that is required in activating disabled-1 in LRP8 [252].

4.2.4.1 CR50 increase eNOS and decrease iNOS protein expression

HCAEC treated with HNP 10-µg/mL in the presence of 10- or 20-µg/mL CR50 for 6 h exhibited a significant recovery of eNOS compared to HNP alone. As reported previously, HNP 10-µg/mL was able to induce a significant decrease in eNOS protein expression (0.068±0.01 vs 0.183±0.02, respectively; p<0.05). Remarkably, the presence of reelin neutralizing antibody (CR50) at 10- and 20-µg/mL was able to block the HNP-induced eNOS attenuation (0.201±0.06 and 0.298±0.03, respectively; p<0.05). The neutralizing effort of the antibody was confirmed when mouse IgG controls at the same dosage revealed a response comparable to stimulation of HNP 10-µg/mL alone (0.094±0.029 at 10-µg/mL and 0.064±0.03 at 20-µg/mL of mouse IgG) (Figure 15A).
Congruently, HCAEC treated with CR50 at 10- and 20-µg/mL for 6 h also rescued the HNP-mediated iNOS elevation. Consistently, endothelial cells treated with HNP 10-µg/mL enhanced iNOS protein expression compared to control (0.258±0.06 vs 0.111±0.03, respectively; p<0.05) (Figure 15B). Moreover, a significant reduction in iNOS protein expression was observed in HCAEC treated with HNP 10-µg/mL in the presence of CR50 (0.12±0.01 at 10-µg/mL and 0.042±0.005 at 20-µg/mL; p<0.05). Once again, the neutralizing effort of CR50 was supported by the increase in iNOS expression in cells treated with HNP 10-µg/mL in the presence of mouse IgG 10- and 20-µg/mL for 6 h. Although only incubation of 10-µg/mL (0.378±0.1), not 20-µg/mL (0.31±0.2), mouse IgG control and HNP 10-µg/mL resulted in a significant increase in iNOS compared to vehicle, however, this may be justified by the low sample size (n=3). Additional repeats of the experiment may off set the presence of outliers.

4.2.4.2 CR50 attenuates HNP-induced nitrotyrosine concentration

The effect of CR50 in HNP-stimulated HCAEC was further supported by the nitrotyrosine concentration. As expected, HNP induced a significant increase in nitrotyrosine concentration from 60.5±5.6-nmol/g protein in control to 116.3±0.3-nmol/g protein (p<0.05). Interestingly, HCAEC stimulated at HNP 10-µg/mL with CR50 10- or 20-µg/mL resulted in a significant decrease in nitrotyrosine concentration to 61.67±9.66- and 51.9±18.6-nmol/g protein, respectively. Compatibly, co-stimulation of HNP 10-µg/mL with 10- or 20-µg/mL mouse IgG control resulted in nitrotyrosine concentrations comparable to HNP alone (117.9±10.9- and 128.77±6.8-nmol/g protein, respectively) (Figure 16).
Figure 15. Reelin neutralizing antibody alleviates HNP-induced eNOS decrease and iNOS increase. HCAEC were stimulated with vehicle control, or HNP 10-µg/mL in the presence of CR50 or mouse IgG control at 10- or 20-µg/mL for 6 h. Protein lysates were collected for the analysis of A. eNOS and B. iNOS protein expression by western blot. *p<0.05 vs control, §p<0.05 vs HNP 10-µg/mL.
Figure 16. Reelin neutralizing antibody reduces HNP-induced nitrotyrosine production. HCAEC were stimulated with vehicle, or HNP 10-µg/mL with CR50 or mouse IgG control at 10- or 20-µg/mL for 6 h. Protein lysates were collected for the quantification of nitrotyrosine. *p<0.05 vs control; §p<0.05 vs HNP 10-µg/mL.

