INFLAMED FAT: immune modulation of adipose tissue and lipid metabolism

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Chapter 1

General introduction and outline

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Targeting white, brown and perivascular adipose tissue in atherosclerosis development.
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OBESITY, ETHNICITY AND RISK OF METABOLIC DISORDERS

According to the World Health Organization, the worldwide prevalence of obesity, defined as a body mass index (BMI) > 30 kg/m^2, has nearly doubled since 1980 and at least 2.8 million people die each year as a result of obesity. This number is expected to further increase over the next decade. Obesity has a great impact on public health as it leads to disorders such as type 2 diabetes and cardiovascular disease (1, 2). It has become evident that besides BMI, ethnicity determines the risk of developing both type 2 diabetes (3-5) and cardiovascular disease (6-9). South Asians, who originate from the Indian subcontinent and compose 20% of the world population, have a higher risk of developing these pathologies compared to white Caucasians. Despite the presence of predisposing classical risk factors such as central obesity, insulin resistance and dyslipidemia in this population (10, 11), the high prevalence of type 2 diabetes and cardiovascular disease in South Asians cannot be explained by these factors alone (9, 12). This suggests that other, non-classical, risk factors underlie their increased susceptibility (13).

LIPID METABOLISM

Obesity develops as a result of a long-term positive energy balance and storage of lipids in several tissues. The most abundant lipid types in our diet are triglycerides and cholesterol. Triglycerides are the storage form of the main source of energy for the body, *i.e.* fatty acids. Fatty acids are burnt by the heart and muscle to generate adenosine triphosphate (ATP), a molecule that carries energy in the form of a covalent bond holding a phosphate group. ATP is required for many cellular and metabolic processes such as muscle contraction (14). Brown adipose tissue (BAT) uses fatty acids to generate heat rather than ATP (15) (**Fig. 1**). When intake of energy exceeds the body's needs, fatty acids are stored as triglycerides in white adipose tissue (WAT), a process that also uses glucose to generate the glycerol backbone to which fatty acids are esterified (16). For humans, cholesterol does not provide energy, but is an essential component of cell membranes and a precursor for synthesis of steroid hormones, vitamin D, and bile acids. Interestingly, cholesterol from the host is also used by certain bacteria such as *Mycobacterium bovis* as energy source when they colonize a host (17, 18).

Lipids are hydrophobic and thus insoluble in blood, for which they are transported in lipoprotein particles. Lipoprotein particles exist of a lipid-rich core containing triglycerides and cholesteryl esters, the storage forms of fatty acids and cholesterol, respectively, surrounded by an amphipathic monolayer of phospholipids and unesterified cholesterol in which specific proteins (*i.e.* apolipoproteins) are embedded. Upon a meal, dietary triglycerides and cholesterol are taken up by intestinal cells, which assemble the lipids into triglyceride-rich lipoproteins carrying apolipoprotein B (apoB) named chylomicrons, that subsequently travel via the lymph to the blood. From the blood, they can provide tissues

with fatty acids. The liver secretes triglyceride-rich lipoprotein particles, named very-low density lipoproteins (VLDL), into the blood to supply tissues for their energy demand as well, which is crucial especially during times of fasting. Chylomicrons and VLDL deliver fatty acids mainly to heart, muscle and adipose tissues as these tissues express lipoprotein lipase (LPL), an enzyme essential for lipolysis of triglycerides within these lipoproteins. Subsequent uptake of the disengaged fatty acids is mediated by expression of cell surface receptors such as CD36. During lipolysis, the triglyceride-rich lipoproteins become enriched with apolipoprotein E (ApoE). ApoE binds the low density lipoprotein receptor (LDLR) or LDL-related protein (LRP) on the liver, ensuring hepatic clearance of the remnant particles (14, 19).

