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The Roles of FABP3 in Endothelial Dysfunction and the Transcriptomic Insights into Cardiovascular Disease

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Abstract:

Heart-type fatty acid binding protein (FABP3) is an effective biomarker for cardiac injuries. However, it has also been tested as a biomarker in patients with peripheral artery diseases; these conditions are complications of atherosclerosis, which is driven by endothelial dysfunction. As FABP3 release is not exclusive to the heart but appears to characterize cardiovascular events, whether FABP3 influences endothelial function is not known. Additionally, the transcriptomic profiles of endothelial cells during cardiovascular stresses remain under-investigated. This thesis investigates the multifaceted role of FABP3 and the transcriptomic alterations in endothelial cells under different cardiovascular stressors, offering novel insights into endothelial dysfunction and atherosclerosis. Through a series of *in vitro* studies, the regulatory dynamics of FABP3 under atherosclerotic stressors, its impact on endothelial cell gene expression, and the effects of Angiotensin II (Ang II) exposure on coding and long noncoding RNAs were demonstrated. The findings of this thesis reveal that FABP3 expression is differentially modulated by oxidative stress, inflammation, and hypertension and highlight its therapeutic potential in cardiovascular diseases through loss of function studies. Additionally, transcriptomic profiling uncovers significant changes in messenger RNAs and long non-coding RNAs, identifying novel pathways involved in endothelial response to Ang II-induced stress. This comprehensive analysis advances the understanding of the molecular mechanisms underlying endothelial dysfunction and opens new avenues for research on intervention in endothelial dysfunction, atherosclerosis, and cardiovascular disease.

Keywords: FABP3, Atherosclerosis, Endothelium, Endothelial Cells, Endothelial Dysfunction, Endothelial Function, Transcriptomic Profile, Oxidative Stress, Inflammation, Hypertension, Cardiovascular Disease.

Summary for Lay Audience:

This thesis examines the protein Heart-type Fatty Acid Binding Protein 3 (FABP3) and the associated genetic changes in the cells lining blood vessels under the context of diseases involving blood vessels. The work addresses our previous observation that FABP3 rises in blood were not exclusive to patients with heart injury, as they were also detected in patients with only vessel disease. We showed that FABP3 in the cells lining the blood vessels reacts uniquely under conditions that lead to heart and vascular diseases, including inflammation, oxidative stress, and lack of oxygen, revealing its treatment potential. Exposure to a hormone associated with high blood pressure, angiotensin II, also alters the genetics of blood vessel linings, including those genes that do not produce proteins but regulate others, which have implications for blood vessel responses to stress. The thesis further identifies new biological pathways activated in stressed conditions by analyzing the entire set of genetic material in the cells of blood vessel linings, opening up new avenues for combating heart and blood vessel diseases. In essence, this study advances the understanding of heart and vessel diseases at the molecular level, suggesting new strategies for prevention and treatment.

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To my father and mother for their unconditional love and support.

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Chapter 1: Background

1.1 The Circulatory System

The intricate circulatory system, vital to our organs and functions by facilitating the delivery of essential nutrients and the removal of toxic biowaste, comprises the heart, blood vessels, and lymphatics. Most of the body's homeostasis is dependent on and maintains proper circulation. The heart pumps blood throughout the body, delivering essential nutrients and oxygen to peripheral tissues through arteries and capillaries. Simultaneously, blood drains into venules and veins, carrying excreted CO2 and metabolic byproducts, such as bicarbonates, from tissues to processing organs, such as the liver, kidneys, spleen, and lungs, before returning to the heart for the next diastole/systole cycle. This repeating cycle ensures all cells receive nutrients and oxygen while toxic byproducts are efficiently removed. Moreover, immune cells circulating in the blood can enter tissues with infections to eradicate pathogens and facilitate recovery. Lastly, blood plays a crucial role in communication between organs via biochemical signals such as hormones [1].

1.1.1 The Heart

Central to this system, the heart comprises 4 semi-symmetrical chambers of cardiac muscles: 2 upper atria and 2 lower ventricles. These chambers maintain a continuous blood volume and operate synchronously to ensure uninterrupted systemic circulation. Deoxygenated blood, returning from the body via the *superior vena cava* (draining the upper half of the body) and the *inferior vena cava* (draining the lower half), enters the *right atrium*. The right atrium contracts and blood moves through the *tricuspid valve* into the right ventricle. The right ventricle pumps deoxygenated blood through the *pulmonary valve* into the *pulmonary arteries*, which bifurcate into the right and left *pulmonary arteries* directed toward the respective lungs. CO2 (metabolic waste) exchange for oxygen occurs within the capillary networks surrounding the lungs' alveoli. The now oxygenated blood drains back to the heart via the *pulmonary veins*, converging into the *left atrium*. The left atrium contracts, and blood flows through the *mitral/bicuspid valve* into the *left ventricle*. Lastly, the left ventricle pumps and propels the oxygenated blood through the *aortic valve* into the *aorta*, which carries blood through the systemic arterial network to deliver oxygen and nutrients to all tissues and organs (**Figure 1.1**).

The heart operates through pulsatile pumping in a synchronized sequence around a repeating diastole/systole or cardiac cycle (**Figure 1.1**). Diastole marks atrial contraction (ventricular filling), and systole marks ventricular contraction (atrial filling). Both sides of the heart operate in a cardiac cycle under markedly different blood pressure. The cardiac cycle initiates with diastole, during which the ventricles relax and the atria contract to transfer blood with minimal pressure into the ventricles. The closure of the aortic (in the left ventricle) or pulmonic (in the right ventricle) valves signals the start of diastole, whereas the mitral (left atrium) or tricuspid (right atrium) valves closing signals its end. Conversely, systole commences with the closing of the mitral or tricuspid valves and concludes with the closure of the aortic or pulmonic valves. Closures of the cardiac valves ensure zero backflow of blood into the preceding chambers; functional impairment of these valves underlies the clinical valvular regurgitation and heart failure [2]. Systole features the contraction of the ventricles that propels blood out into the systemic (ventricle) and pulmonary circulations (right) at high blood pressure (higher in the left ventricle) [3]. Proper functioning of the cardiac cycle forms the basis for the regulation of *blood pressure* via *cardiac output* and *vascular tone* [4]. Cardiac output, the volume of blood pumped by the left ventricle into the systemic circulation per minute, is a function of *stroke volume*, which is the blood volume ejected by the ventricle in a single systole, and *heart rate* [5]. Vascular tone, the state of constriction or dilation of blood vessels, is a key factor of hemodynamics and is regulated by the autonomic nervous system, endothelial factors, and circulating hormones. Vascular tone influences *blood flow resistance* and adjusts in response to physiological needs, thereby affecting cardiac workload [6] [7]. Regulation of blood pressure determines adequate blood perfusion and substance exchange between blood and tissues without causing damage to the vasculature [3]. Modulation of blood pressure is, therefore, central in vascular homeostasis and a regulatory feature of endothelial function [8].

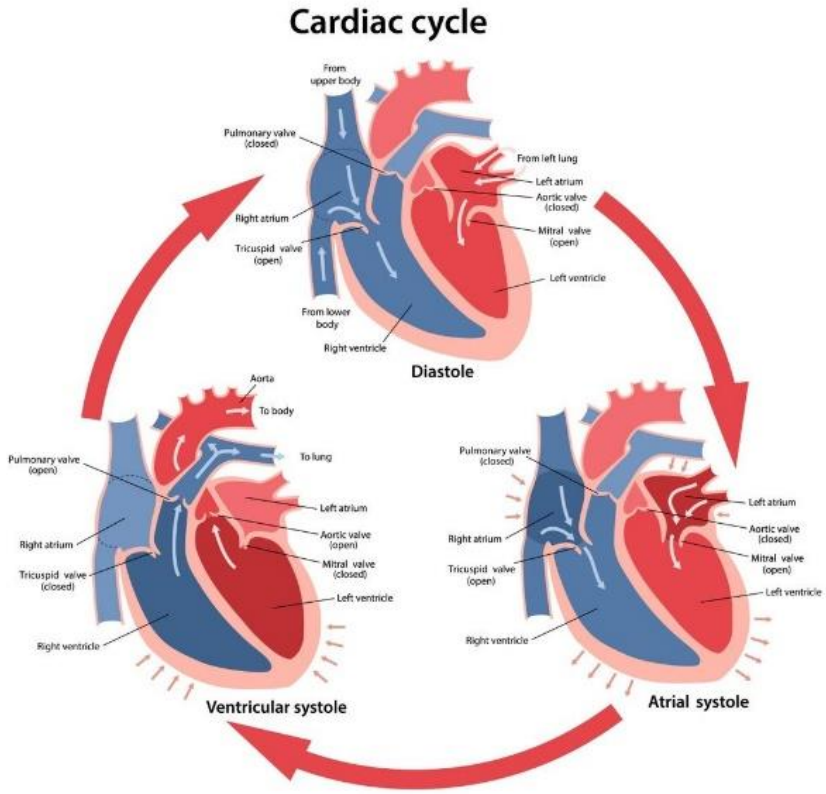


Figure 1.1 The heart and the cardiac cycle

Four semi-symmetrical chambers of the human heart—two atria and two ventricles—work synchronously via cycles of diastole/systole to maintain continuous systemic circulation.

[16] *(CC BY-NC 4.0)*.

1.1.2 Blood and Lymphatic Vessels

Blood vessels constitute the extensive network through which blood circulates away from the heart through *arteries* and *arterioles* and returns via *venules* and *veins*. At the peripheral tissues, the capillary networks facilitate the selective exchange of nutrients, gases, and byproducts. Following this exchange, blood is collected by post-capillary venules and subsequently by veins, through which it travels further towards downstream organs for metabolic processes or back to the right atrium of the heart (**Figure 1.2A**). An essential adjunct to the circulatory pathway is the *lymphatic,*

which also participates in fluid exchange at the tissue level. Excess interstitial fluid from circulatory exchange, carrying proteins and biomolecules, is drained into lymphatic vessels as *lymph*. The lymphatics constitute a secondary circulatory network through which lymph travels to lymph nodes, wherein the adaptive immune system performs surveillance and response functions against pathogens or foreign particles, monitoring the health of tissues. The lymphatic system eventually converges with the venous system, draining into the veins through the *right lymphatic* (right upper part of the body) and *thoracic duct* (lower limbs and left side of the body) and returning processed lymph to the bloodstream (**Figure 1.2B and C**) [9] [10].

Blood vessels, with the exception of capillaries, share common structural features characterized by three distinct layers in their walls, from the outermost to innermost: tunica adventitia, media, and intima (**Figure 1.3**). *Tunica adventitia* (or *external*) primarily consists of connective tissue that provides structural support and flexibility to the vessel; this layer also has nerve fibres and, in larger vessels, nest the *vasa vasorum*, a network of small vessels that supply blood to the walls of larger arteries and veins to ensure that the outer layers receive nutrients and oxygen. *Tunica media* is situated between the adventitia and intima, separated by the *external* and *internal elastic lamina,* respectively. This layer predominantly comprises smooth muscle cells, extracellular matrix, and elastic connective tissues that enable vasotone regulation through vasoconstriction and vasodilation. In larger vessels, typically, the outer 2/3 of the *tunica media* is vascularized by the vasa vasorum from the adventitia; the tunica adventitia and media are too distant to receive nutrients directly from the vessel's lumen. *Tunica intima* is the innermost layer of blood vessels that directly contacts luminal blood. This layer comprises the endothelium resting on a basement membrane and a subendothelial layer with moderate content of extracellular matrix, vascular smooth muscle cells, fibroblasts and myofibroblasts. The endothelium acts as a selective barrier between the blood, the rest of the vessel walls, and tissues (at the capillaries). Notably, capillaries only comprise the tunica intima, and this single lining of endothelial cells forms a resolute barrier between blood and the underlying tissues with heterogenous influences across organ systems over vascular homeostasis, encompassing permeability, hemostasis, and inflammatory responses, among others.

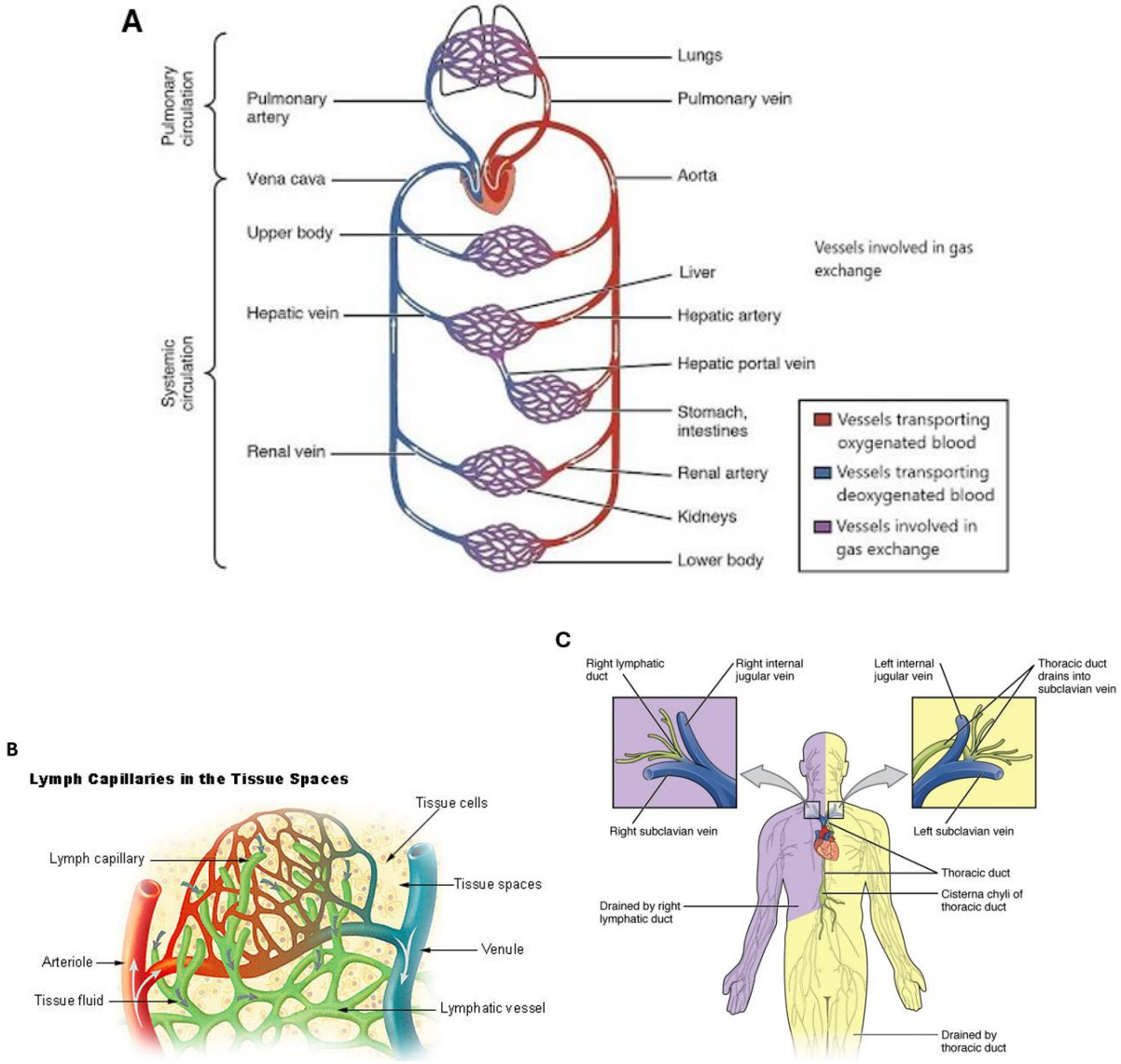


Figure 1.2 Pulmonary, systemic, lymphatic capillaries and circulatory circuits

A) Through pulmonary and systemic circulation, blood flows through arteries and arterioles to peripheral tissues for nutrient and gas exchange and then returns to the heart via venules and veins [17]. B) Capillary and lymphatic networks at the site of blood-tissue and interstitial fluid exchange [18]. C) Lymphatic circulation and the convergence with the venous system; lymph from the right lymphatic duct and thoracic duct is returned to the bloodstream, completing the circulatory cycle [19] *(CC BY-NC 4.0)*.

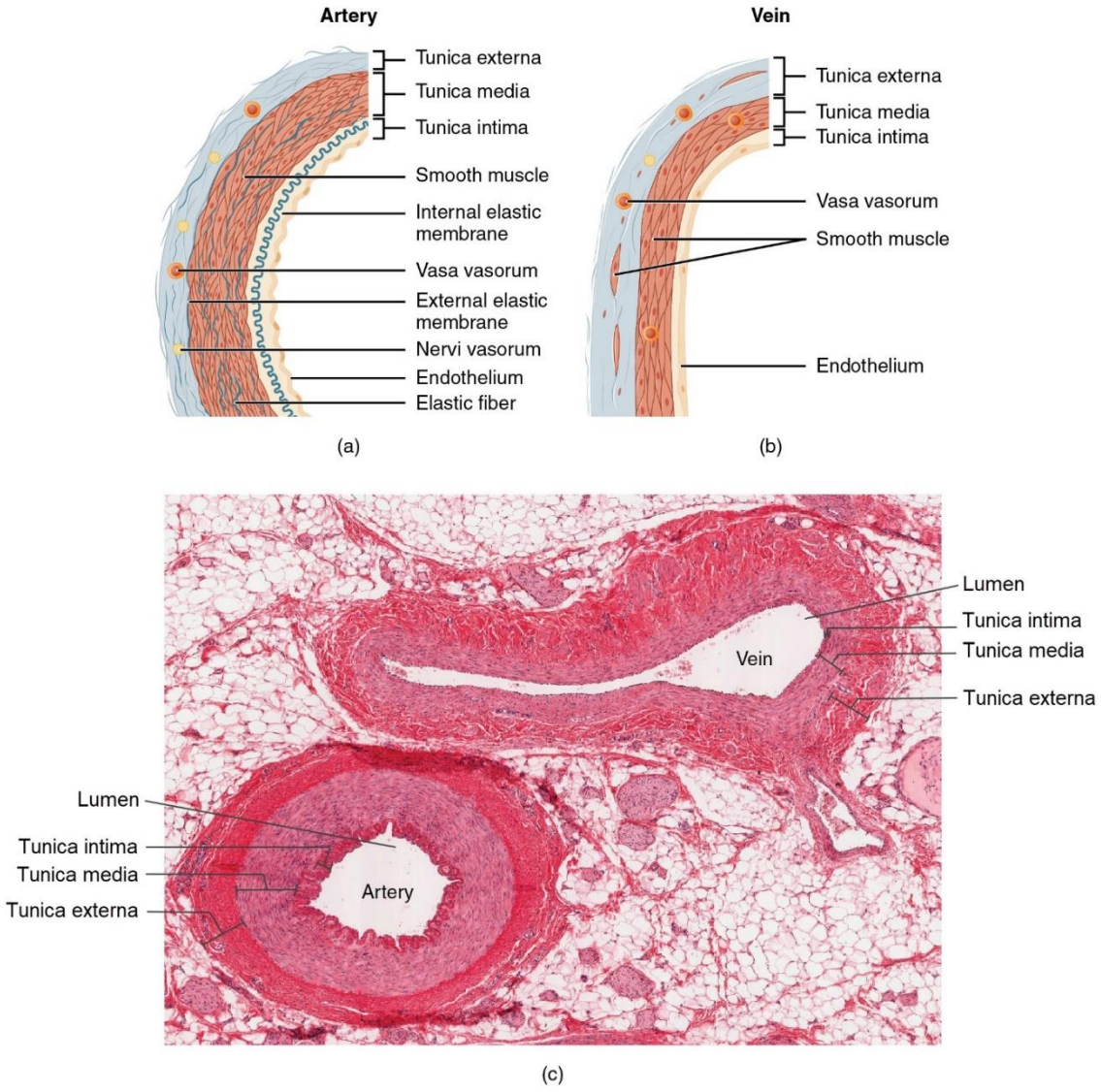


Figure 1.3 Structural layers of blood vessels

Common structural features of artery (a) and vein (b), from the outermost to innermost: tunica adventitia, media, and intima, with histological references (c) [17] *(CC BY-NC 4.0)*.

Variations in the makeup of the three concentric wall layers of vasculatures reflect the different types of vessels and their functions (**Figure 1.4)**. Fundamentally, arteries bring blood away from the heart to all vital organs in the body; this implies characteristics of precise regulation of blood pressure from high enough for transport and low enough for exchanges at the capillaries. On this

basis, the arteries are categorized into three types: *large elastic arteries*, *muscular arteries*, and *arterioles*.

Large elastic arteries (e.g., aorta, pulmonary arteries), the largest of the three, feature a tunica media heavily loaded with alternating elastic fibres (elastin). The elastic property of these arteries enables the accommodation of very high blood pressure coming out of the left ventricle. The vessel can, therefore, easily dilate during diastole, during which the fibres recoil, storing spring energy. In the follow-up systole, the vessel constricts to propel blood forward as the fibres expand and release the energy. Notably, this elasticity becomes compromised with age, leading to larger arteries unable to constrict from lack of media expansion, resulting in arrhythmias and downstream hypoxia, or the inability to dilate from loss of media recoils, resulting in stiff vessels, increased arterial pressures and hypertension.

Muscular arteries (e.g., coronary and renal arteries) possess a *tunica media* that features 1) significantly high smooth muscle cells to extracellular matrix ratio and 2) more distinct internal and external elastic lamina that enable remarkable elasticity [11]. High medial smooth muscle cell levels, arranged in spirals, allow for contractility regulated by the *autonomic nervous system* and local metabolic factors, i.e. vasoconstriction and vasodilation. The body's vascular homeostasis often revolves around these muscular vessels. Notably, endothelial cells regulate medial smooth muscle cells by releasing nitric oxide (causing vasodilation) or endothelin (causing vasoconstriction) [8] [12].

Arterioles and smaller arteries (<2 mm diameter) branch from the larger arteries as units that enter and vascularize end organs. These vessels are barely elastic and, although smaller than mid-sized muscular arteries, they share the high smooth muscle cell-to-matrix ratio feature, indicative of vasotone regulation. Arterioles feature blood flow resistance regulation; changes in wall-thickness of arteriole profoundly affect arteriolar blood pressure (flow resistance is inversely proportional to lumen diameter by the fourth power, i.e., 1/2 diameter increases resistance by 16-fold). This regulatory sensitivity is attributed to the fact that as blood flows into end-organs, blood pressures must drop significantly to steady flow (rather than a pulsatile pattern that propels blood throughout the entire body); this is done by branching into smaller vessels and vasotone regulation at arterioles.

Capillaries are vessels established as complex networks branching from arterioles. Each capillary has a lumen of ~7 μm diameter and an intima lined with endothelial cells but no distinct media or adventitia; instead, the intima is surrounded by a single layer of fibroblast-like pericytes, whose functions remain controversial [13]. Capillary infiltrates the tissue's interstitia and forms the blood-tissue exchange fronts, whereby nutrients and oxygens are absorbed, and toxic byproducts and CO2 are excreted. The branching feature of capillaries plays a crucial role in facilitating their functional feature, as it establishes a very large total cross-sectional surface area to maximize exchange. Additionally, the exchange is also supported by blood pressure regulated to slow and steady flow coming from the arterioles. Notably, metabolically active tissues, such as the heart, tend to have high capillary density [7].

The venous counterparts of capillaries, arterioles, and arteries are post-capillary venules, venules, and veins. Compared to arteries, at each equivalent branching level, the lumen of venous vessels is larger in diameter, and they possess a thinner tunica media with fewer smooth muscle cells. While veins are less regulated for vasotone, their high capacitance property account for ~2/3 of total blood flowing in veins in the body at any time. These structural features enable adaptation to low pressure and high blood flow, which is essential for the roles of veins in delivering blood back to the heart for the next cardiac cycle. Additionally, veins also feature valves that open and close in coordination with flow in order to prevent backflow.

Lastly, while they are not the focus of this thesis, the lymphatic vessels are worth mentioning as they are a crucial component of the body's circulatory system. Lymphatics are thin vessels, also lined with endothelial cells monolayer [14], and operate at the same level as the capillaries; they drain tissue interstitial fluid into a separate circulatory network, which eventually connects back to the venous system via the right lymphatic and thoracic duct (**Figure 1.2B and C**). Tissue fluid draining into the lymphatics carrying antigen-presenting cells and/or immune cells facilitates adaptive immune response at the local lymph nodes; along with blood-tissue exchanges by the vasculatures, the body's circulatory system employs the lymphatics to ultimately establish surveillance of peripheral tissues for infectious agents and regulate the immune response.

Overall, the circulatory system, through cardiac, vascular, and lymphatic functions, ensures healthy physiological conditions for all organs in the body. Interestingly, all vessels of the

circulatory system feature a single-lining endothelium, which is the first barrier exposed to luminal blood. In addition, the ratio of endothelial cells to cardiomyocytes in the heart is ~3:1 [15]. Therefore, endothelial cells are critical players in cardiovascular homeostasis.

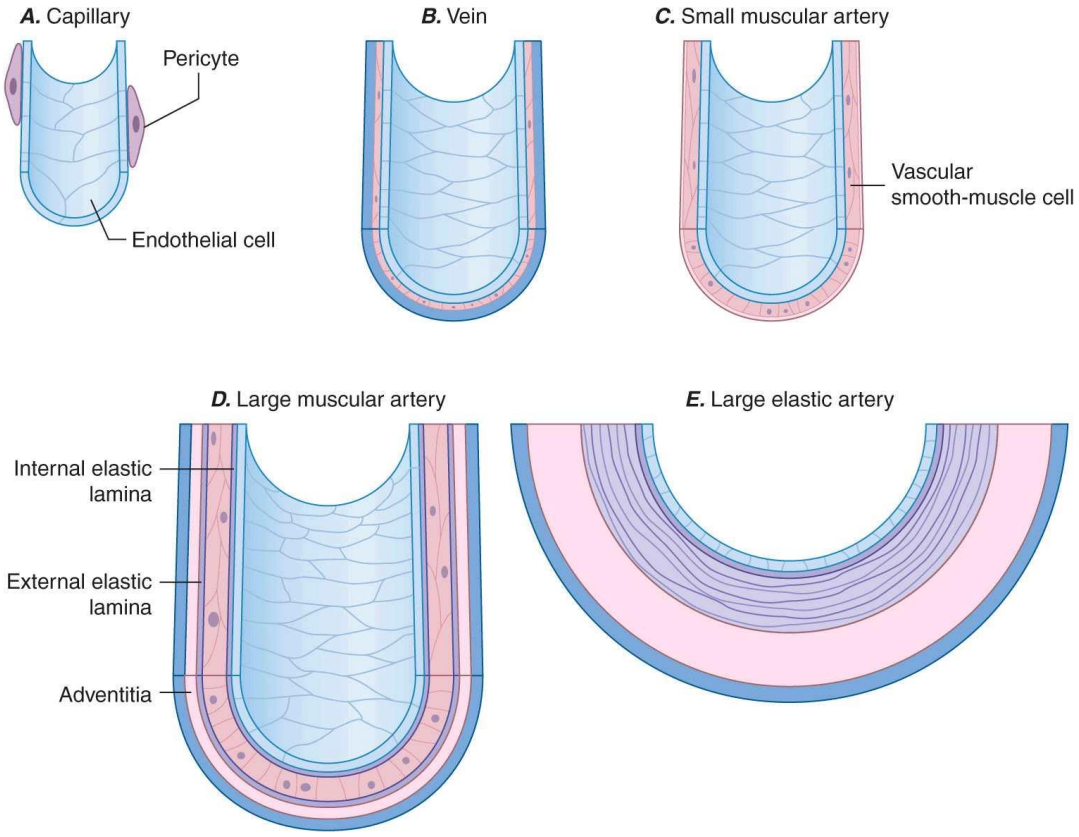


Figure 1.4 Schematics of the structures of various types of blood vessels

Structural variations in the three concentric wall layers reflect the different types of blood vessels. Capillary typically only has the intima (A). Veins generally have a thinner media layer than arteries (B). Different types of arteries vary by the elasticity content, which highlights their roles in vasotone regulation (C, D, E) [20] *(CC BY-NC 4.0)*.

1.2 Cardiovascular Disease

Cardiovascular disease (CVD), the number one cause of global deaths, is a disorder of the circulatory system affecting the blood vessels and the vascularized systems, prominently the heart, brain, and limbs. CVDs can be classified based on whether an ischemic condition is involved, wherein the impairment of blood vessels leads to compromised blood delivery in the corresponding vascularized tissues (ischemia), resulting in tissue injuries or death. Ischemic CVDs are more common and consist of ischemic heart diseases, cerebrovascular diseases, and peripheral vascular diseases, referring to the blockages in the arteries supplying the heart, brain, and peripheral extremities, respectively. These conditions are responsible for serious clinical manifestations such as hypertension, angina (chest pain), acute myocardial infarction, stroke, claudication and limb ischemia [21].

The World Health Organization reported an estimated 17.9 million people died from CVDs in 2019 (32% of all global deaths), an increased from the previous annual death rate of 17.8 million in 2017 [22], with 85% of CVD deaths associated with heart attack and stroke, and 75% with low socioeconomic status. Figures from the Centre for Disease Control and Prevention figures and the American Heart Association indicate that CVD accounted for 931,578 deaths in the United States in 2021 (more deaths than all cancers and chronic lower respiratory diseases combined). Within 2017 - 2020, 127.9 million US adults (48.6%) acquired CVDs; higher prevalence occurring in males than females and older age groups overall (notably over 75 years of age) remains the trend [23]. In 2019 – 2020 alone, the total cost of CVD was $422.3 billion; the direct (prescription and medical services) and indirect (disability, premature death, and losses of employment, earnings, and productivity) costs were $254.3 billion and $168.0 billion, respectively. These figures are up from $351 billion ($213 billion for direct cost and $138 billion for indirect cost) in 2014 and projected to be ~$750 billion by 2035. Additionally, the World Heart Federation projects over 20 million global deaths annually by 2030 if CVDs remain the number 1 cause of death globally. Alarmingly, the 2022 Heart Disease & Stroke report indicates a global death rate of 19.1 million deaths in 2020, with higher mortality rates observed in Eastern Europe, Central Asia, Oceania, North Africa, the Middle East, sub-Saharan Africa, and South and Southeast Asia, and an age-adjusted prevalence rate of ~7354 per 100,000 population. Notably, the rates were lowest in high-income locations in Asia Pacific, North America, Latin America, Western Europe, and Australasia

[24] [25]. The latest mortality figures for Canada, according to Statistics Canada, are more optimistic: an annual 67399 mortality in 2020 (53704 due to heart diseases and 13695 due to stroke) on a downward age-standardized mortality trend for the past 20 years; however, CVDs remain the second leading cause of death in Canada, after cancer.

Risk factors of CVDs are quite common, and many have been identified and categorized into modifiable and non-modifiable risk factor categories. Modifiable risk factors can be improved or prevented by drug treatments or by adopting a healthy lifestyle and social changes such as healthy eating, exercise, and smoking cessation. Physical inactivity and poor diets (high in glucose and saturated- or trans-fats) are the major modifiable risk factors for CVDs as they lead to high blood lipids and cholesterols. These conditions are the developing mechanisms of atherosclerosis, which is the most common cause of CVD. Smoking can promote CVDs by damaging the endothelium, the cell lining of all blood vessels, and the liver. This, in turn, induces endothelial dysfunction and reduces the production of high-density lipoprotein (HDL), which contributes to atherosclerosis and hypercholesterolemia [26],[27].

Non-modifiable risk factors dictate that some individuals are intrinsically more vulnerable than others to cardiovascular complications; such risk factors include older age, male sex, and genetic factors such as the family history of CVDs. In aging individuals, especially those over age 55, the rise in cardiovascular risk is commonly attributed to increasing blood cholesterols [28] and degrading vascular integrities, such as loss of arterial elasticity and reduced arterial compliance [29]. CVD diagnosis in men occurs 10 years earlier than women on average, and the incidence rates for women are substantially lower compared to men of the same age or postmenopausal women [30]. Explanations provided for this phenomenon include the female sex hormone estrogen, which is prominently active in pre-menopausal women and has cardioprotective effects [31]. Women may also have fewer typical CVD symptoms compared to men, making their diagnosis difficult, which further contributes to the sex-specific gap [32],[33]. Genetic risk factors of CVD are associated with vascular health and blood lipid levels, including inherited hypertension and familial hypercholesterolemia, which can lead to atherosclerosis [34]. Other non-modifiable risk factors include ethnicity [35] and socioeconomic status [36]. A significant cause of ischemic CVDs is atherosclerosis [37]. Atherosclerosis is a chronic and inflammatory vascular disorder characterized by lipid-laden plaques that are raised and thickened from the vascular luminal walls.

The development of atherosclerotic plaques can begin in childhood [38] as soft vascular lesions comprising lipid deposits (*fatty streaks*) [39]. As they grow and protrude into the lumen, the maturing plaque (atheroma) narrows the vascular lumen, restricting blood flow and ultimately causing ischemia to the vascularized organs [40]. Late-stage atherosclerosis underlies the common ischemic CVDs, such as coronary artery disease (CAD) and peripheral artery disease (PAD), which arise from the ischemia of the heart or the lower limbs, respectively.

Many risk factors of CVDs culminate in conditions of metabolic syndrome that contribute to the development of atherosclerosis. Metabolic syndrome refers to a cluster of metabolic disorders, including hyperglycemia, insulin resistance, and obesity, characterized by dyslipidemia and hypercholesterolemia. Individuals with exacerbating conditions of the metabolic syndrome are at risk for obesity, type 2 diabetes, and eventually CVDs, among other complications [41].

Type 2 diabetes mellitus and obesity are metabolic disorders characterized by insulin resistance, leading to elevated blood glucose levels. Type 2 diabetes is associated with dyslipidemia, while obesity is associated with hypercholesterolemia, although typically, both disorders share these features [42] [43]. Insulin resistance refers to the unresponsiveness of most cells in the body to insulin, a hormone produced by the beta-cells islets of the pancreas to signal the uptake of blood glucose by cells of various body systems. Many studies have elucidated several mechanisms of obesity that lead to and exacerbate insulin resistance, which in turn promotes hyperglycemia. For instance, type 2 diabetes is quite common in patients with obesity, and insulin resistance is strongly associated with elevated blood lipids, which contribute to dyslipidemia featured in obesity [44]. Individuals with both type 2 diabetes and obesity are at high risk for CVDs, as the most common deaths with the metabolic syndrome are cardiovascular. For example, hypertension is very common among patients with type 2 diabetes [45].

1.3 Atherosclerosis

In atherosclerosis, the affected vasculatures, prominently large arteries, are progressively impaired as the plaque thickens and becomes critically compromised upon a plaque-rupturing event followed by luminal thrombosis [46]. Atherosclerosis can begin at any age, even in childhood. The early soft vascular lesions, referred to as “fatty streaks,” comprise lipid deposits and can be

detected in adults as young as 20 years old [38] [47] [39]. Mechanisms of plaque formation have been elucidated in various animal and human studies [46].

Traditionally, the development of fatty streaks into atheroma is driven by hypercholesterolemia. Circulating low-density lipoproteins (LDL), resembling the body's cholesterols, tend to infiltrate across the endothelium into the blood vessel's intimal layer. LDLs are protein complexes that circulate in the body as the major intermediate lipid carriers. They serve to deliver cholesterols and triglycerides to peripheral tissues and the liver by firstly interacting with the local endothelium and, through specific LDL-receptors (LDLR), become uptake via endocytosis. Optimal expression of LDLR is an important determinant of familial hypercholesterolemia, a risk factor of atherosclerosis [48]. In the vascular wall, where cellular metabolic activities are active, LDLs can also be processed into oxidized LDLs (oxLDL), which introduce local oxidative stress and induce inflammation. Damaged tissues, especially the endothelium, upregulate hemostasis and the recruitment of circulatory monocytes and other leukocytes, such as neutrophils. These immune cells traverse the compromised, leaky endothelium to reach the inflamed vessel wall, participating in the body's innate immune response to repair and relieve oxidative stress. Here, the monocytes differentiate into macrophages and, together with the already presented tissue macrophages, engage in the uptake of oxLDL through scavenging receptors for clearance [39] [49]. As the condition is prolonged, the scavenger macrophages become overwhelmed by oxidative stresses and undergo apoptosis, a process that is also stalled by oxidative stress. These apoptotic/necrotic lipid-laden macrophages (*foam cells*) accumulate in the damaged vascular wall; early concentration of foam cells in the vascular wall mediates the formation of fatty streaks. This chain of events finds the basis for chronic inflammation.

Due to hypercholesterolemia and also to reduced serum levels of the cholesterol-clearing HDL [50], excessive oxLDLs in the vascular wall easily overwhelm the scavenging mechanism, induce apoptosis, followed by necrosis, and increase the foam cell population. This builds up the *necrotic*/*lipid core* of the plaque over time. oxLDLs also stimulate the subendothelial vascular smooth muscle cells to migrate to the injured site and proliferate, forming a structural *fibrous cap* that covers the lipid core and thickens the plaque over time [39].

Late-developing atheromas, already narrowing the vessel's lumen, introduce luminal stenosis and compromise blood flow, giving grounds for hypertension and cardiomyopathies. Other precritical complications include tissue calcification, ulceration, and hemorrhage from local capillaries. Additionally, the plaque becomes increasingly susceptible to rupture. Rupturing of the plaque is the critical stage of atherosclerosis, whereby thrombosis is induced, followed by clot formation at the site of rupture that completely clogs up the vessel's lumen. Tissue ischemia then follows in the downstream vascularized organs, prompting the stage for myocardial infarction, cardiac arrest, coronary artery disease, peripheral artery disease, stroke, and multiple organ failures [49]. Moreover, rupturing releases the plaque contents into circulation, introducing oxidative stress to local vascular regions and potentiating additional atherogenesis. The clot itself may induce thromboembolism, in which pieces of the clots break and circulate with a high risk of lodging, compromising other blood vessels [40] [46] (**Figure 1.7**).

1.4 The Endothelium

The endothelium is the simple squamous specialized epithelial lining that makes up the tunica intima of blood and lymphatic vessels. Endothelial cells are directly exposed to blood, thereby being the first surface to interact with all circulatory *entities*. Different parts of the body adopt a unique interaction with the circulatory system (e.g., gas exchanges at the lungs, nutrient absorptions at the gastrointestinal tracts, and filtration at the livers, spleen and kidney). The corresponding endothelium of each organ system, in turn, adopts unique structural and functional patterns in its roles as an intricate blood-tissue barrier that facilitates permeability-based selective blood-tissue exchanges, communication between tissues and organs (paracrinal & endocrinal signalling) and general body immunity. Through such heterogeneity, endothelial cells can influence the functions of multiple cell types and can be seen as the crucial regulatory player of virtually all mechanisms of systemic metabolism and vascular homeostasis.

The heterogeneity of endothelial cells is demonstrated through unique patterns of differentiation, leading to the formation of endothelia that vary by vessel type and organ specificity. Endothelial cells' differentiations are influenced spatially by intrinsic genetic factors and extrinsic factors, including specific biochemical *milieu*, cell-cell or cell-matrix interactions, pH, mechanical forces, etc., and temporally, whereby heritable or conserved genomic elements, such as epigenetic, and micro-environmental changes (i.e., organ-specific) coordinate early- and late-stage developmental

differentiation, respectively. Moreover, alternative cell lineages have been reported to trans-differentiate into endothelial cells, including adipocytes and mesenchymal stem cells [51].

Structurally, endothelial heterogeneity can be expressed through three general categorizations of the endothelium: continuous, fenestrated, and discontinuous [52]. Continuous endothelium makes up most arteries, veins, and capillaries and features endothelial cells with tight junction cell-cell coupling anchored to a continuous basal membrane, typically providing an impermeable barrier. The most apparent continuous endothelium can be observed in the blood-brain barrier and the equivalent in the reproductive organs, wherein prominent tight junctions establish strict regulation against the diffusion of inflammatory factors from the blood. Notably, hemodynamic stress and vasoactive agents (e.g., histamine) can influence endothelial permeability through these junctions. Fenestrated endothelium is found in tissues specialized for trans-endothelial transport that facilitate increased exchanges or filtration, such as renal, gastrointestinal and pancreatic tissues, adrenal glands, choroid plexus, etc. Fenestrated endothelial cells, similar to continuous endothelium, possess stable and regulatory coupling and a continuous basal membrane but also feature numerous transcellular pores (50–60 nm wide), referred to as fenestrations, sealed by a thin diaphragm as a lesser mean to regulate the increased blood-tissue diffusion. Endothelial cells of discontinuous endothelium, seen in sinusoidal vascular beds of the liver, spleen, and bone marrow, are characterized by dissociated junctions, large intercellular cleft, loosened basal membrane and large fenestrations (100-200 nm wide) without diaphragms. At the level of endothelial cells, structural heterogeneity can manifest through changes in shapes, endocytic patterns (e.g., clathrin-based or caveolin-based) and junction types (e.g., tight junction, cadherin-based, gap-junction, etc.) [52] (**Figure 1.5**).

Functionally, endothelial cells have been described for intricate regulatory capacities through expressing key mediators in vascular homeostatic processes, including barrier/exchange permeability, hemostasis (blood fluidity regulation), inflammation and leukocyte recruitment, and regulation of vascular tone and compliance, wound healing, angiogenesis (formation of new blood vessels from pre-existing ones), and thrombosis (blood-clotting) (**Table 1.1**) The levels of regulation in each process vary depending on specific organs or vascular beds as a feature of functional heterogeneity. For instance, angiogenesis and inflammation occur uniquely rapid and intense in the periodontal tissues due to high levels of vascularization [53]; atherosclerosis, the

pathological formation of lipid-loaded plaque in the vascular wall, tends to affect arteries than capillaries or veins [52].

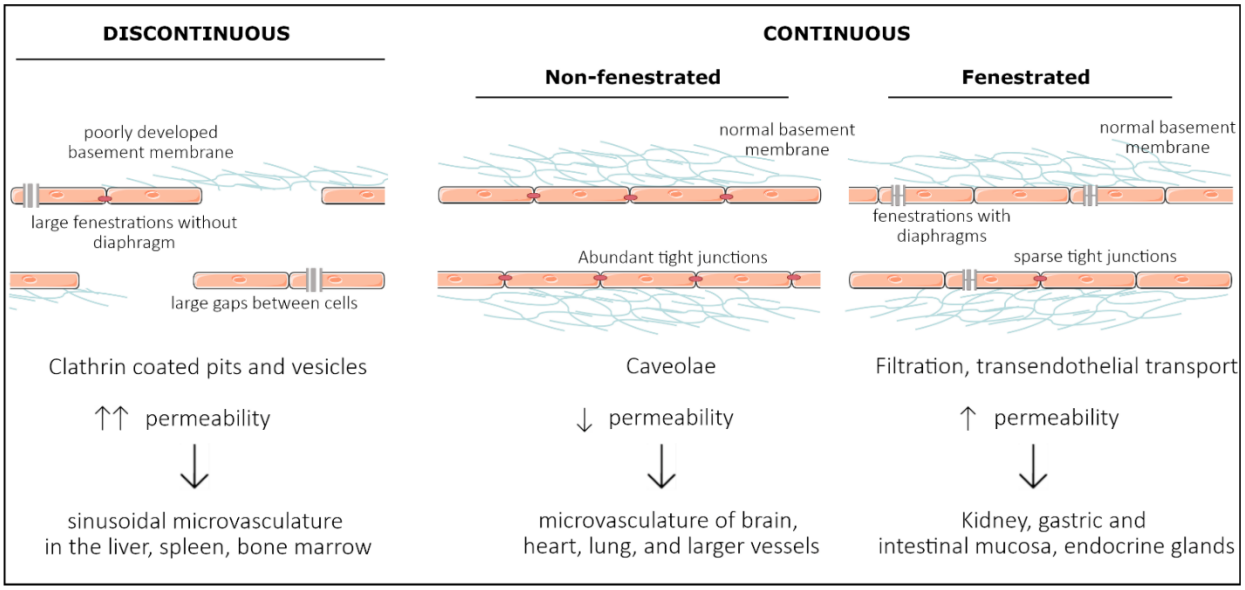


Figure 1.5 Structural/Functional heterogeneity of the endothelium in organ-specific microvasculatures

The structural heterogeneity of endothelial cells features variations in intercellular connections. Continuous endothelium, forming an impermeable blood-tissue barrier in most microvasculatures and larger vessels, is linked by tight junctions and firm basement membranes. Fenestrated endothelium, featuring pores for enhanced transport, is found in organs such as the kidneys and adrenal glands. Discontinuous endothelium, with loose basement membrane and large fenestrations, is present in the liver, spleen, and bone marrow [51] *(CC BY-NC 4.0)*.

