Author: Nahon, J.E.
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General Introduction
Energy metabolism

To function properly, cells in the human body demand energy. This energy is primarily derived from carbohydrates and fat in our meals. After a meal, dietary sugars, fat and cholesteryl esters are digested in the gastro-intestinal tract by mechanic peristaltic actions and enzymatic actions of gastric and pancreatic enzymes. Subsequently, the converted monosaccharides, free fatty acids and free cholesterol are absorbed in the enterocytes of the small intestine for subsequent transport to target tissues via the blood circulation.

Dietary handling of glucose

The brain is an organ with very high energy demand and mainly relies upon glucose as source of energy [1]. Very low glucose levels can therefore threaten normal brain physiology [2]. On the other hand, very high glucose levels are associated with vascular complications [3]. Glucose concentrations are thus kept under strict homeostasis. A complex system of neural pathways, hormones and glucose transport proteins regulate the dietary glucose absorption in the intestine and the glucose disposal by the kidney. In parallel, endogenous glucose production via gluconeogenesis and glycogenolysis and glucose uptake and release by peripheral tissues are controlled. There are many hormones regulating glucose concentrations including corticosteroids and epinephrine [4]. However, under physiological conditions, insulin and its counterpart glucagon are the most important glucose-controlling hormones.

In a postprandial state, glucose levels are reduced by peaking insulin levels inhibiting hepatic gluconeogenesis and adipocyte lipolysis [5]. Simultaneously, insulin induces glucose disposal in skeletal muscle, where it can be used as energy source or stored in the form of glycogen, and in adipose tissue for storage [5]. When the blood glucose concentration drops, insulin production by the pancreatic β-cells in the islets of Langerhans is reduced and glucagon release is initiated from the pancreatic α-cells. Glucagon’s main target organ is the liver where it stimulates gluconeogenesis and glycogenolysis, thereby increasing the hepatic glucose output [6]. In parallel, glucagon inhibits glycogenesis and glycolysis [6]. An overview of glucose homeostasis is schematically depicted in figure 1.

Dietary handling of lipids

Exogenous lipid transport

In contrast to glucose, which is water-soluble and can be transported freely throughout the circulation, lipids are hydrophobic and need to be transported to peripheral tissues through the circulation inside lipoproteins. The hydrophobic core of these lipoproteins contains triglycerides and cholesteryl esters and is surrounded by a phospholipid layer [7]. Within the phospholipid rich outer layer, apolipoproteins reside. These proteins are crucial for the stability of the particles and for the interaction of the particles with cellular receptors and thereby determine the lipoprotein structure, shape and formation, and clearance by tissues [8]. The
largest lipoproteins are chylomicrons, containing apolipoprotein B (ApoB), apolipoprotein C-II (ApoC-II) and apolipoprotein E (ApoE). Dietary lipids are packaged in these large chylomicron particles and circulate as a source of energy for organs and tissues in the periphery. After binding to proteoglycans in the glycocalix, a specialized layer covering the luminal side of the endothelium, the triglyceride-rich chylomicrons donate their lipids for energy use or storage via the interaction of ApoC-II and the enzyme lipoprotein lipase (LPL) [9]. LPL is ex-

Figure 1. Maintenance of glucose homeostasis. Adapted from: Nolan et al. The Lancet, 2011
pressed on endothelial cells, where it hydrolyzes the triglycerides in the chylomicron particle and subsequently breaks them down into free fatty acids and glycerol to be taken up by the cell via cell surface receptors like CD36 [10]. The major recipient tissues are muscles, brown and white adipose tissue. The muscles and brown adipose tissue use the fatty acids for oxidation and generate energy and heat respectively [11]. The white adipose tissue is the major site for lipid storage. By the donation of their triglyceride cargo to cells in the periphery, the chylomicron particles reduce in size. The smaller, triglyceride-deprived particles are now referred to as chylomicron remnants. The chylomicron remnants are cleared by the liver via interaction of ApoE with the low-density lipoprotein (LDL) receptor (LDLR), LDL receptor-related protein (LRP), scavenger receptor class B type I (SR-BI) or other receptors [12,13]. Heparan sulfate proteoglycans are essential in this process, facilitating the interactions between ApoE and the internalization receptors [14].

Endogenous lipid transport
When the dietary supply of lipids is low, the remainder of the exogenous lipids from the chylomicron remnants can be combined with newly synthesized lipids and enter the cycle of endogenous lipid metabolism as very-low-density lipoprotein (VLDL). This process takes place in the liver. The essential apolipoprotein on these VLDL particles is ApoB100, but they also contain ApoE and ApoCII [15]. Similar to the chylomicrons, the VLDL particles circulate as a source for triglyceride-derived fatty acids to be taken up by recipient tissues after hydrolysis by LPL. As the VLDL particles lose their triglyceride content, they diminish in size to become intermediate-density lipoprotein (IDL) particles and subsequently LDL. These IDL and LDL particles are enriched in cholesterol since the triglycerides have been delivered to the peripheral tissues. In the human blood circulation, LDL accounts for 70-80% of the total cholesterol concentration. Finally, the IDL particles are cleared from the circulation by the LDLr and LRP on hepatocytes. LDL is primarily cleared via hepatocyte LDLr-mediated endocytosis. However, extrahepatic tissues can also take up these cholesterol-rich particles. Lipoprotein-derived cholesterol can be used for the synthesis of cellular membranes and steroid hormones specifically in the cells of steroidogenic tissues. In the liver, the internalized remains can again be repackaged into VLDL particles, continuing the cycle [8]. Alternatively, the cholesterol in the liver can be excreted into the bile via ATP-binding cassette (ABC) half-transporters ABCG5 and ABCG8 [16].