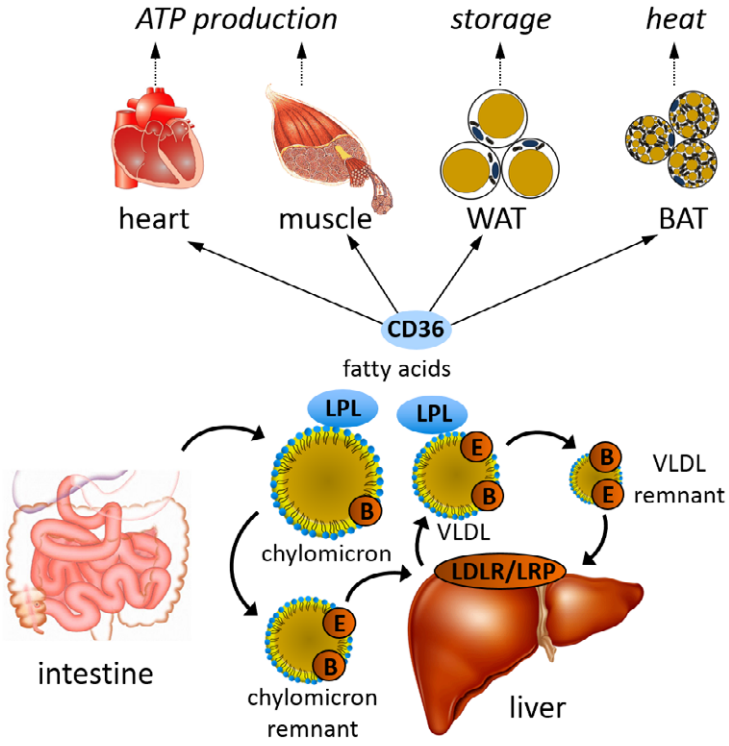

Figure 1. Schematic representation of triglyceride-rich lipoprotein metabolism. *See text for explanation. ATP, adenosine triphosphate; B, apolipoprotein B; BAT, brown adipose tissue; E, apolipoprotein E; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; LRP, low density lipoprotein-related protein; TRL, triglyceride-rich lipoprotein; VLDL, very low density lipoprotein; WAT, white adipose tissue.*

ADIPOSE TISSUE TYPES

Adipose tissue is the main site for energy storage during obesity. WAT is the most abundant adipose tissue type, found throughout the body in different subcutaneous and visceral depots (20). WAT is a major participant in energy regulation of the body, not only by storing excess ingested glucose and fatty acids in the form of triglycerides in the adipocytes but also by releasing fatty acids as a result of intracellular lipolysis to meet the energy needs of other organs. In addition, WAT is an endocrine organ. It responds to hormonal signals and signals from the nervous system and it expresses and releases endocrine factors including leptin, adiponectin, cytokines, chemokines and components of the complement system. This endocrine function of WAT influences essential metabolic processes, including lipid and glucose homeostasis (21). The spherical adipocytes within WAT characteristically contain a single large lipid droplet and a few mitochondria that are dispersed in a thin surrounding layer of cytoplasm.

Another type of adipose tissue is BAT. This tissue is found in the neck, above the clavicles and around the spine in humans (22-24). In contrast to WAT, which stores energy, BAT combusts fatty acids to generate heat and maintain body temperature, a process that is defined as non-shivering thermogenesis. Hence brown adipocytes, that are smaller than white adipocytes, contain many mitochondria and typically hold multiple small lipid droplets (20). Thermogenesis is induced by catecholamine signaling. Catecholamines signal via all types of adrenergic receptors including β1, β2, β3, α1 and α2, although not all of these have stimulatory effects on thermogenesis. Expression of the stimulatory β3-adrenergic receptor on brown adipocytes is likely the most specific and relevant for heat production (25). Apart from adrenergic receptor expression, thermogenesis is dependent on intracellular lipolysis and the presence of uncoupling protein 1 (UCP1). Intracellular lipolysis of triglycerides yields fatty acids, which are used as substrates for heat production and can directly activate UCP1. UCP1 resides in the inner mitochondrial membrane and, upon activation, facilitates proton leak, which disturbs the proton gradient that is generated by oxidative phosphorylation resulting from fatty acid oxidation. This “uncouples” electron transport from synthesis of ATP and leads to production of heat (25, 26). From rodent studies, it is known that besides classical brown adipocytes with high UCP1 expression, brown-like cells with very low basal but highly inducible UCP1 expression exist. These cells usually reside in WAT and are called beige adipocytes, and considerable evidence suggests that human BAT is mainly composed of beige adipocytes (27-29).