Table 1.1 Endothelial Cell Functions

Endothelial Function	Molecular Mediators/Products of Endothelial Cells
Selective permeability barrier	Glycocalyx, Junctional proteins (e.g., Claudins, Occludins)
Anti-coagulant, anti-thrombotic, and fibrinolytic regulators	Prostacyclin, Thrombomodulin, Heparin-like molecules, Plasminogen activator, Antithrombin, Protein C, Protein S
Thrombosis	Von Willebrand factor, Tissue factor, Plasminogen activator inhibitor
Production of extracellular matrix	Collagen, Proteoglycans, Fibronectin
Hemodynamic and vasotone regulation	Endothelin, ACE, Nitric oxide, Prostacyclin
Inflammation/Immunity	IL-1, IL-6, MCP-1, VCAM-1, ICAM-1, E-selectin, P-selectin, TNF-a
Cell cycle/Growth/Angiogenesis	PDGF, CSF, FGF, Heparin, TGF-β, VEGFa
Oxidation of LDL	LDLR, Reactive oxygen species, Nitric oxide

ACE, Angiotensin-converting enzyme; *IL-1*, Interleukin-1; *IL-6*, Interleukin-6; *MCP-1*, Monocyte Chemoattractant Protein-1; *VCAM-1*, Vascular Cell Adhesion Molecule-1; *ICAM-1*, Intercellular Adhesion Molecule-1; *PDGF*, Platelet-Derived Growth Factor; *CSF*, Colony-Stimulating Factor; *FGF*, Fibroblast Growth Factor; *TGF-β*, Transforming Growth Factor-Beta; *VEGFa*, Vascular Endothelial Growth Factor A; *LDLR*, Low-Density Lipoprotein Receptor; *TNF-a*, Tumour necrosis factor alpha [54].

1.5 Endothelial Dysfunction

Physiologically, endothelial cells maintain a non-thrombogenic and anti-inflammatory interface between blood and tissue. In response to physical, chemical, or infectious stressors, endothelial cells undergo activation (**Table 1.2**), transitioning to a state of increased permeability, pro-inflammatory responses, thrombogenesis, and vasoconstriction. Endothelial activation includes several key processes: localized vasoconstriction to direct blood flow to sites of injury, increased permeability and adhesiveness of the endothelium to leukocytes, morphological changes in endothelial cells, and the secretion of inflammatory cytokines. Additionally, endothelial cells release growth factors and a balance of anti- and pro-coagulant factors that regulate angiogenesis and thrombosis, respectively, to facilitate wound healing [55]. Stimuli that activate endothelial cells include hemodynamic or oxidative stresses, advanced glycation end-products, infectious pathogens, and metabolic insults (e.g., hypoxia, obesity, glucose intolerance), among others (**Figure 1.6**). If the stressor is relieved, endothelial activation is reversed. Frequent or prolonged exposure to stressful stimuli, however, can result in endothelial dysfunction [56].

Endothelial dysfunction is characterized by the diminished capacity of endothelial cells to maintain vascular homeostasis, manifesting through a pro-inflammatory and pro-thrombotic state, apoptosis, concentration of reactive oxygen species, and the impairment of proliferation, permeability, and vasotone regulation. Notably, this condition involves a reduction in the bioavailability of endothelium-derived nitric oxide, a critical mediator of vasodilation and endothelial function, alongside an upregulation of vasoconstrictive agents, such as endothelin-1, that underlie hypertension [57].

Table 1.2 Endothelial Function Baseline vs. Activation/Dysfunction

Basal Condition	Endothelial Activation/Dysfunction
Growth and survival expression	Apoptosis and/or necrosis
Barrier and selective permeability	Increased permeability (leaky)
Anticoagulative and anti-thrombotic	Pro-coagulative and pro-thrombotic
Vasodilation	Vasoconstriction
Anti-inflammatory expression	Inflammation

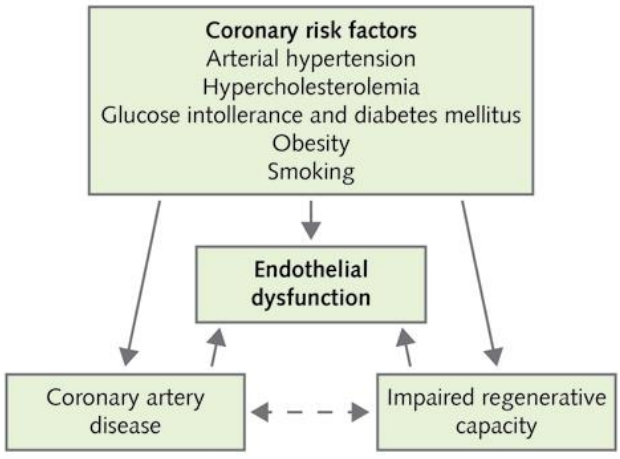


Figure 1.6 Endothelial dysfunction, risk factors, and complications

The relationship between coronary risk factors and endothelial dysfunction, including hypertension, hypercholesterolemia, diabetes, obesity, and smoking. Endothelial dysfunction is, thereby, a critical mediator of coronary artery disease [58] *(CC BY-NC 4.0)*.

1.6 Endothelial Dysfunction and Atherosclerosis

Endothelial dysfunction, in addition to hypercholesterolemia, is the key mechanism behind atherosclerosis and a wide range of CVDs [59]. Moreover, risk factors of CVDs, including obesity, smoking and alcohol abuse, hypercholesterolemia, hyperglycemia, and hypertension, have been shown to influence endothelial dysfunction [60].

In atherosclerotic progression, oxLDL overwhelms the clearance mechanism via oxidative stress, resulting in lipid-laden necrotic macrophages. Chronic inflammation then becomes a source of accumulating foam cells, which build up the necrotic core of the plaque. Meanwhile, continuous bioactivity of vascular smooth muscle cells develops the fibrous cap and raises the plaque, which intensifies physical pressures, blood pressure and hemostasis at the defect site. A key observation of clinical importance is thus revealed: during the development of atherosclerotic plaques, the endothelium is subjected to three driving pathological processes: 1) oxidative stresses, 2) chronic inflammation, and 3) hypertension. The pathophysiology of these processes and their molecular mechanisms in endothelial dysfunction continue to be an active area of research. These aspects are also the main theme of this thesis, which seeks to experimentally expand the current understanding of endothelial dysfunction mechanisms in atherosclerosis development (**Figure 1.7**).

1.6.1 Oxidative Stress

The endothelium is a pathophysiological source of reactive oxygen species (ROS) and related free radicals. Endothelial cells possess endothelial nitric-oxide synthases (eNOS), which can generate ROS as byproducts of nitric oxide synthesis upon pathological imbalance of substrates [61]. Endothelial nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases (NOX), in response to various stressors, including angiotensin-II (Ang II) under hypertension, produce ROS physiologically, and myeloperoxidase, also abundant in phagocytes, generate reactive nitrogen species from NO [61]. Pathologically, excess reactive oxygen species (ROS) in the vessel's wall, a common result of impaired endothelial redox and nitric oxide activities, is associated with assembly disruption of tight junctions and adherens junctions. ROS impairs the interaction between zonula occludens-1 (ZO-1) and occludin proteins in the intercellular space, which is otherwise crucial for assembling and maintaining tight junctions [62]. Common sources of excess ROS from the endothelium include defects in phosphoinositide-3-kinase (PI3K), protein

kinase B (AKT), vascular peroxidase 1 (VPO1) and catalase (CAT) in the redox regulatory pathway, or eNOS of the nitric oxide production pathway [63] [64] [65]. Moreover, tight junction assembly depends on PI3K/AKT's signalling, wherein PI3K interacts with occludin and regulates tight junction modelling through the tyrosine kinase Cellular Sarcoma (c-Src) [66]. Defective tight junction assembling factors, including c-Src and vascular endothelial cadherin (VE-Cadherin), also impair the barrier [67]. An impaired endothelial barrier and a leaky endothelium increase the frequency of invasion by LDL into the tissue. Although LDL can diffuse passively through endothelial cell junctions and the invasion rate is contributed by hypercholesterolemia, retention of LDL in the vascular wall is contributed by the rate of oxidation to oxLDL and the recruitment and presence of scavenging macrophages. Defective redox regulators, including arachidonate-12/15-lipoxygenase (ALOX12/15), NOX2, and superoxide dismutase (SOD), in addition to the above, exacerbate the rate of LDL oxidation (Schmitz & Grandl, 2007). An endothelium more susceptible to inflammation promotes an increased population of scavenging leukocytes. Oxidatively stressed endothelial cells, as in a defective eNOS environment, upregulate monocyte recruitment and differentiation through secreting macrophage colony-stimulating factor (M-CSF) [68].

1.6.2 Inflammation

Endothelial cells are critical mediators of inflammation. An activated endothelium first mounts a vasoconstrictive response by releasing endothelin and introducing localized hemostasis, which promotes the localization of leukocytes. Activated Endothelial cells also upregulate pro-inflammatory molecules such as ICAM-1 and VCAM-1, which facilitate the recruitment and migrations of leukocytes into the assaulted area. In acute inflammation, the events are localized and resolve relatively quickly, involving a regulated physiological balance between pro-inflammatory- (IL-6, IL1b, TNF-a, MCP-1, etc.) and anti-inflammatory (IL-4, IL-10, IL-11, IL-13, etc.) cytokines depending on the degree of damages and the presence of antigens and cellular debris (**Table 1.1**) [69].

Chronic inflammation occurs under the sustained presence of foreign agents, necrotic cellular debris, and endothelial injuries and is characterized by higher expression of vasoconstrictors, pro-inflammatory factors, and excessive activities of leukocytes and their destructive mechanisms, including phagocytosis, ROS production, and secretion of matrix-degrading enzymes, such as the

matrix metalloproteinases (MMP) [70]. Moreover, chronic inflammation puts the immune system into constant overdrive, which impairs the body's overall defense against new antigens, rendering it more vulnerable to infection and sepsis [71].

Inflammation is a major factor in foam cell accumulation and, thereby, atherosclerosis. Notably, an increased risk of atherosclerosis has been associated with allergic individuals, who incur frequent and more severe inflammatory events [72]. Likewise, individuals with diabetes are predisposed to a systemic pro-inflammatory environment by producing glycated circulatory products, which are recognized by and stimulate inflammation through the endothelial receptor for advanced glycation end products [73]. Acute inflammation is a major risk of endothelial dysfunction, altogether impairing the vascular barrier and promoting leukocyte recruitment and oxidative stress. A pro-inflammatory endothelium upregulates several adhesive and chemotactic molecules to promote and aid leukocyte recruitment and retention, including ICAM-1, VCAM-1, E-selectin (ESEL), MCP-1, C-X-C Motif Chemokine Ligand 10 and 11 (CXCL10) (CXCL11) [74] [75]. Leukocytes more prone to inflammation highly express receptors for adhering molecules and chemotaxins, such as Very Late Antigen-4 (VLA-4), P-selectin (PSEL), Monokine Induced by Gamma Interferon (Mig), C-X-C Chemokine Receptor Type 3 (CXCR3), C-C Chemokine Receptor Type 2 (CCR2) [76] [77]. The presence of scavenging macrophages extensively propagates LDL retention in the vascular wall. M-CSF upregulation by oxidatively stressed endothelial cells promotes macrophage recruitment and the expression of the scavenger receptor class A (SR-A) and cluster of differentiation (CD) 36 (CD36) [78] [79]. Likewise, macrophages overexpressing the scavenger receptors exhibited increased lipid content in their foam-cell state [49]. In addition, foam-cell accumulation is also dependent on the rate of scavenger cells undergoing apoptosis, which can exacerbate, under inflammatory TNF-a exposure, ROS-mediated mitochondrial or DNA damage [80] [81].

Development of the fibrous cap is dependent on the rate of vascular smooth muscle cell accumulation and the production of the enclosing extracellular matrix. Under an inflammatory-prone environment, recruited leukocytes in the vascular wall can secrete cytokines and growth factors that stimulate vascular smooth muscle cell migration, proliferation and matrix production. Notably, lymphocyte expression of CD40 and its activation by interacting with its ligands CD154

upregulated by endothelial cells and scavenger macrophages have been demonstrated to stimulate vascular smooth muscle cells and exacerbate fibrous cap formation [82].

Sepsis is a severe inflammatory disorder wherein infectious agents are disseminated in the circulation, leading to systemic inflammation and hyper-production of inflammatory cytokines in tissues and circulation, causing multiple tissue and vascular injuries [83]. Septic shock quickly follows sepsis with clinical complications, including severe fever, diminished cardiac output, hypotension tissue hypoxia, and multiple organ dysfunction or failures. In addition, sepsis can lead to disseminated intravascular coagulation characterized by multiple thrombosis throughout the circulatory system. It's important to note that during atherosclerotic development, activated endothelium can have a prothrombic nature, which can further intensify the complications of both atherosclerosis and septic shock [83].

A well-study agent of sepsis, through which chronic inflammation can be studied, is lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria and a component of the bacterial outer membrane. LPS features a long-chain fatty acid anchor connected to a core sugar chain that forms an antigenic complex on the bacterial outer membrane. LPS is typically recognized by the pattern recognition Toll-like receptors (TLR), particularly TLR4, which proceed to induce inflammation at the site of infection. While the responses to LPS activate protective immunity by upregulating cytokines and chemokines that stimulate T-cell activity, high levels and the maintenance of LPS mediate excessive inflammatory responses and, thereby, chronic inflammation, from which sepsis and septic shock may occur upon the dissemination of LPS into circulation [84].

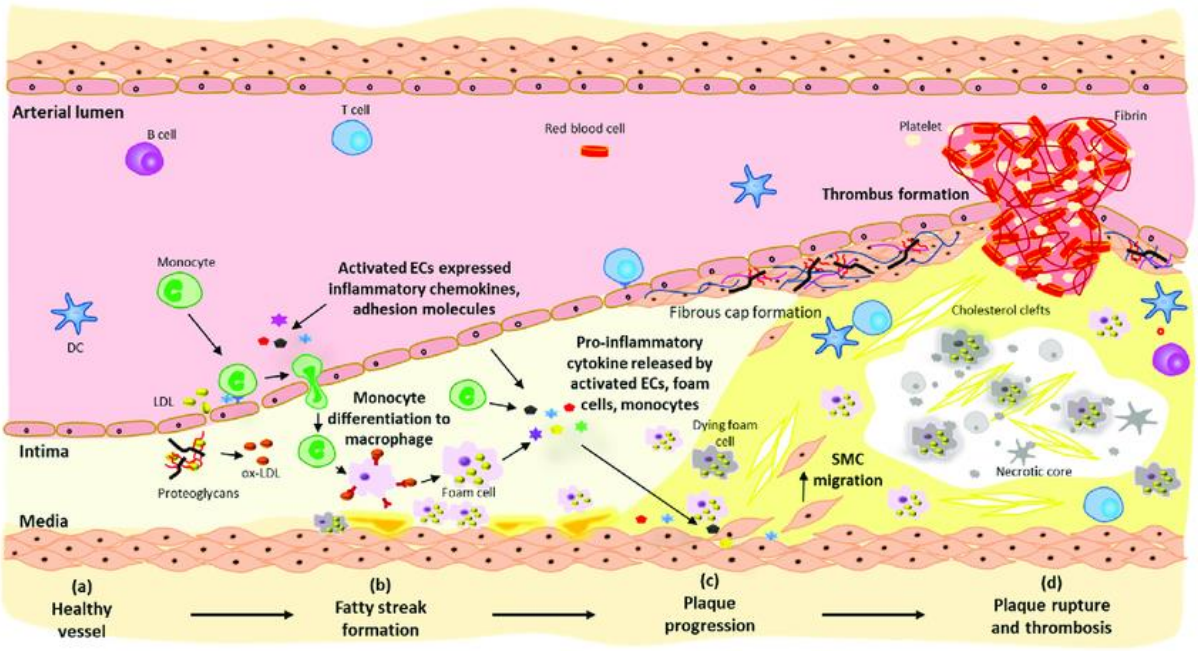


Figure 1.7 Pathogenesis of atherosclerosis, endothelial dysfunction, and the molecular mediators and products of endothelial cells

Hypercholesterolemia-derived LDLs undergo oxidation to oxLDL, instigating oxidative stress and triggering inflammation in the vessel walls. The endothelium responds by recruiting monocytes that transmigrate into the inflamed area and differentiate into scavenging macrophages (a). Chronic oxidative stress overwhelms these macrophages, forming foam cells that contribute to the lipid core and chronic inflammation (b). Concurrently, vascular smooth muscle cells migrate to the site, proliferating and forming a fibrous cap that further thickens the plaque, narrowing the lumen and setting the stage for hypertension and ischemic complications (c). Advanced plaques are prone to rupture, which precipitates thrombosis, leading to complete lumenal blockage and downstream ischemia (d) [111] *(CC BY-NC 4.0)*.

1.6.3 Hypertension

Physiological blood pressure serves to maintain efficient circulation as a function of *cardiac output*, determined by *stroke volume* and *heart rate*, and *vasculature peripheral resistance*, determined by *vasotone* [85] [4]. Blood sodium homeostasis and the renin-angiotensin-aldosterone

(RAS) pathway are the mechanisms by which cardiac blood volume and vascular tones are regulated, thereby being determinants of blood pressure. In physiological RAS, decreased blood pressure results in a low glomerular filtration rate that is detected by cells of the macula-densa of the glomeruli, upon which they secrete renin into circulation. Renin is a proteolytic enzyme that cleaves circulatory angiotensinogen produced from the liver into angiotensin-I (Ang I). The endothelium of multiple tissues, prominently the lungs, releases angiotensin-converting-enzyme (ACE) into circulation to process Ang I into angiotensin-II (Ang II). Ang II is a vasoconstrictor that interacts with the vascular smooth muscle cells of resistance vasculatures to raise blood pressure. Meanwhile, Ang II also targets the adrenal glands to stimulate the secretion of aldosterone, which acts to increase sodium and water reabsorption at the renal distal tubules. Increased reabsorption of water and vasoconstriction, in turn, raise cardiac blood volume and blood pressure. On the other hand, increased blood volume is detected by expansion receptors located in the heart's atria, which secrete the vasodilator *atrial natriuretic peptides,* in turn lowering blood pressure. Additionally, these peptides act on the distal renal tubules to inhibit sodium reabsorption, in turn reducing blood volume [86]. It is important to note that the endothelium forms the first line of contact with circulatory Ang II.

Pathological hypertension, typically chronic and asymptomatic on its own, is a critical risk factor for atherosclerosis. Cardiovascular risks associated with hypertension become more significant with age, and approaches to reduce blood pressure dramatically reduce these risks [87]. Indeed, most pathological hypertension is idiopathic and “essential,” which refers to the inevitable increase in blood pressure as “necessary” to accommodate adult end-organs as a person ages, albeit raising cardiovascular and atherosclerotic risks [88]. Contributing factors of essential hypertension, whether genetic or modifiable, revolve around sodium homeostasis, cardiac output and vascular resistance.

Dysregulation of ACE2, the major determinant of hypertension, is affiliated with increased vascular smooth muscle cell hyperactivity [89] [90]. Increased Ang II has been shown to directly stimulate vascular smooth muscle cell growth and matrix production and upregulate platelet-derived growth factor (PDGF) in endothelial cells, which also stimulates vascular smooth muscle cells [91] [92]. In addition, PDGF upregulates the coagulative *tissue factor* (CD142) in vascular

smooth muscle cells, which renders the fibrous cap vulnerable to rupture and enhances the risk of clot formation [93].

1.6.4 Metabolic and Other Factors

Aging and individuals with cardiovascular-risk, who are more prone to oxidative stress, are also more susceptible to DNA damage, which activates the key pro-apoptotic trigger tumour protein p53 (p53). This suggests a strong implication of atherosclerosis risk in individuals with defective breast-cancer genes (BRCA) 1 or 2, which are key players in DNA repairs. In addition, p53 regulates the inflammatory response, and p53-null mice exhibited pro-inflammatory predisposition [94]. Chronic inflammation, which is imposed in individuals who are pre-disposed to the metabolic syndrome, is also a source of accumulating DNA damage over time [95].

Lastly, a metabolic factor of LDL retention is impaired HDL activities. As part of metabolic homeostasis, the liver produces HDL by the assembly of apolipoprotein A1 (ApoA1), and circulatory HDL controls the cholesterol levels in peripheral tissues and blood [96]. HDLs absorb cholesterols from blood and tissues through the work of the lecithin-cholesterol acyltransferase (LCAT) enzyme and adenosine triphosphates (ATP) binding cassette subfamily A member 1 (ABCA1) transporter, respectively [97]. Notably, HDL interacts with endothelial cells through receptors, including scavenger receptor class B member 1 (SR-B1), ABCA1, and ATP binding cassette subfamily G member 1 (ABCG1) to mediate cholesterols efflux [98]. The induction of ABCA1 also upregulates cyclooxygenase-2 and its production of the anti-inflammatory and anti-thrombotic cytokine prostaglandin I2 (PGI2) [99]. In addition, HDL reduces pro-inflammatory signalling of nuclear factor Kappa B (NF-κB) [100], inhibits the pro-thrombotic expression of PDGF and CD142 [101], and upregulates c-Src and eNOS [102]. Collectively, HDL stimulates the endothelium to suppress inflammation and clot formation and promotes anti-inflammatory measures, junction assembly, endothelial integrity, antioxidant mechanisms, and vasodilation, all of which are compromised in individuals with familial HDL deficiency [96].

1.7 Endothelial Fatty Acids Metabolism

Fatty acids typically serve as a reliable source of long-term energy in many cells. In some cell-types, such as cardiomyocytes, they are the primary source of energy. In others, such as skeletal muscle cells, fatty acids are used as the backup energy in glucose deprivation scenarios. When not

in use, intracellular fatty acids are stored in cytosolic lipid droplets. Upon mobilization, they are liberated from these droplets and subjected to fatty acid oxidation in the mitochondria to produce acetyl-Coenzyme A (acetyl-CoA) that can fuel the tricarboxylic acid cycle and produce ATPs. Other sources of acetyl-CoA include glucose and amino acids such as glutamine [103].

In endothelial cells, fatty acids can be synthesized *de novo* by *fatty-acid synthase* [104] despite the cell's capability to uptake them from circulating lipoproteins [105]. Endothelial fatty acids are notably subjected to the mitochondrial fatty-acid oxidation and tricarboxylic acid cycles primarily for the production of the intermediates oxaloacetate and α-ketoglutarate, from which aspartate and glutamate are derived to be used in deoxyribonucleotides synthesis to support endothelial cells proliferation [106]. In this process, carnitine palmitoyltransferase 1a (CPT1a), which imports fatty acids into the mitochondria, was shown to be a rate-limiting enzyme of endothelial fatty-acid oxidation that selectively stimulates endothelial cell proliferation. Inhibition or silencing of CPT1a resulted in decreased deoxyribonucleotide triphosphate (dNTP) pool and impaired sprouting [106]. Indeed, the retinal vascular network in mice deficient for endothelial-specific CPT1a was compromised for branch point number and radial expansion. However, the migratory property of the endothelial cells in these mice remains normal [106]. Compromising endothelial CPT1 also reduced fatty-acid oxidation and increased endothelial permeability [107]. Moreover, fatty-acid oxidation was demonstrated to be essential in endothelial cell specialization by inhibiting endothelial-to-mesenchymal transition through transforming growth factor-beta (TGF-β) [108]. In addition to serving as lipid storages, the endothelial lipid droplets were also shown to protect against endoplasmic reticulum stress [103]. Mobilized fatty acids in endothelial cells are also utilized for modulating the endothelial cells' membrane lipid composition [109], as well as producing lipid-derived arachidonic acid metabolites, such as prostacyclins, which are regulators of endothelial inflammation, coagulation, and vascular homeostasis [110].

1.8 Fatty Acid Binding Proteins

Lipids are vital components of many biological processes and crucial in the pathogenesis of numerous common diseases, but the specific mechanisms coupling intracellular lipids to biological targets and signalling pathways are not well understood. This is particularly the case for cells burdened with high lipid storage, trafficking and signalling capacity, such as adipocytes and macrophages. Here, we discuss the central role of lipid chaperones—the fatty acid-binding proteins (FABP)—in lipid-mediated biological processes and systemic metabolic homeostasis through the regulation of diverse lipid signals and highlight their therapeutic significance. Pharmacological agents that modify FABP function may provide tissue-specific or cell-type-specific control of lipid signalling pathways, inflammatory responses, and metabolic regulation, potentially providing a new class of drugs for diseases such as obesity, diabetes and atherosclerosis.

The body's lipids are physiologically essential. In addition to serving as effective long-term metabolic energy storage, cellular lipids can have signalling roles in many metabolic and inflammatory pathways. For instance, eicosanoids, such as prostaglandins, are derived from fatty-acids metabolism and mediate the acute inflammatory responses [112]. In addition, lipid levels in adipocytes dictate their production of cytokines and adipokines, such as leptin and adiponectin, that have a potent impact on inflammation and metabolism [113]. Pathologically, as metabolic syndrome is closely linked to cardiovascular risk, diverse lipid signals and cells with high capacity for lipids storage, trafficking and signalling, such as adipocytes and macrophages, are crucial in the pathogenesis of CVDs. Therefore, lipids-related physiology and cardiovascular impacts crucially depend upon the specific processing and management of the bioavailability of cellular lipids. Such roles have been described for the prominently expressed FABPs, lipid-chaperones that regulate many lipid-related processes. The functional aspects of the FABPs are currently being investigated to provide pharmacological or diagnostic targets for controlling the body's lipid signalling and the associated inflammatory and metabolic mechanisms, which helps develop treatment for atherosclerosis and the metabolic syndrome.

The FABPs are small (12–15 kilodaltons) cytosolic proteins abundantly expressed in tissues with active lipid metabolism, such as the heart and liver, or cell types specialized for lipid storage, trafficking and signalling, such as adipocytes and macrophages [114]. They are a multigene family,

well-conserved, and known to be central in a variety of metabolic and cardiovascular disorders, including obesity, diabetes and atherosclerosis [115]. Structurally, all members of the FABP family share a β-barrel signature that consists of a water-filled cavity and a site that binds specific lipid-ligand unique for each member [116] (**Figure 1.8**).

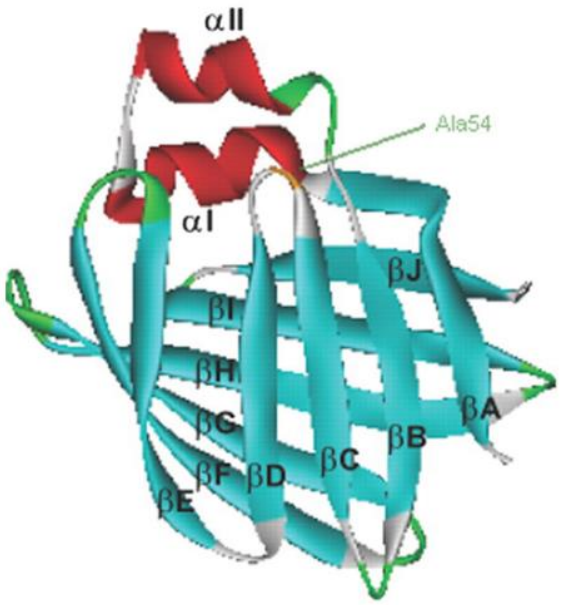


Figure 1.8 NMR-derived structure of human IFABP

A fatty-acid binding protein molecule comprises a β-barrel signature featuring a water-filled cavity and a fatty-acid binding site with a unique affinity that varies across different FABP members [117] *(CC BY-NC 4.0)*.

Nine members of the FABP family have been identified (FABP 1–9) with 20–70% sequence homology among members, and each is named according to the most abundantly expressing tissue in addition to their designed number. For instance, FABP3 is also heart-type FABP, which is most abundant in cardiomyocytes; FABP4 and 5 are adipocyte- and epidermal-type and expressed most prominently in the respective tissues [118]. Despite the unique tissue-expression pattern of each member, in general, tissues with active lipid metabolism tend to express FABPs and, often, more than one isoform. For instance, the small intestine, where active absorption of diet lipids occurs, expresses prominently FABP2, but also FABP1 (liver FABP) and FABP6 (ileal FABP) [119] (**Table 1.3**).

Table 1.3 Fatty-acid binding proteins expression pattern

Fatty Acid-Binding Proteins (FABPs) Family		
Gene Name	**Common Name**	**Expression Tissue**
FABP1	Liver FABP (L-FABP)	Liver, intestine, kidney, pancreas, lung
FABP2	Intestinal FABP (I-FABP)	Small intestine, colon, liver
FABP3	Heart FABP (H-FABP)	Heart, skeletal muscle, brain, kidney, lung, testis, aorta, adrenal glands, mammary glands, placenta, ovary, adipose
FABP4	Adipocyte FABP (A-FABP)	Adipose, macrophages, dendritic cell
FABP5	Epidermal FABP (E-FABP)	Skin, adipose, macrophages, brain, mammary gland, small intestine, colon, kidney, liver, lung, heart, skeletal muscle, testis, retina, spleen
FABP6	Ileal FABP (Il-FABP)	Ileum, small intestine, ovary, adrenal gland
FABP7	Brain FABP (B-FABP)	Brain, glial cells, retina
FABP8	Myelin FABP (M-FABP)	Peripheral nervous system (myelin sheath, Schwann cells)
FABP9	Testis FABP (T-FABP)	Testis, salivary gland, mammary gland

Functionally, the FABPs are known to reversibly interact with hydrophobic ligands with various affinities and mediate their escorts to coordinated sites of lipid metabolisms or signalling, typically serving as intracellular lipid chaperones. Reports up-to-date have documented some of the targeted

sites to be lipid droplets for storage, plasma membrane in lipid import and export, mitochondria for lipid metabolism, as well as the endoplasmic reticulum for phospholipid biosynthesis, specific enzymes for the production of lipid-derived signalling molecules, and the nucleus where their physical interaction with the peroxisome proliferator-activated receptors have been reported [120] (**Table 1.4**). For instance, FABP1 was shown to regulate peroxisome proliferator-activated receptor (PPAR) alpha (PPARα) in mammalian renal COS-7 cells [121]. However, the promoter of the FABP1 gene itself contains a peroxisome-proliferator response element, and, accordingly, FABP1 transcript level was shown to be regulated by intracellular fatty acids, dicarboxylic acids and retinoic acid [122]. The degree of expression may reflect the lipid-metabolizing capacity of a given tissue or cell type and can be modulated upon changes in the bioavailability of lipids, as in lipid exposure or usage processes [123]. While there is a strong regulatory connection between FABP expression and lipid-related signalling, the exact function of different FABP members remains poorly understood as their general mechanism is associated with a vast scope of complex lipid-related regulatory pathways. For example, FABP2 is abundantly expressed in the small intestine and known to bind absorbed saturated long-chain fatty acids with a high affinity. The lipid-intracellular trafficking of FABP2 is thought to be within the lipid-uptake, lipid-sensor, and lipoprotein synthesis pathways. However, complete ablation for FABP2 in mice did not compromise fat absorption but resulted in larger livers and higher triglyceride levels in males and the opposite in females [124]. In another example, studies have shown epidermal FABP5 influencing cell-survival pathways through PPARδ [125] [126] [127]. As the exact functions of each unique FABP member are still under investigation, large resources and attention in metabolic and cardiovascular research are being directed at three members among others: FABP3, 4, and 5.

Table 1.4 General Functions of Fatty-Acid Binding Proteins*

Lipid Metabolism	FABP escort cellular targets
Oxidation	Peroxisome and Mitochondria
Storage	Lipid droplets
Membrane Synthesis/Signaling	Endoplasmic reticulum and enzymes
Transcription (e.g., via PPARγ)	Nucleus

**Summary is based on* [120]

1.8.1 Endothelial Fatty Acid Binding Proteins

Endothelial cells possess extensive lipid-processing mechanisms, including lipid uptake and transport, fatty acid metabolic pathways for the synthesis of deoxyribonucleotide triphosphates that fuel proliferation, and the metabolism of lipid-derived arachidonic acid into various metabolites, such as the prostaglandins, thromboxanes, leukotrienes, etc. [128] [129]. Moreover, cholesterol metabolism was also shown in endothelial cells for their expression of the Niemann–Pick disease type C (NPC) 1 and NPC2 proteins, which mediate cholesterol uptake and trafficking [130]. The activities of the endothelial mammalian target of rapamycin (mTOR), which is a central player of a signalling network regulating cell growth and proliferation, are dependent on intracellular cholesterol trafficking. Pharmacological blockade of cholesterol trafficking by itraconazole or by silencing NPC 1 and 2 led to the inhibition of mTOR activity in endothelial cells [130]. These observations suggest that endothelial cells are active sources of lipid signalling and metabolism. However, few studies have been able to demonstrate the expression and activities of FABP in the endothelium. Nevertheless, two FABP members have been identified in endothelial cells. The expression of FABP3 and FABP5 have been described in microvascular endothelial cells of cardiac tissues and skeletal muscles. In addition, FABP4 and FABP5 expressions were found in the microvasculature of other organs with active fatty acids metabolism, including the liver and adipose tissues [131]–[132]. Moreover, Masouyé *et al.* presented that endothelial cells are capable of fluctuating their expression of FABPs depending on environmental factors, such as between tissue and culture conditions. This study detected FABP5 by immunocytochemistry in cultured human umbilical vein endothelial cells (HUVEC) but not *in vivo* [133].

FABP4 (adipose-FABP) has been described for its role in the development of metabolic syndrome through its mechanisms in adipocytes as well as macrophages. Both the differentiation of functional adipocytes [134] and macrophages [135] involve the regulation of FABP4. Moreover, cellular signals regulating FABP4 in adipocytes and macrophages include fatty acids, agonists of PPARγ, insulin, lipopolysaccharide, and oxLDL [136]. Reduced lipolysis efficiency was observed in adipocyte-specific-FABP4-deficient mice [137], and these mice were also found to have ameliorated insulin resistance during diet-induced obesity ([138],[139]). Moreover, apolipoprotein-E (ApoE)-deficient mice with FABP4 deficiency were found to be protected against atherosclerosis with or without induction by high-cholesterol Western diets [140], but how

FABP4 deficiency can alter insulin resistance and lipid metabolism remain to be revealed. FABP4 was also shown to be released from adipocytes into blood. While their biological roles in the blood remain unknown, serum FABP4 has been suggested as a potential biomarker for metabolic syndrome and CVD [141] [142]. In macrophages, FABP4 was found to modulate inflammation and cholesterol concentration. Administration of the cholesterol-lowering atorvastatin was found to suppress FABP4 expression in macrophages *in vitro* [143]. Additionally, in a study that demonstrated macrophage's FABP4 role in foam-cell formation through regulating the PPARγ/liver-X-receptor-α (LXR-α)/ABCA1 pathway, enhanced cholesterol efflux and upregulated PPARγ were observed in the macrophages of FABP4-deficient mice. The same study also found suppressed production of cytokines and pro-inflammatory enzymes, such as TNF-α and COX2, respectively, in macrophages of FABP4-deficient mice [144].

The epidermal-type FABP5 is most prominently expressed in the skin cells, but it is also expressed in multiple other tissues, including adipocytes and macrophages, as well as the tongue, brain, kidney, liver, lung, and testis [136]. In adipocytes, FABP5 expression is significantly minimal compared to FABP4 [145], but the loss of FABP4 induces the upregulation of FABP5 that, in fact, masks the phenotypic effects of FABP4-deficiency [138]. Unsurprisingly, FABP4 compensatory upregulation is not observed in adipocytes of FABP5-deficient mice due to the presence of a higher level of FABP4 [115]. In macrophages, the expression ratio of FABP4 and 5 is about identical, but no compensatory FABP5 expression is observed in FABP4-deficient mice [146]. Due to this wide and complicated pattern of tissue expression and regulation, the unique function of FABP5 remains unclear. Nevertheless, several *in vivo* phenotypes regarding FABP5 expression are relevant to metabolic syndrome. Transgenic mice overexpressing adipose-specific FABP5 exhibited enhanced lipolysis [147] and reduced insulin sensitivity [115]. On the other hand, increased insulin sensitivity was observed in adipocytes from FABP5-deficient mice [115]. FABP5-deficient mice also appeared healthy, without any changes in the normal epidermal fatty-acid composition [148].

As macrophage accumulation in adipose tissues characterizes the enhanced inflammatory response and risks for insulin resistance and CVDs in obesity [149], the roles of FABP4 and 5 in both adipocytes and macrophages contribute to the inflammatory and metabolic aspects of the metabolic syndrome and atherosclerosis [114]. In general, adipocytes and macrophages in mice deficient for these FABPs were more insulin-sensitive. Obese mice deficient for both FABP4 and

5 exhibited reduced tissue fatty-acid composition and did not develop insulin resistance [150]. Even when the ApoE$^{-/-}$ model was integrated, these mice showed less atherosclerosis development and increased survival compared to the wild-type [151]. The mice deficient for FABP4 and/or FABP5 also exhibited increased fatty acid levels in plasma [152], suggesting that the intracellular bioavailability of lipids, rather than the total body's amount, is more relevant to the development of metabolic and cardiovascular disorders. Recently, we were able to demonstrate that FABP4 levels were elevated in diabetic patients with PAD [142]. The elevation in FABP4 was independent of confounding factors such as age, sex, or prior history of coronary artery disease. Therefore, our work raises the possibility of utilizing FABP4 as a biomarker for diagnosing PAD in diabetic patients.

1.8.2 FABP3

FABP3 (heart-FABP) is expressed most abundantly in myocardiocytes and skeletal muscle. As a lipid chaperone, myocardial FABP3 is essential for the metabolic homeostasis of cardiac function. Physiologically, 70-80% of the energy in the heart is derived from the oxidation of fatty acids within the mitochondria and peroxisomes, which require lipid-trafficking mechanisms [153]. Increased exposures to fatty-acids were shown to upregulate FABP3 in myocytes [154]. FABP3 is also influenced by the metabolic essential PPARα agonists [155]. Diabetic patients, within whom fatty acids become the primary source of energy, also exhibited upregulated cardiac FABP3 [156]. In addition, FABP3-deficient mice showed elevated plasma free fatty acids and were compromised for cardiac fatty acid uptake, resulting in reduced exercise tolerance and a switch toward rapid glucose usage in the heart that leads to cardiac hypertrophy [157]. Meanwhile, similarly to other FABP members, FABP3 was also found in a multitude of other tissues, to a lesser extent, the brain, testis, kidneys, adrenal glands, and others [116]. For this notion, the unique function of FABP3 remains complex and unclear. For instance, while the FABP3 in skeletal muscles mediates the uptake and escorting of fatty acids to the mitochondrial oxidation system as similar to cardiac FABP3, increased apoptosis and exacerbated cardiac dysfunction have been associated with FABP3 overexpression in myocardiocytes [158].

Despite the functional complexity, FABP3 is currently utilized as a clinical biomarker for cardiac injury and heart failure, particularly in the diagnosis of myocardial infarction (MI). MI is characterized by the death of cardiomyocytes from injuries commonly due to atherosclerotic-

mediated cardiac ischemia. The current diagnostic approaches for MI include assessing initial chest pain, characteristic electrocardiography, and the detection of biomarkers for myocardial injury [159]. During a heart injury, myocardiocytes suffer a breakdown of cellular and subcellular components, which is followed by the release of these biomolecules from the injured cells into the circulatory system. Of clinical significance is the leakage of the cytosolic myocardial proteins; their release serves as a pathological biomarker that can be detected and measured in blood to enable early and efficient clinical assessment of cardiac injury. The more effective biomarkers are cardiac-specific and abundantly expressed in the myocardiocytes. Clinical guidelines for MI dictate that detecting/excluding MI within the first 6 h of chest pain would bring about the most effective clinical responses [160]. Currently, the only gold-standard biomarker for MI is the cardiac troponin, particularly the cardiac-specific subunits I and T, which can be detected 2–4 h after the onset of chest pain [161]. FABP3 has been identified as an effective biomarker of myocardial injury. Under normal conditions, the cytosolic to plasma presence ratio of myocardial FABP3 is significantly high, with negligible plasma concentration of FABP3 [162]. Within 30 min of chest pain, blood FABP3 begins to rise and peak in a few hours before returning to baseline due to renal clearance in about 24 hours. This early release of FABP3 from injured myocardium has been observed in both animal models [163] and MI patients [164]. Recently, our research group has demonstrated that patients with PAD have elevated plasma levels of FABP3. Our data demonstrated that the circulating levels of FABP3 increase as the severity of PAD worsens. However, extensive research is still required to validate FABP3 as a biomarker for PAD [165].

1.9 Emergent Roles of Epigenetic Regulation in Atherosclerosis

Non-mitotic cells express their genome in a DNA-protein complex super-structure known as chromatin, which is composed of structural units called nucleosomes. Each nucleosome comprises an octameric histone (H) core (two sets of H2A, H2B, H3 and H4) wrapped around tightly by genomic DNA. In this way, chromatin is initially thought of as a super-packaging entity that thermodynamically stabilizes the fragile and volatile nature of the DNA sequence. Epigenetics, however, reveals a novel nature of the chromatin as a super-regulatory catalogue, wherein specific nucleosomes can be modified to alter the accessibility of transcription factors to specific genes, thereby regulating specific phenotypes that establish the epigenetic imprint. To date, three general mechanisms of epigenetics have been described: 1) DNA methylation, 2) histone modifications,

and 3) post-transcriptional actions of non-coding RNAs [166] [167]. The first two mechanisms feature regulatory, structural opening or closing of specific chromatin segments and influence targeted gene expression. The third mechanism involves the expression of non-coding RNAs other than transfer and ribosomal RNAs that can interact with the targeted gene's mRNAs and influence whether they are properly translated to proteins, thereby affecting phenotypes without touching the targeted gene's sequences.

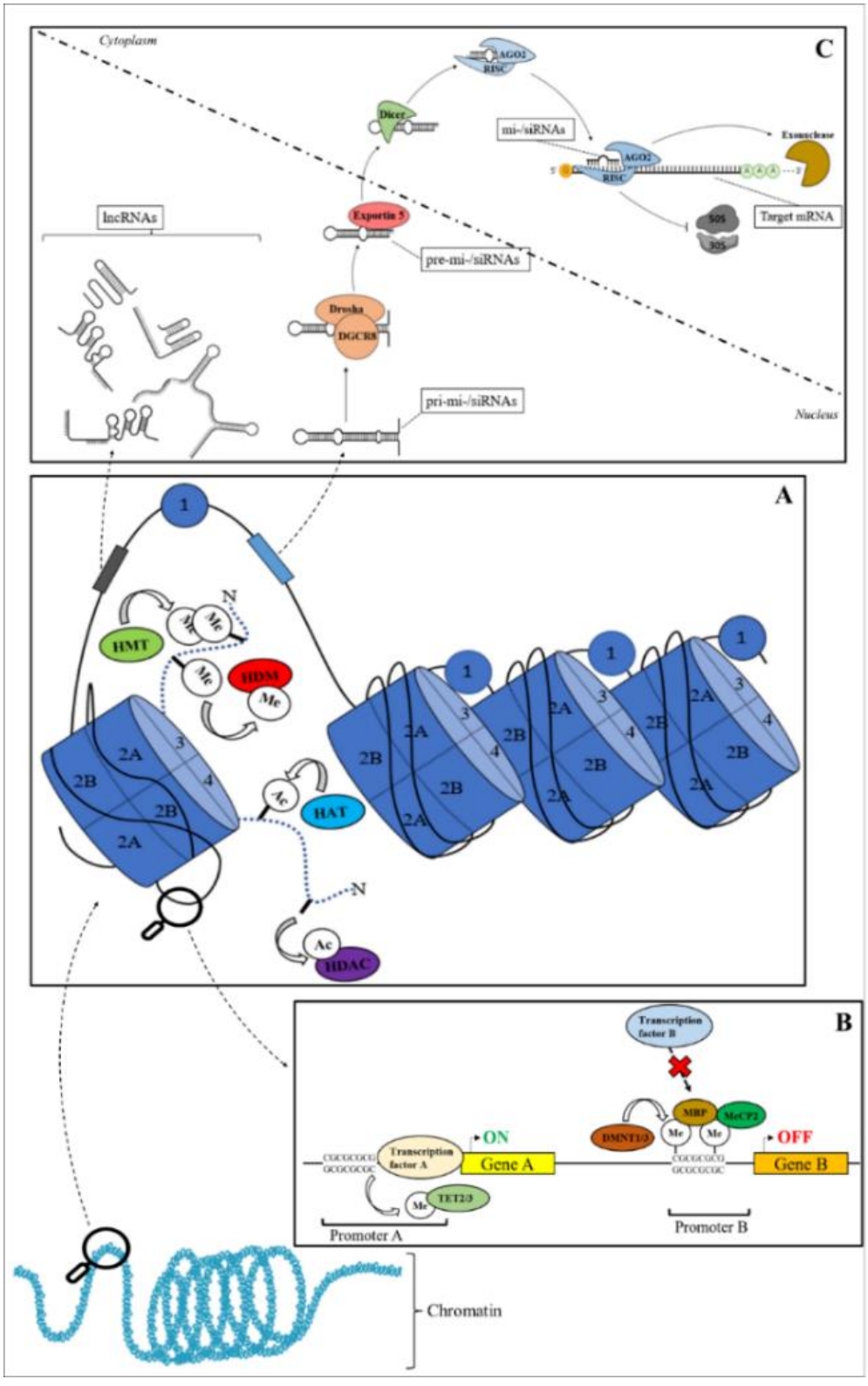
The current research focuses on the regulatory aspect of epigenetic mechanisms in various functional pathways (**Figure 1.9**). Broadly, epigenetic regulation has been identified in essential biological processes such as cell growth, differentiation, inflammation, etc. There has been a growing scientific interest in investigating the complex interactions of epigenetic mechanisms and their impact on the regulation of specific gene expressions. For instance, multiple methylation signatures at lysine K9 residues of histone H3 (H3K9) appear to induce DNA methylation [168]. Epigenetic cross-talks in actions of non-coding RNAs, however, remain a novel field of study [169] [170]. In line with the accumulating interest in epigenetic mechanisms, several therapeutic drugs have been developed to target epigenetic factors and modulate pathogenic gene expression in recent years [171]. Overall, epigenetics has emerged as the novel layer of the cellular regulatory gene-expression network and a new frontier for pharmaceutical development.