Reverse cholesterol transport
Excessive cholesterol in peripheral tissues can be eliminated via high-density lipoprotein (HDL)-mediated reverse cholesterol transport. During this process, cholesterol efflux mediators ABCA1 and ABCG1 on cells in the periphery interact with apolipoprotein A-I (ApoA-I) on the HDL particles shuttling their excess cholesterol out of the cell [17]. The lipid poor discoidal ApoA-I particles are produced by the liver and intestine. This ApoA-I
is already partly lipidated by the liver. Further enrichment in cholesterol is achieved in the periphery by cholesterol efflux out of the peripheral tissue, mostly the brain, intestine, kidney and macrophages, forming the phospholipid-rich HDL particles. The esterification of the cholesterol inside the HDL particles into cholesterol esters is mediated by the enzyme lecithin–cholesterol acyltransferase (LCAT). An enzyme called cholesterol ester transfer protein (CETP) exchanges cholesterol esters carried in the core of HDL particles with triglycerides from ApoB-containing lipoproteins in the circulation. Hereby, cholesterol from the reverse cholesterol transport pathway is transferred back into the endogenous cholesterol pathway [18]. Interestingly, mice do not express this enzyme. The mature HDL particles circulate back to the liver where they selectively transfer the cholesteryl esters from their core to hepatocytes via SR-BI [19]. After selective cholesterol ester delivery, the smaller cholesterol-depleted HDL particle is released from the receptor and can re-enter the circulation for another round of reverse cholesterol transport.

Hepatic lipid metabolism
The liver plays a pivotal role in lipid metabolism. Therefore, intra-hepatic cholesterol homeostasis is imperative to total body lipid homeostasis. The intra-hepatic cholesterol concentration is maintained by multiple balancing pathways. Hepatic free cholesterol levels increase due to the clearance of lipoproteins from the circulation and de-novo synthesis from acetyl-CoA. In contrast, the hepatic free cholesterol levels are decreased as a result of esterification of free cholesterol for storage in the cholesteryl ester pool, the production of VLDL, the efflux of cholesterol to ApoA-I via ABCA1 to produce HDL particles and the excretion of cholesterol via the bile; either direct via ABCG5/G8 or after catabolism of free cholesterol to bile acids by cholesterol 7α-hydroxylase (CYP7A1). An important regulator of these processes is the nuclear transcription factor liver X receptor (LXR). Upon activation by oxysterols, LXR forms a hetero-dimer with the retinoic X receptor (RXR) and subsequently binds to the specific LXR responsive elements within the DNA. This DNA binding stimulates target gene expression [20]. The net effect of LXR activation is the reduction of hepatic free cholesterol levels. This is accomplished by activating the pathways involved in excreting cholesterol into the bile via increased expression of CYP7A1 [21,22] and ABCG5/G8 [23,24]. In addition to controlling hepatic cholesterol efflux, LXR also promotes fatty acid synthesis by the liver. This is accomplished by increasing the expression of the lipogenic transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) and its target genes, including stearoyl-CoA desaturase 1 (SCD1), fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase (ACC). Fatty acid synthesis uses the cholesterol precursor acetyl-CoA as a common substrate, reducing its use in the mevalonate pathway synthesizing cholesterol de novo. Moreover, fatty acids can be esterified with free cholesterol into cholesteryl esters, providing a safe means of cholesterol storage. A schematic overview of intra-hepatic cholesterol homeostasis is depicted in figure 2.
White adipose tissue lipid metabolism

Traditionally, the white adipose tissue is ascribed one main physiological role: serving as an energy repository. However, white adipose tissue plays a critical role in energy metabolism as it actively mediates postprandial lipid flux to maintain homeostasis of lipid concentrations in the circulation [25]. Similar to the maintenance of glucose homeostasis, insulin plays an important role in this context. In a postprandial state, insulin inhibits the actions of the key lipolysis enzyme hormone-sensitive lipase (HSL) to reduce the lipolysis of lipid droplets and subsequent release of free fatty acids into the circulation [26]. In a low nutritional state, this is counteracted by the actions of catecholamines and sympathetic nervous system innervation of the white adipose tissue [26]. Insulin also stimulates the uptake of fatty acids in adipocytes from triglyceride-rich particles in the circulation via LPL [27]. Additionally, insulin stimulates the fatty acid esterification pathway in adipocytes in concert with acylation stimulating protein (ASP), thereby reducing the concentrations of lipids in the circulation [28,29]. Notably, white adipose tissue also functions as an endocrine organ. The major adipokine secreted by adipocytes is leptin [30]. Leptin centrally regulates food intake and energy expenditure, but also has local effects on glucose and lipid metabolism in liver, muscle and pancreas [31,32].