In addition to adipocytes, adipose tissue is composed of pre-adipocytes, endothelial cells, fibroblasts, nerve tissue and immune cells. This contributes to the complexity and metabolic and endocrine functions of adipose tissue. Adipose tissue not only responds to hormonal and neural signaling, it also expresses and secretes a range of factors named adipokines. These factors include cytokines, chemokines and complement components (21). The secretion of adipokines accounts for the role of adipose tissue in systemic signaling pathways, such as regulation of energy balance and inflammation (30, 31). Differences exist in the complexity and endocrine functions of adipose tissue depots. For example, BAT is

more densely vascularized and innervated than WAT (20) whereas the transcript levels of inflammatory genes in BAT are generally lower than in WAT (32, 33).

WHITE ADIPOSE TISSUE INFLAMMATION IN OBESITY

Increased storage of lipids in WAT during obesity leads to hypertrophy of adipocytes, hypoxia, and eventually cell death, causing adipose tissue dysfunction and fibrosis. Dysfunctional and dying adipocytes change the local microenvironment by leakage of fatty acids and other products. The altered microenvironment causes release of adipokines (*e.g.* IL-6, TNF, CCL2) and recruitment of inflammatory cells by WAT (31, 34).

In WAT of lean mice, 10-15% of the cells are macrophages, whereas WAT of obese mice contains 45-60% macrophages (35). Resident macrophages in lean WAT have a predominant anti-inflammatory or M2 phenotype. In obesity, inflammatory monocytes are recruited to WAT, where they differentiate, acquire a pro-inflammatory or M1 phenotype and form the majority of macrophages (35, 36). Other myeloid cells that play a role in WAT include neutrophils and eosinophils. Neutrophils are very short-lived cells that infiltrate the WAT within 3 days after exposure to a high-fat diet (37, 38). The amount of eosinophils is inversely correlated with adiposity, and depletion of eosinophils in mice results in obesity and insulin resistance (39).

The repertoire of immune cells found in WAT also comprises lymphoid cells. More specifically, 10% of the stromal vascular fraction of lean WAT consists of T cells. During obesity, the total amount of T cells, $CD8^+$ cytotoxic T cells and $CD4^+$ effector T cells in WAT increase, whereas $CD4^+$ Tregs decrease (40). Furthermore, pro-inflammatory Th1 T cells increase while more anti-inflammatory Th2 T cells decrease, altogether resulting in increased inflammation in obese WAT (39, 41-43). The chronic low-grade inflammation in obese WAT includes the recruitment of B cells, natural killer cells and mast cells (44, 45). Overall, a variety of immune cells infiltrate WAT during obesity, inducing a switch from a homeostatic anti-inflammatory environment to a state of chronic low-grade inflammation.

Due to the extensive communication between adipocytes and immune cells, chronic inflammation as occurs in obesity disturbs insulin signalling in the tissue (34, 46) thereby contributing to development of type 2 diabetes as will be further described below.

IMMUNE MODULATION OF BROWN AND BEIGE ADIPOSE TISSUE

In contrast to the well-established presence of different immune cell types in WAT, the presence and role of the immune system in the development, function and activity of BAT is still largely unknown. Interestingly, many factors that modulate energy expenditure, the hallmark of BAT activity, affect inflammation too, all in all suggesting that the immune

system is involved in BAT function. These factors include environmental temperature (47, 48), the biological clock (49, 50), hormones (51, 52) and food intake (53).