Recent studies have unearthed a newfound interest in the roles and potential therapeutic implications of epigenetics in atherosclerosis development (**Table 1.5**). Early understanding describes epigenetics as intrinsic cellular mechanisms by which the expression of specific phenotypes is altered without direct interactions with the DNA sequences of the associated specific genotypes. Recent advancements in the field have revealed that genes regulated by epigenetics establish an expression pattern that is unique to each individual, referred to as the epigenetic imprint, which can be influenced by environmental factors and be inherited [172]. Thus, a cardiovascular risk assessment that takes epigenetic imprints into account emphasizes the importance of considering the patient's family cardiovascular history and socioeconomic status in diagnosis or research-based demographic assessment. An additional benefit of epigenetic imprints in assessing cardiovascular risk is that previously established modifiable or genetic risk factors can further be interpreted and directed toward the individual based on their unique imprint, enabling target therapeutic strategies [173].

1.9.1 Long Non-Coding RNAs

A more recent novel player of the epigenetic imprint is the non-coding RNAs (ncRNA). The majority of sequences of euchromatin have been documented to be transcribed but not translated; these transcripts were initially thought to serve no value or, at most, be processed and modulate gene expression with transcription factors. Recent findings have demonstrated the epigenetic regulatory and clinical implication of ncRNAs through two types: short- and long-non-coding RNAs (**Table 1.5**). Extensive research continues to elucidate vast amounts of ncRNA candidates that participate in atherosclerosis development.

Long non-coding RNAs (lncRNAs) are transcripts of about 200 nucleotides in length with complex structure. Recent findings have suggested the roles of lncRNAs in a broad catalogue of regulatory actions, including working in both the nucleus and cytoplasm, influencing histone modification, working in tandem with micro RNAs (miRNAs), interacting with transcription factors and mRNAs, etc. [174]. In this notion, they are relatively less understood than their short counterparts in terms of mechanisms. However, in the context of atherosclerosis, several lncRNAs have been identified and investigated *in vitro* and *in vivo* for roles and signalling partners in multiple steps of atherogenesis. For instance, the long non-coding RNA lncLSTR was shown to affect blood cholesterols through regulating bile acid biosynthesis, particularly interacting with TAR DNA-binding protein 43 (TDP-43) and cytochrome P450 family 8 subfamily B member 1 (Cyp8b1) and increasing production of ApoC2 [175]. In the liver, the lncRNA LeXis interacts with the nuclear receptor liver X receptor (LXR) in regulating cholesterol production [176]. As their mechanisms remain obscure, lncRNAs have roughly been categorized into multiple functional modes surrounding transcriptional regulation [177]. Nonetheless, multiple lncRNA candidates have been identified as being involved in multiple steps of atherogenesis (**Table 1.5**). The mechanisms of lncRNAs and their functional implications in cardiovascular disease are therefore warranted as subjects of future studies.


C
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Exonuclease
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lncRNAs
Exportin 5
Target mRNA
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30S
pre-mi-/siRNAs
Drosha
DGCR8
pri-mi-/siRNAs
Nucleus
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HDM
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2A
2B
3
4
Ac
HAT
N
Ac
HDAC
B
Transcription factor B
MBP
MeCP2
DNMT1/3
Me
Me
ON
OFF
Transcription factor A
Gene A
Gene B
TET2/3
Me
Promoter A
Promoter B
Chromatin

Figure 1.9 Regulatory Mechanism of Epigenetic Imprints

A) Histone modification: chromatin is organized into nucleosomes that can be modified to regulate between euchromatin ("opened") or heterochromatin ("close") arrangement. Methylation and acetylation, mediated by histone methylase (HMT) or demethylase (HDM) and acetylase (HAT) or deacetylase (HDAC), respectively, are conducted onto lysine or arginine residues of the histone subunit's N-terminal tails, commonly on the H3 and H4 subunits. **B) DNA methylation**: DNA methylase and demethylase, such as DMNT1/3 and TET2/3, regulate the methylation imprints on CpG sequences of specific gene promoters, controlling transcription factors. **C) Non-coding RNAs**: a majority of euchromatin transcribe vagabond RNAs that regulate specific gene expression through complementary base-pairing, including long non-coding RNAs (lncRNAs), microRNAs and small interfering RNAs (mi-/siRNAs). LncRNAs are known to function in the nucleus, although their mechanisms remain under-investigated. Endogenous miRNAs or siRNAs are expressed first as primary miRNAs (pri-miRNAs), then processed to precursor miRNAs (pre-miRNAs) and exported to the cytoplasm by Drosha and Exportin. Pre-miRNAs are then processed to mature miRNAs, loaded onto RISC-AGO2, and guided the complex to the targeted gene's mRNAs. RISC-AGO2 arrests the targeted transcript and inhibits its translation; then, the degree of base-pairing at the 'seed' region determines whether (miRNAs) exonuclease is recruited to degrade the arrested transcript (siRNAs). Created with BioRender.com (Agreement # RW26K0OMO0).

Table 1.5 Roles of Epigenetic and Non-coding Mechanisms in CVD

Epigenetic Signatures / *Atherogenesis processes*	**Altered DNA-methylated genes promoters:**	**Histone methylation/acetylation markers & affected genes:**	**Regulatory lncRNAs**	**Regulatory miRNAs**
Hypertension and endothelial injury	*EGFR, MAP1B, MYH10*	H3K9 (*AGTR1*)		
Impaired lipid metabolism & oxidative stress	*PXDN*	H3K4me3 (*FOXO*), H3K27me3 (*FOXO*), HDAC9 (*ABCA1, ABCG1, PPAR-γ*)	*ANRIL, LeXis, MeXis, Gas5,*	*miR-200c, miR-33, miR-302, miR-21*
Endothelial dysfunction	*MIR23b, HOXB3, A2BP1, DAAM1, PLA2G10*	H3K9me2 (*ICAM1*), H3K4me3 (*ICAM1)*	*SNHG12, SENCR*	*miR-34a, miR-217, miR-146a*
Inflammatory cytokines/chemokines upregulation	*HOXA9*	H3K4me1 (*NF-κB*), H3K4me2 (*MCP-1, NF-κB*), H3K4me3 (*IL-6, IL-18, IRF8, MCP1*), H3K9 (*IL-6, MCP-1, MIP-1α,, MIP-1β*), H3K27me3 (*IRF8*), HDAC7 (IL-6, MCP-1), HDAC9 (PPAR-γ)	*RP5-833A20.1*	*miR-126a, miR-21, miR-19a, miR-19b, miR-146a, miR-128, miR-185, miR-365, miR-305*
Leukocyte recruitment and scavenger fate	*PDGFD*	H3K4me3 (*TNFα, CD36, KLF4*), H3K27me3 (*KLF4, TNFα*), H3K9me3 (*TNFα*)	*TUG1, NEXN-AS1, MANTIS, RNCR3, MALAT1, RAPIA, NEAT1*	*miR-21, miR-33*
VSMCs stimulation	*CALD1, KCNMA1, RPTOR, PRRX1, PTK2, TSC22D1*	H3K9 (*ROCR2*), H3K4me3 (*MMP9, MMP2*), HDAC9 (*MMP12*)		*miR-143/145, miR-21, miR-1, miR-10a, miR-126, miR-22, miR-26a, miR-34a, miR-103a, miR-221*
Pro-thrombotic processes	*FBN2, PRKCE, PLAT*			
References:	(Zaina et al., 2014)	(Jiang et al., 2018b)	(Z. Zhang et al., 2018)	(Lu et al., 2018)

1.10 References

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Chapter 2: Heart-type Fatty Acid Binding Protein is Differentially Regulated in Endothelial Dysfunction and Atherosclerosis

2.1 Abstract

FABP3 is a biomarker for cardiac injuries. Recently, an increased level of circulatory FABP3 was reported in peripheral artery disease (PAD) in the absence of cardiac injury; however, the source of circulatory FABP3 in PAD is not clear. Endothelial dysfunction, driven by factors like oxidative stress, inflammation, and hypertension, is central to the pathogenesis of PAD or atherosclerosis and associated cardiovascular complications. The expression and functional roles of FABP3 in endothelial cells remain under investigation. We aimed to determine the expression of FABP3 in endothelial cells under various stressors associated with endothelial dysfunction and atherosclerosis. Our results showed that FABP3 is basally expressed and differentially modulated in response to treatments of oxidized low-density lipoproteins (oxidative stress), angiotensin-II (hypertensive milieu), and the endotoxin lipopolysaccharide (inflammation), suggesting dynamic regulatory mechanisms associated with endothelial function. Understanding FABP3 regulation in endothelial cells provides insights into the endothelium's role in the pathogenesis of PAD and other atherosclerotic complications, potentially leading to targeted therapeutic interventions for cardiovascular diseases.

2.2 Introduction

Fatty acid-binding proteins (FABPs) are a family of transport proteins for fatty acids and other lipophilic substances between extra/intra-cellular membranes and receptors and play an essential role in regulating lipid homeostasis [1]. FABP3 is currently investigated as an effective biomarker for cardiac injuries for the following features described in both animal models and heart failure patients: 1) low concentration in blood and a notably high ratio of cytosolic to blood concentration at rest; 2) an increase in blood levels noticed within 30 minutes of chest pain, peaking in a few hours, then returning to normal levels through renal elimination, all within 24 hours [2] [3] [4]. In addition, FABP3 has also been tested as a biomarker in patients with peripheral artery diseases (PAD) in the absence of cardiac injury or coronary artery disease background. Notably, the upregulation of FABP3 in skeletal muscle cells has been identified in patients with critical limb ischemia (the most severe form of PAD) compared to healthy individuals [5]. However, whether

skeletal muscle cells are the sole source of FABP3 in PAD or whether FABP3 is also released by other cell types, such as endothelial cells, is unknown. Nonetheless, these findings suggest that FABP3 release is not exclusive to cardiac injury and may signal earlier cardiovascular events.

PAD and heart failure are cardiovascular complications of atherosclerosis. Atherosclerosis, the primary cause of cardiovascular diseases, which represents an epidemic and the top-leading cause of death worldwide, is characterized by the build-up of lipid-laden plaques in the vascular inner walls, which leads to compromised circulation, downstream ischemia, hypoxia, and organ failures. Clinical complications associated with atherosclerosis include heart failure, stroke, and peripheral artery diseases. Endothelial dysfunction is the central mechanism of atherogenesis, influenced by three prominent pathophysiological processes: oxidative stresses, chronic inflammation, and hypertension [6] [7].

The endothelium comprises specialized epithelial cells called endothelial cells that form a single lining that coats the inner luminal wall of blood vessels to serve as the first metabolic and regulatory barrier between blood and tissues [8]. As endothelial cells are active lipid-metabolizing cells, the roles of FABPs in endothelial function remain under investigation [9]. Moreover, endothelial cells are one of the first cell types to be exposed to circulatory FABP3 during a cardiovascular event; however, the regulatory implications between FABP3 and endothelial cells that are related to endothelial function remain unexplored. Given this context and the fact that endothelial dysfunction drives atherosclerosis and underlies cardiovascular complications, we hypothesize that endothelial cells are a source of FABP3 with regulatory implications under endothelial dysfunction.

Our goal was to evaluate the expression of FABP3 in the endothelium at baseline and under atherosclerosis-related stress. Endothelial cells are cultured and exposed to oxidized low-density lipoprotein (oxLDL), lipopolysaccharide (LPS), and angiotensin-II (Ang II), which are prominent agents of oxidative stress, inflammation, and hypertension, respectively [10] [11] [12]. Our data show that FABP3 is expressed at baseline in endothelial cells and is differentially regulated under stress in a context-dependent manner.

2.3 Methods

Cell Culture - Human Umbilical Vein Endothelial Cells **(**HUVECs, Lonza # CC-2519, pooled), a standard model to study endothelial cell function *in vitro* [13] [14] [15] [16] [17], were grown in endothelial-cell complete growth medium-2 (EGM™-2 Bulletkit™; Lonza) under standard conditions (37°C, 5% CO2, and 90% humidity). Frozen newly obtained HUVECs (passage#1-2) were thawed rapidly at 37°C prior to transferring into warmed (37°C) complete growth media in tissue-cultured treated flasks, appropriated for culture area by seeding density in biosafety cabinet. The very first media change was done after 4-5 hours to replace the media with the preservatives from the frozen container. From then on, the media was changed every 24-48 hours until the cells became confluent (1.5 mL media per 5 cm^2 when confluency was below 45%, which increased to 2 mL per 5 cm^2 when confluency was greater than 45%). Typically, 70-85% confluent cells were readied for experiments, passaging, or storing, prior to which a cell suspension was obtained. In establishing cell suspension, the cell flask was aspired for old media and washed with phosphate-buffered saline (PBS) twice, followed by the addition of trypsin (0.25% with EDTA, #25200056, Gibco) and incubation for 3-5 minutes in 37°C to detach and suspend adherent HUVECs. Next, 20% fetal bovine serum (FBS) media was added at a 1:1 ratio to deactivate the trypsin. The cells were then flushed down to the bottom of the flask and harvested into centrifugation tubes. Centrifugation was performed at 300 g for 5 minutes at room temperature to pellet the cells, followed by the removal of the trypsin-containing supernatant. The pellet was then suspended in new, fresh and complete growth media. Cells were then counted and applied to downstream processing (freezing, experiments, passaging for expansion). Passage#1-2 cells were sub-cultured to passage#3 for long-term storage and expansion. For storage, cells during passage#1-3 were suspended as above, counted, and combined with 80% complete growth media, 10% FBS, and 10% dimethyl sulfoxide (DMSO) to establish the freezing media. This mixture was transferred into cryovials and chilled on ice or at 4ºC for at least 5 minutes. The cryovials were then stored in a -80ºC freezer overnight. The next day, they were transferred into a liquid nitrogen container. On the other hand, starting with passage#4, cells were sub-cultured to perform experiments. In sub-culturing, the media was changed every 24-48 hours, similarly to the above, until the cells became 70-85% confluent and ready for another round of passaging or experimentation.

For Human Skeletal Muscle Cells (SkMCs), the culture conditions for primary SkMCs (PCS-950-010, ATCC) were 37°C, 5% CO2, and 95% humidity. Primary SkMC stem cells were received and stored in the *vapour phase* of liquid nitrogen (<-130ºC). During application, media or solutions (differentiation, saline, trypsin, etc.), except for cell lysis, were warmed to 37ºC before addition into the cultures. Cells were seeded initially at the recommended 2500-5000 cell/cm^2 culture (92.7% post-freeze cell viability) in Complete Expansion Medium (CEM), combined from 1X Mesenchymal Stem Cell Basal Medium (ATCC PCS-500-030) and 1X Primary Skeletal Cell Muscle Growth Kit (ATCC PCS-950-040), into pre-warmed (37ºC) culture flasks/plates following manufacturer's specific manual. To maintain the culture, old media was replaced with new, warmed CEM. When confluence was < 80%, media change was repeated once every 48 hours. Cells were sub-cultured, assayed, or frozen when the confluence was 80-90%. Subculturing and freezing were conducted similarly to the above, with CEM used as the complete growth media. In sub-culturing, trypsin (0.25% with EDTA, #25200056, Gibco) and CEM (containing fetal bovine serum) were used to obtain cell suspension. Cells were passed into new cultures with CEM. Prior to assays and after 24 hours of culturing, primary SkMCs were differentiated using the Primary Skeletal Differentiation Tool (ATCC PCS-950-050) for 96 hours, with differentiation solution replaced every 48 hours, on culture plates coated with fibronectin (F-0895-1mg), following the manufacturer's instruction. Experimental treatment and assays were only conducted on differentiated skeletal muscle cells.

Treatments: HUVECs were treated with either LPS (Sigma-Aldrich), commercial oxLDL (Invitrogen), Ang II (Sigma) or diluent (PBS) in MCDB-131 (+1% FBS) media. Dose- and time-dependent experiments were conducted using PBS as a diluent to establish serial treatment concentrations and as the control samples.

Cell Counting: Counting cells and obtaining the cell concentration of the cell suspension were necessary to either freeze cells for storage, seed cells for experiments, or passage cells into new cell culture flasks. The Corning Cell Counter (Cytosmart) was employed for automated cell counting. 1:1 mixing the cell suspension with trypan blue and loading of 10 µL mixture per chamber of a hematocytometer were achieved prior to live-image cell-counting. Both chambers were counted eight times each, and the overall averages were taken as the cell concentration of the suspension.

RNA Extraction: Total RNAs were extracted and quantified using the Trizol standard method (Invitrogen) [18]. In brief, from the tissue cultures, media was removed, cells were washed with cold PBS, and then Trizol was added at a calibrated volume (following the manufacturer's recommendation). The plates were shaken and left to rest at room temperature for 10 minutes, allowing for the dissociation of RNA-protein complexes. Lysates per wells were further homogenized by pipetting and transferred into a 1.5 mL centrifuge tube. Chloroform was added at 20% volume of Trizol to each sample, and samples were then mixed vigorously, allowing for phase separation at room temperature for 3 minutes. As phase separation formed, the tubes were centrifuged at 12000 g for 15 minutes at 4°C, after which an aqueous layer, an interphase layer, and a coloured organic layer were clearly defined. Next, the RNA-phenol pure aqueous layer of each sample was transferred to a corresponding tube with isopropanol at a 1:1 volume ratio. The tubes were then inverted to mix and incubated at room temperature for 10 minutes to allow for the precipitation of RNAs. Afterward, the tubes were centrifuged at 12000 g for 10 minutes at 4°C. The phenol supernatant of each sample was decanted, and the RNA pellets were washed with 75% Ethanol in RNAase-free water [diethyl pyrocarbonate (DEPC)-treated water] two times, with a round of centrifugation at 7500 g for 5 minutes at 4°C after each wash. The ethanol supernatant was decanted, and each sample's RNA pellet was air dried 5-10 minutes before suspension in 20 µL of RNAse-free water. On the NanoDrop ND-1000 spectrophotometer (Thermo Scientific), 2 µL of each RNA sample were then measured for RNA concentration and purity assessment via the 260/280 and 260/230 wavelength absorbance ratio (pure RNA was considered with a ratio of about 2 and 1.8, respectively).

cDNA Synthesis: Complementary DNAs (cDNA) were synthesized from isolated RNAs for each sample by the QuantiTect Reverse Transcription Kit (Qiagen). Briefly, an equal amount of RNAs from each sample (1 µg) was mixed with 2 µL of the provided genomic DNA wipeout buffer and incubated at 42°C for the total removal of DNA from the samples. Next, 6 µL of the master mix, prepared for all RNA samples from the kit-provided reverse transcriptase, enzyme buffer and primer mix at a ratio of 1:4:1, respectively, was added to each sample. The samples were incubated at 42°C for 30 minutes to undergo cDNA synthesis, followed by 95°C for 3 minutes to inactivate reverse transcriptase and complete the reactions. The cDNAs from each sample were then diluted to 10 ng/µL for quantitative real-time polymerase chain reaction.

Quantitative Real-Time PCR: Quantitative polymerase chain reactions (qPCR) were conducted to evaluate the expression of targeted genes from each sample. Commercial qPCR master-mix with SYBR Green dye (BioRad) combined with primers with sequences (forward and backward) targeting specific genes (following the formula from the manufacturer's manual) was added to the cDNA pool of each experimental sample (up to 30 ng) on a 96 well-plate (3 wells per sample, equivalent to 3 technical replicates). Non-template controls containing no cDNAs were also established in triplicates. The prepared plate was sealed, spun to bring each well's mixture to the bottom, and shaken to mix solutions prior to being processed by the QuantStudio-3 Real-Time PCR thermocycler (Applied Biosystem) with the recommended thermal cycling program: 1) 10 minutes at 95ºC for activation; 2) 15 seconds at 95ºC for denaturation; 3) 1 minute at 60ºC for annealing or extension at which real-time dye signal is collected and charted; then repeat with denaturation again for 40 cycles. Sequences for primers used to amplify gene targets are summarized in **Table 2.1**.

Table 2.1 Sequences for the primers used to amplify the gene targets

Gene targets	***Sequence***	
	Forward	***Reverse***
GAPDH	5′-CACCAGG GCTGCTTTTAACTCTGGTA-3′	5′-CCTTGACGGTGCCATGGAATTTGC-3′
FABP3	5'-CATGACCAAGCCTACCACAAT-3'	5'-CCCCAACTTAAAGCTGATCTCTG-3'
FABP4	5'-ACTGGGCCAGGAATTTGACG-3'	5'-CTCGTGGAAGTGACGCCTT-3'
FABP5	5'-TGAAGGAGCTAGGAGTGGGAA-3'	5'-TGCACCATCTGTAAAGTTGCAG-3'

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); Fatty acid binding protein- 3, 4, 5 (FABP-3, 4, 5).

Immunofluorescence: Cells were grown (37°C, 5% CO2, 95% humidity) and treated on specialized cell-culture 4-chamber slides (Nunc Lab-Tek II CC^2), with each chamber containing 2.5 x 10^4 cells in 1 mL of complete growth medium. When cells were ~70-80% confluent, media was removed, and cells were *rinsed* with 500 µL PBS twice, followed by fixation using 400 µL fixative (4% Paraformaldehyde, pH 7.4) for 10-20 minutes at room temperature and washed thrice with PBS. Next, cells were permeated for 30 minutes at room temperature by the addition of 400 µL

permeabilizer (0.3% Triton X-100 in 1X PBS) per chamber, followed by another 3 rounds of washing. The cells were then incubated in blocking buffer (1% bovine serum albumin in 1X PBS) for 1 hour at room temperature, followed by incubation with primary antibodies (300 µL per chamber, 1:200 diluted in 0.1% BSA in 1X PBS) for 3 hours at room temperature (or overnight at 4ºC). Next, the chambers were washed 3 times, using washing buffer (0.05% Tween 20 in 1X PBS, 500 µL per chamber), before incubation in fluorophore-tagged secondary antibody (300 µL per chamber, 1:1000 diluted in 0.1% BSA in 1X PBS) for 1 hour at room temperature while protected from light. After another 3 rounds of washing buffer, using the provided tools, the chambers' walls were removed, followed by one/two drops of DAPI Mounting Medium into the central regions of each chamber. Next, a cleaned coverslip was placed carefully (ensuring no air bubbles) on top of the entire slide. The slides were immediately stored in the dark overnight at 4ºC (for imaging next day) or -20C (long-term). Confocal microscopy was employed to capture fluorescing signals of the targeted proteins, and images were analyzed using ImageJ [19].

Statistical Analysis: Differences between the means of two groups and more than two groups were calculated using the Student's T-test and Analysis of Variance (ANOVA) statistical analysis, respectively. ANOVA significant results were further validated using the post-hoc Tukey's test. Data are presented as mean ± SD unless otherwise indicated.

2.4 Results

OxLDL and Ang II treatments downregulate FABP3 in endothelial cells. HUVECs express a basal level of FABP3 transcript under standard *in vitro* conditions, which was previously believed to be predominantly expressed by cardiomyocytes (**Figure 2.1**). When subjected to oxidative stress, FABP3 expression in oxLDL-treated HUVECs appears to be downregulated in a dose-dependent manner**.** Additionally, FABP4 and 5, known to be expressed by endothelial cells, are also downregulated similarly by oxLDL after 24h of treatment (**Figure 2.1A, B, C**). Time-wise, FABP3 is initially upregulated by 6h of oxLDL treatment but downregulated at 12h and 24h, suggesting a chronological sensitivity pattern **(Figure 2.1D).** When exposed to angiotensin-II, which is upregulated in circulation during vascular hypertension and, thereby, representing the biomolecular milieu of hypertension, FABP3's transcript levels in HUVECs are downregulated in a time-dependent manner (**Figure 2.1E**), at a dose of 1 µM (**Figure 2.1F**). These findings suggest a directional, regulatory dynamic of FABP3 expression at the transcript levels in endothelial cells

under atherosclerotic stresses and a trend of downregulating the general FABP function in endothelial cells under oxidative stress, respectively.

LPS upregulates FABP3 in endothelial cells, similar to human skeletal muscle cells under hypoxic stress. In contrast to oxidative stress and Ang II treatments, HUVECs FABP3 is upregulated at the protein level by LPS treatment at 100 ng/mL for 24 hours (**Figure 2.2**). As hypertension, oxidative stress, and inflammation often occur together in atherosclerosis development, this opposite observation suggests a dynamic regulatory implication beyond a common metabolic response by FABP3 expression in cardiovascular stresses. Likewise, SKMCs under 6h hypoxia, representative of injured skeletal muscle cells in PAD patients, also upregulate FABP3 (**Figure 2.3**), which may be associated with similar regulatory implications of FABP3. However, future work is required to focus on SKMCs to validate this implication.

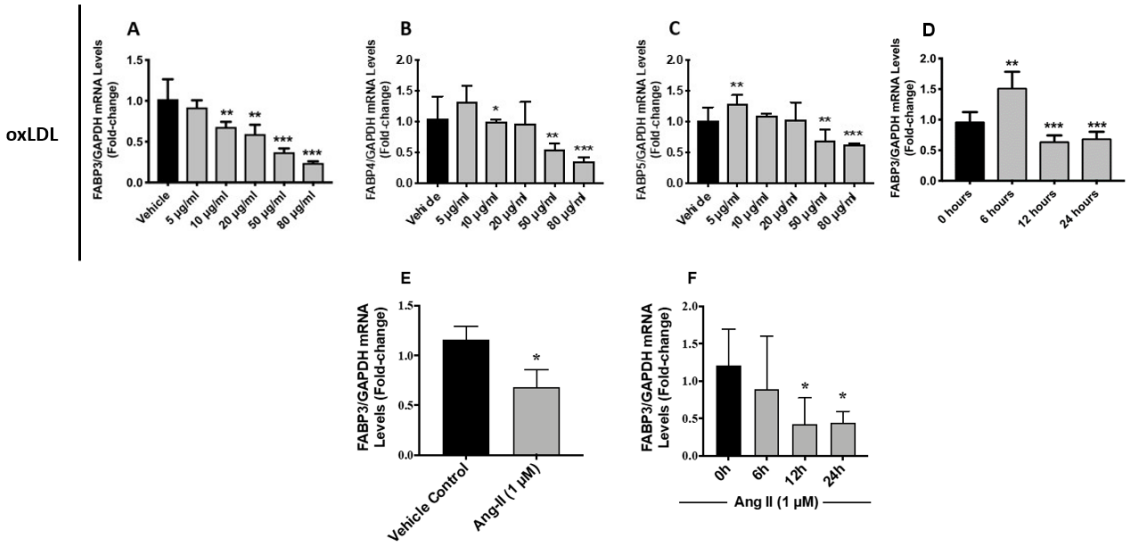

Figure 2.1 HUVECs' FABP3 expression under oxidative and hypertensive environments

qPCR data demonstrating that HUVECs' FABP3 expression is downregulated by oxLDL in a dose-dependent manner (24h treatment) (A). Timewise, FABP3 appears to be upregulated in oxLDL-treated HUVECs after 6h treatment but downregulated in the subsequent hours (80 μg/mL) (D). FABP3 in HUVECs is downregulated by Ang II at 1 μM (24h treatment) (F) and in a time-dependent manner (1 μM) (E). As FABP4 and 5 have also been found in microvasculatures, FABP4 and FABP5 were also downregulated by oxLDL (B, C). *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* Vehicle, $N \geq 3$ in triplicates. Data is represented as mean + SD.

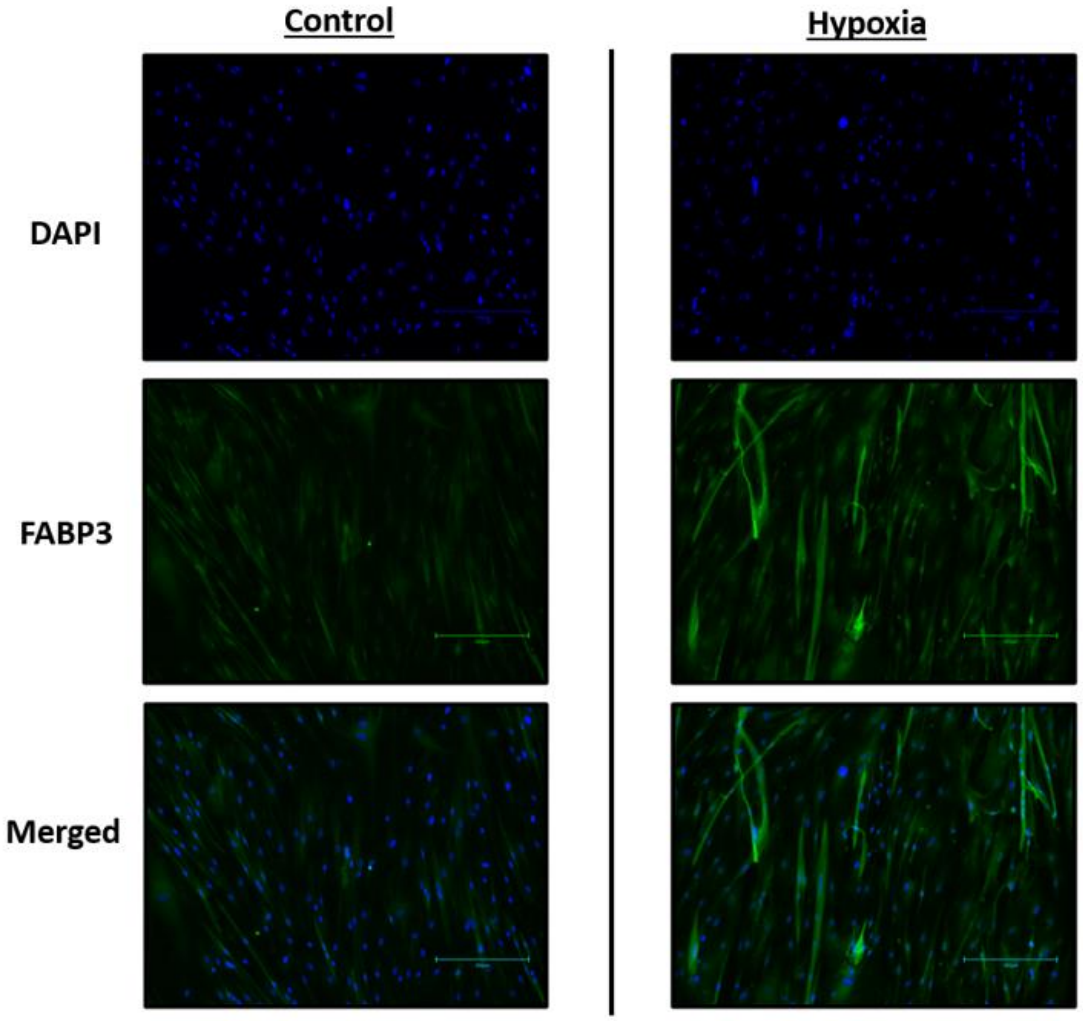

Figure 2.2 Hypoxia-induced (6h, 1% O2) FABP3 upregulation in human skeletal muscle cells

Immunofluorescence staining for FABP3 (green signals) in skeletal muscle cells subjected under hypoxia. Slides were counter-stained with DAPI (blue signals) for nuclei. N = 4.

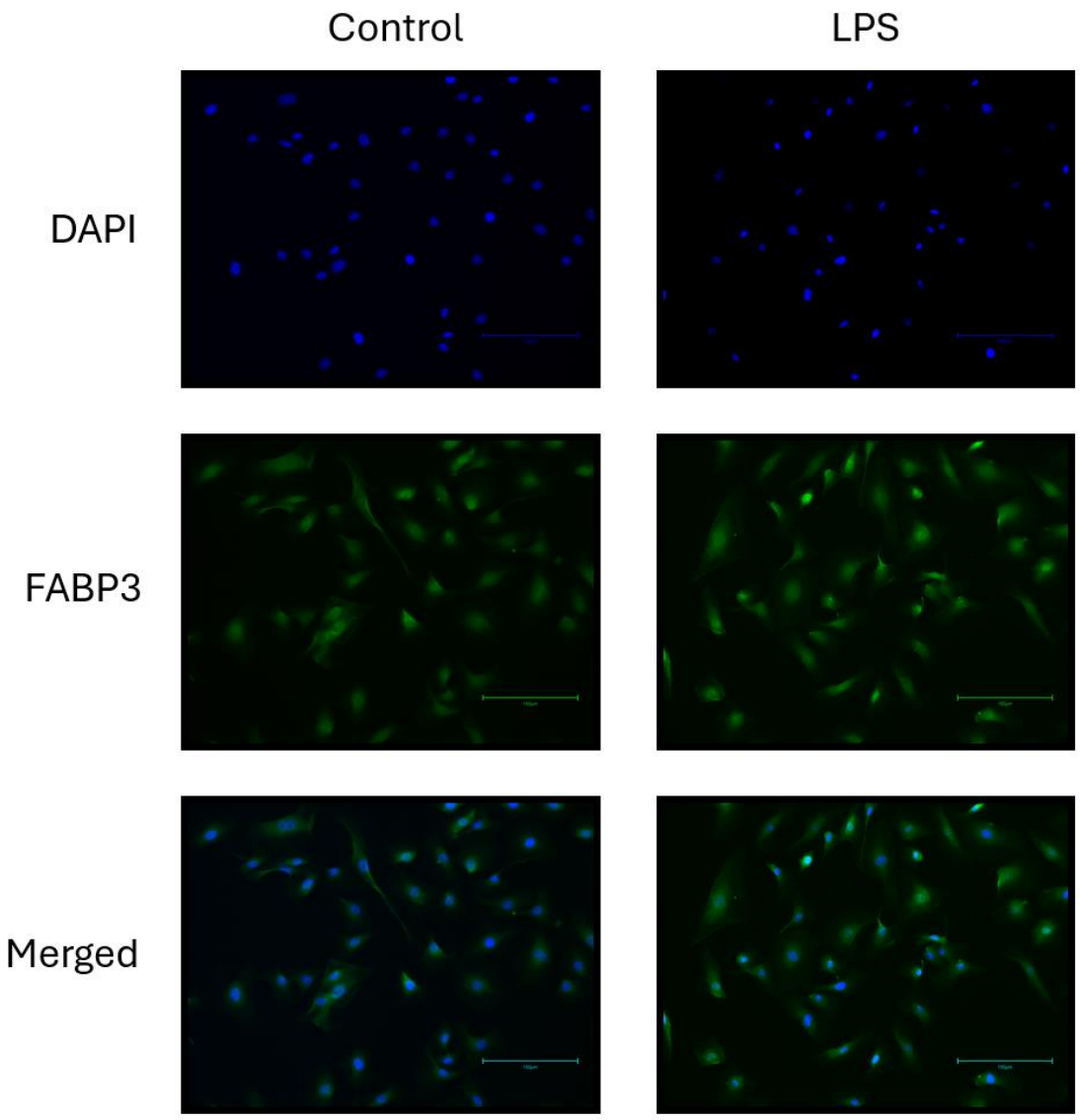

Figure 2.3 Endothelial FABP3 is upregulated in endothelial dysfunction induced by LPS

Immunofluorescence staining for FABP3 (green signals) in HUVECs subjected under LPS. Slides were counter-stained with DAPI (blue signals) for nuclei. N = 2.

2.5 Discussion

Endothelial cells are a source of lipid-derived arachidonic acid metabolites, such as prostacyclins, which are regulators of endothelial inflammation, coagulation, and vascular homeostasis [20] [21].

While the endothelium primarily uses glucose for energy, endothelial cells also rely on lipid uptake, transport, and metabolism to synthesize building-block and signalling lipids such as deoxyribonucleotide triphosphate and arachidonic acid metabolites [22]. Endothelial cells also express Niemann–Pick disease type C (NPC) 1 and NPC2 proteins, which mediate cholesterol uptake and trafficking [23]. However, only a few studies have characterized the roles of FABPs, which regulate intracellular lipid-bioavailability, in the endothelium [24] [25] [26] [27].

The FABPs are small (12–15 kDa) cytosolic proteins constituting a well-conserved multigene family. Nine family members have been identified (FABP 1–9) with 20–70% sequence homology among isoforms [1]. When discovered, each isoform is named according to the most abundantly expressing tissue; however, tissues with active lipid metabolism generally express more than one FABP member. Structurally, all members share a β-barrel signature featuring a water-filled cavity and a site that binds a specific lipid ligand unique to each member [1]. Functionally, the FABPs interact reversibly with hydrophobic ligands with varying affinities and serve to escort fatty acids to the sites of lipid metabolisms (e.g., lipid droplets, nucleus, endoplasmic reticulum, enzymes, or adaptors, etc.) [28]. The regulation of FABP expression is strongly associated with lipid-related signalling. This is exemplified by the modulation of FABP1's transcript level by intracellular fatty acids, dicarboxylic acids, and retinoic acid, indicating lipid bioavailability impacting FABP expression [29]. However, the precise functions of different FABP members remain poorly understood [30] [27].

It has been traditionally accepted that FABP3 is primarily expressed in cardiomyocytes. We show that HUVECs express FABP3, in addition to FABP4 and 5, at baseline under standard *in vitro* conditions, contributing to the understanding that a specific cell type may express multiple members of the FABP family, and a FABP member may be found expressed in multiple cell-types (**Figure 2.1**). We also demonstrate a nuanced regulatory pattern of FABP3 in response to varying stressors: downregulation by oxidized low-density lipoprotein (oxLDL) and angiotensin-II (Ang II). Specifically, oxLDL leads to a dose-dependent decrease in FABP3 expression, with an initial upregulation at 6 hours followed by a subsequent downregulation at 12 and 24 hours, suggesting a complex regulatory dynamic of FABP3 in endothelial cells under oxidative stress (**Figure 2.1A and D**). Ang II, a mediator known to be elevated in circulation during hypertensive states, also downregulates FABP3 in a more linear, time-dependent manner **(Figure 2.1E)**. However, our

evaluation of the dose-response relationship of FABP3 to Ang II differs from that for oxLDL treatment. Our Ang II data show a single-dose effect at 1 μM Ang II (**Figure 2.1F**) instead of the effects of various doses. This dose has been established previously to induce pronounced oxidative stress and inflammation [31], and it is shown here to downregulate FABP3 in endothelial cells, strengthening the regulatory role of endothelial FABP3 in hypertension. However, a dose-dependent effect on FABP3 expression would elucidate the precise behaviour of FABP3 in endothelial cells under hypertension. Overall, these varying expression patterns indicate a modulatory adaptive mechanism of endothelial FABP3 in response to oxidative stress and hypertension, implying that endothelial dysfunction under these stresses involves FABP3's function. Moreover, the downregulation of FABP4 and 5, which are known to be highly expressed in endothelial cells in response to oxLDL (**Figure 2.1B and C**), following a similar trend of FABP3, suggests that they share common regulatory pathways with FABP3 and may participate collectively in the endothelial response to oxidative stress.

Extensive research has been conducted on the roles of FABP4 and FABP5 in the development of metabolic syndrome and atherosclerosis [32]. FABP4, the adipocyte type, is regulated by fatty acids, agonists of PPAR-γ, insulin, LPS, and oxLDL in the differentiation of adipocytes [26] and macrophages [33]. In fact, FABP4 deficiency leads to reduced lipolysis efficiency *in vivo* [34], while in macrophages, it modulates inflammation and cholesterol concentration *in vitro* [35]. FABP5, the epidermal type, is also found at a lower level in adipocytes compared to FABP4 [36]. However, loss of FABP4 in adipocytes induces FABP5 upregulation that can mask the phenotypes of FABP4 deficiency [37]. Interestingly, mice overexpressing adipose-specific FABP5 exhibited enhanced lipolysis [38]. In macrophages, the FABP4 and 5 expression ratios are identical, and no compensatory regulation was observed [39]. FABP4 and FABP5 deficiency in adipocytes and macrophages generally protect against atherosclerosis. FABP4- and FABP5-deficient mice exhibited protection against atherosclerosis with ameliorated insulin resistance, enhanced cholesterol efflux and insulin sensitivity, and reduced cytokines, pro-inflammatory enzymes production, and tissue fatty-acid composition [28] [37] [40] [41] [42] [43] [44] [45]. Nevertheless, the FABP4 and FABP5 expressions in endothelial cells remain under investigation [46] [9]. Here, we demonstrated the common downregulation trends of the endothelial FABPs (FABP3, 4 and 5) in oxidative-stress-induced endothelial dysfunction, respectively (**Figure 2.1**). These findings suggest a regulatory role of endothelial FABPs in endothelial dysfunction, possibly linked to the

development of atherosclerosis. Assessment of FABP4 and FABP5 in endothelial cells under inflammation and the hypertensive biochemical milieu would also contribute to this comprehension and is a subject for future studies.

Additionally, the upregulation of FABP3 protein expression by LPS in our immunofluorescence data (**Figure 2.2**) indicates that FABP3 may have a regulatory role in inflammatory-induced endothelial dysfunction. This notion diverges from our premise that FABP3 is upregulated in hypoxic skeletal muscle cells, suggesting tissue-specific regulatory implications of FABP3 (**Figure 2.3**). Overall, these findings provide a comprehensive demonstration of the regulatory pattern of endothelial FABP3 under atherosclerotic stress.

2.6 Conclusion

We have demonstrated that FABP3 is basally expressed in endothelial cells and that FABP3 expression is regulated under different stress conditions relevant to endothelial dysfunction and atherosclerosis. These findings support our initial hypothesis and underscore the necessity for further research into the functional roles of FABP3 in the context of vascular health and disease. Our findings expand the current knowledge of FABP3's clinical significance within the cardiovascular system, warranting future comprehensive studies that focus on each atherosclerotic stress condition that regulates endothelial FABP3, further evaluating its potential impact on cardiovascular diseases. Further investigation of the regulatory relationship between FABP3 and the endothelium may enhance our understanding and therapeutic approach to endothelial dysfunction in the context of atherosclerotic disorders, potentially leading to novel treatments for PAD and related vascular conditions.

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Chapter 3: Loss of fatty acid binding protein 3 ameliorates lipopolysaccharide-induced inflammation and endothelial dysfunction

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See Appendix B for information.

3.1 Abstract

Circulating fatty acid binding protein 3 (FABP3) is an effective biomarker of myocardial injury and peripheral artery disease (PAD). The endothelium, which forms the inner-most layer of every blood vessel, is exposed to higher levels of FABP3 in PAD or following myocardial injury, but the pathophysiological role of endothelial FABP3, the effect of FABP3 exposure on endothelial cells, and related mechanisms are unknown. Here, we aimed to evaluate the pathophysiological role of endothelial FABP3 and related mechanisms *in vitro*. Our molecular and functional *in vitro* analyses show that: 1) FABP3 is basally expressed in endothelial cells; 2) inflammatory stress in the form of lipopolysaccharide (LPS) upregulated endothelial FABP3 expression; 3) loss of endogenous FABP3 protected endothelial cells against LPS-induced endothelial dysfunction; however, exogenous FABP3 exposure exacerbated LPS-induced inflammation; 4) loss of endogenous FABP3 protected against LPS-induced endothelial dysfunction by promoting cell survival and anti-inflammatory and pro-angiogenic signalling pathways. Together, these findings suggest that gain-of endothelial FABP3 exacerbates, whereas loss-of endothelial FABP3 inhibits LPS-induced endothelial dysfunction by promoting cell survival and anti-inflammatory and pro-angiogenic signalling. We propose that an increased circulating FABP3 in myocardial injury or PAD patients may be detrimental to endothelial function and, therefore, therapies aimed at inhibiting FABP3 may improve endothelial function in diseased states.

3.2 Introduction

The fatty acid-binding proteins (FABPs) are a family of transport proteins for fatty acids and other lipophilic substances between extra- and intracellular membranes and receptors and play an important role in the regulation of lipid homeostasis [1]. FABPs are also involved in the production of cellular phospholipid membranes in the endoplasmic reticulum and various enzymatic activities in the cytosol [2]. The FABP protein superfamily is encoded by nine different genes, and different FABPs have usually been named according to their dominant expression in certain tissues [3], of which FABP4 and 5 are reported to be expressed in the endothelial cells [1] [4], where they play overlapping and non-redundant roles. They are pro-angiogenic proteins and modulate important signalling pathways, including p38, eNOS, and PPARδ signalling [1] [4].

The myocardial isoform, heart-type fatty acid-binding protein (H-FABP), is encoded by the FABP3 gene. Besides its abundant expression in the cardiomyocytes, FABP3 is also expressed significantly in other cell types [5]. Their lipid-trafficking mechanism is essential for the metabolic homeostasis of cardiac function [6]. For their unique cardiac expression profile, FABP3 has been proposed as an effective biomarker of myocardial injury [7] as FABP3 is readily released from heart muscles into the blood following a heart attack [8] [9] [10]. The release of FABP3 from the injured myocardium has been observed in both animal models [11] and myocardial infarction patients [12]. Aside from the general lipid-trafficking mechanism and its feature as a cardiac biomarker, the unique function of FABP3 remains largely unknown, particularly its roles in cardiovascular diseases (CVDs). Systemic infections, or sepsis, have been reported to exacerbate cardiac injuries in atherosclerotic patients [13]. Physiologically, the body's lipids contribute not only as an efficient source of energy but also as a source of regulatory signals maintaining proper systemic functions or homeostasis, such as hormonal balance [14] and inflammation [15]. Pathologically, lipids bioavailability and their interacting factors are the driving agents of the metabolic syndrome [16]. Moreover, the bioavailability of lipids and their interacting factors have been employed as biomarkers for cardiovascular-related complications [17].