Figure 2. Intra-hepatic cholesterol balance. Adapted from: Jakobsson et al. Trends in Pharm. Sci., 2012

Universiteit Leiden | General Introduction
Metabolic disturbances and associated pathologies

Dysglycemia

Diabetes mellitus is a group of pathologies characterized by hyperglycemia. In general, two major categories of this metabolic disorder can be distinguished based on the etiopathogenesis: type 1 diabetes mellitus and type 2 diabetes mellitus (T2D). Although environmental factors do play a role, there is a strong genetic component determining the susceptibility for type 1 diabetes [33]. In type 1 diabetic patients, autoimmune destruction of pancreatic beta cells results in insulin deficiency and thus a disturbed glucose homeostasis. The disease onset appears abrupt and usually occurs during childhood. The global prevalence is approximated at 0.4% [34]. Since the major problem in type 1 diabetes is the production of insulin, insulin-replacement therapy is the current method for treatment. In contrast, type 2 diabetes has an unclear etiology and a gradual progression into full blown disease. Initially, a failing glucose homeostasis is asymptomatic. Based on clinical tests for diagnosis of type 2 diabetes mellitus, two intermediate stages are identified. Individuals with impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are at risk to progress gradually into type 2 diabetes and are therefore referred to as prediabetic [35,36]. The glucose homeostasis in type 2 diabetes patients is disturbed at three points: insulin secretion in the pancreas is decreased, insulin sensitivity in peripheral tissues is impaired, and the hepatic glucose production is increased [37]. It has been shown that the pancreatic beta cell dysfunction is the major determinant for the development of type 2 diabetes [38]. As the beta cells eventually fail to compensate for the increased need for insulin as a result of decreased insulin sensitivity, the pancreatic islets deteriorate and go into apoptosis [37]. The International Diabetes Federation estimated the global prevalence at 6.4% in 2010. However, this percentage is rapidly increasing to an estimated 7.7% in 2030 [39]. The chronic hyperglycemia causes micro- and macrovascular complications. Type 2 diabetes is additionally associated with increased risk of cardiovascular disease, partly by its relation to obesity and covariate risk factors [40]. Prediabetes development into type 2 diabetes can be prevented by lifestyle changes [41]. However, lifestyle changes alone are not sufficient to overcome the clinical burden of full blown type 2 diabetes [42]. To achieve glycemic control of type 2 diabetes, the drug metformin is used. Reduced plasma glucose is achieved upon metformin treatment via increased glucose clearance and suppressed endogenous glucose production in the liver via activation of the AMP-activated protein kinase pathway, thereby increasing insulin sensitization [43,44]. Despite glycemic control, the impaired function of the pancreatic beta cells leads to progressive loss of these cells. Therefore, there is a need for therapeutics that directly target pathological mechanisms rather than merely normalize the hyperglycemia in type 2 diabetic patients. Thiazolidinediones are a class of compounds that stimulate the nuclear transcription factor peroxisome proliferator-activated receptor gamma (PPARγ). They stimulate the classical insulin receptor pathway, thereby improving insulin sensitivity in liver, muscle and adipocytes [45]. In addition, PPARγ enhances the
differentiation of adipocytes, thereby improving insulin sensitivity [46,47]. Importantly, thiazolidinediones prevent the decline of pancreatic beta cells [48]. Another pathway that can be stimulated is the incretin hormone glucagon-like peptide-1 (GLP-1) pathway. Both GLP-1 receptor agonists and dipeptidyl peptidase-4 (DDP-4) inhibitors are developed which inhibit the breakdown of endogenous hormone levels. These compounds stimulate insulin secretion and inhibit glucagon secretion in a glucose-dependent manner [49]. They also stimulate beta cell function directly and reduce appetite via the brain [50]. Both the thiazolidinediones and the incretin stimulating compounds are not used in clinical practice at a regular basis yet.

**Dyslipidemia**

A disturbed lipid homeostasis can present itself as dyslipidemia. High levels of circulating triglycerides, LDL cholesterol and/or low levels of HDL cholesterol are important markers of metabolic health. Dyslipidemia is associated with pathological conditions such as obesity and cardiovascular disease.