Macrophages, the most abundant immune cells in WAT, are present in BAT as demonstrated by flow cytometry and qPCR (54). Evaluation of gene networks in BAT shows that immune cell trafficking is upregulated upon high-fat diet feeding (55). Furthermore, like WAT, BAT secretes adipokines (*e.g.* IL-6). Although the transcript levels of pro-inflammatory cytokines in BAT are lower compared to WAT, augmented expression of pro-inflammatory cytokines has been found in obese compared to lean BAT. These pro-inflammatory factors are thought to diminish BAT function (30).

The limited research that has been done into the role of immune cells in BAT may suggest that macrophages play a role in non-shivering thermogenesis. Cold exposure increases M2 macrophages in both WAT and BAT, which release the catecholamine noradrenaline that in turn activates BAT and induces browning of WAT (54, 56). Eosinophils indirectly contribute to thermogenesis by promoting activation of M2 macrophages within adipose tissue (56, 57). Besides macrophages, type 2 innate lymphoid cells are important mediators of beiging of WAT (58). So, although knowledge about the role of immune cells in BAT and beiging WAT lags behind, inflammation is probably an important player in brown adipocyte function as well.

INFLAMMATION SIGNIFICANTLY CONTRIBUTES TO TYPE 2 DIABETES AND CARDIOVASCULAR DISEASE

A key mechanism by which inflammation leads to insulin resistance is inhibition of insulin signaling pathways in several metabolic tissues (59). After a meal, insulin is released by the pancreas and acts on its target tissues including muscle, liver and adipose tissues. Stimulation of the insulin receptor induces the uptake of glucose by these tissues (60, 61). The inflammatory cytokines TNF and IL-6, and the acute phase protein C-reactive protein (CRP) can directly inhibit the insulin pathway and thereby induce insulin resistance (62-64). Moreover, inflammation activates a number of intracellular serine/threonine kinases such as c-Jun N-terminal kinases (JNKs) and IκB kinase (IKK) which inhibit insulin signaling. Inhibition of insulin signaling leads to peripheral insulin resistance and subsequent hyperglycemia, which can result in development of type 2 diabetes (59). On top of that, since insulin normally reduces VLDL production, decreased insulin sensitivity in the liver leads to enhanced production of VLDL and thereby to hyperlipidemia (65).

Atherosclerosis is the pathology underlying cardiovascular disease, and the main risk factor for the development of atherosclerosis is hypercholesterolemia. Atherosclerosis development is initiated by retention of low-density lipoproteins (LDL) and very-low-density lipoprotein (VLDL) remnant particles in the vessel wall. In response, the vessel wall releases oxidative and pro-inflammatory factors that modify these particles, for example by oxidation, resulting in oxidized LDL (oxLDL) (66, 67). LDL is also modified by hydrolyzing enzymes that are present in the lesions (68, 69). Immune cells, mainly monocytes, are