Accordingly, we recently identified increased circulating levels of FABP3 in peripheral arterial disease (PAD) patients with severe inflammation and particularly undergoing critical limb ischemia, who were negative for any signs of cardiac damage[18]. The endothelium lines the inner

walls of all blood vessels and is in direct contact with blood and regulates tissue-blood metabolic and signalling exchanges, vascular homeostasis, and inflammation; impaired endothelial function or endothelial dysfunction is a key mechanism behind CVDs [19] [20] [14]. It is important to note that in both myocardial ischemia and PAD patients, endothelial cells are directly exposed to higher levels of FABP3 [8] [9] [10]. However, the source of FABP3 and its effect on the endothelium remains largely unknown, and the role of endothelial FABP3 has not been fully characterized at baseline and under stress conditions. Accordingly, our objective is to evaluate 1) the endothelium as a potential source of FABP3, 2) the role of endothelial FABP3 in endothelial function and survival, and 3) the effect of increased FABP3 exposure on endothelial cell function and inflammation at baseline and after stress, and 4) related mechanisms.

Chronic inflammation is the central driving mechanism between endothelial dysfunction and CVDs [21] [22]. Inflammation is also a common factor between myocardial ischemia/heart failure [23] and PADs [24], which are associated with increased circulatory FABP3 and thereby increased FABP3 exposure to endothelial cells. Lipopolysaccharide (LPS), a Gram-negative bacterial endotoxin, is known to induce severe inflammation and endothelial dysfunction [25]; accordingly, LPS is extensively used in experimental models to study inflammation and associated endothelial dysfunction *in vitro* and *in vivo* [26] [25] [27] [28].

Our data demonstrate that endothelial cells basally express FABP3; inflammation, in the form of LPS treatment, significantly up-regulates endothelial FABP3 expression. Furthermore, loss- of endothelial FABP3 inhibits LPS-induced endothelial dysfunction by promoting cell survival and anti-inflammatory and pro-angiogenic pathways. In contrast, the gain of endothelial FABP3 appears to exacerbate inflammation and endothelial function. Our results suggest that elevated FABP3 in myocardial injury or PAD may be detrimental to the endothelium; therefore, therapies aimed at inhibiting serum FABP3 may improve endothelial function in diseased states.

3.3 Methods

Animals - Wild-type C57BL/6J (Charles River Laboratories, Quebec, Canada) mice were used in accordance with the Guide to Care and Use of Animals of the Canadian Council of Animal Care (CCAC). The use of animals was approved by the Animal Care Committee at Western University, Canada (AUP# 2020-128D).

Cell Culture, FABP3 Silencing and LPS treatment - Human Umbilical Vein Endothelial Cells **(**HUVECs, Lonza # CC-2519, pooled, passage # 4-7), a standard model to study endothelial cells function *in vitro* [28] [55] [53] [56] [54], were grown in endothelial-cell complete growth medium-2 (EGM™-2 Bulletkit™; Lonza). HUVECs were reverse transfected with either siFABP3 (optimized to 5nM, sense strand: 5'-GCUAAUUGAUGGAAAACUCTT -3' and antisense strand: 5'-GAGUUUUCCAUCAAUUAGCTC-3') or scrambled control (Ambion™ Silencer™ Select Pre-Designed siRNA) using Lipofectamine RNAi-max (Invitrogen) and OptiMEM (Gibco). Following 24 hours of reverse transfection, HUVECs were treated with either LPS (Sigma-Aldrich) or diluent (PBS) in MCDB-131 low-serum media (+1% FBS) for different time points. HUVECs were starved overnight in the MCDB-131 low-serum media before treatment.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR – Following transfection and treatment, total RNAs were extracted and quantified using the Trizol standard method (Invitrogen) [107]. Total RNA was quantified using NanoDrop (Thermo Scientific). Complementary DNAs (cDNA) were synthesized from RNAs using the Quantitect kit (Qiagen). Quantitative polymerase chain reactions (qPCR) were conducted to evaluate the expression of targeted genes using SYBR (BioRad), primers and QuantStudio-3 Real-Time PCR system (Applied Biosystems). All protocols were conducted in accordance with the manufacturer's instructions. The qPCR was performed for GAPDH, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin [108], p21, eNOS [53], FABP3 (forward 5'-CATGACCAAGCCTACCACAAT-3' and reverse 5'-CCCCAACTTAAAGCTGATCTCTG), FABP4 [109], FABP5 [109], IL1b (forward 5'-GAAGCTGATGGCCCTAAACA-3' and reverse 5'- AAGCCCTTGCTGTAGTGGTG-3'), Il6 (forward 5'-AGTGAGGAACAAGCCAGAGC-3' and reverse 5'-GTCAGGGGTGGTTATTGCAT-3'), MCP1 (forward 5'-GCCTCCAGCATGAAAGTCTC-3' and reverse 5'-AGGTGACTGGGGCATTGAT-3') and AKT (forward 5' -TCTATGGCGCTGAGATTGTG-3' and reverse 5'-CTTAATGTGCCCGTCCTTGT-3').

Western Blot – Cultured HUVECs were collected in RIPA buffer to isolate total proteins [110]. An equal amount of proteins from each sample were loaded onto sodium dodecyl sulfate (SDS) polyacrylamide gels, which were then subjected to electrophoresis. Proteins were then transferred onto PVDF membranes (BioRad), and the following antibodies were employed to detect for the

proteins of interest (Cell Signalling Technology): ICAM-1 (4915S, dilution 1:1000), VCAM-1 (13662S, dilution 1:1000), eNOS (32027S, dilution 1:1000), phospho (p)-eNOS (Millipore, 07-428-I, dilution 1:1000), Akt (4691S, dilution 1:1000), p-Akt (4060S, dilution 1:1000), cleaved-caspase 3 (9664S, dilution 1:1000), p21 (2947S, dilution 1:1000) and GAPDH (5174S, dilution 1:1000). Western blot for FABP3 was performed using polyclonal antibody (Thermo Fisher, PA5-92386, dilution 1:1000) and wild-type mouse total heart protein was used as a positive control. Western blots were developed using chemiluminescence substrates (BioRad) and the Licor-Odyssey XF Imaging System. Densitometry was performed to measure the band intensities using the Image Studio™ Lite.

Cell Counting - In reverse transfection with either siFABP3 or scrambled control, HUVECs were seeded at a density of 2×10^5 cells/well in a 6-well plate prior to LPS or diluent control treatment for 24 h. Cells from each well were then harvested and counted using an Automated Cell Counter (CytoSmart).

Scratch Assay – In reverse transfection with either siFABP3 or scrambled control, HUVECs were seeded at a density of 2×10^5 cells/well in a 6-well plate and allowed to grow to 70 to 80% confluency. Each well was then administered a consistent straight scratch prior to LPS- or diluent control treatment. Phase-contrast microscopy using an adapted camera (Optika) was employed to take pictures of cells in each well migrating into the scratch over time to evaluate for migrating capacity as described [111]. The experiment was performed in triplicates.

In vitro Tube-formation Assay - The *In vitro* Angiogenesis Kit (Millipore) was employed to evaluate endothelial angiogenic properties. HUVECs were transfected and seeded at a density of 2×10^5 cells/well in a 6-well plate and allowed to grow to ~75% confluency. The kit-provided matrix solution was added into designated wells of a 96-well plate. Transfected cells from the previous preparation were then harvested and seeded at an equal density of $1\text{-}1.5 \times 10^4$ cells/well onto the designated wells in EGM-2 supplemented with LPS or vehicle diluent. Phase-contrast microscopy (Optika) was employed to obtain pictures of cells under phase-contrast in each designated well over time to monitor tube formation, and quantification was performed according to the manufacturer's instructions.

Exogenous Recombinant FABP3-treatment – HUVECs grown in endothelial cell complete growth medium-2 were exposed to different doses of human-recombinant FABP3 (Cayman Chemical) or diluent (PBS) in low-serum MCDB-131 media. Following 1 h of exposure, HUVECs were treated with either LPS or diluent control for 6 h for RNA extraction to perform qPCR and 24 h for protein extraction to perform western blotting.

Enzyme-linked Immunosorbent Assay (ELISA)– HUVECs were cultured and treated with 100 ng/mL of LPS or vehicle control for 24 h following 80% confluency. Later, the culture medium was collected, and ELISA for FABP3 was performed using concentrated culture medium and analyzed as instructed by DuoSet ELISA Development System and Ancillary Reagent Kit 2 (R&D Systems, Cat. # DY1678 and DY008). ELISA for circulating FABP3 was performed following 4 hours of intraperitoneal injection of LPS (4 mg/kg) or vehicle (PBS) to the wild-type mice (N = 6/group, C57BL/6 12-15 weeks old male – Charles River Laboratories, Quebec, Canada). Blood was collected in heparinized tubes, centrifuged, and the supernatant was collected to isolate plasma. A total of undiluted 100 μL of mouse plasma was used to perform ELISA as instructed by the Mouse FABP3 ELISA Kit (Froggabio, Cat #MOES01684).

Isometric Tension Myography Studies of Isolated Aortas – Wild-type male mice (N = 4, 12-15 weeks of age) were euthanized by overdose inhalation of isoflurane. Descending thoracic aortas were removed from mice, placed in ice-cold Krebs HEPES buffer, and cleaned of adherent fat and connective tissues. Krebs HEPES buffer (pH 7.4, 37 °C) was composed of 114 mM NaCl, 4.7 mM KCl, 0.8 mM KH_2PO4, 1.2 mM $MgCl_2$ $6H_2O$, 2.5 mM, $CaCl_2$ $2H_2O$, 11.0 mM D-Glucose, 20 mM $NaHCO_3$ and 5 mM HEPES hemisodium salt. Krebs buffer was bubbled continuously with 95% O_2/5% CO_2 during myograph experiments. In brief, we used DMT 620M myograph chambers with the methods and conditions described in [41] for continuous measuring and recording of isometric tension with mouse aortas. The aorta from each mouse was divided into two groups: control (vehicle, PBS) and treatment (rhFABP3, 45 ng/mL). We tested the viability of aorta preparations (1-3 mm lengths) using 90 mM KCl. Viable tissue contractions were >1 mN. We assessed acetylcholine-induced relaxations of phenylephrine-contracted aortas under isometric tension conditions as we described previously [41]. Aortic rings mounted in the DMT620M chambers were exposed to treatments for 20 min, then contracted with phenylephrine (3 μM), and then the acetylcholine dose-response curves were constructed.

Prime qPCR Array – RNAs extracted from HUVECs transfected with either siFABP3 or scrambled control and treated with LPS or vehicles were subjected to a prime qPCR array screening a library of vascular disease-related genes (BioRad, Vascular disease, tier 1, H384, cat#10038720). The expression levels of the differentially expressed mRNAs (DE mRNAs) targets were measured and then organized to outline the topmost upregulated or downregulated gene targets. HUVECs were treated with either LPS or diluent in MCDB-131, and RNAs were extracted to perform qPCR validation. Validation of the outlined targets was then conducted by regular qPCR procedure using the primers listed in **Table 3.6** as described in the *RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR section*. The targets were also analyzed by gene-ontology enrichment using Enrichr software to highlight the biological processes or pathways affected by the differentially expressed genes.

Data and Statistical Analysis - Differences between the means of two groups and more than two groups were calculated using the Student's t-test and Analysis of Variance (ANOVA) statistical analysis, respectively. ANOVA significant results were followed by the post-hoc Tukey's test. Data are presented as mean ± S.D. unless otherwise indicated. N = number of biological replicates. In myograph experiments, relaxation (%) by acetylcholine was calculated as the reversal of tension induced by the contractile agonist (phenylephrine). Acetylcholine-induced relaxations were analyzed using 2-way ANOVA with Bonferroni post-hoc test for pairwise comparisons. $p < 0.05$ was considered significant.

3.4 Results

LPS upregulates FABP3 expression in endothelial cells - Our FABP3 qPCR data on vehicle-treated (control) endothelial cells confirmed the basal expression of FABP3 in HUVECs (**Figure 3.1A**). Next, to evaluate the effect of inflammation in endothelial cells in the form of LPS treatment on FABP3 expression, we treated endothelial cells with different doses of LPS (10, 20, 50, 100 and 200 ng/mL) or vehicle control for 24 hours and then measured the FABP3 expression. Our qPCR data show significant up-regulation of FABP3 in endothelial cells by all the doses of LPS treatment (**Figure 3.1A**). Maximum but similar FABP3 expression was observed for 100 and 200 ng/mL of LPS, and accordingly, 100 ng/mL was chosen to be the experimental dose to evaluate the effect of loss of FABP3 on LPS-induced endothelial dysfunction. A similar dose has been used

by many other comparable studies in endothelial cells [29] [30]. We also evaluated the effect of time on LPS-induced FABP3 upregulation and observed that the FABP3 was up-regulated as early as 1-hour post-treatment (**Figure 3.1B**). We then tested whether LPS-induced FABP3 up-regulation is associated with increased secretion of FABP3 in the culture medium and observed increased LPS-treatment-induced secretion of FABP3 in the culture medium (**Figure 3.1C**).

Endothelial cells' loss of FABP3 protects against LPS-induced endothelial dysfunction and apoptosis - To understand the effect of LPS-induced upregulation of FABP3 on endothelial function, we successfully silenced FABP3 in HUVECs and observed ~90% reduction at the transcript level (**Figure 3.1D**). FABP3-silencing was also confirmed at the protein level by western blotting for FABP3 (**Figure 3.1E**). We then treated FABP3-silenced and scrambled-control endothelial cells with 100 ng/mL of LPS and evaluated endothelial function in the form of tube-forming, migratory and proliferative potential of endothelial cells. To our surprise, the loss of FABP3 significantly increased the number of nodes and tube length in FABP3-silenced *vs.* control endothelial cells (**Figure 3.1F-H**). LPS treatment is known to inhibit tube-forming potential [31]; accordingly, we also observed significant inhibition of tube formation in LPS-treated scrambled-transfected *vs.* vehicle-treated scrambled-transfected control endothelial cells (**Figure 3.1F-H**). Interestingly, loss of FABP3 significantly restored tube length in LPS-treated FABP3-deficient cells compared to LPS-treated control endothelial cells (**Figure 3.1F, H**). However, the loss of FABP3 showed no effect on the LPS-induced inhibition of the number of nodes in HUVECs (**Figure 3.1F, G**). Next, to understand the effect of LPS treatment on the migratory capacity of FABP3-deficient endothelial cells, we measured migratory capacity *via* scratch assay [32]. Loss of FABP3 and LPS treatment appeared to inhibit and up-regulate endothelial cell migration, respectively (**Figure 3.1I, J**). LPS-induced upregulation of endothelial cell migration has been previously reported depending on specific dosages [33]; however, loss of FABP3 was able to attenuate LPS's effect on endothelial cell migration (**Figure 3.1I, J).** We observed increased LPS-induced migration, but the difference was non-significant, and that can be attributed to the sensitivity of the method used. We then evaluated the effect of loss of FABP3 and LPS on the proliferative capacity of endothelial cells by measuring the cell count using the Cytosmart Automated Cell Counter. Loss of FABP3 appeared not to affect endothelial cell population; however, LPS treatment significantly reduced endothelial cell numbers, which was, interestingly, rescued in the FABP3-silenced and LPS-treated in comparison to LPS-treated scrambled control-

transfected endothelial cells (**Figure 3.1K**). Next, to understand whether LPS-induced reduced cell proliferation is associated with increased cell death and whether the loss of FABP3 is associated with the restoration of cell proliferation due to increased survival, we measured apoptosis in FABP3-silenced and LPS-treated endothelial cells. Our western blot data demonstrated the absence of cleaved caspase-3 protein in the siFABP3-transfected endothelial cells, suggesting that LPS-induced apoptosis in endothelial cells was inhibited by loss of FABP3 in LPS-treated endothelial cells (**Figure 3.1L**). Overall, these data indicate that loss of FABP3 protects against LPS-induced endothelial dysfunction by restoring angiogenic, migratory and proliferative potential and by inhibiting LPS-induced apoptosis of endothelial cells.

Endothelial cell loss of FABP3 restores LPS-induced endothelial nitric oxide synthase expression and activation - To understand the effect of loss of FABP3 and LPS on the molecular and regulatory level in endothelial cells, we evaluated the expression and activation of the essential regulators of endothelial function. Endothelial nitric oxide synthase (eNOS) and protein kinase B (AKT) are the two key regulators of endothelial function [34]. LPS is known to inhibit eNOS expression and activation [35], and accordingly, we also observed a reduction in the eNOS protein expression and activation levels in LPS-treated endothelial cells (**Figure 3.2A-C**). Interestingly, we observed a significantly higher protein level of eNOS in FABP3-silenced endothelial cells, which was associated with increased phosphorylation of eNOS in LPS-treated FABP3-silenced cells (**Figure 3.2A-C**). This indicates that LPS-associated inhibition of eNOS expression and activation was restored in LPS-treated FABP3-silenced endothelial cells. Given that the PI3K/AKT/eNOS signalling pathway is critical for the maintenance of endothelial function and that activated AKT can directly activate eNOS [34], we next measured total and activated AKT levels in FABP3-silenced and LPS-treated endothelial cells. LPS has been shown to compromise AKT activation [36]; accordingly, we also observed reduced AKT activation in LPS-treated endothelial cells (**Figure 3.2D**). However, to our surprise, when we quantified and evaluated the activated AKT *vs.* total AKT, the inhibition was not significant between the LPS-treated siFABP3- and scrambled-transfected HUVECs (**Figure 3.2E**). Next, we questioned whether this lack of difference is due to the inhibition of total AKT expression by LPS treatment in endothelial cells and quantified total AKT. As expected, LPS significantly inhibited total AKT expression in endothelial cells (**Figure 3.2F**). Interestingly, AKT expression was restored in LPS-treated FABP3-silenced endothelial cells (**Figure 3.2F**), and when we quantified activated AKT, we

observed a significant up-regulation again for both FABP3-silenced endothelial cells and LPS-treated FABP3-silenced endothelial cells (**Figure 3.2D, H**). Protein p21, a cell cycle inhibitor, is known to regulate endothelial cell proliferation physiologically and also in pathological conditions [37]. Most importantly, LPS-mediated inhibition of cell proliferation has been previously attributed to p21-upregulation [38]. Accordingly, we measured the p21 expression in FABP3-silenced and LPS-treated endothelial cells. Our transcript data showed a significant reduction in p21 transcript level in FABP3-silenced endothelial cells; p21 transcript and protein appeared to be up-regulated in LPS-treated endothelial cells, whereas the p21 expression was restored in LPS-treated FABP3-silenced endothelial cells in comparison to LPS-treated scrambled control-transfected endothelial cells (**Figure 3.2I-K**). These data indicated that loss of FABP3-associated restoration of endothelial function in LPS-treated endothelial cells is mediated by increased AKT/eNOS signalling and inhibition of LPS-associated p21 expression.

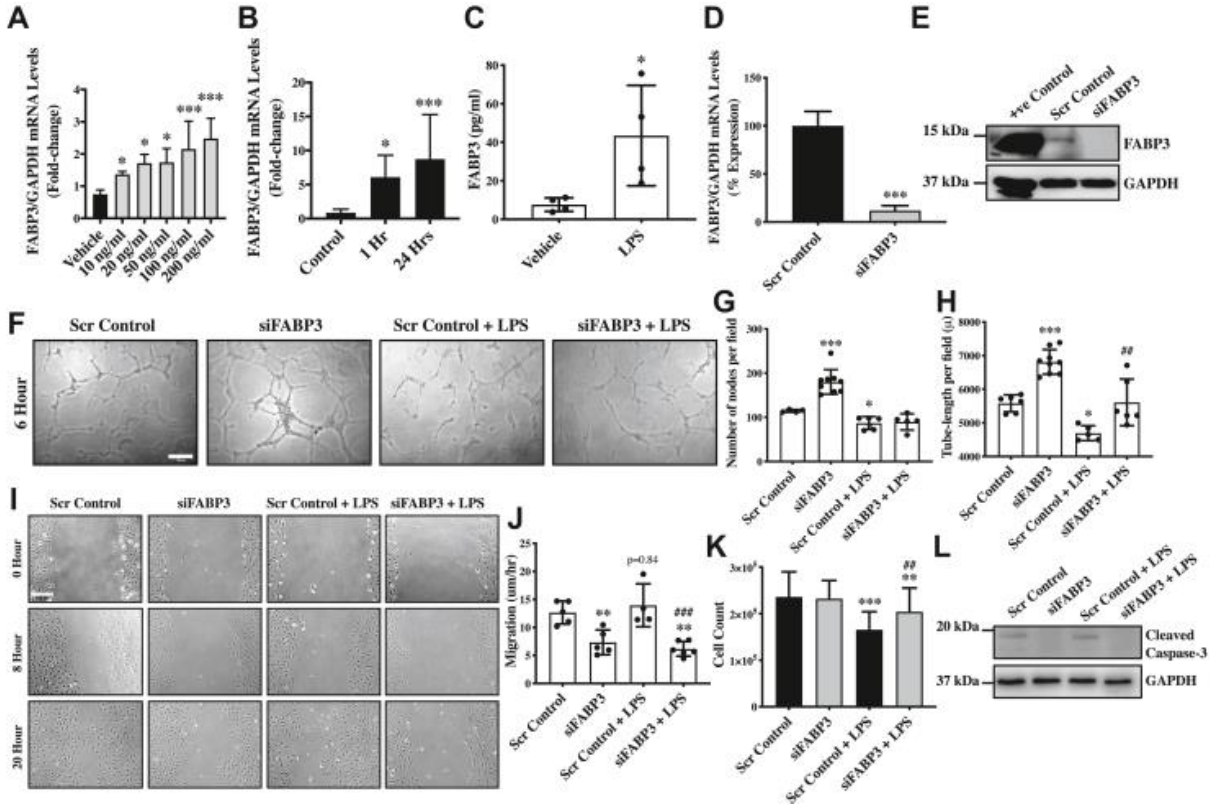

Figure 3.1 LPS-induced FABP3 modulates endothelial function

A, HUVECs were treated with different doses of LPS, and RNA was extracted 24 h posttreatment to perform qPCR for *FABP3*. *B*, HUVECs were treated with 100 ng/mL of LPS, and RNA was extracted 1h and 24 h post-treatment to perform qPCR for *FABP3*. *C*, HUVECs were treated with 100 ng/mL of LPS for 24 h, and culture media were collected to perform ELISA for FABP3. *D* and *E*, HUVECs were transfected with either scrambled control or siFABP3, and RNAs and proteins were extracted to perform qPCR and immunoblot, respectively, for FABP3; GAPDH was used as a control. *F–H*, HUVECs were transfected with either scrambled control or siFABP3 and seeded on Matrigel in the presence of vehicle or LPS for 6 h, and tube formation was assessed microscopically (F); the number of nodes (*G*) and tube lengths (*H*) were quantified (scale bar = 100 μm). *I* and *J*, HUVECs were transfected with either scrambled control or siFABP3, and 24 h post-transfection, a scratch was made, and cell migration was assessed using phase contrast light microscopy at 0, 8, and 20 h, scale bar = 200 μm (*I*), and migratory capacity was calculated (*J*). *K*, HUVECs were transfected with either scrambled control or siFABP3 for 24 h, and the live cells were counted using Cytosmart automated cell counter. *L*, HUVECs were transfected with either scrambled control or siFABP3, and then proteins were extracted to perform immunoblot for cleaved-CASPASE3 and GAPDH (loading control). Differences between the means of two groups and more than two groups were calculated using the Student's *t*-test and one-way ANOVA with Tukey's multiple comparison test, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *versus* Vehicle, control and Scr Control. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ *versus* Scr Control + LPS. N = 3 in triplicates for qPCR. Data are represented as mean ± SD for C, G, H, and J and as mean + SD for A, B, D and K. FABP3, fatty acid–binding protein 3; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide; qPCR, quantitative polymerase chain reaction.

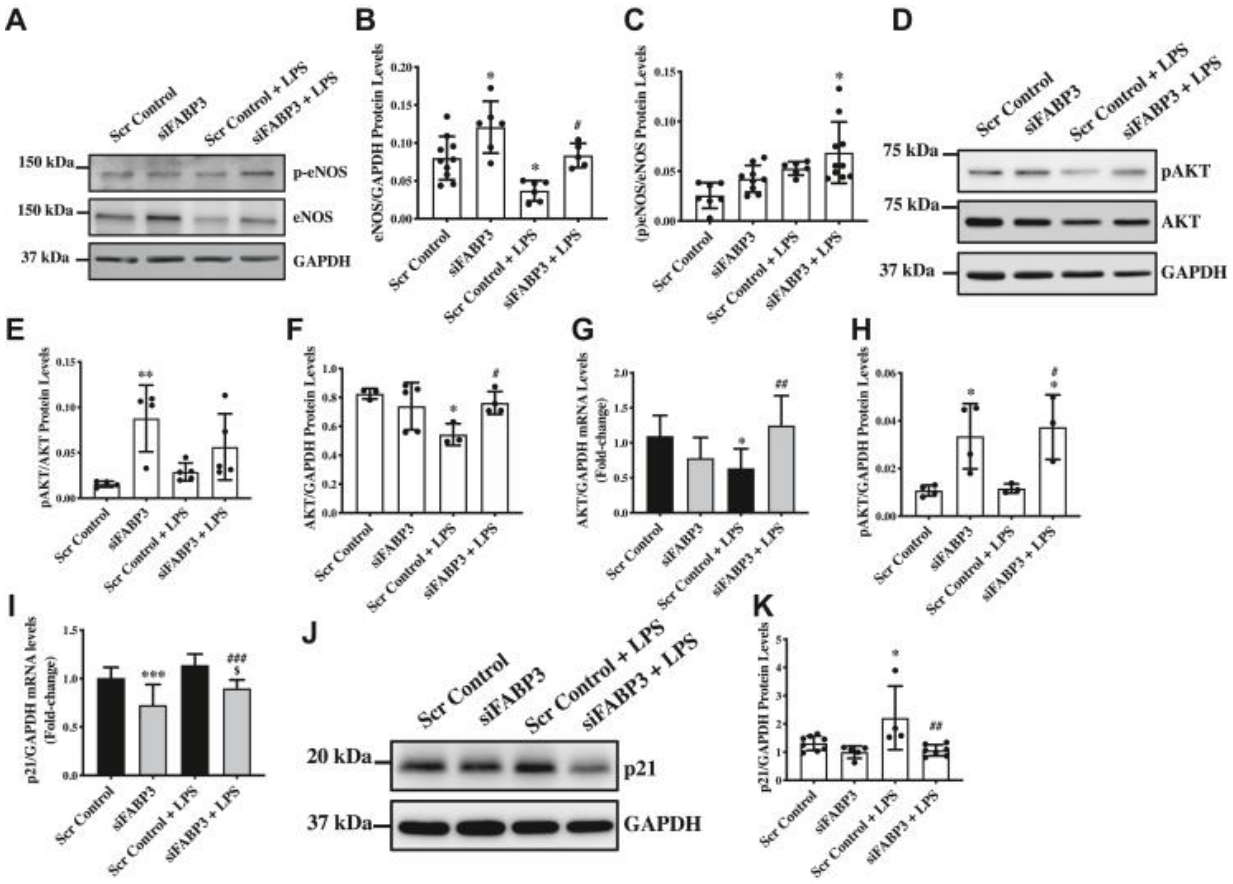

Figure 3.2 Endothelial cell loss of FABP3 promotes eNOS expression and activation

HUVECs were transfected with either scrambled control or siFABP3 for 24 h and treated for an additional 24 h with LPS, and then protein and RNA were extracted to perform immunoblot and qPCR, respectively. *A–C*, immunoblotting for eNOS, p-eNOS, and GAPDH (*A*), and quantification for eNOS (*B*) and p-eNOS/eNOS ratio (*C*). *D*, *E*, *F* and *H*, immunoblotting for AKT, pAKT, and GAPDH and quantification for pAKT/AKT ratio (*E*), AKT (*F*), and pAKT (*H*). *J* and *K*, immunoblot (*J*) and (*K*) quantification for p21. *G* and *I*, qPCR was performed for *AKT* (*G*) and *p21* (*I*). Differences between the means of groups were calculated using one-way ANOVA with Tukey's multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *versus* Scr Control. $\#p < 0.05$, $\#\#p < 0.01$, ###<p0.001 *versus* Scr Control+LPS. $\$p < 0.05$ *versus* siFABP3. N = 3 in triplicates for qPCR. Data are represented as mean ± SD except for G and I, whose data are presented as mean + SD. AKT, protein kinase B; eNOS, endothelial nitric oxide synthase; FABP3, fatty acid–binding protein 3; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide.

Endogenous FABP3-deficiency ameliorates LPS-induced inflammation in endothelial cells - To assess the role of FABP3 in endothelial inflammation, we evaluated the expression level of key inflammatory markers, including the ICAM-1, VCAM-1, and E-selectin and the secretory inflammatory cytokines, such as IL1b, IL6 and MCP-1 in FABP3-silenced and LPS-treated endothelial cells. LPS treatment is known to induce ICAM-1 and VCAM-1 expression [39]; accordingly, we also observed a significant induction of ICAM-1 (**Figure 3.3A-C**) and VCAM-1 (**Figure 3.3D-F)** in the LPS-treated scrambled control-transfected endothelial cells. Loss of FABP3 significantly inhibited LPS-induced expression of ICAM-1 at both the transcript and protein levels in HUVECs (**Figure 3.3A-C)**. LPS-induced VCAM-1 transcript level also appeared to be inhibited by loss of FABP3 in endothelial cells; however, to our surprise, this data did not translate to the protein levels, where we observed a further increased level of VCAM-1 in the LPS-treated FABP3-silenced endothelial cells *vs.* LPS-treated scrambled control-transfected endothelial cells (**Figure 3.3D-F**). Similar to ICAM-1 and VCAM-1, the expression level of E-selectin was induced by LPS, which was again restored by loss of FABP3 in LPS-treated FABP3-silenced endothelial cells **(Figure 3.3G).** LPS is also known to promote the expression of inflammatory cytokines, such as the interleukins (IL1b, IL6) and the chemoattractant factor MCP-1 [40]. Accordingly, we observed LPS-induced significant upregulation in the expression level of IL1b and IL6 along with the expression of MCP-1 in endothelial cells (**Figure 3.3H-J**). Interestingly, loss of FABP3 was successfully able to significantly inhibit the expression of all these studied inflammatory molecules in LPS-treated FABP3-silenced endothelial cells (**Figure 3.3H-J**). Taken together, these data indicate that loss of FABP3 protects against LPS-induced inflammation in endothelial cells.

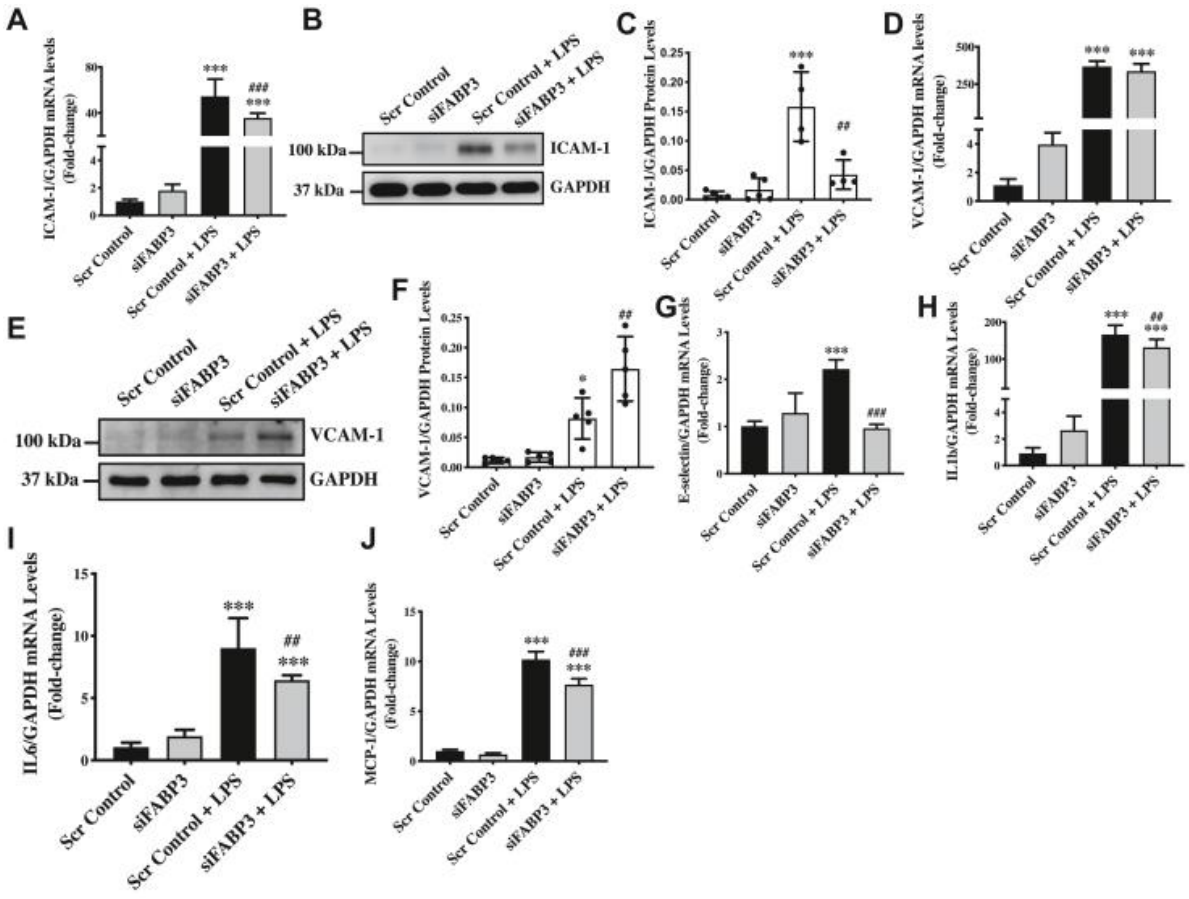

Figure 3.3 Inflammatory markers modulated by loss of FABP3's function in LPS-treated endothelial cells

HUVECs were transfected with either scrambled control or siFABP3 for 24 h and treated for an additional 6 h and 24 h with LPS to isolate RNA and protein, respectively. *A*, *D*, *G–J*, bar graphs representing qPCR data for *ICAM-1* (*A*), *VCAM-1* (*D*), *E-SELECTIN* (*G*), *IL1b* (*H*), *IL6* (*I*), and *MCP-1* (*J*). *B*, *C–F*, immunoblot and quantification for ICAM-1 (*B* and *C*) and ICAM-1 (*E* and *F*). Differences between the means of groups were calculated using one-way ANOVA with Tukey's multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *versus* Scr Control. $\#\#p < 0.01$, $\#\#\#p < 0.001$ *versus* Scr Control+LPS. N = 3 in triplicates for qPCR. Data are represented as mean + SD, except for C and F, whose data are presented as mean ± SD. FABP3, fatty acid–binding protein 3; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide; qPCR, quantitative polymerase chain reaction.

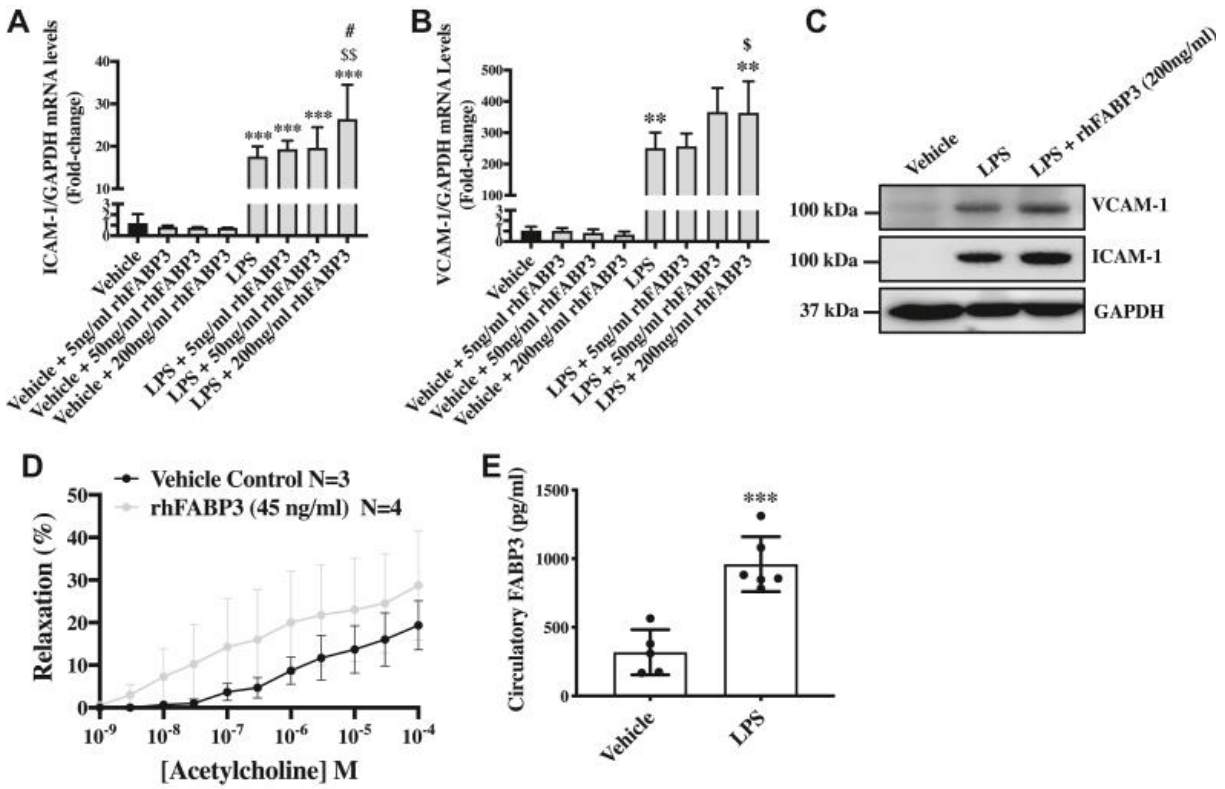

Figure 3.4 Exogenous FABP3 treatment exacerbates LPS-induced inflammation in endothelial cells, and LPS treatment upregulates circulatory FABP3 levels in wild-type mice

HUVECS were cultured, and following 60 to 70% confluency, these cells were pretreated with different doses of recombinant human FABP3 for 1-h before treatment with 100 ng/mL of LPS. Later, 6 h and 24 h posttreatment, RNA and proteins, respectively, were extracted. *A* and *B*, bar graphs show the qPCR quantification for *ICAM-1* (*A*) and *VCAM-1* (*B*). *C*, the qPCR data were further confirmed by immunoblotting for ICAM-1 and VCAM-1, which also showed exacerbation of ICAM-1 and VCAM-1 expression in rhFABP3 pretreated and LPS-treated endothelial cells. *D*, isometric tension data from myograph experiments using acetylcholine to show relaxation (%) of the phenylephrine-contracted aorta in control (PBS) *versus* rhFABP3 (45 ng/mL, 20 min) groups ($p > 0.05$). *E*, wildtype mice were treated with vehicle (N = 5) or LPS (N = 6, 4 mg/kg), and plasma was collected 4 h posttreatment to perform ELISA for *FABP3*. Differences between the means of groups were calculated using one-way ANOVA with Tukey's multiple comparison tests (*A* and *B*), two-way ANOVA with Bonferroni's multiple comparison tests (*C*), and Student's *t*-test (*D*). $**p <$ 0.01, $***p < 0.001$ *versus* Vehicle. $\$p < 0.05$, $\$\$p < 0.01$ *versus* LPS + 50 ng/mL rhFABP3, $\#p <$

0.005 *versus* LPS + 5 or 50 ng/mL rhFABP3. Data are represented as mean + SD, except for D and E, whose data are presented as mean ± SEM and mean ± SD, respectively. FABP3, fatty acid–binding protein 3; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide; rhFABP3, recombinant human FABP3.

Exogenous exposure to FABP3 exacerbates LPS-induced inflammation in endothelial cells - Next, to understand the effect of exogenous exposure to FABP3 on endothelial inflammation basally and after LPS stimulation, we treated endothelial cells with different doses of recombinant human FABP3 (rhFABP3) and LPS, and then measured the expression level of ICAM-1 and VCAM-1. Recombinant human FABP3 alone did not significantly affect the inflammation, measured in the form of ICAM-1 and VCAM-1 expression; however, rhFABP3 significantly increased ICAM-1 and VCAM-1 expression in LPS-treated endothelial cells, demonstrating an additive effect (**Figure 3.4A, B**). Given the observed discrepancy between transcript and protein levels in LPS-treated FABP3-deficient endothelial cells, we measured the expression level of FABP3 in rhFABP3 and LPS-treated endothelial cells. However, we observed an expected result, where VCAM-1 and ICAM-1 proteins were increased in rhFABP3 and LPS-treated endothelial cells in comparison to LPS-only treated endothelial cells (**Figure 3.4C**). Next, to assess the effect of rhFAB3 exposure on endothelial cell function *in vivo*, we measured acetylcholine-induced relaxations using myography with isolated aortas from wild-type mice [41]. There appears to be increased relaxation (<10%) of phenylephrine-contracted aortas by acetylcholine in the rhFABP3-treatment group vs. controls; however, the difference was not significant (p=0.5878) (**Figure 3.4D**). In order to confirm whether LPS-induced FABP3 expression in endothelial cells *in vitro* also occurs *in vivo*, we treated wild-type mice with 4 mg/kg [42] of LPS or diluent for 4 hours, as we have previously observed that 4 hours of LPS treatment is sufficient to induce circulatory cytokines [43] and measured circulatory FABP3 level in mouse plasma. Our data showed significantly increased circulatory FABP3 levels in LPS-treated *vs.* vehicle-treated mice (**Figure 3.4E**). Taken together, our data indicate that FABP3 exposure exacerbates LPS-induced inflammation *in vitro* and may cause endothelial dysfunction *in vivo* in endothelial cells.

Loss of FABP3 protects endothelial cells against LPS-induced endothelial dysfunction by promoting cell survival and pro-angiogenic pathways and by inhibiting inflammatory pathways – Given the increased circulatory level of FABP3 in myocardial injury [9] and PAD

[18], the obscurity about the role of endothelial FABP3, and the observed complexity about the role of FABP3 in LPS-treated endothelial cells from our data, for clarity, we performed a qPCR array containing 84 endothelial and vascular disease-related genes. Our prime qPCR array data in FABP3-silenced *vs.* scrambled control demonstrated a total of 15 up-regulated genes (cut-off <2 fold) (**Table 3.1**). These genes included pro-angiogenic and pro-survival genes, such as COL1A2, BDNF, FN1, BCL2, EGFR, VEGFA, EGR1, CDK1 and BIRC5 (**Figure 3.5**). PTGS2 was the most-up-regulated gene identified in the FABP3-silenced endothelial cells. Validation qPCR was performed for 5 of the upregulated genes to validate the qPCR array data (**Table 3.1**). LPS-treatment upregulated a total of 10 genes (mainly pro-inflammatory, such as IL6, IL1b, CCL2, CCL5, TLR2 and ICAM-1) and downregulated 18 genes (mainly pro-survival and pro-angiogenic, such as STAT1, IGFBP3, CAV1, STAT3, BIRC5, AURKA, COL1A2, CDK1, KDR and FGF2) in comparison to vehicle-treated control (cut-off <2 fold) (**Table 3.2 and 3.3, Figure 3.5**. IL1b and MMP7 were the most up- and down-regulated genes in LPS-treated endothelial cells **(Table 3.2 and 3.3)**. Validation performed for 4 of the upregulated genes and 5 of downregulated genes demonstrated a similar trend as the qPCR array (**Table 3.2 and 3.3**). The prime qPCR array data for LPS *vs.* vehicle-treated FABP3-silenced endothelial cells showed a total of 15 up-regulated and 8 downregulated genes (**Table 3.4 and 3.5**). Most of the upregulated genes in LPS-treated FABP3-silenced endothelial cells were pro-survival and pro-angiogenic and the most down-regulated genes were pro-inflammatory in nature **(Table 3.4 and 3.5, Figure 3.5)**. Overall, our PCR array data indicated that loss of FABP3 promotes endothelial cell function and survival and protects against LPS-induced toxicity by promoting pro-angiogenic and pro-survival pathways and by inhibiting inflammation.