**Obesity**

A failure of the body’s systems to properly cope with the environmental, behavioral, physiological and genetic influences that affect energy homeostasis, results in accumulation of body fat mass [25]. The World Health Organization defines the increased accumulation of body fat mass that will consequently negatively affect an individual’s health as obesity. Obesity is a growing health problem with an almost tripled prevalence in 2016 as compared to 1975, reaching up to 13% of the world’s adult population [51]. Obesity is associated with increased risk of pathological conditions clustered under the name metabolic syndrome including dyslipidemia, hypertension, hepatic steatosis, type 2 diabetes and cardiovascular disease [52]. Adipose tissue is a central player in obesity. Similar to diabetes being a consequence of dysregulated glucose fluxes, obesity is a consequence of dysregulated fatty acid fluxes. As previously mentioned, adipocytes receive the fatty acids they store as triglycerides from triglyceride-rich lipoproteins in the circulation. Besides the uptake of fatty acids after hydrolysis by LPL, adipocytes can release fatty acids generated during lipolysis. These are the two major contributors to the flux of fatty acids. In an obese state, the omental adipocyte depots increase. Omental adipose tissue depots are more sensitive to lipolysis. On the other hand, fatty acid uptake by adipocytes and subsequent incorporation into triglycerides for storage is reduced [29]. As a consequence, the level of fatty acids and triglyceride-rich chylomicron remnants in the circulation increases. The excessive free fatty acids in the circulation cause lipotoxic side effects when taken up by hepatocytes, muscle and pancreatic beta cells [53]. Oxidized fatty acids in the liver stimulate VLDL production, hepatic gluconeogenesis and reduce the uptake of insulin [54,55]. Ultimately this can lead to dyslipidemia and insulin resistance [56]. As a protective mechanism to counteract the influx of free fatty acids in the liver, triglyceride synthesis is increased [57]. The accumulation of triglycerides in the liver is termed hepatic steatosis [58].
Cardiovascular disease

Initial atherosclerotic lesions

The endothelial cell layer inside the blood vessels is a specialized barrier protecting the integrity of the arterial wall. This monolayer of cells is challenged by local hemodynamic factors. At areas like the inner curvature of the coronary arteries where the shear stress is low or near bifurcations where the shear stress is oscillatory, endothelial cells respond by altering gene expression [59]. Cardiovascular disease risk factors such as smoking and diabetes also contribute to endothelial dysfunction [60,61]. These changes translate in morphological alterations of the endothelial cells, compromising the barrier function of the cells [62]. There is an increased turn-over of endothelial cells, increased permeability and increased expression of adhesion molecules for leukocytes on the surface of the cells [63]. Macromolecules such as LDL can now also readily enter the sub-endothelial space called the arterial intima. The arterial intima is rich in proteoglycans, highly charged macromolecules. By binding of proteoglycans to the apolipoproteins on the LDL particles, these lipoproteins are trapped in the intima and undergo modifications [64]. Enzymes that are present at the site can oxidize the LDL molecules resulting in the very atherogenic oxidized LDL moieties (oxLDL). Oxidized LDL aggravates the local pro-inflammatory environment by increasing the expression of adhesion molecules on endothelial cells such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), P-selectin and E-selectin [65]. Furthermore, inflammatory markers aiding in the recruitment and maturation of inflammatory cells like C-C Motif Chemokine Ligand 2 (CCL2), Macrophage Colony Stimulating Factor 1 (MCSF) and Granulocyte-Macrophage Colony Stimulating Factor (GMCSF) are produced as reaction to the presence of oxLDL [66]. Monocytes circulating in the blood are “captured” by the adhesion molecules presented by the dysfunctional endothelial cells and subsequently migrate into the arterial intima [67]. Under the influence of the locally produced inflammatory mediators like MCSF, these monocytes differentiate into macrophages. These macrophages recognize the oxidation-specific epitopes on the LDL particles via their scavenger receptors and engulf the oxLDL. The lipid-laden macrophages are now referred to as foam cells; the hallmark of initial atherosclerotic lesions [68]. The accumulation of lipids and foam cells inside the arterial intima is called a fatty streak. Fatty streaks are developed at susceptible sites throughout the arteries from a young age, however they are clinically silent [69]. The process of initial atherosclerotic lesion formation is schematically depicted in figure 3.

Advanced atherosclerotic lesions

As the foam cells within the fatty streak take up more oxLDL, they expand and eventually die, leaving the cytotoxic cellular debris exposed to the microenvironment. In atherosclerosis, the process of clearing the dead cells called efferocytosis, is insufficient leading to the formation of a necrotic core consisting of the cellular debris, lipids and cholesterol crystals [70]. This further aggravates the pro-inflammatory environment, leading to the recruitment and accumulation
of leukocytes. Besides macrophages, immune cells from both the innate and adaptive immune system are attracted to the atherosclerosis site [71]. The presence and interactions of dendritic cells and T-lymphocytes increase the levels of cytokines and chemokines inside the lesions [72]. Essentially, this system is in place to manage the damaging processes in the arterial wall. Besides the attraction of leukocytes, smooth muscle cells (SMC) are recruited to the atherosclerotic lesion. Differentiation and proliferation of SMC initiated by the microenvironment, leads to the formation of a layer of SMC covering the lesion in a fibrous cap [73]. The lesion is further stabilized by the production of extracellular matrix components by the SMC. The growth of the necrotic core and fibrous cap of the atherosclerotic lesions can result in the reduction of the arterial lumen, called stenosis. Severe stenosis can clinically present itself as stable angina pectoris [74]. Partial obstruction of the blood flow through the coronary arteries results in chest pain during exercise in patients suffering from stable angina pectoris. Stable atherosclerotic lesions can be destabilized by activated T-lymphocytes, macrophages and mast cells that produce enzymes degrading the extracellular matrix. Furthermore, SMC apoptosis induced by pro-inflammatory cytokines leads to thinning of the fibrous cap [75]. This results in a vulnerable lesion that is prone to rupture. As a result of the rupture, the content of the atherosclerotic lesion is exposed to the circulation and initiates thrombus formation. The total obstruction of the arterial lumen leads to severe ischemic events such as myocardial infarction and cerebral stroke.