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<th>Condition</th>
<th>Nitrotyrosine (nmol/g protein)</th>
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<td>Veh</td>
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<td>HNP 10 µg/mL</td>
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4.2.4.3 CR50 attenuates HNP-induced platelet aggregation

The inhibitory effect of CR50 was evaluated in platelet aggregation. Human PRP was primed with 0.5\(\mu\)M ADP for 1 min prior to the co-stimulation of HNP 10-\(\mu\)g/mL and CR50 10-\(\mu\)g/mL. Compared to HNP alone, the presence of CR50 significantly reduced the end aggregation from 50.87±14.4% to 29.16±12.2% (p<0.05) (Figure 14). Although the decrease in aggregation elicited by CR50 at 10-\(\mu\)g/mL was not significantly different from the response elicited by reelin at 5-\(\mu\)g/mL, however, a dose-dependent curve with increasing CR50 concentration may address this concern.

4.3 Result summary

In summary, we have shown that HNP induces reelin protein expression and release in HCAEC. The effect of reelin was specific to HNP since the blocking of P2Y\(_6\)-receptor attenuated the HNP-induced reelin expression. Reelin was also shown to attenuate eNOS and elevate iNOS protein expression in HCAEC. This was consequently found to increase nitrotyrosine concentrations. Moreover, in platelets, reelin was shown to induce a second-wave of platelet aggregation following ADP priming. The effect of reelin in inducing endothelial activation and platelet aggregation was significantly attenuated when cells were treated with a reelin-neutralizing antibody (CR50).
Chapter 5: Discussion

5.1 Main findings

In this study, we have demonstrated a novel mechanism of HNP inducing endothelial activation and platelet aggregation mediated through an LRP8 ligand, reelin. HNP was shown to activate the purinergic receptor, P2Y6, on human coronary artery endothelial cells (HCAEC), and induce endothelial-derived reelin expression and release. Consequently, reelin down-regulated eNOS and up-regulated iNOS protein expression in HCAEC. This was paralleled with an observed increase in nitrotyrosine content, suggesting the occurrence of nitrosative stress in the endothelial cells. In isolated human platelets, reelin was able to sustain ADP-mediated platelet aggregation by inducing a second wave of platelet aggregation. Lastly, to confirm the specificity of reelin, the functional activity of reelin was blocked using a reelin neutralizing antibody: CR50. Indeed, in the presence of CR50, HNP-induced endothelial dysfunction and platelet aggregation was significantly attenuated.

5.2 Potential mechanism of HNP-induced reelin expression and release

We have demonstrated the ability of HNP to induce a significant increase in reelin 300-kDa, and a decrease in reelin 400-kDa protein expression in HCAEC. Reelin is a large extracellular matrix protein that exists in a multitude of fragments after proteolytic cleavage by tPA or matrix metalloproteinase [244-248, 250, 262]. Correspondingly, studies have determined that it is the central-containing segment that binds and activates the receptor LRP8 [244, 245, 262], with the N-terminus responsible for forming the compulsory functional reelin homodimer [245-248, 250, 271]. Therefore, to exert the reelin signaling pathway, much attention is directed to focus on full-length reelin and reelin fragment 300-kDa. Previous studies have already documented the overexpression of reelin 300-kDa in patients with liver cirrhosis [257] and ocular injury [258], suggesting the involvement of reelin 300-kDa in mediating
inflammatory events. However, the mechanism by which HNP induces reelin expression requires additional analysis.

5.2.1 HNP mediates reelin expression

In our study, we have shown that upon HNP stimulation, HCAEC up-regulates the reelin protein expression, specifically the reelin 300-kDa fragment. However, the mechanism of HNP-induced reelin expression remained to be further examined. Over the last 10 years, research has been conducted to determine the various different extracellular signal molecules that could induce reelin expression. Among them are TGF-β, thyroid hormone, retinoic acid, β-amyloid, corticosterone, and 17β-estradiol [272-277]. Interestingly, even injury to the adult mice brain would elicit an increase in reelin expression [278]. The common ground of this induction is the epigenetic regulation of the reelin promoter. In a study conducted by Chen et al, it was shown that reelin expression was dependent on the methylation status of its promoter. Using the methylation inhibitor, aza-2'-deoxycytidine, reelin mRNA was shown to exhibit a 60-fold increase [279]. Similarly, the use of histone deacetylase inhibitors (trichostatin A) and valproic acid also induced the expression of the endogenous reelinsw promoter [279]. Moreover, the start site of the human reelin RNA is very GC-rich (75%) and forms large CpG islands, a genomic region with high frequencies of cytosine and guanine linked by a phosphodiester bond [279]. This provides the notion that the reelin promoter is epigenetically regulated, specifically through changes in the DNA cytosine methylation [280-283]. Unfortunately, currently there is a deficiency of knowledge of the relationship between HNP or P2Y6 in regulating histone deacetylase. In addition to histone deacetylases, future studies may focus on the relationship between HNP or P2Y6 and transcriptional regulators such as Sp1 and Pax6. In a study conducted by Chen et al in 2007, the group found that Sp1
and Pax6 was able to bind to the enhancer site of reelin to induce an increase expression of reelin mRNA [274].