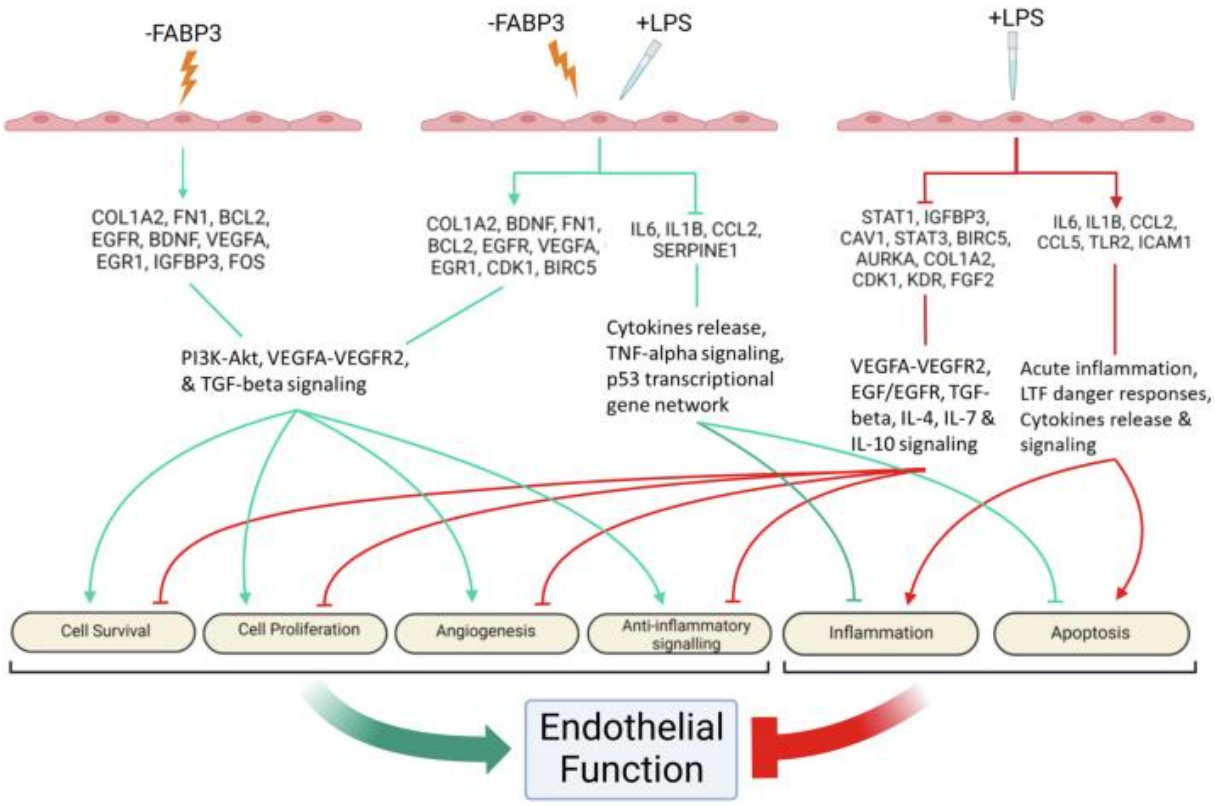

Figure 3.5 Endothelial cell loss of FABP3 protects against LPS-induced endothelial dysfunction

The illustration summarizes the prime qPCR array data on the differentially expressed genes, their regulatory implications, and the proposed effects derived from gene ontology and pathway enrichment analyses in FABP3-deficient endothelial cells under LPS-induced stress. LPS-treated endothelial cells undergo dysfunction, inflammation, and injuries through upregulating and downregulating proinflammatory and pro-survival genes, respectively. Loss of FABP3 function ameliorates cellular impairment induced by LPS in endothelial cells through upregulation of pro-survival targets and downregulation of inflammatory and senescent factors. FABP3, fatty acid–binding protein 3; LPS, lipopolysaccharide. Created with BioRender.com (agreement # BY26K0OSEO).

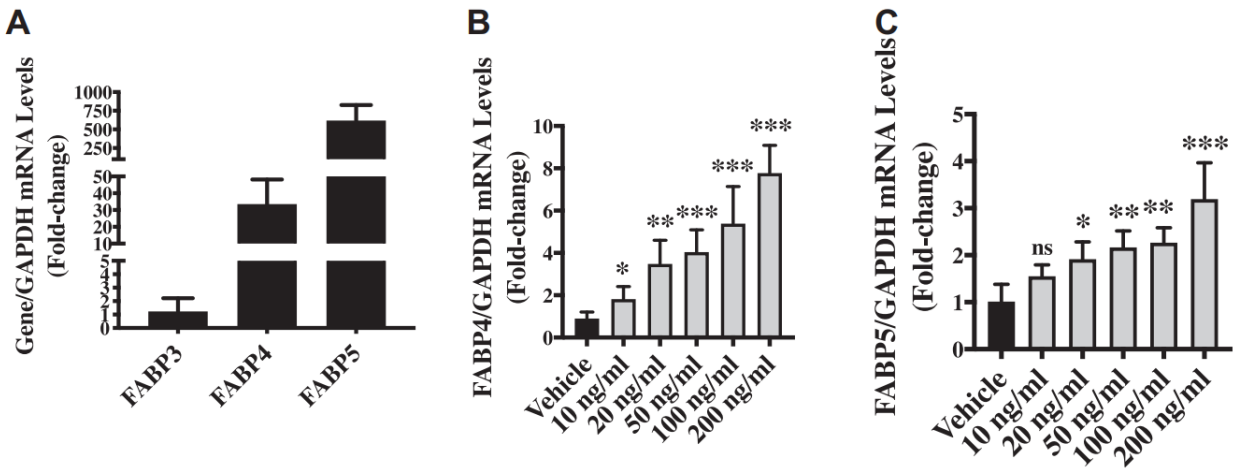

Figure 3.6 Relative expression of FABP3, FABP4, and FABP5 in cultured endothelial cells

A, HUVECs were cultured and following 70 to 80% confluency, RNA was extracted to perform qPCR for *FABP3*, *FABP4*, and *FABP5*. *B* and *C*, cultured HUVECs were treated with 100 ng/mL of LPS, and RNA was extracted 24 h posttreatment to perform qPCR for *FABP4* and *FABP5*. N = 3 in triplicates. Data presented as mean + SD. Differences between the means of groups were calculated using ANOVA with Tukey's multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *versus* vehicle. FABP3, fatty acid–binding protein 3; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide.

3.5 Discussion

The FABPs are cytosolic lipid-chaperones abundantly expressed in active lipid-metabolizing tissues, such as the heart and liver, or cell types specialized in lipid storage, trafficking and signalling, such as adipocytes and macrophages [44]. The FABP family consists of nine members (FABP 1-9), each with unique tissue-expression patterns [45], although lipid-metabolizing tissues or cells can be found with more than one isoform [46]. The degree of FABP expression in a tissue or cell type may reflect their lipid-metabolizing capacity, which can be modulated by changes in lipid bioavailability [47]. All FABPs are generally known to reversibly interact and escort hydrophobic ligands with various affinities to sites of lipid metabolism or signalling (e.g., lipid droplets, plasma membrane, mitochondria, etc.) [48]. However, 20–70% sequence homology

exists among the nine members [45], and the unique functional features of each member remain poorly understood [49]. The FABPs expressed in adipocytes and macrophages have been associated with metabolic and inflammatory regulation [44].

Endothelial cells are known to metabolize fatty acids for energy through mitochondrial oxidation processes [50]. Parenchymal absorption of circulating lipids is mediated by endothelial cells [51]. Moreover, fatty acids in endothelial cells also have signalling roles impacting cell differentiation, endothelial function and dysfunction in diseases, although the underlying mechanisms remain largely unclear outside the metabolic diseases. A single recent study by our group has identified FABP3 in human coronary artery endothelial cells and suggested their interaction with the peroxisome proliferator-activated receptors gamma (PPARγ) through binding fatty acids in regulating transcriptional activities [5]. PPARγ is a central component in the inflammatory response mounted by endothelial cells. In addition, the PPAR family of nuclear receptors/transcription factors is expressed in endothelial cells to mediate endothelial function [52]. In this notion, we planned to evaluate endothelial FABP3 and investigate its connection to endothelial function. To induce inflammation and endothelial dysfunction, we treated cells with lipopolysaccharide (LPS), an *in vitro* model, to study inflammation [26]. We used the standard *in vitro* endothelial cell model, HUVECs [53] [54] [55] [56] [53], and confirmed basal FABP3 expression, which was upregulated upon LPS treatment, suggesting a regulatory role of FABP3 in the endothelial response to LPS (**Figure 3.1A-C**). LPS binding to endothelial cells elicits endothelial activation, which encompasses the upregulation of inflammatory cytokines and adhesion molecules and the modulation of several critical pathways, including NF-κB, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways [57] [58] [59] [60].

Aspects of endothelial function include angiogenesis, migration, proliferation, nitric oxide (NO) production and mounting the inflammatory responses [53] [54] [55] [56]. In our tube-formation and migratory assessment, FABP3-silenced HUVECs demonstrated better tube-forming potential, but the migratory potential was reduced relative to scramble-controls in both following vehicle or LPS treatment (**Figure 3.1F-J**). Although how FABP3 is oppositely influencing the two functional aspects remains inconclusive, our data strongly suggest a consequential role of endothelial FABP3 in angiogenesis and endothelial migration. Endothelial inflammatory activation is marked by an

increased migratory response [61]. Loss of FABP3 appeared to reduce cell migration at baseline and after LPS -treatment, suggesting an independent effect of loss of FABP3 on endothelial cell migration (**Figure 3.1I, J**). Endothelial NO synthase is a key regulator of endothelial functions by its influence on NO production, which is essentially involved in oxidative homeostasis and, thereby, influencing many aspects of endothelial function [62]. In endothelial cells, AKT is an upstream regulator of eNOS [34]. Assessment of these two key regulators of endothelial function revealed an increased eNOS expression in FABP3-silenced HUVECs and the restoration of eNOS expression in FABP3-deficient endothelial cells following LPS treatment (**Figure 3.2A-C**). AKT's activity, measured by the levels of its phosphorylated and total AKT expression ratio, appeared to be upregulated in both FABP3-silenced endothelial cells and FABP3-silenced endothelial cells treated with LPS (**Figure 3.2D, E, H)**. Data from both eNOS and AKT assessments suggest their activities are upregulated by the loss of FABP3 in endothelial cells, thereby improving endothelial function. Moreover, LPS has previously been reported to inhibit AKT in endothelial cells [36], but we, for the first time, show that LPS also significantly inhibits total AKT expression, which was salvaged in endothelial cells with loss of FABP3's function (**Figure 3.2D, E, G**). Likewise, our proliferative and survival assessments indicated improved endothelial proliferation and survival in FABP3-silenced LPS-treated endothelial cells (**Figure 3.1K**). P21 is a cyclin-dependent kinase that inhibits the cell cycle and, thereby, proliferation in endothelial cells [63]. LPS is known to promote p21 expression and inhibit cell proliferation [38]. In line, we also observed increased p21 expression and reduced proliferation in LPS-treated endothelial cells (**Figure 3.2I-K**). However, the loss of FABP3 downregulated p21 both under the vehicle- and LPS treatment, implying enhanced proliferation and unmasking the effect of LPS (**Figure 3.2I-K**). Lastly, our western blotting data for cleaved-caspase 3 showed induction of apoptosis in LPS-treated cells (**Figure 3.1L**) as previously reported [64]; however, the LPS-induced apoptosis was prevented by loss of FABP3 in endothelial cells (**Figure 3.1L**). Overall, it appears that in this scenario, both reduced p21 expression and increased survival contribute to the restoration of endothelial cell proliferation in FABP3-deficient LPS-treated endothelial cells.

In the inflammatory response, activated endothelial cells express adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin, that function primarily to recruit circulatory leukocytes and mediate their trans-endothelial migration toward the site of acting antigen [65]. Activated endothelial cells also secrete the chemokines, such as MCP-1, and the interleukins (e.g., IL1b, IL6,

etc.), which mediate the chemotaxis of neutrophils and amplify the inflammatory response, respectively [61]. These inflammatory molecules were evaluated in our assessments of endothelial function. LPS is known to induce the expression of endothelial cells' inflammatory markers, such as ICAM-1, VCAM-1, and E-selectin [66]. Accordingly, we also observed a significant up-regulation of these markers in LPS-treated *vs.* vehicle-treated endothelial cells (**Figure 3.3A-G**). To our surprise, loss of FABP3 significantly reduced LPS-induced ICAM-1 and E-selectin expression; however, interestingly, opposite to VCAM-1 transcript expression, the VCAM-1 protein expression was significantly exacerbated in LPS-treated FABP3-silenced *vs.* LPS-treated control endothelial cells (**Figure 3.3D-F**). The observed increase in VCAM-1 protein might be due to a higher accumulation of the protein's stability in endothelial activation, as previously reported [67]; however, this remains to be explained in this case. LPS-induced expression of MCP-1, IL1b and IL6 were also reduced in FABP3-deficient endothelial cells following LPS-treatment **(Figure 3.3H-J**).

Establishing the gain of FABP3's function through exogenous treatment with rhFABP3 revealed a reverse trend for ICAM-1 and VCAM-1; rhFABP3 exacerbated LPS-induced upregulation of ICAM-1 and VCAM-1 in endothelial cells, reinforcing the inflammatory role of FABP3 (**Figure 3.4A-C**). ICAM-1, E-selectin, and VCAM-1 in an activated endothelium all function in leukocyte-endothelial adhesion *via* interaction with leukocytes' LFA-1 [68], PSGL1 [69], and ITGA4/ITGB1 complexes [70], respectively, that are present on leukocytes. Of the three, ICAM-1 is notably also expressed in leukocytes, an active source of fatty acid signalling [71]; such interaction may imply a role related to FABP3 in leukocyte-endothelial interaction in an activated endothelium. E-selectin and VCAM-1 are more specific to endothelial cells, and both are notable for their additional roles in angiogenesis [72]. Most interestingly, compared to E-selectin and ICAM-1 in endothelial cells, which are localized primarily on the cell membrane, VCAM-1 is expressed both intra-cellularly in addition to the cell surface [73] [74] [75]. This and the vastly diverse regulatory implications of cellular fatty acids [76] may be attributed to the complicated behavior of VCAM-1 in our siFABP3-transfected endothelial cells under LPS-induced inflammation. Lastly, the elevation of all three pro-inflammatory markers is associated with cardiovascular and atherosclerotic risk [77]. Overall, our data indicate a regulatory anti-inflammatory role of FABP3 in endothelial cells. To extend these findings and start to explore the acute effect of exogenous rhFABP3 on vasoreactivity, we performed a myography experiment with isolated aortas from wild-type mice. We treated these

aortas with either vehicle or rhFABP3 and measured acetylcholine-induced relaxations. These relaxations did not differ significantly between the controls and the treatment group using 45 ng/mL rhFABP3 (**Figure 3.4D**). Although the human and mouse forms of FABP3 are highly conserved, we argue that a larger sample-sized study using mouse FABP3 with time-course studies accounting for both sexes and other blood vessel types warrants attention as we did observe a small effect of increasing relaxation (<10%). In a clinical scenario, FABP3 released into the circulation following ischemia may help by a vasodilatory effect to increase the blood flow to the impacted tissues. In this scenario, an increase in acute circulatory FABP3 may be beneficial, for example, after acute myocardial infarction; however, a chronic presence of circulatory FABP3 in PAD patients may be beneficial for similar reasons but is countered by the detrimental additive effect to increase the severity of PAD. Accordingly, in PAD, inhibiting FABP3 might prove to be beneficial. LPS is used in an *in vitro* model to study inflammation and in an *in vivo* model to study sepsis [78]. To evaluate the relevance of our LPS-associated *in vitro* data in an animal model of sepsis, we treated wild-type mice with LPS and measured circulatory FABP3. Not only we observed baseline circulatory FABP3, but also a significant increase of FABP3 levels in response to LPS stimulation *in vivo* (**Figure 3.4E**). The source of LPS-induced FABP3 in mouse plasma is still unknown, but if true in humans, then FABP3 might also provide a biomarker for the severity of sepsis in humans, which warrants future investigations. FABP3 as a biomarker is of particular interest as we were also able to find an association between urinary FABP3 and PAD [79].

Given that FABP4 and FABP5 are the known predominant FABPs in endothelial cells [1] [4]. We assessed the relative expression of FABP3, FABP4 and FABP5. As expected, out of these three FABPs, FABP5 was the most-, and FABP3 was the least expressed FABP in endothelial cells (**Figure 3.6A**). These data show that FABP3 is basally expressed at a low level; however, FABP3 is up-regulated in stress conditions such as LPS treatment in endothelial cells. Next, we also tested the effect of LPS on FABP4 and 5 and observed a similar up-regulation of these genes to that of FABP3 (**Figure 3.6B, C**). FABP4 and 5 are known to be co-expressed and to play overlapping as well as non-redundant roles [1] [4]. A similar pattern observed for LPS-induced upregulation of FABP3, 4 and 5 in endothelial cells indicates that these molecules may be co-expressed; however, distinct effects of loss- and gain-of FABP3 in endothelial cells warrant similar investigations following the loss- and gain- of FABP4 and 5 in endothelial cells under LPS-treatment or inflammation.

To understand the complexity of FABP3-action and to expand our endothelial function assessment, the regulatory roles of endothelial FABP3 were conducted using a prime qPCR array to evaluate endothelial cell-specific genes known to play roles in vascular disease. Among the upregulated genes in siFABP3-transfected endothelial cells, COL1A2 encodes for collagen type I, which composes the extracellular matrix (ECM) and surrounding connective tissues. The expression of COL1A2, as well as that of the fibronectin-encoding FN1 gene, are featured in the focal adhesion processes of endothelial cells, which promote endothelial cells' integrity, growth and survival through the TGF-beta and PI3K-Akt signalling pathways [80] [81]. EGFR's gene product, which is the receptor for the epidermal growth factors (EGFs), is also activated in endothelial focal adhesion and promotes cell growth through the PI3K-Akt signalling pathway [82]. IGFBP3, which encodes for a component of the complex carrier of the IGFs, stimulates endothelial cells' proliferation through EGFR [83] [84]. VEGFA is an inducer of endothelial cell growth required for angiogenesis and vasculogenesis, as well as general endothelial function and the maintenance of vessel integrity [85]. As we observed upregulated EGR1, VEGFa also promotes EGR1 [86], which has been linked to activated PDGF-A that regulates endothelial function [87]. FOS's gene product, which composes the transcription factor complex AP-1, promotes endothelial cell growth [88]. BDNF, whose gene product is essential in the survival and differentiation of neurons [89], regulates vessel integrity and promotes angiogenesis in endothelial cells [90]. Lastly, BCL2 is the pro-survival factor in apoptosis, which functions to inhibit caspase activity, thereby promoting cell survival [91]. Overall, the upregulation of these genes in HUVECs with compromised FABP3 indicates a positive regulation of endothelial cells' growth, function, and survival from the loss of FABP3's function (**Table 3.1, Figure 3.5**).

Under LPS treatment, endothelial cells respond negatively with impaired function and survival and a state of inflammation (**Table 3.2 and 3.3, Figure 3.5**). As expected, in LPS-treated HUVECs, genes encoding for the pro-inflammatory cytokines (IL6, IL1B) and chemokines (CCL2, CCL5) were upregulated, as well as TLR2, which encodes for the receptor responding against foreign agents [25], and the leucocyte-adhesive inflammatory marker ICAM-1 [92]. Likewise, among the downregulated genes were IGFBP3 and COL1A2, suggesting a downregulation in endothelial proliferation and survival in response to LPS. This notion is further supported by the downregulated AURKA, FGF2, and CDK1, whose gene products are key regulators of cellular proliferation [93][94][95]. The gene products of STAT1 and STAT3 are transcription factors of the

STAT protein family known to be regulated by the interferons and EGFs [96][97]; their roles have also been implied in VEGFa and EGFR signalling [98][99], as well as the production of the anti-inflammatory cytokines IL-4, IL-7, and IL-10 [100]. Downregulated STAT1 and STAT3 in LPS-treated endothelial cells, therefore, indicated compromised cell growth and a state of pro-inflammation. Impaired endothelial function is further suggested by the downregulation of CAV1, a mediator of cellular transcytosis essential for many cell-signalling pathways [101], and KDR, which encodes for VEGFR, the receptor for VEGFa [102]. LPS-mediated apoptosis is also indicated by downregulated BIRC5, whose gene product, Survivin, is a member of the inhibitor of apoptosis (IAP) protein family [103].

From the differentially expressed genes in HUVECs with both siFABP3 and LPS treatments, we observed a remarkable ameliorating effect by the loss of FABP3's function (**Table 3.4 and 3.5, Figure 3.5**). Salvaged endothelial integrity and survival were indicated by the upregulation of COL1A2 & FN1 and BCl2 & BIRC5, respectively. Upregulated EGFR and CDK1 further suggested increased cell proliferation, and upregulated BDNF, EGR1, and VEGFA indicated a promotion of endothelial functions. On the other hand, CCL2, IL6 and IL1B were downregulated, indicating reduced inflammation. Interestingly, SERPINE1, whose gene product is an inhibitor of fibrinolysis [104], was downregulated. Activated SERPINE 1 also promotes cellular senescence downstream of the p53 regulatory network [105]. Reduced activity of SERPINE1, therefore, suggests that loss of FABP3's function prompts endothelial cells away from a state of senescence and improves clot breakage, providing benefits against cardiovascular risk in aging and dysregulated clot-formation in atherosclerosis [106].

Table 3.1 Top upregulated DE mRNAs in HUVECs transfected with siFABP3 vs. scrambled-controls

qPCR array data				Validation data	
Nr	**Gene symbol**	**Fold change**	***p value***	**Fold change**	***p value***
1	*PTGS2*	5.93	0.001058	3.75 ± 0.61	5.42E-06
2	*COL1A2*	4.04	0.000606	5.68 ± 1.79	3.21E-05
3	*PLAU*	4.01	0.000419	2.00 ± 0.18	0.010158
4	*BDNF*	3.48	0.000012	3.09 ± 0.37	5.01E-08
5	*BCL2*	3.12	0.000104		
6	*CCL5*	2.99	0.03108	2.37 ± 0.32	0.000321
7	*EGR1*	2.93	0.001455		
8	*TLR2*	2.91	0.0044		
9	*EGFR*	2.71	0.003101		
10	*FN1*	2.54	0.005133		
11	*TOP2A*	2.4	0.001877		
12	*RRM2*	2.35	0.005011		
13	*IGFBP3*	2.23	0.010228		
14	*FOS*	2.13	0.025431		
15	*VEGFA*	2.01	0.012069	–	–

Abbreviations: *PTGS2*, prostaglandin-endoperoxide synthase 2; *COL1A2*, collagen type I alpha 2 chain; *PLAU*, plasminogen activator, urokinase; *BDNF*, brain-derived neurotrophic factor; *BCL2*, BCL2 apoptosis regulator; *CCL5*, C-C motif chemokine ligand 5; *EGR1*, early growth response 1; *TLR2*, toll-like receptor 2; *EGFR*, epidermal growth factor receptor; *FN1*, fibronectin 1; *TOP2A*, DNA topoisomerase II alpha; *RRM2*, ribonucleotide reductase regulatory subunit M2; *IGFBP3*, insulin-like growth factor binding protein 3; *FOS*, Fos proto-oncogene, AP-1 transcription factor subunit; *VEGFA*, vascular endothelial growth factor A; DE, differentially expressed.

Table 3.2 Top upregulated DE mRNAs in HUVECs treated with LPS vs. Vehicle

qPCR array data				Validation data	
Nr	**Gene symbol**	**Fold change**	***p value***	**Fold change**	***p value***
1	*IL1b*	20.01	0.001069	15.1 ± 0.21	0.00007
2	*IL8*	14.87	0.000030	41.1 ± 20.12	0.00193
3	*CCL5*	5.38	0.008772	8.44 ± 1.56	8.42E-05
4	*SOD2*	3.58	0.003152		
5	*ICAM1*	2.82	0.001696	3.47 ± 0.32	0.00021
6	*TLR2*	2.31	0.020022		
7	*CCL2*	2.18	0.035624		
8	*PTGS2*	1.97	0.030868		
9	*IL6*	1.89	0.002871		
10	*PLAU*	1.59	0.039647		

Abbreviations: *IL1b*, interleukin 1 beta; *IL8*, C-X-C motif chemokine ligand 8; *CCL5*, C-C motif chemokine ligand 5; *SOD2*, superoxide dismutase 2; *ICAM1*, intercellular adhesion molecule 1; *TLR2*, toll-like receptor 2; *CCL2*, C-C motif chemokine ligand 2; *PTGS2*, prostaglandin-endoperoxide synthase 2; *IL6*, interleukin 6; *PLAU*, plasminogen activator, urokinase.

Table 3.3 Top downregulated DE mRNAs in HUVECs treated with LPS vs. Vehicle

qPCR array data				Validation data	
Nr	**Gene symbol**	**Fold change**	***p value***	**Fold change**	***p value***
1	*MMP7*	−4.79	0.012612	0.678 ± 0.14	0.091
2	*RRM2*	−4.77	0.000825		
3	*TOP2A*	−4.26	0.000580		
4	*FGF2*	−3.79	0.000975	0.469 ± 0.09	0.0051
5	*CDK1*	−3.68	0.000090	0.661 ± 0.14	0.0013
6	*IL18*	−3.67	0.019809		
7	*BIRC5*	−3.66	0.000739		
8	*COL1A2*	−3.40	0.001588	0.404 ± 0.01	0.0155
9	*TACC3*	−2.92	0.007174		
10	*IGFBP3*	−2.85	0.028628	0.768 ± 0.08	0.068
11	*AURKA*	−2.82	0.019969		
12	*ABCB1*	−2.77	0.000632		
13	*KDR*	−2.45	0.018919		
14	*TCF7L2*	−2.25	0.024388		
15	*RB1*	−2.19	0.006922		
16	*CAV1*	−2.13	0.000385		
17	*STAT1*	−2.13	0.011240		
18	*STAT3*	−2.13	0.032995		

Abbreviations: *MMP7*, matrix metallopeptidase 7; *RRM2*, ribonucleotide reductase regulatory subunit M2; *TOP2A*, DNA topoisomerase II alpha; *FGF2*, fibroblast growth factor 2; *CDK1*, cyclin-dependent kinase 1; *IL18*, interleukin 18; *BIRC5*, baculoviral IAP repeat containing 5; *COL1A2*, collagen type I alpha 2 chain; *TACC3*, transforming acidic coiled-coil containing protein 3; *IGFBP3*, insulin-like growth factor binding protein 3; *AURKA*, aurora kinase A; *ABCB1*, ATP binding cassette subfamily B member 1; *KDR*, kinase insert domain receptor; *TCF7L2*, transcription factor 7 like 2; *RB1*, RB transcriptional corepressor 1; *CAV1*, caveolin 1; *STAT1*, signal transducer and activator of transcription 1; *STAT3*, signal transducer and activator of transcription 3.

Table 3.4 Top upregulated DE mRNAs in LPS-treated HUVECs transfected with scrambled-controls vs. siFABP3

siFABP3- *versus* scramble-transfected and LPS-treated HUVECs				siFABP3- *versus* scramble-transfected HUVECs		LPS- *versus* vehicle-treated HUVECs	
Nr	Gene symbol	Fold change	*p value*	Fold change	*p value*	Fold change	*p value*
1	*COL1A2*	5.46	0.000518	4.04	0.000606	−3.40	0.001588
2	*BDNF*	4.48	0.002061	3.48	0.000012	−1.28	0.045815
3	*RRM2*	3.71	0.000630	2.35	0.005011	−4.77	0.000825
4	*PTGS2*	3.61	0.001994	5.93	0.001058	1.97	0.030868
5	*CCL5*	3.53	0.016385	2.99	0.031080	5.38	0.008772
6	*TOP2A*	3.29	0.004118	2.40	0.001877	−4.26	0.000580
7	*BIRC5*	3.25	0.004793	1.94	0.001717	−3.66	0.000739
8	*BCL2*	3.16	0.006861	3.12	0.000104	−1.27	0.200420
9	*CXCL10*	2.92	0.046103	1.30	0.621431	−1.29	0.685949
10	*EGR1*	2.78	0.008167	2.93	0.001455	−1.30	0.123815
11	*FN1*	2.35	0.013405	2.54	0.005133	−1.83	0.022166
12	*CDK1*	2.33	0.011819	1.42	0.011762	−3.68	0.000090
13	*EGFR*	2.10	0.012967	2.71	0.003101	−1.01	0.939513
14	*TACC3*	2.02	0.019845	1.31	0.184808	−2.92	0.007174
15	*VEGFA*	1.49	0.039754	2.01	0.012069	−1.14	0.488615

Abbreviations: *COL1A2*, collagen type I alpha 2 chain; *BDNF*, brain-derived neurotrophic factor; *RRM2*, ribonucleotide reductase regulatory subunit M2; *PTGS2*, prostaglandin-endoperoxide synthase 2; *CCL5*, C-C motif chemokine ligand 5; *TOP2A*, DNA topoisomerase II alpha; *BIRC5*, baculoviral IAP repeat containing 5; *BCL2*, BCL2 apoptosis regulator; *CXCL10*, C-X-C motif chemokine ligand 10; *EGR1*, early growth response 1; *FN1*, fibronectin 1; *CDK1*, cyclin-dependent kinase 1; *EGFR*, epidermal growth factor receptor; *TACC3*, transforming acidic coiled-coil containing protein 3; *VEGFA*, vascular endothelial growth factor A.

Table 3.5 Top downregulated DE mRNAs in LPS-treated HUVECs transfected with scrambled-controls vs. siFABP3

siFABP3- *versus* scramble-transfected and LPS-treated HUVECs				siFABP3- *versus* scramble-transfected HUVECs		LPS- *versus* vehicle-treated HUVECs	
Nr	Gene symbol	Fold change	*p value*	Fold change	*p value*	Fold change	*p value*
1	*MMP1*	−2.21	0.006971	−1.67	0.001441	−1.29	0.023254
2	*CCL2*	−2.01	0.028389	−1.23	0.461531	2.18	0.035624
3	*SERPINE1*	−1.82	0.029037	1.18	0.321398	1.52	0.068618
4	*SOD2*	−1.68	0.045407	−1.22	0.319038	3.58	0.003152
5	*CTBP2*	−1.63	0.047921	−1.25	0.436409	−1.85	0.072752
6	*IL1B*	−2.55	0.054269	1.78	0.208236	20.01	0.001069
7	*IL8*	−1.59	0.062081	1.09	0.388103	14.87	0.000030
8	*IL6*	−1.47	0.058008	1.60	0.005547	1.89	0.002871

Abbreviations: *MMP1*, matrix metallopeptidase 1; *CCL2*, C-C motif chemokine ligand 2; *SERPINE1*, serpin family E member 1; *SOD2*, superoxide dismutase 2; *CTBP2*, C-terminal binding protein 2; *IL1B*, interleukin 1 beta; *IL8*, C-X-C motif chemokine ligand 8; *IL6*, interleukin-6.

Table 3.6 List of primers used to amplify respective genes

Targets	Forward primer's sequence	Reverse primer's sequence
PTGS2	5'-CTGGCGCTCAGCCATACAG-3'	5'-CGCACTTATACTGGTCAAATCCC-3'
COL1A2	5'-GGCCCTCAAGGTTTCCAAGG-3'	5'-CACCCTGTGGTCCAACAACTC-3'
PLAU	5'-GGGAATGGTCACTTTTACCGAG	5'-GGGCATGGTACGTTTGCTG-3'
BDNF	5'-GGCTTGACATCATTGGCTGAC-3'	5'-CATTGGGCCGAACTTTCTGGT-3'
IL8	5'-ACTGAGAGTGATTGAGAGTGGAC-3'	5'-AACCCTCTGCACCCAGTTTTC-3'
SPARC	5'-CCCATTGGCGAGTTTGAGAAG-3'	5'-CAAGGCCCGATGTAGTCCA-3'
MMP7	5'-GAGTGAGCTACAGTGGGAACA-3'	5'-CTATGACGCGGGAGTTTAACAT-3'
SERPINE	5'-GCACCACAGACGCGATCTT-3'	5'-ACCTCTGAAAAGTCCACTTGC-3'
TYMS	5'-GGAGTGAAAATCTGGGATGCC-3'	5'-ACTGGAAGCCATAAACTGGGC-3'
FGF2	5'-AGTGTGTGCTAACCGTTACCT-3'	5'-ACTGCCCAGTTCGTTTCAGTG-3'
CDK1	5'-GGATGTGCTTATGCAGGATTCC-3'	5'-CATGTACTGACCAGGAGGGATAG-3'
RB1	5'-TTGGATCACAGCGATACAAACTT-3'	5'-AGCGCACGCCAATAAAGACAT-3'
KRAS	5'-GGACTGGGGAGGGCTTTCT-3'	5'-GCCTGTTTTGTGTCTACTGTTCT-3'
VEGFR2	5'-GTGATCGGAAATGACACTGGAG-3'	5'-CATGTTGGTCACTAACAGAAGCA-3'
IGFBP3	5'-AGAGCACAGATACCCAGAACT-3'	5'-GGTGATTCAGTGTGTCTTCCATT-3'

source: https://pga.mgh.harvard.edu/primerbank/

3.6 Conclusion

In summary, our data demonstrate that FABP3 is expressed in endothelial cells and that loss of endothelial FABP3 inhibits LPS-induced endothelial dysfunction by modulating cell survival and inflammatory and angiogenic signalling pathways. We also observed exacerbation of LPS-induced inflammation in endothelial cells. We were able to provide a global view of the pathways associated with FABP3; however, these findings warrant further detailed investigations. We observed a rather low expression of FABP3 in endothelium and an increased level of circulating FABP3 in LPS-treated mice; however, it remains to be seen whether the endothelium is a significant source of FABP3 in LPS-treated mice and in PAD patients. To this aim, we are generating endothelial cell-specific FABP3 knockout ($FABP3^{endo}$) mice. We will measure circulating FABP3 following LPS treatment to $FABP3^{endo}$ and wild-type mice. Circulating FABP3 will also be measured following crossing $FABP3^{endo}$ mice with $ApoE^{null}$ mice ($FABP3^{endo}$:$ApoE^{null}$) and feeding them a high-fat diet to induce atherosclerosis. A decreased level of circulating FABP3 in $FABP3^{endo}$ following LPS treatment or in $FABP3^{endo}$:$ApoE^{null}$ mice following high-fat diet treatment will confirm endothelium as a significant source of FABP3 *in vivo*. As of present, our data indicate that an increase in circulating FABP3 may be detrimental to endothelial function, and therefore, therapies aimed at inhibiting FABP3 may improve endothelial function in diseased states, particularly in the cases with chronic elevation of FABP3, such as PAD.

3.7 References

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Chapter 4: The Transcriptomic Profile of FABP3 Exposure on Human Endothelial Cells: Implications for Cardiovascular Pathophysiology

4.1 Abstract

Heart-type fatty acid binding protein 3 (FABP3) is released into the circulation following myocardial infarction, and elevated levels are also observed in peripheral artery disease (PAD) patients, potentially exposing endothelial cells to higher levels of FABP3. Our recent research has shown that loss of endothelial FABP3 protects against inflammation-induced endothelial dysfunction, but the effects of FABP3 exposure on endothelial cells are poorly understood. Here, we treated cultured human endothelial cells with recombinant human FABP3 (rhFABP3) or vehicle control, extracted total RNAs and performed RNA-seq analysis. Differential gene expression, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to identify differentially expressed genes, cellular functions and pathways affected by rhFABP3 exposure. Our results indicate that kinesin family member 26b (KIF26B) and survival motor neuron 2 protein-coding genes were the most up- and down-regulated, respectively, in rhFABP3-treated endothelial cells compared to vehicle-treated cells. Many differentially expressed genes were associated with endothelial cell motility, immune response, and angiogenesis. GO and KEGG analyses potentially highlighted crucial pathways affected by rhFABP3 exposure, notably "Regulation of leukocyte-mediated cytotoxicity" and "Natural killer cell-mediated cytotoxicity," suggesting possible FABP3's involvement in endothelial cells' response to cardiovascular stress. Conclusively, we demonstrated the rhFABP3-induced transcriptomics in human endothelial cells. Our findings reveal novel genes and potential processes and pathways influenced by FABP3 exposure, expanding current knowledge on FABP3's role in endothelial biology and cardiovascular diseases. Further research is warranted to validate our findings and fully elucidate FABP3's implications in cardiovascular pathology.

4.2 Introduction

Lipids-related physiology linked to cardiovascular impacts crucially depends upon the bioavailability of cellular lipids, which is implicated by the role of metabolic syndrome in cardiovascular diseases (CVDs) [1] [2] [3]. Central to the regulation of cellular lipid bioavailability and signalling are a family of intracellular lipid-chaperones, the fatty acid binding proteins

(FABPs). Heart-type FABP, or FABP3, mainly known to be expressed in myocardiocytes, is integral to cardiac metabolic homeostasis [4] [5] [6] [7]. Meanwhile, FABP3 can also be found in many other tissues, notably skeletal muscles and, to a lesser extent, the brain, testis, kidneys, adrenal glands, and others [8]. Indeed, the unique function of FABP3 remains complex and unclear [9].

Nonetheless, FABP3 is investigated as a biomarker for cardiac injuries, having been characterized as follows in both animal models and heart failure patients: 1) negligible plasma concentration and significantly high cytosolic to plasma ratio at rest; 2) blood elevation detectable within 30 min of chest pain, peak in a few hours, and returning to baseline via renal clearance, all within 24 h [10] [11] [12]. In addition to heart failure, FABP3 has also been tested as a biomarker in patients with peripheral artery diseases (PAD). Interestingly, in the absence of cardiac injury and regardless of diabetic and coronary artery disease background, FABP3's circulatory levels elevate and notably correlate with the severity of PAD [13]. Notably, the same study reported a significant upregulation of FABP3 in skeletal muscle cells in PAD patients compared to healthy individuals. Recently, we reported FABP3 basal and inflammation-induced expression in endothelial cells; we also demonstrated that endothelial cell-specific loss of FABP3 protects endothelial cells against inflammation-induced endothelial dysfunction and apoptosis [14]. Overall, these findings suggest that FABP3 release is non-specific to cardiac injury and may signal earlier cardiovascular events.

PAD and heart failure are cardiovascular complications of atherosclerosis, a chronic vascular inflammatory disorder characterized by circulatory blockage due to the build-up of lipid-laden plaques in the vascular inner walls, leading to downstream ischemia, hypoxia and organ failures [15]. Atherosclerosis is the primary cause of CVDs and is driven by endothelial dysfunction [16].

The inner lumenal walls of virtually all blood vessels (tunica intima) constitute the endothelium, a specialized simple squamous epithelial lining comprising endothelial cells. Endothelial cells are versatile and in direct contact with blood. They establish a delicate semi-permeable blood-tissue barrier known to extensively regulate selective exchanges and vascular homeostasis at varying capacities across organ systems. They oversee the production of signalling agents that maintain or mediate vasotone (vasodilation vs. vasoconstriction), vessel compliance, barrier/exchange permeability, blood fluidity, inflammation, wound healing, angiogenesis, and thrombosis [17]. In

CVD pathogenesis, endothelial cells are often impaired by various stresses, including elevated oxidative radicals, advanced glycation end products, endotoxin, hypertension, and hypoxia. Stressed endothelial cells are activated into a hyper-functional state to alleviate the source of stress, favouring permeability in the blood-tissue barrier, inflammatory signalling, thrombosis, hemostasis, and vasoconstriction. When prolonged, endothelial activation becomes endothelial dysfunction, featuring a leaky and oxidative barrier that exacerbates injuries and propagates the damaging agents, a hyper-inflammatory environment leading to chronic inflammation, dysregulated metabolism, diminished vasotone, and impaired vascular homeostasis [18].

Given the remarkable capacity of FABP3 as a biomarker, albeit not specific to cardiac injury, and that endothelial cells are one of the first cells to be exposed to elevated levels of circulatory FABP3 in conditions like heart failure and PAD, the impacts of circulatory FABP3 on endothelial cells and endothelial function are yet to be elucidated. As endothelial dysfunction is central in atherosclerosis and CVD, the mechanisms by which FABP3 influences endothelial function warrant an investigation. This study explores the transcriptomic profiles of endothelial cells subjected to FABP3 exposure under the hypothesis that circulatory FABP3 regulates endothelial function. We aim to pursue insights into the link between FABP3 and endothelial dysfunction, potentiating the development of novel clinical applications of FABP3 in the cardiovascular field.

4.3 Methods

Cell Culture and Treatment - Human umbilical vein endothelial cells (HUVECs, pooled, Lonza; passage 4) were cultured in endothelial cell growth medium-2 (EGM-2 Bulletkit; Lonza) supplemented with growth factors, serum and antibiotics at 37^0C in humidified 5% CO2. Confluent HUVECs were maintained in six-well plates and starved overnight before treating with either a vehicle (PBS) or rhFABP3 (50 ng/mL; Cayman Chemical) for 6 hours.

RNA sequencing (RNA-seq) and Analysis - Total RNA was extracted from HUVECs using TRIzol (Invitrogen) reagent and quantified and assessed for purity with the NanoDrop ND-1000 spectrophotometer. RNA sequencing was performed at The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada. Sequencing was conducted on the Illumina HiSeq2500 platform, using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760; New England Biolabs) and bcl2fastq2 v2.20 for paired-end reads (125 base pairs). Reads

were generated in FASTQ format and, on the Compute Canada platform, subjected to 1) trimming of low-quality reads using Trimmomatic based on the adapters TruSeq3-PE [19], 2) quality assessment using FASTQC [20], and 3) Kallisto transcriptome pseudo-alignment using the open-access GRCh38 (Genome Reference Consortium human genome build 38) indices [21] [22]. Differential gene expression analysis was conducted using edgeR on R version 4.3.1 [23] [24]. P-values were generated using edgeR's model for discrete count data that includes dispersion estimation, and the Benjamini-Hochberg method was applied for false discovery rate (FDR) adjustment. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using Bioconductor packages in R [25]. To enable a broader assessment in cases with a limited number of statistically significant genes, unadjusted p-values from edgeR were considered for GO and KEGG analysis, acknowledging the trade-off of potentially increasing the rate of Type-1 errors and that future multiple testing adjustment is overall essential to tease out true positives, regardless of scientific intention [26] [27].

4.4 Results

Quality Assessment: RNA integrity, quantity and purity were assessed with the NanoDrop ND-1000. The A260 /A280 optical density (OD) ratios yielded values of about 2.0, which confirmed the purity of our RNAs (**Table 4.4**). The intensity of the 28S ribosomal RNA was about twice (indicated by % of total Area) that of the 18S ribosomal RNA, confirming the integrity of RNAs (**Figure 4.3 and 4.4**) used in this study. Overall, the RNAs used to perform RNA-seq were pure and not degraded.

FABP3 Exposure Induced Differential Gene Expression in Endothelial Cells: The impact of rhFABP3 exposure on HUVECs' gene expression was examined via the *RNA-seq*. A genome-wide expression of 15,688 genes in rhFABP3-treated HUVECs vs. vehicle controls was tested in a comprehensive differential gene expression (DGE) analysis. Principle component analysis (PCA) on the first and second principle components, capturing 58.71% and 20.7% variance, respectively, showed distinct samples clustering between the rhFABP3-treated and vehicle-treated groups, suggesting changes in gene expression profiles in HUVECs due to rhFABP3 exposure (**Figure 4.1A**). DGE analysis using Volcano and Manhattan plots identified 11 genes with significant differential expression that satisfy a log (2) fold-change threshold of 1 and -1 (equivalent to one

doubling of gene expression up or downward) in rhFABP3-treated group vs. vehicle controls; genes with FDR-adjusted p-values < 0.05 are considered significantly differentially expressed, and 7 genes were upregulated, and 4 were downregulated (**Figure 4.1B and C**) (**Table 4.1**). Accordingly, differentially expressed genes are distributed between chromosomes 1, 2, 5, 6, 12, 16, 19 and 21, and the *IMPDH1P10* processed-pseudogene is the most upregulated (log2 fold-change of 8.75), followed by the protein-coding genes: *KIF26B* (7.10), *NCR1* (4.17), and *DNAJC14* (1.95). *CENPBD1P* pseudogene is the most downregulated (log2 fold-change of -8.98), followed by the protein-coding genes: *ENSG00000269242* (-3.45), *CFAP298-TCP10L* (-3.10), and *SMN2* (-2.28).