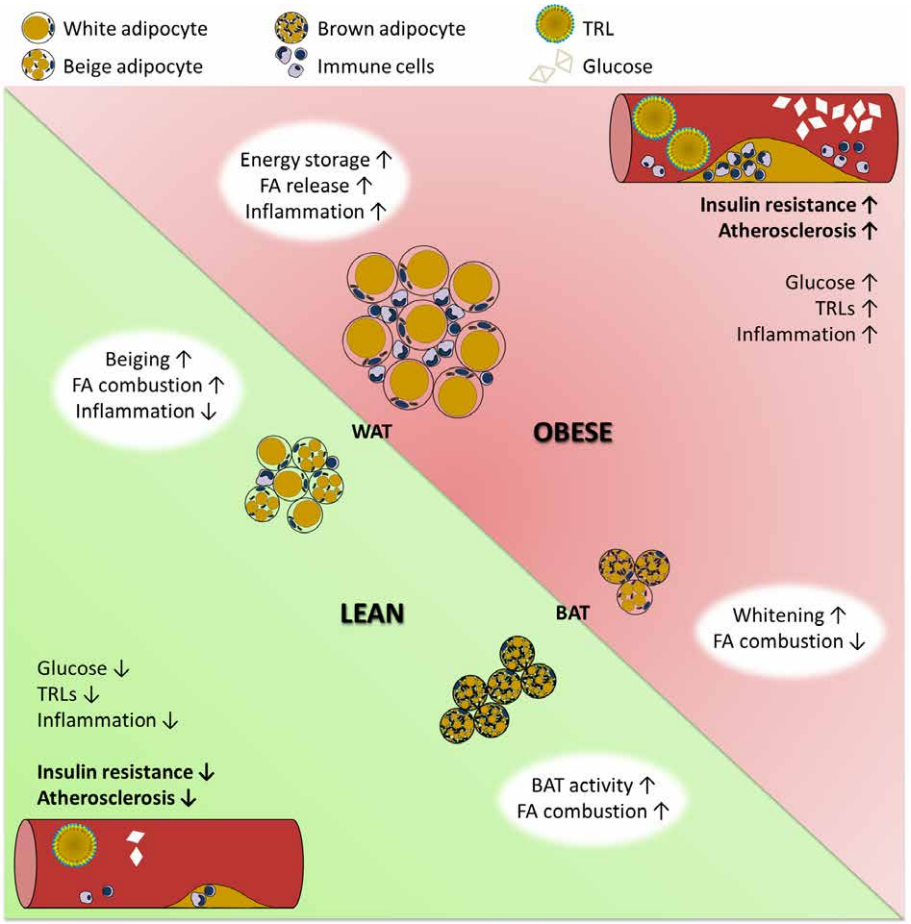

Figure 2. The proposed role of white and brown adipose tissue in the development of insulin resistance and atherosclerosis during obesity. *In obesity, both energy storage and fatty acid release are enhanced in white adipose tissue (WAT). Immune cells infiltrate the WAT, causing systemic inflammation. Brown adipose tissue (BAT) displays a whitened phenotype in obesity, resulting in decreased combustion of fatty acids. Therapeutic strategies to reduce insulin resistance and atherosclerosis should on the one hand focus on activation of brown adipocytes and beiging of white adipocytes to reduce circulating triglyceride-rich lipoproteins, and on the other hand on reducing adipose tissue and systemic inflammation. BAT, brown adipose tissue; FA, fatty acid; TRL, triglyceride-rich lipoprotein; WAT, white adipose tissue.*

recruited from the circulation upon the expression of chemoattractants like tumor necrosis factor (TNF) (70) and monocyte chemoattractant protein-1 (MCP-1) in the lesions (71). Locally produced factors, such as monocyte-colony stimulating factor (M-CSF) (72), promote the differentiation of monocytes into macrophages. Macrophages scavenge the accumulating and modified lipoproteins thereby developing into foam cells. Foam cells augment the inflammatory response, resulting in additional recruitment of immune cells

into the atherosclerotic plaque (66, 67). Ultimately, plaques can rupture and/or occlude the vessel, leading to a cardiovascular event in *e.g.* the heart (myocardial infarction) or brain (stroke) (71). In many cell types that are involved in buildup of the atherosclerotic plaque, intracellular inflammatory pathways (*e.g.* JNK and IKK) and transcription factors (*e.g.* nuclear factor κ-light-chain-enhancer of activated B cells; NFκB) control the expression of adhesion molecules and inflammatory chemokines and cytokines. Besides local inflammation in the vasculature, other peripheral sources of inflammation can augment the development of atherosclerosis, including chronic infection (73, 74) and obese liver and WAT (75).

Taken together, inflammation is an important factor in the development of both type 2 diabetes and cardiovascular disease. Together with therapeutic interventions that activate BAT or induce browning, resulting in enhanced nutrient combustion, strategies that reduce inflammation may help to mitigate these pathologies (**Fig. 2**).