**Immune system and atherosclerosis**

Atherosclerosis is considered a chronic low-grade inflammatory disease in response to lipids. It has been shown that patients with chronic inflammatory diseases like arthritis, systemic
lupus erythematosus and psoriasis have increased risk for developing cardiovascular disease [76]. As indicated above, both the innate and the adaptive immune system are involved in the pathophysiology. From thorough characterization of atherosclerotic lesions, it has been found that the most abundant cell types are macrophages and T-lymphocytes [77]. These major leukocyte classes and their role in atherosclerosis are highlighted in detail below.

Macrophages
Macrophages are central to the initiation of atherosclerosis as a result of their combined actions when activated: regulating lipid uptake in the intima, producing pro-inflammatory mediators, interacting with cells from the adaptive immunity and contributing to vascular repair and remodeling processes. Two major macrophage subtypes can be distinguished: M1-type pro-inflammatory macrophages and M2-type anti-inflammatory macrophages. Under the influence of the atherosclerotic microenvironment, macrophage polarization occurs constantly. One of the major contributors to the microenvironment in developing lesions are modified lipids and lipoproteins. Cholesterol crystals, oxLDL and oxidized cholesteryl esters activate intracellular cascades polarizing macrophages to an M1 type [78,79]. In line, histological analysis has revealed that M1 macrophages have been found mostly in proximity to the lipid core, while M2 macrophages are located in the shoulder regions of human atherosclerotic lesions [80]. Furthermore, transcriptomic analysis of murine atherosclerotic regression models have shown that regressing lesions are especially enriched in M2 macrophages [81]. Although the classical M1 and M2 (including M2a, M2b, M2c, and M2d subtypes) account for the majority of macrophage subtypes identified in atherosclerotic lesions, also other populations of macrophages have been identified such as the Mox, M4 and Mhem's [82,83]. Each subtype has its own markers and contribution to atherosclerosis. M1 and M4 seem to be predominantly linked to pro-atherogenic effects while M2, Mhem and Mox seem mostly atheroprotective. However, it is important to note that although M2 macrophages are considered atheroprotective based on their anti-inflammatory properties in later stages of the disease, in the initial stage of atherosclerosis these cells are more prone to become foam cells which results in a switch of the M2 macrophages to a proinflammatory/pro-atherogenic phenotype [79]. It is still under debate whether these different macrophage subsets are derived from local polarization or the infiltration of different monocyte precursors or a combination [83]. As the techniques identifying and characterizing these different subsets improve, the research field will keep expanding, becoming more and more complex.

T-lymphocytes
Antigen presenting cells (APCs) such as macrophages and dendritic cells, engulf and process antigens from their environment. Subsequently, these cells migrate to the draining lymph node where they activate T-lymphocytes via interaction with T-cell receptors present on the cell surface. There are two major T-lymphocyte subsets: CD4+ and CD8+ T-lymphocytes.
Each subset is defined by their interaction with the APCs. The majority of T-lymphocytes present in atherosclerotic lesions are CD4+ T-lymphocytes, although CD8+ T-lymphocytes can also be found [84]. Depending on co-stimulatory signals present on both the APCs and T-lymphocytes as well as cytokines, T-lymphocytes can become activated effector, anergic, regulatory or memory T-lymphocytes. Activated T-lymphocytes proliferate in the lymph node and migrate towards the inflammatory site. Depending on environmental factors, T-lymphocytes can differentiate into cell subsets such as cytotoxic T-lymphocytes, T-helper cells (Th1, Th2, Th17 etc.) or T-regulatory cells [85]. Total body depletion of all T-lymphocytes using anti-CD3 treatment reduces initial atherosclerotic lesion formation, lesion progression and increased lesion regression in mice [86,87]. Additionally, different immune-deficient mouse models lacking T-lymphocytes show significant reductions in atherosclerotic lesion development [88,89]. However, it should be acknowledged that each specific T-lymphocyte subset has its unique contributions to the physiopathological processes, some atheroprotective and some atherogenic. In addition, modulation of the activity of one T-lymphocyte subset can indirectly affect another. The different T-lymphocyte subsets and their effects are nicely reviewed by Ketelhuth and colleagues [90]. Although we can conclude that T-lymphocytes impact on atherosclerosis susceptibility, more research into the specific contribution of the individual subsets is needed to obtain a complete picture.