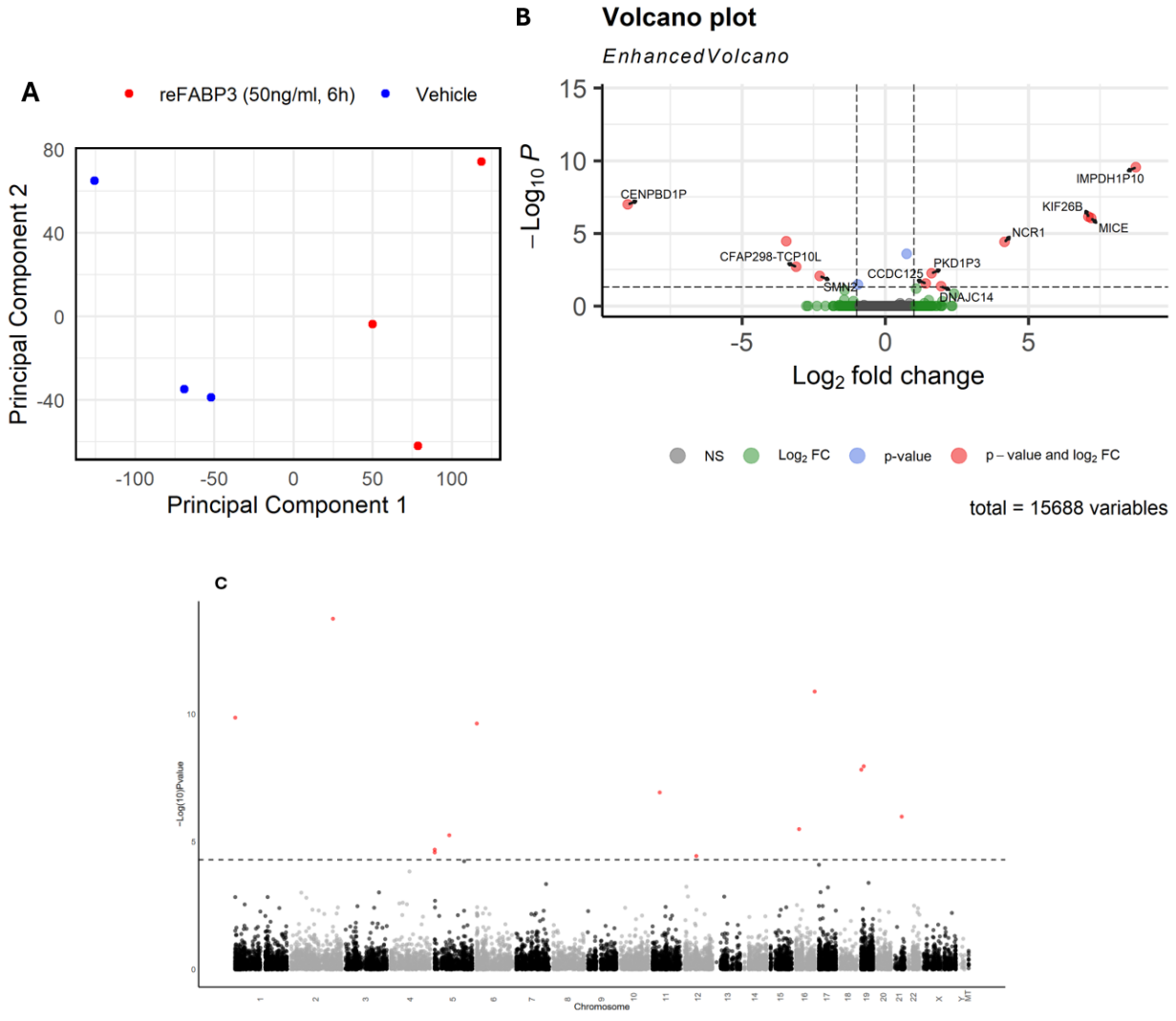


Figure 4.1 Differential gene expression (DGE) analysis of HUVECs treated with rhFABP3 (50ng/mL) for 6 hours vs. Vehicle

(A) Principle Component Analysis (PCA) plot clustering the samples of HUVECs treated with rhFABP3 (50ng/mL) for 6 hours vs. Vehicle (red = rhFABP3; blue = Vehicle), accessing their global expression of 15688 genes derived from RNA-sequencing results; the x- and y-axes represent the first and second principal components, which capture the most (58.71%) and second-most (20.70%) variance within the data, respectively. Volcano (B) and Manhattan (C) plots of DGE genes in HUVECs treated with rhFABP3 (50ng/mL) for 6 hours vs. vehicle controls. (B) Log (2) fold change is plotted against –log (10) False Discovery Rate (FDR) adjusted p-values; genes with FDR-adjusted p-values less than 0.05 (dashed y-intercept) that pass the log (2) fold-change of 1 or

-1 (dashed x-intercepts) in differential expression are labelled (red). (C) DGE genes tested are localized to their chromosomes (x-axis); genes with FDR-adjusted p-values less than 0.05 (y-intercept; dashed line represents the threshold for FDR-adjusted statistical significance) are highlighted (red). N = 3 biological replicates per group. HUVEC = Human Umbilical Vein Endothelial Cells; rhFABP3 = recombinant human FABP3, MT = mitochondria.

Table 4.1 Summary of top-differentially-expressed genes in FABP3-treated HUVECs vs. Vehicle

HUGO Gene Nomenclatures*	Gene name**	Gene ID*	Gene Biotype*	Log2 Fold Change	Fold Change	p-value***	Chromosome/Strand
Top Up-regulated Differentially Expressed Genes							
KIF26B	*Kinesin Family Member 26B*	ENSG00000281216	protein_coding	7.10	137.08	7.16E-07	1/+
NCR1	*Natural Cytotoxicity Triggering Receptor 1*	ENSG00000275156	protein_coding	4.17	18.02	3.89E-05	19/+
DNAJC14	*DnaJ Heat Shock Protein Family (Hsp40) Member*	ENSG00000135392	protein_coding	1.95	3.86	4.31E-02	12/-
CCDC125	*Coiled-Coil Domain Containing 125*	ENSG00000277868	protein_coding	1.42	2.68	2.90E-02	5/-
IMPDH1P10	*Inosine Monophosphate Dehydrogenase 1 Pseudogene 10*	ENSG00000232133	processed_pseudogene	8.75	431.03	2.87E-10	2/-
MICE	*Unnamed*	ENSG00000251581	transcribed_unprocessed_pseudogene	7.19	146.26	9.12E-07	6/-
PKD1P3	*Polycystin 1, Transient Receptor Potential Channel Interacting Pseudogene 3*	ENSG00000183458	transcribed_unprocessed_pseudogene	1.62	3.07	5.55E-03	16/+
Top Down-regulated Differentially Expressed Genes							
SMN2	*Survival Of Motor Neuron 2, Centromeric*	ENSG00000205571	protein_coding	-2.28	0.21	8.64E-03	5/+
CFAP298-TCP10L	*CFAP298-TCP10L Readthrough*	ENSG00000265590	protein_coding	-3.10	0.12	2.02E-03	21/-
Unnamed	*Glycosyl Hydrolases Family 38 C-Terminal Beta Sandwich Domain-Containing Protein*	ENSG00000269242	protein_coding	-3.45	0.09	3.44E-05	19/-
CENPBD1P	*CENPB DNA-Binding Domain Containing 1, Pseudogene*	ENSG00000177946	transcribed_unitary_pseudogene	-8.98	0.00	1.02E-07	16/-

*Human Genome Organization (HUGO) classification utilized in the gene annotation database. A total of 15688 genome-wide genes were validated from processed RNA-seq results and tested for differential gene expression.

**Gene names are derived from genecards.org

*** Summary of FDR-significant ($p < 0.05$) top-differentially-expressed genes by at least log (2) fold-change of 1, including top up- and down-regulated DE protein-coding genes, in HUVECs treated with rhFAB3 (50 ng/mL, 6h) vs. vehicle controls. N = 3 biological replicates per group

FABP3 Exposure Affects Potential Biological Functions/Pathways in Endothelial Cells: Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to assess the biological significance of the observed differential gene expression in rhFABP3-treated HUVECs. Due to the low number of significant genes meeting our FDR/fold-change filter, GO/KEGG analyses were performed with 80 differentially expressed genes (38 upregulated and 42 downregulated) selected using the unadjusted edgeR p-values < 0.05 and log (2) fold change of lesser or greater than -1 or 1, respectively, against a total of 15,688 DGE-tested genes. The analyses revealed several cellular functions and pathways potentially impacted by rhFABP3 exposure, suggesting a multifaceted role of FABP3 in endothelial cell physiology (**Table 4.2 and Table 4.3**). GO and KEGG results are predominantly related to immune response and cell cytotoxicity biological processes for the upregulated differentially expressed genes, with the most significant being "Regulation of leukocyte mediated cytotoxicity (p = 2.08e-04)" and "Natural killer cell-mediated cytotoxicity (p = 9.17e-03)", respectively. On the other hand, the downregulated DE genes are associated with GO and KEGG terms of complex regulatory implication, including "RNA processing and inflammatory and immune systems mechanisms, with SMN complex (cellular component) (p = 1.67e-04)" and "NOD-like receptor signalling (p = 2.92e-02)" pathway being the most significant. Additionally, a broader assessment of gene expression patterns, which shows distinct clusters of differential expression patterns in rhFABP3-treated HUVECs, was conducted via two separated heatmaps: one visualizing the 80 differentially expressed genes selected for GO and KEGG analyses (**Figure 4.2**) and another examining 231 genes with the protein-coding category and edgeR-derived unadjusted p-values < 0.05 out of the 15,688 DGE-tested genes (**Figure 4.5**). These findings illustrate the specific gene expression signatures associated with FABP3 exposure and underscore the potential functional impacts on endothelial cells.

Table 4.2 Top up- and down-regulated gene ontologies impacted in HUVECs under rhFABP3 exposure

GO Terms	*GO Categories	Counts	**p-value
Associated with Upregulated DE Genes			
Regulation of leukocyte mediated cytotoxicity	BP	3	2.08E-04
Regulation of cell killing	BP	3	3.07E-04
Leukocyte mediated cytotoxicity	BP	3	5.25E-04
Positive regulation of T cell mediated cytotoxicity	BP	2	1.37E-03
Regulation of lymphocyte mediated immunity	BP	3	1.62E-03
Regulation of T cell mediated cytotoxicity	BP	2	2.30E-03
Positive regulation of leukocyte mediated cytotoxicity	BP	2	2.71E-03
Positive regulation of cell killing	BP	2	3.14E-03
T cell mediated cytotoxicity	BP	2	3.14E-03
Associated with Downregulated DE Genes			
SMN complex	CC	2	1.67E-04
Canonical inflammasome complex	CC	2	2.44E-04
Gemini of coiled bodies	CC	2	2.44E-04
SMN-Sm protein complex	CC	2	4.99E-04
DNA-templated transcription termination	BP	2	1.01E-03
Pyroptosis	BP	2	1.18E-03
Positive regulation of interleukin-1 beta production	BP	2	2.52E-03
Spliceosomal snRNP assembly	BP	2	2.65E-03
Positive regulation of interleukin-1 production	BP	2	3.21E-03
Immune response-regulating signaling pathway	BP	4	3.82E-03

*BP = Biological processes; CC = Cellular Component.

**Gene ontology (GO) analysis is conducted by enriching 38 upregulated and 42 downregulated genes with unadjusted p-values less than 0.05 and a log2 fold-change of at least 1 or -1 (one gene expression doubling increase or decrease) against 15688 RNA-seq genes tested for differential gene expression.

Table 4.3 Top up- and down-regulated functional pathways impacted in HUVECs under rhFABP3 exposure

KEGG Pathway Terms	Counts	**p-value
Associated with Upregulated DE Genes		
Natural killer cell mediated cytotoxicity	2	9.17E-03
JAK-STAT signaling pathway	2	1.63E-02
Associated with Downregulated DE Genes		
NOD-like receptor signaling pathway	2	2.92E-02

**Kyoto Encyclopedia of Genes and Genomes (KEGG) based pathways analysis is conducted by enriching 38 upregulated and 42 downregulated genes with unadjusted p-values less than 0.05 and a log2 fold-change of at least 1 or -1 (one gene expression doubling increase or decrease) against 15688 RNA-seq genes tested for differential gene expression.

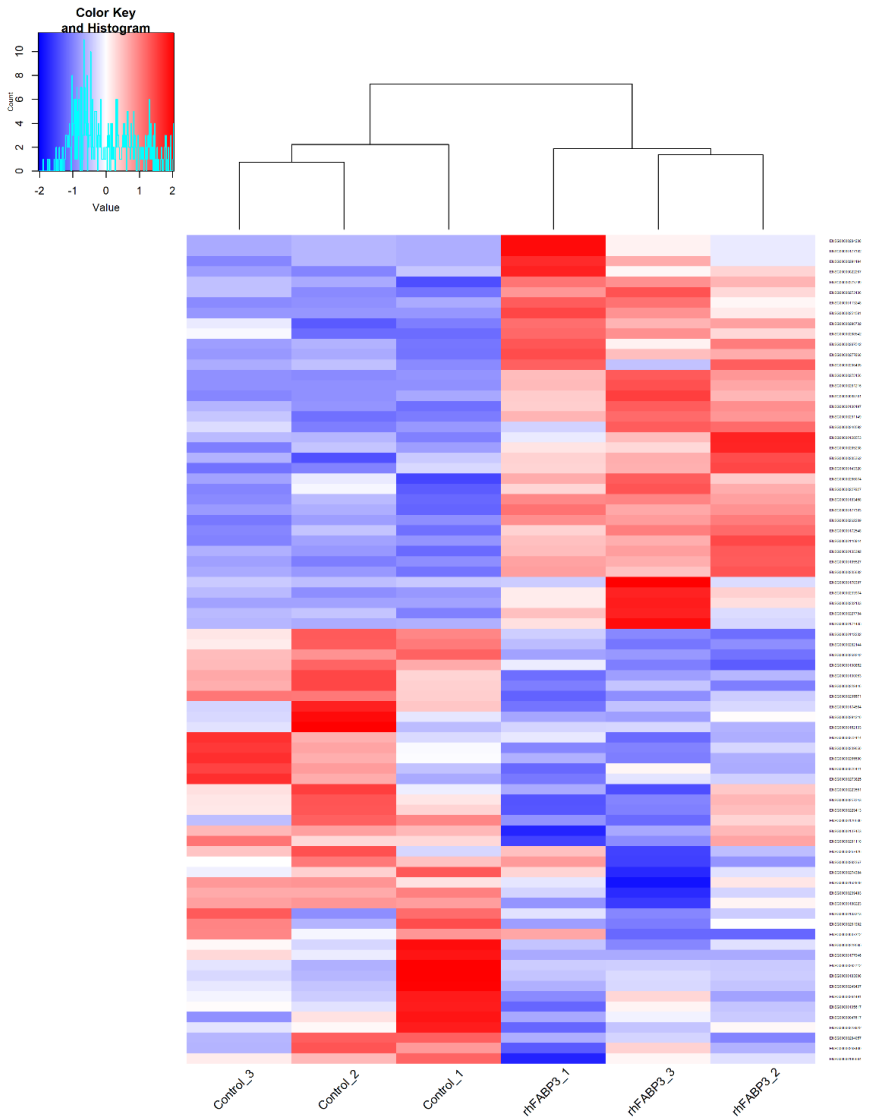
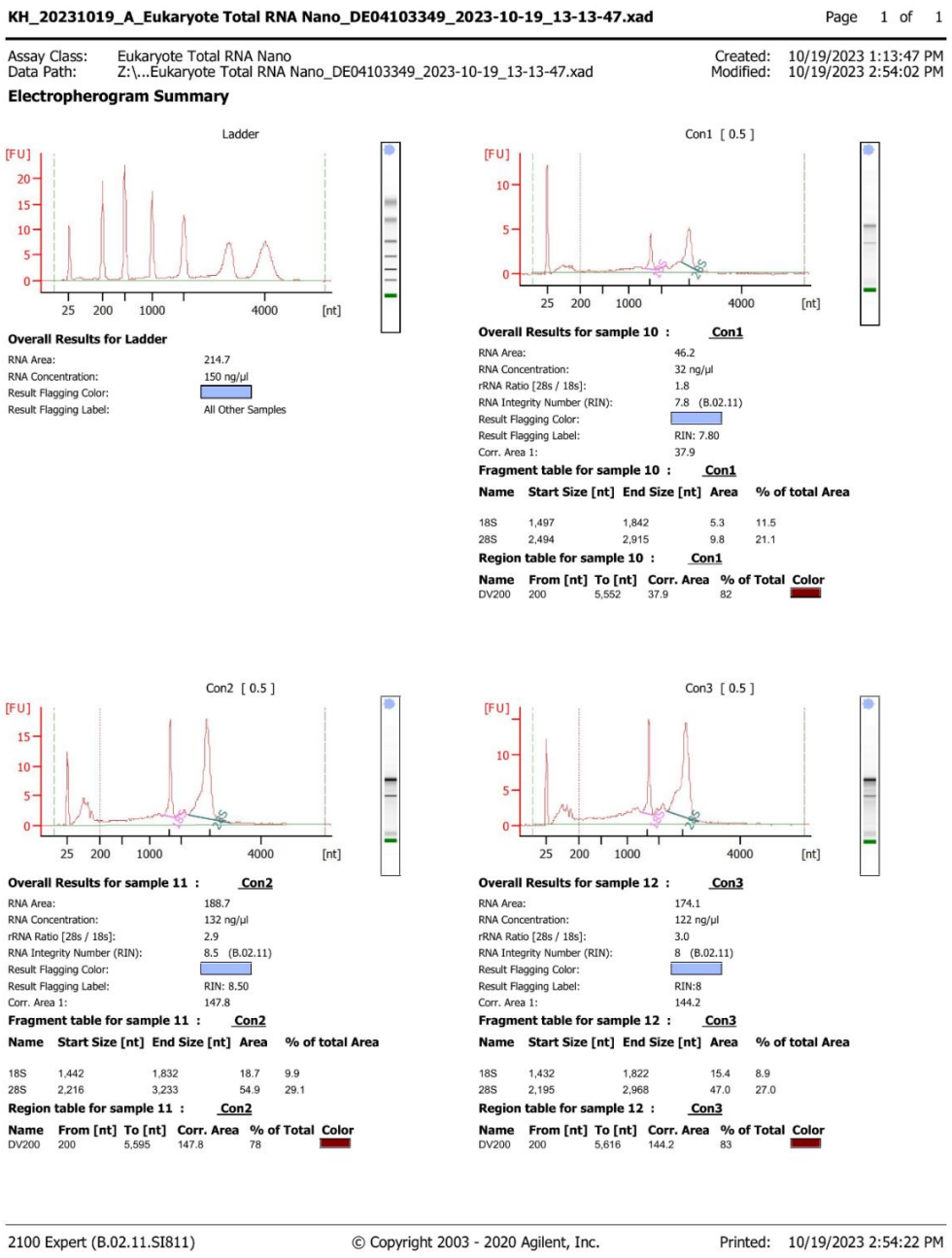


Figure 4.2 Functional impacts of exogenous FABP3 on endothelial cells via 80 up- and down-regulated genes

Gene expression heatmap showing clusters of samples from HUVECs treated with rhFABP3 (50ng/mL) for 6 hours vs. Vehicle groups. The analysis illustrates 80 up- and down-regulated genes that pass the log (2) fold-change of 1 or -1 with unadjusted p-values < 0.05 (all of which were subjected to GO and KEGG analyses) out of 15688 genes tested for differential gene expression. N = 3 biological replicates per group. HUVEC = Human Umbilical Vein Endothelial Cells; rhFABP3 = recombinant human FABP3.



KH_20231019_A_Eukaryote Total RNA Nano_DE04103349_2023-10-19_13-13-47.xad Page 1 of 1

Assay Class: Eukaryote Total RNA Nano
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Created: 10/19/2023 1:13:47 PM
Modified: 10/19/2023 2:54:02 PM

Electropherogram Summary

Ladder

Overall Results for Ladder

RNA Area:	214.7
RNA Concentration:	150 ng/µl
Result Flagging Color:	
Result Flagging Label:	All Other Samples

Con1 [0.5]

Overall Results for sample 10 : Con1

RNA Area:	46.2
RNA Concentration:	32 ng/µl
rRNA Ratio [28s / 18s]:	1.8
RNA Integrity Number (RIN):	7.8 (B.02.11)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.80
Corr. Area 1:	37.9

Fragment table for sample 10 : Con1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,497	1,842	5.3	11.5
28S	2,494	2,915	9.8	21.1

Region table for sample 10 : Con1

Name	From [nt]	To [nt]	Corr. Area	% of Total	Color
DV200	200	5,552	37.9	82	

Con2 [0.5]

Overall Results for sample 11 : Con2

RNA Area:	188.7
RNA Concentration:	132 ng/µl
rRNA Ratio [28s / 18s]:	2.9
RNA Integrity Number (RIN):	8.5 (B.02.11)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.50
Corr. Area 1:	147.8

Fragment table for sample 11 : Con2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,442	1,832	18.7	9.9
28S	2,216	3,233	54.9	29.1

Region table for sample 11 : Con2

Name	From [nt]	To [nt]	Corr. Area	% of Total	Color
DV200	200	5,595	147.8	78	

Con3 [0.5]

Overall Results for sample 12 : Con3

RNA Area:	174.1
RNA Concentration:	122 ng/µl
rRNA Ratio [28s / 18s]:	3.0
RNA Integrity Number (RIN):	8 (B.02.11)
Result Flagging Color:	
Result Flagging Label:	RIN:8
Corr. Area 1:	144.2

Fragment table for sample 12 : Con3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,432	1,822	15.4	8.9
28S	2,195	2,968	47.0	27.0

Region table for sample 12 : Con3

Name	From [nt]	To [nt]	Corr. Area	% of Total	Color
DV200	200	5,616	144.2	83	

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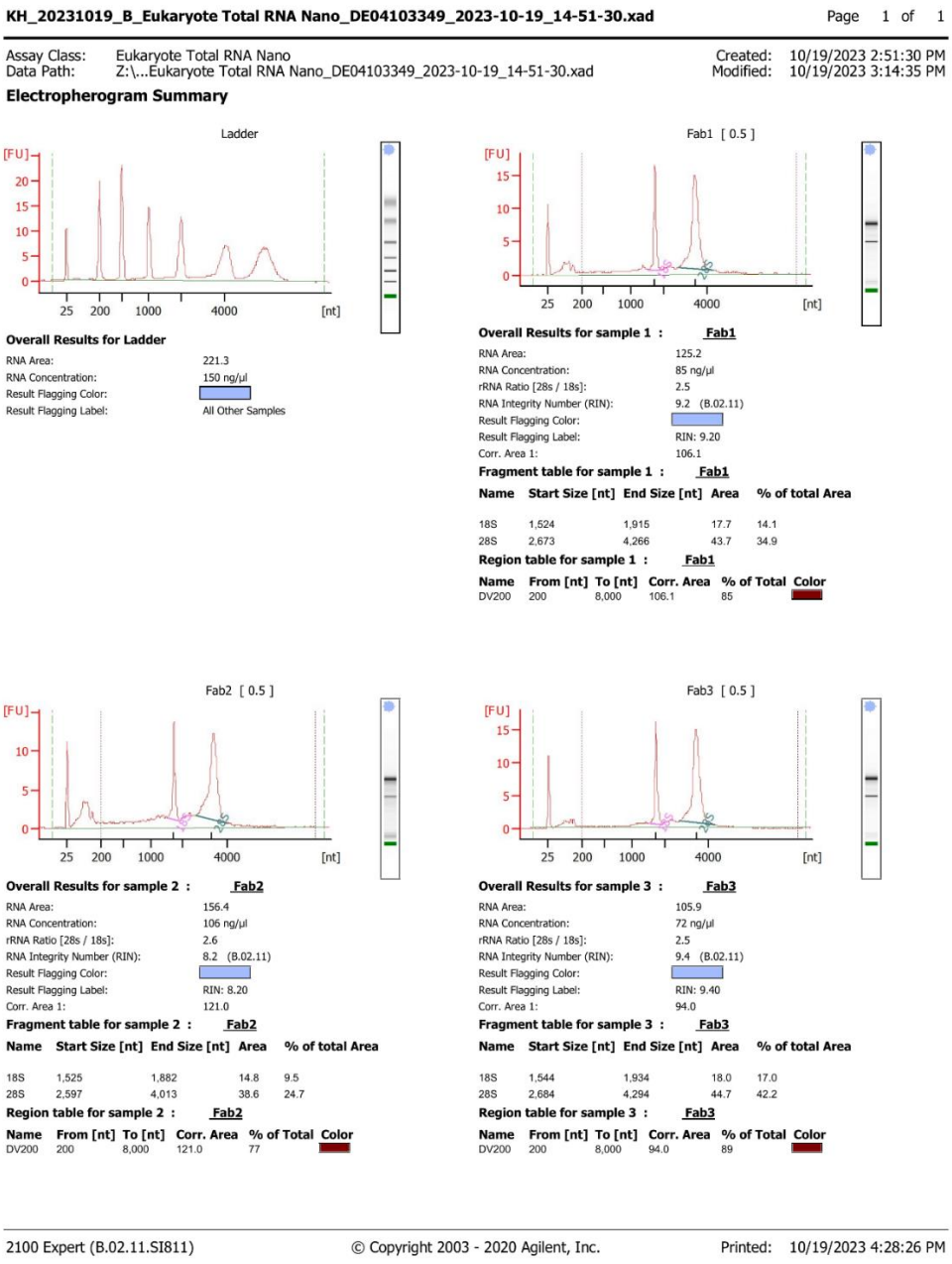
Figure 4.3 Confirmation of RNA purity for Vehicle-treated HUVECs



KH_20231019_B_Eukaryote Total RNA Nano_DE04103349_2023-10-19_14-51-30.xad Page 1 of 1

Assay Class: Eukaryote Total RNA Nano
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Modified: 10/19/2023 3:14:35 PM

Electropherogram Summary

Ladder

[FU] 20 15 10 5 0 — 25 200 1000 4000 [nt]

Overall Results for Ladder

RNA Area:	221.3
RNA Concentration:	150 ng/µl
Result Flagging Color:	
Result Flagging Label:	All Other Samples

Fab1 [0.5]

[FU] 15 10 5 0 — 25 200 1000 4000 [nt]

Overall Results for sample 1 : Fab1

RNA Area:	125.2
RNA Concentration:	85 ng/µl
rRNA Ratio [28s / 18s]:	2.5
RNA Integrity Number (RIN):	9.2 (B.02.11)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.20
Corr. Area 1:	106.1

Fragment table for sample 1 : Fab1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,524	1,915	17.7	14.1
28S	2,673	4,266	43.7	34.9

Region table for sample 1 : Fab1

Name	From [nt]	To [nt]	Corr. Area	% of Total	Color
DV200	200	8,000	106.1	85	

Fab2 [0.5]

[FU] 10 5 0 — 25 200 1000 4000 [nt]

Overall Results for sample 2 : Fab2

RNA Area:	156.4
RNA Concentration:	106 ng/µl
rRNA Ratio [28s / 18s]:	2.6
RNA Integrity Number (RIN):	8.2 (B.02.11)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.20
Corr. Area 1:	121.0

Fragment table for sample 2 : Fab2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,525	1,882	14.8	9.5
28S	2,597	4,013	38.6	24.7

Region table for sample 2 : Fab2

Name	From [nt]	To [nt]	Corr. Area	% of Total	Color
DV200	200	8,000	121.0	77	

Fab3 [0.5]

[FU] 15 10 5 0 — 25 200 1000 4000 [nt]

Overall Results for sample 3 : Fab3

RNA Area:	105.9
RNA Concentration:	72 ng/µl
rRNA Ratio [28s / 18s]:	2.5
RNA Integrity Number (RIN):	9.4 (B.02.11)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.40
Corr. Area 1:	94.0

Fragment table for sample 3 : Fab3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,544	1,934	18.0	17.0
28S	2,684	4,294	44.7	42.2

Region table for sample 3 : Fab3

Name	From [nt]	To [nt]	Corr. Area	% of Total	Color
DV200	200	8,000	94.0	89	

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Figure 4.4 Confirmation of RNA purity for rhFABP3-treated HUVECs

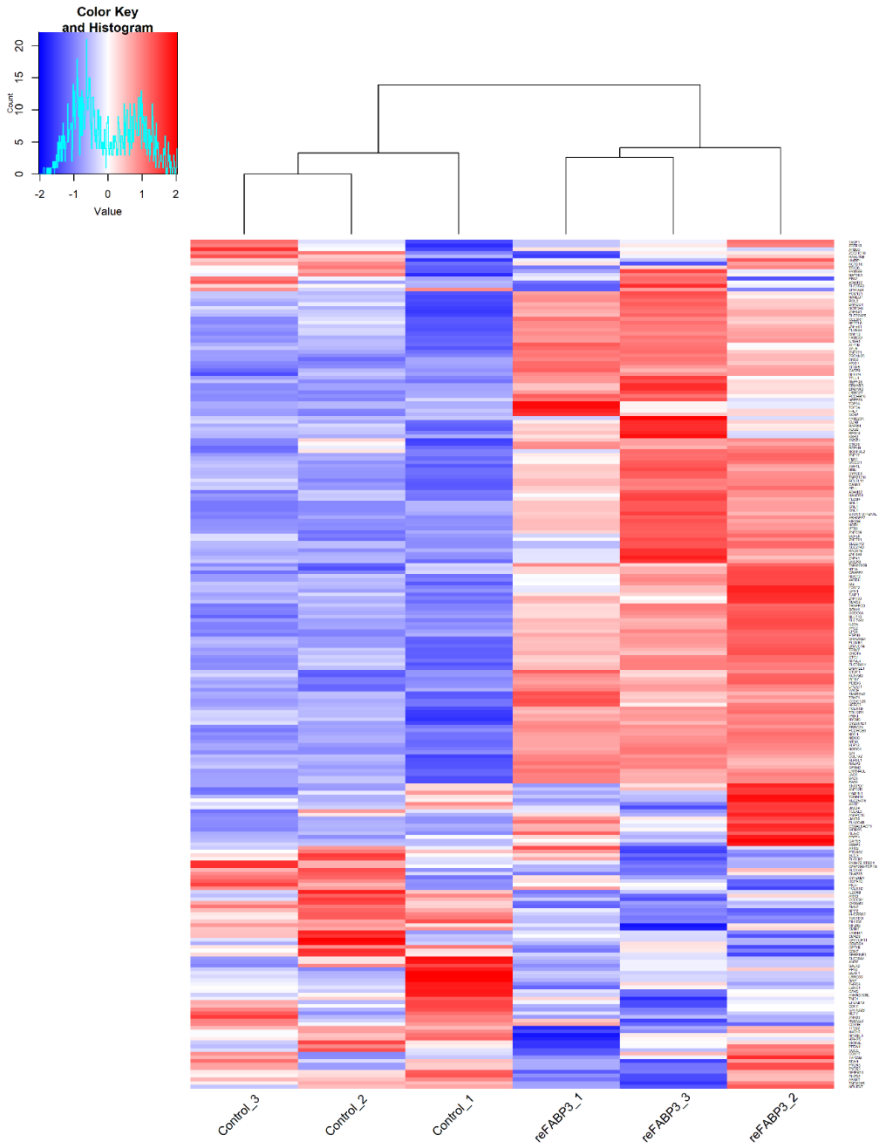

Figure 4.5 Functional impacts in endothelial cells due to FABP3 exposure via protein-coding genes

Gene expression heatmap showing clusters of samples from HUVECs treated with rhFABP3 (50ng/mL) for 6 hours vs. Vehicle groups. The analysis was conducted on 231 protein-coding genes out of 15688 RNA seq genes that were tested for differential gene expression (DGE), with unadjusted p-values of less than 0.05. N = 3 biological replicates per group. HUVEC = Human Umbilical Vein Endothelial Cells; rhFABP3 = recombinant human FABP3.

4.5 Discussion

Our RNA seq and DGE analyses identify several significantly impacted genes in rhFABP3-treated HUVECs, which underline the complex nature of endothelial cells' response to circulatory FABP3. Of the upregulated genes, *KIF26B* is an oncogene that has been studied in breast, gastric, colorectal, and hepatocellular cancers; its upregulation correlates with risk of metastases, stage progression, and poor prognosis, suggesting capacities as a biomarker [28]. KIF26B is regulated by miR-372 [29] and essential in developmental processes, implicated in the adhesion and polarization of mesenchymal cells [30]. In cancer, KIF26B is involved in the VEGF signalling pathway that prompts angiogenesis [31]. The upregulation of KIF26B in rhFABP3-treated endothelial cells suggests induced mobilization of cellular motility and possibly angiogenesis, suggesting a regulatory metabolic impact of FABP3 exposure on endothelial cells. It is also important to note that FABP3 is released in circulation mainly during ischemic/hypoxic stress [32], and increased angiogenesis may be a compensatory response of endothelial cells. The upregulated *NCR1* gene encodes an activating receptor on natural killer cells, which imposes innate cytotoxicity and surveillance against bacteria, virally infected cells, and tumour cells [33]. NCR1 is known to mediate the pathogenesis of cancer, autoimmune disorders, and infectious diseases, being a target for immunomodulation and immunotherapy [33]. Regulatory factors of NCR1 include cytokines, transcription factors, microRNAs, and post-translational modifications [34] [35] [36] [37]. While the NCR1's expression is a main feature of natural killer cells, NCR1 has been found in other cell types, such as T-cells [38]. Endothelial NCR1 is poorly understood, and our detection of NCR1's expression in endothelial cells suggests a novel regulatory link between the innate immune system and the endothelium. Particularly, NCR1 upregulation in HUVECs indicates stimulation of the innate immune system by FABP3 exposure. Next, the upregulated gene *DNAJC14* encodes a member of the DNAJ family of intracellular heat-shock chaperone proteins, which are engaged in the cellular stress response and protein quality controls [39]. In particular, they interact with the Hsp70 chaperone proteins via the distinguishing J-domain and assist Hsp70 in re-folding misfolded proteins [40]. While the specific roles of DNAJC14 remain under investigation, aberrant expression of DNAJC14 has been implicated in multiple diseases, including viral infections and neurodegenerative diseases, particularly in the context of misfolded proteins [41] [42]. The upregulation of DNAJC14 in endothelial cells under FABP3 exposure indicates stress response mechanisms, reinforcing the metabolic impact of circulatory FABP3 on the

endothelium. Among the downregulated genes, survival of motor neuron 2 (*SMN2)*, canonically crucial in motor neuron functions and spinal muscular atrophy, encodes a more truncated and less functional protein than the full-length version expressed by SMN1 [43]. SMN proteins mediate the assembly of small nuclear ribonucleoproteins of spliceosomes, thereby regulating RNA splicing, post-transcriptional processing, and non-coding RNAs [44]. SMN2 downregulation in rhFABP3-treated endothelial cells indicates a negative modulation of RNAs in the endothelium, suggesting a regulatory effect on transcript levels. The rest of the identified DE genes are less characterized. *CCDC125* encodes for a protein that is not well-characterized and may be involved in cellular motility according to its Uniprot profile. The human genome GRCh37 ensembl profile of *CFAP298-TCP10L* (*ENSG00000265590*) indicates that it is a protein-coding readthrough transcription between the neighboring chromosome 21 open reading frame 59 and TCP10L (t-complex 10 like) that hasn't been investigated for any functions. *Glycosyl Hydrolases Family 38 C-Terminal Beta Sandwich Domain-Containing Protei*n (*ENSG00000269242*) is a novel transcript with gene ontology annotations related to carbohydrate binding and mannosidase activities, according to its gene-card profile. *IMPDH1P10*, *ENSG00000251581*, *PKD1P3*, and *CENPBD1P* are pseudogenes that remain functionally elusive. Overall, although some DE genes suggest a metabolic and immunity-based response in rhFABP3-treated endothelial cells, a notable amount are pseudogenes, and more than half are currently not characterized. Future validation, such as via qPCR, and characterization of the identified DE genes are necessary to establish more robust mechanistic implications.

To our surprise, only 11 significantly differentially expressed genes were identified from our RNA-seq and differential gene analyses (a total of 15688 genes tested) that meet a log (2) fold-change of 1.0 cut-off. This presents an ostensive limitation of the study for broader assessment that is, therefore, worth a reassessment of our experimental treatment.

The limitation may be attributed to our low dose of FABP3 (50 ng/mL for 6h). In a study that evaluated 2287 patients with acute coronary syndromes, 332 patients (14.5%) were found with elevated circulatory H-FABP levels (>8 ng/mL). This elevation was associated with an increased risk of death and major cardiac events through a 10-month follow-up period, including recurrent myocardial infarction and congestive heart failure. From the elevated H-FABP cohort, the median level of H-FABP3 in circulation was 16 ng/mL, ranging from 8 to 434 ng/mL [45]. In our previous

PAD patients study that identified a robust positive correlation between the severity of PAD and blood FABP3 levels, severe PAD patients (ABI < 0.4) exhibited up to an average of 7.22 ng/mL of blood FABP3 [13]. Therefore, our 50 ng/mL of FABP3 is informed by existing clinical data, fitting within these variable ranges of circulatory FABP3 reported in human patients. However, from our study on the loss of FABP3 in endothelial dysfunction, 200 ng/mL of rhFABP3, but not 50 ng/mL, was found to exacerbate ICAM1 and VCAM1 upregulation in HUVECs stressed by LPS for 6h [14]. This not only suggests a negative inflammatory role of FABP3 exposure but also that our current FABP3 dose may fall short in inducing a pronounced gene expression response, overall implying that a higher dose should be considered within the provided clinical ranges for future investigation. Nevertheless, our suggestive previous findings substantiated the current attempt to analyze the total RNAs from rhFABP3-treated endothelial cells using RNA-seq. Hence, the transcriptomic analysis was conducted at our selected dosing regimen to clarify how endothelial cells are affected by FABP3 exposure. Due to the difficulty in identifying genes with statistically significant differences after adjusting for multiple comparisons, unadjusted p-values are considered a trade-off between sensitivity and specificity, increasing Type-1 errors. However, the aim was not to overlook potentially interesting genes due to stringent statistical corrections while acknowledging that this choice might introduce more uncertainty into the analysis. This approach enables 80 up- and downregulated genes and 231 protein-coding genes with unadjusted p-values <0.05 to be available for GO and KEGG pathways and heatmap cluster analyses, providing potential biological functions and pathways to be examined (**Table 4.1**, **Table 4.2**, **Figure 4.2,** and **Figure 4.5**). While the analytical accuracy due to the trade-off is compromised, FABP3's exposure impact on endothelial cell physiology is indicated by the distinguishing clustering of gene expression shown in the PCA plot and heatmap (**Figure 4.1A, Figure 4.2,** and **Figure 4.5**). Overall, the outcomes of the trade-off provide directions for future insightful investigations on the roles of FABP3 in the endothelium, with improvement on replicate numbers and optimization for the dosing regimen.

4.6 Conclusion

In conclusion, the endothelial genome-wide alterations in response to FABP3 exposure were delineated. Differentially expressed gene analyses highlighted several genes associated with endothelial cells' metabolic and immune-related stress response invoked by FABP3 treatment,

suggesting that FABP3 release during cardiovascular events impacts endothelial function. While the roles of less characterized genes remain to be elucidated, we provide a transcriptomic profile for future research into FABP3's impact on endothelial biology. Our analysis's trade-off between sensitivity and specificity underscores the need for protein validation. Given that endothelial cells form the first interaction with circulatory FABP3, future studies should expand on these findings, exploring the therapeutic and diagnostic applications of FABP3 within the vascular system.

Table 4.4 RNA quantity and purity were assessed with the NanoDrop ND-1000

Sample Name	Nucleic Acid(ng/uL)	A260/A280 Ratio
Vehicle -1	302.076	1.872
Vehicle-2	286.097	1.95
Vehicle-3	248.249	1.928
rhFABP3-1	278.835	1.985
rhFABP3-2	300.089	1.93
rhFABP3-3	207.784	1.922

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Chapter 5: Transcriptomics of Angiotensin II-induced Long Noncoding and Coding RNAs in Endothelial Cells

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See Appendix B for information.

5.1 Abstract

Angiotensin II (Ang II) is implicated in endothelial dysfunction and hypertension, critical factors in developing cardiovascular diseases and atherosclerosis. Long noncoding RNAs (lncRNAs) have emerged as crucial regulators in cardiovascular pathobiology; however, their regulatory mechanisms in response to the Ang II-rich environment in endothelial cells remain under-investigated. To assess the expression profiles of lncRNAs and coding RNAs in endothelial cells following Ang II treatment, we cultured and treated human umbilical vein endothelial cells (HUVECs) with Ang II (1 μM, 24 h), and their expression profiles of lncRNAs and mRNAs were analyzed using the Arraystar Human lncRNA Expression Microarray V3.0. We reveal significant changes in the expression of both lncRNAs and mRNAs in HUVECs following Ang II treatment. Out of 30,584 lncRNA targets screened, 25 were significantly upregulated and 69 downregulated. Of 26,106 mRNA targets screened, 28 were significantly upregulated and 67 downregulated. LncRNAs RP11-354P11.2 and RP11-360F5.1 were the most pronounced upregulated and downregulated, respectively. Likewise, RAB11FIP4 and DNAJA2 mRNAs were the most upregulated and downregulated, respectively. Functional analysis revealed the involvement of differentially regulated genes in nucleotide excision repair and ECM-receptor interaction pathways. We provide the first comprehensive profiling of the transcriptomic changes for both lncRNAs and mRNAs in Ang II-treated human endothelial cells, expanding current understanding of the molecular mechanisms and cardiovascular implications underlying Ang II-induced

endothelial dysfunction. Our findings highlight potential novel therapeutic targets for managing cardiovascular disorders associated with Ang II signalling.

5.2 Introduction

Endothelial cells play essential roles in maintaining vascular homeostasis by forming a macromolecular barrier between the blood and vessel wall and by regulating essential gaseous/nutrient exchange under physiological conditions. However, under pathological conditions, the endothelium undergoes structural and functional alterations, a condition referred to as 'endothelial dysfunction.' Endothelial dysfunction is often accompanied by platelet adherence, leukocyte migration, smooth muscle cell proliferation, apoptosis and impaired vascular tone, causing various cardiovascular diseases (CVDs), such as atherosclerosis, hypertension, myocardial infarction and heart failure [1,2]. The major risk factor related to impaired vascular tone is the imbalanced bioavailability of nitric oxide, an endogenous vasodilator. Nitric oxide biology has been extensively studied [3], where nitric oxide has been shown to have protective effects on the cardiovascular system. Furthermore, the loss or inhibition of nitric oxide has been associated with the development of CVD, such as atherosclerosis [4].

Angiotensin II (Ang II), an endogenous vasoconstrictor [4], was originally viewed as a hormone involved in the renin-angiotensin system (RAS) regulating blood pressure [5]; however, mounting evidence now suggests its roles beyond hemodynamic regulation. Ang II has been shown to have a role in the pathobiology of vascular diseases [5]. For example, elevated Ang II reduces nitric oxide bioavailability and induces oxidative stress, leading to impaired endothelium-mediated vasorelaxation in response to endothelial dysfunction [6]. Particularly, Ang II activates NADH/NADPH oxidase, which is the major source of superoxide in the vessel wall, leading to reduced nitric oxide production [7]. Moreover, Ang II triggers pro-inflammatory events by activating the NF-κB pathway, and subsequently, VCAM-1 and ICAM-1; Ang II also stimulates reactive oxygen species (ROS) production [5]. Ang II is involved in vascular remodelling *via* modulating the expression of matrix metalloproteinases, vascular endothelial growth factor receptors and vascular cell migration [8]. High levels of Ang II and Ang II converting enzyme (ACE) have been implicated in many vascular diseases [5]. Moreover, Dzau [9] reported that during the process of plaque build-up in atherosclerosis, there was an

upregulation of ACE, which leads to more Ang II production, creating a positive feedback loop or a vicious cycle exacerbating endothelial dysfunction. To put it all together, Ang II can decrease nitric oxide production, increase oxidative stress and amplify endothelial dysfunction, suggesting a close link between its expression and CVDs [5].

Recently, long noncoding RNAs (lncRNAs) have been shown to regulate endothelial functions [10]. LncRNAs are transcripts that are more than 200 nucleotides in length that actively modulate the expression of genes involved in the pathobiology of CVDs [11]. The most well-established mechanism is 'miRNA sponging,' wherein lncRNAs sequester miRNAs that target mRNAs to indirectly modulate the expression of these mRNAs [11], resulting in either activation or inhibition of downstream molecular pathways [12]. LncRNAs can also directly bind to their target mRNAs to regulate their translation. Both Ang II and lncRNAs are involved in regulating endothelial function and CVDs; however, the effect of Ang II on lncRNA expression in endothelial cells has never been investigated. Accordingly, for the very first time, we aim to profile the expression pattern of lncRNAs and mRNAs following Ang II treatment of endothelial cells to provide a better insight into the mechanisms behind Ang II-associated adverse effects on endothelial cells and their plausible roles in cardiovascular pathobiology.

5.3 Methods

Cell Culture: Human umbilical vein endothelial cells (HUVECs, pooled, Lonza; passage 4–7), which is a standard model to study endothelial cells *in vitro*[13,14], were cultured in endothelial cell growth medium-2 (EGM-2 Bulletkit; Lonza) supplemented with growth factors, serum and antibiotics at 37°C in humidified 5% CO2. Confluent HUVECs were maintained in six-well plates and starved overnight before treating with either a vehicle (PBS) or Ang II (10^{-6} mol/L, Sigma); this dose has previously induced consistent oxidative stress and inflammation in endothelial cells [15,16].

