The handle http://hdl.handle.net/1887/20887 holds various files of this Leiden University dissertation.

**Author:** Vroegrijk, I.O.C.M.
**Title:** Fatty acid metabolism and metabolic inflammation: two important players in the development of insulin resistance
**Issue Date:** 2013-05-16
The metabolic syndrome is defined as a cluster of multiple metabolic abnormalities including obesity, insulin resistance, dyslipidemia and hypertension that co-occur more often than might be expected by chance. Since these abnormalities are all well documented risk factors for cardiovascular disease, the increasing prevalence of the metabolic syndrome in Western societies is a cause for major concern. Diet (e.g. consumption of fruit and vegetables and alcohol), lifestyle factors (e.g. smoking and physical exercise) and genetic makeup influence the susceptibility to develop the metabolic syndrome.

Obesity, in particular visceral obesity, is one of the defining components of the metabolic syndrome. Obesity is a condition in which body fat has accumulated to such an extent that it may impair health. Body Mass Index (BMI), weight in kilograms divided by the square of the height in meters (kg/m²), is a simple index to classify overweight and obesity in adults. Adults with a BMI ≥ 25 are defined as overweight and adults with a BMI ≥ 30 are classified as obese, according to the guidelines of the World Health Organization (WHO). In 2008 1.5 billion adults were overweight. Of these, over 200 million men and nearly 300 million women were obese.

Adipose tissue plays a central role in the development of the metabolic syndrome. Under normal, healthy conditions, adipose tissue stores the excess of nutrients in the form of fat and releases energy under conditions of shortage. Under pathophysiological conditions fat may be disposed in non-adipose tissue (ectopic fat deposition), which will contribute to the pathogenesis of the metabolic syndrome. Excessive adipose tissue expansion may also lead to the development of pathogenic adipose tissue, characterized by chronic low-grade inflammation, which eventually may affect the systemic inflammatory status. This systemic pro-inflammatory status can also contribute to the pathogenesis of the metabolic syndrome by affecting peripheral organs such as the liver, causing hypertriglyceridemia or the vasculature, causing atherosclerosis. In this thesis, I have focused on changes in fatty acid (FA) metabolism and inflammatory status to investigate the effects on obesity, fat deposition, insulin resistance and hypertriglyceridemia, all components of the metabolic syndrome.

Insulin resistance is a condition in which tissues of the body do not respond adequately to the actions of insulin. As a result, insulin induced transport of glucose across the cell membrane into muscle, adipose tissue and heart is impaired. In the liver, insulin inhibits glucose production and insulin resistance compromises the inhibition of glucose production by the liver. This results in fasting and postprandial hyperglycemia. Besides the effects on glucose metabolism, insulin resistance also influences lipid metabolism. Insulin resistance is associated with increased secretion of triglycerides (TG) by the liver and with increased secretion of FA from adipose tissue. Both of these altered conditions can result in hypertriglyceridemia.

In this chapter, FA metabolism is introduced and the role of FA in the pathology of insulin resistance is discussed. Subsequently, metabolic inflammation is introduced, as well as its role in insulin resistance.
Dyslipidemia is an important characteristic of the metabolic syndrome and specifically, elevated plasma TG and decreased high density lipoprotein (HDL) cholesterol. TG, which is composed of glycerol and three FA, and cholesterol are common lipids in our diet and are essential for life because they provide energy and are needed for proper cellular functioning. Lipids are hydrophobic molecules that are insoluble in an aqueous environment such as blood. Therefore, after absorption in the intestine, TG and cholesterol are packed into water-soluble particles called lipoproteins. These lipoproteins have a hydrophobic inner core, containing TG and cholesteryl esters (CE), that is covered by a shell of hydrophilic phospholipids (PL), unesterified cholesterol and proteins, termed apolipoproteins. Lipoproteins are divided into 5 classes, according to their origin and density: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The metabolism of these lipoproteins and the subsequent distribution and cellular processing of TG derived FA is described in the following sections and a schematic overview is depicted in Figure 1. In addition, the role of FA in the pathogenesis of insulin resistance will be discussed.