Therapeutic strategies for cardiovascular disease

The primary treatment strategy of patients with coronary heart disease is a combination of lifestyle changes and medical therapy. As high levels of cholesterol cause endothelial dysfunction and is essential for inducing foam cell formation, reducing the dietary intake of lipids and cholesterol can potentially lower the risk of atherosclerotic lesion formation. Furthermore, physical exercise reduces the risk of cardiovascular events, presumably by reducing risk factors like hypertension, insulin resistance and obesity [91]. To support dietary and lifestyle interventions, pharmacological reduction of cholesterol levels by statins is often used. Statins inhibit the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), which is the rate limiting enzyme for cellular cholesterol synthesis. By decreasing the production of cholesterol, the circulating cholesterol levels are indirectly lowered [92]. As a result of reduced cholesterol production in the liver, the expression of the LDL receptor is increased, strongly decreasing LDL-cholesterol levels in the circulation [93]. Besides its lipid lowering properties, statins have off-target side effects that benefit the therapeutic goals as reviewed by Bellosta at al [94]. Despite the therapeutical value of statins, it is noteworthy that the cardiovascular disease risk is only reduced with 25% by statin treatment [95].

In addition to statins or as an alternative for patients that have side-effects from statin use, a novel cholesterol lowering therapy has been developed, inhibiting pro-protein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 is an enzyme that is involved in the intracellular recycling of the LDLr. By promoting the degradation of the LDLr in endosomal and lysosomal
vesicles, less LDLr is available at the cell surface of the liver \cite{96}. Variations in the PCSK9 gene in humans are associated with high cholesterol and increased cardiovascular disease \cite{97,98}. While different approaches are undertaken to reduce the levels of PCSK9 for therapeutic purposes, antibody treatment against PCSK9 is in the most advanced stages of development promising effective and safe treatment \cite{99}. Besides lowering cholesterol levels, a successful trial has shown that lowering the inflammatory component in cardiovascular disease by antibody treatment targeting interleukin 1β, lowers the risk of events on top of regular statin use \cite{100}. However, the safety and efficacy of these novel treatment approaches should be evaluated on the long-term.

Surgical intervention might be required to prevent future cardiovascular events or to restore blood flow to the affected areas after an event. Removing the atherosclerotic lesion as a whole during endarterectomy surgery is a possibility \cite{101}. However, especially for patients with complex multiple occluded vessels, coronary-artery bypass grafting has been the preferred treatment option since the introduction in 1968 \cite{102}. Alternatively, balloon angioplasty or placing a stent are less invasive methods to resume blood flow through the occluded vessels \cite{103}. Unfortunately, localized endothelial damage caused by the surgical intervention can cause excessive SMC proliferation and neointima formation, narrowing the lumen of the blood vessel in a process called restenosis \cite{104}. To overcome these problems, drug eluding stents have been used to reduce SMC proliferation and thereby the extent of restenosis. However, poor arterial healing and thrombosis remain common complications.

There is clearly a high need of novel therapeutic options to treat cardiovascular diseases. Therefore, understanding the pathophysiology is essential. Most pre-clinical research is done in murine models for cardiovascular disease because of the extensive knowledge on mouse genetics and biology as well as the low costs of purchase, breeding and housing. However, using murine models in atherosclerosis research has its challenges. Mice have a majorly different lipoprotein profile with high HDL-cholesterol levels as compared to humans that exhibit high LDL-cholesterol levels under physiological conditions. To create mice that are susceptible for atherosclerosis, their lipoprotein profile is made hyperlipidemic (an increase in the atherogenic LDL/VLDL-cholesterol levels) rather than dyslipidemic as seen in patients with cardiovascular disease (high triglyceride and low HDL-cholesterol levels). A combination of increased dietary cholesterol in a Western-type diet (containing 0.25%kcal cholesterol in addition to 15% fat as cocoa butter) and a genetic predisposition for hyperlipidemia is used to induce atherosclerosis in mice. The most widely used genetic models are the LDLr knock-out (KO) mice and ApoE KO mice. The LDLr KO mice are developed based on the genetic cause of a severe hyperlipidemia seen in humans as a result of mutations in the LDLr: familial hypercholesterolemia. Due to defective uptake of LDL particles from the circulation, LDL-cholesterol levels rise. On a Western-type diet, LDLr KO mice develop severe hypercholesterolemia and atherosclerosis \cite{105}. ApoE is the apolipoprotein found on remnant lipoproteins. As the ligand for lipoprotein uptake receptors in the liver is lacking as a result of the ApoE
deficiency, the lipoproteins in ApoE KO mice cannot be cleared from the circulation [106]. This leads to hypercholesterolemia, mainly driven by an increase in circulating VLDL and chylomicron remnants, and the development of atherosclerotic lesions even without dietary intervention. To study gene specific effects in cardiometabolic disease in vivo, double KO (dKO) mice can be generated. Hereto, knockout mice of the gene of interest are crossbred to one of the atherosclerosis-prone mouse models. As a more rapid and cost-effective alternative to crossing with hypercholesterolemic mice, PCSK9 - adeno-associated viruses (AAV) have been generated [107,108]. After a single AAV injection carrying a gain-of-function PCSK9 mutant in combination with Western-type diet feeding, mice show a sustained hyperlipidemic profile with increases in both LDL-cholesterol and VLDL-cholesterol, leading to the development of atherosclerotic lesions [108]. Macrophages and other bone marrow-derived cells are of special interest in the field of atherosclerosis as they are essential in the disease etiology. As a relatively fast method to investigate the effect of a genetic deletion specifically in bone marrow-derived cells on atherosclerotic lesion development, a bone marrow transplantation procedure using bone marrow from genetically modified animals into hyperlipidemic mice is a well-established model [109]. After eradication of the endogenous bone marrow cells by whole body Röntgen-irradiation, bone marrow derived from donor mice is injected into the tail vein of the irradiated recipients. Hereafter, the donor bone marrow cells eventually home to the bone marrow niche of the recipient mice where they replace the function of the endogenous bone marrow.