Microarray Profiling: Total RNA was isolated from HUVECs using TRIzol (Invitrogen) reagent and quantified with the NanoDrop ND-1000 spectrophotometer. RNA integrity was confirmed by standard denaturing agarose gel electrophoresis as described previously (**Figure 5.3 and 5.4**) [17]. The expression profile of 30,584 human lncRNAs and 26,106 protein-coding transcripts was conducted with the Arraystar Human LncRNA Microarray V3.0 apparatus (Arraystar Inc.,

Rockville, Maryland, USA). Sample labelling and array hybridization were performed on the Agilent Array platform. Briefly, the total RNA from each sample was amplified and transcribed into fluorescent cRNA (Arraystar Flash RNA Labeling Kit; Arraystar Inc.) before 1 μg of each labelled cRNA was hybridized onto the microarray slide. The hybridized arrays were washed, fixed and scanned with the Agilent DNA Microarray Scanner (Product# G2505C; Agilent Technologies, Santa Clara, California, USA). The acquired array images were analyzed with the Agilent Feature Extraction software (version 11.0.1.1). Quantile normalization and subsequent data processing were performed with the GeneSpring GX v11.5.1 software package (Agilent Technologies). For differentially expressed genes, the *P* values were calculated by *t*-test and adjusted for multiple testing using the Benjamini-Hochberg method to minimize the false discovery rate. Volcano plot filtering, set at a threshold of at least 2.0 folds, was used to screen for lncRNAs and mRNAs that exhibited significantly different ($P < 0.05$; unpaired *t*-test) expression levels in the two study groups. Pathway analysis was based on the current Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Gene Ontology (GO) analysis was performed with the topGO package of the Bioconductor system.

Validation qPCR: HUVECs were cultured and treated with either vehicle or Ang II for 24 h, and then total RNA was extracted using TRIzol. Complementary DNA was synthesized using Quantitect reverse transcription kit (Qiagen, Germantown, Maryland, USA), and qPCR was performed using forward and reverse primers (**Table 5.7**) and CFX Opus (Biorad, Hercules, California, USA) qPCR machine. Data was analyzed using the $2^{-\Delta\Delta Ct}$ method and Student's *t*-test. A value of *P* less than 0.05 was considered statistically significant.

5.4 Results

Quality assessment of lncRNA and mRNA data: RNA integrity was evaluated using denaturing agarose gel electrophoresis. The intensity of the upper 28S ribosomal RNA bands was about twice that of the lower 18S band, confirming the integrity of RNAs. The absence of smears above the 28S band attests to the purity of the RNA samples (**Figure 5.3**). RNA quantity and purity were also assessed with the NanoDrop ND-1000. The A260 /A280 and A260/A230 optical density (OD) ratios yielded values of about 2.0 and 1.8, respectively, which further confirmed the purity of our

RNAs. Our Box plots (10th and 90th percentile) showed comparable distributions of expression values after normalization (**Figure 5.4**).

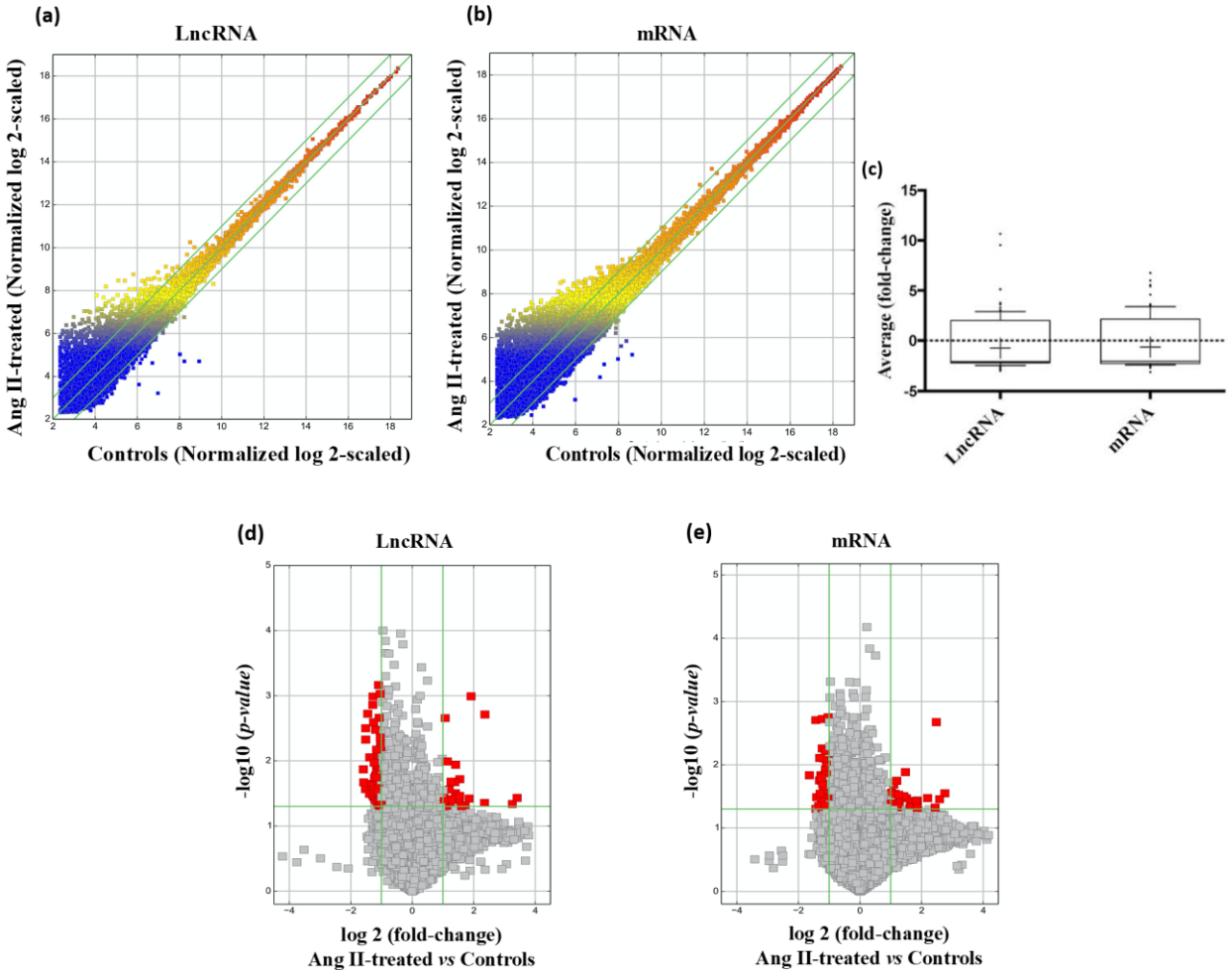

Figure 5.1 LncRNA and mRNA expression profiles in HUVECs treated with Ang II (10^{-6} mol/L) vs. vehicle-treated Control

(**a, b**) Scatter plots comparing the variation in lncRNA and mRNA expression. The values plotted are the averaged normalized signal values (log2 scaled) for the control (x-axis) and the Ang II-treatment (y-axis) groups. The green lines indicate fold change. LncRNAs and mRNAs above the top green line and below the bottom green line exhibit at least a 2.0-fold difference between the two study groups. (**c**) Box-and-Whisker plots (10th, 90th percentile) showing average fold-change of lncRNAs and mRNAs. Median intensity is denoted with a '-' and mean intensity is denoted with a '+' sign. (**d, e**) Volcano plots detailing the magnitude of expression difference. The vertical green

lines correspond to 2.0-fold upregulation and 2.0-fold downregulation of expression. The horizontal green line indicates an adjusted *P* value of ≤0.05. Red points represent lncRNAs and mRNAs with statistically significant differential expressions (fold-change ≥ 2.0, adjusted *P*≤0.05). 94 differentially expressed lncRNAs and 95 differentially expressed mRNAs in comparison to vehicle-treated control samples. N = 3 biological replicates per group.

Expression assessment of Ang II-induced lncRNAs and mRNAs in endothelial cells: Scatter plots provided a profile of HUVEC lncRNAs (**Figure 5.1a**) and mRNAs (**Figure 5.1b**) that were upregulated, downregulated or unaffected by Ang II treatment. Overall, the average fold-changes were similar across the group for the differentially expressed lncRNAs and mRNAs under the study conditions (**Figure 5.1c**). Subsequent volcano plot filtering uncovered 25 significantly upregulated and 69 significantly downregulated lncRNAs in HUVECs treated with Ang II in comparison to the vehicle-treated control samples (**Figure 5.1d**). LncRNAs that demonstrated the greatest differences in expression ranged from 11,100 to 200 bp. Tables 1 and 2 list the 10 most upregulated and downregulated lncRNAs depending upon the fold-change expression, respectively. In particular, RP11-354P11.2 (RNA length: 224 bp, chromosome 17) was the most upregulated lncRNA (∼11-fold), and RP11-360F5.1 (RNA length: 946 bp, chromosome 4) was the most downregulated (∼3-fold) lncRNA in HUVECs subjected to Ang II-treatment. We validated our findings by performing qPCR for selected upregulated (RP11-354P11.2, RP13-507I23.1, TTC28-AS1 and RP11-506G7.1) and downregulated (RP11-360F5.1, XLOC_008554, GLG1, AC073130.3 and SLC7A11-AS1) lncRNAs (Tables 1 and 2). Among Ang II-induced upregulated lncRNAs, RP13-507I23.1 (1.91 ± 0.20-fold, *P* = 3.62E-06), TTC28-AS1 (1.45 ± 0.38-fold, *P* = 0.016) and RP11-506G7.1 (2.30 ± 0.88-fold, *P* = 0.004) demonstrated similar expression pattern as observed in the array (Table 1). We were unable to obtain quantifiable data for RP11-354P11.2. Among Ang II-associated downregulated lncRNAs, GLG1 (0.78 ± 0.24-fold, *P* = 0.11), AC073130.3 (0.73 ± 0.25-fold, *P* = 0.044) and SLC7A11-AS1 (0.45 ± 0.32-fold, *P* = 3.22E-05) showed similar expression patterns as observed in the lncRNA array (Table 2). We were unable to obtain quantifiable data for RP11-360F5.1 and XLOC_008554. Ang II-associated changes at the transcript level in HUVECs were also noted amongst the 95 screened mRNAs, with 28 upregulated and 67 downregulated mRNAs (**Figure 5.1e**). Tables 3 and 4 list the 10 most upregulated and

downregulated mRNAs depending upon the fold-change expression, respectively. Upon Ang II treatment, intracellular transport protein RAB11 family-interacting protein 4 (*RAB11FIP4)* was the most upregulated transcript (∼7-fold), while the cell cycle progression restoration 3 (*DNAJA2)* was the most downregulated mRNA (∼3-fold). Later, validation qPCR was performed for upregulated genes Collagen Type XIII, Alpha-1 (*Col13A1)*, Potassium Channel, Voltage-gated, Subfamily G, Member 3 (*KCNG3)* and Neutralized E3 Ubiquitin Protein Ligase 4 (*NEURL4)*; and downregulated gene *DNAJA2* (Tables 3 and 4). The validation qPCR demonstrated significant upregulation for *Col13A1* (2.00 ± 0.67-fold, $P = 0.01$), *KCNG3* (3.70 ± 1.44-fold, $P = 0.0005$) and *NEURL4* (2.41 ± 1.16-fold, $P = 0.01$), and downregulation for *DNAJA2* (0.63 ± 0.22-fold, $P = 0.006$) as observed in the array (Tables 3 and 4).

LncRNA chromosomal distribution and subtype analysis: Figure 5.5 shows the dendrograms (heatmap) generated for the hierarchical analysis of clustered lncRNAs that were differentially expressed in HUVECs treated with Ang II in comparison to vehicle-treated controls. Although the lncRNAs modulated by Ang II treatment were abundant and found on every human chromosome, chromosomes 1, 2, 3, 7, 8, 12, 16 and 17 were noted to have the highest number of lncRNAs (**Figure 5.2a**). Further probing revealed that although these differentially expressed lncRNAs are expressed along the entire length of the chromosomes, there is a notable clustering of lncRNAs, particularly on chromosome 2, 15 and 22 (**Figure 5.2b**). A lncRNA subgroup analysis, which helps identify the functional relationship between lncRNAs and their associated protein-coding genes, demonstrated that the majority (∼50%) of lncRNAs were intergenic in origin, followed by natural and intronic antisense lncRNAs (**Figure 5.2c**)**.** We also identified bidirectional, exon sense-overlapping and intron sense-overlapping lncRNAs (**Figure 5.2c**).

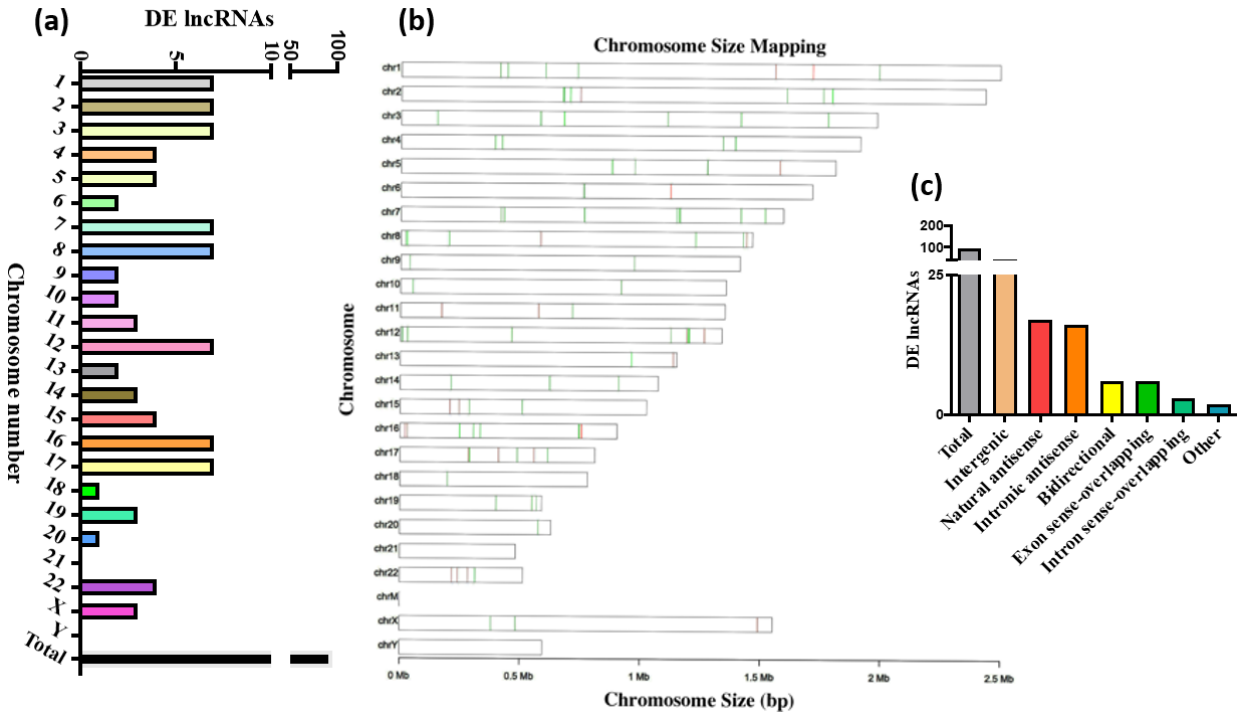

Figure 5.2 Distribution, location and classification of differentially expressed lncRNAs in HUVECs treated with Ang II (10^{-6} mol/L) vs. vehicle-treated control

Demonstration of (**a)** numbers and (**b)** chromosomal location of differentially expressed (DE) lncRNAs on different chromosomes. (**c)** Bar-graph representing types of differently expressed lncRNAs, depending upon their genomic location. N = 3 biological replicates per group

Bioinformatics analyses: Pathway analysis with the current KEGG database yielded interesting findings, wherein we observed significant downregulation for pathways involved in nucleotide excision repair and ECM-receptor interaction (**Table 5.5**). The results of the GO analysis grouped the differentially expressed mRNAs under the following three categories: Biological Processes, Cellular Components and Molecular Function. GO terms most broadly associated with upregulated mRNAs were regulation of single organism process, localization and transport. GO terms associated with downregulated mRNA were mainly enriched in metabolic process, cell periphery and ion binding (**Table 5.6**).

Table 5.1 Ten most upregulated lncRNAs in HUVECs upon Ang II (10^{-6} mol/L) stimulation in comparison to vehicle-treated controls

Top 10 upregulated DE lncRNAs in Ang II treated vs. control endothelial cells						
No.	**Gene symbol**	**Fold change**	***P***	**Associated gene**	**RNA length**	**Chr/ Strand**
1	RP11-354P11.2	10.6534177	0.036898809	*BLMH*	224	17/+
2	RP13-507I23.1	9.5334709	0.045867677	*CXorf40B*	547	X/+
3	TTC28-AS1	5.1482452	0.001933328	*PITPNB*	565	22/+
4	RP11-706C16.8	5.1002669	0.043965255	*LY6D*	602	8/+
5	RP11-506G7.1	3.7693643	0.001020892		743	17/+
6	LINC00281	3.6061733	0.037690972		3215	22/-
7	AK025511	3.2931008	0.04829421	*ATP11A*	5344	13/+
8	XLOC_009078	3.0441687	0.049303053		327	11/+
9	LOC400084	2.9207082	0.019073615		2822	12/-
10	AC005606.14	2.9034744	0.034347729	*SYNGR3*	545	16/-

ATP11A, ATPase Class VI Type 11a; BLMH, bleomycin hydrolase; LY6D, lymphocyte antigen 6 complex locus D; PITPNB, phosphatidylinositol transfer protein beta; SYNGR3, Synaptogyrin 3.

Table 5.2 Ten most downregulated lncRNAs in HUVECs upon Ang II (10^{-6} mol/L) stimulation in comparison to vehicle-treated controls

Top 10 downregulated DE lncRNAs in Ang II-treated vs. control endothelial cells						
No.	**Gene symbol**	**Fold change**	***P***	**Associated gene**	**RNA length**	**Chr/ Strand**
1	RP11-360F5.1	3.0136135	0.013442124	*KLHL5*	946	4/-
2	XLOC_008554	2.9839886	0.021356487		613	10/+
3	RP11-415D17.3	2.8673817	0.027062378	*FILIP1*	506	6/+
4	GLG1	2.860294	0.00469547	*GLG1*	9448	16/-
5	AC073130.3	2.8562164	0.003143427	*TES*	842	7/-
6	SLC7A11-AS1	2.7342087	0.001890476		817	4/+
7	AC068610.3	2.6243215	0.031952981		784	7/+
8	RP11-455B3.1	2.615349	0.008796369		264	5/+
9	RP11-342C23.4	2.4772416	0.02233984	*FBP2*	1080	9/+
10	RP11-439C15.4	2.4696443	0.02712366	*KBTBD11*	557	8/-

FBP2, Fructose-1,6-bisphosphatase 2; FILIP1, Filamin A-interacting Protein 1; GLG1, Golgi Apparatus Protein 1; KBTBD11, Kelch repeat- and btb/poz Domain-containing Protein 11; KLHL5, kelch-like 5; TES, Testis-derived Transcript.

Table 5.3 Ten most upregulated mRNAs in HUVECs upon Ang II (10^{-6} mol/L) stimulation in comparison to vehicle-treated controls

Top 10 upregulated DE mRNAs in Ang II-treated vs. control endothelial cells			
No.	**Gene symbol**	**Fold change**	***P***
1	*RAB11FIP4*	6.7547128	0.028130498
2	*COL13A1*	6.0001995	0.034844245
3	*KCNG3*	5.5657981	0.002100061
4	*PRKAR1A*	5.4190279	0.047401117
5	*NEURL4*	4.5848951	0.033462694
6	*MAPT*	3.6389586	0.036545522
7	*C22orf39*	3.6371241	0.047490119
8	*FAM123A*	3.5522304	0.046210964
9	*TNS1*	3.5285042	0.037949728
10	*FAM178B*	3.2582795	0.037095586

COL13A1, Collagen, Type XIII, Alpha-1; FAM123A, Family with Sequence Similarity 123, Member a; KCNG3, Potassium Channel, Voltage-gated, Subfamily g, Member 3; MAPT, Microtubule-associated Protein Tau; NEURL4, Neutralized e3 Ubiquitin Protein Ligase 4; PRKAR1A, Protein Kinase, Camp-dependent, Regulatory, Type I, Alpha; RAB11FIP4, Rab11 Family-interacting Protein 4; TNS1, Tensin 1.

Table 5.4 Ten most downregulated mRNAs in HUVECs upon Ang II (10^{-6} mol/L) stimulation in comparison to vehicle-treated controls

Top 10 downregulated DE mRNAs in Ang II-treated vs. control endothelial cells			
No.	**Gene symbol**	**Fold change**	***P***
1	*DNAJA2*	3.1199056	0.01457991
2	*KIDINS220*	2.7126928	0.001954717
3	*MARCH7*	2.6986797	0.049530097
4	*EIF2A*	2.6547819	0.032343461
5	*CNOT4*	2.5378737	0.044541049
6	*HNRNPA1L2*	2.4920336	0.017606208
7	*FAM221A*	2.480581	0.007758564
8	*NOSTRIN*	2.4622312	0.028365697
9	*B4GALT6*	2.4142459	0.014564299
10	*CLEC2A*	2.393869	0.029924369

B4GALT6, Beta-GlcNac beta-1,4-galactosyltransferase, Polypeptide 6; CLEC2A, C-type Lectin Domain Family 2, Member A; CNOT4, ccr4-not Transcription Complex, Subunit 4; DNAJA2, DNAj/hsp40 Homolog, Subfamily a, Member 2; EIF2A, Eukaryotic Translation Initiation Factor 2a; KIDINS220, Kinase d-interacting substrate, 220-kd; MARCH7, Membrane-associated Ring-ch Finger Protein 7; NOSTRIN, Nitric Oxide Synthase Trafficker.

Table 5.5 Results of bioinformatics analyses on down-regulated pathways in HUVECs after Ang II (10^{-6} mol/L) stimulation in comparison to vehicle-treated controls

Downregulated pathways in Ang II-treated vs. control endothelial cells				
Nr	**Pathways**	**Count**	***P***	**Genes**
1	Nucleotide excision repair	5	0.01596065	*ERCC6//POLE*
2	TGF-beta signaling pathway	4	0.04291208	*LTBP1//TGFBR1*
3	ECM-receptor interaction	3	0.04888822	*COL5A2//ITGA1*

Table 5.6 Results of bioinformatics GO (gene ontology) enrichment analyses to determine the roles of differentially expressed mRNAs in GO term

Upregulated				**Downregulated**			
Go Term	**Count**	***% Genes***	***P***	**Go term**	**Count**	***% Genes***	***P***
Biological process							
Single-organism cellular process	21	23	0.02430	Metabolic process	47	56	0.01103
Localization	13		0.01052	Organic substance metabolic process	44		0.01498
Transport	12		0.00496	Primary metabolic process	43		0.01587
Establishment of localization	12		0.00601	Cellular metabolic process	42		0.01965
Single-organism transport	10		0.01343	Macromolecule metabolic process	38		0.00778
Cellular component							
Endoplasmic reticulum	5	23	0.03790	Cell periphery	21	52	0.02490
Ribonucleoprotein granule	2		0.01118	Plasma membrane	20		0.03871
Cytoplasmic ribonucleoprotein granule	2		0.01118	Transferase complex	5		0.02612
Endoplasmic reticulum lumen	2		0.02414	Endosome	5		0.04589
Chromatoid body	1		0.01586	Late endosome	3		0.01792
Molecular function							
Ion transmembrane transporter activity	4	22	0.02158	Ion binding	29	58	0.03808
Substrate-specific transmembrane transporter activity	4		0.02716	Small molecule binding	16		0.01985
Enzyme regulator activity	4		0.02976	Transferase activity	15		0.00566
Transmembrane transporter activity	4		0.03650	Nucleotide binding	14		0.03571
Substrate-specific transporter activity	4		0.04337	Nucleoside phosphate binding	14		0.03582

5.5 Discussion

This is the first report profiling Ang II-induced differential expression of mRNAs and lncRNAs in cultured endothelial cells. Differentially expressed lncRNAs and mRNAs were regulated with similar average fold-changes, and our volcano plots uncovered 94 differentially expressed lncRNAs and 95 differentially expressed mRNAs in comparison to vehicle-treated control samples (**Figure 5.1**). The differentially expressed lncRNAs were distributed on all chromosomes, mainly on chromosomes 1, 2, 3, 7, 8, 12, 16, and 17 (**Figure 5.2**). Similar distributions of lncRNA have been previously reported in endothelial cells [17]. The majority of differentially expressed lncRNAs were intergenic, which is also in line with previous reports [17].

In the array data, we were able to identify several novel differentially expressed lncRNAs and mRNAs, which have not yet been characterized. However, on the basis of other relevant studies, we discuss their plausible roles in Ang II-mediated effects in cardiovascular disease. The most significantly upregulated lncRNA is RP11-354P11.2 (11-fold), which is not characterized but is located near the coding region of miR-423, whose expression was found to be strongly correlated with the clinical diagnosis of heart failure (**Table 5.1**) [18]. In addition, Rizzacasa *et al.*[19] found that miR-423 was differentially expressed in patients with acute myocardial infarction compared with healthy controls. There are no reports on the effect of Ang II on miR-423; however, it is possible that Ang II-induced adverse effects are mediated *via* an interaction between RP11-354P11.2 and miR-423. The second most upregulated lncRNA is RP13-507I23.1, which is associated with the gene CXorf40B and, thereby, the complex locus MT1A and MT2A (**Table 5.1**) [20]. MT1A and MT2A encode for proteins metallothionein 1A and metallothionein 2A and have anti-inflammatory, antioxidative and antiapoptotic roles [21]. This correlates well, as Ang II induces oxidative stress, which may modulate MT2A via its interaction with RP13-507I23.1. The third most upregulated lncRNA is TTC28-AS1, which is associated with the gene encoding phosphatidylinositol transfer protein (PITPNB). PITPNB binds to and transfers phosphatidylinositol from the endoplasmic reticulum to other membrane compartments [22]. Phosphatidylinositol is an important precursor of many signalling molecules that regulate cell proliferation, migration and differentiation. Phosphatidylinositol also plays an important role in cellular mechano-transduction, which tightly regulates the function of cardiovascular cells and has been implicated in the pathogenesis of CVDs [23]. Among other upregulated lncRNAs, Lei *et*

al.[24] have previously found that RP11-706C16.8 is involved in the AKT signalling pathway that promotes tumour formation in pancreatic cancer. AK025511 is associated with the gene *ATP11A*, which encodes for ABCA1, an ATP-binding cassette transporter that transports ions across the cell membrane. ATP11A was also found to be a susceptibility locus for the development of pulmonary fibrosis [25]. There is no literature linking Ang II and AK025511, but Budinger *et al.*[26] suggested that increasing Ang II metabolism or the activity of angiotensin-converting enzymes may protect patients from pulmonary fibrosis. Although AC005606.14 has not been characterized, it may be involved in dopamine reuptake and the regulation of exocytosis from neurotransmitters (**Table 5.1**) [27].

The most significantly downregulated (3-fold) lncRNA is RP11-360F5.1 and is located near the protein-coding gene Kelch Like Family Member 5 (*KLHL* 5), which is involved in class I MHC-mediated antigen processing and presentation [28], suggesting a connection of this lncRNA to the innate immune response following Ang II treatment (**Table 5.2**). RP11-415D17.3 associates with the gene *FILIP1* (FilaminA interacting protein 1), which interacts with filamin A, a protein that facilitates branching of actin filaments and anchors transmembrane proteins to the actin cytoskeleton [29]. FILIP1 possibly promotes the migration of neuroblasts and is implicated in glioblastoma. GLG1 is associated with the gene GLG1 (Golgi Glycoprotein 1), which binds to fibroblast growth factor and E-selectin [30]. E-selectin is uniquely expressed in endothelial cells following an inflammatory response and is responsible for controlling leucocyte accumulation [31]. Skaletz-Rorowski *et al.*[32] found that the Angiotensin AT1 receptor can upregulate the expression of fibroblast growth factor in human coronary smooth muscle cells, which might explain the upregulation of GLG1 in HUVECs following Ang II treatment. AC073130.3 associates with the gene *TES* (Testin LIM Domain Protein), which might be involved in regulating cell proliferation and cancer. Tatarelli *et al.*[33] found that TESTIN mRNA expression is absent in 22% of cancer cell lines compared to universal expression in normal human cell lines. Luo *et al.*[34] found that the expression of SLC7A11-AS1 is significantly downregulated in patients with gastric cancer, which could promote tumour formation; this effect is mediated *via* the ASK1-p38MAPK/JNK pathway, which has been suggested to promote cardiac fibrosis *in vivo*[35]. RP11-342C23.4 associates with the gene *FBP1* (fructose biphosphatase-2), which is responsible for catalyzing the hydrolysis of fructose 1-6 biphosphate to fructose 6

phosphate [36]. Uncontrolled fructose metabolism could result in the production of uric acid, a major risk factor for hypertension [36] (**Table 5.2)**.

Transcriptome analysis following Ang II treatment revealed differential expression of mRNAs. The most upregulated mRNA is *RAB11FIP4*, which regulates the formation, targeting and fusion of intracellular transport vesicles. RAB11FIP4 directly interacts with RAB11A, which belongs to the small GTPase family and is responsible for intracellular membrane trafficking [37] (**Table 5.3)**. Vascular endothelial-cadherin is important for maintaining vascular integrity and is an essential component of the vascular barrier and recycling. RAB11A is also involved in vascular endothelial-cadherin recycling in endothelial cells [38]. Yan *et al.* [38] found that silencing *RAB11A* prevented the recycling process of vascular endothelial-cadherin and the presentation of vascular endothelial-cadherin on the plasma membrane. Moreover, inactivating RAB11A prevented reannealing of junctions following vascular inflammation, and blocking the expression of RAB11A in pulmonary microvessels in mice resulted in vascular leakage [39]. Given that Ang II also induces pulmonary microvascular endothelial barrier injury [39], the RAB11A upregulation appears to promote a compensatory mechanism to protect against Ang II-induced endothelial injury. *COL13A1* was also upregulated in Ang II-treated endothelial cells, which is involved in organ fibrosis. Ang II also promotes fibrosis, and it is possible that Ang II promotes fibrosis via COL13A1 upregulation (**Table 5.3)**. COL13A1 could also bind to heparin, an anticoagulant, which is believed to offset the vasoconstrictive effect of Ang II [40]. KCNG3 encodes for voltage-gated potassium channels that are responsible for regulating neurotransmitter release, heart rate, insulin release and smooth muscle contraction [41]. Although there is no existing literature linking Ang II and KCNG3, they share a common function, which is regulating vascular smooth muscle contraction [41,42]. Both elevation of Ang II and closing of potassium channels lead to vasoconstriction. PRKAR1A is involved in the regulation of lipid metabolism. In addition, PRKAR1A is highly expressed in the heart; Liu *et al.*[43] found that PRKAR1A deficiency in young patients with Carney complex disorder had reduced left ventricular mass and suppression of cardiomyocyte hypertrophy. It is possible that elevated levels of PRKAR1A could exacerbate the development of cardiac hypertrophy on top of the effect of elevated Ang II [44]. NEURL4 (Neutralized E3 Ubiquitin Protein Ligase 4) acts as a substrate for HERC2, and together, they modulate the centrosome architecture *via* a ubiquitin-dependent pathway [45]. Hashimoto-Komatsu *et al.*[46] found that Ang II can induce microtubule reorganization in endothelial cells,

and as the centrosome is an integral part of the reorganization process, it would be interesting to investigate the link between Ang II and NEURL4. Similarly, MAPT was also shown to be upregulated, and it encodes for protein TAU, which is a microtubule-stabilizing protein important in cytoskeletal remodelling [47]. There is no literature on C22orf39, but what is known is that it interacts with CEP76, which is another centrosomal protein correlating with the other upregulated mRNAs (**Table 5.3)**. FAM123A is a novel negative regulator of the Wnt/β signalling pathway during neuroectodermal patterning, a critical process of embryonic development [48]. It is interesting to note that Ang II and the Wnt/β signalling pathway have an antagonistic pleiotropy relationship in the aging process. The blocking of the renin-angiotensin pathway, which slows the process of ageing, is accompanied by the downregulation of Wnt/β signalling, which is critical for survival [49]. Tensin 1 (TNS1) is an actin-binding protein important in focal adhesion; the abnormal cleavage of TNS1 results in abnormal cell morphology [50]. Bernau *et al.*[51] found that TNS1 expression is induced in pulmonary fibrosis and might serve as a therapeutic target for tissue fibrosis. As elevated Ang II also serves as a risk factor for pulmonary fibrosis, it is possible that upregulated TNS1 following Ang II treatment exacerbates the development of pulmonary fibrosis. Although there is no existing literature for FAM178B, its paralog SLF2 plays an important role in repairing DNA damage [52] (**Table 5.3)**. Elevation of Ang II leads to increased oxidative stress and, subsequently, DNA damage [53], and FAM178B might play a protective role.

The most downregulated mRNA is cell cycle progression restoration 3 (*DNAJA3*), which is responsible for regulating molecular chaperone activity [54] (**Table 5.4)**. This is in line with a previous finding, wherein postprandial sera treatment to cultured endothelial cells caused reduced endothelial function and DNAJA3 expression [55]. It is likely that Ang II-associated reduced DNAJA3 expression participates in Ang II-induced endothelial dysfunction. KIDINS220 (Kinase D-interacting Substrate of 220 kDa) participates in the MAP-kinase signalling *via* activating Rap-1 and is preferentially expressed in the nervous system [56]. Scholz-Starke and Cesca [57] summarized the function of KIDINS220 in neuronal survival, outgrowth of neuronal process and neuronal activity. KIDINS220 also plays an important role in the cardiovascular system, as mice with KIDINS220 knockout exhibited cardiovascular abnormalities. KIDINS220 also targets and interacts with vascular endothelial growth factor receptors, which acts upstream to eNOS and regulates nitric oxide release [58]. It would be interesting to investigate whether KIDINS220 is involved in mediating the inhibitory effect of Ang II on nitric oxide release.

Furthermore, KIDINS220 has been implicated in immunomodulation, as indicated by co-immunoprecipitation of KIDINS220 and intercellular adhesion molecule-3 in primary T-lymphocytes [59]. MARCH7 is an E3 ubiquitin ligase, and it has been shown to interact directly with another E3 ubiquitin ligase, Mdm2, to maintain its stability. Mdm2 specifically targets p53 for degradation [60], which regulates the cell cycle and apoptosis [60]. Downregulation of MARCH7 following Ang II treatment might result in destabilization of Mdm2 and, subsequently, an imbalanced p53 in cells (**Table 5.4)**. EIF2A participates in the initial translation process of some mRNAs by binding the initiator tRNA to the 40S ribosomal subunit [61]. CNOT4 (CCR4-NOT Transcription Complex Subunit 4) has E3 ubiquitin ligase activity and represses RNA polymerase II transcription [62]. In addition, CNOT4 also enhances the JAK/STAT pathway, which is important for innate immunity and response to stress signals in human Hela cells [63], which might be involved in the immune responses following Ang II treatment. NRNPA1L2 is involved in the processing of mRNA and RNA splicing [64]. Treatment of Ang II might lead to increased modification of some mRNAs, as Kobori *et al*. [65] found that increased circulating Ang II led to increased production of angiotensinogen mRNA. NOSTRIN protein is found to be expressed in vascular endothelial cells and directly interacts with eNOS to inhibit nitric oxide release [66]. Nitric oxide is a critical mediator of many biological processes, including neurotransmission, vascular homeostasis and inflammation [66]. An imbalance of nitric oxide levels in endothelial cells could lead to endothelial dysfunction, which is implicated in many CVDs [4]. As was mentioned previously, elevated Ang II reduces nitric oxide bioavailability, and NOSTRIN might be one of the mediators of this effect. CLEC2A is expressed in various immune cells and can increase the survival of T cells by increasing their stability [67]; CLEC2A may also participate in the immune responses elicited by Ang II treatment.

Pathway analysis with the current KEGG database reveals two main downregulated pathways in ECs following Ang II stimulation: the nucleotide excision repair (NER) pathway and the ECM-receptor interaction pathway (**Table 5.5 and 5.6)**. NER is one of the major DNA repair pathways that can remove a broad range of helix-distorting DNA lesions from the genome [68]; defects in NER are linked to many CVDs [69]. Durik *et al.*[70] found that mice with defective NER genes showed increased vascular dysfunction, vascular cell senescence, increased blood pressure and vasodilator dysfunction at a young age compared with wild-type controls. As previously mentioned, Ang II can induce oxidative stress in VSMCs and increase ROS, which is implicated

in stress-induced premature senescence (SIPS) [71] and replicative senescence via telomere attrition [72]. Telomeres are long stretches of DNA that get shortened with age. DNA is extremely vulnerable to ROS-mediated damage, and unfixed DNA damage can result in SIPS [72]. What is important to note is that senescent cell phenotypes and telomere shortening are involved in the pathobiology of many vascular diseases, such as atherosclerosis [72]. It is also known that Ang II treatment in endothelial cells causes inflammation and increased ROS, which leads to increased DNA damage and, subsequently, SIPS and age-related senescence. This is the first report linking Ang II with the NER pathway in endothelial cells, and it appears that Ang II-mediated downregulation of the NER pathway is associated with increased accumulation of DNA damage, accelerated endothelial senescence, dysfunction and apoptosis. Extracellular matrix (ECM) is a critical component and support for vascular endothelium [73]. By interacting with integrins on endothelial cells, ECM forms a scaffold necessary to maintain vascular organization. More importantly, the adhesion of endothelial cells to the ECM is crucial for maintaining endothelial function, including proliferation, migration and angiogenesis [73]. There is no previous report about the effect of Ang II on pathways for ECM interaction; however, reduced ECM interaction may disrupt the vascular networks and eventually lead to endothelial dysfunction and vascular diseases. Here, we report the novel interactions between Ang II and NER and ECM pathways with their plausible role in the endothelium, but further studies are needed to exactly delineate the role of these pathways in Ang II-associated endothelial phenotype.

The present study is the first to demonstrate the expression profile of lncRNAs and mRNAs in human endothelial cells following treatment with Ang II. We identified several lncRNAs that have previously not been characterized and associated with the endothelium and, therefore, present as novel targets. This study also provided information about chromosomal locations and sequences of lncRNAs, which is not only an indicator of the reproducibility and credibility of this study but, most importantly, it also provides geneticists with the necessary information to identify causative genes, lncRNAs or SNPs in the chromosomal loci that have already been associated with hypertension or CVDs in genome-wide association studies [74–76]. This is particularly evident in the genome-wide association studies that independently identified a susceptibility locus of coronary artery disease on human chromosome 9p21, which was further associated with an SNP in lncRNA ANRIL [76–78]. Our bioinformatic analyses shed light on some of the pathways that might govern endothelial function following Ang II treatment, particularly the NER pathway,

which appears promising and has not been investigated in association with Ang II in ECs or any other cell types. There are also some limitations to the present work. HUVECs are an established representative cell type for endothelial research *in vitro*[13,14]; however, Ang II is known to induce hypertension *via* RAS that has systemic and differential effects on micro-vascular and macro-vascular function [79,80]. Therefore, it is important to validate our findings in endothelial cells of other micro-vascular and macro-vascular origin. Lastly, a major challenge is that most of the identified differentially expressed lncRNAs are not characterized; therefore, it is difficult to associate the relevance of these changes with Ang II and associated endothelial phenotype. Thus, further characterization of these novel lncRNAs is recommended.

5.6 Conclusion

Overall, this is the first study to profile the Ang II-induced differentially expressed lncRNAs and mRNAs in human endothelial cells. Our results reveal novel targets and substantially extend the list of potential candidate genes involved in Ang II-induced endothelial dysfunction and cardiovascular diseases.

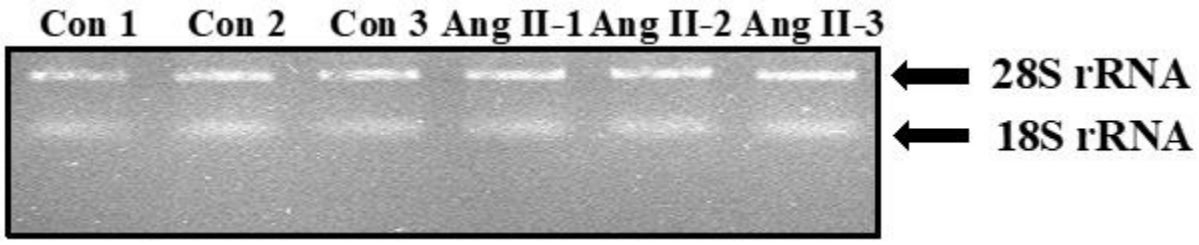

Figure 5.3 Quality Assessment of RNA Samples

Denaturing agarose gel (0.5%) electrophoresis was used to assess RNA integrity and genomic DNA contamination. The gel image shows clear and intact 28S and 18S rRNA bands. Con 1, 2 and 3 correspond to vehicle-treated control samples, and Ang II-1, 2 and 3 correspond to the Ang II-treated (10^{-6} μM) samples.

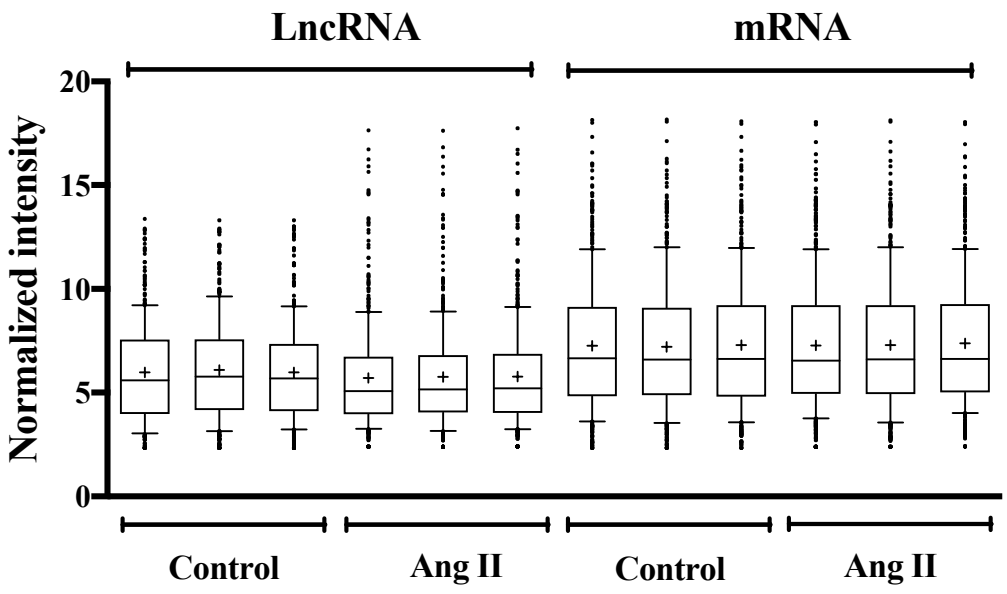

Figure 5.4 Summary of Differential Expression in lncRNAs and mRNAs Data

Box-and-Whisker plots (10^{th}, 90^{th} percentile) showing the normalized intensity of gene expression levels for the 6 study samples to quickly visualize the distribution of the dataset. Mean intensity is denoted with a "+" sign. Control represents the vehicle-treated, and Ang II represents the Ang II-treated (10^{-6} μM) group.

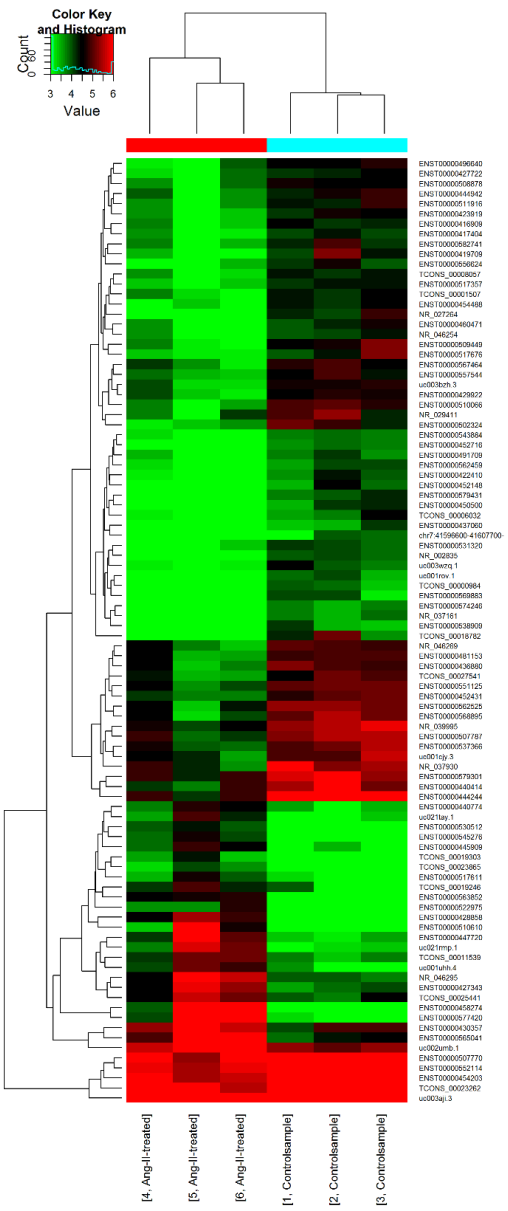

Figure 5.5 Heat map and hierarchical clustering of differences in lncRNA expression from HUVECs treated with Ang II (10^{-6} µM) vs. control

The dendrogram shows the relationships among the expression levels of samples. Hierarchical clustering that was performed based on 'differentially expressed lncRNAs' shows a distinguishable lncRNA expression profiling among samples. N = 3 biological replicates per group.