Figure 1. Schematic overview of lipoprotein metabolism. See text for explanation. CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; FA, fatty acids; LCAT, lecithin:cholesterol acyltransferase; LDLr, LDL receptor; LPL, lipoprotein lipase; LRP, LDLr related protein; PL, phospholipid; PLTP, phospholipid transfer protein; TG, triglycerides.
Formation of lipoproteins

In the intestine, dietary lipids are emulsified by bile acids and subsequently lipolysed by pancreatic lipase to glycerol and FA. FA are absorbed by the epithelial cells of the small intestinal villi, named enterocytes. Inside the enterocytes FA are re-esterified to TG and chylomicrons are formed: very large particles that consist mainly of TG, but also of cholesterol, phospholipids and apoproteins (apoAI, apoAIV, apoB48, apoCI, apoCII, apoCIII and apoE). Chylomicrons are secreted into the lymph, from which they are transported to the blood circulation.

In the fasting state, the liver is the main site of cholesterol and TG secretion. The liver secretes lipids in the form of VLDL particles. VLDL particles are formed within the endoplasmatic reticulum (ER) of hepatocytes where microsomal triglyceride transfer protein (MTP) transfers lipids onto apoB, thereby forming a pre-VLDL particle. This pre-VLDL particle undergoes a second step of lipidation in the Golgi system and is subsequently secreted as mature TG-rich particle. The lipids that are used for loading onto apoB originate from lipoprotein remnants, adipose tissue or can be synthesized de novo by the liver itself. When the VLDL particles have entered the bloodstream, they become enriched with apoCI, apoCII, apoCIII and apoE.

HDL is needed for the removal of excess cholesterol from peripheral tissues back to the liver. Nascent HDL is produced by the liver and intestine from apoAI and PL. This HDL particle can take up cholesterol from various tissues via ATP-binding cassette transporter A1 (ABCA1). Cholesterol is subsequently esterified by lecithin:cholesterol acyltransferase (LCAT) into CE that are stored in the core of HDL. Due to cholesterol accumulation, the HDL particle expands and matures into spherical HDL. HDL-derived cholesterol can be taken up by the liver and secreted in the bile, thus maintaining cholesterol homeostasis. HDL can exchange lipids with other lipoproteins via interaction with phospholipid transfer protein (PLTP), which facilitates the transport of PL from chylomicrons and VLDL to HDL and cholesteryl ester transfer protein (CETP), which can exchange CE from HDL with TG from apoB-containing lipoproteins. When CE is transferred from HDL to apoB containing lipoproteins, a more atherogenic profile is created with low levels of HDL cholesterol and high levels of LDL cholesterol.

Lipolysis of lipoproteins

Lipoprotein lipase (LPL) is the main enzyme responsible for lipolysis of chylomicron and VLDL TG into glycerol and FA. LPL hydrolyses the ester bonds of mono-, di-, and triglycerides. After TG depletion, the resulting chylomicron remnants are taken up by the liver mainly via apoE specific recognition sites such as the LDL receptor (LDLr) and the LDLr related protein (LRP). IDL and LDL particles, formed after lipolysis of VLDL particles, can also be cleared by the LDLr via apoE. In addition, LDL particles can be taken up by extra-hepatic tissues that need the cholesterol from LDL for proper cellular functioning and steroid hormone synthesis.

LPL is expressed in almost all tissues, though most abundantly in tissues that utilize FA for energy (e.g. heart and skeletal muscle) or storage (adipose tissue). LPL is not expressed...
in adult livers. LPL is formed in parenchymal cells and translocated to the luminal surface of endothelial cells to which it becomes anchored to interact with TG-rich lipoproteins. Regulation of LPL is tissue-specific and dependent on nutritional status. Adipose tissue LPL activity is high in the postprandial state, since in this state FA are primarily used for storage. In contrast, during fasting when FA are needed as an energy source, heart and muscle LPL activity is high. The activity of LPL is also influenced by apolipoproteins. They can either stimulate LPL activity (e.g. apoCII and apoAV), or inhibit the activity (e.g. apoCIII and apoCI), thereby influencing plasma TG and FA levels.