Alternatively, the reelin promoter has four potential sites for transcriptional regulation, one of which is NF-κB [284]. Interestingly, P2Y6 has previously been implicated to activate the NF-κB signaling pathway [182, 285, 286]. This may provide an alternative mechanism through which HNP-activated P2Y6 may govern the expression of reelin.

5.2.2 HNP mediates reelin processing
As mentioned previously, reelin can undergo proteolytic cleavage by tPA or matrix metalloproteinase [244-248, 250, 262, 287]. Therefore, a potential mechanism of elevating reelin 300-kDa is by increasing the cleavage of the full-length molecule. This can be potentially accomplished by: 1) HNP directly cleaving reelin; or 2) HNP increases the expression and/or activity of tPA or matrix metalloproteinase. Of the two possibilities, the former mechanism may be of least likelihood. Unlike their sister family, the serine protease serprocidins, HNP lacks enzymatic activity [288, 289], which renders them unable to conduct proteolytic cleavage on other molecules. Furthermore, reelin’s proteolytic processing occurs at the N- and C-terminus, which recent studies have shown to have specific requirements. Proteases involved in the N-terminus cleavage of reelin require a zinc ion for its catalytic activity, contains a furin-like proprotein convertase activity, and has a high binding affinity for heparin [246]. HNP, once released into the extracellular milieu upon neutrophil activation, is an active molecule that does not require a co-factor for its proper function. Also, due to the high basicity of reelin’s C-terminus [279], it is likely that HNP’s cationic charge would be electrostatically repelled from the cleavage site. Collectively, this would strongly suggest that
HNP may mediate reelin processing indirectly, perhaps by increasing the expression or activity of tPA or matrix metalloproteinase.

Indeed, in a study conducted by Higazi et al in 1996, the group showed that HNP was able to modulate tPA binding to fibrin and endothelial cells [290], suggesting HNP could alter the plasmin activity through a direct interaction [260]. Moreover, in a study that was recently published by Ahn et al, the group showed that HNP-1 regulated the production of MMPs, a process that was dependent on the regulation of the JNK, ERK and NF-κB pathway [291]. Therefore, HNP-stimulated HCAEC could have triggered a signaling cascade that resulted in an increase in protease expression that was responsible for the increase in reelin processing.

Although it is important to recognize that HNP stimulation of HCAEC induced an increase in reelin 300-kDa, however, our experiments conducted could not differentiate if the observed increase was due to: a) an increase in proteolytic activity on basal level of full-length reelin; or b) an increase in full-length reelin expression that was subsequently subjected to processing to generate the large pool of reelin 300-kDa fragment; or c) a concurrent experience of both a and b. Further research should be performed to elucidate the mechanism governing the downstream pathways affecting reelin expression and processing. Such studies may include analyzing the mRNA content of reelin using qRT-PCR after HNP stimulation.

**5.2.3 HNP mediates reelin release**

The role of reelin acting as a secretory molecule was chiefly established by their localization in the golgi apparatus and secretory organelles [292, 293]. In addition to neuronal cells, secretion of reelin has also been found in lymphatic endothelial cells and liver [255, 256]. However, the mechanism underlying the release is unknown.
In our study, we have shown that HNP was able to induce HCAEC reelin release, since the presence of a reelin neutralizing antibody (CR50) in the culture medium was able to inhibit HNP-mediated eNOS attenuation and iNOS elevation. This phenomenon then raises the question of what is the relationship between HNP and secretory organelles? In a study by Befus et al, the group showed that HNP was able to induce histamine release in mast cells that was dependent on a G-protein coupled receptor [294]. Likewise, our lab demonstrated that HNP binds to the purinergic G-protein coupled receptor, P2Y$_6$, on human lung epithelial cells to induce IL-8 expression and release [185]. Correspondingly, by knocking down P2Y$_6$, HNP-mediated IL-8 expression was significantly attenuated [185]. These provide an insight in the role of purinergic receptors in shuttling secretory organelles to the plasma membrane and facilitate exocytosis of granular content.