The interplay between different pathologies: cardiometabolic disease

From epidemiological studies, it is clear that an association exists between diabetes, obesity and cardiovascular disease [110,111]. In large cohorts, the incidence of cardiovascular disease is positively correlated with increased weight. Similarly, in the highest quintiles of body mass index (BMI) the mortality from cardiovascular disease is increased. Increased body weight is often accompanied by increased risk of hypertension, dyslipidemia and diabetes and early atherosclerotic lesions, also in children [112]. This actually predisposes them for cardiometabolic diseases later on in life. The common coherence of these risk factors was first described by Reaven and colleagues and is now commonly known as metabolic syndrome [113]. The cardiovascular disease mortality is increased 2-fold, while the incidence of an ischemic cardiovascular event such as heart attack or stroke is tripled in individuals with metabolic syndrome [114].

It is widely discussed whether the metabolic syndrome has one unified physiopathology that eventually adds up to cardiovascular disease. Evidence from a large epidemiological study suggests that obesity is the instigator of the metabolic syndrome [115]. This hypothesis is supported by studies investigating the underlying pathophysiological processes. The increased free fatty acid levels and adipokines that are released from the white adipose tissue under obese conditions are found to impair insulin actions in peripheral tissues and are implicated
in insulin resistance [116,117]. In addition to its proposed effects on insulin resistance, free fatty acids and adipokines directly affect vasodilatation and vascular production of nitric oxide, thus providing a direct link between obesity, hypertension and cardiovascular disease [118]. In another hypothesis, inflammation is the link connecting the risk factors in metabolic syndrome as reviewed by Lee and colleagues [119]. The intake of macronutrients has been shown to induce inflammatory signaling pathways directly [120,121]. Low-grade chronic inflammation forms the common ground as underlying cause for type 2 diabetes as well as atherosclerosis. However, the pathways involved in the physiological and pathophysiological regulation of metabolism are plethoric and complex. Therefore it is challenging to determine the existing interrelationships and the direction of the causal pathways. Further insight in these processes is essential in order to unravel a potential common underlying casual mechanism. A schematic overview of the interactions between tissues and pathologies is presented in figure 4.

As for atherosclerosis, murine models are often used to investigate these processes. Unfortunately, mice on a regular diet do not develop cardiometabolic disease. This phenotype can be induced by diet or by genetic modulation. High fat diets are used to induce obesity, insulin resistance and hypertension [122]. These diets contain either 45% kcal or 60% kcal fat as lard in complement to 20% kcal protein and either 35% kcal or 20% kcal carbohydrates. In general,
these diets simulate the situation in humans as the diet is the major instigator of metabolic syndrome also in humans. As a downside, the relative susceptibility is dependent on the background strain. The most widely used obesity-prone murine models for metabolic syndrome are derived from spontaneous mutations showing an obese phenotype with insulin resistance. These models include leptin deficient (Lep\textsuperscript{ob/ob}), leptin receptor deficient (LepR\textsuperscript{db/db}) mice and the agouti yellow obese Ay/a mice. In combination with the obese and insulin resistant phenotype, these mice show increased plasma lipid levels [123]. Unfortunately, the increase in plasma lipid levels are merely an increase in the naturally present HDL levels. These mice therefore do not spontaneously develop atherosclerosis [123]. By cross-breeding these mice onto an atherosclerotic prone background like LDLr KO or ApoE KO mice, a more complete model for cardiometabolic disease can be created. Alternatively, adding a high fat component to these hypercholesterolemic murine models will induce obesity and insulin resistance in these mice, providing a tool to study cardiovascular disease under metabolic syndrome conditions [107,124].