Mouse models with tissue specific deletion or over expression of LPL have indicated an important role for LPL in the metabolic syndrome. For instance, LPL over expression in muscle has been associated with increased TG storage in the muscle, reduced high fat diet (HFD)-induced obesity and may affect insulin sensitivity. Deletion of LPL in adipose tissue reduced bodyweight in a genetically obese mouse model. Modifiers of LPL action play a role in the development of the metabolic syndrome as well. For example, mice that lack the LPL inhibitor apoCIII are more obese and develop more severe insulin resistance after HFD feeding, whereas genetically obese mice that over express the LPL inhibitor apoCI are less obese and less insulin resistant than control genetically obese mice. Overexpression of apoAV, a stimulator of LPL, however did not affect obesity or insulin resistance development in mice subjected to a HFD.

**Cellular uptake of fatty acids**

Uptake, transport and storage of TG-derived FA into tissues are highly regulated since unbound FA are cytotoxic. FA have been shown to passively transfer across cell membranes, however several fatty acid binding proteins exist to facilitate the cellular entry of FA. CD36, also called FA translocase, is such an important fatty acid binding protein that facilitates FA uptake. CD36 is an 88 kDa integral membrane protein and is expressed in many tissues, including adipose tissue, heart, skeletal muscle and intestine, but also in endothelial cells, platelets and macrophages. In liver, CD36 is not a main fatty acid transporter. In addition to its role in facilitating FA transport, CD36 can also function as class B scavenger receptor. As scavenger receptor, CD36 plays a central role in development of different metabolic abnormalities associated with the metabolic syndrome such as hypertriglyceridemia, atherosclerosis, inflammation and insulin resistance. CD36-deficient mice are protected against HFD-induced obesity and this thesis. However, studies investigating the effects of variants in the CD36 gene on obesity in humans have yielded conflicting results. Choquet et al. found no effect of CD36 single nucleotide polymorphisms (SNPs) on onset of obesity, whereas Heni et al. found other SNPs that were associated with waist circumference and BMI. Whether CD36-deficiency protects against insulin resistance is controversial. CD36-deficient mice are insulin resistant in the liver, but remain insulin sensitive in muscle. In a specific CD36-deficient Japanese population insulin sensitivity was decreased, although another study did not find an effect on insulin sensitivity in similar CD36-deficient subjects. In addition some SNPs in the CD36 gene are associated with increased insulin resistance, although another is not.
**Intracellular processing of fatty acids**

Inside the cell, diacylglycerol acyltransferases (DGATs) catalyze the re-esterification of FA that are not utilized for energy, so that they can be stored as TG intracellularly. In the fed state, adipose tissue is the main site for TG storage. In the adipose tissue there is a continuous cycle of lipolysis and re-esterification of FA that is regulated by several enzymes. Adipose triglyceride lipase (ATGL) catalyzes the first step in TG lipolysis: the breakdown of TG into diacylglycerol (DG) and FA. Hormone sensitive lipase (HSL) catalyzes the breakdown of DG into monoacylglycerol (MG) and FA. In the fed state, HSL activity is inhibited by insulin resulting in intracellular net uptake of FA and accumulation of TG in adipose tissue. During fasting or enhanced energy demand hormones such as glucagon stimulate HSL activity, which results in the release of FA into the circulation. The released FA can be taken up by other tissues and used as energy source. Interestingly, decreased ATGL and HSL mRNA and protein levels have been reported in obese individuals with insulin resistance.

**Role of fatty acids in the pathology of insulin resistance**

The increased prevalence of the metabolic syndrome in our society is driven by an increasing disbalance between energy intake and energy expenditure. The expanded adipose tissue mass that is characteristic of obesity is thought to play an important role in the pathology of the metabolic syndrome. Adipose tissue continuously takes up and secretes FA in a so called futile cycle as discussed in the previous paragraph. Under normal, healthy conditions, the net balance of absorption and secretion is tightly regulated. The absolute amount of the adipose tissue determines the contribution to the futile cycle. With increasing adipose tissue mass, the contribution of the futile cycle to basal plasma FA levels will increase and it has been hypothesized that this increase in cytotoxic FA is one of the causes driving the metabolic syndrome.