Certainly, P2Y$_6$-signaling has previously been shown to activate PLC to increase intracellular concentrations of DAG and IP$_3$ in pancreatic β-cells [295]. The resultant increase in Ca$^{2+}$ content enabled the shuttling of insulin into the extracellular milieu [295]. The importance of elevating Ca$^{2+}$ ions to mediate P2Y$_6$-dependent exocytosis was further supported in a study conducted by Kottgen et al. The group shown that P2Y$_6$ receptor in colonic epithelial cells stimulated NaCl secretion by a synergistic increase of intracellular Ca$^{2+}$ and cAMP [296]. It is therefore possible that secretion of reelin into the extracellular milieu is reliant on the P2Y$_6$ activated Ca$^{2+}$-dependent exocytosis.

5.3 Reelin-induced endothelial activation

5.3.1 HNP-induced endothelial activation
Disruption of vascular integrity is a primitive event observed in early atherosclerosis [1-3, 67, 297]. Under a quiescent state, endothelial cells seldom attract leukocytes, however, upon endothelial activation, expression of surface adhesion molecules provide a stepping stone for leukocyte recruitment [23, 298-300]. These surface adhesion molecules include ICAM-1 and VCAM-1. To elucidate the role of HNP in inducing endothelial activation, our lab has previously demonstrated that stimulation of HCAEC with HNP elicited an increase in VCAM-1 expression [174]. In addition to endothelial activation, our group has also shown that HNP was able to induce epithelial activation. Using SAEC and A549 epithelial cells, stimulation of HNP induced a 2- and 3-fold increase in ICAM-1 expression in SAEC and A549, respectively [162]. These provide support for the role of HNP in inducing cell activation.

5.3.1.1 HNP-mediated eNOS and iNOS protein expression

Whilst the expression of surface adhesion molecules, another commonly observed activity during endothelial activation is the attenuation of endothelial nitric oxide synthase (eNOS) protein expression, the hallmark enzyme that governs basal regulatory function in the vasculature [301]. Consequentially, the cell begins to encounter a diminished pool of nitric oxide, but an elevation of reactive oxygen or nitrogen species. This activation of the endothelium eventually leads to the condition endothelial dysfunction. Endothelial dysfunction is frequently shown to exhibit an attenuation of eNOS and an elevation of iNOS protein expression [302-304].

Our study has shown that in the presence of HNP, HCAEC responds with a decrease in eNOS and an increase in iNOS protein expression. These results are in congruency with results published by Kougias et al in 2006, whereby it was shown that HNP stimulation on
porcine coronary arteries elicited a decrease in eNOS mRNA [201]. Consequently, the endothelium-dependent vasorelaxation was attenuated [201].

Concurrently, we also observed that HNP-stimulated HCAEC had a profound increase in nitrotyrosine content, a resultant product from the nitrosylation of proteins by the reactive nitrogen species, peroxynitrite. The source of the NO that drove the synthesis of peroxynitrite is believed to be from the increased expression of iNOS. When we quantified the NO content indirectly by measuring the nitrate/nitrite concentration through the Griess assay, we found that HNP induced a significant increase in nitrate/nitrite concentration. Since eNOS protein expression was significantly attenuated upon HNP stimulation, it would suggest that iNOS would be the major contributor of NO, and eventually, peroxynitrite. iNOS has previously been shown to induce peroxynitrite production which consequently induced myocardial contractile failure [305]. Moreover, the rise in peroxynitrite could provide a positive feedback loop to further increase iNOS protein expression through the activation of NF-κB [306], therefore, supplementing endothelial activation. Akin to our findings, Kougias et al also found that stimulation of porcine coronary arteries with HNP also induced an increase in superoxide anion concentration [201], a trait indicative of oxidative stress and endothelial activation. Now, the molecular mechanism of HNP-induced endothelial activation has been weakly explored. However, our study may provide insights on the mechanism, through the signaling pathway of reelin.