Table 5.7 Details of primers used in validation qPCR

Nrs.	Primer	Sequences
1	Hs-KCNG3-F2	5'-GAGTCACCTTGAGGGTACTTAGA-3'
2	Hs-KCNG3-R2	5'-CATAACCATCTCTCGGTAGCAAC-3'
3	Hs- COL13A1-F	5'-GGAGACGGCTATTTTGGGACG-3'
4	Hs- COL13A1-R	5'-TCCTTGAGTGGAGCTTCCATT-3'
5	Hs-NEURL4-F1	5'-GCATCGACCGCAAGGTCAA-3'
6	Hs-NEURL4-R1	5'-CAGAGCGTCCATCTCTCAGC-3'
7	Hs-DNAJA2-F2	5'-TACGGAGAGCAAGGTCTTCG-3'
8	Hs-DNAJA2-R2	5'-CATGCACTACAGAGCACATTCTT-3'
9	Hs-GLG1-F2	5'-TGATGAGGAATTTGGTTCTTGA-3'
10	Hs-GLG1-R2	5'-GGTTGCAGGGAGCTGAGAT-3'
11	Hs-AC073130.3-F1	5'-GATTCCTAACAACTTGGGGGTA-3'
12	Hs-AC073130.3-R1	5'-CAGCATATGACTTTGAGGAAACA-3'
13	Hs-SLC7A11-AS1-F	5'-TGTGCTATATTTCATGCATGCTC-3'
14	Hs-SLC7A11-AS1-R	5'-GAGCAAATAACAGATGGGACTGT-3'
15	Hs-RP13-507I23.1-F1	5'-CCAAAGGTCTGGCAACTAGC-3'
16	Hs-RP13-507I23.1-R1	5'-TCCCATCTTCGGGAGTAGGT-3'
17	Hs-TTC28-AS1-F1	5'-CCTCCCTTCAGCTTTGGTC-3'
18	Hs-TTC28-AS1-R1	5'-ACTGGAAAACTCACCGGAAA-3'
19	Hs-RP11-506G7.1-F1	5'-AGCTGGGGGAGGTACCAG-3'
20	Hs-RP11-506G7.1-R1	5'-TTGTAGATGCTGGTGCTACTAGGT-3'

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Chapter 6: Discussion/Final Remarks

6.1 Endothelial FABP3 is differentially expressed by atherosclerotic stressors

Chapter 2 provides an overview assessment of the FABP3 regulation in endothelial cells under atherosclerotic stress conditions, contributing insights and clinical relevance into the role of FABP3 in endothelial dysfunction and its implications in atherosclerosis. **Chapter 2-Figure 1** illustrates the diverse regulation of FABP3 expression in endothelial cells under various atherosclerotic stresses, suggesting a nuanced regulatory interplay of FABP3 expression in endothelial dysfunction. Oxidized low-density lipoprotein (oxLDL) and Angiotensin II (Ang II) treatments lead to the downregulation of FABP3 in endothelial cells. Specifically, FABP3 expression in HUVECs treated with oxLDL for 24 hours appears to decrease dose-dependently, akin to the downregulation observed in FABP4 and FABP5, which are known to be expressed by endothelial cells. Notably, at a concentration of 80 μg/mL of oxLDL, FABP3 exhibits a transient increase after 6 hours of oxLDL treatment, followed by a subsequent decrease at 12 and 24 hours, indicating a fluctuating temporal sensitivity pattern. Exposure to Ang II results in a time-dependent downregulation of FABP3 transcript levels in HUVECs at a dose of 1 μM. This intricate regulatory response underscores the multifaceted role of FABP3 in endothelial function under conditions of atherosclerotic stress, hinting at its potential as a therapeutic target for cardiovascular diseases. Interestingly, immunofluorescence staining conducted in HUVECs not only confirms the basal expression of FABP3 in endothelial cells but also visually suggests an increase in FABP3 expression upon LPS treatment (**Chapter 2-Figure 3**). These findings reinforce the dynamic regulatory role of FABP3 in endothelial cells under stress conditions and underscore its potential significance in the pathophysiology of cardiovascular diseases.

The rationale for Chapter 2 was grounded in the need to elucidate the role of Fatty Acid-Binding Protein 3 (FABP3) in endothelial cells, particularly in the context of atherosclerotic stressors that contribute to endothelial dysfunction. Traditionally confined to cardiomyocytes, FABP3's detection in human umbilical endothelial cells under *in vitro* conditions prompts a re-evaluation of its role beyond the myocardium. Moreover, we unveiled a nuanced regulatory pattern of FABP3 in response to atherosclerosis-associated stressors. Oxidized low-density lipoprotein and angiotensin II were found to downregulate FABP3 in a complex, dynamic regulatory mechanism.

Conversely, gram-negative endotoxin lipopolysaccharide-induced FABP3 upregulation suggests FABP3's role in inflammatory endothelial dysfunction (**Chapter 2-Figure 3**). Overall, this chapter elucidates the regulatory modulation of endothelial FABP3 under atherosclerotic stresses, reinforcing the hypothesis that FABP3 is regulated in endothelial dysfunction and the pathogenesis of atherosclerosis. This implication paves the way for targeted therapies aimed at mitigating endothelial dysfunction to manage cardiovascular complications.

The study's strength lies in its novel identification of FABP3 expression in endothelial cells and the comprehensive analysis of its regulatory dynamics under various stressors. The integration of different stress models (oxidative stress, hypertension, and inflammation) offers a multifaceted perspective on endothelial FABP3 regulation in the setting of atherosclerosis, although the experimental environment was strictly *in vitro*. The dose-response relationship of FABP3 to Ang II would benefit from further dose-optimization in addition to using a previously established dosage. Moreover, there's a lack of affirming that endothelial cells were under stress by the treatments; future work could evaluate ROS levels and representative molecular expression of endothelial cells under inflammatory (e.g., VCAM1) [1] and Ang II-rich environments (e.g., eNOS) [2]. These limitations also suggest that while the data are highly suggestive, the quantitative aspects of the findings should be interpreted with caution until future validation with greater statistical power. Nonetheless, the significance of this chapter lies in its revelation of the dynamic regulation of FABP3 in response to multiple atherosclerotic settings. Future studies should aim to consolidate these findings, examine the mechanistic pathways of FABP3 regulation, and extend the investigation to *in vivo* models to fully comprehend the therapeutic potential of targeting FABP3 in cardiovascular diseases.

In conclusion, Chapter 2 provides evidence that FABP3 expression in endothelial cells is subject to complex regulation by various atherosclerotic stressors, including oxidative stress, hypertensive milieu, and inflammatory stimuli. The differential expression patterns in response to these stressors suggest novel comprehensions for the capacities of FABP3 as a biomarker and therapeutic target in endothelial dysfunction and atherosclerotic complications.

6.2 FABP3 regulates endothelial cell response to inflammation

Previously, Chapter 2 established the foundation of the regulatory modulation of FABP3 expression in response to oxidative stress, inflammation, and hypertension, which are the features of atherosclerosis. Endothelial FABP3 was basally expressed and intricately responsive to external inflammatory stimuli, such as LPS. Chapter 3 extends the investigations on this aspect, evaluating endothelial function under the loss of endothelial FABP3 in LPS-treated endothelial cells. Moreover, how the presence of exogenous FABP3, such as in the bloodstream during cardiovascular conditions (e.g., cardiac injuries, PAD, endothelial dysfunction), affects the endothelium is also examined. Further mechanistic insights are provided, particularly how key molecular players of endothelial functions are regulated by the loss and gain of FABP3's function. Essentially, this chapter examines whether the FABP3 expression and/or exposure is protective or detrimental to the endothelium and endothelial function. Overall, Chapter 3 shifts the focus from an overview of endothelial FABP3's regulatory trends in Chapter 2 to functional consequences. In this notion, Chapter 3 closely examines the potential of FABP3 as a therapeutic target, approaching the implication that modulating FABP3 levels could affect endothelial dysfunction.

6.2.1 Loss of FABP3 protects against inflammatory endothelial dysfunction

Chapter 3-Figure 1 demonstrates that LPS treatment at increasing concentrations significantly upregulates FABP3 expression in cultured human endothelial cells. LPS-induced FABP3 upregulation occurs as early as 1 hour after treatment and also manifests through increased FABP3 secretion from LPS-treated endothelial cells into the culture medium. Moreover, loss of FABP3 improves and rescues impaired tube formation in LPS-treated endothelial cells, indicating that FABP3 contributes to LPS-induced dysfunction in angiogenesis. The scratch-assay assessment shows that loss of FABP3 appears to inhibit migration, suggesting that FABP3 mediates endothelial cells' migratory processes, such as in wound healing. Cell counting results suggest that the loss of cell population induced by LPS is rescued by the loss of endothelial FABP3. This and the reduced expression of cleaved-CASPASE3 in FABP3-silenced endothelial cells indicate that FABP3 positively regulates apoptosis, serving a detrimental role to endothelial cells under inflammation-induced endothelial dysfunction. Overall, LPS induces upregulation of FABP3 in endothelial cells, which is associated with various detrimental effects on endothelial function. Silencing FABP3 seems to confer protection against these LPS-induced effects, improving the

angiogenic and proliferative capacity of endothelial cells and reducing apoptosis. **Chapter 3-Figure 2** investigates the effects of the loss of Fatty Acid-Binding Protein 3 (FABP3) and lipopolysaccharide (LPS) treatment on the molecular mechanisms underlying endothelial cell function. LPS compromises the expression and activation (phosphorylation) of endothelial nitric oxide synthase (eNOS) and protein kinase B (AKT), which are key regulators of endothelial function. Total AKT expression, at both the transcript and protein levels, was significantly inhibited by LPS. Conversely, this was restored in FABP3-silenced endothelial cells. Activated AKT (p-AKT) was also significantly upregulated in both FABP3-silenced endothelial cells and those treated with LPS. The expression and activation of eNOS were also significantly upregulated by loss of endothelial FABP3, even in the presence of LPS. These findings indicate that loss of FABP3 counteracts-, and hence, FABP3 exacerbates the negative effects of LPS on eNOS and Akt, thereby impairing endothelial function. **Chapter 3-Figure 2** also illustrates that LPS treatment is associated with the upregulation of p21, a cell cycle inhibitor. FABP3-silenced endothelial cells with or without LPS treatment showed a significant reduction in p21 expression, indicating downregulation of p21 levels by the loss of endothelial FABP3 and that FABP3 is involved in the inhibition of endothelial cell proliferation. This notion is further strengthened by the previously reduced expression of cleave-CASPASE3 in FABP3-silenced endothelial cells, which together may have potentiated their rescuing of cell viability and proliferative capacities under LPS stress (**Chapter 3-Figure 1)**. Overall, **Chapter 3-Figure 2** data demonstrate that increased AKT and eNOS signalling, along with the inhibition of p21 expression due to the loss of FABP3, collectively contribute to the restoration of endothelial function from adverse events in an LPS-treated environment. On the other hand, while eNOS was examined, the more ubiquitously expressed inducible nitric oxide synthase (iNOS) lacks assessment, which is also a source of NO and ROS and is involved in vascular homeostasis and inflammation [3]. Future studies should address the involvement of iNOS as its regulatory connection to FABP3 would further elucidate the roles of FABP3 on endothelial function under stress conditions.

6.2.2 Loss- and gain- of FABP3, functional implications in endothelial inflammation

Chapter 3-Figure 3 presents the effects of endogenous FABP3 deficiency on inflammation in endothelial cells in response to LPS-induced endothelial dysfunction. At both transcript and protein levels, loss of FABP3 in endothelial cells rescues LPS upregulation of inflammatory

mediators ICAM-1 but exacerbates that of VCAM-1. ICAM-1 and VCAM-1 are key inflammatory mediators of endothelial cells, suggesting the regulatory dynamic of FABP3 between these inflammatory markers. It is known that VCAM-1 plays a more dominant role in initiating atherosclerosis compared to ICAM-1. ICAM-1 also does not compensate for reduced VCAM-1 in atherosclerosis, and deficiencies in both these factors don't appear to alter lesion formation [4]. Through loss-of-function studies, our findings suggest that FABP3 positively regulates endothelial inflammation through ICAM-1, and in the case that this was compromised, VCAM-1 upregulation becomes over-compensatory, in turn exacerbating inflammation, providing novel insights. Moreover, the induction of E-SELECTIN, another adhesive inflammatory marker, by LPS is restored to lower levels by loss of FABP3 in LPS-treated endothelial cells; such rescuing effect also occurs for the inflammatory cytokines, IL1b and IL6, and chemokine MCP-1. Overall, these findings suggest that FABP3 exacerbates the inflammatory response in endothelial cells induced by LPS. Conversely, the deficiency or silencing of FABP3 can substantially mitigate this response by modulating the levels of key inflammatory markers and cytokines. **Chapter 3-Figure 4** examines the impact of exogenous FABP3 in endothelial cells under LPS stress. Treatment with recombinant human FABP3 (rhFABP3) alone did not significantly alter the expression of inflammation markers ICAM-1 and VCAM-1 in endothelial cells. However, when endothelial cells were treated with both LPS and rhFABP3, there was a significant increase in the transcripts and proteins for both ICAM-1 and VCAM-1, indicating that rhFABP3 exacerbates LPS-induced inflammation. In the case of VCAM-1, inflammation is exacerbated in both loss- and gain- of FABP3, prompting future investigation on other inflammatory markers for exogenous FABP3 treatment, as in FABP3 silencing, to further clarify the roles of endothelial FABP3 in inflammation. In assessing the effect of rhFABP3 on vascular function, wire myography shows a minor increase in relaxation in rhFABP3-treated aortas compared to wild-type mice. As this is not statistically significant, future studies should aim to increase replicates to validate this observation. On the other hand, circulatory FABP3 levels were significantly higher in the LPS-treated mice compared to vehicle-treated ones after 4h of treatment, confirming the *in vitro* induction of FABP3 by LPS and strengthening the observation that inflammation upregulates FABP3 release. Overall, **Chapter 3-Figure 4** demonstrates that while exogenous FABP3 does not independently induce inflammation, it significantly amplifies LPS-induced inflammatory responses in endothelial cells, as shown by increased expression of key inflammatory markers. Additionally, the trend that

suggests a slight vascular effect of rhFABP3 on aortic relaxation beckons future increases in the number of mice for assessment. Lastly, LPS increases circulatory FABP3 levels both *in vitro* and *in vivo*. These findings indicate that exogenous or circulatory FABP3 is involved in inflammation and contributes to inflammatory endothelial dysfunction.

6.2.3 Gene ontology of inflammatory FABP3-deficient human endothelial cells

Endothelial biology gene arrays, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of Chapter 3 provide insight into how the loss of FABP3 influences the genetic expression profile related to endothelial function and inflammation, especially in the context of LPS-induced stress. In *FABP3*-silenced endothelial cells, among the most upregulated genes (more than two-fold change) are genes associated with angiogenesis and cell survival (*COL1A2, BDNF, FN1, BCL2, EGFR, VEGFA, EGR1, CDK1,* and *BIRC5*), verifying the negative regulatory implication of FABP3 on endothelial function in **Chapter 3-Figure 2**. LPS treatment alone upregulates primarily pro-inflammatory genes (*IL6, IL1b, CCL2, CCL5, TLR2,* and *ICAM-1*) and downregulates genes involved in survival and angiogenesis (*STAT1, IGFBP3, CAV1, STAT3, BIRC5, AURKA, COL1A2, CDK1, KDR,* and *FGF2*). Conversely, the upregulated genes in the LPS-treated *FABP3*-silenced cells were mainly related to survival and angiogenesis, while the downregulated genes were predominantly pro-inflammatory. These analyses are summarized in **Chapter 3-Figure 5**, indicating that the absence of FABP3 in endothelial cells may lead to a favourable shift in gene expression for endothelial function under inflammatory stress, promoting angiogenic and survival pathways while suppressing inflammatory pathways. These findings reinforce that FABP3 release may harm the endothelium under inflammation and during cardiovascular events.

Overall, Chapter 3 demonstrates that loss of FABP3 in endothelial cells improves angiogenesis and cell survival and reduces inflammatory responses, providing insights into FABP3's role in regulating inflammatory endothelial dysfunction at the phenotypic and genotypic levels. This chapter utilizes a combination of loss- and gain-of-function approaches in both *in vitro* and *in vivo* settings to elucidate the role of FABP3 in endothelial function. A comprehensive analysis of gene expression changes associated with FABP3 and LPS treatment is also provided, demonstrating regulatory links between FABP3 expression and endothelial cell function under inflammatory conditions. However, the exact mechanisms by which FABP3 influences

inflammatory endothelial dysfunction and the observed effects *in vivo* (particularly the non-significant myography) remain to be further validated by increasing replicates and statistical power. In addition, results for *in vitro* migratory capacities and proliferation could be reinforced using more advanced assays, such as the Transwell Migration assay [5] and the WST-1 proliferation assay [6]. Regarding proliferation, future works could rely on additional assays beyond cell counts and cleave-CASPASE3 evaluation to distinctly address cell proliferation (e.g., BrdU assay) [7] and viability (e.g., MTT assay) [8]. Accurate functional assessment of FABP3 on endothelial cell survival would provide a robust understanding of the essential of FABP3 in endothelial functions. Nonetheless, results from Chapter 3 highlight the complexity of endothelial FABP3 functions, which could inform future research and suggest that modulating FABP3 levels poses therapeutic potential for inflammatory endothelial dysfunction, sepsis, atherosclerosis and cardiovascular diseases. However, the precise functions of FABP3 and its interactions with other endothelial cell processes warrant further investigations.

6.3 Elevated FABP3 impacts differential endothelial cell gene expression.

As Chapter 3 elucidated the protective role of endothelial FABP3 loss against inflammation-induced dysfunction, Chapter 4 pivots to further examine the effects of increased FABP3 exposure on endothelial cells, a condition observed in the bloodstream post-cardiac injuries and in peripheral artery disease (PAD) patients and suggested to be harmful to endothelial cells in Chapter 3. Chapter 4 aims to address this discrepancy by employing a comprehensive transcriptomic approach to understand the consequences of the endothelium subjected to FABP3 exposure. RNA sequencing, differential gene expression (DGE), GO and KEGG analyses were employed to capture a transcriptome-wide spectrum of differential gene expression and cellular function and pathways potentially impacted in cultured endothelial cells treated with recombinant human FABP3 (rhFABP3), providing insights into the roles of FABP3 in endothelial function and cardiovascular diseases.

In **Chapter 4-Figure 1**, a total of 15,688 genes were identified from processed RNA-seq data obtained from total RNAs of rhFABP3-treated human endothelial cells vs. vehicle groups. Principal component analysis (PCA) on the first two principal components (79.41% total variance captured) showed clear clustering of the samples that indicates a distinct change in the gene

expression profiles of rhFABP3-treated human endothelial cells. The DGE analysis identified 11 genes with significant differential expressions by at least 2-fold; 7 genes were upregulated and 4 downregulated. Among the most upregulated genes are *KIF26B, NCR1,* and *DNAJC14*. Among the most downregulated is *SMN2*.

Chapter 4-Figure 2 shows the GO and KEGG pathway analyses that interpret the significance of the differentially expressed genes. The study was limited by deficient genes from the DGE analysis, which have their significance adjusted for False Discovery Rate (FDR) and cannot be meaningfully subjected to GO and KEGG analyses. As we aimed to assess the potential transcriptomic impact of FABP3 exposure, the FDR adjustment was lifted, enabling the enrichment of 80 genes (38 upregulated and 42 downregulated). At the same time, the increase in Type-1 error was acknowledged ("analytical noise"). With this caution, GO analysis showed the association of upregulated genes mainly with immune response and cell cytotoxicity, the most notable functions being the "regulation of leukocyte-mediated cytotoxicity" and "natural killer cell-mediated cytotoxicity." The downregulated genes are linked to RNA processing and the inflammatory systems; the most significant terms for the downregulated genes are related to the SMN complex, a cellular component involved in RNA processing, and the NOD-like receptor signalling pathway, which is involved in inflammation [9]. The GO and KEGG findings suggest that, while considering statistical noises, FABP3 exposure may stimulate the endothelium to engage in the immune response. Additionally, a broader assessment of gene expression patterns was conducted to offset the lack of FDR adjustment. A heatmap of 231 protein-coding genes non-FDR-adjusted shows distinct clusters of gene expression in rhFABP3-treated HUVECs compared to vehicle treatment, highlighting the potential functional impacts of FABP3 exposure on the physiology of endothelial cells.

Chapter 4 reports that FABP3 exposure induces notable transcriptomic changes in gene expression. Specifically, 11 genes showed significant differential expressions, which may influence cellular processes such as angiogenesis, immune response, and stress mechanisms. The upregulation of *KIF26B* suggests a role in cellular motility and angiogenesis, potentially acting as a biomarker for cancer prognosis [10]. *NCR1* upregulation indicates a novel link between the innate immune system and endothelial cells, potentially enhancing immune surveillance [11]. *DNAJC14* upregulation is suggestive of a stress response within the endothelial cells [12]. The downregulated

SMN2 gene, which is involved in RNA splicing and processing, suggests a transcript modification effect of FABP3 on endothelial cells. Likewise, GO and KEGG suggest innate immune responses involving natural killer cells may be impacted upon FABP3 exposure. While these findings expand the current understanding of endothelial cell biology under cardiovascular events, this Chapter encountered considerable obstacles. Limited numbers of significantly differentially expressed genes might be attributed to the semi-arbitrary dosing regimen of rhFABP3 (50 ng/mL for 6h); however, this was reasoned to be within the clinical range based on past studies (7.22 – 434 ng/mL) [13] [14]. This obstacle potentiated the analytical trade-off that arises when unadjusted p-values from DGE analysis were utilized for GO and KEGG enrichment, leading to results that are likely to be affected by Type-1 errors. Moreover, many differentially expressed genes identified by DGE analysis are not well characterized, necessitating further research to incorporate their functional implication in the context of rhFABP3-treated endothelial cells. Overall, Chapter 4 attempts to elucidate the transcriptomic profile of endothelial cells under rhFABP3 exposure, with the aim of drawing potential clinical implications for cardiovascular diseases. While the findings highlight the complexity of endothelial responses to FABP3 and open avenues for future validating research, the Chapter also acknowledges the limitations and trade-offs inherent in the study design. The strength of the Chapter lies in its comprehensive methodology, detailed analysis and the identification of novel gene expressions and pathways, while the weakness revolves around the dosing regimen and the analytical constraints. Future research is suggested to overcome these limitations, such as optimizing the FABP3 dosage and employing more stringent statistical analyses to solidify these preliminary findings and enhance the understanding of FABP3's implications in cardiovascular pathology.

6.4 Endothelial cells transcriptomic profile under Angiotensin II exposure

Endothelial function is central in maintaining vascular homeostasis, and dysfunction drives adverse vascular events that underlie atherosclerosis, such as hypertension. Chapters 2 to 4 have investigated the regulatory roles of FABP3 in endothelial dysfunction and highlighted specific upregulated and downregulated genes and functional pathways potentially impacted in endothelial cells subjected to FABP3 deficiency or exposure. From Chapter 4, many of the identified significant differential expressed genes under rhFABP3 exposure were not well-characterized transcripts, such as unprocessed transcripts. Long non-coding RNAs, RNA molecules greater than

200 nucleotides in length that do not translate, have been recently recognized for their regulatory roles in endothelial function [15]. The previous chapter's insights into FABP3's effects also set the stage for a pilot investigation into how other potent molecules like Ang II, a vasoconstrictor implicated in hypertension and cardiovascular diseases, may impact endothelial dysfunction genome-wide. This Chapter harnesses and advances the methodology from Chapter 4, particularly utilizing the Arraystar Human lncRNA Expression Microarray [16], to establish a novel transcriptomic profile of differentially expressed lncRNAs and mRNAs in endothelial cells exposed to Ang II, bridging the knowledge gap between the cardiovascular implication of lncRNAs and hypertension.

Chapter 5 provides a comprehensive analytical profile of the differentially expressed lncRNAs and mRNAs in Ang II-treated human endothelial cells, with discussed cardiovascular implications. From **Chapter 5-Figure 1** and **Chapter 5-Table 1-4**, 25 lncRNAs and 28 mRNAs were significantly upregulated, while 69 lncRNAs and 67 mRNAs were downregulated. Of the top 10 significantly upregulated and downregulated lncRNAs, RP11-354P11.2 and RP11-360F5.1 are the most up- and downregulated, respectively. Among the top significant differentially expressed mRNAs, *RAB11FIP4* and *DNAJA2* are the most up- and downregulated, respectively. The significant differentially expressed lncRNAs are distributed pronouncedly over chromosomes 1, 2, 3, 7, 8, 12, 16, and 17, as shown in **Chapter 5-Figure 2**. Notably, chromosomal mapping indicates a significant clustering of significant differentially expressed lncRNAs on chromosomes 2, 15, and 22 in Ang II-treated human endothelial cells. Subgroup analysis, which helps interpret the functional ties between lncRNAs and protein-coding genes, found that about half of the lncRNAs are intergenic, with a substantial portion also being natural and intronic antisense lncRNAs. The chapter highlights the cardiovascular implications of the differentially expressed lncRNAs and mRNAs and the lncRNAs' chromosomal distribution in Ang II-treated human endothelial cells, with a focus on potential modulatory roles in heart failure, immune responses, intracellular transport, cellular structure integrity, and inflammation. GO and KEGG analyses follow up by sorting the differentially expressed mRNAs in Ang II-treated human endothelial cells into corresponding significant cellular functions and pathways. Particularly, GO analysis associates the upregulated mRNAs mostly with the regulation of single organism processes, localization, and transport, whereas downregulated mRNAs were predominantly with metabolic processes, cell periphery, and ion binding, as summarized in **Chapter 5-Table 6**. KEGG analysis,

summarized in **Chapter 5-Table 5**, highlights the pathways related to nucleotide excision repair and ECM-receptor interaction that are associated with the most downregulated mRNAs. The strength of this chapter lies in its comprehensive bioinformatic approach to both mRNA and lncRNA profiling, outlining possible overlaps in endothelial function, pathways, and cardiovascular implications. These approaches build a multidimensional picture of the endothelial cell response to Ang II and hypertension. However, validation in different types of endothelial cells remains necessary for distinguishing between microvascular and macrovascular functions. Additionally, the uncharacterized nature of many of the differentially expressed lncRNAs limits the ability to link these molecules to Ang II-related endothelial phenotypes. Overall, Chapter 5 presents a foundational study of endothelial cells' transcriptomic responses to Ang II exposure, having identified numerous potential targets for further investigation on hypertensive endothelial dysfunction.

6.5 Final Remarks & Limitations:

Chapters 2 to 5 delineate the multifaceted role of FABP3 within the endothelial cell context, particularly under atherosclerotic stressors and inflammatory conditions, with further exploration into the transcriptomic alterations induced by Angiotensin II (Ang II) exposure. This body of work provides insights into the dynamic interplay between FABP3 expression and endothelial dysfunction and the potential therapeutic of FABP3 in managing cardiovascular disease.

Chapter 2 sets the foundation by elucidating the regulatory modulation of FABP3 in response to various atherosclerotic stressors, establishing a central role of FABP3 in endothelial dysfunction. The novel identification of FABP3 basal expression in endothelial cells and its dynamic regulation under oxidative stress, hypertension and inflammation underscore FABP3's roles in the pathophysiology of cardiovascular diseases. However, the chapter acknowledges the inherent limitations of being strictly *in vitro* alongside a constrained statistical power due to limited biological replicates. Moreover, biomarkers indicative of each mode of stress should be assessed in future studies to confirm endothelial cells are under stress. It is also necessary to affirm endothelial genotypes (e.g., qPCR for CD31, an endothelial-specific molecular marker) in the experimental setting, ensuring that the switching of media for treatment doesn’t alter basal endothelial function.

Chapter 3 further investigates FABP3's role, emphasizing the effects of loss- and gain- of FABP3's function in endothelial cell response to inflammation. The findings suggest that FABP3 may exacerbate inflammatory responses, and its deficiency could offer protective effects against endothelial dysfunction. This chapter also underscores the complexity of endothelial FABP3 functions, hinting at its potential as a therapeutic target. Nevertheless, the chapter also concurs with the limitation of an *in vitro* focus and the lack of investigation across different endothelial cell types. Alternative versions of several endothelial function assays should also be considered in future studies to confirm the findings; in particular, endothelial proliferative capacity needs to be distinguished from viability and survival. Moreover, while potential interaction targets of FABP3 were outlined (**Chapter 3-Table 1-6**), the mechanism of FABP3 actions remains under-investigated and is a subject of future work, particularly how it was uptake by HUVECs in the experimental context to exert effects on ICAM-1 and VCAM-1 **(Chapter 3-Figure 4).**

Chapter 4 shifts to focus on the impact of elevated FABP3 on endothelial cell gene expression, identifying significant transcriptomic changes that might contribute to cardiovascular stress responses. This chapter encounters considerable obstacles despite identifying novel gene expressions, which hint toward the upregulation of stress response and natural killer cell toxicity and pathways potentially influenced by FABP3 exposure. The chapter grapples with analytical limitations, particularly the possibility of inefficient rhFABP3 dose, time of exposure and low biological replicates number, and the necessity for further research to solidify these preliminary findings. Future work should address these statistical limitations utilizing various dosages and times of FABP3 treatments, as well as validating the identified significant DE genes with qPCR and immunoblot. The approach of this chapter is essential to identify the interaction targets of FABP3 in endothelial cells, considering that Chapter 3 has identified significant exacerbation of inflammatory signalling in endothelial cells under LPS stress by exogenous FABP3 treatments.

Chapter 5 expands the thesis's scope by delving into the transcriptomic profile changes under Ang II exposure, highlighting the modulatory implications of long non-coding RNAs in hypertension-induced endothelial dysfunction. The results enrich the thesis with a novel transcriptomic perspective on endothelial cell responses to hypertensive conditions; however, it is not without gaps for future improvement. This chapter faces the challenge of extending its findings to *in vivo* models and across diverse endothelial cell types.

Through these chapters, the thesis presents a compelling narrative on the critical role of FABP3 in endothelial cell biology, particularly under stress conditions relevant to cardiovascular diseases. The overarching strengths of this work lie in its novel insights into FABP3 regulation, the comprehensive analysis of endothelial responses to various stressors, and the identification of potential therapeutic targets.

Conversely, major weaknesses stem from the strictly *in vitro* nature of the investigations, limited exploration across endothelial cell types, and concerns regarding statistical power and replicability. HUVECs are veinous and, thereby, compromised for functional relevance regarding vasculatures under atherosclerotic stresses; for instance, endothelial cells of branching arteries are particularly vulnerable to atherosclerosis [17]. Moreover, using only pooled HUVECs lacks accounting for the effects of sex, age, and endothelial cells' functional heterogeneity across the vascular tree [18]. Findings of this thesis would, therefore, greatly benefit from additional in vitro models, such as human coronary and pulmonary artery endothelial cells, microvascular endothelial cells from various tissues, and individuals of distinct age and sex groups [19]. This diversifying approach will provide a more accurate understanding of the effects of FABP3 on endothelial function and vascular biology. Lastly, while valuable insights were provided, there's a need to translate the thesis's *in vitro* findings into *in vivo* experiments to consolidate its scientific meaning and provide practical clinical implications. On this aim, employing an endothelial cell-specific FABP3 knockout mice vulnerable to atherosclerosis (e.g., $ApoE^{-/-}$ mice) would profoundly highlight the role of endothelial FABP3 in atherosclerosis [20]. Additionally, comparing circulatory FABP3 levels in knockout versus wild-type mice could further determine if endothelial cells are a significant source of FABP3 and how its absence affects atherosclerotic development. Moreover, studying endothelial cells harvested ex vivo from these models will provide insights into how FABP3 influences endothelial function in different vascular regions, further addressing the roles of endothelial heterogeneity.

In conclusion, this thesis addresses the primary processes of endothelial dysfunction and supports the hypothesis that *FABP3 is a critical regulator of endothelial dysfunction*. Our findings, therefore, offer significant contributions to cardiovascular research on the topics of circulatory FABP3, endothelial dysfunction and atherosclerosis, particularly in understanding FABP3's role in endothelial dysfunction. Critical areas for future investigation are also highlighted. Addressing the

noted limitations through *in vivo* studies, expanding the investigation to different endothelial cell types to account for the endothelium's inherent heterogeneity, and enhancing statistical robustness through increasing replicates, treatment optimization, and employing *in vivo* models will be the crucial steps forward. Ultimately, this work underscores the importance of FABP3 as a potential modulatory therapeutic target in endothelial dysfunction and atherosclerosis, marking significant steps toward more effective treatments and interventions.

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Appendices:

Appendix A: Abbreviations

Chapter 1 Abbreviations

acetyl-CoA, Acetyl Coenzyme A; ABCA1, ATP Binding Cassette Subfamily A Member 1; ABCG1, ATP Binding Cassette Subfamily G Member 1; ACE, Angiotensin-converting enzyme; AGE, Advanced glycation end products; AKT, Protein Kinase B; ALOX12/15, Arachidonate-12/15-Lipoxygenase; Ang I, Angiotensin-I; Ang II, Angiotensin-II; ApoA1, E, C2, Apolipoprotein A1, E, C2; ATP, Adenosine Triphosphate; CAD, Coronary Artery Disease; CAT, Catalase; c-Src, Cellular Sarcoma; CCR2, C-C Chemokine Receptor Type 2; CD36, 40, 154, Cluster of Differentiation 36, 40, 154; CD142, Tissue Factor; CO2, Carbon Dioxide; COX2, Cyclooxygenase 2; CPT1a, Carnitine Palmitoyltransferase 1a; CSF, Colony-Stimulating Factor; CVD, Cardiovascular Disease; CXCL10, 11, C-X-C Motif Chemokine Ligand 10, 11; CXCR3, C-X-C Chemokine Receptor Type 3; Cyp8b1, Cytochrome P450 Family 8 Subfamily B Member 1; dNTP, Deoxyribonucleotide Triphosphate; eNOS, Endothelial Nitric Oxide Synthase; E-/P-SEL, E-/P-Selectin; FABP, Fatty Acid-Binding Protein; FGF, Fibroblast Growth Factor; H2A, H2B, H3, H4, Histones 2A,2B, 3 and 4; H3K9, Histone H3 Lysine 9 specific site of methylation; HDL, High-Density Lipoprotein; HUVEC, Human Umbilical Vein Endothelial Cells; ICAM-1, Intercellular Adhesion Molecule-1; IL-1b, IL-4, IL-6, IL-10, IL-11, IL-13, Interleukin 1-beta, 4, 6, 10, 11, 13; LCAT, Lecithin-Cholesterol Acyltransferase; LDL, Low-Density Lipoproteins; LDLR, Low-Density Lipoprotein Receptor; lncRNAs, Long non-coding RNAs; LPS, Lipopolysaccharide; LXR-α, Liver X Receptor-Alpha; MCP-1, Monocyte Chemoattractant Protein-1; M-CSF, Macrophage Colony-Stimulating Factor; Mig, Monokine Induced by Gamma Interferon; miRNAs, MicroRNAs; MMP, Matrix Metalloproteinases; mRNAs, Messenger RNAs; NADPH, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; ncRNA, Non-coding RNA; NF-κB, Nuclear Factor Kappa B; NO, Nitric Oxide; NOX, NADPH Oxidases; NOX2, NADPH Oxidase 2; NPC, Niemann–Pick Disease Type C; MI, Myocardial Infarction; mTOR, Mammalian Target of Rapamycin; oxLDL, Oxidized Low-Density Lipoproteins; PAD, Peripheral Artery Disease; PDGF, Platelet-Derived Growth Factor; PGI2, Prostaglandin I2; PI3K, Phosphoinositide-3-Kinase; PPARα, δ, γ, Peroxisome Proliferator-Activated Receptor Alpha, -Delta, -Gamma; RAS,

Renin-Angiotensin-Aldosterone System; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase; SR-A, Scavenger Receptor Class A; SR-B1, Scavenger Receptor Class B Member 1; TDP-43, TAR DNA-binding protein 43; TGF-β, Transforming Growth Factor-Beta; TLR, Toll-like Receptors; TNF-a, Tumor Necrosis Factor-alpha; VCAM-1, Vascular Cell Adhesion Molecule-1; VE-Cadherin, Vascular Endothelial Cadherin; VEGFa, Vascular Endothelial Growth Factor A; VLA-4, Very Late Antigen-4; VPO1, Vascular Peroxidase 1; ZO-1, Zonula Occludens-1.

Chapter 2 Abbreviations

FABPs, Fatty Acid-Binding Proteins; PAD, Peripheral Artery Diseases; HUVECs, Human Umbilical Vein Endothelial Cells; oxLDL, Oxidized Low-Density Lipoprotein; LPS, Lipopolysaccharide; Ang II, Angiotensin-II; NPC, Niemann–Pick Disease Type C Proteins; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; SkMCs, Skeletal Muscle Cells; qPCR, Quantitative Real-Time Polymerase Chain Reaction; ELISA, Enzyme-Linked Immunosorbent Assay; VEGFa, Vascular Endothelial Growth Factor-alpha.

Chapter 3 Abbreviations

PTGS2, Prostaglandin-Endoperoxide Synthase 2; COL1A2, Collagen Type I Alpha 2 Chain; PLAU, Plasminogen Activator, Urokinase; BDNF, Brain-Derived Neurotrophic Factor; BCL2, BCL2 Apoptosis Regulator; CCL5, C-C Motif Chemokine Ligand 5; EGR1, Early Growth Response 1; TLR2, Toll-Like Receptor 2; EGFR, Epidermal Growth Factor Receptor; FN1, Fibronectin 1; TOP2A, DNA Topoisomerase II Alpha; RRM2, Ribonucleotide Reductase Regulatory Subunit M2; IGFBP3, Insulin-Like Growth Factor Binding Protein 3; FOS, Fos Proto-Oncogene, AP-1 Transcription Factor Subunit; VEGFA, Vascular Endothelial Growth Factor A; IL1b, Interleukin 1 Beta; IL8, C-X-C Motif Chemokine Ligand 8; SOD2, Superoxide Dismutase 2; ICAM1, Intercellular Adhesion Molecule 1; CCL2, C-C Motif Chemokine Ligand 2; IL6, Interleukin 6; MMP7, Matrix Metallopeptidase 7; FGF2, Fibroblast Growth Factor 2; CDK1, Cyclin Dependent Kinase 1; IL18, Interleukin 18; BIRC5, Baculoviral IAP Repeat Containing 5; TACC3, Transforming Acidic Coiled-Coil Containing Protein 3; AURKA, Aurora Kinase A; ABCB1, ATP Binding Cassette Subfamily B Member 1; KDR, Kinase Insert Domain Receptor; TCF7L2, Transcription Factor 7 Like 2; RB1, RB Transcriptional Corepressor 1; CAV1, Caveolin

1; STAT1, Signal Transducer and Activator of Transcription 1; STAT3, Signal Transducer and Activator of Transcription 3; CXCL10, C-X-C Motif Chemokine Ligand 10; MMP1, Matrix Metallopeptidase 1; SERPINE1, Serpin Family E Member 1; CTBP2, C-Terminal Binding Protein 2; AKT, Protein Kinase B; CVD, Cardiovascular Diseases; eNOS, Endothelial Nitric Oxide Synthase; FABPs, Fatty Acid–Binding Proteins; HUVECs, Human Umbilical Vein Endothelial Cells; LPS, Lipopolysaccharide; NO, Nitric Oxide; PAD, Peripheral Artery Disease; PPAR, Peroxisome Proliferator-Activated Receptor; qPCR, Quantitative Polymerase Chain Reaction; rhFABP3, Recombinant Human FABP3.

Chapter 4 Abbreviations

FABP3, Heart-type fatty acid binding protein; *KIF26B*, Kinesin family member 26b; *SMN2*, Survival of motor neuron 2; *NCR1*, Natural cytotoxicity triggering receptor 1; *DNAJC14*, DnaJ heat shock protein family (Hsp40) member C14; *ENSG00000269242*, Glycosyl Hydrolases Family 38 C-Terminal Beta Sandwich Domain-Containing Protein; *CFAP298-TCP10L*, Cilia and flagella associated protein 298-T-complex 10 like; *IMPDH1P10*, IMPDH1 pseudogene 10; *CENPBD1P*, Centromere protein B direct repeat 1 pseudogene; *PKD1P3*, Polycystic kidney disease 1 (autosomal dominant) pseudogene 3; FABPs, fatty acid–binding proteins; HUVECs, human umbilical vein endothelial cells; PAD, peripheral artery disease; qPCR, quantitative polymerase chain reaction; rh/re-FABP3, recombinant human FABP3.

Chapter 5 Abbreviations

Ang II, angiotensin II; ACE, Angiotensin-Converting Enzyme; CVDs, Cardiovascular Diseases; DE, Differentially Expressed; ECM, Extracellular Matrix; GO, Gene Ontology; HUVECs, Human Umbilical Vein Endothelial Cells; KEGG, Kyoto Encyclopedia of Genes and Genomes; NER, Nucleotide Excision Repair; OD, Optical Density; PBS, Phosphate-Buffered Saline; RAS, Renin-Angiotensin System; ROS, Reactive Oxygen Species; SIPS, Stress-Induced Premature Senescence; lncRNAs, Long Noncoding RNAs.

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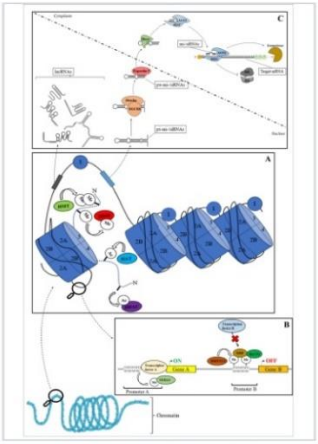

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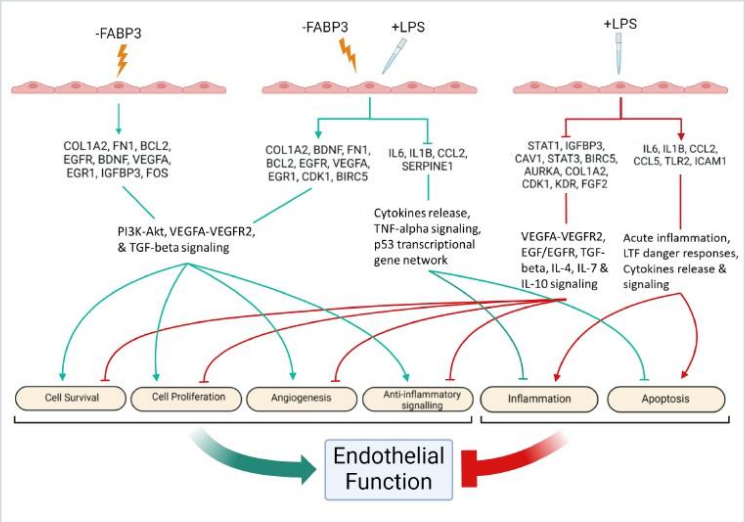

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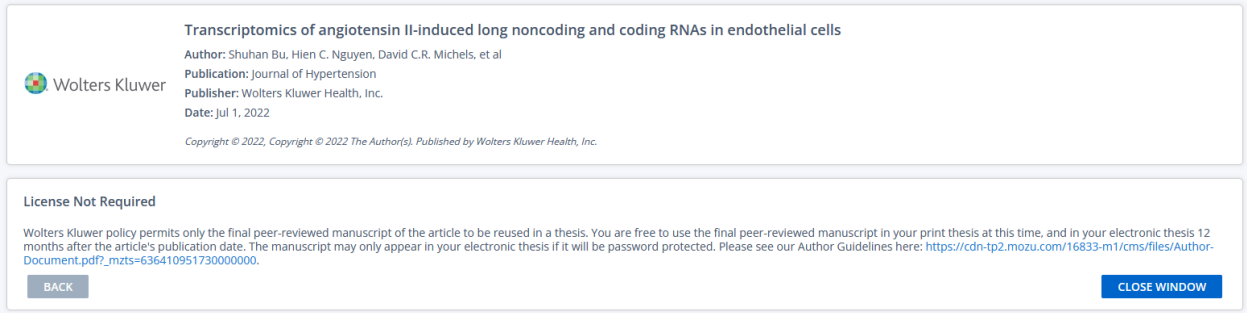

Transcriptomics of angiotensin II-induced long noncoding and coding RNAs in endothelial cells

Author: Shuhan Bu, Hien C. Nguyen, David C.R. Michels, et al
Publication: Journal of Hypertension
Publisher: Wolters Kluwer Health, Inc.
Date: Jul 1, 2022

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1. Roles of Fatty Acid Binding Protein-3 in Endothelial Function. London Health Research Day (2022), London, Canada.

2. The Essential Role of Fatty-acid Binding Protein 3 in Endothelial Function. Robarts Research Retreat (2022), London, Canada.

3. Fatty acid-binding Protein-3 (FABP3) is a Novel Regulator of Endothelial Function. Experimental Biology (2021), United States of America.

4. Fatty acid-binding Protein-3 (FABP3) Roles in Endothelial Dysfunction. Anatomy and Cell Biology's Research Day (2021), London, Canada.

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