The lipid overload at the onset of HFD-induced obesity has been related to the onset of insulin resistance. In mice and humans, intravenous lipid infusions cause insulin resistance. Excess lipids may be delivered to non-adipose tissues (ectopic fat deposition) that are not suited for fat storage (i.e. skeletal muscle and the liver), thereby increasing the formation of specific fatty acid metabolites such as fatty acyl CoA, diacylglycerol, and ceramides that may directly impair insulin signaling. Normally, insulin stimulates tyrosine phosphorylation of insulin receptor substrate (IRS) proteins that mediate insulin signaling. In insulin resistance, serine residues of IRS are phosphorylated instead, which interferes in insulin receptor signaling. In mice and humans it was found that increased intramyocellular diacylglycerol activates protein kinase C isoforms, which can inhibit insulin signaling by phosphorylating serine residues of IRS-1. Moreover, increased ceramide levels in plasma and muscle of mice and human also inhibit insulin signaling. In addition to ectopic fat deposition, high levels of FA can also lead to metabolic inflammation, another key event that can impair insulin signaling which will be further discussed in the following paragraphs.
Classically, inflammation is described as the primary response of the body against injury. This classical response is strong, acute and limited. In contrast to this acute inflammatory response, it has been discovered in the past decades that many obese subjects have slightly increased levels of inflammatory markers in plasma, which remain elevated over a longer period of time. This kind of inflammation is referred to as low-grade-, chronic- or metabolic inflammation, since it is hypothesized that this inflammation is triggered by a surplus of nutrients.

The immune response consists of 2 branches, the innate response that is relatively non-specific and the adaptive response, which is specific. Leukocytes such as macrophages and mast cells are the main cells of the innate immune system. T- and B-lymphocytes are the main cells of the adaptive immune system. T-lymphocytes have diverse roles. CD4 T-lymphocytes activate other cells of the immune system such as B-lymphocytes and macrophages. CD8 T-lymphocytes are cytotoxic and kill cells that are infected with pathogens. The primary function of B-lymphocytes is to produce antibodies that can bind to pathogens so that they become tagged for destruction. Both the innate and the adaptive arms of the immune response are involved in metabolic inflammation. In the following sections adipose tissue inflammation and the role of different immune cells herein is described. A schematic overview is depicted in Figure 2. In addition, the role of metabolic inflammation in the pathology of insulin resistance is discussed.

Adipose tissue inflammation
This section specifically focuses on the role of immune cells that reside in adipose tissue. Nowadays, adipose tissue is no longer regarded as a simple depot for excess calories. Adipose tissue secretes hormones such as leptin and adiponectin, acute-phase proteins, cytokines, chemokines, growth factors and components of the complement system. These peptides all influence insulin sensitivity and inflammation. Normally the liver and the lymphoid organs are the main production sites of inflammatory mediators, but when adipose tissue expands, adipose tissue also becomes an important producer of these inflammatory mediators. Besides secretion of pro inflammatory molecules, obese adipose tissue contains more immune cells such as macrophages, T-lymphocytes and activated B-lymphocytes. Finally, hypertrophic adipocytes secrete more FA, which can have a pro inflammatory effect via Toll Like Receptor (TLR) 2 or 4. All these aspects contribute to the pro inflammatory phenotype that is highly prevalent in obese subjects.

Role of macrophages in adipose tissue inflammation
When there is a surplus of nutrients, adipose tissue needs to expand to increase its storage capacity. Adipocyte hyperplasia (increase in adipocyte number) and adipocyte hypertrophy (increase in adipocyte size) can contribute to this process. The process of adipose tissue expansion requires tissue remodeling such as extracellular matrix degradation and new
blood vessel formation, since expansion of adipose tissue is physically limited by nutrient and oxygen supply. Lack of nutrients and oxygen result in hypoxia, adipocyte stress and adipocyte cell death, which are characterized by enhanced chemokine secretion and deregulation of FA fluxes. These processes at the onset of adipose tissue expansion can lead to increased macrophage recruitment within adipose tissue. Indeed, macrophages are increased in obese adipose tissue. These adipose tissue macrophages (ATMs) are not a uniform population of cells, but can exhibit a range of activation states. ATMs that reside in lean adipose tissue are predominately alternatively activated macrophages (M2 macrophages). Alternative activation of macrophages occurs in response to IL-4 and IL-13 and promotes tissue repair and remodeling. Alternative activated macrophages are anti-inflammatory and secrete the anti-inflammatory cytokine IL-10. In contrast, in obese adipose tissue, macrophages are more polarized towards classical activated macrophages (M1 macrophages). Classical activation occurs in response to IFN-γ and results in pro inflammatory macrophages that
secrete e.g. IL-6 and TNF-α. These pro-inflammatory cytokines can act on adipocytes to induce insulin resistance (this is further discussed in the section on the role of metabolic inflammation in the pathology of insulin resistance).