5.3.2 Reelin and NOS

5.3.2.1 Use of mouse recombinant reelin on human cells

To address the molecular mechanism of HNP-induced endothelial activation, we employed a recombinant reelin to stimulate HCAEC. Prior to discussing the results of the reelin-stimulated
HCAEC, I would like to address the matter regarding the use of a mouse recombinant protein on human cells: 1) although we conducted an interspecies stimulation, however, when we ran a BLAST search to compare the protein sequence of reelin between human and mouse, it revealed a 98% homology shared between the two species [Figure 17]; 2) studies have shown that the human and mouse cDNAs of reelin share an 88% nucleotide identity as well as a high degree of sequence similarity on the transcription start site [249, 279], and 3) in consistent with a recently published article by Lutter et al, the group utilized the same recombinant protein to induce MCP-1 production in primary human lymphatic endothelial cells [255]. This suggests the functional activity of the recombinant protein to elicit the activation of appropriate signaling pathways in endothelial cells to mediate an inflammatory response. Nevertheless, the responses provoked by the mouse recombinant reelin protein in our experiments should be confirmed with a human recombinant reelin protein.

5.3.2.2 Reelin and NOS

We have shown that stimulation of HCAEC with mouse recombinant reelin exhibited a comparable response to HNP. Like HNP, mouse recombinant reelin down-regulated eNOS and up-regulated iNOS protein expression. Moreover, the nitrotyrosine concentration was also significantly elevated by treatment of reelin in comparison to vehicle control. The specificity of reelin was confirmed with the use of the reelin neutralizing antibody (CR50). To our amazement, the presence of CR50 significantly attenuated HNP-induced endothelial activation.

Due to the prominent expression of reelin in the brain, attention has been more focused on the relationship between reelin and neuronal NOS (nNOS). nNOS is a constitutively expressed nitric oxide synthase, with structural and functional similarities to eNOS [41].
Figure 17. Mouse reelin is 95% homologous to human reelin. A protein sequence blast search was conducted to determine the percentage homology between mouse reelin and human reelin.
Nevertheless, the relationship between reelin and nNOS may provide us with insight to the relationship between reelin and eNOS, and iNOS. In a study conducted by Romay-Tallon et al, reelin was found to be co-expressed with nNOS in the dentate gyrus neuron of mice [307]. Moreover, in a study by Herrman et al, it was shown that reeler mice have an attenuated nNOS protein expression in the olfactory bulb [308]. This suggests the potential role of reelin in modulating NOS protein expression. Unfortunately, further study is necessary to elaborate on the mechanism in which reelin can modulate NOS protein expression.

5.3.2.3 Reelin-mediated endothelial activation through its receptors

Currently, there is a deficiency of knowledge on how reelin may directly modulate the expression of eNOS and iNOS. However, this does not negate the possibility of reelin indirectly modulating the NOS expression through the activation of reelin receptors. Recently, much attention has been focused on the role of LRP8 in mediating the development of atherosclerosis. Interestingly, LRP8 is a conventional reelin receptor.

In a crucial study conducted by Ramesh et al, the group showed that activation of the LRP8 receptor in vivo yielded a significant increase in leukocyte recruitment to the vascular endothelium [230]. It was later found that LRP8 increased the activity of the PP2A phosphatase, which reduced the eNOS protein activity and endorsed endothelial activation [230]. This was in parallel with our study published in 2011, whereby LRP8−/− mice showed a significant attenuation in leukocyte rolling when given a bolus of HNP in comparison to wild-type mice [174]. Interestingly, LRP8 is a surface protein that is commonly found in the caveolae of cell membrane, a location in which eNOS is also localized in [309, 310]. This could provide the grounds for upon LRP8 activation, possibly through reelin, in endothelial
cells to inactivate eNOS and induce endothelial activation. Nevertheless, future studies may want to study the role of LRP8 in inhibiting eNOS protein expression, on a genomic level.