**Thesis outline**

The metabolic syndrome is a major risk factor for developing severe metabolic diseases such as hepatic steatosis and coronary heart disease. Current treatment of cardiometabolic diseases relies mainly on lifestyle changes and therapeutic lowering of plasma lipid levels. However, there is a considerable amount of residual risk, leaving patients vulnerable for cardiovascular events. Understanding the underlying mechanisms is deemed crucial to identify specific targets for the development of novel effective therapeutic strategies. There are numerous different approaches to target identification, however nowadays they are mostly founded on the enormous progress in gene sequencing and information technology. Identifying disease-modifying genes allows for the understanding of disease etiology on a fundamental level; the genetic control of cellular functions [125]. Classically, these genetic studies were pedigree-based. In this approach, genetic variants are analyzed within families displaying extreme disease phenotypes such as pre-mature atherosclerosis. With the advances in genetic screening, the feasibility and affordability of genome-wide screening in large study populations resulted in the rise of Genome-wide association studies (GWAS). GWAS identifies genetic loci that are associated with a predisposition for a specific disease [126]. In addition to these broad approaches, a more focused approach can be undertaken, investigating gene expression in a tissue or cell specific-model for the disease. Diseased tissue can often be identified based on its gene expression profile. Compared to tissues in a physiological state, genes can be over- or under expressed or in extreme cases new genes can be expressed or gene transcripts can be completely absent. This phenomenon is studied using microarrays or RNA-Seq on whole tissues such as atherosclerotic lesions or white adipose tissue [127] or in
specific cells such as foam cells or pancreatic islet of Langerhans cells [128]. It is important to note that post-transcriptional modification and regulation can hamper the direct translation of gene expression levels to physiological activity. Alternatively, proteomic approaches instead or complimentary to the genomic-based approaches could be valuable for the identification of potential therapeutic targets [129].

In this thesis, we have combined multiple of these approaches to select three different targets for validation in cardiovascular and metabolic disease. A microarray of murine atherosclerotic lesion material in different stages of development was used to identify differentially expressed genes. From this data set we identified Proteoglycan 4 (Prg4) as one of the most regulated genes in initial atherosclerotic lesion formation. More specifically, Prg4 expression was 92-fold induced during the first 2 weeks of lesion development. Interestingly, genetic variations within this gene were also identified in a pedigree with premature atherosclerosis (J.C. van Capelleveen, G.M. Dallinga-Thie and K.G. Hovingh, AMC Amsterdam, unpublished data). Prg4 is a secreted and ubiquitously expressed proteoglycan. Although the proteoglycan has been implicated in multiple processes such as lubrication of joints and hematopoietic cell proliferation, a role in cardiovascular disease has not yet been described [130,131]. Importantly, other proteoglycans are established contributors to atherosclerosis [132–134]. In addition to its associations in cardiovascular disease, proteomic and transcriptomic approaches have also implicated Prg4 in the metabolic syndrome. In obese study populations, associations have been described between plasma PRG4 levels and measures of glucose tolerance, plasma lipid levels and body weight in a clinical setting [135–137]. This combination made Prg4 an interesting candidate gene for validation as potential therapeutic target in both cardiovascular and metabolic disease. we investigated the role of macrophage specific Prg4 deficiency in atherosclerosis in Chapter 3. We further expanded on this research by investigating the role of total-body Prg4 deficiency in atherosclerosis in Chapter 4. Furthermore, the potential role of Prg4 in the development of metabolic syndrome in was investigated in Chapter 5.

In addition to the microarray on developing atherosclerotic lesions, we used a microarray to identify genes that are differentially expressed during macrophage foam cell formation. Stabilin-1 (Stab-1) appeared to be downregulated upon loading of macrophages with oxLDL. Subsequent verification of Stab-1 in the microarray of the atherosclerotic lesions, revealed that Stab-1 was also differentially expressed upon lesion development. Interestingly, similar to Prg4, genetic variations in the Stab-1 gene were also identified in the genetic analysis of a family with premature atherosclerosis (J.C. van Capelleveen, G.M. Dallinga-Thie and K.G. Hovingh, AMC Amsterdam, unpublished data). Based on these combined findings, we selected Stab-1 for validation as a potential therapeutic target in cardiovascular disease. Notably, Stab-1 is a scavenger receptor that is expressed on sinusoidal endothelial cells and macrophages [138]. Among the ligands of Stab-1 is oxLDL [139]. The role of macrophage scavenger receptors has been extensively studied, highlighting their relevance in atherosclerosis [140–142]. Therefore, we investigated the effect of macrophage Stab-1 deficiency on atherosclerosis outcome in
Chapter 2. Another target we identified based on its differential expression in foam cells is protein arginine methyl transferase 3 (PRMT3). Within the microarray, it is one of the most upregulated genes upon foam cell induction. We verified that PRMT3 was present and differentially expressed in murine atherosclerotic lesions in the microarray of the developing atherosclerotic lesions. Upon further literature research we found that PRMT3 might interact with LXR, thereby modulating its activity [143]. LXR is one of the master transcriptional regulators of macrophage cholesterol metabolism as well as hepatic cholesterol and lipid metabolism [144]. Because of its central role, LXR has been implicated in both cardiovascular and metabolic disease [145]. This information combined with the differential gene expression in both microarrays, made us select PRMT3 as a potential therapeutic target. Therefore, we aimed to investigate the role of PRMT3 specifically in liver and macrophage lipid metabolism in Chapter 6 and in a more broader context of metabolic syndrome including hepatic steatosis and atherosclerosis in Chapter 7.
REFERENCES