Cytokines produced by ATMs can have endocrine cross talk with skeletal muscle and liver; two other organs that have increased macrophage content in obese patients and thus can also develop inflammation-driven insulin resistance.

Role of T-lymphocytes in adipose tissue inflammation
In addition to macrophages, other immune cells like T-lymphocytes are also increased in obese adipose tissue. Two main populations of T-lymphocytes exist: T-helper lymphocytes, expressing co-receptor CD4 and cytotoxic T-lymphocytes, expressing co-receptor CD8. T-helper lymphocytes are important for the development of an antigen-specific B-lymphocyte response (which will be discussed in the following paragraph). The population of T-helper lymphocytes can roughly be subdivided into pro-inflammatory cells (T_h1, T_h17) and anti-inflammatory and regulatory sublineages (T_h2, Foxp3+). In obese subjects the number of T_h1 and CD8+ T-lymphocytes in adipose tissues is increased, whereas the number of regulatory T-lymphocytes is decreased. Recent studies have suggested that T_h1 lymphocytes and CD8+ T-lymphocytes help recruit macrophages into adipose tissue and stimulate the M1 macrophage inflammatory activation status. It is speculated that these lymphocytes respond to unique antigens that are generated in obese adipose tissue during HFD feeding. Gain- and loss-of-function studies have indicated that these T_h1 and CD8+ T-lymphocytes are associated with increased insulin resistance.

Role of B-lymphocytes in adipose tissue inflammation
B-lymphocytes are also recruited to adipose tissue shortly after initiation of HFD and activation of B-lymphocytes is increased in patients with type 2 diabetes. B-lymphocytes produce antibodies, also named immunoglobulins (Ig) in response to antigens. These antibodies have a so-called constant part (Fc region) that can bind to C1q, which is the recognition component of the classical complement pathway. Upon binding to C1q, the complement system is activated and processes such as phagocytosis are initiated to clear pathogens. The Fc region can also bind to Fc receptors (FcRs). FcRs can be found on most hematopoietic cells, such as neutrophils, natural killer cells, dendritic cells, mast cells and monocytes/macrophages. Upon binding to FcRs, a range of cellular responses is initiated, such as phagocytosis, antigen-dependent cellular cytotoxicity and secretion of cytokines. FcRs and the complement system thus form a link between the innate and the adaptive immune system. FcRs are multi-subunit receptors, in which the ligand-binding α-chain associates with the signal transducing γ-chain that contains an immunoreceptor tyrosine-based motif (ITAM). In addition to signaling, the γ-chain is also essential for normal surface expression of FcαRI, FcγRI and III and FcεRI.

Recently, it has been shown that B-lymphocytes from HFD-fed mice can activate CD8+ and CD4+ T-lymphocytes. When these T-lymphocytes are activated, they start to produce
pro inflammatory cytokines such as INF-γ that stimulate M1 macrophage polarization, which can eventually lead to insulin resistance. The requirement of T-lymphocytes in this process suggests that the antigen presenting function of B-lymphocytes is important for the development of insulin resistance. In line with this idea, it was found that B-lymphocytes from HFD-fed mice produce IgG antibodies that via their Fc portion elicit a pro inflammatory cytokine response. It is possible that the source of the antigens responsible for the detrimental effect on insulin sensitivity is formed by stressed apoptotic adipocytes, since IgG antibodies concentrate in so called crown-like structures within adipose tissue. Crown-like structures are composed of dead adipocytes surrounded by activated adipose tissue macrophages.

Antibodies produced by B-lymphocytes are thus important for the development of HFD-induced insulin resistance, however the role of antibody effector pathways in this process is not clear yet 75.