5.3.2.3.1 Alternative reelin receptors

As a conventional LRP8 ligand, it is postulated that reelin could act on the LRP8 receptor to attenuate eNOS and increase iNOS protein expression, however, we have yet to confirm that LRP8-signaling pathway was indeed activated. Future studies may consider quantifying the tyrosine phosphorylation of Dab-1 on LRP8 to confirm the activation of the signaling pathway. Moreover, although reelin is widely known to bind to LRP8, however, reelin is also known to bind to LRP8's sister receptor: VLDL receptor. We did not specify if the signaling pathway induced by reelin was due to LRP8 or VLDL receptor in our experimental model. However, our lab has previously shown that in mice deficient in all LDL receptor and LRP (LDLR⁻/⁻/LRP⁻/⁻), which also includes the deficiency in VLDL receptor, there was no significant difference in leukocyte recruitment after stimulation with HNP in comparison to LRP8⁻/⁻ mice [174]. This suggests that although other LDL receptor family members may play a role in leukocyte recruitment to the endothelium, it is primarily LRP8 that mediates this response. This response may also be similar in the reelin-induced endothelial activation. However, to provide a more evidentiary support, future studies should repeat the experiment with HCAEC transfected with VLDL receptor siRNA such that the only reelin receptor expressed on the cell is LRP8.

5.4 Reelin-mediated platelet aggregation

5.4.1 Reelin mediates HNP-induced platelet aggregation

We performed a platelet aggregation experiment to determine if HNP-induced platelet aggregation was mediated through the reelin molecule. After priming the platelet rich plasma
with ADP, HNP was shown to sustain the platelet aggregation responses, confirming the role of HNP as a novel platelet agonist [174]. Interestingly, when recombinant reelin was used instead of HNP, the sustenance of platelet aggregation was also observed. Although not as significant as the HNP stimulation, however that could be due to the discrepancy in molar-molar stimulation. While 10-µg/mL of HNP was used to stimulate the platelets, recombinant reelin was only stimulated at 5-µg/mL. Moreover, since HNP is a smaller molecule (~3-4-kDa) than reelin (300-400-kDa), the molar concentration of HNP used at 10-µg/mL was substantially higher than reelin at 5-µg/mL. Therefore, the discrepancy in molar ratio could account for the variance observed in the platelet aggregation effect between HNP and reelin. Nevertheless, the role of reelin in facilitating HNP-induced platelet aggregation was supported by the observation that platelet aggregation was attenuated when platelets were co-stimulated with HNP and CR50. This suggests that HNP induced a release of reelin from platelets, and it was the reelin that induced the platelet aggregation.

5.4.1.1 Reelin mediates HNP-induced platelet aggregation through LRP8 signaling

It is possible that the platelet aggregation induced by reelin were mediated by the LRP8 signaling pathway since LRP8 is the only LRP family member expressed in platelets [223]. Previously, it has been suggested that activation of LRP8 by LDL could elicit an activation of the p38 MAPK pathway, which would mediate a rise in cPLA2 activity and arachidonic acid content. This rise in membrane phospholipids would then facilitate the production of thromboxane A2, a potent platelet agonist known to induce a conformational change of αIIbβ3-integrin from inactive to active form [228]. Once active, αIIbβ3-integrin can bind to soluble fibrinogen and form platelet aggregates. Therefore, activation of LRP8 signaling pathway mediated by reelin could induce platelet aggregation by increasing the content of
thromboxane A₂. Alternatively, activation of LRP8 by reelin may have also down-regulate platelet eNOS protein expression, like the response found in reelin-treated HCAEC. Consequently, there would have been a diminished content of NO, which coincidentally, also plays a role in inhibiting platelet aggregation [226].

5.4.2 Reelin expression in platelets

Although platelets have been documented to express reelin [254], however, it is to our knowledge that it is not known how the expression of reelin is regulated in platelets. Equally to be elucidated is that it is not known which granules would reelin be stored in to mediate its release since glycoproteins can be found in both the α- and dense granules [311]. Moreover, it is also unknown how HNP could induce platelet granular release.