OUTLINE OF THIS THESIS

As is evident from this chapter (**chapter 1**) of this thesis, inflammation plays an important role in the development of insulin resistance and atherosclerosis by disturbing both WAT and BAT, and targeting inflammation may thus hold therapeutic potential. Although widely used compounds to treat type 2 diabetes and cardiovascular disease, such as respectively metformin and statins, have pleiotropic anti-inflammatory effects (76-78), no strategies currently exist that primarily target inflammation in the treatment of these disorders. A better understanding of the interaction between inflammatory pathways in metabolic tissues and metabolic derangements is a prerequisite for the development of novel compounds that dampen inflammation to treat individuals with these pathologies.

The research described in this thesis was performed to address three key objectives: 1) gain more insight into the role of the immune system in obesity, insulin resistance, dyslipidemia and atherosclerosis, 2) study inflammation in a human population with a particularly high risk to develop type 2 diabetes and cardiovascular disease, and 3) study the therapeutic potential of reducing inflammation by pharmacological strategies on obesity and glucose and lipid metabolism in pre-clinical models, with emphasis on BAT and WAT.

To address the first key objective, we studied the effect of a potent inflammatory trigger on lipid metabolism and atherosclerosis. Bacille-Calmette-Guérin (BCG), prepared from attenuated live *Mycobacterium bovis*, is the only licensed and widely used vaccine against tuberculosis and modulates atherosclerosis development via immunomodulatory mechanisms. However, previous studies are conflicting with regards to whether BCG protects against or enhances atherosclerosis and did not take into account the effect of BCG on metabolism of cholesterol, the main driver of atherosclerosis. The aim of **chapter 2** therefore was to elucidate the effect of BCG on cholesterol metabolism and atherosclerosis development. In the next chapter, we switched our focus from immune modulation of lipid metabolism and atherosclerosis to glucose metabolism and type

2 diabetes. During the development of obesity, B cells accumulate in WAT and produce pathogenic IgG antibodies, which contribute to the development of glucose intolerance. IgG antibodies signal by binding to Fcγ receptors (FcγR) and by activating the complement system. In **chapter 3**, the purpose was to investigate whether activation of FcγR and/or complement pathway mediate the development of glucose intolerance in WAT.

To meet our second key objective, we performed a human study in which we compared Dutch South Asians and Dutch white Caucasians. These ethnic groups were selected because of the strikingly elevated risk of developing type 2 diabetes that South Asians have when compared to white Caucasians. Even though their increased risk of type 2 diabetes is well-established and inflammation is known to contribute to the development of this disease, inflammatory pathways in South Asians have not been extensively studied previously. The intent in **chapter 4** was therefore to investigate the inflammatory state of blood and metabolic tissues in Dutch South Asians compared to Dutch white Caucasians.

In the final part of this thesis, studies are described that focus on the treatment of metabolic inflammation and disease by targeting BAT and WAT (key objective 3). Salsalate is an anti-inflammatory drug that improves glucose intolerance and dyslipidemia in type 2 diabetic patients, but the mechanisms behind these effects are unclear. The research in **chapter 5** was designed to unravel these mechanisms. We assessed the effects of salsalate on lipid metabolism and adipose tissue inflammation *in vivo*, and investigated the molecular pathways mediating direct effects of salsalate on brown adipocytes *in vitro*. We then aspired to assess the therapeutic potential of targeting GPR120, a fatty acid receptor that mediates anti-inflammatory and insulin-sensitizing effects. Interestingly, cold exposure markedly increases the expression of GPR120 in BAT and WAT. These immunomodulatory and fat-related properties of GPR120 prompted us to investigate the effects of GPR120 deficiency and treatment with a GPR120 agonist on obesity and lipid metabolism in **chapter 6**.

Finally, the results from these studies and their implications for the development of therapeutic strategies are discussed in **chapter 7**.

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