The role of metabolic inflammation in the pathology of insulin resistance

Thus, in addition to FA and their metabolites as discussed above, tissue specific and systemic inflammatory signals likely play determinant roles in the pathology of insulin resistance. TNF-α, IL-6 and IL-1β secreted by classical activated macrophages can activate Jun N-terminal kinase (JNK) and inhibitor of κB kinase (IKKB) in adipocytes, myocytes and hepatocytes. Like FA metabolites, these kinases are able to phosphorylate the serine residue of IRS, thereby inhibiting insulin signaling 92 93 94. Besides acting on IRS, these kinases also activate transcription factor activator protein 1 (AP1) (c-Jun/Fos) and nuclear factor NF-κB, which induces further inflammatory gene expression 95. Mouse models that lack either JNK1 or IKKB in myeloid cells are protected against obesity-induced insulin resistance 96 84.

Saturated FA may also have a direct pro inflammatory effect. Innate immune cells possess recognition receptors that sense invading pathogens and subsequently start to stimulate pro inflammatory signaling pathways in order to recruit other immune cells. TLRs form such a family of recognition receptors. Recently it has been found that in addition to pathogens, saturated FA can also interact with TLR4 and TLR2 and activate pro inflammatory signaling pathways 64, 97. Accordingly, TLR4 and TLR2 deficient mice have reduced HFD-induced inflammation, as well as reduced HFD-induced insulin resistance 64, 98, 99. In addition to TLRs, the inflammasome, a multiprotein complex consisting of a member of the Nod-like receptor family (e.g. NLRP3), and the inflammasome adaptor molecule ASC, can also recognize pathogens. When the inflammasome becomes activated, caspase-1 is recruited and activated which results in activation of the pro inflammatory cytokines IL-1β and IL-18 100, 101. Recently, it was discovered that HFD feeding results in activation of caspase-1 in adipose tissue of mice. In these HFD-fed mice increased IL-1β and IL-18 protein levels were observed 102. In line with these results caspase-1 deficient mice demonstrated reduced HFD-obesity, HFD-induced-insulin resistance and HFD-induced inflammation in adipose tissue 103.
The research described in this thesis focuses on TG/FA metabolism and inflammation, two key processes that are deranged during the development of the metabolic syndrome. We specifically focus on insulin resistance, an important metabolic abnormality of the metabolic syndrome. In Chapter 2 we studied obesity development, fat deposition, adipocyte development and functioning and insulin sensitivity in a murine model deficient for a major FA transporter, CD36. Mice deficient in CD36 have increased fasting plasma FA, TG and total cholesterol levels and decreased fasting plasma glucose levels on normal chow diet[^104], indicating a role for CD36 in lipoprotein, FA and glucose metabolism. Because CD36 facilitates FA uptake and FA uptake is important in adipogenesis, the CD36<sup>−/−</sup> mice is an appropriate model to study fat deposition, adipocyte development and functioning and its effect on insulin sensitivity. In Chapter 3 and 4 we determined how different pharmacological interventions in inflammation affect lipid metabolism (Chapter 3) or insulin sensitivity (Chapter 4). In Chapter 3 we investigated the mechanism of TG lowering by the non-steroidal anti-inflammatory drug aspirin. Human apolipoprotein CI-expressing mice (APOC1<sup>+</sup>) were used as a model for hypertriglyceridemia. APOC1<sup>−/−</sup> mice have increased plasma TG due to diminished clearance of VLDL particles through apoCI-mediated inhibition of LPL. In Chapter 4 we investigated whether an anti-inflammatory compound derived from an extract of <em>Humulus lupulus</em> L., META060, had the capacity to improve insulin sensitivity in a HFD fed mouse. In Chapter 5 we investigated a novel mechanism underlying the inflammation that is associated with the metabolic syndrome. We used the FcR γ-chain<sup>−/−</sup> mice, characterized by diminished activation of IgG and IgE antibody effector pathways, to study the role of functional FcRs in the development of obesity-induced insulin resistance. It has been discovered that HFD-induced IgG play a role in the development of insulin resistance. However, the role of functional FcRs in this process has not been investigated yet. The last chapter provides an overall discussion and conclusion about this PhD project (Chapter 6).

### REFERENCES


87. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Otsu M, Hara K,