5.4.3 HNP-induced platelet aggregation

Recently, a paper published by Horn et al found that HNP induced platelet aggregation through the release of thrombospondin-1 [312]. Thrombospondin-1 is a platelet agonist found primarily in the α-granules of platelets [313], which can stimulate platelet aggregation by inhibiting the anti-thrombotic NO/cGMP signaling pathway [314]. The study itself was a ground breaking study that supported HNP’s role in inducing platelet aggregation, however, it left many questions that perhaps our study could provide additional insight. Firstly, although HNP was shown to bind to gel-filtered platelets, however, the study did not address the receptor to which HNP bounded to. This perhaps could have been P2Y₆ since our lab has previously elucidated the ligand-receptor relationship between HNP and P2Y₆. Secondly, prior to the release of granular content, platelets must be activated by an agonist. Although HNP itself could act as an agonist, it appears that it is reelin that plays the predominant role as the
platelet agonist. Purinergic receptors, P2Y₁ and P2Y₁₂, are commonly expressed on platelets to elicit platelet activation in response to ADP [315-318]. Although expressed on platelet surface in small quantities, P2Y₆ itself does not induce platelet activation [315]. Therefore, perhaps through the same mechanism observed in endothelial cells, HNP binds to platelet surface P2Y₆ to induce reelin expression and release. Subsequently, reelin may again act in an autocrine manner to bind to platelet LRP8 and induce platelet aggregation. Moreover, in a previous study conducted by Urbanus et al, it was shown that platelet activation required the downstream signaling pathway induced by LRP8 activation, including Dab-1 phosphorylation [319]. It was perhaps through the activation by reelin in which thrombospondin-1 was released, as observed by the study conducted by Horn et al. To further provide support for reelin’s role as the mediator of HNP-induced platelet aggregation, future studies may examine platelet aggregation in the presence of MRS compound, such that HNP would no longer be able to induce reelin secretion by P2Y₆.
Chapter 6: Summary and future studies

6.1 Summary

Since the discovery of defensins, HNP has recently gained much attention to its role in promoting atherosclerosis. While the physiological consequences of HNP in endothelial cells, platelets and leukocytes have been widely studied, only a handful of published research has dedicated in finding the mechanism in which HNP induces its pro-atherogenic events. In this study, we have revealed the molecular mechanism in which HNP induced endothelial activation and platelet aggregation. By binding to endothelial or platelet P2Y_6, HNP can induce reelin protein expression and release into the extracellular milieu. The released reelin then acts on endothelial or platelet LRP8 in an paracrine manner to induce endothelial activation or platelet aggregation, respectively. The induction of the LRP8 signaling pathway then induces an endothelial decrease in eNOS and increase in iNOS. Although nitric oxide is generated by iNOS, however, NO may quickly be converted into peroxynitrite and mediate nitrosative stress. Likewise, the release reelin is able to sustain platelet aggregation by sustaining the “first-wave” and inducing the “second-wave” of platelet aggregation. The observed responses mediated by reelin were significantly attenuated in the presence of reelin neutralizing antibody. Thus, with this knowledge by which reelin is the perpetrator of HNP-mediated atherogenesis, future therapeutic strategies targeting patient cardiovascular disease should include the possibility of inhibiting reelin.

6.2 Future Direction

6.2.1 In vivo studies

Lastly, to translate our in vitro model into an in vivo model, which would provide a more physiological depiction of reelin, we would use our previously established atherosclerotic mouse model, ApoE^{-/-}/HNP^{(+), and continuously inject the reelin neutralizing antibody by an
osmotic Alzet pump. Following five weeks of high fat diet, the animal will be sacrificed to analyze for atherosclerotic lesion formation, leukocyte recruitment and platelet aggregation (Figure 18). These mice will be compared with mice that received a vehicle control, a placebo that would replace the reelin neutralizing antibody. This would provide a more humanized representation of atherosclerosis in mice, and allow us to examine the potential therapeutic effect of CR50 as an intervention for HNP-mediated atherogenesis.
Figure 18. Experimental design for the potential therapeutic effect of CR50 against HNP-mediated atherogenesis in vivo.

5-week old male ApoE<sup>−/−</sup>/HNP<sup>(−)</sup>

Osmotic Pump Implantation (Vehicle or CR50)

High Fat Diet

Platelet aggregation

Leukocyte recruitment

Atherosclerotic lesion

Plasma cytokine profile

Week 9

Week 10

Sacrifice

92
References:


