Factors influencing obesity-associated low-grade inflammation in atherosclerosis

The role of fat distribution, sex and the gut microbiota

Inge C.L. van den Munckhof
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The role of fat distribution, sex and the gut microbiota

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Colofon

The research presented in this thesis was performed at the department of Internal Medicine of the Radboud university medical center in Nijmegen and carried out within the Radboud Institute for Health Sciences, the Netherlands.

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Success is not the key to happiness. Happiness is the key to success.
If you love what you are doing, you will be successful.
Albert Schweitzer
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CHAPTER 1

General introduction, aim and outline of the thesis
History of cardiovascular disease

Atherosclerosis is a pathophysiological process in the arteries leading to accumulation of inflammatory cells, lipid and calcium in arterial walls. Atherosclerosis ultimately results in stenosis of arteries and cardiovascular disease (CVD). Leonardo Da Vinci (1452–1519) already described in his finding on the autopsy on a centenarian that ‘vessels in the elderly restrict the transit of blood through thickening of the tunics’ (1). In 1799 Caleb Hillier Parry, a British physician (1755–1822), was the first to describe atherosclerosis in the autopsy of a patient suffering from chest pains. Upon examination of the heart he concluded that the coronaries had become bony canals. These observations were the basis for his subsequent book ‘Inquiry into the symptoms and causes of ‘syncope anginosa’, commonly called angina pectoris’.

Traditional assessment of cardiovascular risk

At present, CVD, and specifically ischemic heart disease and stroke, have become leading causes of death worldwide (2). The most common underlying pathophysiological process for CVD is atherosclerosis, which may ultimately result in vascular occlusion with myocardial infarction or stroke as a result. The detection of an increased risk for CVD in a specific individual up till now is mainly based on the presence of major cardiovascular risk factors: male sex, dyslipoproteinemia, hypertension, smoking and diabetes mellitus, which were found previously in a large population-based study, the Framingham study (3). In subjects with obesity a number of these risk factors, namely hypertension, dyslipoproteinemia, hyperglycemia and abdominal obesity cluster and are called the ‘metabolic syndrome’. This is associated with a twofold higher risk for CVD (4). Further research was performed to identify additional factors that contribute to the pathophysiology of atherosclerosis.

A few decades ago, atherosclerosis was generally considered a bland arterial wall collection of cholesterol with smooth muscle accumulation, although already in 1856 Virchow et al. had hypothesized that immune cells might be involved in the pathogenesis of atherosclerosis. It took however more than 125 years to test this hypothesis (5). A research group from the Göteborg University was the first to identify immune cells in atherosclerotic plaques (6). Although these findings were initially met with skepticism (7), inflammation has now been recognized as a key regulatory process in the pathogenesis of atherosclerosis.

Whereas the detrimental roles of systemic chronic low-grade inflammation have been studied intensively during the past 30 years, the primary cause of proinflammatory changes in patients at increased risk of CVD has not been elucidated. Individuals with obesity that develop CVD are often characterized by a status of chronic low-grade inflammatory (8, 9). It has been hypothesized that differences in inflammatory status might explain the individual differences in cardiovascular risk among subjects with overweight and obesity.

Low-grade inflammation in atherosclerosis

The immune system is a host defense mechanism that involves a complex cellular and humoral network and helps the body fight against infections. It consists of an innate part, which
embody non-specific defense mechanisms that act within hours after the presence of certain antigens; and of an adaptive part, which is antigen-specific and performed by the B- and T-cells. Cells from both the innate and specific immune system have been shown to contribute to the initiation, progression and destabilization of atherosclerotic plaques (10). Triggers such as hypertension and hyperglycemia can initiate the expression of adhesion molecules such as vascular adhesion molecule-1 (VCAM-1) by endothelial cells. This process is stimulated by oxidized lipids and pro-inflammatory cytokines. The intima layer will subsequently be infiltrated by various immune cells such as monocytes and lymphocytes. Monocytes have shown to play a pivotal role in atherosclerosis development. Monocytes mature into macrophages and engulf modified lipoproteins, becoming foam cells. This induces a pro-inflammatory state in the atherosclerotic plaque which attracts other immune cells that further exacerbate the inflammation. As foam cells accumulate, a process of necrosis is initiated in the core of the plaque. Due to accumulation of lipid coalescences and death of macrophages and smooth muscle cells, a necrotic core is created, which provokes inflammation further. Fibrous tissue is added to form a fibrous cap between this necrotic core and the endothelium. As inflammation blocks the creation of new collagen fibers and stimulates the destruction of existing collagen, partly by stimulating the production of matrix metalloproteinases by macrophages, the fibrous cap becomes thin and ruptures. This event in the atherosclerotic plaque is the cause of the majority of acute coronary syndromes (11).

Low-grade inflammation in obesity
Since the discovery that tumor necrosis factor (TNF)-α is overexpressed in the adipose tissue of mice with obesity, numerous studies have focused on the role of adipose tissue in the development of inflammation (12). One of the current hypotheses linking obesity to increased inflammation suggests that fat mass expands due to a positive energy balance, which leads to local hypoxia at adipocyte level and cell death. Cell death results in the release of danger-associated molecular patterns (DAMPs) that can induce an inflammatory reaction of both adipocytes and surrounding tissue macrophages. While initially considered to be of little relevance for metabolic dysregulation and cardiovascular risk in obesity, the modest increases in circulating inflammatory mediators have in recent years been found to be strongly associated with the development of NAFLD, type 2 diabetes mellitus and atherosclerosis (13, 14).

One of the most commonly used biomarker of low-grade inflammation is C-reactive protein (CRP). In 2010 in a meta-analyse of 160 000 asymptomatic individuals, CRP levels were associated with risk of coronary heart disease, ischaemic stroke and cardiovascular mortality (15). The increased CVD risk imposed by CRP was comparable to that of hypertension and dyslipoproteinemia. Another important observation came from a large study by Ridker et al (16). They randomized healthy individuals with an LDL-cholesterol of < 3.4 mmol/L but an increased hsCRP levels of > 2.0 mg/L to 20 mg rosvastatin or placebo and followed for occurrence of CVD. In these ‘healthy’ individuals with increased hsCRP levels, rosvastatin
significantly reduced the incidence of major cardiovascular events, proving again the importance of low-grade inflammation in CVD. Most mechanistic studies suggest that CRP itself is unlikely the underlying to be pathogenic, but its precursor IL-6 and probably even more important the members of the IL-1 family are the causative factors(17). The inflammasome has been shown to play a pivotal role in the production of IL-1 family cytokines.

The inflammasome

The inflammatory process that leads to the low-grade inflammation state in individuals with obesity is not initiated by an infection, but by metabolic factors and is therefore also called ‘metaflammation’ (18). This metaflammation is present in the adipocytes, but also in other metabolically active organs like the liver, pancreas and the central nerve system (19). A key player in this process is the inflammasome, which consists of intracellular sensor molecules, of which the NOD-like receptor family pyrin domain containing-3 (NLRP3) is probably one of the most important ones. Activation of this inflammasome is mediated via recognition of sterile danger ligands (danger associated molecular pattern, DAMP) or pathogen-derived ligands (pathogen associated molecular pattern, PAMP) by pattern recognition receptors (PRRs). Activation depends on two signals. Glucose, oxidized LDL-cholesterol, and uric acid, all

![Diagram of inflammasome activation](image.png)

Figure 1 – Activation of the NLRP3 inflammasome by different danger associated molecular patterns (DAMPs) ©Inge van den Munckhof
increased in obesity, have been demonstrated to activate the NLPRP3 (20, 21). After activation of the inflammasome an intracellular cascade starts leading to activation of caspase-1. Caspase-1 cleaves multiple proteins, including pro-interleukin(IL)-1β and pro-IL-18, which then become biologically active (22). Individuals with an increase in BMI but who are metabolically healthy are characterized by less NLRP3 inflammasome activation compared to individuals with the metabolic syndrome (23).

The role of IL-1, IL-6 and IL-18
Multiple lines of evidence have shown a causal role for the IL-1 family in atherosclerosis. The strongest evidence was found in the CANTOS trial investigating the effect of canakinumab, a human monoclonal antibody that inhibits the pro-inflammatory cytokine IL-1β in subjects with a previous myocardial infarction. Treatment with canakinumab reduced the incidence of cardiovascular death, non-fatal myocardial infarction and non-fatal stroke during their 3.5 year follow-up (24). Next to IL-1β, IL-18 is another important cytokine from the IL-1 family (25). Besides IL-1β and IL-18, several modulators have been found to influence IL-1 related pathways, including IL-18 binding protein (IL-18BP) (26), alpha-1-anti-trypsin (AAT), and adipokines.

However not only this inflammatory pathway is important in the pathogenesis of atherosclerosis, as very recently, the anti-inflammatory drug colchicine has also shown to reduce the risk of cardiovascular events in individuals with chronic coronary disease (27). Colchicine has broad cellular effects that include inhibition of tubulin polymerization and alteration of leukocyte responsiveness.

Adipokines
Cytokines produced by the adipocyte, adipokines, represent another way by which the adipose tissue could contribute to the inflammatory process during metabolic dysregulation and obesity. Adipokines mediate the crosstalk between adipose tissue and the other metabolic organs. Leptin and adiponectin are the best known adipocytokines. Leptin regulates body weight by signaling nutritional status to other organs, especially the hypothalamus that then produces neuropeptides which modulate food intake and energy expenditure (28). The leptin level is closely related to the adipose tissue mass. Recombinant leptin however cannot be used as weight losing drug in the general population, as individuals with obesity are resistant to leptin due to hyperleptinemia. Leptin acts pro-inflammatory via macrophages, T cells, and other immune cells to stimulate the production of a wide spectrum of cytokines (28, 29). Adiponectin has been shown to have various anti-inflammatory actions: it suppresses TNF-α production in obese mice; it can enhance the clearance of apoptotic cells by facilitating their opsonization and uptake by macrophages and has been shown to NFκB activity leading to reduced monocyte adhesion to endothelial cells (28). The adiponectin levels are negatively associated with adiposity and fasting glucose (30). Circulating levels of adiponectin and leptin are generally higher in women than in men. This is at least partly caused by a higher percentage of adipose tissue in women (31).
**Systemic and adipose tissue inflammation measurement**

Low-grade inflammation is usually assessed by measurement of circulating cytokines and related peptides such as high sensitivity C-reactive protein (hsCRP). As mentioned above, the adipokines can also easily be measured in the blood. The immune cell count and differentiation reflect another way of measuring low-grade inflammation. In addition to systemic inflammation, there is accumulating evidence that also the phenotype of circulating immune cells impact on the development of atherosclerosis. Circulating monocytes have an enhanced cytokine production capacity in patients with risk factors for atherosclerosis, including familial hypercholesterolemia and elevated lipoprotein (a) (32, 33), and in patients with established coronary atherosclerosis (34, 35). The capacity to produce cytokines can be assessed through ex vivo stimulation experiments of immune cells, this represents another way to investigate and detect low-grade inflammation.

Next to measurement of low-grade inflammation markers in the blood, direct analysis of the metabolically active tissues could provide new insights. Especially, the subcutaneous adipose tissue compartment can be relatively easily reached by biopsy. During obesity different processes are histologically visible in the adipose tissue. Firstly, the adipocyte cell size increases to store additional energy. Secondly, immune cells are attracted. The presence of these macrophages in the adipose tissue has been mainly related to obesity-associated complications such as diabetes mellitus (36). The presence of three or more macrophages around one adipocyte is called a crown-like structure. These structures were found to be increased 30-fold in obese diabetic mice compared to lean mice (37). The number of crown-like structure is associated with a more pro-inflammatory phenotype via activation of the inflammasome. As the phenotype by which inflammation in the adipose tissue presents is quite heterogenous (20,21), several parameters can be combined into an adipose tissue inflammation score.

**Role of obesity in cardiovascular disease**

The prevalence of obesity has dramatically increased over the last decades. In 2018 almost half of the Dutch population was overweight and 15.4% were obese (38). These numbers are even higher in other countries such as the USA with an obesity rate above 40%. The worldwide pandemic of obesity has led to an increased prevalence of cardiovascular risk factors (diabetes mellitus, hypertension and dyslipidemia). As mentioned, the cluster of these obesity related cardiovascular risk factors is called the ‘metabolic syndrome’.

Interestingly however, not all individuals with obesity develop the metabolic syndrome and resulting CVD. Approximately 20-30% of these individuals are metabolically healthy and their risk for CVD is substantially lower than in individuals with metabolically unhealthy obesity.
An important factor that contributes to the metabolic dysregulation is the fat distribution.

**Fat distribution**
As the volumes of the different fat compartments are not easily measurable, indirect measures are usually used to assess adiposity. Calculation of body mass index (BMI), which is the sum of the weight (kg) divided by the square of the length (m\(^2\)), is the most commonly used method to diagnose overweight and obesity. A BMI between 25 and 30 kg/m\(^2\) is defined as overweight and a BMI > 30 kg/m\(^2\) is defined as obesity.

There is a strong correlation between BMI and CVD, however this is not a linear relationship. This is mainly due to the fact that the body composition itself is not taken into account. Not only does the amount of muscles influence the cardiovascular risk, also does the distribution of fat within the body. Especially the fat around the abdominal organs, the visceral adipose tissue (VAT) is associated with diabetes mellitus and CVD (41). The results of studies investigating the atherogenic risk of subcutaneous adipose tissue (SAT) have been conflicting. Once regarded as a single entity, the abdominal SAT can actually further be divided by the scarp fascia into the deep (dSAT) and superficial adipose tissues (sSAT). The adipocytes from dSAT have higher lipolytic activity than superficial SAT adipocytes and contribute substantially to free fatty acid (FFA) levels in the circulation (42). However, the separate role of the distinct subcutaneous adipose tissue compartments with regard to cardiovascular risk is not clear.

Fat can also be stored in the liver. Hepatic steatosis is defined by a hepatic fat content above 5.6% and is seen as the hepatic equivalent of the metabolic syndrome. Hepatic steatosis is the first stage of non-alcohol fatty liver disease (NAFLD) and is usually benign (43). However, in 10-20% of these individuals, NAFLD progresses to non-alcohol steatohepatitis (NASH) which can result in cirrhosis. The cardiovascular risk in both NAFLD and NASH is substantially higher (44, 45). The relation between SAT and hepatic steatosis is not clear, because the separate layers of the SAT have not been taken into account in this relationship.

If fat is mainly distributed abdominally, known as the ‘apple shape’ in men, this will increase the waist circumference, which is associated to a higher cardiovascular risk. On the other hand, fat that is mainly distributed around the hip region, the ‘pear shape’ in women has been related to a lower CVD risk (46). The anthropometric marker waist-hip-ratio has been
developed to take the effect on risk of both fat compartments into account. Mainly in men, the waist circumference and waist-hip-ratio have been shown to be superior to BMI in its relation to non-invasive markers of atherosclerosis. However, in women, the data on the relation of these anthropometric markers with metabolic and cardiovascular risk factors are conflicting. This suggest a sex-specific role of the adipose tissue compartments in metabolic dysregulation and atherosclerosis.

**Sex differences**

Although women develop CVD approximately eight years later than men (47, 48), the annual CVD mortality rate is about the same for women as for men (49, 50). In most historical cardiovascular research studies women were excluded. The knowledge about CVD in women has largely been extrapolated from studies investigating these relations in men. However, differences are already present in the anatomy of (atherosclerotic) arteries. Women generally have smaller arteries, with less plaque volume, but a higher stenosis grade (51). It is known that the microvasculature is more important in the pathophysiology of CVD in women than in men (52). Another important contribution to the prognosis of CVD in women is a delay in diagnosis and treatment, partly because of a different set of symptoms (53). Next to these factors, a difference in the fat distribution, as discussed above, could contribute to sex-specific differences in the atherosclerotic process. Besides, these differences in chronic low-grade inflammatory state and it’s metabolic and vascular consequences could also contribute to atherosclerosis.

Sex-specific differences in inflammation have been described before. In general, both the innate and the adaptive immune responses to pathogens are higher in women than in men (54). Also circulating cytokine levels differ between women and men. In healthy individuals IL-1Ra levels were higher in women compared to men, whereas IL-18BP levels were lower in women compared to men (55). Colleagues have shown that in stimulation experiments in healthy individuals the production of pro-inflammatory cytokines from monocytes was higher in men after stimulation with several agents (55). The exact aetiology behind these differences is not yet fully known.

**Role of gut microbiota in obesity and cardiovascular disease**

Several studies suggest that obesity-related metabolic dysregulation and systemic inflammation is at least partly driven by an altered gut microbial composition and function (56). The human gut microbiota consists of approximately \(10^{13}-10^{14}\) microbes, including bacteria, viruses, fungi and protozoa. The gene content of the gut microbiota (metagenome) in the human gut may exceed that of the host by at least 100-fold (57). Besides their function in intestinal epithelial homeostasis, development of the immune system, protection against pathogens and energy homeostasis (58-61), the gut microbiota also plays a role in pathophysiological mechanisms associated with different diseases (62).
Role of gut microbiota in cardiovascular disease
Karlsson et al. was the first to demonstrate several associations between the gut microbiota and carotid atherosclerosis. In patients with symptomatic atherosclerosis the genus *Collinsella* was enriched, whereas *Roseburia* and *Eubacterium* were enriched in healthy controls. In coronary atherosclerosis patients, the phylum *Bacteroidetes* was significantly decreased, while the order *Lactobacillus* was significantly increased in these patients (63).

Role of gut microbiota in obesity
The relationship between obesity and the gut microbiome composition was first reported in 2005. Genetically induced obese mice appeared to have less phylum *Bacteroidetes* and more *Firmicutes* compared to lean phenotypes (64). A further major observation came from Turnbaugh et al. who reported that the core gut microbiome of individuals affected by obesity has an increased capacity for energy harvesting (65). A low gut microbial diversity has previously been reported in individuals with obesity compared to lean individuals (66). However, not just the diversity of gut microbiota, but also the presence of specific species has been related to metabolic dysregulation and chronic low-grade inflammation markers.

Many potential underlying mechanisms have been proposed. Possible ways by which the gut microbiome could influence inflammatory mechanisms have been reviewed extensively (67), which is part of this thesis. Besides the impact on inflammatory pathways, it is also known that the gut microbiota is related to metabolites, which are small molecules essential in particular metabolic processes. Previous studies have shown that the gut microbiota explain a substantial amount of lipoprotein variance (68). Next to these indirect pathways, it has been suggested that trimethylamine N-oxide (TMAO), a gut microbial-derived metabolite, could directly influence the atherosclerotic process. In 2013 increased TMAO levels were found to be associated with an increased risk of incident major adverse cardiovascular events (69).

So far, the effect of the above-mentioned factors influencing obesity-associated low-grade inflammation in atherosclerosis is not fully known, and clearly more detailed information regarding these effects is needed. In this thesis I propose that fat distribution, sex differences, the IL-1 family and the gut microbiome play an important role in the pro-inflammatory state in individuals affected by obesity.
Aim and outline of the thesis
As described in the previous paragraphs, obesity is importantly linked to the development of metabolic disorders and CVD. However, some of the individuals with obesity are metabolically healthy and their risk for CVD is substantially lower. The central hypothesis in our studies is that differences in inflammatory status partly explain the individual differences in cardiovascular risk among subjects with overweight and obesity. The overall aim of this thesis is to gain more insight into factors influencing obesity-associated low-grade inflammation in atherosclerosis. A better understanding of these factors and the role of low-grade inflammation in atherosclerosis might offer novel diagnostic tools and eventually new treatment strategies to prevent or treat CVD. This thesis therefore provides several studies in women and men with overweight and obesity on:

- The impact of fat distribution and its relation to metabolic dysregulation.
- The contribution of fat distribution on low-grade inflammation.
- The impact of sex on the relation between low-grade inflammation markers and the presence of the metabolic syndrome.
- The relation of circulating low-grade inflammation markers with non-invasive measurements of atherosclerosis.
- The relation between the gut microbiota, plasma metabolome and non-invasive measurements of atherosclerosis.

Research population
Much of the work presented in this thesis has been established in the 300-OB study, in which in total 302 individuals aged between 55 and 82 were enrolled in the period between 2014 and 2016 in the Radboud university medical center. All individuals were overweight or obese and half had the metabolic syndrome. Most of these individuals (n = 227) had also participated in the Nijmegen Biomedical Study – Non-Invasive Measurements of Atherosclerosis 1 (NBS-NIMA1) study, a population-based survey of Nijmegen residents (70). Additionally, we recruited 75 participants, acquaintances of earlier included subjects, fulfilling the inclusion criteria (age > 55 years and BMI > 27 kg/m²). Exclusion criteria were: a recent cardiovascular event (myocardial infarction, transient ischemic attack, or stroke less than six months before), history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral or subcutaneous anti-coagulant therapy, use of thrombocyte aggregation inhibitors other than acetylsalicylic acid and carbasalate calcium or a contra-indication for MRI. Participants who used lipid-lowering therapy, temporarily discontinued this medication four weeks prior to the measurements. All women were postmenopausal and did not use hormonal replacement therapy.
Part of the findings were validated in the 500 Functional Genomics (500FG) cohort, consisting of healthy individuals with a mean age 29 years and mean BMI 22.7 kg/m². The analysis on the gut microbiome were also performed in a part of the LifeLines Deep study, we included 978 subjects (567 women and 411 men) with an average BMI of 25 kg/m².

Methods
All individuals filled out an extensive questionnaire about life style, medication use, and previous medical history. Blood sampling was performed in the morning after an overnight fast to measure blood glucose, triglycerides, total cholesterol and high-density lipoprotein cholesterol. The inflammatory status was phenotyped extensively by measurement of circulating cytokines, cell counts, stimulation experiments of PBMCs and whole blood and analysis of subcutaneous adipose tissue. We used a high-throughput targeted Nuclear Magnetic Resonance (NMR) metabolomics platform (Nightingale's Biomarker Analysis Platform) to generate spectra for the quantification of 231 lipid and metabolite measures. Fecal samples were collected within one-two days after blood samples were taken. Metagenomic shotgun sequencing was performed using the Illumina HiSeq platform (Illumina, San Diego, California). The profile of microbial composition was determined using MetaPhlan 2.2 and the abundance of metabolic pathways was determined using HUMAnN2.

All individuals underwent comprehensive cardiovascular assessment, including the measurement of carotid intima-medial thickness (cIMT), plaque presence and maximum plaque thickness. Abdominal and thigh fat distribution and liver fat content were determined by magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (MRS), respectively.
Research questions
The overall aim of this thesis is to obtain more insight in factors influencing obesity-associated low-grade inflammation in atherosclerosis. In this thesis we therefore investigated the following research questions:

- Are sex-specific differences present in the distribution of fat within the subcutaneous adipose tissue layer? Does the amount of sSAT and dSAT relate to hepatic steatosis, the metabolic syndrome and its individual traits?
- Are sex-specific differences in fat distribution associated with different inflammatory profiles? And how does the fat distribution relate to adipose tissue inflammation?
- Does sex impact the relation between inflammation and metabolic syndrome in individuals with overweight and obesity. What is the role of circulating metabolites and hormones on this relation?
- How do systemic inflammatory mediators, especially from the IL-1 family, relate to non-invasive markers of atherosclerosis? Is there a circulating cytokine that is associated with adipose tissue inflammation?
- Does the gut microbiota impact on the plasma metabolome? And is this related to cardiometabolic phenotypes and individual metabolic risk of cardiovascular disease?
- How is the gut microbiota related to markers of low-grade inflammation in humans (systemic review)? What are the possible mechanisms by which the gut microbiota can influence chronic low-grade inflammation and thereby contribute to the development and progression of ACVD?

Outline of the thesis
To address the research questions, this thesis continues with chapter 2, in which we evaluated the sex-specific associations of the deep and superficial SAT and the VAT with hepatic steatosis and the metabolic syndrome traits.

We investigated this in 285 individuals with overweight and obesity from the 300-OB study of whom MRI and MRS measurements were present.

In chapter 3, we investigated the relation between adipose tissue distribution and markers of systemic and adipose tissue inflammation in a sex-specific manner. This study was performed in the 300-OB cohort. MRI measurements were performed to assess the fat distribution in detail. Systemic low-grade inflammation was assessed via three groups of inflammatory parameters: plasma adipokine and cytokine concentrations, immune cell subpopulations in the blood, and the ex-vivo cytokine production capacity of circulating immune cells (such as monocytes and T cells). Subcutaneous fat biopsies were taken to analyze the adipose tissue inflammation. The different adipose tissue compartment volumes were related to all these inflammatory markers to see whether sex-specific differences in fat distribution were associated with different inflammatory profiles.
Recently, the importance of anti-inflammatory therapies has been shown in CVD. However, these anti-inflammatory therapies in CVD are being developed under the assumption that inflammatory pathways are identical in women and men, but it is not known if this is indeed the case. In chapter 4, we investigated the impact of sex on the relation between inflammation and metabolic syndrome in overweight women and men. All individuals from the 300-OB cohort were included. The three groups of inflammation markers were related to the presence of the metabolic syndrome and the different metabolic traits in women and men. We also investigated the role of circulating metabolites, which were analyzed via Nuclear Magnetic Resonance metabolomics platform, and sex hormones on this relation.

In chapter 5, our aim was to investigate the impact of systemic inflammatory mediators, especially from the IL-1 family of cytokines, on atherosclerosis in individuals with overweight and obesity. We therefore performed ultrasound measurements of the carotid artery in all 300-OB participants to assess the intima-medial thickness, plaque presence and maximum plaque thickness. These cardiovascular parameters were related to circulating cytokines. We also assessed whether these inflammatory mediators were related to adipose tissue inflammation. Part of the findings were validated in the 500FG cohort, consisting of healthy individuals with a mean age 29 years and mean BMI 22.7 kg/m².

Another factor which relates to obesity and cardiovascular risk is the gut microbiome. However, it is not fully known by which (path)physiological process the gut microbiota influences the cardiovascular risk. Therefore, we related the microbial species and pathways in two different human studies to the plasma metabolome, which mainly included lipoprotein composition, but also inflammatory markers, and related it to atherosclerotic manifestations. Next to the 300-OB cohort, 978 individuals from the LifeLines Deep study, taken from a general Dutch population, were included in these analyses. The gut microbiota was analyzed via metagenomic shotgun sequencing. Plasma metabolites were analyzed using nuclear magnetic resonance (NMR) and the Nightingale's Biomarker Analysis Platform. The results of this study can be found in chapter 6.

Up till now, many gut microbiome studies have been carried out in mice. As the composition of the gut microbiota in mice differs significantly from humans, the relation with obesity-associated low-grade inflammation could also be affected in a different way. In chapter 7, we therefore performed a systematic review of all articles on the relation between the gut microbiome and markers of low-grade inflammation in humans. Next to that, we speculated on possible mechanisms through which the gut microbiota can affect low-grade inflammation and thereby CVD.

Finally, chapter 8 discusses the findings described in the previous chapters by comparing them to the literature. We discuss the strengths and limitations of our research and provide implications for practice that derived from the findings in this thesis.
Figure 5 – Schematic overview of the thesis
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CHAPTER 2

Superficial versus deep subcutaneous adipose tissue: sex-specific associations with hepatic steatosis and metabolic traits


Submitted
ABSTRACT

Objective
Subcutaneous adipose tissue (SAT) is not homogeneous, as the fascia scarpa separates the deep SAT (dSAT) from the superficial SAT (sSAT). The aim of this study was to evaluate the sex-specific associations of sSAT and dSAT with hepatic steatosis and the metabolic syndrome in individuals with overweight.

Methods
We recruited 285 individuals with a BMI ≥27 kg/m², aged 55-81 years. Abdominal magnetic resonance imaging was performed around level L4-L5 to measure VAT, dSAT and sSAT volumes. The amount of hepatic fat was quantified by MR spectroscopy.

Results
Men had significantly higher volumes of VAT (122.6 cm³ versus 98.7 cm³, p < 0.001) and had only half of the volume of sSAT compared to women adjusted for BMI (50.3 cm³ in men versus 97.0 cm³ in women, p < 0.001). However, just a small difference in the volume of dSAT was observed between sexes. In men, dSAT correlated significantly with hepatic fat content in univariate analysis (stand. β = 0.190, p < 0.05), while VAT correlated significantly with hepatic steatosis in a multivariate model, adjusted for age, alcohol use and other abdominal fat compartments (stand. β = 0.184, p = 0.037). Moreover, dSAT in men correlated negatively with HDL-c (stand. β = -0.165, p = 0.038) in multivariate analyses. In women with a BMI between 30-40 kg/m² (but not a lower BMI), in a multivariate model adjusted for age, alcohol use and other abdominal fat compartments, VAT correlated positively (stand. β = -0.404, p = 0.003), and sSAT negatively (stand. β = -0.300, p = 0.04) with hepatic fat content.

Conclusions
In men, dSAT is associated with hepatic steatosis and adverse metabolic traits, such as lower HDL-cholesterol levels; while in women with obesity sSAT shows a beneficial relation with respect to hepatic fat content.
INTRODUCTION

The prevalence of obesity has nearly doubled worldwide since 1980 (1). This increase has led to a pandemic increase in obesity-related disorders such as hypertension, dyslipidaemia and diabetes mellitus, which are all components of the metabolic syndrome. Especially the expansion of the visceral adipose tissue stores is related to the metabolic dysregulation, and subsequently leads to an increased risk for cardiovascular morbidity and mortality (2, 3). Visceral adipose tissue (VAT) is an important factor in the development of non-alcoholic fatty liver disease (NAFLD), also seen as the hepatic equivalent of the metabolic syndrome, both in women and men. Approximately 75% of individuals with obesity have increased hepatic fat, defined as a hepatic fat fraction above 5.6% as measured by magnetic resonance spectroscopy (4-6). Hepatic steatosis is the first stage of NAFLD and is usually benign and reversible (7). However, this condition can progress to non-alcoholic steatohepatitis (NASH), resulting in cirrhosis in 1-2% of patients (8). Both NAFLD and NASH are related to a higher risk for cardiovascular disease (9, 10).

Previous studies have reported that VAT is an important factor in the development of NAFLD and contributes to an increased cardiovascular risk (2, 3, 11). However, a relative increase in subcutaneous adipose tissue (SAT) compared to VAT is related to decreased severity of NAFLD (12). Currently, there are two hypotheses to explain the difference between VAT and SAT in the pathogenesis of NAFLD. The “portal theory” proposes that the liver is exposed to excessive free fatty acids (FFA) and pro-inflammatory factors released from the visceral fat into the portal vein of patients with obesity, which promotes the development of hepatic insulin resistance and hepatic steatosis (13). However, only 5-30% of the delivered FFA to the liver originates from visceral fat (14, 15), while at least 50-60% of the FFA comes from the systemic circulation and thus from subcutaneous fat (16). The second hypothesis is the “ectopic fat hypothesis” (17), which proposes an indirect mechanism whereby increased energy storage in peripheral subcutaneous fat exerts a protective effect by decreasing fat deposition in the liver, muscle and heart. However, although subcutaneous fat seems to be related to a more beneficial metabolic profile than visceral fat, it is important to realize that subcutaneous fat is not a homogeneous compartment, and we now hypothesize that there is a differential contribution to fatty liver disease of the two different subcutaneous fat compartments.

Once regarded to be a single entity, the abdominal SAT is divided by the scarpa fascia into the superficial (sSAT) and the deep subcutaneous adipose tissue (dSAT). The adipocytes from dSAT have higher lipolytic activity than superficial SAT adipocytes and contribute substantially to FFA levels in the circulation (18). It has been suggested that abdominal dSAT exhibits an intermediate phenotype between VAT and abdominal sSAT (19). Moreover, in patients with type 2 diabetes mellitus, a higher relative distribution of abdominal fat in sSAT was associated with a lower cardiovascular risk, whereas dSAT was related to a higher blood pressure and lower heart rate variability (20).

Although different studies have shown that dSAT and sSAT are evidently distinct abdominal adipose tissue depots, in the majority of the studies investigating the association between abdominal adipose tissue distribution and hepatic steatosis or the metabolic syndrome, no
distinction is made between dSAT and sSAT. As adipocytes from dSAT and sSAT have a different metabolic and inflammatory profile (21), we hypothesized that dSAT, but not sSAT, is associated with hepatic steatosis. We also investigated the associations between the different components of the metabolic syndrome and the various adipose tissue compartments (VAT, dSAT, sSAT), taking into account the sex differences in adipose tissue distribution.

RESEARCH DESIGN AND METHODS

Study population
We enrolled 302 individuals with overweight (BMI above 27 kg/m²), aged 55-80 years, in the period between 2014 and 2016 (300-OB study). The majority of the individuals (n = 227) also participated in the NBS-NIMA1 study, a population-based survey of Nijmegen residents (22). We recruited another 75 participants, acquaintances of previously included individuals, who fulfilled the inclusion criteria of age > 55 years and BMI > 27 kg/m². Exclusion criteria were: a recent cardiovascular event (myocardial infarction, transient ischemic attack, or stroke less than six months before), history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral or subcutaneous anticoagulant therapy, use of thromboocyte aggregation inhibitors other than acetylsalicylic acid and carbasalate calcium or a contra-indication for MRI. Participants who used lipid-lowering therapy, temporarily discontinued this medication four weeks prior to the measurements. All women were postmenopausal and did not use hormonal replacement therapy. All participants received detailed written and oral information and provided written informed consent. The Arnhem-Nijmegen Medical Ethics Committee approved the study protocol (in accordance with the Declaration of Helsinki).

Clinical parameters
Individuals filled out an extensive questionnaire about lifestyle, medication use, and previous medical history. Blood sampling was performed in the morning after an overnight fast. Blood glucose, triglycerides (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-c) were measured by standard laboratory procedures. Weight and height were measured and body mass index was calculated as body weight (kg) divided by the square of height (m). Waist circumference (WC) was measured at the level of the umbilicus to the nearest 0.1 cm. Hip circumference was measured at the level of the trochanter major. Waist/hip ratio (WHR) was calculated by dividing the waist circumference by the hip circumference. The diagnosis of metabolic syndrome was made using the clinical criteria of the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) (23).

MRI and MRS data acquisition
Abdominal fat distribution and liver fat content were determined by magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (MRS), respectively. The
combined MR examinations were performed on a 3.0 T Magnetom Skyra or Trio MR system (Siemens, Erlangen, Germany). Individuals were examined in the supine position with their arms positioned parallel to the lateral sides of the body. For the determination of visceral and subcutaneous fat distribution a series of T1-weighted FLASH 2D axial MR images were acquired from a region extending from 4 cm above to 4 cm below the fourth and fifth lumbar interspace (16 slices, matrix size 192/256, field of view 300-353 / 400-470 mm², slice thickness 5 mm, breath-hold, TR/TE 80 ms/2.46 ms). Breathing commands were used to avoid motion-induced artifacts.

For the MRS measurement, a single voxel of 27 cm³ was positioned in the right lobe of the liver, avoiding the biliary tree and large blood vessels. A STEAM (24) localization sequence without water suppression was used for data acquisition. To minimize relaxation effects on signal intensity long repetition time (TR = 3 s) and short echo time (TE = 20 ms) were used. Six scans were averaged during breath holding for 15 seconds. No pre-scans were used.

VAT, SAT, SSAT and dSAT analysis

The images acquired were retrieved from the MR scanner and analyzed with software developed in the IDL 6.0 environment, called HIPPO FAT (version 1.3, V. Positano) (25). Due to the T1-weighting, fatty tissues are represented with signal intensity in these images. VAT, SAT, dSAT and sSAT volumes were measured on 8 separate slices, with an interslice distance of 5 mm, around the L4-L5 intervertebrate level. HIPPO FAT automatically generates three contour lines at each image provided by an active fuzzy clustering algorithm (26) that allowed the separation of SAT from VAT: (1) along the outer margin of the SAT, (2) along the inner margin of the SAT and (3) around the smallest possible region in the visceral region that included all VAT. A histogram of signal intensities in the VAT region was provided, in which a Gaussian curve automatically fitted the high-intensity peak, which identified the visceral fat. After automatic segmentation, the analyst (TB), if necessary, manually adjusted both the contour lines and the shape of Gaussian curve by eyeballing. The MRI scan allows visualization of the scarp fascia as a fine black line. To divide sSAT from dSAT, a line was drawn manually over the scarp fascia. Adipose tissue pixels between this line and the outer margin of the SAT were defined as sSAT. dSAT was calculated by the total subcutaneous adipose tissue pixels minus the superficial subcutaneous adipose tissue pixels (dSAT = SAT – sSAT). Interclass correlation coefficients for inter-observer comparisons were 0.799 for VAT, 0.999 for SAT, 0.998 for dSAT and 0.999 for sSAT based on n = 11.

Quantification of hepatic fat content

All MR spectra were post-processed using the jMRUI software v3.0 package and the AMARES algorithm (27) to determine water (4.7 ppm) and methylene (1.3 ppm) resonance areas. Intrahepatic TG content was expressed as the fraction of the methylene signal in the combined signal of methylene and water (%). No correction for relaxation differences was applied. Based on the European guidelines (28) we considered NAFLD to be present when the ratio methylene to methylene and water was equal or higher than 5.6%.
Statistical analysis
Quantitative variables are shown as the mean ± standard deviation. As hepatic fat content is not normally distributed, the univariate analyses on the relations to hepatic fat content are performed by non-parametric tests (Spearman correlation), while all other univariate analyses are performed by Pearson correlations (Table 2 and 3). The association between the various abdominal adipose tissue compartments and the characteristics of the metabolic syndrome were investigated using linear regression analysis, adjusted for sex, age, alcohol intake and medication use (antidiabetic, antihypertensive drugs and statins). The statistical analysis was performed using SPSS version 22 (Armonk, NY: IBM Corp). A p-value less than 0.05 was considered to indicate statistical significance.

RESULTS
Baseline characteristics
In seven individuals MRI was not performed because of claustrophobia. In ten individuals it was not possible to calculate any abdominal adipose tissue volume due to insufficient MRI data (mostly movement artefacts). Our study cohort thereby consisted of 285 individuals (126 women and 159 men). In seven individuals it was not possible to calculate the VAT volumes due to low quality images and eighteen individuals could not be classified due to missing data for liver fat as MR spectroscopy could not be performed in these individuals due to technical difficulties.

Subjects with missing data on VAT or liver fat were excluded from analyses including these variables. The mean age was 67.1 ± 5.3 years and mean BMI was 30.6 ± 3.3 kg/m². 44.6% of the study population was treated for hypertension (54 women and 73 men), 25.3% were on lipid lowering medication (26 women and 46 men), 7.4% used oral antidiabetic medication (6 women and 15 men), and 1.8% used insulin. There was no significant correlation between the units of alcohol per day and the amount of hepatic steatosis. The baseline characteristics of the study population are shown in Table 1. A total of 153 individuals met the NCEP ATP III criteria for metabolic syndrome with no significant difference in the prevalence between women and men. The prevalence of a hepatic fat content ≥5.6% as measured by magnetic resonance spectroscopy was 48% in women and 54% in men.
Table 1 - Baseline characteristics of the study population (n = 285).

Abdominal adipose tissue distribution

Men had a higher weight (95.7 kg versus 83.3 kg), larger WC (109.2 cm versus 102.3 cm), and higher WHR (0.99 versus 0.91) than women, all p < 0.001. There was a clear sex difference in the abdominal adipose tissue distribution. On the eight 5 mm MRI slices as described before, men had a significantly higher volume of VAT (122.6 cm³ versus 98.7 cm³, p < 0.001) and a lower volume of SAT (137.1 cm³ versus 193.8 cm³, p < 0.001) compared to women, despite no difference in BMI. This resulted in a higher VAT/SAT ratio in men compared to women (0.97 versus 0.54). When adjusted for height, the difference between women and men of VAT and SAT remained significant. In particular, there was a profound difference in the volume of sSAT (50.3 cm³ in men versus 97.0 cm³ in women, p < 0.001), whereas the difference in dSAT volume was much smaller, but still significant (86.9 cm³ in men versus 96.8 cm³ in women, p = 0.007). This resulted in a higher sSAT/dSAT ratio in women compared with men (1.06 versus 0.61, p < 0.001).
Abdominal adipose tissue distribution in relation to metabolic syndrome

We next assessed whether the volume of the separate adipose tissue compartments and the hepatic fat content correlated with the metabolic parameters. In all individuals with metabolic syndrome, VAT and VAT/SAT ratio were significantly higher compared to individuals without the metabolic syndrome, both in women and in men. The hepatic fat content was twice as high in men with the metabolic syndrome (12.9% vs 6.6%, \( P < 0.001 \)), and this was also significantly higher in women with metabolic syndrome (12.1% vs 6.6%, \( P = 0.016 \)). Furthermore, in men with metabolic syndrome dSAT was significantly higher than in men without metabolic syndrome (94.0 cm\(^3\) vs 78.9 cm\(^3\), \( p = 0.001 \)), whereas no difference was found for sSAT.

In univariate analysis, both VAT and hepatic fat content showed a strong association with the separate components of the metabolic syndrome. In men, dSAT was associated negatively with HDL-c (Table 2). After adjustment for age, alcohol, and medication use, only in men dSAT was associated negatively with HDL-c in the plasma (Fig. 1). In contrast, we found no association between these markers and sSAT both in women women. As expected, VAT associated positively both in women and men with glucose and TG levels and was negatively associated with HDL-c. Hepatic fat content was associated in women with glucose and triglycerides and inversely with HDL-c, in men only glucose and TG correlated with hepatic liver fat (Fig. 1).

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<tr>
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<th>Volume VAT</th>
<th>Volume sSAT</th>
<th>Volume dSAT</th>
<th>Hepatic fat content</th>
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<td></td>
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<td>.513***</td>
<td>.400****</td>
<td>.558***</td>
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<td>-.096</td>
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<td>-.229**</td>
<td>-.075</td>
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<td>Glucose</td>
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<td>.213**</td>
<td>.106</td>
<td>-.027</td>
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Table 2 – Univariate correlations of hepatic fat content and the different abdominal fat compartments with the metabolic syndrome traits. Relation with hepatic fat content is based on spearman correlations, relation with abdominal fat compartment volumes are based on pearson correlation. VAT Visceral Adipose Tissue; sSAT superficial Subcutaneous Adipose Tissue; dSAT deep Subcutaneous Adipose Tissue. HDL-c High-Density Lipoprotein cholesterol; BP Blood Pressure. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).
Figure 1 – Associations of metabolic parameters with the different abdominal adipose tissue compartments, adjusted for age, alcohol intake, medication use and stratified for sex. On the Y-axis the standardized β coefficients are reported. Blue bars represent women and white bars represent men. Every metabolic parameter is adjusted for specific medication use (A for antidiabetic medication, B and C for lipid lowering medication).

VAT Visceral Adipose Tissue; dSAT deep Subcutaneous Adipose Tissue; sSAT superficial Subcutaneous Adipose Tissue. HFC Hepatic Fat Content *P < 0.05.
Abdominal adipose tissue distribution in relation to hepatic fat content

In all individuals, there was a significant correlation between VAT and hepatic fat content. This association remained significant, also after adjustment for age, alcohol intake and other abdominal fat compartment volumes (sSAT and dSAT in this case), with a standardized beta of 0.184 for men ($p = 0.037$) and a standardized beta of 0.304 for women ($p = 0.001$). Only in men however, dSAT was positively associated with both VAT and hepatic fat content in a bivariate analysis (Table 3).

As the amount of total adipose tissue could influence the relation between the different adipose tissue compartments and hepatic steatosis, we investigated the associations with VAT, dSAT and sSAT for different BMI categories (BMI 27 – 30 kg/m$^2$ and BMI 30 – 40 kg/m$^2$; data not shown) after correction for age, alcohol intake and other abdominal fat compartment volumes. In men, the association between different adipose tissue compartments and hepatic steatosis was not significant anymore. However, large differences were observed between women with overweight and obesity. VAT was strongly positively associated with hepatic fat content, whereas sSAT was significantly negatively associated with hepatic fat content in women with a BMI between 30 and 40 kg/m$^2$ with a standardized beta of -0.300, $p = 0.04$. In women with overweight (BMI between 27 and 30 kg/m$^2$) however, no associations were seen between the abdominal fat compartments and hepatic fat content.

**DISCUSSION**

In this study, we observed profound sex differences in the abdominal fat distribution and its correlations with hepatic steatosis and components of the metabolic syndrome. Although women and men had similar BMI, men had significantly higher volumes of VAT and relatively more dSAT compared to women. Within the SAT compartment, women had a much higher ratio of superficial to deep SAT compared to men. Importantly, only in men, dSAT was positively associated with hepatic fat content and negatively associated with HDL-c, whereas

<table>
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<th>Hepatic fat content</th>
<th>Volume dSAT</th>
<th>Volume sSAT</th>
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<tbody>
<tr>
<td></td>
<td>Women (n = 120)</td>
<td>Men (n = 147)</td>
<td>Women (n = 126)</td>
</tr>
<tr>
<td>Volume VAT</td>
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<td>.322***</td>
<td>.148</td>
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<tr>
<td>Volume sSAT</td>
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<td>.498***</td>
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<td>Volume dSAT</td>
<td>.113</td>
<td>.190*</td>
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</table>

Table 3 – Univariate correlations between hepatic fat content and between different abdominal fat compartments. Relation with hepatic fat content is based on spearman correlations, relation with abdominal fat compartment volumes are based on pearson correlation.

**VAT** Visceral Adipose Tissue; **sSAT** superficial Subcutaneous Adipose Tissue; **dSAT** deep Subcutaneous Adipose Tissue. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.**
in women, dSAT did not show any associations with these parameters. In contrast, only in women with obesity, sSAT was negatively associated with hepatic fat content.

A sex difference in SAT distribution has been reported by Golan et al. (20), who investigated VAT, total SAT and sSAT measurements by MRI. In line with our results, they observed that men had a higher VAT/SAT ratio and women had a higher proportion of abdominal sSAT. Furthermore, our results confirm the results of another study, who suggested that in men adiposity is characterized by disproportionate expansion of dSAT compared with sSAT, whereas in women both dSAT and sSAT expand similarly in obesity (29). A greater proportion of dSAT in men at any level of subcutaneous or total body fat compared with women was previously observed, in contrast with our results (30). However, in that study, single slice computed tomography was used to evaluate abdominal adipose tissue compartments, which may explain the differences with our findings. A possible explanation for the higher VAT content and lower dSAT and sSAT content in men could be the difference in hormonal status. Hyperandrogenism in normal weight PCOS women was associated with preferential visceral fat deposition and moreover an increased proportion of small subcutaneous abdominal adipocytes, which could limit subcutaneous fat storage (31). Moreover, six months of testosterone therapy in aging men with a low normal bioavailable testosterone decreased subcutaneous fat on the abdomen and lower extremities, while visceral fat remained unchanged (32).

In all individuals, VAT correlated significantly with hepatic fat content. As previous studies have shown that fat volumes could be influenced by medication use (33-35), we also adjusted the analysis of VAT and hepatic fat content for antidiabetic, antihypertensive and lipid lowering medication, however, VAT still remained positively associated with hepatic fat content in all individuals. It would be interesting to investigate whether the duration of medical therapy would influence this relation. Importantly, in men, dSAT also correlated positively with hepatic fat content, while in women with obesity sSAT correlated negatively with hepatic fat content. Yaskolka Meir et al. found in a group of 275 individuals selected on a high waist circumference or dyslipidemia, only a significant positive association for VAT with hepatic fat content, while sSAT was inversely associated with hepatic fat content in an age, sex and waist circumference adjusted model (36). However they did not perform analysis stratified by sex, which could explain the differences with our results. Another study related abdominal adipose tissue volumes in a small group of type 2 DM patients with peripheral and hepatic insulin resistance. They found that dSAT was only in men associated with peripheral and hepatic insulin resistance (37). An explanation for the clear difference in women with obesity between dSAT and sSAT in its association with hepatic steatosis and metabolic syndrome may be the contrasting morphological cellular aspects of dSAT and sSAT. As mentioned before, dSAT is more lipolytically active than sSAT, which can contribute to elevated levels of FFA in the circulation (18). This would also explain the correlation with hepatic fat content in men. A potentially protective effect of sSAT in the development of
hepatic steatosis is its high level of adiponectin, compared to dSAT (21). Adiponectin has been considered to be able to protect hepatocytes from TG accumulation, probably by increasing β-oxidation of FFA and/or decreasing de novo FFA production within hepatocytes (38, 39). Adipocytes in sSAT may have an increased cell size because they have an increased ability for fatty acid storage. In contrast, the presence of smaller cells in dSAT may be a reflection of their relative impairment to store fat, subsequently leading to inflammation and stress signals (40). In addition, dSAT and sSAT have different mRNA expression of inflammatory, lipogenic and lipolytic target genes (21, 29); ADIPOR1, an mRNA expression of adiponectin, is higher in sSAT compared with dSAT, while the mRNA expressions of leptin, hormone sensitive lipase and lipoprotein lipase (LPL) showed higher expression levels in dSAT compared with sSAT. The expression of genes involved in inflammatory pathways, such as interleukin-6 and MCP1, and genes involved in fatty acid synthesis, such as FASN, are higher in dSAT compared with sSAT (21, 29). In addition, Walker et al. also found an intermediate phenotype of dSAT with respect to the gene expression profiles of 11beta-HSD1, leptin and resistin (41).

Based on the multivariate correlations between the abdominal adipose tissue compartments and the circulating concentrations of the metabolic parameters glucose, TG and HDL-c, dSAT seems to be an intermediate phenotype between sSAT and VAT, especially in men. This finding in men has been described earlier by Marinou et al. (29). In their study, fasting glucose was only associated with dSAT in men. Another study showed that both VAT and dSAT were associated with insulin-stimulated glucose utilization, HDL-c and TG, while sSAT showed weaker association with all these parameters (42). This study performed also no sex-stratified analysis.

The main strength of our study is the large population size, which enabled us to investigate the differences between women and men. The assessment of both adipose tissue compartment volumes and hepatic fat content by MRI is another strength of the present study. Our study also has a number of limitations. Because of the cross-sectional design, no causal interpretations can be made of the associations observed. We only included participants of Western European ancestry, which precludes extrapolation of our results to other ethnic groups. As we included individuals who were 55-80 years of age and all women were postmenopausal, our results cannot be extrapolated to other age groups. Next to this, we only included individuals with overweight and obesity which could weaken the strength of the associations found in comparison to analyses of cohorts including subjects across a wide range of BMI.

In conclusion, VAT as measured by MRI is positively correlated with hepatic steatosis in women and men. Men have relatively more dSAT compared to women, and only in men, dSAT correlated positively with hepatic fat content. Moreover, dSAT in men associated negatively with HDL-c in multivariate analyses; while in women with obesity sSAT correlated negatively with hepatic fat content. dSAT seems to be an intermediate metabolic phenotype between
sSAT and VAT. Therefore in future studies it is important to distinguish between dSAT and sSAT and between women and men, as we have shown a different impact on metabolic traits.
REFERENCES


CHAPTER 3

Female-specific influence of adipose tissue distribution on systemic and adipose tissue inflammation


* Contributed equally

To be Submitted
ABSTRACT

Introduction
Women have more subcutaneous adipose tissue and less visceral adipose tissue than men. The aim of our study was to determine whether these differences in body fat distribution account for sex-specific differences in low-grade inflammation in individuals with obesity and overweight.

Methods
We recruited 285 individuals with a BMI≥27 kg/m², aged 55-81 years. Abdominal magnetic resonance imaging was performed around level L4-L5 to measure visceral adipose tissue (VAT), deep subcutaneous adipose tissue (SAT) and superficial SAT volumes. Circulating cytokines, leukocyte count and differentiation were measured and peripheral blood mononuclear cells (PBMCs) were stimulated ex vivo. Adipose tissue biopsies were taken from the superficial SAT and analysed for histology and gene expression of inflammatory genes.

Results
In women, SAT volumes showed positive associations with leukocyte number, monocytes and circulating IL-6, hsCRP and IL-18BP. In addition superficial SAT was positively associated with IL-6 and IL-1Ra production of stimulated PBMCs in women. Next to that, the gene expression level of different pro-inflammatory genes were related mainly to the volume of the superficial SAT in women. In contrast in men, VAT was positively associated to leukocyte number, circulating IL-6, hsCRP and IL-18BP. To investigate whether this association originates from the adipose tissue itself, we characterized adipocyte cell size and gene expression and found in men a positive association between the adipocyte cell size, VAT and SAT.

Conclusion
In an overweight and obese cohort, in women, SAT is the main contributing adipose tissue compartment for the low-grade inflammatory status. While in men mainly VAT is positively associated with markers of low-grade inflammation in men.
INTRODUCTION

Fat distribution is known to play an important role in the propensity of an individual to develop CVD, for instance increased visceral adipose tissue (VAT) is related to an increased cardiovascular risk (1). Importantly, fat distribution is sex-specific, women tend to have less VAT and more subcutaneous adipose tissue (SAT). Chronic low-grade inflammation in the adipose tissue has been proposed to be one of the causal links between obesity and atherosclerosis (2). Although this inflammation is present in low levels, even these modest increases in circulating inflammatory mediators have been strongly associated with the development of type 2 diabetes and CVD (3, 4). The CANTOS trial showed that inhibiting the pro-inflammatory cytokine IL-1β by canakinumab, reduces cardiovascular death, non-fatal myocardial infarction and non-fatal stroke (5). Very recently, the anti-inflammatory drug colchicine has also shown to reduce the risk of cardiovascular events in individuals with chronic coronary disease (6). These studies confirmed that chronic low-grade inflammation is a causal factor in the progression of atherosclerosis.

We have previously shown sex-specific patterns in obesity-associated inflammation during metabolic dysregulation. Women with obesity-induced metabolic dysregulation were characterized by a deficiency of the anti-inflammatory adiponectin, whereas men with the metabolic syndrome have higher levels of the pro-inflammatory IL-6 and leptin (7). We also found in women that adiponectin was negatively associated with increased adipose tissue inflammation. In contrast in men, adipose tissue inflammation was only positively associated with circulating leptin and IL-6 only. This suggests a sex-specific role for adipose tissue on the obesity associated low-grade inflammatory state. Until now most studies investigating the effects of fat distribution on chronic low-grade inflammation have been performed in men. Therefore the aim of the study presented here was to investigate sex-specific differences in the association between the various fat compartments and measures of systemic inflammation and adipose tissue inflammation in a group of overweight and obese individuals.

METHODS

Study population

We enrolled 302 individuals aged between 55 to 80 with a BMI above 27 kg/m² at screening in the 300-Obesity (300-OB) cohort study at the Radboud university medical center as previously described (7). Subjects with a recent cardiovascular event (MI, transient ischemic attack, stroke < 6 months), a history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral subcutaneous anticoagulant therapy, use of thrombocyte aggregation inhibitors other than acetylsalicylic acid and carbasalate calcium were excluded. All subjects filled out questionnaires about lifestyle and medication use. All lipid-lowering medication, when used, were discontinued for four weeks prior to the measurements. All participants received detailed printed and oral information and provided written informed consent. The study was approved by the Ethical
Committee of the Radboud University (nr. 34462.091.10). Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

**MRI data acquisition**
As previously described (8), abdominal fat distribution were determined by magnetic resonance imaging (MRI). A 3.0 T Magnetom Skyra or Trio MR system (Siemens, Erlangen, Germany) was used to perform the MR examinations. Individuals were examined in the supine position with their arms positioned parallel to the lateral sides of the body. At the L4-L5 level, sixteen axial T1-weighted MRI slices of 0.5cm were acquired for each subject. Breathing commands were used to avoid motion-induced artifacts.

VAT, SAT, sSAT and dSAT analysis
The images acquired were retrieved from the MR scanner and analyzed with software developed in the IDL 6.0 environment, called HIPPO FAT (version 1.3, V. Positano) (9). Due to the T1-weighting, fatty tissues are represented with signal intensity in these images.

VAT, SAT, dSAT and sSAT volumes were measured on 8 separate slices around the L4-L5 intervertebrate level, with an interslice distance of 5 mm. HIPPO FAT automatically generates three contour lines at each image provided by an active fuzzy clustering algorithm (10) that allowed the separation of SAT from VAT: (1) along the outer margin of the SAT, (2) along the inner margin of the SAT and (3) around the smallest possible region in the visceral region that included all VAT. A histogram of signal intensities in the VAT region was provided, in which a Gaussian curve automatically fitted the high-intensity peak, which identified the visceral fat. After automatic segmentation, the analyst (TB), if necessary, manually adjusted both the contour lines and the shape of Gaussian curve by eyeballing. The MRI scan allows visualization of the scarpa fascia as a fine black line. A line was drawn manually over the scarpa fascia to divide sSAT from dSAT. Adipose tissue pixels between this line and the outer margin of the SAT were defined as sSAT. dSAT was calculated by the total subcutaneous adipose tissue pixels minus the superficial subcutaneous adipose tissue pixels (dSAT = SAT – sSAT).

**Biochemical analyses**
Biochemical characteristics were determined as described previously (7). Blood sampling was performed in the morning after an overnight fast. Blood glucose, triglycerides (TG) and high-density lipoprotein cholesterol (HDL-c) were measured using standard laboratory procedures. Cell count data - Immune cell counts were determined in fresh whole blood EDTA samples using the Sysmex XE-5000. Circulating cytokines - Plasma concentration of circulating cytokines were determined using enzyme-linked immunosorbent assays (ELISA).

Stimulation experiments
**PBMC stimulation experiments** - Isolation of peripheral blood mononuclear cells (PBMCs) was performed as described in Oosting et al (11).
**Whole blood stimulation experiments** - A volume of 100 μl of heparin blood was added to a 48 well plate (Corning) containing 400 μl stimulus (final volume 500μl/well) for 48 hours at 37°C and 5% CO2.

**ELISA analysis** - Cytokine concentrations after stimulation were measured using commercially available ELISA kits.

**Stimuli and cytokines**
Table 1 in the supplementary material lists the concentrations of the stimuli used. The choice of pro-inflammatory mediators was based on the extensive body of evidence linking cytokines and adipokines to inflammation and CVD complications. Stimulation of PBMCs was performed with a comprehensive set of stimuli containing both purified innate immune stimuli that are associated with chronic inflammation (e.g. LPS, oxidized low-density lipoprotein (LDL)) and microorganism that are the source of microbial ligands that translocate in the circulation at the intestinal level. Interleukin (IL)-1β, IL-6, TNF-α, and IL-1Ra were measured after 24-hour stimulation with these stimuli. After 7 days of stimulation for C. albicans and S. aureus, IFN-γ, IL-17, and IL-22 were measured.

**Protein measurements**
Circulating plasma inflammatory markers were measured as previously described (12). In short, we used the commercially available Olink Proteomics AB (Uppsala Sweden) Inflammation Panel (92 inflammatory proteins), using a Proceek © Multiplex proximity extension assay (13). In this assay proteins are recognized by pairs of antibodies coupled to cDNA strands, which bind when they are in close proximity and extend by a polymerase reaction. A pooled plasma sample and an interplate control were included on each plate in triplicate to correct for batch differences. Detected proteins are normalized and measured on a log2-scale as normalized protein expression values.

**Adipose tissue analysis**
After an overnight fast, subcutaneous adipose tissue biopsies were obtained under local anaesthesia by needle biopsies performed 6-10cm lateral to the umbilicus in the right lower quadrant.

*Adipocyte size* - The morphometry of individual fat cells was assessed using digital image analyses as described previously (14). For each participant, the adipocyte cell diameters of all adipocytes in four microscopic fields of view were counted and measured. On average, 219 adipocytes (range 113 - 330) were measured per field.

*Immunohistochemistry* - To detect macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Serotec, Oxford, UK). Sections were preincubated with 20% normal horse serum followed by overnight incubation at 4 °C with the primary antibody diluted 1:40 in phosphate-buffered saline, 1% bovine serum albumin. After incubation with the primary antibody (mouse anti-human), a horse anti-mouse IgG conjugated to horseradish peroxidise (Vector labs brunschwigh) was used as a secondary antibody. Visualization of the
complex was done using 3,3’-diaminobenzidine for 12 min. Negative controls were used by omitting the primary antibody. Hematoxylin and eosin staining of sections was done using standard protocols. The percentage of macrophages was expressed as the total number of macrophages divided by the total number of adipocytes counted in 15 random microscopic fields of view. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages (15).

**Adipose tissue inflammation score** – To robustly quantify adipose tissue inflammation among study participants using histology, several parameters were assessed and combined into an adipose tissue inflammation score (AT score), as the phenotype by which inflammation in the adipose tissue presents is quite heterogenous (16, 17). This assessment led to a score including following parameters: a mean adipocyte diameter above the average diameter of the cohort (> 51.7um (mean diameters in the 300-OB cohort) was defined as one point in the AT score, the percentage of macrophages above the average of the cohort (> 12.6% (mean in the 300-OB cohort) was defined as one point in the AT score and the presence of crown-like structures (CLS) was defined as one point in the AT score. Hence, the adipose tissue inflammation score could range from 0 (no inflammation) to 3 (severely inflamed) (as shown in Figure 1).

**Figure 1** – Immunohistochemically illustration by CD68 staining of individual with a high level of adipose tissue inflammation, a crown-like structure is indicated by an arrow (adipose tissue score 3; mean adipocyte diameter of 60 um, 13% CD68 cells present and 5 crown-like structures in the analysed fields).

**RNA isolation and qPCR analysis** – Total RNA was isolated from adipose tissue using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Concentrations and purity of RNA samples were determined on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Landsmeer, The Netherlands). In total 1μg of RNA was used for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was done to study the mRNA expression levels of leptin, adiponectin, CD68, MCP-1, IL-1α, IL-1Ra, IL-18, IL-18BP, IL-18Rα, IL-18Rβ and IL-37. The gene expression of IL-1Ra, IL-18, IL-18BP and IL-18Ra were present in low quantities in the majority of the individuals, as completion of the standard curve was not possible, we did not include these genes in our analyses. The sequence-specific primer pairs of the other genes are listed in Table 2 in the supplementary material. qPCR was performed by platinum Taq polymerase (Invitrogen) and SYBR Green using an iCycler PCR machine (Bio-Rad). Melt curve analysis was included to assure a single PCR product was formed. The mRNA expression of all genes reported was normalized to Ribosomal Protein L37a (RPL37A) gene expression.
Statistics
Correlations and corresponding p-values were calculated using the rank-based Spearman correlation as implemented in the “cor.test()” function of the “R” programming language (R Foundation for Statistical Computing, Vienna, Austria).
Regression analysis was performed using the ‘lm’ function, which is part of the ‘stats’ package, as implemented in the ‘R’ language. Before the regression analysis, the circulating cytokines of inflammation and were normalized using logarithmic transformation to get a distribution more closely resembling a normal distribution. Multiple testing correction was performed using the Benjamini – Hochberg False Discovery Rate method as implemented in the “p.adjust” function of the “stats” package in the R programming language (18). Standardized regression Coefficients ($\beta$) were calculated based on objects of class ‘lm’ using the ‘lm.beta’ function part of the ‘QuantPsyc’ package in the R programming language. The covariates included in all regression models are: age, seasonality and pack years.

RESULTS
Baseline characteristics
The 300-OB cohort consists of 302 individuals of Western-European ancestry with a BMI higher than 27 kg/m2. We were unable to acquire MRI data in fourteen individuals, as seven individuals were claustrophobic and in seven individuals the MRI data were insufficient to calculate any abdominal adipose tissue volumes (mostly due to movement artefacts). So in total 128 women and 160 men were selected for these analyses. Incomplete MRI data were obtained from eleven individuals as we could not calculate VAT volumes due to low quality images. Baseline characteristics are shown in Table 1. There were no significant differences between women and men with regard to age, BMI, antihypertensive, lipid-lowering and antidiabetic drugs. Tobacco use was significantly higher in men.

Sex differences in adipose tissue distribution
BMI did not differ between women (30.5 kg/m2) and men (31.0 kg/m2). Women had significant more total abdominal fat, the sum of VAT and SAT, (288 cm$^3$ vs 257 cm$^3$, p < 0.0001) and more hip subcutaneous fat (158 cm$^3$ vs 88 cm$^3$, p < 0.0001). With regard to the separate abdominal adipose tissue compartments, women had less VAT (99 cm$^3$ vs 123 cm$^3$, p < 0.0001), but more superficial SAT (97 cm$^3$ vs 49 cm$^3$, p < 0.001) and slightly more deep SAT and 98 cm$^3$ vs 87 cm$^3$, p < 0.01).
<table>
<thead>
<tr>
<th></th>
<th>Total (n = 288)</th>
<th>Women (n = 128)</th>
<th>Men (n = 160)</th>
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<tbody>
<tr>
<td>Age (y)</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>30.6 +/- 3.4</td>
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<td>WC (cm)</td>
<td>106.2 +/- 9.6</td>
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<td>Pack years</td>
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<td>Total cholesterol (mmol/L)</td>
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<td>6.6 +/- 1.0</td>
<td>6.0 +/- 1.1</td>
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<tr>
<td>LDL-c (mmol/L)</td>
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<td>4.4 +/- 0.9</td>
<td>3.9 +/- 1.0</td>
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<td>HDL-c (mmol/L)</td>
<td>1.3 +/- 0.3</td>
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<td>TG (mmol/L)</td>
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<td>SBP (mmHg)</td>
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<td>DBP (mmHg)</td>
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<td>Lipid lowering med. (%)</td>
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<td>Antihypertensive med. (%)</td>
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<td>43.8%</td>
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<td>Antidiabetic med.(%)</td>
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<td>6.3%</td>
<td>9.4%</td>
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<td>hsCRP (ug/mL)</td>
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<td>3.65 +/- 3.73</td>
<td>2.39 +/- 2.61</td>
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<tr>
<td>IL-6 (pg/mL)</td>
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<tr>
<td>IL-18 (pg/mL)</td>
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<td>IL-18BP (ng/ml)</td>
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</table>

Table 1 - Baseline characteristics of 300-Obesity Cohort presented as mean +/- standard deviation or n (%) in total cohort, women and men. WC: waist circumference; TG: Triglycerides; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Ns Not significant.

Contribution of adipose tissue compartments to systemic inflammation is sex-specific

Relation to white blood cell count and components

One of the markers of systemic low-grade inflammation is a high white blood cell count of which especially the monocytes and neutrophils play a key role in the progression of atherosclerosis (19). In women, no significant relation was present between the VAT volume and total leukocyte count or individual components. However, there was a strong association present between the SAT with the total leukocyte count. Looking at the leukocyte subtypes, the association with monocytes was the strongest with a stand. β of 0.34, p < 0.0001. Significant positive associations with total SAT were also present for neutrophils and basophils (see Table 2). In contrast, in men not SAT but VAT was positively associated with the leukocyte count. Further subdividing this into the individual leukocyte subtypes, in men no associations are present anymore. To summarize, in women SAT was positively associated with leukocyte number, while in men VAT was the only compartment that showed a positive association with leukocyte number.
Table 2 – Heatmap of correlation between different adipose tissue compartment volumes and white blood cell count and component in women (A) and men (B). Standardized betas after adjustment for age, pack years and season. The intensity of the red and blue colours represent the strength of the significance.

VAT Visceral adipose tissue; SAT Subcutaneous adipose tissue; sSAT Superficial subcutaneous adipose tissue; dSAT Deep subcutaneous adipose tissue; SAT_hip Subcutaneous adipose tissue at the hip region; VAT+SAT Visceral plus subcutaneous adipose tissue.

Relation to circulating cytokines

Leptin levels were strongly associated with SAT volumes in both women and men with stand. $\beta$ of 0.60 vs 0.62 respectively, $p < 0.0001$ (see also Table 3). Adiponectin levels were also sex-independently associated with VAT and total abdominal fat. For the other cytokines we observed significant sex-specific differences in the relation with fat compartments.

Levels of IL-6 were associated in women only with SAT volume (stand. $\beta = 0.30$, $p < 0.001$), whereas in men this related to VAT and SAT (stand. $\beta = 0.30$, $p < 0.0001$). Also for IL-18BP, which we have previously shown to be associated with carotid atherosclerosis (van den Munckhof et al, under revision), the associations were sex-specific: in men IL-18BP levels were related to VAT (stand. $\beta = 0.16$ with $p < 0.05$), while in women these levels were only related to the amount of SAT (stand. $\beta = 0.22$ with $p < 0.001$). hsCRP levels showed a comparable association with the SAT and VAT volume in women and men. The amount of hip SAT in both women and men were significantly related to IL-6 levels (stand. $\beta$’s of 0.34 and 0.30 respectively, $p < 0.0001$) and weaker, but still significantly, with hsCRP.
### Women

<table>
<thead>
<tr>
<th></th>
<th>VAT</th>
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<th>dSAT</th>
<th>SAT_hip</th>
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<td>dSAT</td>
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<td>Leptin</td>
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<td>Adiponectin</td>
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### Men

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**Table 3 - Heatmap of correlation between different adipose tissue compartment volumes and a panel of circulating inflammatory mediators in women (A) and men (B). Standardized betas after adjustment for age, pack years and season. The intensity of the red and blue colours represent the strength of the significance. VAT Visceral adipose tissue; SAT Subcutaneous adipose tissue; sSAT Superficial subcutaneous adipose tissue; dSAT Deep subcutaneous adipose tissue; SAT_hip Subcutaneous adipose tissue at the hip region; VAT+SAT Visceral plus subcutaneous adipose tissue; hsCRP High sensitivity C-reactive protein; IL- Interleukin--; VEGF Vascular endothelial growth factor; AAT Alpha-1 antitrypsin.**

**Relation to cytokine production capacity**

Cytokine production capacity of PBMC’s is an indicator of the intrinsic inflammatory responsiveness and has previously been related to the presence of atherosclerosis (20, 21). Production of the pro-inflammatory cytokine IL-6 after stimulation of PBMC’s with C. albicans and LPS is associated with the volume of the SAT in women (stand. β 0.25 and 0.17 respectively, p < 0.01 and p < 0.05 respectively). The production of the anti-inflammatory cytokine IL-1Ra after stimulation with C. Albicans are both in men and women positively
associated SAT and total abdominal fat. All associations are shown in Table 3 in the supplementary material.

**Sex-specific contribution of fat distribution to low-grade inflammation measured with proteomics**

In addition to immunophenotyping, the quantification of circulating cytokines levels and stimulation experiments, we also measured the proteins levels of 92 different inflammatory proteins. After correction for multiple testing, in women 7 proteins were significantly associated with adipose tissue volume versus 12 in men (Table 4). Supporting our other findings, most inflammatory proteins were positively related to the amount of SAT in women, whereas in men mainly the VAT showed positively associations. Both in women and men the strongest associations are seen between SAT and VAT respectively with IL-6, hepatocyte growth factor (HGF) and macrophage colony-stimulating factor (M-CSF). In women the relation with SAT was almost exclusively for sSAT, while in men the association with IL-6, HGF and M-CSF were the strongest for VAT and for dSAT, but not for sSAT.

Table 4 – Heatmap of correlation between top 7 protein levels in relation to different adipose tissue compartment volumes in women (A) and men (B). Standardized betas after adjustment for age, pack years and season and multiple testing. The intensity of the red colour represent the strength of the significance. VAT Visceral adipose tissue; SAT Subcutaneous adipose tissue;
sSAT Superficial subcutaneous adipose tissue; dSAT Deep subcutaneous adipose tissue; SAT_hip Subcutaneous adipose tissue at the hip region; VAT+SAT Visceral plus subcutaneous adipose tissue; HGF Hepatocyte growth factor; M-CSF1 Macrophage colony-stimulating factor 1; IL- Interleukin; TNF-r sm9 Tumor necrosis factor- receptor superfamily member 9; C-CMC19 C-C motif chemokine 19; TNF-1 Tumor necrosis factor- ligand; FGF-21 Fibroblast growth factor-21.

In conclusion, sex-specific differences are present in the relation between the various fat compartments with inflammatory mediators. The mechanism behind this difference is not yet known. As adipose tissue inflammation is an hallmark of chronic low-grade inflammation, we took fat biopsies from the sSAT in all our individuals and investigated the relation between the fat distribution and markers of adipose tissue inflammation.

**Sex-specific adipose tissue distribution in relation to adipose tissue inflammation**

We only found a higher expression level of leptin in the adipose tissue biopsies in women compared to men. Furthermore, no significant differences were found between women and men in the histology and gene expression profiles. When focusing on the relation of sSAT, of which the fat biopsies were taken, we found increased expression in women with increasing volume of sSAT, both for the pro-inflammatory leptin and TNF-alpha, as for the anti-inflammatory adiponectin and the macrophage-marker CD68 (Table 5). In men, the gene expression of some cytokines were also related to the volume of sSAT, although less strongly. The size of adipocytes however showed quite strong associations with mainly the volume of SAT and dSAT in men, but not showed no significant associations in women.

<table>
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<th>SAT at hip</th>
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Table 5 – Heatmap of correlation between adipose tissue histology and adipose tissue gene expression of different adipokines and pro- and anti-inflammatory (after normalization to Ribosomal Protein L37a (RPL37A) gene expression) and different adipose tissue compartment volumes in women (A) and men (B). Standardized betas after adjustment for age, pack years and season. The intensity of the red and blue colours represent the strength of the significance. VAT Visceral adipose tissue; SAT Subcutaneous adipose tissue; sSAT Superficial subcutaneous adipose tissue; dSAT Deep subcutaneous adipose tissue; SAT_hip Subcutaneous adipose tissue at the hip region; VAT+SAT Visceral plus subcutaneous adipose tissue; CLS Crown-like structure; ATscore Adipose tissue score; MCP-1 Monocyte Chemoattractant Protein 1; TNF- Tumor necrosis factor-α; IL- Interleukin-; IL-18Rβ Interleukin-18 Receptor β.

We previously reported that in women adiponectin was negatively associated with the AT score, while in men adipose tissue inflammation was positively associated with circulating leptin and IL-6 (7). We now expanded these analysis to all circulating markers and found that the relation between the AT score and adiponectin was dependent on the adipocyte cell size and number of crown-like structures. In women only IL-18 showed a positive association with the adipocyte cell size and AT score. The relation between the AT score and leptin in men, was mainly dependent on the adipocyte cell size (Table 6).
### Table 6 – Heatmap of correlation between different adipose tissue inflammation histological parameters and circulating cytokines in women (A) and men (B). Standardized betas after adjustment for age, pack years and season. The intensity of the red and blue colours represent the strength of the significance. CLS Crown-like structure; ATscore Adipose tissue score; hsCRP High sensitivity C-reactive protein; IL- Interleukin-; VEGF Vascular endothelial growth factor; AAT Alpha-1 antitrypsin.

**DISCUSSION**

In the present study, we found clear sex-specific differences in the association between adipose tissue distribution, markers of chronic low-grade inflammation, and adipose tissue inflammation in overweight and obese individuals. We observed that in women SAT is mainly related to white blood cell count, circulating levels of inflammatory markers (hsCRP, IL-6 and IL-18BP) and higher levels of the production of pro-inflammatory cytokines after ex vivo stimulation experiments. We also found that SAT, mainly sSAT, was related to higher levels of the proteins IL-6, HGF and M-CSF. In contrast, in men VAT showed the strongest association with the number of leukocytes, whereas VAT and SAT were equally associated with circulating cytokines (hsCRP and IL-6). The relation with protein levels of IL-6, HGF and M-CSF was...
significant positive with VAT volume and dSAT, but not sSAT, in men. This suggests that the hypothesis about a beneficial effect of SAT appears not to be applicable to women, at least not to women with overweight or obesity. We hypothesized that the possible mechanism behind this sex-specific difference would be present in the adipose tissue itself. We therefore took adipose tissue biopsies and analyzed them histologically and performed analysis on the expression of RNA. Indeed, in women the volume of sSAT showed a stronger association to the tissue expression level of inflammatory proteins, such as CD68 and TNF-α, than in men. In addition, as previously described we found significant sex-specific differences in the relation between the adipose tissue histology, in which in women the AT score was negatively related to adiponectin, while in men adipose tissue inflammation was related to leptin, hsCRP and IL-6. In the current analysis we found in women a positive association between IL-18 and the AT score, which was not found in men. These findings call for a sex-specific approach with regard to interpretation of adipose tissue distribution and its contribution to low-grade inflammation.

A number of studies investigated the contributing role of VAT and SAT on markers of low-grade inflammation (22-25). However, a sex-stratified analysis to investigate sex-specific differences in the association between fat distribution and systemic inflammation was performed only in a minority of studies. A larger contribution of SAT to a pro-inflammatory status in women has previously been described. In a group of 145 healthy women and 208 healthy men a mean age of 36.8 years and 42.2 respectively, and mean BMI of 26.6 and 26.0 kg/m² respectively, CRP levels were higher in women. Whereas the CRP concentrations in men were largely influenced by VAT, SAT was the key correlate of CRP in women (26). We extend these finding by showing that these sex differences in the role of different fat compartments are not only restricted to systemic inflammatory markers such as CRP, but can be found for different parameters of inflammation, including circulating leukocyte counts and an extensive panel of circulating inflammatory markers. The strong association in both women and men between SAT and VAT respectively with M-CSF and HGF suggest a possible relation between the adipose tissue and bone marrow. Both the M-CSF and HGF have been shown to be produced in the adipose tissue (27, 28). Possibly in women the leukocytes/monocytes that are present in the SAT could produce M-CSF and HGF, which stimulates the bone marrow to produce more pro-inflammatory cells, while in men mainly the leukocytes in the VAT are responsible for this upregulation. A previous study has shown that obesity is associated with HGF (27). A recent study suggested that HGF inhibits the vicious cycle of adipocytes and macrophages through the inhibition of macrophage-mediated pro-inflammatory cytokines and upregulation of adiponectin in adipocytes (29). M-CSF on the other hand has long been known to play a role in adipocyte hyperplasia and the physiological regulation of adipose tissue growth (28). Maybe the sex-specific differences between fat distribution and inflammatory markers originates partly from differences in adipose tissue production of M-CSF and HGF in SAT and VAT in women and men. The relation that SAT in women and VAT in men are respectively the most important fat compartment for a pro-inflammatory status is also supported by another large study.
investigating a cohort of 4,261 women and men, aged 25-70 years. They found that a considerably higher percentage of variability in inflammatory markers was explained by body composition in women compared to men (30). One limitation is that the fat measurements were performed via bioelectric impedance. For IL-6, waist circumference explained in both women and men the highest percentage of variability. As it has been shown previously that WC in men is mainly influenced by VAT, whereas in women WC is mainly determined by the amount of SAT (31), this would suggest a higher contribution of SAT to IL-6 in women. However, conflicting data with regard to the contributing role of VAT and SAT to low-grade inflammation has been reported. In 1250 patients from the Framingham Heart risk study, VAT and SAT contributed similarly to CRP in women and men, while the contribution of both compartments in women was higher compared to men (32). Another large cohort of healthy elderly (Health ABC) found that after correction for BMI, VAT was most consistently associated with higher IL-6 and CRP concentrations in both women and men (33). However, as BMI is part of the causal chain of the relation between VAT, SAT and low-grade inflammation, we did not adjust for BMI as a confounding factor. When concentrating on this relation in the Health ABC study, SAT showed comparable associations as VAT to CRP and IL-6 levels in women, while in men SAT was only significantly related to CRP in black men.

Etiology of systemic low-grade inflammation

A contributing role of adipose tissue to inflammation was discovered by Hotamisligil et al. and colleagues more than 25 years ago. They found increased mRNA and protein levels of TNF-α in epididymal adipose tissue of rodent models of obesity and diabetes (34). Since then a growing body of evidence has shown a prominent role for low-grade inflammation in the pathogenesis of atherosclerosis. The current hypothesis on the obesity-associated low-grade inflammation and further cardiovascular risk states that fat mass expands due to a positive energy balance, which leads to adipocyte hypertrophy and finally to local hypoxia at adipocyte level. This leads to an altered secretion of adipokines and induces cell death, which provokes a pro-inflammatory reaction of both adipocytes and surrounding immune cells. During a healthy state, the adipose tissue constitutes for ~50% adipocytes and ~50% other cells as preadipocytes, endothelial cells, and mainly M2 macrophages (35). During obesity other inflammatory cells are attracted and cytokines produced by TH1 lymphocytes or bacterial products as LPS can mature the macrophages to a pro-inflammatory M1 macrophage (36). Both the adipocytes and the macrophages are important for cytokine production and probably act synergistically to amplify local inflammation (37). There are two important players in the pro-inflammatory process. The first is the inflammasome, which consists of intracellular sensor molecules, of which the NOD-like receptor family pyrin domain containing-3 (NLRP3) is one of the most important ones (38). The exact triggers for NLRP3 activation are not fully clear, but free fatty acids, reactive oxygen species, glucose, LPS and oxidized LDL-cholesterol among others have been demonstrated (39, 40). This results in caspase-1 activation, which cleaves multiple proteins, including pro-interleukin(IL)-1β and pro-IL-18, which then become biologically active (41). Caspase-1 and these cytokines have been shown to play a crucial role
in insulin resistance and adipocyte differentiation (42). The importance of the inflammasome has recently been demonstrated by the CANTOS trial. Inhibition of IL-1β by canakinumab in subjects with a previous myocardial infarction reduced the incidence of cardiovascular death, non-fatal myocardial infarction and non-fatal stroke during their 3.5 year follow-up (5). The second important player in the obesity-associated low-grade inflammation process is the production of adipokines. Next to the production of pro-inflammatory cytokines by the adipocytes and inflammatory cells, the adipocytes produce several kind of adipokines such as leptin and adiponectin. Leptin shows a linear relation with body fat. As SAT constitutes normally ~80% of the body fat, this explains the high relation between leptin and SAT (43). Leptin is a pro-inflammatory adipokine that regulates body weight by signaling nutritional status to other organs, especially the hypothalamus that then produces neuropeptides which modulate food intake and energy expenditure (44). Leptin acts pro-inflammatory via macrophages, T cells, and other immune cells to stimulate the production of a wide spectrum of cytokines (44, 45). Leptin levels are seldom used in clinical care, as individuals with obesity are resistant to leptin due to hyperleptinemia. Adiponectin has been shown to have different anti-inflammatory actions: it suppresses TNF-α production in obese mice; it can enhance the clearance of apoptotic cells by facilitating their opsonization and uptake by macrophages and has been shown to decrease NFκB activity leading to reduced monocyte adhesion to endothelial cells (44). Circulating levels of adiponectin and leptin are in general higher in women than in men. This is at least partly caused by a higher percentage of adipose tissue in women (47). We found that adiponectin showed the strongest negative association with the adipose tissue score in women, while in men the strongest association was found between the adipocyte cell size with leptin. This is in line with our previous finding that overweight women with the metabolic syndrome are mainly characterized by a deficiency of the adiponectin, while men with the metabolic syndrome have a pro-inflammatory phenotype with increased levels of IL-6 and leptin (7).

As shown above, the adipose tissue plays a crucial role in obesity associated systemic low-grade inflammation in obesity. We therefore also took fat biopsies from the sSAT. We first examined whether the volume of the sSAT is associated with histological markers of tissue inflammation. In men, we found a significant association between the adipocyte cell size with the volume of sSAT, but also with the VAT and dSAT volume. This is in line with previous studies that found that men have a more hypertrophic type of white adipose tissue expansion compared to women (48). In contrast in women, while no association was seen with the histology at all in women, a stronger positive association is seen with the upregulation of multiple pro-inflammatory genes. This could explain the possible detrimental effect of sSAT in women compared to men.
Hormonal differences
In addition to possible sex-specific differences that are present within the adipose tissue, the hormonal status has previously been shown to influence immune function and adipose tissue volumes (49). During puberty significant changes appear in body fat percentage and distribution. Women show an increase in adipose tissue mass and mainly in the SAT compartment, while men show an increase in the lean body mass during these years (50). During menopause, the amount of VAT increase in women (51, 52). It is hypothesized that the lower estrogen levels play an important causal factor in this body shape development. As all our women were post-menopausal, we hypothesize that estrogen would not add much to the sex-specific differences in the relation between fat distribution with inflammation. The transgender population is an intriguing population to investigate the interaction between gender, sex hormones and fat distribution. Transwomen (transformation from male to female) develop an increase in fat mass, with significantly more gynoid fat during a 1 year follow up study. Transmen (transformation from female to male) showed a significant decrease in total fat mass, while the android fat mass stayed the same (53). Another study found that the cross-sex hormone administration in transwomen resulted in an increase in the SAT of 38%, but also an increase in VAT of 18%, while transmen have a reduction in SAT of 22% and increase in VAT of 13% (54). These studies underestimate a possible role for sex hormones in the distribution of fat, which could influence the relation between fat distribution and low-grade inflammation.

Limitations
Our study has a number of limitations. Due to the cross-sectional design of our study, no causal relations can be drawn from our 300-OB study. As we only include individuals of Western European ancestry, extrapolation of our results to other ethnic groups are uncertain. Furthermore, as we included individuals who were 55-80 years of age and all women were postmenopausal, our results cannot be extrapolated to other age groups and a possible role of sex hormones cannot be extracted from our data. Lastly, we only included individuals with overweight or obesity, this could weaken the strength of the associations found and extrapolation to lean individuals is not possible.

Conclusion
In the present study, we found clear sex differences in the relation between adipose tissue distribution and markers of chronic low-grade inflammation in overweight and obese individuals. In women SAT was linked to a pro-inflammatory state, also showing increased gene expression in the adipose tissue of pro-inflammatory genes. In contrast, in men VAT showed the strongest association with pro-inflammatory cells and comparable associations with circulating cytokines as SAT in men. In the adipose tissue histology, adipocyte hypertrophy was strongly associated to VAT and SAT in men. Combined, these findings call for a sex-specific approach with regard to interpretation of adipose tissue distribution and its contribution to low-grade inflammation.
REFERENCES


SUPPLEMENTARY MATERIAL

24h PBMC stimulations + RPMI

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7d PBMC stimulations + RPMI + 10% human serum

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24h Whole Blood stimulations + RPMI

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Supplementary Table 1 – Concentrations of stimuli used in the cytokine production capacity experiments. PBMC Peripheral blood mononuclear cells; Pam3Cys Tripalmitoyl cysteine; Poly I:C Polyinosinic:polycytidylic acid; LPS Lipopolysaccharide; PHA Phytohemagglutinin
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Supplementary Table 2 - Forward and reverse sequence-specific primers of the household gene RPL37A and investigated genes to perform the real-time PCR to study the mRNA expression levels.
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Supplementary Table 3 – Heatmap of correlation between top 7 protein levels in relation to different adipose tissue compartment volumes in women (A) and men (B). Standardized betas after adjustment for age, pack years and season and multiple testing. The intensity of the red colour represent the strength of the significance.

VAT Visceral adipose tissue; SAT Subcutaneous adipose tissue; sSAT Superficial subcutaneous adipose tissue; dSAT Deep subcutaneous adipose tissue; SAT_hip Subcutaneous adipose tissue at the hip region; VAT+SAT Visceral plus subcutaneous adipose tissue; C.albicans.yeast Candida albicans yeast; LPS.100ng Lipopolysaccharide 100ng; MSU monosodium urate; P.Gingivalis Porphyromonas gingivalis; Pam3Cys Tripalmitoyl cysteine; Poly.I.C Polyinosinic:polycytidylic acid; S.Aureus Staphylococcus Aureus; PHA Phytohemagglutinin; PBMC Peripheral blood mononuclear cells; 24h 24 hours; 48h 48 hours; 7d seven days; IL.1b Interleukin-16.

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**P-value**

- P < 0.05
- P < 0.01
- P < 0.001
CHAPTER 4

Sex-specific regulation of inflammation and metabolic syndrome in obesity

* Contributed equally

ABSTRACT

Objective
Metabolic dysregulation and inflammation are important consequences of obesity, and impact susceptibility to cardiovascular diseases (CVD). Anti-inflammatory therapy in CVD is being developed under the assumption that inflammatory pathways are identical in women and men, but it is not known if this is indeed the case. In this study, we assessed the sex-specific relation between inflammation and metabolic dysregulation in obesity.

Approach and results
302 individuals were included, half with a BMI 27-30kg/m² and half with a BMI>30kg/m², 45% were women. The presence of metabolic syndrome was assessed according to the NCEP-ATPIII criteria, and inflammation was studied using circulating markers of inflammation, cell counts and ex vivo cytokine production capacity of isolated immune cells. Additionally, lipidomic and metabolomic data were gathered, and subcutaneous fat biopsies were histologically assessed. Metabolic syndrome is associated with an increased inflammatory profile that profoundly differs between women and men: women with metabolic syndrome show a lower concentration of the anti-inflammatory adiponectin, whereas men show increased levels of several pro-inflammatory markers such as interleukin-6 and leptin. Adipose tissue inflammation showed similar sex-specific associations with these markers. Peripheral blood mononuclear cells isolated from men, but not women, with metabolic syndrome display enhanced cytokine production capacity.

Conclusions
We identified sex-specific pathways that influence inflammation in obesity. Excessive production of pro-inflammatory cytokines was observed in men with metabolic syndrome. In contrast, women typically showed reduced levels of the anti-inflammatory adipokine adiponectin. These different mechanisms of inflammatory dysregulation between women and men with obesity argue for sex-specific therapeutic strategies.
INTRODUCTION
The prevalence of overweight and obesity has increased dramatically over the last few decades. In 2016, globally 39% of adults were overweight (BMI > 25 kg/m\(^2\)) and 13% were obese (BMI > 30 kg/m\(^2\)) (1), while in developed countries the percentages are often even higher. Overweight and obesity are strongly associated with atherosclerotic cardiovascular diseases (CVD), diabetes, and several types of cancer (2). Two important mechanisms that drive CVD development in obesity are metabolic dysregulation and systemic inflammation, with low-grade chronic inflammation contributing to the development of metabolic syndrome and its complications (3). The metabolic syndrome serves as an indicator of obesity-related metabolic dysregulation and is strongly associated with atherosclerotic CVD (4).

There are important sex differences in the pathophysiology of atherosclerotic CVD, with sex-specific risk factors and differences in the prevalence and treatment (5). Differences in sex hormones and sex-specific effects of adipokines are all implicated in these differences, but knowledge on sex-specific regulation of inflammation and metabolic dysregulation in obesity is sparse.

We have previously shown that sex, age, genes and the environment are important determinants of systemic inflammatory processes in healthy individuals (6, 7). While it is implied that the same mechanisms control inflammation in women and men with regard to CVD, this has never been systematically investigated. In this study, we investigated the impact of sex on the relation between inflammation and metabolic syndrome in overweight women and men. We further explored the role of circulating metabolites (metabolome) and circulating hormones on sex-specific effects. We specifically focused on three groups of inflammatory parameters: plasma adipokine and cytokine concentrations, immune cell subpopulations in the blood, and the ex vivo cytokine production capacity of circulating immune cells (such as monocytes and T cells). These distinct inflammatory phenotypes are all known to be important in the pathophysiology of CVD, but are differentially regulated (8).

METHODS AND MATERIALS
Cohort and measurements
Anonymized data and materials have been made publicly available at the Human Functional Genomics Project (HFGP) website and can be accessed at https://hfgp.bbmri.nl/. As part of the HFGP (9), we recruited a cohort of 302 individuals affected by overweight or obesity between 55 and 82 years of age, with a BMI > 27 kg/m\(^2\) at screening, of mostly Western European ancestry, termed the 300-Obesity (300-OB) cohort. Most of these participants previously took part in the Nijmegen Biomedical Study – Non-Invasive Measurements of Atherosclerosis 1 (NBS-NIMA1) (10). Detailed information about the cohort is available in the Methods in the supplementary material. All participants were included between the year 2014 and 2016.
All individuals filled out questionnaires, which included questions about life style, environmental factors and medication usage. If used, participants temporarily discontinued lipid-lowering therapy four weeks prior to the measurements. Blood samples were taken in the morning following an overnight fast. All women were postmenopausal and were not receiving hormonal replacement therapy. All participants received detailed printed and oral information and subsequently gave written informed consent. The study was approved by the Ethical Committee of the Radboud University (nr. 46846.091.13). Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

The metabolic syndrome
In our study, the metabolic syndrome is defined by the NCEP ATP III criteria as the presence of any three of the following five traits (11):
- Abdominal obesity, defined as a waist circumference in men ≥102 cm (40 in) and in women ≥88 cm (35 in)
- Serum triglycerides (TG) ≥150 mg/dL (1.7 mmol/L) or drug treatment for elevated TG
- Serum high-density lipoprotein cholesterol (HDL-C) < 40 mg/dL (1 mmol/L) in men and < 50 mg/dL (1.3 mmol/L) in women or drug treatment for low HDL-C
- Blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure
- Fasting plasma glucose ≥100 mg/dL (5.6 mmol/L) or drug treatment for elevated blood glucose

Blood glucose, Triglycerides (TG), total cholesterol and HDL-C were measured using standard laboratory procedures. Before measuring systolic and diastolic blood pressure, participants took 30 minutes of supine rest.
Detailed information on the measurements is available in the Methods in the supplementary material.

Circulating mediators
Circulating mediators (cytokines and adipokines) were measured in human EDTA plasma using Enzyme Linked Immunosorbent Assay (ELISA).

Stimulation experiments

PBMC stimulation experiments
Isolation of peripheral blood mononuclear cells (PBMCs) was performed as described in Oosting et al (12).

Whole blood stimulation experiments
A volume of 100 μl of heparin blood was added to a 48 well plate (Corning) containing 400 μl stimulus (final volume 500ul/well) for 48 hours at 37°C and 5% CO₂.
**ELISA analysis**
Cytokine concentrations after stimulation were measured using commercially available ELISA kits.

**Stimuli and cytokines**
Table 1 in the online supplementary material lists the concentrations of the stimuli used. Interleukin (IL)-1β, IL-6, TNF-α, and IL-1Ra were measured after 24-hour stimulation with these stimuli, and IFNγ, IL-17, and IL-22 were measured after 7 days of stimulation only for *C. albicans* and *S. aureus* stimulation. The choice of pro-inflammatory mediators was based on extensive literature linking cytokines and adipokines to inflammation and CVD complications. Stimulation of PBMCs was performed with a comprehensive set of stimuli containing both purified innate immune stimuli that are associated with chronic inflammation (e.g. LPS, oxidized low-density lipoprotein (LDL)) and microorganism that are the source of microbial ligands that translocate in the circulation at the intestinal level.

**Cell count data**
Immune cell counts were determined in fresh whole blood EDTA samples using the Sysmex XE-5000.

**Metabolomics**

*Untargeted metabolomics*
Blood was collected in EDTA tubes and plasma was extracted. Flow injection electrospray time-of-flight mass spectrometry was performed by General Metabolomics (1 Broadway, Cambridge MA 02142) to identify metabolic features based on m/z. Details of the procedure can be found in Fuhrer et al (13). 1339 m/z signals could be assigned to one or more metabolites.

*Lipidomics*
A high-throughput Nuclear Magnetic Resonance (NMR) metabolomics platform (Nightingale's Biomarker Analysis Platform) was used to quantify a total of 231 lipid and metabolite measures. Most of these measures were very highly correlated to other measures from the same platform. Groups of lipoprotein particle characteristics were therefore made based on a correlation between variables of at least r>0.75 and expert knowledge. This led to 17 groups (see Table 2 in the online supplementary material), for each group a representative variable was selected to represent the whole group of measurements. This led to easier interpretation and less strict multiple testing correction.

**Adipose tissue analysis**
Subcutaneous adipose tissue biopsies were obtained under local anesthesia by needle biopsies performed 6-10cm lateral to the umbilicus in the right lower quadrant, after an overnight fast. The morphometry of individual fat cells was assessed using digital image analyses as described.
For each participant, the adipocyte cell diameters of all adipocytes in four microscopic fields of view were counted and measured. To detect macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Serotec, Oxford, UK). The percentage of macrophages was expressed as the total number of macrophages divided by the total number of adipocytes counted in 15 random microscopic fields of view. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages (15). To robustly quantify adipose tissue inflammation among study participants using histology, several parameters were assessed and combined into an adipose tissue inflammation score, as the phenotype by which inflammation in the adipose tissue presents is quite heterogeneous (16, 17). This assessment led to a score including the following parameters: a mean adipocyte diameter above the average diameter of the cohort (> 51.7um (mean diameters in the 300-OB cohort) was defined as one point in the AT score, the percentage of macrophages above the average of the cohort (>12.6% (mean in the 300-OB cohort) was defined as one point in the AT score and the presence of crown-like structures (CLS) was defined as one point in the AT score. Hence, the adipose tissue inflammation score ranged from 0 (no inflammation) to 3 (severely inflamed).

Quantification and statistical analysis

Data and software availability

The R code (via R programming language (18)) used for the analyses will be made available upon request. Multiple testing correction was performed using the Benjamini-Hochberg FDR procedure (19).

For the metabolic pathway analysis, we used an adaptation (Fast Gene Set Enrichment Analysis (FGSEA) (20)) of Gene Set Enrichment Analysis (GSEA) (21). The pathways provided by the KEGG pathway database (22) were used for enrichment analysis. Interesting pathways were visualized using Pathview (23).

For details on the statistical analysis see supplemental methods.

RESULTS

300-OB cohort

Figure 1 provides an overview of the clinical characteristics of the cohort, separated by sex: 55% are men and 45% are women, with a similar BMI distribution in both sexes (Figure 1B and S). Metabolic syndrome is defined as having three out of five cardiovascular risk factors mentioned in figure 1E till I (11). The prevalence of metabolic syndrome was 54.8% in women and 53.9% in men (Figure 1C), and a larger percentage of women had abdominal obesity and low HDL-C (Figure 1G and H). Some individuals also had other obesity-related comorbidities (Figure 1D).
Association between markers of inflammation in individuals with overweight or obesity

The sex-specific values of circulating inflammatory markers and adipokines are depicted in Figure 2A. We previously reported the effects of age and sex on inflammation in a healthy cohort (7) (n = 489, age 27.4 ± 12.5 years, BMI 22.7 ± 2.7 kg/m²). Overall, in the present study we observed similar effects of age (Figure 2B and Table 3 in the online supplementary material), i.e. interleukin(IL)-6 and IL-18BP circulating concentrations increase with age. In addition, we observed an effect of sex on various parameters; higher levels of hsCRP, leptin and adiponectin were seen in women (Figure 2B).

To better understand the regulation of inflammation in obesity, we evaluated the association between different markers of inflammation using FDR corrected p-values based on linear
regression models. Age, sex and season were added as co-factors, since they are known to have an influence on the immune system (7). Most markers of inflammation show positive associations with one another, specifically IL-6, IL-18, IL-18BP, high-sensitive CRP (hsCRP), VEGF and leptin (Figure 2C and Table 4 in the online supplementary material). IL-6 concentrations show particularly strong associations with leptin (Figure 2D, adjusted $\beta = 0.31$) and hsCRP (adjusted $\beta = 0.53$), which is in line with previous studies (24, 25). We observed a negative association of adiponectin with several inflammatory markers (Figure 2C), in line with its known anti-inflammatory properties and its decrease with increasing BMI (26). Associations of these markers of inflammation are mostly the same between women and men (Table 5 in the online supplementary material).

In addition to circulating inflammatory markers, we also measured immune cell populations. Absolute numbers of neutrophils and monocytes increase with age, and men generally have higher numbers of neutrophils, eosinophils and especially monocytes (Figure 2E and Table 7 in the online supplementary material), in line with previous findings (26, 27).

We found strong associations between most inflammatory markers and the number of leukocytes, neutrophils and monocytes, most of them positive (Figure 2F, Table 7 in the online supplementary material).
Figure 2 - Associations of various immune and host parameters in the 300-OB cohort. (A) Mean and standard deviations of several circulating inflammatory markers for women and men. (B) P-values of associations between age and sex with markers of inflammation* (BH-FDR multiple testing corrected). (C) P-values of associations between markers of inflammation, corrected for age, sex and season (BH-FDR multiple testing corrected). (D) Example association between IL-6 and leptin. (E) P-values of associations between age and sex with immune cell counts* (BH-FDR multiple testing corrected). (F) P-values of associations between immune cell numbers and circulating markers of inflammation, corrected for age, sex and season (BH-FDR multiple testing corrected). Note: All p-values were calculated using linear regression and testing the null hypothesis that $\beta = 0$ for relationship between $x$ and $y$, where $x$ and $y$ are the parameters of
interest. The data in the example plots (D) was corrected for age, sex and season and transformed using IRT (see methods). * = Associations with age corrected for sex and season, associations with sex corrected for age and season.

The impact of sex on metabolic syndrome and inflammation

The presence of metabolic syndrome was positively associated with several inflammatory markers IL-6, IL-18, IL-IL-18BP, hsCRP, leptin and VEGF (Figure 3A and Table 8 in the online supplementary material). Importantly, IL-6 and leptin show profound sex-specific effects: only in men, these are higher in the presence of the metabolic syndrome, whereas in women there is no association with the metabolic syndrome (Figure 3A, B, E and F). Similar trends are observed for hsCRP and IL-18, though the interaction between sex and metabolic syndrome was not significant when testing based on a linear regression model with an interaction term (Figure 3A and B, Table 8 and 9 in the online supplementary material). In contrast to the pro-inflammatory mediators, the anti-inflammatory adiponectin was lower in individuals with metabolic syndrome. Interestingly, this effect was significantly stronger in women compared to men, as opposed to IL-6 and leptin (Figure 3A, B and G). To validate this finding, we measured adiponectin levels in a subset of the NBS-NIMA1 cohort (28) of 441 participants, filtering for a BMI>27kg/m². The individuals in this cohort were on average 4.4 years younger than the 300-OB cohort (2.8 years younger on average for men and 6.0 years for women). In this cohort, 33% of the subjects suffered from the metabolic syndrome (Table 10 in the online supplementary material). We were able to confirm the same pattern of a lower concentration of adiponectin in women with the metabolic syndrome, but not in men (Figure 3H).

In addition, as inflammation originating from the adipose tissue is one of the main driving forces for systemic low-grade inflammation present in obesity (29), we examined the possible existence of sex-specific differences in the relation between circulating leptin, adiponectin and adipose tissue inflammation. We calculated AT scores for the 300-OB cohort based on fat tissue taken from the abdominal subcutaneous fat depot. After adjustment for age, pack years and season (7), AT scores showed significant association with circulating levels of leptin and IL-6 in men but not women (standardized β\textsubscript{men} = 0.22, \textit{p}\textsubscript{men} < 0.001, \textit{p\textsubscript{interaction\_sex\_leptin}} = 0.02 for leptin; β\textsubscript{men} = 0.25, \textit{p\textsubscript{men}} < 0.001, \textit{p\textsubscript{interaction\_sex\_IL6}} = 0.07 for IL-6), while in women AT scores were more strongly negatively associated with circulating adiponectin (standardized β\textsubscript{women} = -0.28; \textit{p\textsubscript{women}} < 0.0001). This provides further evidence of sex-specific regulation of systemic low-grade inflammation.

Since IL-6 and leptin concentrations are correlated, and the concentrations of these markers are elevated specifically in men with metabolic syndrome, we assessed the strength of this correlation separately by sex and presence/absence of metabolic syndrome. We observed that leptin and IL-6 are only correlated in men but not in women, and most strongly in men without metabolic syndrome (Figure 3I).
The five factors (Figure 1E till I) that define metabolic syndrome might not have an equal contribution to the sex-specific effects described in the previous paragraphs. We therefore evaluated each of these factors separately, using the cut-off levels as defined by NCEP ATP-III criteria (see methods). Having high vs. normal TG levels appears to be the most important parameter in explaining the sex-specific effects of metabolic syndrome on both IL-6 in men and adiponectin in women (Figure 3D, compare to Figure 3B). The sex-specific changes in leptin with metabolic syndrome cannot be explained by any single parameter, and the full definition of metabolic syndrome is needed to explain that effect.

We also observed changes in the number of immune cell subtypes in individuals with metabolic syndrome: specifically, total leukocytes, lymphocytes and monocytes numbers are higher in individuals with metabolic syndrome (Figure 3E). Importantly, these changes in both absolute amounts of cell subtypes with metabolic syndrome are not sex-dependent (Table 11 in the online supplementary material).

Figure 3 - The effect of metabolic syndrome (MetS) on immune parameters. (A) Significance of the difference in levels of several inflammatory markers between individuals with and without MetS. Red indicates that the marker is significantly higher in individuals with metabolic syndrome and
blue means it is higher in those without. The first column is for all individuals, and the second and third are for women and men respectively (FDR corrected per column). (B) Significance of the interaction effect between sex and metabolic syndrome. Red indicates that the increase of women with metabolic syndrome vs. those without was significantly lower than men with metabolic syndrome vs. those without, i.e. red means men show a stronger increase than women or women show a stronger decrease than men. (C) Interaction effects for each individual medical condition used to score metabolic syndrome with sex. Each individual is scored as either having the condition or not, and interaction effects are calculated in a similar way as in (B). Similarly, red means men show a stronger increase than women, or women show a stronger decrease than men; blue means the opposite. The left part shows the p-values not corrected for multiple testing, and the right part shows the FDR corrected p-values. (D) Same as A, but for cell counts. (E)-(H) Example plots split into women and men with and without metabolic syndrome. (E) IL-6, (F) leptin, (G) adiponectin. (H) Similar to (G), but for the validation cohort. (I) Plot showing Spearman correlations between leptin and IL-6 for the 4 categories: men with MetS, men without MetS, women with MetS and women without MetS.

Note: P-values in (A and D) were calculated using linear regression by testing the null hypothesis that $\beta = 0$ for relationship between metabolic syndrome status (independent variable) and a continuous parameter (dependent variable), see methods for details. The interaction effects of (B and C) were calculated using linear regression, with the null hypothesis that $\beta = 0$ for the interaction effect between sex and metabolic syndrome status (independent variable) and a continuous parameter (dependent variable).

Differential regulation of cytokine production capacity in women and men

Cytokine production capacity of PBMCs, as an indicator of the intrinsic inflammatory responsiveness, has previously been linked to the presence of atherosclerosis (30, 31). Production of the pro-inflammatory cytokines IL-1β and IL-6 and the anti-inflammatory cytokine IL-1Ra show associations with circulating levels of IL-6 and hsCRP. Interestingly, in line with higher inflammatory markers in men with metabolic syndrome, there is a strong trend towards higher monocyte-derived inflammatory cytokine production capacity specifically in men, but not women, with metabolic syndrome (Figure 4A(left) and B, Table 12 and 13 in the online supplementary material). This effect does not appear to be limited to a single PBMC stimulation, and is most apparent for the cytokines IL-6 and IL-1β, and less so for TNF-α. Again, high TG is the main factor of metabolic syndrome showing sex-specific effects, with only men with high TG showing increased levels of cytokine production capacity (Figure 4A and C, Figure 2 and Table 14 in the online supplementary material). There were no differences in the cytokine production capacity of IL-1Ra and the lymphocyte derived cytokines IL-22, IL-17 and IFNγ between women or men with or without metabolic syndrome (Data not shown).
Changes in the metabolome of individuals with metabolic syndrome

Metabolic dysregulation and systemic inflammation are important mechanisms that drive CVD development in obesity. To further explore whether the composition of circulating lipoproteins could contribute to the sex-specific association of the metabolic syndrome with IL-6, leptin, adiponectin, and cytokine production capacity, we measured lipoprotein subclasses, lipoprotein particles sizes, apolipoproteins and lipoprotein content using the Nightingale platform (Table 2 in the online supplementary material). A principle component analysis (PCA) shows separation between both women and men and between individuals with and without metabolic syndrome (Figure 5A). The PCA and a linear regression analysis show that women have larger concentrations of HDL particles, HDL triglycerides, ApoA1 and cholesterol (Figure 5B and Table 15 in the online supplementary material) and many lipoproteins have altered composition and concentration in subjects with metabolic syndrome, e.g. decreases in HDL particles and increases in LDL particles, with an increase in triglycerides (Figure 5C[left]). Women and men show the
same patterns of change when comparing individuals with and without metabolic syndrome (Figure 5C[right]).

To further explore metabolic changes associated with the metabolic syndrome beyond lipidomics, we used untargeted metabolomics assay that detected 1339 metabolite signals. We evaluated the association of metabolic syndrome with these metabolites. First, we applied PCA to these data to see if metabolic syndrome and/or sex impact on the variation of this data. PC2 shows a clear separation between women and men (Figure 5D). However, using these untargeted analyses, no large differences between individuals with and without metabolic syndrome can be observed. Nonetheless, using a regression model we found many metabolites that were associated with metabolic syndrome. The associations for all metabolite hits with metabolic syndrome are listed in Table 16 in the online supplementary material. The top pathway found in a pathway analysis was “valine, leucine and isoleucine biosynthesis” though it did not reach statistical significance after multiple testing correction (Table 17 in online supplementary material). It is interesting to note that 5 out of the 7 metabolites of the “valine, leucine and isoleucine biosynthesis” pathway that are significantly associated with metabolic syndrome, also show significantly different levels between women and men (Figure 5E and Table 18 in the supplementary material). However, these metabolites did not show a sex-specific association with metabolic syndrome (Figure 5E).

**Hormonal changes do not explain sex differences**

The cause of the differences in inflammatory profiles between women and men with metabolic syndrome is not yet known, but one hypothesis is that it might involve differential homeostasis of sex hormones. To evaluate the potential role of sex hormones in the metabolic syndrome we measured baseline levels of several steroid hormones: testosterone, androstenedione, cortisol, 11-deoxycortisol and 17-hydroxyprogesterone (Table 19 in the supplementary material). We found no significant difference in hormone levels associated with the metabolic syndrome in both women and men (Figure 5F).
Figure 5 - (A) PCA plot of PC1 vs PC2 for the lipidomics data. (B) Lipid groups that are significantly different between women and men. Blue indicates higher levels in women. (C). [left] significance (FDR corrected p-values) of the difference of lipid concentrations between individuals with or without metabolic syndrome. Calculated for the whole 300-OB cohort and calculated for women a women separately. [right] FDR corrected P-values for the interaction effect between metabolic syndrome and sex in predicting the lipids, i.e. showing if the increase/decrease of these lipids seen in metabolic syndrome is the same in women and men. (D) PCA plot of the metabolomics data after inverse rank transformation and standardization. The first two PCs are plotted, and the samples are split into four groups: women without metabolic syndrome (“Female_NoMetS”), women with metabolic syndrome (“Female_YesMetS”), men without metabolic syndrome (“Male_NoMetS”), men with metabolic syndrome (“Female_YesMetS”). PC2 shows some separation between women and men. (E) Significance of sex differences for the 7 metabolites from the “valine, leucine and isoleucine biosynthesis” that showed significant association with metabolic syndrome. Several of these metabolites also show clear differences between women and men. (F) Significance (FDR corrected p-values) of the difference in hormone concentrations between individuals with or without metabolic syndrome.
Note: P-values in (B) and (E) were calculated using linear regression by testing the null hypothesis that $\beta = 0$ for relationship between sex (independent variable) and a continuous parameter (dependent variable), see methods for details. P-values in (C[left]) and (F) were calculated using linear regression by testing the null hypothesis that $\beta = 0$ for relationship between metabolic syndrome status (independent variable) and a continuous parameter (dependent variable), see methods for details. The interaction effect of (C[right]) was calculated using linear regression, with the null hypothesis that $\beta = 0$ for the interaction effect between sex and metabolic syndrome status (independent variable) and a continuous parameter (dependent variable).

XL- Very large; L- Large; M- Medium; S- Small; XS- Extremely small; VLDL Very-Low-Density Lipoproteins; IDL Intermediate-Density Lipoproteins; LDL Low-Density Lipoproteins; HDL High-Density Lipoproteins; P Concentration of particles; L Total lipids; PL Phospholipids; C Total cholesterol; CE Cholesterol esters; FC Free cholesterol; TG Triglycerides; D Mean diameter; Serum-C Serum cholesterol.

Plasma metabolome effects on inflammatory markers
To explore whether circulating metabolites influence inflammatory parameters and account for the sex-differences in inflammation in relation to metabolic syndrome, we evaluated the relationship between metabolites and inflammation. We see the strongest associations for adiponectin and IL-18BP, but hsCRP, resistin and IL-6 also show many associations with metabolites (Figure 6A and Table 20 in the online supplementary material).

The results for the top 10 pathways that correlate with inflammatory markers are displayed in Figure 6B. Pathway analysis was performed using an adapted version of Gene Set Enrichment Analysis (21). “Central Carbon Metabolism in Cancer”, is particularly interesting, since an important part of it is the TCA cycle, which in the last years has been shown to have a strong impact on inflammation and the function of immune cells (32, 33). However, although we identified important correlations between metabolites and inflammation, these associations were not sex-specific and could not explain the sex-differences in inflammatory markers in individuals with metabolic syndrome (Table 21 in the online supplementary material).
Figure 6 - Metabolic comparison between normal weight and overweight individuals. (A) Visualization of the top 5% of metabolites (lowest p-values) associated with each of the circulating markers of inflammation to give an impression of the number of significant metabolites. For each marker the FDR corrected p-values were sorted independently to show the relative number of significant metabolites for each marker. (B) Top 10 metabolic pathways associated with circulating markers of inflammation in the 300-OB cohort. See methods for details. (C) Example pathway plot of “Central Carbon Metabolism in Cancer” for VEGF in the 300-OB cohort. Significance is indicated as -log10(p-values), any p-value below p = 1e-4 is rounded up to 1e-4 for this visualization. Red indicates a positive association, blue indicates a negative association and white indicates that the metabolite was not present in our data. Visualization was performed using Pathview (23). The portion of the figure showing the TCA cycle is shown as a zoomed-up inset.
DISCUSSION

In this study, we aimed to understand the various regulatory layers of the adverse immunometabolic effects of overweight and obesity, and in particular which parts of that regulation differ between women and men. Therefore, we characterized in detail a cohort of 302 individuals (approximately equally distributed between women and men, with or without metabolic syndrome) with overweight and obesity in terms of circulating inflammatory markers, immune cell counts, immune cell responsiveness, circulating metabolomics/lipidomics, and histological analysis of subcutaneous fat biopsies. The most important finding was that there is a sex-specific association of metabolic syndrome with various inflammatory parameters: metabolic syndrome is associated with lower circulating concentrations of the anti-inflammatory adiponectin, whereas only in men the presence of metabolic syndrome is associated with increased monocye-derived circulating cytokines (mainly IL-6), and increased leptin. For adiponectin, these sex-specific associations were validated in an independent cohort. Adipose tissue inflammation is a central feature of metabolic syndrome, and we found that adipose tissue inflammation was positively associated with circulating leptin and IL-6 only in men, while in women adiponectin was negatively associated with the AT score. In addition, metabolic syndrome was associated with hyper-responsive circulating immune cells only in men. This suggests that the role of inflammation and the immune system in the adverse cardiometabolic consequences of obesity is different in women and men. Further analyses revealed sex-specific differences circulating metabolites, lipoprotein composition, and hormones, but these differences did not explain the differential association of inflammatory markers with metabolic syndrome between women and men. These findings call for a sex-specific approach with regard to inflammation as a pharmacological target to prevent CVD in individuals with obesity and for further research to unravel the mechanisms that drive the sex-specific differences.

The metabolic syndrome serves as an indicator of metabolic dysregulation, is strongly associated with the development of atherosclerotic CVD and is often accompanied by a chronic low-grade inflammation (34). Differences in a selection of inflammatory parameters between women and men that have metabolic syndrome have been reported previously in small studies. Sarbijani et al. showed in a group of 40 subjects that men with metabolic syndrome have higher IL-6 levels than those without (35), though it should be noted that for women and men the characteristics of the case and control groups were different in this study. Ahonen et al. showed that absolute differences in adiponectin in individuals with and without the metabolic syndrome were larger in women than men, though the significance of this difference was not reported (36). However, a systematic overview of the differences between women and men with metabolic syndrome is still lacking. The strength of our current study, is that it provides a more comprehensive description of the sex-specific associations of metabolic syndrome with circulating inflammatory markers and immune cell phenotype. Additionally, we strengthened these findings by studying
adipose tissue inflammation and an independent cohort, and explored potential underlying mechanisms. One of the most important observations of our study is the difference in the dysregulation of inflammation between women and men: in women, the presence of metabolic syndrome is characterized by a lack of the anti-inflammatory adipokine adiponectin, while in men it presents as an excess of pro-inflammatory mediators such as IL-6 and leptin. Interestingly, IL-6 and leptin are only correlated in men, further suggesting a differential regulation of these inflammatory markers in women. We were able to validate the sex specific associations of adiponectin levels in women and men with metabolic syndrome in an independent cohort. These different inflammatory phenotypes in women and men with metabolic syndrome can have important therapeutic consequences, suggesting sex-specific approaches.

In addition to systemic inflammation, there is accumulating evidence that the phenotype of circulating immune cells contributes to adverse cardiometabolic effects. We and others have recently reported that circulating monocytes have an enhanced cytokine production capacity in patients with risk factors for atherosclerosis, including familial hypercholesterolemia and elevated lipoprotein (a) (37, 38), and in patients with established coronary atherosclerosis (30, 31). Interestingly, in the 300-OB cohort, a similar hyperresponsiveness of circulating PBMCs was present in men with metabolic syndrome compared to men without metabolic syndrome, whereas in women, these differences were not observed. In men, the presence of metabolic syndrome was associated with a higher production of IL-1β and IL-6 after stimulation with a wide range of inflammatory stimuli. This might well be related to the observation that circulating IL-6 is higher in men with metabolic syndrome, since IL-6 is associated with ex vivo production of IL-1β and IL-6 in response to several stimuli.

To gain insight into the mechanism responsible for the sex-specific association of metabolic syndrome with adipokines, and inflammatory parameters, we first studied the association between adipose tissue inflammation and circulating adipokine and cytokine levels. Leptin and adiponectin are both produced in adipose tissue. Leptin is at the interface between metabolism and inflammation in fat tissue; leptin production by the adipose tissue facilitates the secretion of proinflammatory cytokines, and these, in turn, promote the release of leptin from adipocytes (39). Adipose tissue inflammation is a well-known feature of metabolic syndrome. Of great interest, we were able to demonstrate a positive association of adipose tissue inflammation with leptin and IL-6 specifically in men and a negative association of adipose tissue inflammation with adiponectin in women. However, the causality between adipose tissue inflammation and these circulating adipokines and cytokines, needs to be further established. We hypothesize that the increased adipose tissue inflammation associated with metabolic syndrome in men results in peripheral leptin resistance, which in turn leads to increased leptin levels and also impacts other inflammatory parameters. Conversely, in women the increased adipose tissue inflammation
limits adiponectin production. It is important to realize that the adipose tissue biopsy was obtained from the abdominal subcutaneous fat depot in both sexes, while women and men are known to have a difference in (abdominal) fat distribution: with the same BMI, men have on average a higher ratio of visceral/subcutaneous adipose tissue compared to women (40). This difference might impact on systemic inflammation and needs to be included in future analyses.

Additionally, to gain more insight into what specific features of the metabolic syndrome drive the observed associations with circulating adipokines and cytokines, we individually evaluated the five parameters defining metabolic syndrome. We showed that circulating TG concentration appear to be the most important condition driving the sex-specific association. In women with high TGs, adiponectin levels are lower, whereas in men with high TGs, plasma IL6 is higher. In addition, also the augmented cytokine production capacity of PBMCs in men with metabolic syndrome is mainly dependent on the presence of high TGs. Further lipidomic analyses showed that this differential effect is not due to sex-differences in the composition of circulating lipoproteins. Following high-fat loads, circulating triglycerides are associated with circulating cytokine levels such as IL-6 and with activation of circulating innate immune cells (41, 42). However, it is currently unclear why triglycerides are associated with higher IL-6 and higher cytokine production capacity only in men.

As has been shown previously that for example testosterone can have immunomodulatory effect (43), we tested the hypothesis that circulating hormones contribute to the observed sex-specific differences in inflammatory parameters by measuring the serum concentrations of five circulating steroid hormones, of which four show strong differential concentrations in women and men, including 17-OH-progesteron and testosterone. For these hormones, we did not observe any differential association with triglycerides or the metabolic syndrome, nor an association with circulating cytokines and cytokine-production capacity that could explain our findings. It is important to realize that we were only able to test a selection of hormones, and that we cannot exclude effects of progesterone or estrogens. Also, though changes in sex hormones with the metabolic syndrome do not explain the observed sex differences, contrasting baseline levels of steroid hormones between women and men might still play a role these differences (Table 19 in the online supplementary material).

Following these observations of a differential association of adipokines, inflammatory markers and immune cell responsiveness, we further explored the role of other circulating metabolites that might play a role in metabolic syndrome. Metabolites are the end-point of many biological processes, and the metabolome thereby provides a snap-shot of the current physiological state. We observed strong differences between women and men in circulating metabolites. In our study the “valine, leucine and isoleucine biosynthesis” pathway was associated with the presence of
metabolic syndrome, and is likely to be the most biologically relevant in terms of association with inflammatory parameters. This pathway has previously linked to metabolic syndrome, poor metabolic health, insulin resistance and type II diabetes (44, 45). However, women and men showed the same associations between these metabolite concentrations and metabolic syndrome. Lipid profiles of women and men with metabolic syndrome showed similar patterns: while levels of most lipid markers were different between women and men and these levels changed with metabolic syndrome, these changes were the same in women and men. This suggests that even though metabolites play an important role in metabolic syndrome, this role is similar in both sexes.

There are several limitations to the current study. First, this is an observational study, and although we validated some of the major findings in an independent cohort, our data do not provide information about causality and mechanisms. Secondly, all individuals in the two cohorts studied here were of Western European descent, and it is difficult to extrapolate these data to other populations. Thirdly, we used metabolic syndrome as indicator of cardiometabolic dysregulation. It is important to realize that there are various definitions for metabolic syndrome and this syndrome is heterogeneous. To optimize external validation, we used the most frequently used definition of the NCEP ATP III. In addition, we performed all analyses separately for the various components of the metabolic syndrome, indicating triglycerides as major factor that contributes to the sex-specific differences. Finally, we have only studied a selection of circulating hormones and cannot exclude modulating effects of unmeasured hormones as an explanation of our findings.

In conclusion, in this first study, we comprehensively analyzed the regulation of inflammation in overweight individuals, and we showed that there is as strong sex-dependent association of metabolic syndrome with circulating markers of inflammation. Importantly, we demonstrate that inflammatory dysregulation in women and men with obesity and metabolic syndrome is mediated by different mechanisms, which relate to adipose tissue inflammation. Women show defective anti-inflammatory mechanisms (adiponectin), whereas men have higher concentrations of pro-inflammatory mediators (leptin, IL-6) and their myeloid cells show a hyper-responsive phenotype. These findings strongly argue for more in vitro and in vivo studies aimed at unraveling mechanisms that underlie this sex-specific inflammatory regulation. Moreover, these findings suggest that women and men might benefit from a differential sex-specific anti-inflammatory pharmacological intervention to prevent the adverse cardiometabolic effects of obesity.
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SUPPLEMENTAL MATERIAL

Supplementary Methods

Measurements and sample collection details

The 300-Obesity (300-OB) cohort study consists of 302 individuals between 55 and 80 years of age with a BMI > 27 kg/m² at screening. All participants were included between the year 2014 and 2016. This 300-OB cohort described in this study is part of the Human Functional Genomics Project (HFGP) at the Radboud university medical center, which contains a collection of cohorts with various backgrounds. Most of these participants previously took part in the Nijmegen Biomedical Study – Non-Invasive Measurements of Atherosclerosis 1 (NBS-NIMA1), a population-based survey of inhabitants of the municipality of Nijmegen, The Netherlands. 949 of the 1,517 participants of the NBS-NIMA1 study (with a BMI > 25 kg/m² at the time of NBS-NIMA1) were invited to participate. 472 subjects were excluded, mainly because they did not fulfill the criterion BMI > 27 kg/m². Another 250 subjects refused. This resulted in 227 individuals willing to participate and fulfilling all inclusion criteria. Additionally, we recruited 75 participants that were acquaintances of first 227 participants, fulfilling the inclusion criteria (age > 55 years and BMI > 27 kg/m²). Exclusion criteria for the 300-OB cohort were:

- a recent cardiovascular event (MI, transient ischemic attack or stroke < 6 months)
- prior bariatric surgery or bowel resection
- inflammatory bowel disease (IBD)
- renal dysfunction
- increased tendency for bleeding
- use of anti-coagulant therapy (oral or subcutaneous)
- use of thrombocyte aggregation inhibitors other than acetylsalicylic acid or carbasalate calcium

All subjects filled out questionnaires, which included questions about lifestyle, environmental factors and medication usage. If used, participants temporarily discontinued lipid-lowering therapy four weeks prior to the measurements. Blood samples were taken in the morning following an overnight fast. All women were postmenopausal and were not receiving hormonal replacement therapy. All participants received detailed printed and oral information and subsequently gave written informed consent. The study was approved by the Ethical Committee of the Radboud University (nr. 46846.091.13). Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

The metabolic syndrome

We used criteria defined in the National Cholesterol Education Program (NCEP) to diagnose the metabolic syndrome. The metabolic syndrome at the time of this study is defined by the NCEP ATP-III criteria as the presence of any three of the following five traits:

- Abdominal obesity, defined as a waist circumference in men ≥102 cm (40 in) and in women ≥88 cm (35 in)
- Serum triglycerides (TG) ≥150 mg/dL (1.7 mmol/L) or drug treatment for elevated TG
- Serum high-density lipoprotein cholesterol (HDL-C) < 40 mg/dL (1 mmol/L) in men and < 50 mg/dL (1.3 mmol/L) in women or drug treatment for low HDL-C
- Blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure
- Fasting plasma glucose ≥100 mg/dL (5.6 mmol/L) or drug treatment for elevated blood glucose
Blood glucose, TG, total cholesterol and HDL-C were measured using standard laboratory procedures. Before measuring systolic and diastolic blood pressure, participants took 30 minutes of supine rest. Hypertension was defined as a systolic blood pressure (SBP) level > 140 mmHg and/or diastolic blood pressure (DBP) > 90 mmHg, or currently undergoing treatment for hypertension. Type 2 diabetes mellitus was defined as a glucose level over 7.0 mmol/L after an overnight fast or currently undergoing treatment for type 2 diabetes. Weight and height were determined during visitation, and this was used to calculate BMI. The BMI was calculated as body weight (kg) divided by the square of height (m). Waist circumference was measured at the level of the umbilicus and hip circumference was measured at the level of the trochanter major. Both of these were rounded to the nearest first decimal.

**Circulating mediators**

Circulating mediators (cytokines and adipokines) were measured in human EDTA plasma using Enzyme Linked Immunosorbent Assay (ELISA). Concentrations of circulating mediators resistin, leptin, adiponectin, hsCRP and alpha-1 antitrypsin (AAT) were determined in EDTA plasma using the R&D Systems ELISA kits following the Manufacturer’s protocol. Plasma interleukin(IL)-18, IL-6 and VEGF were measured in Simple Plex cartridges using the Ella apparatus (Protein Simple, San Jose, CA). IL-18 binding protein (IL-18BP) concentrations were measured using R&D Duoset kits following the manufacturer’s standard protocol.

**Stimulation experiments**

**PBMC stimulation experiments**

At baseline, 20 mL EDTA venous blood was collected. Isolation of peripheral blood mononuclear cells (PBMCs) was performed as described in Oosting et al.\(^3\) In short, the PBMCs were isolated within 6 hours by density gradient centrifugation of PBS diluted blood (1:1) over Ficoll-Paque, washed twice with PBS and subsequently resuspended in Dutch modified RPMI 1640 medium (Invitrogen) supplemented with 50 µg/mL gentamicin (Centrafarm), 2 mM GlutaMAX and 1 mM pyruvate (Life Technologies). PBMC stimulations were performed with 5x10^5 cells/well in round-bottom 96-wells plates (Greiner) at 37 °C and 5% CO\(_2\). Supernatants were collected after 24 hours and 7 days and stored at -20 °C until ELISA measurements were performed. The different stimuli and cytokines that were measured, are shown in Table 1 and 2.

**Whole blood stimulation experiments**

At baseline, 10 ml heparin blood was collected. A volume of 100 µl of heparin blood was added to a 48 well plate (Corning) containing 400 µl stimulus (final volume 500ul/well) for 48 hours at 37°C and 5% CO\(_2\). Supernatants were collected and stored in -20°C until ELISA measurements were performed. The different stimuli and cytokines that were measured, are shown in Table 1 and 2.

**ELISA analysis**

Cytokine concentrations after stimulation were measured using commercially available ELISA kits. For the 24-hour PBMC stimulation and whole blood experiments the following kits were used:

- IL-1β: R&D systems, DY201
- IL-1Ra: R&D systems, DRA00B
- TNFα: R&D systems, DY210
- IL-6: Sanquin, M9316

For the 7-day PBMC stimulation experiments the following kits were used,
- IL-17: R&D systems, DY317
- IL-22: R&D systems, DY782
- IFNγ: Sanquin, M9333

All measurements were done according to the protocols supplied by the manufacturer.

**Cell count data**
Immune cell counts were determined in fresh whole blood EDTA samples using the Sysmex XE-5000. The Sysmex XE-5000 was used in a laboratory automation configuration part of a routine clinical setting. All analyses were preformed following the manufacturer’s protocol. Details about the XE-5000 and automatic measurements can be found on the manufacturer’s website.

**Metabolomics**

*Untargeted metabolomics*
Blood was collected in EDTA tubes and plasma was extracted. Plasma samples were frozen and stored at -80°C before extraction. Prior to extraction plasma samples were allowed to thaw on ice for 30-60 minutes. 20 µL of serum/plasma was aliquoted into a labeled 2 mL microtube and then 180 µL of aq. 80% LCMS-grade methanol was added. The samples were thoroughly mixed on a vortex mixer for 15 seconds to precipitate protein and afterwards allowed incubate for 1 hour at 4°C. Samples were centrifuged (room temperature) at > 14,000g for 15 minutes to pellet the precipitate. 100 µL of the supernatant was transferred to a fresh microtube. Samples were stored at -80°C prior to shipping.

Flow injection electrospray time-of-flight mass spectrometry was performed by General Metabolomics (1 Broadway, Cambridge MA 02142) to identify metabolic features based on m/z. Details of the procedure can be found in Fuhrer et al. The total number of m/z signals that could be assigned to one or more metabolites was 1339 for the 300-OB cohort and 1979 for the 500FG cohort.

**Lipidomics**
A high-throughput Nuclear Magnetic Resonance (NMR) metabolomics platform (Nightingale’s Biomarker Analysis Platform) was used to quantify a total of 231 lipid and metabolite measures in EDTA plasma. Most of these measures were very highly correlated to other measures from the same platform. Groups of lipoprotein particle characteristics were therefore made based on a correlation between variables of at least r > 0.75 and expert knowledge. This led to 17 groups comprising of lipoprotein subclasses, lipoprotein particle sizes, apolipoproteins and cholesterol (Table 12). For each of these 17 groups a representative variable was selected to represent the whole group of measurements. This led to easier interpretation and less strict multiple testing correction.

**Adipose tissue analysis**

*Adipose tissue biopsies*
Subcutaneous adipose tissue biopsies were obtained under local anesthesia by needle biopsies performed 6-10cm lateral to the umbilicus in the right lower quadrant, after an overnight fast. Abdomen biopsies were rinsed with PBS after they were taken with a Sterican
Adipocyte size
Haematoxylin and Eosin (H&E) staining was done to determine adipocyte size. The slides were first incubated two times in xylene (Depot) for 5 minutes and subsequently 5 minutes in 100%, 96% and 70% ethanol, respectively. The slides were then incubated for 20 minutes in haematoxylin (haematoxylin was prepared using the following protocol: first, 1 g haematoxylin (VWR) was added in 10 mL ethanol 96%. Then 200 mg sodiumlodate (VWR), 50 g potassiumaluin (VWR) and the complete dissolved haematoxylin(10 mL) was added to 1000 mL aquadest. Dissolved o/n at RT and 50 g chloralhydrate (VWR) and 1 g citric acid (Sigma) was added. Solution was mixed well, used after 2 days and filtered before use.) and rinsed in running tap water for 10 minutes. After that, the slides were incubated for 5 minutes in eosin (eosin was prepared using the following protocol: first, 1 g eosine-G (VWR) was added in 100 mL aquadest, then 100 mL 96% ethanol was added. Before used, solution was diluted 1:5 with aquadest), rinsed short in 70% ethanol and incubated for 5 minutes in 96% and 100% ethanol, respectively. At last the slides were incubated two times in xylene (Depot) for 5 minutes. Finally, the slides were sealed with Permount (Fischer Scientific) and a cover slide. After staining, adipocytes were scored on their size with the KS-400 software. This was done with the microscope Zeiss Axiophoto.

The morphometry of individual fat cells was afterwards assessed using digital image analyses as described previously. For each participant, the adipocyte cell diameters of all adipocytes in four microscopic fields of view were counted and measured. On average, 219 adipocytes (range 113 - 330) were measured per field.

Presence of macrophages
To detect macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Serotec, Oxford, UK). Sections were preincubated with 20% normal horse serum followed by overnight incubation at 4 °C with the primary antibody diluted 1:40 in phosphate-buffered saline, 1% bovine serum albumin. After incubation with the primary antibody (mouse anti-human), a horse anti-mouse IgG conjugated to horseradish peroxidise (Vector labs brunschwig) was used as a secondary antibody. Visualization of the complex was done using 3,3'-diaminobenzidene for 12 min. Negative controls were used by omitting the primary antibody. Hematoxylin and eosin staining of sections was done using standard protocols. The percentage of macrophages was expressed as the total number of macrophages divided by the total number of adipocytes counted in 15 random microscopic fields of view. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages.

Quantification and statistical analysis
Data preprocessing and scaling
For circulating markers and cytokines produced after stimulation, any value above or below a detection limit was set to that detection limit. Metabolomic data, lipidomic data, cytokine production capacity data, circulating inflammatory marker data were transformed using the
rank based inverse normal transformation (INT) before analysis unless stated otherwise. The following R code was used (R programming language):

\[
\text{transformed} = \text{rank(original)}
\]
\[
\text{transformed} = \text{qnorm(} \text{transformed} \big/ (\text{length(} \text{transformed} \text{)}+0.5)\text{}),
\]
where “transformed” is the transformed data and “original” the original data.

**Metabolic syndrome and sex**

Analysis was performed in the R programming language, using the ‘lm’ function from the “stats” package. A linear model was created with the following formula:

\[
\text{markerOfInterest} \sim \text{intercept} + \beta_1*\text{age} + \beta_2*\text{sex} + \beta_3*\text{season} + \beta_4*\text{metabolicSyndromeStatus}
\]

Details for the seasonality correction are provided below. The model was run 3 times, once for all data, once for just men and once for just women. In case the model was run for just one sex, the correction factor “sex” was removed. Each run p-values for regression coefficients of the relevant markers were corrected for multiple testing using the Benjamini-Hochberg FDR procedure, using FDR < 0.05 after correction as the threshold for statistical significance.

To assess if any of our biological markers (circulating markers of inflammation, cell subsets, cytokine production capacity) showed significant differences between women and men when comparing individuals with and without metabolic syndrome, we calculated interaction effects between someone’s “metabolic syndrome status” and sex:

\[
\text{markerOfInterest} \sim \text{intercept} + \beta_1*\text{age} + \beta_2*\text{sex} + \beta_3*\text{season} + \beta_4*\text{metabolicSyndromeStatus} + \beta_5*\text{sex:metabolicSyndromeStatus}
\]

The p-values were calculated for the estimate of the interaction term. Specifically, the “lm” function calculates a t-statistic to test whether the corresponding regression coefficient is different from 0.

The significance of the correlation between leptin and IL-6 was calculated using Spearman correlation and the “cor.test” function in the R programming language. The null hypothesis in this test is that \( \rho = 0 \).

Similar approaches were taken for each of the five factors that make up metabolic syndrome. In that case “metabolicSyndromeStatus” is replaced by one of these factors, for instance “highTriglycerides”.

**Associations between host factors, circulating markers, metabolomics, cell subtypes**

Associations between parameters were calculated using the ‘lm’ function from the “stats” package in the R programming language. The following data-types were associated to one another in this way for different analyses:

- age and sex with circulating markers of inflammation, cell subsets (absolute and percentages), cytokine production capacity
- circulating markers of inflammation with circulating markers of inflammation and cell subsets (absolute and percentages), metabolomics

A linear model was created with the following formula:

\[
\text{parameter1} \sim \text{intercept} + \beta_1*\text{age} + \beta_2*\text{sex} + \beta_3*\text{season} + \beta_4*\text{parameter2}
\]

here, parameter1 and parameter2 are the parameters of interest. So, e.g. in the association between circulating markers of inflammation and cell subsets, one comparison would be:

\[
\text{IL-6} \sim \text{intercept} + \beta_1*\text{age} + \beta_2*\text{sex} + \beta_3*\text{season} + \beta_4*\text{neutrophils%}
\]
So, the association between IL-6 and neutrophils% is evaluated, whilst correcting for age, sex and season. Seasonality correction is explained below. The function “lm” in R provides the p-values. This function calculates a t-statistic to test whether the corresponding regression coefficient is different from 0. A hypothesis test using this t-statistic generates the p-value. Multiple testing correction was performed using the Benjamini–Hochberg FDR procedure.

**Seasonality correction**

The seasonality correction was performed similar to Ter Horst et al.\(^9\). This was introduced to correct for seasonal differences in levels of inflammatory markers and cytokine production capacity. In short, the following terms were added to the regression formula:

- \( \sin(2 \times \pi \times \text{numDaysFromJan2014} / 365) \)
- \( \cos(2 \times \pi \times \text{numDaysFromJan2014} / 365) \)
- \( \text{numDaysFromJan2014} \)

So, in the regression formula the following would be inserted:

\[
\beta_1 \sin(2 \times \pi \times \text{numDaysFromJan2014} / 365) + \beta_2 \cos(2 \times \pi \times \text{numDaysFromJan2014} / 365) + \beta_3 \text{numDaysFromJan2014}
\]

where \( \sin(2 \times \pi \times \text{numDaysFromJan2014} / 365) \) and \( \cos(2 \times \pi \times \text{numDaysFromJan2014} / 365) \) combined capture seasonality patterns with a periodicity of one year. The linear term “numDaysFromJan2014”, which indicates how many days after January 1st 2014 the sample was collected, was added to correct for potential sample storage degradation. For the 500FG cohort the same corrections were applied, though here numDaysFromJan2014 was replaced with numDaysFromJan2013, since samples were collected a year earlier.

**PCA and tSNE**

Principal component analysis (PCA) was applied to the inflammatory markers and circulating metabolite data. Any individuals with missing values for any marker were excluded from this analysis. The data was first inverse rank transformed followed by mean centering and standardization. PCA was performed in the R programming language using the “prcomp” function that is part of the “stats” package.

**Metabolic association analysis**

Associations between metabolite levels and circulating markers of inflammation were calculated using a linear model correcting for age, sex and seasonality. Seasonality corrections were performed as described in Ter Horst et al.\(^9\). The linear models were constructed using the “lm” function of the “stats” package in the R programming language. All p-values were calculated using linear regression by testing the null hypothesis that \( \beta = 0 \) for relationship between a metabolite and a marker of inflammation. Both the cytokine data and the metabolite data were transformed using a Rank-Based Inverse Normal Transformation. Associations were corrected for multiple testing using the Benjamini–Hochberg FDR procedure.

**Metabolic pathway analysis**

Metabolic pathway analysis was performed using an adaptation of Gene Set Enrichment Analysis (GSEA), as originally developed by Subramanian et al.\(^10\). A fast implementation in the R programming language called Fast Gene Set Enrichment Analysis (FGSEA)\(^11\) was used, using the -log10(p-value) for each metabolite as the score.
Each mass/charge-ratio detected by the metabolomics could be the result of one or more metabolites. In the pathway analysis, all these metabolites were assigned the same score. However, if they mapped to any common pathways, the mass/charge-ratio was only counted once for that pathway, to avoid artificial enrichment. This was achieved by removing all but one of the metabolites with the same m/z value from each pathway.

The pathways provided by the KEGG pathway database were used for enrichment analysis. Interesting pathways were visualized using Pathview, plotting the -log10(p-values) for the metabolites, setting any p-value smaller than 1e-4 to exactly 1e-4 for visualization purposes. Negative associations are indicated in blue in these plots and positive associations with red.

Top pathways for the circulating markers of inflammation were selected by taking the average of the lowest three p-values over all circulating markers and ordering them by these p-values.

**Supplementary references**


**Supplementary tables**

For the supplementary tables: check the online supplementary.
CHAPTER 5

IL-18 binding protein: a novel biomarker in obesity-related atherosclerosis that modulates lipoprotein metabolism


* Contributed equally

Submitted
ABSTRACT

Objective
The aim of the present study was to investigate the impact of systemic inflammatory mediators, especially from the IL-1 family of cytokines, on atherosclerosis in individuals with overweight and obesity.

Background
Although obesity is a well-known risk factor for metabolic dysregulation and atherosclerotic cardiovascular diseases, a sizeable number of individuals affected by obesity do not develop these complications. It is unknown whether inter-individual variation of chronic inflammation explain these differences in atherosclerotic risk in the obese population.

Methods
We measured a panel of circulating inflammatory markers, among which many related to the interleukin-1 pathway, and correlated them to indicators of carotid atherosclerosis and circulating lipoprotein particles in 302 individuals of European origin with a BMI ≥ 27 kg/m² (300-Obesity cohort).

Results
We identified that interleukin-18 binding protein (IL-18BP) showed a stronger relation to the presence of carotid atherosclerosis than currently used markers of inflammation (CRP, interleukin-6). IL-18BP correlated strongly with the lipoprotein particles from the very-low-density lipoprotein (VLDL) groups and with the number of atherogenic lipoprotein particles (measured by apolipoprotein B levels). The association between IL-18BP and VLDL was validated in a cohort of young healthy individuals. The molecular mechanism may involve a decreased expression of genes known to be involved in hepatic lipid metabolism including PPP1R3G and ENHO regulated by the IL-18 pathway.

Conclusions
These findings identify IL-18BP as a novel biomarker of atherosclerosis in individuals with obesity, and the IL-18/IL-18BP pathway as a functional modulator of VLDL metabolism and atherosclerosis both in lean individuals and individuals with obesity.
INTRODUCTION

The epidemic increase in obesity affects both developed and developing countries, making cardiovascular disease (CVD) the world’s leading cause of death (1). The cluster of obesity related cardiovascular risk factors is called the metabolic syndrome. Interestingly, approximately 20-30% of the obese population is metabolically ‘healthy’ with a substantially lower risk for CVD (2). The factors that account for the heterogeneity within the obese population are not known.

It is well established that obesity, especially visceral fat accumulation, leads to a state of chronic low-grade inflammation, which is a key regulatory process in the development of atherosclerosis (3-5). In approximately four out of ten people, the risk of recurrent cardiovascular events after myocardial infarction (MI) is directly related to increased systemic inflammation associated with atherosclerosis (6). Inflammation has been linked to endothelial dysfunction, fatty streak development, and plaque vulnerability (7). Recently, the CANTOS trial demonstrated that inhibition of the pro-inflammatory cytokine interleukin(IL)-1β lowers cardiovascular event rate in patients after MI (6). In addition to IL-1β, IL-18 is another cytokine from the IL-1 family (8), and important modulators of this pathway include IL-18 binding protein (IL-18BP) (9), alpha-1-antitrypsin (AAT), and adipokines. IL-18 is associated with obesity, insulin resistance and dyslipidemia (10-13), while IL-18BP binds IL-18 and thereby inhibits its function. IL-18BP production is regulated under influence of interferon (IFN) gamma via a negative feedback loop with IL-18 (8).

The aim of the present study was to investigate the impact of systemic inflammatory mediators, especially from the IL-1 family of cytokines, on atherosclerosis in individuals with obesity. We hypothesize that the propensity of an individual with obesity to display a more pro-inflammatory profile has an important impact on the likelihood to develop atherosclerosis.

MATERIALS AND METHODS

Human studies

300-Obesity study

We enrolled 302 individuals aged 55-80 years with a BMI > 27 kg/m² at screening in the 300-Obesity (300-OB) cohort study. This study was performed in the period between 2014 and 2016 at the Radboud university medical center and is part of the Human Functional Genomics Project (14). Individuals with a recent cardiovascular event (MI, transient ischemic attack or stroke < 6 months), a history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral or subcutaneous anti-coagulant therapy, use of thrombocyte aggregation inhibitors other than acetylsalicylic acid and carbasalate calcium were excluded. The majority of the individuals was derived from the cohort of the Nijmegen Biomedical Study – Non-Invasive Measurements of Atherosclerosis study, a population-based survey of residents of Nijmegen, the Netherlands (15). Of the original 1,517 participants from this study, 949 (with a BMI > 25 kg/m² at that time) were...
invited to participate, resulting in 227 individuals fulfilling all the inclusion criteria and willing to participate. 444 individuals were excluded, mainly because of a BMI < 27 kg/m². Another 249 individuals refused. During the 300-OB study, we additionally recruited 75 participants, fulfilling the inclusion criteria (age > 55 years and BMI > 27 kg/m²). All women were postmenopausal and did not use hormonal replacement therapy. All participants provided written informed consent. The study was approved by the Ethical Committee of the Radboud University (nr. 46846.091.13). Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

**Baseline characteristics**
All individuals filled out questionnaires about life style and medication use. We took blood samples in the morning following an overnight fast. Participants who used lipid-lowering therapy, temporarily discontinued this medication 4 weeks prior to the measurements. Blood glucose, triglycerides (TG), total cholesterol, high-density lipoprotein (HDL) cholesterol and apolipoprotein B (ApoB) were measured using standard laboratory procedures. Systolic and diastolic blood pressure were measured after 30 minutes of supine rest. Hypertension was defined by systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg or current treatment for hypertension. Diabetes mellitus type 2 (DM 2) was defined as a glucose level > 7.0 mmol/L after an overnight fast or current treatment for DM 2. We used clinical criteria as defined in the National Cholesterol Education Program (NCEP) to diagnose the metabolic syndrome (16). The BMI was calculated as body weight (kg) divided by the square of height (m). Waist circumference was measured at the level of the umbilicus to the nearest 0.1cm. Hip circumference was measured at the level of the trochanter major.

**Cardiovascular phenotyping**
All individuals underwent comprehensive cardiovascular assessment, including the measurement of carotid intima-medial thickness (cIMT), plaque presence and maximum plaque thickness. After a resting period of at least 30 minutes, baseline resting diameter, wall thickness, plaque presence and maximal plaque thickness of both carotid arteries were assessed by ultrasound (Esaote Biomedica, Genoa, Italy). The cIMT and diameter measurements were performed in the proximal 1cm straight portion of the carotid artery in three different angles (90°, 120° and 180°) for 6 heartbeats. The outcome variable was defined as the mean cIMT of the 3 different angles. Subsequently the presence of plaque and the thickness of plaques in the common carotid, internal carotid, or external carotid artery or at the carotid bulb were measured. The presence of a plaque was defined as focal thickening of the wall of at least 1.5 x the mean cIMT or a cIMT > 1.5 mm.

**Circulating mediators**
Cytokines and circulating mediators were measured in human EDTA plasma using Enzyme Linked Immunosorbent Assay (ELISA). Adiponectin, leptin, resistin, AAT and C-reactive protein (CRP) were measured using kits from R&D Systems following manufacturer’s instructions. IL-
6 and IL-18 were measured by Simple Plex cartridges using the Ella apparatus (Protein Simple, San Jose). Levels of plasma IL-18BP were measured using the DuoSet ELISA (R & D Systems).

Metabolomics
We used a high-throughput targeted Nuclear Magnetic Resonance (NMR) metabolomics platform (Nightingale's Biomarker Analysis Platform) (17) to generate spectra for the quantification of 231 lipid and metabolite measures, either as absolute concentrations of each metabolic measure or as ratios. In this study, we focus on the lipoprotein profile with total lipid concentrations of 14 lipoprotein subclasses, lipoprotein particles sizes, apolipoproteins and cholesterol. Groups of lipoprotein particle parameters were made based on a correlation between variables of \( r > 0.75 \) (see online supplementary material from chapter 4).

Adipose tissue analysis
Subcutaneous adipose tissue biopsies were obtained under local anaesthesia by needle biopsies performed 6-10cm lateral to the umbilicus in the right lower quadrant, after an overnight fast. The morphometry of individual fat cells was assessed using digital image analyses as described previously (18). For each participant, the adipocyte cell diameters of all adipocytes in four microscopic fields of view were counted and measured. To detect macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Serotec, Oxford, UK). The percentage of macrophages was expressed as the total number of macrophages divided by the total number of adipocytes counted in 15 random microscopic fields of view. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages (19). To robustly quantify adipose tissue inflammation among study participants using histology, several parameters were assessed and combined into an adipose tissue inflammation score, as the phenotype by which inflammation in the adipose tissue presents is quite heterogenous (20,21). This assessment led to a score including following parameters: a mean adipocyte diameter above the average diameter of the cohort (> 51.7um (mean diameters in the 300-OB cohort) was defined as one point in the AT score, the percentage of macrophages above the average of the cohort (> 12.6% (mean in the 300-OB cohort) was defined as one point in the AT score and the presence of crown-like structures (CLS) was defined as one point in the AT score. Hence, the adipose tissue inflammation score could ranges from 0 (no inflammation) to 3 (severely inflamed).

500FG cohort
We validated the association between IL-18BP and lipoproteins in 500 healthy individuals from the 500 Functional Genomics (500FG) cohort (mean age 29 years and mean BMI 22.7 kg/m\(^2\)) using the same methodology for analysis of circulating mediators and metabolomics.(14) The cohort was previously described in detail in Ter Horst et al. (22).
Statistics
Correlations and corresponding p-values were calculated using the rank-based Spearman correlation as implemented in the “cor.test()” function of the “R” programming language (R Foundation for Statistical Computing, Vienna, Austria). Before the regression analysis, the circulating markers of inflammation and the lipidomics data were normalized using logarithmic transformation to get a distribution more closely resembling a normal distribution. Regression analysis was performed using the “Rfit” package, as implemented in the “R” language. Multiple testing correction was performed using the Benjamini – Hochberg False Discovery Rate method as implemented in the “p.adjust” function of the “stats” package in the R programming language (23).

Mice studies
Mice
IL-18 deficient (IL-18-/−) mice were generated on a C57Bl/6J background as previously described (24). Body weight of the mice was recorded every month. In an additional study, lean wild type (WT) C57Bl/6 mice were treated with recombinant IL-18 or phosphate buffered saline (PBS) for 18 hours. At the end of the experiments, animals were sacrificed and organs were isolated and used for subsequent analysis as described below. All animal studies were approved by the animal ethical committee of the Radboudumc.

RNA isolation and qPCR analysis
RNA from the liver was isolated using Trizol Reagent (Invitrogen) following manufacturer’s instructions. RNA concentration and purity was determined using a Nanodrop (Thermo Fisher Scientific, USA).

Transcriptomics analysis
Two datasets were used for transcriptome analysis. The first dataset consists of pooled samples of 4 treatment groups (WT vs IL-18-/− and WT-treated with PBS vs WT-treated with recombinant mouse IL-18 (18 hours of treatment)) that were subjected to expression profiling by microarray. To this end, purified total RNA of n = 5 mouse liver samples per group was pooled and labelled with the Affymetrix GeneChip RNA One cycle Amplification Kit (Affymetrix, Santa Clara, CA, U.S.; P/N 900652) and hybridized to Affymetrix Mouse Genome 430 2.0 arrays (Affymetrix). Quality control and data analysis pipeline have been described in detail previously (25). A second dataset consisting of raw expression data of livers of WT and IL-18-/− mice of 12 weeks of age were extracted from the Gene Expression Omnibus (GSE64309) (26). Data was background corrected using the normexp+offset method (27), followed by quantile normalized using functions in the library limma (28). Next differentially expressed genes were identified by using linear models and an intensity-based moderated t-statistic. Probe sets that satisfied the criterion of P < 0.05 were considered to be regulated. To identify IL-18 regulated genes, both datasets were combined using a FC > 1.3 and a P-value < 0.05 as selection criteria.
RESULTS

300-OB cohort
The 300-OB cohort consisted of 302 individuals of Western-European ancestry with a BMI above 27 kg/m². Approximately half fulfilled the criteria for metabolic syndrome and half had carotid atherosclerosis (Figure 1A through C). No differences were present between the individuals from the originally NBS-NIMA1 study and the additionally recruited subjects.

![Figure 1 - Baseline characteristics of 300-Obesity Cohort. (A) Sex and current smoking status as assessed by questionnaires. Plaque presence as measured by ultrasound of both carotid arteries. (B) The distribution of age, BMI, triglycerides and HDL-cholesterol. (C) The prevalence of cardiovascular diseases.](image)

**BMI** Body Mass Index; **MI** Myocardial Infarction; **CVA** Cerebrovascular Accident; **PTCA** Percutaneous Transluminal Coronary Angioplasty; **TIA** Transient Ischemic Accident; **PAD** Peripheral Arterial Disease.

**IL-18 binding protein is a marker of carotid atherosclerosis in individuals with obesity**
The correlations between all circulating markers and indices of atherosclerosis are shown in figure 2. After adjustment for factors known to influence cytokine production (age, sex, BMI, smoking, and season) (22), only IL-18BP concentrations remained positively associated with maximum plaque thickness (Figure 2A). There was a dose dependent association between plaque thickness and the IL-18BP concentration (Figure 2B). In contrast, IL-18, IL-6 or CRP concentrations showed no association with plaque thickness. Markers of metabolic status, adiponectin, leptin and resistin did also not show an association with atherosclerosis after adjustment for factors known to influence cytokine production. To gain insight into whether the association between IL-18BP and atherosclerosis was independent of the classical risk factors, we additionally adjusted for systolic blood pressure, glucose level, HDL cholesterol and low-density lipoprotein (LDL) cholesterol, antihypertensive, antidiabetic and lipid-lowering drugs. The association between IL-18BP and maximum plaque thickness remained
statistically significant after adjustment confirming an independent link between IL-18BP as marker of inflammation and atherosclerosis (supplementary material Figure 1).

Figure 2 - (A) Heatmap of correlation between a panel of circulating inflammatory mediators and the mean carotid intima-medial thickness and maximum plaque thickness. Red indicates a significant positive association. Age, sex, BMI, smoking and season were taken into account as adjustment variables. (B) Box plot for relation between IL-18BP concentrations and maximum plaque thickness in mm.

AAT Alpha-1 antitrypsin; CRP C-reactive protein; IL-18 Interleukin-18; IL-18BP interleukin-18 binding protein; IL-6 Interleukin-6; cIMT carotid intima-medial thickness; maxPlaqueThickness maximum plaque thickness in millimeter.

**IL-18 and IL-18BP correlate with lipoprotein particle composition and number**
To investigate the underlying mechanism by which IL-18BP could be related to plaque thickness, we investigated the relation between IL-18, IL-18BP concentrations and the lipoprotein particles using a metabolomics platform focusing on CVD. Parameters of lipoprotein particles were first divided into seventeen groups based on a correlation between variables of $r > 0.75$ (see online supplementary material from chapter 4). We found a strong and significant univariate association between IL-18BP concentrations and very-low-density lipoprotein (VLDL) particles; namely the very large VLDL particles, the small-to-large VLDL particles, and very small VLDL particles, group 1, 2 and 3 respectively (Figure 3A). The strongest associations were seen within group 2 (Figure 3D). Furthermore, we observed a positive association between IL-18BP concentration and part of group 4, the intermediate-density lipoproteins (IDL) and LDL particles, with the non-HDL/non-LDL cholesterol (remnant) and free cholesterol from group 5. A strong association with the number of lipoprotein particles, as measured by ApoB in group 6, was seen with IL-18BP. IL-18 showed the same profile, but weaker correlations were seen with group 1, 2 and 3. Within group 4, IL-18 concentration was mainly associated with TG particles in the IDL and LDL lipoproteins. In this cohort of individuals
with obesity, we found no association between the lipoprotein profile and IL-6 or CRP plasma concentrations (supplementary material figure 2 through 5). Importantly, the association between IL-18BP and atherosclerosis was lost after adjustment for triglycerides and VLDL cholesterol (supplementary material Figure 1).

Figure 3 - **IL-18BP is strongly associated with components of the VLDL lipoproteins.** (A) Heatmaps with correlations between total IL-18, IL-18BP, CRP, IL-6 and lipoprotein particle composition in the 300-OB Study. The intensity of the red color represents the strength of the significance. (B) Validation of this relation in healthy individuals from the 500FG Study. (C) Heatmap with the correlation between lipoprotein particles and markers of atherosclerosis. (D) Scatterplots of IL-18BP lipoprotein relation.

XXL.VLDL.P Concentration of chylomicrons and extremely large VLDL particles; .L Total lipids; .PL Phospholipids; .C Total cholesterol; .CE Cholesterol esters; .FC Free cholesterol; .D Diameter; TG Triglycerides; XL.VLDL very large VLDL; .L Large; M. Medium; S. Small; XS. Extreme small; VLDL very-low-density lipoproteins; IDL intermediate-density lipoproteins; LDL low-density lipoproteins; HDL high-density lipoproteins; Remnant.C Remnant cholesterol (non-HDL, non-LDL -cholesterol); Est Esterified; IL-18BP Interleukin-18 binding protein; IL-18 Interleukin-18; CRP C-reactive protein; IL-6 Interleukin-6. cIMT carotid intima-medial thickness; maxPlaqueThickness maximum plaque thickness in millimeter.
IL-18 binding protein is a marker of adipose tissue inflammation in individuals with obesity

One of the main driving forces for systemic low-grade inflammation present in obesity is inflammation originating from the adipose tissue (29). To investigate whether IL-18BP could serve as a marker for this adipose tissue inflammation, we analyzed subcutaneous fat taken from the abdomen in individuals with obesity. The AT score associated significantly with IL-18BP; individuals with more adipose tissue inflammation had higher IL-18BP levels (Figure 4). This suggests that IL-18BP is a good systemic marker of adipose tissue inflammation.

Figure 4 – IL-18BP is strongly associated with adipose tissue inflammation. (A) Box plots of adipose tissue inflammation score in relation to IL-18BP level. Components of the adipose tissue inflammation score; adipocyte diameter, percentage of macrophages, crown-like structures. (B,C) Immunohistochemically illustration of individual without adipose tissue inflammation (score 0) versus individual with extreme adipose tissue inflammation (score 3). AT score Adipose tissue inflammation score; IL-18BP Interleukin-18 binding protein.
Validation in cohort of lean individuals
To investigate whether the association found between IL-18BP and lipoprotein particle profile was also present in young, lean individuals, we investigated this relation in the individuals of the 500FG cohort (30). In this cohort, the same associations between IL-18BP, IL-18 concentrations and the different VLDL, LDL groups, serum TG, free and remnant cholesterol and ApoB were found (Figure 3B). In the 500FG cohort we found an association between CRP concentration and VLDL, most strongly for small VLDL and LDL. This is in contrast to the individuals of the 300-OB in which only the TG particles in the IDL and LDL lipoproteins were associated with CRP.

Lipoproteins in relation to carotid atherosclerosis
To investigate whether the association between IL-18BP and atherosclerosis could be attributed to a change in lipoprotein particle characteristics, we investigated the relationship between lipoprotein particle composition and atherosclerosis in the 300-OB cohort. Analysis on the relation with these lipoproteins was possible in 301 out of 302 individuals of whom we had all the data. In line with the data on IL-18/IL-18BP pathway, the strongest association with the maximum plaque thickness was observed for lipoprotein groups 1 and 2, including the small-to-very large VLDL particles (Figure 3C). The maximum plaque thickness was also related with the TG in the small LDL particles, but not with TG in other LDL particles, nor with LDL-cholesterol, showing that in this obese population, remnant cholesterol has a stronger relation to atherosclerosis than LDL-cholesterol. A significant association with atherosclerosis was also seen for the non-HDL/non-LDL cholesterol (remnant), free cholesterol, as well as for apoB concentrations. The associations between other lipoprotein clusters and atherosclerosis are shown in supplementary material figure 6 through 9. Based on these correlations, we hypothesized that IL-18BP impacts on atherosclerosis via modulation of lipoprotein metabolism.

IL-18 induced changes in liver gene expression profiles
One of the hypothesis on the relation between IL-18BP and atherosclerosis is that it influences lipoprotein metabolism in the liver by affecting IL-18 activity due to its binding to IL-18. To directly investigate the effect of the IL-18 pathway on lipoprotein metabolism in the liver, we used two mouse models with genetic/pharmacological disturbances in this pathway. In the combined analysis, we included liver samples from WT and IL-18/-/- mice and WT animals treated with recombinant IL-18 with the ultimate goal to identify genes controlled by IL-18. Analysis of the gene expression profile of the liver showed 11 genes (absolute FC > 1.3, p-value < 0.05) involved in lipoprotein metabolism to be upregulated in WT vs IL-18/-/- mice. Interestingly, two genes were consistently upregulated in the WT vs IL-18/-/- mice and in the presence of (higher) IL-18 levels (Figure 5). Both genes Protein Phosphatase 1 Regulatory subunit 3G (PPP1R3G) and Energy-homeostasis associated (ENHO) have been found to play an important role in lipid metabolism (31,32).
Figure 5 – Effect of IL-18 on transcriptome of the liver. Transcriptome analysis of the liver in WT vs IL-18-/- animals and WT animals treated once with PBS vs recombinant IL-18 via intraperitoneal administration (data is presented as log2 fold changes).
DISCUSSION

In the present study, we found that IL-18BP is strongly related to presence and severity of carotid atherosclerosis in individuals with a BMI above 27 kg/m². We identified that in individuals with obesity, circulating IL-18BP concentrations showed a stronger association with the presence of atherosclerosis than the currently used markers of inflammation CRP or IL-6. By further investigating this association using a large metabolomics platform, we were able to show that IL-18BP was strongly positively linked to triglyceride-rich lipoprotein particles, especially VLDL particles. This association between IL-18BP and VLDL particles was confirmed in a cohort of younger and leaner, healthy individuals. In addition, we showed that IL-18BP was a good systemic marker of adipose tissue inflammation.

The causal role for inflammation and in particular the IL-1 pathway in atherosclerotic cardiovascular disease was demonstrated by the positive results of the CANTOS trial (6). We now demonstrate that IL-18BP, also a member of the IL-1 family inflammatory pathway, is linked to adipose tissue inflammation, metabolic and cardiovascular complications in obesity. We hypothesize that the IL-18/IL-18BP system impacts on atherogenesis via modulation of VLDL metabolism in the liver. This finding might identify potential future targets for pharmacological strategies to prevent cardiovascular disease.

IL-18BP is strongly associated with VLDL lipoprotein particles, an association we found both in overweight and lean individuals. We found that remnant cholesterol, and in particular the small-to-large VLDL particles showed the strongest association with carotid atherosclerosis in our obese cohort. A recent study by Lawler et al. identified VLDL as the lipoprotein particle most strongly related to CVD in individuals treated by statins (33). On statin therapy, levels of the smallest VLDL particle subclass were associated with a 68% per-SD (standard deviation) increase in residual cardiovascular risk. Pechlaner et al. also found VLDL associated apolipoproteins to be the strongest predictor for cardiovascular events in a large prospective population based study (34). In our cohort, IL-18BP was also strongly related to apoB levels, which is also associated with cardiovascular risk (35). Together with the results of the validation studies in mice, these data support the hypothesis of an IL-18/IL-18BP pathway/lipoprotein axis with an important impact on cardiovascular complications.

As IL-18BP binds IL-18, we investigated the effect of IL-18 on gene expression in the liver. To explain the effect of the IL-18 pathway on lipoprotein metabolism, we investigated which hepatic genes were regulated by IL-18 using both IL-18/-/- vs WT animals, and WT animals treated with recombinant IL-18. We identified several hepatic genes with a known role in lipid metabolism to be regulated by IL-18. Two of those genes, PPP1R3G and ENHO, were of particular interest. PPP1R3G has previously been linked to glucose homeostasis and lipid metabolism (36). Transgenic mice with liver-specific expression of PPP1R3G demonstrated increased hepatic glycogen accumulation with increased clearance rate of postprandial blood glucose (31). In addition to PPP1R3G, ENHO was identified as a potential IL-18 regulated gene.
This gene encodes the peptide adropin, which has been shown to be able to attenuate steatohepatitis and insulin resistance independently of the effects on adiposity or food intake (32). Multiple studies have suggested a protective role for adropin in human plasma in atherosclerosis (37) and coronary artery disease (38). IL-18BP might impact on atherogenesis by limiting the circulating IL-18 availability, which subsequently increases VLDL formation in the liver.

On the other hand, in our analyses we found a stronger relation between IL-18BP and atherosclerosis than for IL-18, indicating that the effect of IL-18BP might be moderated through other factors than only changes in IL-18 bioactivity. Another potential mechanism to explain the association of high IL18BP with high cardiovascular (CV) risk is modulation of the function of the anti-inflammatory cytokine IL37, as IL18BP binds to IL37 as well (39). Due to the high affinity of IL18BP, the large 10-20 fold molar excess of IL18BP over IL18, this results in near total binding of IL18, leaving the levels of IL18 in both health and disease in the low picogram level (8,30). As the concentrations of IL18BP increase, there may be greater binding to endogenous IL37. Since IL37 is a fundamental inhibitor of both innate inflammation and acquired immunity (40), a reduced bioavailability of endogenous IL37 with modulatory activity may become a risk factor for immune-mediated diseases. In fact, low levels of IL37 in adipose tissues of humans are linked to high insulin resistance and mice expressing human IL37 are protected against obesity-associated inflammation and insulin resistance (41). The current methodologies for measurement of circulating IL-37 concentrations are lacking sufficient accuracy, but future development of better methods and assessment of IL-37 circulating concentrations as well as its production by peripheral blood mononuclear cells (PBMC) are warranted.

Limitations, strengths and conclusions
There are some limitations to our study. First, the cohorts studied are relatively small (302 and 500 individuals). Although the fact that we validated our findings in an independent cohort strongly supports the validity of our data, larger studies are needed to investigate the possibility of IL-18BP as a biomarker of atherosclerosis. Another limitation is related to the cross-sectional design of our study group. No causal relations can be drawn from the 300-OB study directly, experimental studies will need to confirm the causal relation. Finally, a third limitation is that we related the IL18 pathway to the presence of asymptomatic carotid plaques, and the relation to clinical atherosclerotic cardiovascular disease remains to be established. Importantly, we related the circulating IL-18BP concentration to the incidence of major adverse cardiovascular events in a population of 927 patients undergoing coronary angiography. In this separate cohort IL-18BP was independently predictive for cardiovascular morbidity and mortality (article under review).

The strength of our study is the focus on individuals with obesity, with or without cardiovascular and metabolic complications with a deep phenotyping of clinical and
immunological parameters. Another important strength is the validation of the IL-18BP relation with the lipoprotein groups in another cohort of mostly younger healthy individuals, the 500FG cohort. This study has important implications for understanding the factors that influence metabolic and atherosclerotic derangement in individuals with obesity: we provide strong arguments that inflammation in general and the IL-18/IL-18BP pathway in particular, plays an important role. To translate this pathway to novel pharmacological strategies to prevent cardiovascular disease, it is necessary to further elucidate the underlying mechanism by which the IL-18 pathway influences lipoprotein metabolism and the atherosclerotic process.
REFERENCES

Supplemental Methods
For additional supplementary methods see chapter 4.

Cardiovascular phenotyping
The measurements were performed after an overnight fast or in the afternoon six hours after a standardized breakfast. Participants were asked to abstain from caffeinated products for at least twelve hours and they were asked not to smoke for 12 hours before the visit. Testing was performed in a quiet, temperature-controlled room with the patients in supine position. After a resting period of at least 30 minutes, baseline resting diameter, distensibility, and wall thickness of the carotid artery were assessed by a well-trained sonographer. A 7.5-MHz transducer of a Mylab Class C ultrasound device (Esaote Biomedica, Genoa, Italy) connected to a computer with a data acquisition board (Art.lab). Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. The cIMT and diameter measurements were performed in the proximal 1cm straight portion of the carotid artery in three different angles (90°, 120° and 180°) for 6 heart beats. The measurements were recorded during the diastolic phase. Measurement of the cIMT was performed using an automatic boundary detection system based on RF processing-based measurement (Art.lab, Esaote Europe BV, Maastricht, Netherlands) (1). Analysis of the cIMT and diameter was performed by an independent blinded researcher. The primary outcome variable was defined as the mean cIMT of the 3 different angles (2). Subsequently the presence of plaque and the thickness of plaques in the common carotid, internal carotid, or external carotid artery or at the carotid bulbus were measured.

500FG cohort
The validation of the IL-18BP relation with the lipoproteins was performed in 500 healthy individuals from the 500FG cohort (mean age 29 years and mean BMI 22.7 kg/m²). This cohort is part of the Human Functional Genomics Project (HFGP; 500FG cohort, www.humanfunctionalgenomics.org) (3).

Mice
Five male IL18-/- and five male wild type (WT) mice were generated on a C57Bl/6J background as previously described (4). Body weight of the mice was recorded every month. To induce obesity, animals were fed a high-fat diet (HFD) containing 45% energy from fat (D12451, Research diets, New Brunswick, USA). In an additional study, ten lean female WT C57Bl/6 mice were treated with recombinant IL-18 or PBS for 18 hours. At the end of the experiments, when the mice were aged 10-14 weeks, animals were sacrificed and organs were isolated and used for subsequent analysis as described below. All animal studies were approved by the animal ethical committee of the Radboudumc.

RNA isolation and qPCR analysis in mice study
RNA from animal tissues was isolated using Trizol Reagent (Invitrogen) following manufacturer’s instructions. RNA concentration and purity was determined using a Nanodrop (Thermo Fisher Scientific, USA). 500 ng or 1 μg of RNA was used for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands).
Transcriptomics analysis mice study

Two datasets were used for transcriptome analysis. The first dataset consists of pooled samples of 4 treatment groups (WT vs IL-18-/- and WT-treated with PBS vs WT-treated with recombinant IL-18 (18hrs of treatment)) that were subjected to expression profiling by microarray. To this end, purified total RNA of n = 5 mouse liver samples per group was pooled and labelled with the Affymetrix GeneChip RNA One cycle Amplification Kit (Affymetrix, Santa Clara, CA, U.S.; P/N 900652) and hybridized to Affymetrix Mouse Genome 430 2.0 arrays (Affymetrix). Quality control and data analysis pipeline have been described in detail previously (5). Briefly, normalized expression estimates of probe sets were computed by the robust multi-array analysis (RMA) algorithms implemented in the Bioconductor library AffyPLM (6). Probe sets were redefined using current genome information according to Dai et al (7) based on annotations provided by the Entrez Gene database, which resulted in the profiling of 18,075 unique genes (custom CDF v21). Genes that differed more than 1.3 fold between IL-18/- and WT, or between IL-18 and PBS treatment were selected for subsequent analysis. A second dataset consisting of raw expression data of livers of WT and IL-18/- mice of 12 weeks of age were extracted from the Gene Expression Omnibus (GSE64309) (8). Data was background corrected using the normexp+offset method (9) followed by quantile normalized using functions in the library limma (10). Next differentially expressed genes were identified by using linear models and an intensity-based moderated t-statistic. Probe sets that satisfied the criterion of P < 0.05 were considered to be regulated. To identify IL-18 regulated genes, both datasets were combined using a FC > 1.3 and a P-value < 0.05 as selection criteria. Pooled samples of the 4 study groups (male mice (10-14 weeks of age): WT, IL-18/-, and female mice (10-14 weeks of age): WT-PBS, WT-recombinant IL18 (18hrs of treatment) were subjected to expression profiling by microarray. To this end, purified total RNA of 2 (WT vs. IL18/-) or 4 (WT-PBS vs. WT-rIL18) liver samples per study group was pooled and labelled with the Affymetrix GeneChip RNA One cycle Amplification Kit (Affymetrix, Santa Clara, CA, U.S.; P/N 900652) and hybridized to Affymetrix Mouse Genome 430 2.0 arrays (Affymetrix). Quality control and data analysis pipeline have been described in detail previously (5). Briefly, normalized expression estimates of probe sets were computed by the robust multiarray analysis (RMA) algorithm as implemented in the Bioconductor library AffyPLM (6). Probe sets were redefined using current genome information according to Dai et al. (7) based on annotations provided by the Entrez Gene database, which resulted in the profiling of 18,075 unique genes (custom CDF v21). Genes that differed more than 1.3 fold between IL18/- versus WT, or PBS versus recombinant IL-18 treatment were selected for subsequent analysis.

Raw expression data of livers from wild-type and IL18/- male mice of 12 weeks of age were extracted from the Gene Expression Omnibus (GSE64309) (8). Data was background corrected using the normexp+offset method (9) followed by quantile normalized using functions in the library limma (10, 11). Next, differentially expressed genes were identified by using linear models and an intensity-based moderated t-statistic (10, 11). Probesets that satisfied the criterion of moderated P < 0.05 were considered to be regulated. Results from our and the published dataset were compared as follows: all genes that were differentially expressed (moderated P < 0.05) in livers of the 12 weeks-old IL18/- mice (GSE64309) were selected. Next, for these 52 genes, the log2FC were retrieved from our datasets and together with the log2FC of the published dataset visualized in a heatmap. The colors in the heatmap directly correspond to the log2FC values and no row-scaling was applied.
Statistics
Regression analysis was performed using the “Rfit” package, as implemented in the “R” language. Rfit is a regression method that is more robust to outliers in response space than the standard linear models. Depending on the analyses, different factors were included as covariates to adjust for the effect of e.g. age, gender, BMI, smoking, drug usage and season. P-values are within Rfit calculated using the t-statistic, which is the ratio between the estimate and it’s standard error. The p-value is based on a t distribution with n-p-1 degrees of freedom where p is the number of regression parameters. Seasonality was corrected using the method described in Ter Horst et al. (12)

Supplementary References
Supplemental Figure 1 – *IL-18BP is independently associated with maximum plaque thickness.* IL-18BP was in an univariate analysis associated with maximum plaque thickness. In model 1, after adjustment for age, sex and smoking (pack years). In model 2, after additional adjustment for the classical cardiovascular risk factors (systolic blood pressure, glucose level, HDL and LDL cholesterol, lipid lowering, antihypertensive and antidiabetic drugs) IL-18BP remained significantly associated. However in model 3, after additional adjustment for triglycerides and VLDL cholesterol, the relation disappeared.
Supplemental Figure 2 - Unadjusted relation between all lipoprotein groups, IL-18BP, IL-18, CRP and IL-6. IL-18 and IL-18 binding protein show the strongest positive spearman correlations with the small to large VLDL particles. Next to that they show strong positive associations with apoB levels and with the triglycerides in extremely large HDL. Both IL-18BP and IL-18 are negatively associated with HDL-cholesterol. Neither CRP nor IL-6 showed a significant association.

XXL VLDL Chylomicrons and extremely large VLDL particles; XL Very large; L. Large; M. Medium; S. Small; XS. Extreme small; VLDL Very Low Density Lipoproteins; IDL Intermediate Density Lipoproteins; LDL Low Density Lipoproteins; HDL High Density Lipoproteins; L Total lipids; TG Triglycerides; PL Phospholipids; D Mean diameter; Serum.C Serum cholesterol; ApoB Apolipoprotein B; HDL3.C Total cholesterol in HDL3; ApoA1 Apolipoprotein A1; IL-18BP Interleukin-18 binding protein; IL-18 Interleukin-18; CRP C-reactive protein; IL-6 Interleukin-6.
Supplemental Figure 3 - Relation of lipids in HDL groups with IL-18BP, IL-18, CRP and IL-6.

Heatmap with relation between lipids in HDL groups, group 9, 11 and 13 of the metabolites, and IL-18BP, IL-18, CRP and IL-6.

L. Large; M. Medium; S. Small; HDL High Density Lipoproteins; .P Concentration of particles; .L Total lipids; .PL Phospholipids; .C Total cholesterol; .CE Cholesterol esters; .FC Free cholesterol; .TG Triglycerides; .D Mean diameter; Serum.C Serum cholesterol; HDL2.C; IL-18BP Interleukin-18 binding protein; IL-18 Interleukin-18; CRP C-reactive protein; IL-6 Interleukin-6.
Supplemental Figure 4 – Relation of HDL triglycerides with IL-18BP, IL-18, CRP and IL-6.

XL. Very large; L. Large; M. Medium; S. Small; XS. Extreme small; HDL High Density Lipoproteins; IL-18BP Interleukin-18 binding protein; IL-18 Interleukin-18; CRP C-reactive protein; IL-6 Interleukin-6.
Supplemental Figure 5 - Relation between all lipoprotein groups, IL-18BP, IL-18, CRP and IL-6 adjusted for influencing factors. (A) Heatmap with the correlation between the lipoprotein group, IL-18binding protein, IL-18, hsCRP and IL-6, adjusted for age, sex, smoking and season. (B) With additional adjustment for BMI.

XXL.VLDL Chylomicrons and extremely large VLDL particles; XL. Very large; L. Large; M. Medium; S. Small; XS. Extreme small; VLDL Very Low Density Lipoproteins; IDL Intermediate Density Lipoproteins; LDL Low Density Lipoproteins; HDL High Density Lipoproteins; .L Total lipids; .TG Triglycerides; .PL Phospholipids; .D Mean diameter; Serum.C Serum cholesterol; ApoB Apolipoprotein B; HDL3.C Total cholesterol in HDL3; ApoA1 Apolipoprotein A1; IL-18BP Interleukin-18 binding protein; IL-18 Interleukin-18; CRP C-reactive protein; IL-6 Interleukin-6.
Supplemental Figure 6 - Unadjusted relation between all lipoprotein metabolites and atherosclerosis markers. Relation between all lipoprotein metabolites with the mean intima-medial thickness of both carotid arteries and the maximum plaque thickness assessed by carotid ultrasound.

XXL. VLDL Chylomicrons and extremely large VLDL particles; XL. Very large; L. Large; M. Medium; S. Small; XS. Extreme small; VLDL Very Low Density Lipoproteins; IDL Intermediate Density Lipoproteins; LDL Low Density Lipoproteins; HDL High Density Lipoproteins; .L Total lipids; .TG Triglycerides; .PL Phospholipids; .D Mean diameter; .Serum.C Serum cholesterol; ApoB Apolipoprotein B; HDL3.C Total cholesterol in HDL3; ApoA1 Apolipoprotein A1; cIMT carotid intima-medial thickness; maxPlaqueThickness maximum plaque thickness in millimeter.
Supplemental Figure 7 - Relation of lipids in HDL groups with atherosclerosis markers.
Heatmap with relation between lipids in HDL groups, group 7, 9, 11, 13 and 14 of the metabolites, and the mean intima-medial thickness of both carotid arteries and the maximum plaque thickness assessed by carotid ultrasound.
L. Large; M. Medium; S. Small; HDL High Density Lipoproteins; .P Concentration of particles; .L Total lipids; .PL Phospholipids; .C Total cholesterol; .CE Cholesterol esters; .FC Free cholesterol; .TG Triglycerides; .D Mean diameter; Serum.C Serum cholesterol; HDL2.C; IMT intima-medial thickness; maxPlaqueThickness maximum plaque thickness in millimeter.
Supplemental Figure 8 - Relation of triglycerides in large HDL particles with atherosclerosis markers. Triglycerides in extremely large and medium HDL particles, group 8 and 12 of the metabolites respectively, are positively associated with maximum plaque thickness assessed by carotid ultrasound.

L. Large; HDL High Density Lipoproteins; TG Triglycerides; IMT intima-medial thickness; maxPlaqueThickness maximum plaque thickness in millimeter.
Supplemental Figure 9 - Relation between all lipoprotein groups and atherosclerosis markers, adjusted for influencing factors. (A) Heatmap with the correlation between the lipoprotein group, the mean intima-medial thickness of both carotid arteries and the maximum plaque thickness assessed by carotid ultrasound adjusted for age, sex, smoking and season. (B) With additional adjustment for BMI.

XXL: VLDL Chylomicrons and extremely large VLDL particles; XL: Very large; L: Large; M: Medium; S: Small; XS: Extreme small; VLDL: Very Low Density Lipoproteins; IDL: Intermediate Density Lipoproteins; LDL: Low Density Lipoproteins; HDL: High Density Lipoproteins; .L: Total lipids; .TG: Triglycerides; .PL: Phospholipids; .D: Mean diameter; Serum.C: Serum cholesterol; ApoB: Apolipoprotein B; HDL3.C: Total cholesterol in HDL3; ApoA1: Apolipoprotein A1; cIMT: Carotid intima-medial thickness; maxPlaqueThickness: Maximum plaque thickness in millimeter.
CHAPTER 6

Gut Microbial Associations to Plasma Metabolites Linked to Cardiovascular Phenotypes and Risk: A Cross-Sectional Study


* Contributed equally

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ABSTRACT

Rationale
Altered gut microbial composition has been linked to cardiovascular diseases (CVD), but its functional links to host metabolism and immunity in relation to CVD development remain unclear.

Objectives
To systematically assess functional links between the microbiome and the plasma metabolome, cardiometabolic phenotypes and CVD risk and to identify diet-microbe-metabolism-immune interactions in well-documented cohorts.

Methods and Results
We assessed metagenomics-based microbial associations between 231 plasma metabolites and microbial species and pathways in the population-based Lifelines-DEEP cohort (n = 978) and a clinical obesity cohort (n = 297). After correcting for age, gender and BMI, the gut microbiome could explain up to 11.1% and 13.4% of the variation in plasma metabolites in the population-based and obesity cohorts, respectively. Obese-specific microbial associations were found for lipid compositions in the VLDL, IDL and LDL lipoprotein subclasses. Bacterial L-methionine biosynthesis and a Ruminococcus species were associated to cardiovascular phenotypes in individuals with obesity, namely atherosclerosis and liver fat content, respectively. Integration of microbiome-diet-inflammation analysis in relation to metabolic risk score of CVD in the population cohort revealed 48 microbial pathways associated to CVD risk that were largely independent of diet and inflammation. Our data also showed that plasma levels rather than fecal levels of short chain fatty acids were relevant to inflammation and CVD risk.

Conclusions
This study presents the largest metagenome-based association study on plasma metabolism and microbiome relevance to diet, inflammation, CVD risk and cardiometabolic phenotypes in both population-based and clinical obesity cohorts. Our findings identified novel bacterial species and pathways that associated to specific lipoprotein subclasses and revealed functional links between the gut microbiome and host health that provide a basis for developing microbiome-targeted therapy for disease prevention and treatment.
INTRODUCTION

The human gut is colonized by a highly complex community of microorganisms called the microbiome. The gut microbiome interacts closely with the host and is involved in digestion and degradation of nutrients, maintenance of digestive tract integrity, stimulation of the immune system and modulation of the host metabolism (1,2). Recent studies indicate a strong link between the gut microbiota and the development of various human diseases, including obesity (3,4), insulin resistance and type 2 diabetes (5,6), as well as gastrointestinal (7,8), autoimmune (9–11), and cardiovascular (12,13) disorders (CVD). Various studies have provided evidence that host-microbe interactions contribute to the etiology of many of these diseases through their impact on metabolism and immunity. Lower bacterial richness (a reduction in the number of different species or bacterial genes) has been associated with an overall increase in adiposity, insulin resistance, dyslipidemia and inflammatory phenotypes (3). The distinct gut microbiome profile found in overweight individuals has been shown to have an increased capacity to harvest nutrients from food (14). Moreover, the gut microbiome is also associated with an individual’s cytokine production capacity in response to different pathogens (15). Despite a large body of evidence from cross-sectional association studies, the underlying mechanisms are largely unknown and several putative mechanisms and functional links have been proposed. For instance, the impact of the gut microbiome on insulin sensitivity and glucose homeostasis may be mediated via microbial biosynthesis of branched-chain amino acids (5), short-chain fatty acids (SCFAs) and N-acyl amides (16,17). The fermentation products of dietary fibers, in particular SCFAs, also have potential roles in the host’s innate and adaptive immunity through modulation of cell proliferation and differentiation (18,19), hormone secretion (20), G protein-coupled receptor activation and regulation of colonic Treg cell homeostasis (21–23). Finally, inhibition of gut-microbiome-induced trimethylamine-N-oxide (TMAO) production can attenuate atherosclerosis development in mice (12,24).

However, our understanding of the functions of gut microbes and of diet-microbe-metabolism-immune interactions in CVD remains limited, leaving a knowledge gap that greatly delays clinical translation. Evaluating the complex interactions between the gut microbiome, host metabolism and immune system—as affected by intrinsic host and external factors (diet, medication)—requires multi-omics, systems-biology-based approaches (25).

To do so, we examined both a population-representative and an overweight patient cohort, collectively comprising 1,275 individuals. The obese cohort was deeply phenotyped for cardiometabolic traits, fat distribution and plasma level of TMAO, while the population-based cohort was deeply phenotyped for inflammation, diet and SCFAs (Figure 1A). We first aimed to identify microbial species and metabolic pathways associated with plasma metabolite profiles (Fig 1B). We then identified the relevance of these metabolism-related microbial factors to cardiometabolic phenotypes in the obese patient cohort. Finally, we assessed individual metabolic risk of CVD in the population-based cohort, and identified bacterial pathways associated to the CVD risk score and assessed the diet-microbe-metabolism-immune interactions in CVD risk (Fig 1B).
Figure 1 – Study overview. A. This study included a population-representative cohort (LLD) with an average BMI of 25 kg/m² and a cohort of individuals with overweight and obesity (300-OB study) with an average BMI of 31 kg/m². In addition to metagenomics-sequencing data and plasma metabolomics generated for all individuals, unique phenotypic information was collected in each cohort. In the population-representative LLD, detailed lifestyle and phenotypic information was collected, including 78 dietary factors, 12 inflammatory markers and stool levels of 5 short-chain fatty acids (SCFAs). In the 300-OB study, detailed cardiometabolic phenotyping was conducted, including assessment of carotid artery plaques and the amount of subcutaneous and visceral adipose tissue and liver fat measured using magnetic resonance imaging. B. Analysis scheme. The whole analysis can be divided into four steps: 1) a metagenome-wide association study (MWAS) to explore pair-wise association between 231 metabolic traits and metagenomics-based 188 species and 562 pathways in LLD and 300-OB, respectively, 2) an MWAS to assess the relevance of metabolome-associated microbial features to cardiometabolic phenotypes in the 300-OB cohort, 3) an MWAS to identify microbial factors associated with the metabolic risk score (MRS) of CVD in LLD, which was constructed using 33 metabolic biomarkers, and 4) an integration analysis to assess the relevance of 78 dietary factors, inflammatory cytokines, and SCFAs in microbial association of MRS in LLD.

METHODS

All metagenomics and metabolism data have been made publically available at the European Genomics-phenome Archive (EGA) at accession number EGAS00001001704 for the LifeLines-DEEP cohort and EGAS00001003508 for the 300-Obese cohort. Because of the sensitive nature of clinic data collected for this study, requests to access clinical phenotypic data of the Lifelines-DEEP cohort and 300-Obese cohort may be sent to the LifeLines cohort study at research@lifelines.nl and to the Human Functional Genomics project at martin.jaeger@radboudumc.nl, respectively.

Cohorts
LifeLines-DEEP cohort (LLD)
LLD is a sub-cohort of the large, prospective, population-based LifeLines cohort (167,729 subjects) (26). A subset of 1,539 participants with deep omics profiling makes up LLD (13,27).
Participants volunteered to participate in LLD from April to August 2013, with blood and fecal samples collected in the same period (within 2 weeks). High quality metagenomic data and detailed dietary (78 dietary factors) information are available for 1,135 LLD participants (13), and 1,046 LLD individuals were profiled for plasma metabolites and inflammation data (see sections below). We further excluded 57 participants who were taking antibiotics or lipid-lowering medication and 11 non-fasting subjects. This left 978 subjects (411 males and 567 females) for further analyses.

**300-Obesity cohort (300-OB)**

Between 2014 and 2016, 302 individuals aged 55 to 80 years were enrolled in the 300-OB study at the Radboud University Medical Center (RUMC), Nijmegen, the Netherlands. All had a body mass index (BMI) > 27 kg/m², and the majority (n = 227) had participated in the Nijmegen Biomedical Study–Non-Invasive Measurements of Atherosclerosis 1 (NBS-NIMA1) study, a population-based survey of Nijmegen residents (28). We recruited another 75 participants, acquaintances of previously-included subjects, who fulfilled the inclusion criteria of age > 55 years and BMI > 27 kg/m². Most of these new participants were unrelated subjects, with only nine being family members of previously-included subjects; we therefore did not separately evaluate or incorporate the potential clustering of subjects. Subjects with a recent cardiovascular event (myocardial infarction, transient ischemic attack, stroke < 6 months), a history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral or subcutaneous anti-coagulant therapy, use of thrombocyte aggregation inhibitors other than acetylsalicylic acid and carbasalate calcium, or a contra-indication for magnetic resonance imaging (MRI) were excluded from the study. Participants who used lipid-lowering therapy temporarily discontinued this medication 4 weeks prior to the measurements. All women were postmenopausal and did not use hormonal replacement therapy. All subjects completed questionnaires about lifestyle, medication use and previous diagnosis of hypertension and diabetes. For all participants, blood samples for metabolomics analysis were collected in the morning following an overnight fast. All underwent comprehensive assessment of cardiovascular profile and fat distribution, as detailed below. Five samples of metagenomic data failed to pass the quality control, leaving 297 individuals for further analyses.

**Cardiovascular phenotyping in the 300-OB cohort**

Cardiovascular assessment was performed at Radboud UMC. Vascular studies included the measurement of carotid intima-medial thickness (cIMT), carotid plaque presence and maximum plaque thickness. Measurements were performed after an overnight fast or in the afternoon 6 hours after a standardized breakfast. Participants were asked to abstain from caffeinated products for at least 12 hours and to not smoke for 12 hours before the visit. Testing was performed in a quiet temperature-controlled room with patients in supine position. After a resting period of at least 30 minutes, baseline resting diameter, distensibility and wall thickness of the carotid artery were assessed by a well-trained sonographer using a
7.5-MHz transducer of a Mylab Class C ultrasound device (Esaote Biomedica, Genoa, Italy) connected to a computer with a data acquisition board (Art.lab, Esaote Europe BV, Maastricht, Netherlands). Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. The cIMT and diameter measurements were performed in the proximal 1 cm straight portion of the carotid artery in three different angles (90°, 120° and 180°) for 6 heartbeats. Measurements were recorded during the diastolic phase. Measurement of cIMT was performed using an automatic boundary detection system based on RF processing-based measurement (Art.lab) (29). The primary outcome variable was defined as the mean cIMT of the different angles (30).

Subsequently, the presence of plaque and the maximum thickness of plaques in the common carotid, internal carotid, external carotid artery and at the carotid bulb were measured. Presence of plaque was defined as focal thickening of the wall of at least 1.5x the mean IMT or an IMT > 1.5mm, according to the Mannheim intima-media thickness consensus (31).

Furthermore, fat distribution was assessed using MRI, including volumes of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT), divided into deep and superficial subcutaneous adipose tissue (dSAT and sSAT), respectively. Hepatic fat content was quantified using localized proton magnetic resonance spectroscopy (1H-MRS) (detailed methods see online supplementary material).

**Microbiome data profiling**

*Metagenomic shotgun sequencing*

High quality metagenomics data were already available for the 1,135 LLD participants (13). For this study, we performed metagenomic sequencing of the 300-OB cohort using a similar protocol and analysis pipeline. In brief, fecal and blood samples were collected within 2 weeks for LLD participants and within 1-2 days for 300-OB participants in order to reduce potential bias introduced by sampling. Further processing of all sample sets was identical to LLD. In brief, DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany; cat. #80204) with the addition of mechanical lysis. Metagenomic shotgun sequencing was performed using the Illumina HiSeq platform (Illumina, San Diego, California), with an average of 3.0 Gb data (around 32.3 million reads) obtained per sample. Reads were quality-filtered using our in-house pipeline. Sequencing adapters were removed using Trimmomatic (v.0.32) (32). Human reads were removed by mapping the data to the human reference genome (version NCBI37) with Bowtie2 (v.2.1.0).

*Identifying microbial taxa abundances*

The profile of microbial composition was determined using MetaPhlan 2.2 (33), and it reported 1,772 microbial taxonomies in our data. We normalized the taxonomy data using log- and inverse rank sum transformation and further corrected for age and sex with linear regression. We confined the analysis to the 188 common microbial species (online supplementary material Table 1) present in > 10% of samples.
Identifying abundances of bacterial metabolic pathways

The abundance of metabolic pathways was determined using HUMAnN2 (http://huttenhower.sph.harvard.edu/humann2), which maps reads to a customized database of functionally annotated pan-genomes. This analysis mapped reads to UniProt Reference Clusters (UniRef50, http://www.uniprot.org), then further grouped them to 773 pathways from the MetaCyc metabolic pathway database (www.metacyc.org). Only pathways present in > 25% of samples (562 pathways, online supplementary material Table 2) were used in our downstream analysis. For the non-zero gene counts per MetaCyc pathway, we performed log- and inverse rank sum transformations, followed by correction for the effects of age and sex using linear regression.

Plasma metabolome profiling

For both cohorts, we profiled a wide range of plasma metabolites using nuclear magnetic resonance (NMR) and Nightingale’s Biomarker Analysis Platform (34). This platform provides measures of 231 plasma metabolome traits (online supplementary material Table 3), including total lipid concentrations and relative compositions of 14 lipoprotein subclasses, lipoprotein particle sizes, apolipoproteins, cholesterol, glycerides and phospholipid concentrations, various glycolysis components, fatty acid composition, inflammation, fluid balance, ketone bodies and amino acids. The NMR metabolomics platform has recently been used in several epidemiological, genome-wide association and functional genetic studies (35–37). To further validate platform precision, we compared NMR measures of several traits with corresponding routine lipid measurements, including concentrations of HDL, LDL, triglycerides and total cholesterol (supplementary material Figure 1). For these traits we observed very high correlation rates (R > 0.89), in agreement with earlier platform validation results (37). There were ~2.2% missing values across all data. Given the high correlation structure of metabolites, missing values could be imputed using the Principal Components Imputation method implemented in the “missMDA” package for R, using the first 10 principal components. Prior to microbiome-metabolome association analysis, due to non-linear dependency of metabolomic traits to covariates, we used locally weighted scatterplot smoothing (LOESS) to correct for sex-dependent age and BMI effects.

Adipokine and cytokine profiling in LLD

The panel of cytokines for the LLD cohorts (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α) was measured by ProcartaPlexTM multiplex immunoassay (eBioscience, San Diego, California, USA) according to protocols described before (38,39). Other inflammation markers were measured by using commercially available sandwich ELISA kits (R&D systems, Minneapolis, Minnesota, USA), including leptin, adiponectin, IL-18, IL-18BP, resistin and alpha-1 antitrypsin (AAT).
SCFA profiling in LLD
For LLD, we measured fecal levels of acetate, propionate, butyrate, valerate and caproate by gas chromatography-mass spectrometry following the method of García-Villalba et al (2012) (40). The abundance of acetate in plasma was obtained through plasma metabolome profiling using NMR. All SCFA measurements were corrected for age and gender using LOESS.

TMAO, choline, betaine and citrulline profiling in 300-OB
TMAO, choline, L-carnitine, betaine and γ-butyrobetaine in plasma were analyzed by ultra-high performance liquid chromatography in combination with isotope dilution tandem mass spectrometry (UPLC-MS/MS). In short, 25μL plasma was pipetted into 96-well plates, 25μL internal standard solution was added (containing TMAO-D9, choline-D9, L-carnitine-D3 and betaine-D11), followed by 300μL 80% acetonitrile (ACN) and 1% formic acid (FA) in Millipore water. Mass spectrometric detection was performed on a XEVO TQ-s system (Waters). Analytes were detected in positive mode and selected reaction monitoring mode. The respective quantifier ion transitions were as follows: m/z 76.15 > 58.3 for TMAO, m/z 104.2 > 60.3 for choline, m/z 162.2 > 103.25 for L-carnitine, m/z 118.2 > 59.3 for betaine and m/z 146.25 > 60.3 for γ-butyrobetaine. All analytes were baseline separated from each other.

Statistical analysis
Microbiome-metabolome association and explained variance
(a) To identify the associations between metabolic and microbial factors, association analyses were performed in both LLD and 300-OB. Microbiome features included both microbial pathways and species. After adjusting for age, sex, BMI and smoking, Spearman correlation of metabolic traits and microbiome features was used to identify associations. The analysis was confined to pair-wise non-zero values. False discovery rate (FDR) was estimated using 100x permutations. We also calculated the permutation-based family-wise error rate (FWER) and report the top association at FWER < 0.05 level. (b) To estimate the proportion of variation in plasma metabolism explained by microbial factors, we used a LASSO shrinkage model (41) that included all identified microbial pathways and species as predictors. The independent and most dominant microbial features were selected automatically, and the variation of each metabolite explained by the selected microbial factors was then estimated by LASSO. Moreover, we used the same LASSO algorithm to estimate the proportion of metabolic variation in 300-OB explained by microbial factors identified in LLD.

Association to cardiometabolic phenotypes
The bacterial pathways and species identified were further tested for association with cardiometabolic phenotypes in 300-OB, including fat distribution and atherosclerosis phenotypes. The analysis was performed using linear regression, with cardiometabolic phenotype as outcome and microbiome feature as predictor, and treating age, sex and BMI as covariates. Quantitative outcomes were adjusted using inverse rank-sum transformation; for binary outcomes, logistic regression was used instead of linear regression. Furthermore,
to assess to what extent bacterial-derived TMAO and its relevant metabolites could underlie microbial associations to cardiometabolic phenotypes, we performed extra analysis with adjustment for the plasma levels of TMAO, choline, betaine and L-carnitine. Significant associations for each phenotypic trait are reported at empirical FDR < 0.05 level based on 100x permutations.

**Microbiome association to metabolic risk of CVD**

To estimate individual metabolic risk for CVD development in the population cohort, we used 33 established metabolic biomarkers for CVD measured using the same NMR platform and associated with future CVD incidents in three perspective cohorts (36). We first constructed each individual’s CVD metabolic risk score (MRS) using a weighted risk model:

\[ MRS = \sum_{i=1}^{33} b_i M_i \]

where \( M_i \) is the scaled level of the \( i \)-th metabolic marker in serum, not adjusted for phenotypes, and \( b_i \) is a hazard ratio for the corresponding effect of each metabolic marker on the CVD risk as reported by Würtz et al (36). The MRS score showed a normal distribution. We then tested MRS association to microbial pathways and species, adjusting for age, sex, BMI and smoking. The significance was controlled at FDR < 0.05 based on 100x permutations. For microbiome pathways, we also examined to what extent these pathways are driven by specific taxa by calculating Spearman correlations between pathways abundance and taxa abundance.

**Integration association with dietary factors, inflammatory markers and stool levels of SCFAs**

To better understand the functional properties of the MRS-associated bacterial pathways in relation to metabolism, inflammation, diet and SCFAs, we conducted an integration analysis with 12 inflammatory markers, 78 dietary factors and stool levels of 5 SCFAs. First, we computed pair-wise associations between MRS-associated pathways and each of these factors, using linear regression adjusted for age, sex, BMI and smoking and controlling for FDR 0.05 using 100x permutations per dataset (cytokines, diet, SCFA) separately. All traits were transformed using inverse rank sum transformation prior to analysis. Second, to elaborate if pathway-cytokine associations were dependent on MRS, we recalculated these associations using linear regression, additionally adjusting for MRS as a covariate. Finally, to explore if the microbial pathways were associated to variance of MRS independently of inflammatory markers and diet, we performed stepwise model selection for each pathway, with MRS as outcome and pathway, age, sex, BMI, smoking, all pathway-associated cytokines and all pathway-associated diet categories as predictors. This was done using the stepAIC function from R package MASS. At each selection step, predictors were selected by both forward and backward direction using AIC value (Akaike information criterion) as an indicator of goodness-of-fit. The model with the highest AIC was selected as the best model. Pathways that survived in the best-fit model were considered independent predictors.
RESULTS

Gut microbiome associated with plasma metabolomics in healthy individuals and individuals with obesity

This study included 1,275 individuals from two independent Dutch cohorts: 978 subjects from the population-based cohort LifeLines-DEEP in the Northern part of the Netherlands (the provinces of Groningen, Drenthe and Friesland), with an average age of 44.5 years (18-81 years), an average BMI of 25 (16-45) and 42% male and 297 individuals were from the 300-OB cohort on the territory of Nijmegen province, with an average age of 67 (54-81 years), an average BMI of 30 (26.3-45.5) and 55% male. Both the LLD and 300OB cohorts followed cohort-specific, disease- and drug-specific exclusion criteria (see Methods). We measured both serum metabolomics and gut metagenomics profiles in both cohorts (Figure 1A). After quality check and imputation of ~2.2% missing values, a total of 188 microbial species (online supplementary material Table 1), 562 bacterial metabolic pathways (online supplementary material Table 2) and 231 metabolic traits (online supplementary material Table 3) were subjected to association analysis. After correcting for age, gender and BMI, microbial associations were detected for 210 metabolites in LLD at FDR 0.05 level, with 64 associations to 12 species and 4,135 associations to 308 pathways. To evaluate if imputation of missing values in the metabolite data (see Methods) introduced any systematic bias, we re-conducted association analyses by removing missing values following two approaches, and found concordant results (supplementary material Figure 2). For instance, after removing missing values, 3,952 significant associations were detected at FDR 0.05 level, and 3,597 of these overlapped with 4,135 associations revealed by missing values imputation. In the 300-OB cohort, microbial associations were detected for 35 metabolites, with three associations to one species and 72 associations to 16 pathways. All significant associations at FDR < 0.05 level can be downloaded from https://github.com/alexa-kur/NMR_microbiome. Most of the microbial factors identified showed very modest effects and could jointly explain, on average, 3.7% of the variation in LLD and 7.7% of the variation in 300-OB (Figure 2). The highest levels of variation explained were 11.1% for glycoprotein N-acetylgs (Gp) in LLD and 13.4% for IDL_C_percent in 300-OB. The smaller number of associations in 300-OB was likely due to its smaller sample size, but our data also show that the microbial factors identified in LLD generally had lower predictive value for metabolic variation in 300-OB (Figure 2). This indicates there are some genuine differences in microbial associations between the population and the obese cohort. We further compared association strength and directions for the top associations from both cohorts (supplementary material Figure 3 and 4), including 17 associations to microbial species (online supplementary material Table 4) and 297 associations to pathways (online supplementary material Table 5) at FWER 0.05 level. We observed some obese-specific associations, particularly for the relative composition of lipoprotein subclasses. For instance, we found significant associations of Ruminococcus species sp_5_1_39BFAA to XXL-VLDL_PL_percent (r = 0.31, P = 2.3x10^{-7}) and XL_VDL_CE_percent (r = -0.29, P = 1.25x10^{-6}) in 300-OB that were completely absent in LLD (P > 0.05), even with its much larger sample size (online supplementary material Table 4). We
also observed several opposite associations between LLD and 300-OB. For instance, the pathway of pyruvate fermentation to acetate and lactate (PWY-5100) was positively associated to S_VLDL_C_percent in LLD ($r = 0.15$, $P = 1.35 \times 10^{-6}$), yet showed a negative association in 300-OB ($r = -0.2$, $P = 4.3 \times 10^{-4}$) (online supplementary material Table 5).
Figure 2 – Variation of plasma metabolites explained by the identified gut microbiome. Bar plot shows the variation of 231 metabolites explained by identified microbial factors in LLD (left) and in 300-OB (right). Each bar represents one metabolite. Bar color indicates type of metabolite according to the color key at right. We also assessed to what extent the microbial factors identified in the LLD cohort could explain the metabolic variation in the 300-OB cohort. This data is shown by the gray bars on the right.

Metabolism-related microbial factors linked to clinical phenotypes in subjects with obesity

To investigate whether the microbial factors found to be associated with plasma metabolites were related to end-point phenotypes, we focused on the top associated species and pathways from both cohorts (online supplementary material Table 4-5) and further tested their associations to 27 cardiometabolic phenotypes in the 300-OB cohort (Figure 1B). After correcting for age, sex and BMI, two microbial factors were identified with FDR < 0.05 (online supplementary material Table 6): higher abundance of bacterial L-methionine biosynthesis (HSERMETANA-PWY) was significantly associated with the presence of plaque (P = 0.001) and Ruminococcus sp_5_1_39BFAA was positively associated to hepatic fat content (Liver-fat) (r = 0.21, P = 5.0x10^-4) (Figure 3). Previous studies have suggested that gut-microbiome-derived TMAO can increase CVD risk. We observed that plasma level of TMAO was positively associated to visceral fat (r = 0.167, P = 0.002) but not to atherosclerosis phenotypes and hepatic fat (online supplementary material Table 7), nor was it associated to metabolite-associated species and bacterial pathways (online supplementary material Table 8). After correcting for TMAO and its related metabolites (L-carnitine, choline and betaine), associations of L-methionine biosynthesis and Ruminococcus sp_5_1_39BFAA to cardiometabolic phenotypes remained similar (online supplementary material Table 6).

A. Abundance of the L-methionine synthesis pathway (HSERMETANA-PWY) is significantly higher in individuals with carotid atherosclerotic plaques. B. Ruminococcus sp_5_1_39BFAA is significantly correlated with liver fat content measured by magnetic resonance spectroscopy. Each dot presents one individual and the fitted line is shown in black.
Microbial pathways linked to metabolic risk of CVD in the LLD general population cohort

We further assessed the association of the gut microbiome to individual MRSs of CVD in LLD (Figure 1B). To do so, we constructed CVD MRSs using 33 established CVD metabolic biomarkers that have been associated with future CVD incidents and that were independent of other known risk factors like age, gender, BMI and smoking status (36). At FDR 0.05 level, 48 associations of MRS with microbial pathways were detected (Figure 4, online supplementary material Table 9).

Figure 4 - Association of 48 microbial pathways with metabolic risk score of CVD, SCFAs in stool, cytokines and dietary factors. Bar plot on the right shows the associations of 48 microbial metabolic pathways with MRS of CVD. Y-axis refers to the association strength in terms of beta-value. Red bars indicate positive associations and blue bars indicate negative associations. The 3-panel heatmap on the left shows the associations of MRS-associated pathways with plasma levels of cytokines, stool levels of SCFAs and dietary factors, respectively. Blue cells indicate negative associations. Red cells indicate positive associations. White stars indicate significance at FDR < 0.05. The color key at the bottom shows association strength and direction in terms of t-value.
The top associated pathways were related to GDP-mannose biosynthesis (PWY-7323), which was negatively associated with CVD MRS (beta = -0.122, P = 7.5x10^{-6}). Interestingly, a large number of associated pathways were involved in amino acid (AA) metabolism, including metabolism of glutamate-family AAs (L-proline, L-arginine, L-histidine, L-histidine), branched-chain AAs (L-valine), hydrophobic AAs (L-threonine), aromatic AAs (L-phenylalanine and L-tyrosine) and sulfur-containing AAs (L-methionine). Most AA pathways were associated to lower MRS score, except for positive associations detected for L-methionine and L-threonine. Other associated pathways were mostly involved in, among others, fermentation, carbohydrates and sugar derivatives metabolism (online supplementary material Table 9). We also assessed to what extent specific bacterial taxa can drive MRS-related microbial pathways. For this purpose, we identified the top taxon for each pathway that showed the strongest association between the abundances of the taxon and the pathway (online supplementary material Table 9). What we found is that the relative contribution of the top taxa varied greatly: the correlation coefficients between top taxa and pathways ranged from 0.26 to 0.89, with an average value of 0.60. This suggests that some pathways are driven by one dominant bacterial player, while others may be attributed to many different players. For instance, phylum Bacteroidetes—including class Bacteroida and families Bacteroidaceae and Rikenellaceae—is the major player in 17 of 31 lower-MRS-associated pathways, in particular GDP-mannose biosynthesis and glutamate-family AAs (r > 0.8). In contrast, the top players in L-methionine metabolism, the Ruminococcus genus and Actinobacteria phylum, only contributed a very modest effect (online supplementary material Table 9).

The linkage of MRS-related pathways to inflammation and diet

To gain deeper insight into the contribution of host-microbe-diet interactions to metabolism and inflammation, which both underlie susceptibility to CVD, we conducted a systematic integration analysis between the 48 MRS-associated bacterial pathways and the plasma level of 12 cytokines (as a read-out of low-grade inflammation, online supplementary material Table 10) and 78 dietary factors (see Methods). After adjustment for age, sex and BMI, 14 associations between 12 pathways and 5 cytokines were significant at FDR 0.05 level (Figure 4, online supplementary material Table 10). The associations detected were also largely independent of MRS and remained significant after adjusting for MRS (online supplementary material Table 11). Most associations were found to interleukins members, namely 8 associations to IL-10, 3 associations to IL-6, one to IL-12P70 and one to IL-18BP. Elevated levels of these interleukin members have previously been linked to increased risk of CVD (42). The pathways associated with these interleukins were related to bacterial AA biosynthesis (proline, ornithine, threonine, citrulline, tyrosine, arginine), although IL-10 and IL-18BP were also associated to GDP-mannose metabolism glycolysis and homolactic fermentation. Moreover, the bacterial glycolysis pathway (GLYCOYSIS) was positively associated to plasma level of adiponectin, which is known to be involved in glucose metabolism regulation. This finding shows a possible interaction between the host and the gut microbiome in glucose metabolism.
Diet is known to be an important factor that affects metabolism, CVD risk and the gut microbiome. Among 78 dietary factors, 34 were associated to MRS-associated microbial pathways at FDR 0.05 level, after adjustment for age, sex and BMI and smoking (online supplementary material Table 12). The dietary factors linked with lower-CVD-associated-pathways included higher intake of fruits, vegetables, nuts, fish, tea and red wine and a protein-rich or gluten-free diet. Higher intakes of carbohydrates, fat, total calories, sweetened drinks, bread and dairy products were associated with microbial pathways linked to higher CVD risk (Figure 4).

To further estimate to what extent the microbiome-MRS associations were dependent on effects of diet and inflammation, we included diet and cytokines in the stepwise regression model and selected the best model with the highest AICs (see Methods). This analysis showed that 43 of 48 pathways survived feature selection and were included as predictors, which indicates that they were significantly associated to MRS variation independent of diet and inflammation (online supplementary material Table 13).

**Plasma levels rather than stool levels of SCFAs are more relevant to CVD**
As fruit and vegetable intake were mostly associated to MRS-associated pathways, we hypothesized that bacterial production of SCFAs by fiber fermentation may underlie the mechanism. We measured stool levels of five different SCFAs (online supplementary material Table 10) and found they were associated to most of the MRS-associated microbial pathways (Figure 4). To our surprise, the association directions we observed were in contrast to the previously suggested beneficial effect of SCFAs. Furthermore, no significant associations were found between stool levels of SCFAs and MRS (online supplementary material Table 14). Since 95% of the SCFAs produced in the gut are rapidly absorbed by colonocytes, and only 5% are actually secreted into the feces (18), we hypothesized that blood levels of SCFAs might be more relevant to CVD risk. We therefore extracted the plasma level of acetate, the most abundant SCFA, from the NMR-based metabolic profiling. Indeed, the plasma level of acetate was associated to lower MRS (Beta = -0.09, P = 7.2 x10^-4) and to 29 MRS-associated pathways at P < 0.05, all with the expected effect directions (online supplementary material Table 15).

**DISCUSSION**
This study presents a comprehensive exploration of the relationship of the gut microbiome with plasma metabolites, metabolic risk of CVD and cardiometabolic phenotypes in 1,275 individuals from a population cohort and a cohort of overweight individuals with cardiovascular and metabolic complications. We conducted an integrated analysis of diet, metagenome, plasma metabolites, inflammatory cytokines and stool SCFAs. Previously, relationships of the gut microbiome to plasma metabolites and metabolic traits were investigated using 16S rRNA sequencing (43). To our knowledge, this study is the largest metagenome-based study to date and provides richer information into the functional link between the gut microbiome and CVD risk. It is also the first study to simultaneously address microbial association with cardiometabolic phenotypes in obesity and with metabolic risk for
future development of CVD in a population-based non-patient cohort. Due to the scope of the NMR-based metabolic platform, this study primarily focused on lipid-related traits, including various lipoprotein subclasses and fatty acids. Microbial associations to a broader spectrum of metabolism therefore still need to be identified.

While our group had previously established the association of the gut microbiome with routine blood lipid level, our current data show that it is not only levels of lipoproteins but also their size and composition that are important and differentially associated with microbiome taxonomic and functional composition. After correcting for age, gender, BMI and smoking, microbial associations were identified for 210 metabolic traits in the LLD population cohort and for 35 metabolic traits in the 300-OB obesity cohort. The microbial factors identified showed modest effects, jointly explaining up to 11.1% of the variation in plasma level of glycoprotein acetyl in LLD and up to 13.4% of variation in IDL_C_percent in 300-OB. Our data also show obese-specific microbial associations, in particular for lipid compositions in VLDL, IDL and LDL lipoprotein subclasses. Furthermore, our data identified that bacterial L-methionine biosynthesis and the species Ruminococcus 5_1_39BFAA were associated to atherosclerosis and liver fat content, respectively, in our obese cohort, and that 48 bacterial pathways were associated to metabolic risk score of CVD in our population cohort. Finally, we integrated the microbiome and metabolomics with diet, SCFAs and low-grade inflammation, with most associations being detected to fruit and vegetable intake, plasma levels of adiponectin and several interleukin family members (IL-10, IL-6, IL-2P70 and IL-18BP). We also observed that the microbiome-MRS associations identified were largely independent of diet and cytokines. However, the current dietary information was obtained from food questionnaires, and the inaccuracy of self-reported data can attenuate the power in dietary analysis. Our data further shows that plasma level of SCFAs is more relevant to CVD risk than stool SCFA levels, but we did not confirm the previously observed association of TMAO to CVD. Among the bacterial associations we identified, the bacterial pathways of L-methionine biosynthesis showed consistent links with plasma metabolites, MRS of CVD and atherosclerotic plaques, and these pathways were driven by lower fruit intake. These observations are consistent with some previous findings. For instance, supplementation of the glutamine-family amino acids is predicted to have a beneficial effect towards decreasing CVD risk (45,46), while L-methionine and its metabolic products S-adenosyl-L-methionine (SAM-e) and L-homocysteine have been associated with CVD incidence and complication (47). Methionine has an essential role in a number of cellular processes, including the initiation of protein synthesis, the methylation of DNA and metabolism of xenobiotics. It is also a crucial factor in the biosynthesis of cysteine, phospholipids and polyamine (48). It is hypothesized that L-methionine induces atherosclerosis by increasing plasma homocysteine levels, as L-methionine can be converted to homocysteine directly or through SAM-e. Hyperhomocysteinemia has also been related to CVD development (49,50). A recent meta-analysis addressing the effects of low homocysteine by folic acid supplementation found a 10% reduction in risk for stroke and a 4% reduction in risk for CVD (51). Individuals with homocysteinuria, a genetic disorder characterized by severe hyperhomocysteinemia, suffer
from severe atherosclerotic disease at a young age (52). A number of mechanisms have been proposed to explain the induction of atherosclerosis by elevated homocysteine levels, e.g., through endothelial dysfunction, an increase in proliferation of vascular smooth muscle cells, oxidative damage with deterioration of arterial wall elastic material (53), and a reduction of HDL-cholesterol levels (54). Furthermore, homocysteinemia has been shown to promote the attraction of monocytes and production of pro-inflammatory cells (55). Homocysteine also induces macrophage maturation in vessel walls with enhanced vascular inflammation (56). Recently, Wang et al. revealed a pro-inflammatory status via NLRP3 inflammasome activation in hyperhomocysteinemia induced by a high methionine diet in apoE-deficient mice (57). Our data thus highlight that bacterial metabolism of L-methionine is also associated to the development of CVD in humans, an observation that was confirmed in both our population-representative cohort and the CVD-enriched cohort of overweight individuals. The association between bacterial metabolism of L-methionine and atherosclerotic plaques was observed to be independent of TMAO metabolism (L-carnitine, choline, betaine and TMAO) and BMI.

Our study also identified several functional links between the gut microbiome and metabolic profile that may predict future CVD events, in particular the association of bacterial pathways related to metabolism of amino acids, carbohydrates and polysaccharides (specifically, GDP-mannose) with MRS. This highlights the potential of microbiome-targeting therapy for CVD prevention and treatment. Some of these pathways are dominantly driven by a specific taxon such as class Bacteroidida. However, there are several pathways, including bacterial pathways of L-methionine metabolism, which are driven by many different taxa, each with modest or small effect. This suggests potential applications of different microbiome-targeting approaches in controlling a certain taxon or bacterial pathway, e.g., through personalized dietary control combined with prebiotic and ‘post-biotic’ treatment (58,59). Interestingly, the top players in MRS-related bacterial metabolism we identified were in line with previous findings. For instance, genus Faecalibacterium, Subdoligranulum, species Methanobrevibacter smithii, Eubacterium eligens and others were top players in lower-MRS associated pathways. Previous studies have suggested their health-promoting properties: Faecalibacterium members have been associated to lower intestinal and adipose-tissue inflammation (60,61), lower levels of members of Rikenellaceae family have been associated to liver disease and obesity (62,63), and levels of different members of Bacteroidaceae family have been associated with numerous host properties, acting as both mutualistic and pathogenic cohabitants (64).

We acknowledge several limitations in our study. Firstly, both the LLD and 300-OB cohorts comprised of participants of Dutch ethnicity. The reported results are thus likely biased towards a region-specific genetic background and diet, and both are known to affect both metabolism and the gut microbiome. Secondly, this was an association analysis based on a cross-sectional design, which means that the underlying causality and mechanism remain unclear. The Mendelian randomization (MR) approach is considered to be a powerful method to assess causality. However, several recent studies have shown that genetics and microbiome likely exert independent additive effects on CVD-related phenotypes that include, among
others, blood lipid levels, CVD-related proteins and BMI (44,65,66). This may limit the power of our MR analysis to illustrate underlying causality. Longitudinal studies and further functional studies are thus essential to reveal the underlying mechanism and causality.

Conclusions
Our study presents an integrated analysis of the relationship between the gut microbiome and host metabolomics, cardiometabolic phenotypes and metabolic risk of CVD in humans. Importantly, we investigated microbial association in both a population-based and an obese cohort.

We identified numerous associations of functional properties and microbial species in the gut microbiome with plasma metabolic traits, including lipoprotein particle composition, fatty acid saturation and glycoprotein N-acetyls. Some of the microbial factors identified were also linked with clinical outcomes in individuals with obesity, including hepatic fat content and atherosclerosis. In our population-representative cohort, the combined metabolic risk that represents the probability of having a CVD event in future is associated with numerous microbiome functional parameters such as biosynthesis and degradation of amino acids, fermentation, and carbohydrate and sugar derivative metabolism.

Altogether, our study highlights microbial associations to current and future clinical outcomes related to CVD. The microbial factors identified and their interactions with diet and inflammation, such as association of bacterial L-methionine biosynthesis with CVD risk and current atherosclerosis, provide a rationale for the future studies, including intervention and prospective experiments. These may contribute to the development of preventive or therapeutic strategies aimed at modulating the microbiome to reduce the burden of cardiovascular events.
REFERENCES


SUPPLEMENTAL MATERIAL

Supplemental Figure 1 – Correlation between blood lipids levels measured by NMR and blood lipid levels measured using biochemical methods. Each dot represents one individual. Spearman correlation coefficients are shown. Blood lipid levels measured by NMR were highly correlated to measurements made using routine biochemical methods.
Supplemental Figure 2 – Comparison of P values between imputing and removing missing values. Each dot represents one correlation between two metabolites. Looking at the number of significant associations at FDR 0.05 level, the number of associations when excluding missing values was 3,952, which is slightly lower than the 4,135 significant associations we identified with imputed data.
Supplemental Figure 3 – Top microbial associations to metabolites in LLD.
A. 282 associations between bacterial pathways and metabolites at FWER 0.05 level. Y-axis shows bacterial pathway. X-axis shows metabolic traits. B. 15 significant associations between bacterial species and metabolites at FWER 0.05 level. Y-axis shows bacterial species. X-axis shows metabolic traits. Association strength and direction are shown by the color key. Red indicates positive association and blue indicates negative association. Significant association and its consistency with 300-OB is indicated by the white symbols: an open circles indicates no significant association in 300-OB; an asterisk indicates association in 300-OB at $P < 0.05$ with the same effect direction; a cross indicates association in 300-OB at $P < 0.05$ with opposite direction.
Supplemental Figure 4 – Top microbial associations to metabolites in 300-OB. 
A. 282 associations between bacterial pathways and metabolites at FWER 0.05 level. Y-axis shows bacterial pathway. X-axis shows metabolic traits. B. 15 significant associations between bacterial species and metabolites at FWER 0.05 level. Y-axis shows bacterial species. X-axis shows metabolic traits. Association strength and direction are shown by the color key. Red indicates positive association and blue indicates negative association. Significant association and its consistency with 300-OB is indicated by white symbols: an open circle indicates no significant association in LLD; an asterisk indicates association in LLD at \( P < 0.05 \) with same effect direction; a cross refers to association in LLD at \( P < 0.05 \) with opposite direction.
CHAPTER 7

Role of gut microbiota in chronic low-grade inflammation as potential driver for atherosclerotic cardiovascular disease: a systematic review of human studies


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ABSTRACT

A hallmark of obesity is chronic low-grade inflammation, which plays a major role in the process of atherosclerotic cardiovascular disease (ACVD). Gut microbiota is one of the factors influencing systemic immune responses, and profound changes have been found in its composition and metabolic function in individuals with obesity. This systematic review assesses the association between the gut microbiota and markers of low-grade inflammation in humans. We identified 14 studies which were mostly observational and relatively small (n = 10 to 471). The way in which the microbiome is analyzed differed extensively between these studies. Lower gut microbial diversity was associated with higher white blood cell counts and high sensitivity C-reactive protein (hsCRP) levels. The abundance of Bifidobacterium, Faecalibacterium, Ruminococcus and Prevotella were inversely related to different markers of low-grade inflammation such as hsCRP and interleukin(IL)-6. In addition, this review speculates on possible mechanisms through which the gut microbiota can affect low-grade inflammation and thereby ACVD. We discuss the associations between the microbiome and the inflammasome, the innate immune system, bile acids, gut permeability, the endocannabinoid system and TMAO. These data reinforce the importance of human research into the gut microbiota as potential diagnostic and therapeutic strategy to prevent ACVD.
INTRODUCTION

The worldwide pandemic of obesity has led to an increased prevalence of cardiovascular risk factors (diabetes mellitus, hypertension and dyslipoproteinemia) and atherosclerotic cardiovascular disease (ACVD). This complex of cardiovascular risk factors is labeled ‘the metabolic syndrome’, which is associated with a twofold higher risk for ACVD (1). Losing weight is notoriously difficult and most often only temporary. Interestingly however, not all individuals with obesity develop the metabolic syndrome and resulting ACVD. Approximately 20-30% of the individuals with obesity are metabolically healthy and their risk for ACVD is substantially lower than in individuals with metabolically unhealthy obesity (2, 3). A better understanding of metabolically healthy obesity might offer novel treatment strategies to prevent ACVD in patients that are overweight (4). Individuals with obesity that develop ACVD are often characterized by a chronic low-grade inflammatory status, described as ‘meta-inflammation’ (5, 6). It has been hypothesized that differences in inflammatory status partly explain the individual differences in cardiovascular risk.

Since the discovery that tumor necrosis factor (TNF)-α is overexpressed in the adipose tissue of mice with obesity, numerous studies have focused on the role of adipose tissue in the development of inflammation (7). The current hypothesis states that fat mass expands due to a positive energy balance, which leads to a pro-inflammatory reaction of both adipocytes and surrounding immune cells. While initially considered to be of little relevance for metabolic dysregulation and cardiovascular risk in obesity-related disease, the modest increases in circulating inflammatory mediators have in recent years been found to be strongly associated with the development of non-alcoholic fatty liver disease, type 2 diabetes and atherosclerosis (8, 9). These pro-inflammatory pathways are partly mediated via pattern recognition receptors (PRRs). The beneficial effects of blocking inflammatory pathways on ACVD were recently underscored by the results of the CANTOS trial. Canakinumab, a human monoclonal antibody that inhibits the pro-inflammatory cytokine interleukin(IL)-1β reduced cardiovascular death, non-fatal myocardial infarction and non-fatal stroke (10). Despite this progress, the initial triggers of inflammation in patients with metabolic dysregulation remain obscure.

Several studies suggest that the obesity-related systemic inflammation is at least partly driven by an altered gut microbial composition and function (11). The human gut microbiota consists of approximately 10^{13}-10^{14} microbes, including bacteria, viruses, fungi and protozoa. Besides their function in intestinal epithelial homeostasis, development of the immune system, protection against pathogens and energy homeostasis (12-15), the gut microbiota also plays a role in pathophysiological mechanisms associated with different diseases (16). Profound changes have been observed in the composition and metabolic function of gut microbiota in individuals with obesity. Obesity was first reported to be associated with a change of gut microbiota by Ley et al. in 2005; characterized by obesity, ob/ob mice appeared
To have less phylum Bacteroidetes and more Firmicutes compared to lean phenotypes (17). A further major observation came from Turnbaugh et al. who reported that the core gut microbiome of individuals affected by obesity has an increased capacity for energy harvesting (18).

To explore whether the gut microbiota contributes to chronic inflammation as driver for ACVD in humans, we conducted a systematic review of studies investigating the role of the gut microbiome in chronic low-grade inflammation. In this review we also discuss possible mechanisms by which the gut microbiota can influence chronic low-grade inflammation and thereby contribute to the development and progression of ACVD.

METHODS

Identification and selection of articles
A systematic review of peer-reviewed studies examining the role of the gut microbiome on chronic low-grade inflammatory markers in human populations was undertaken. A protocol was developed a priori, outlining the review aim and procedure. The literature search was conducted using Medline (Pubmed), CINAHL and the Cochrane Library from inception until November 2017. Key MeSH subject terms and keywords pertaining to the gut microbiome and inflammation in correlation to obesity or ACVD were included. The following search string was employed: (‘Gastrointestinal Microbiome’ OR ‘Gut microbiome’) AND (‘Inflammation’ OR ‘Inflammation Mediators’) OR (‘Host-Pathogen Interactions’) OR (‘Immune System’) OR (‘Immunity’) OR (‘Adaptive Immunity’) AND (‘Obesity’ OR (‘Overweight’) OR (‘Cardiovascular Diseases’) OR (‘Coronary Disease’) OR (‘Atherosclerosis’)) Filters: Humans.

Two reviewers (I.v.d.M. and J.R.) independently evaluated eligibility of studies based on the title and abstract using the following inclusion criteria: (i) participants older than 18 years of age; (ii) investigation of the gut microbiome; (iii) investigation of a quantitative measure of inflammation, e.g. inflammatory cell count, hsCRP, cytokines or a potential trigger for inflammation like circulating lipopolysaccharide (LPS); (iv) full text availability in the English language. The presence of infectious or inflammatory diseases (i.e. inflammatory bowel disease, HIV and rheumatoid arthritis) in the study population was the only exclusion criterion. Afterwards, full-text articles were assessed independently by two reviewers (I.v.d.M. and J.R.). We also checked for cited articles in original research articles and reviews that addressed the mechanistic links between the gut microbiome and low-grade inflammation.

Study Quality Assessment
The included observational studies were assessed on their quality using an adapted scale from the Newcastle-Ottawa scale for quality assessment of cohort and case-control studies (NOS scale). This scale is a modified version of the NOS scale, as also used by several other studies (19, 20). For the included non-randomized intervention studies, we used the conventional
NOS scale. A study with a NOS score of 7 or more can be considered to be a study of ‘good’ quality (21). For the randomized intervention studies we used the Cochrane risk of bias2.0 (22).

RESULTS

Data extraction and quality assessment

The search process identified 629 articles for potential inclusion. In total 260 manuscript contained original research data. After reviewing the titles and abstracts 61 studies met the initial inclusion criteria (Figure 1).

Figure 1 - PRISMA Flow Diagram with schematic presentation of the study assessment and exclusion stages
Following the initial selection, another 51 studies were excluded, mainly because they were not original studies or the study did not investigate the direct relation between the gut microbiome and inflammatory markers at baseline, leading to inclusion of 10 studies. We also checked the reference list of the 61 non-review studies that initially met the inclusion criteria. Additionally, we checked 369 reviews, of which 49 were of interest and of which we checked the reference list. In total 34 original manuscripts met the inclusion criteria. However, the majority of these studies were excluded, because they did not report on microbiome analysis or used a surrogate marker to assess the gut microbiome. An additional 5 articles were identified via these reference lists for cited articles. We identified one study that presented a data set that was published more than once (trial registered as ISRCTN88720134). For this data set, we selected the study reporting on the largest cohort (23). Two other studies used the same data set, however they performed different analyses (24, 25), therefore both studies were included. Finally, this resulted in 14 unique manuscripts. The 14 articles included 21 populations with a total of 1,418 individuals (see Table 1 and supplementary material Table 1 for detailed information of each study). For the intervention studies, we only included the data at baseline in order to overcome the effect of the intervention on the relation between the gut microbiome and inflammation.

**Table 1 – Detailed information of all studies included in this systematic review.** BMI body mass index; qPCR quantitative polymerase chain reaction; 16S rRNA 16S ribosomal ribonucleic acid.

<table>
<thead>
<tr>
<th>Study - year</th>
<th>Country</th>
<th>Population details</th>
<th>Mean age</th>
<th>Mean BMI</th>
<th>Study design</th>
<th>Statistical analyses</th>
<th>Method microbiome analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schirmer et al. – 2016 (27)</td>
<td>The Netherlands</td>
<td>471 healthy subjects</td>
<td>29 ± 14</td>
<td>23 ± 3</td>
<td>Observational</td>
<td>Spearman correlation with Benjamini-Hochberg correction</td>
<td>Quantitative metagenomics</td>
</tr>
<tr>
<td>Dao MC et al. – 2016 (24)</td>
<td>France</td>
<td>49 subjects with overweight</td>
<td>42 ± 12</td>
<td>33 ± 1</td>
<td>dietary intervention</td>
<td>Kruskal-Wallis test with Bonferroni correction</td>
<td>Quantitative metagenomics</td>
</tr>
<tr>
<td>Radilla-Vázquez RB et al. - 2016 (35)</td>
<td>Mexico</td>
<td>32 subjects with obesity and 32 control subjects</td>
<td>21 ± 2 and 21 ± 2</td>
<td>21 and 35</td>
<td>Observational</td>
<td>Kruskal-Wallis test</td>
<td>qPCR</td>
</tr>
<tr>
<td>Study</td>
<td>Country</td>
<td>Participants</td>
<td>Number</td>
<td>Study Design</td>
<td>Statistical Method</td>
<td>Analytical Method</td>
<td></td>
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<tr>
<td>Rajkumar H et al. – 2014</td>
<td>India</td>
<td>60 subjects</td>
<td>49</td>
<td>Randomized controlled</td>
<td>ANOVA</td>
<td>Culturing</td>
<td></td>
</tr>
<tr>
<td>et al. (33)</td>
<td></td>
<td>with overweight</td>
<td>29</td>
<td>trial with probiotic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Le Chatelier E et al. – 2013</td>
<td>Denmark</td>
<td>169 subjects</td>
<td>57</td>
<td>Observational</td>
<td>Linear model adjusting for</td>
<td>Quantitative metagenomics</td>
<td></td>
</tr>
<tr>
<td>(26)</td>
<td></td>
<td>with obesity and 123 control subjects</td>
<td>30</td>
<td>age and sex;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotillard A et al. – 2013</td>
<td>France</td>
<td>38 subjects</td>
<td>44 ± 2</td>
<td>Randomized cross-over</td>
<td>Mann-Whitney test</td>
<td>Quantitative metagenomics</td>
<td></td>
</tr>
<tr>
<td>(25)</td>
<td></td>
<td>with obesity and 11 subjects with overweight</td>
<td>46 ± 3</td>
<td>trial with alcohol intervention</td>
<td></td>
<td></td>
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<tr>
<td>Clemente-Postigo M et al. - 2013</td>
<td>Spain</td>
<td>10 subjects</td>
<td>48 ± 2</td>
<td>Randomized cross-over</td>
<td>Pearson's correlation test</td>
<td>16S rRNA gene analysis</td>
<td></td>
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<tr>
<td>(23)</td>
<td></td>
<td></td>
<td>28 ± 3</td>
<td>trial with dietary intervention</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Martínez I et al. – 2013</td>
<td>USA</td>
<td>28 healthy subjects</td>
<td>26 ± 6</td>
<td>Randomized cross-over</td>
<td>Pearson's correlation test</td>
<td>16S rRNA gene analysis</td>
<td></td>
</tr>
<tr>
<td>(31)</td>
<td></td>
<td></td>
<td>25 ± 5</td>
<td>trial with dietary intervention</td>
<td></td>
<td></td>
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<tr>
<td>Claesson MJ et al. – 2012</td>
<td>Ireland</td>
<td>165 older and 38 subjects</td>
<td>78 ± 8</td>
<td>Observational</td>
<td>Linear quantile regression</td>
<td>16S rRNA gene analysis</td>
<td></td>
</tr>
<tr>
<td>(30)</td>
<td></td>
<td>young subjects</td>
<td>36 ± 6</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Brignardello J et al. – 2010</td>
<td>Chile</td>
<td>6 subjects with obesity and 6 control subjects</td>
<td>34 ± 12</td>
<td>Observational</td>
<td>Spearman rank test</td>
<td>G+C peak content analysis</td>
<td></td>
</tr>
<tr>
<td>(52)</td>
<td></td>
<td></td>
<td>30 ± 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mikelsaar M et al. – 2010</td>
<td>Estonia</td>
<td>38 older subjects</td>
<td>72 ± 5</td>
<td>Observational</td>
<td>Linear multiple regression</td>
<td>qPCR</td>
<td></td>
</tr>
<tr>
<td>(28)</td>
<td></td>
<td></td>
<td>27 ± 4</td>
<td>analysis adjusted for age, sex and BMI</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Eight out of the eleven observational and non-randomized studies were considered to be studies of good quality based on the NOS score (supplementary material Table 2 and 3). The methodological quality of the randomized intervention studies ranged from low risk to high risk for bias (three studies; supplementary material Table 4).

**Circulating immune cell counts**

Three studies investigated circulating immune cell counts as marker of inflammation. The details of these studies and the results are summarized in Table 2a. A low gene count (LGC) of the gut microbiome correlated with a higher white blood cell count in a large Danish population-based study employing metagenomics analysis (26). Another large Dutch cohort study that included mainly healthy younger individuals did not find a relationship between the gut microbiome composition and white blood cell count (27). In a small study in elderly, a higher white blood cell count correlated with the presence of the species *Lactobacillus reuteri* (*L. reuteri*) as analyzed by quantitative polymerase chain reaction (qPCR) (28).

**Table 2a – Summary of findings of included studies in this systematic review on gut microbiome measurements in relation to white blood cell counts**

<table>
<thead>
<tr>
<th>Study</th>
<th>Summary of finding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Le Chatelier et al.</strong> (26)</td>
<td>The phenotype of low gene count people was associated with a more marked inflammatory phenotype with higher white blood cell counts, model adjusted for age and sex (<em>q</em> = 0.014; <em>P</em> = 0.002).</td>
</tr>
<tr>
<td><strong>Schirmer et al.</strong> (27)</td>
<td>No effect of microbial composition on the most important immune cell population (T/B lymphocytes, monocytes, neutrophils, NK cells) was detected.</td>
</tr>
<tr>
<td><strong>Mikelsaar M et al.</strong> (28)</td>
<td>The higher white blood cells count was positively related (<em>r</em> = 0.434, <em>P</em> = 0.007) to the presence of <em>L. reuteri</em>, also after adjustment for age, sex, and BMI (adj. <em>R</em>² = 0.193, <em>P</em> = 0.027).</td>
</tr>
</tbody>
</table>
C-reactive protein
The majority of studies investigating the relation between the gut microbiome and low-grade inflammation used (hs)CRP as marker. In Table 2b we provide an overview of the details and results of these studies. An LGC phenotype was associated with increased hsCRP levels in the aforementioned Danish population-based study (26), and revealed a tendency towards an increased hsCRP in another smaller diet intervention study (25). The total bacterial cell counts related inversely with hsCRP in another study (29). Six studies examined the relationship between the phylum or species abundance with (hs)CRP levels. The largest study included 165 older individuals and specifically measured 16S rRNA by massive parallel sequencing finding that lower levels of the Oscillibacter co-abundance group and higher levels of the Bacteroides co-abundance group coincided with increased levels of CRP (30). Two other small studies reported that lower levels of the genera Faecalibacterium and Ruminococcus, and Faecalibacterium prausnitzii (F. prausnitzii) species correlated with increased hsCRP levels (31, 32). These studies were fairly similar in both samples sizes and methods of microbiome analysis. The first study was a randomized cross-over trial with four week diet intervention that included 28 young adults and used 16S rRNA gene sequencing to reconstruct microbiome taxonomic composition (31). The second study was an intervention study with bariatric surgery in 30 individuals with obesity. They analyzed the gut microbiota by 16S rRNA gene sequencing combined with qPCR analysis (32). For the current review, we only considered the correlation results at baseline from both studies. Next to that, the study by Rajkumar et al. who found that lower levels of the genera Lactobacillus, Bifidobacterium and Streptococcus and higher levels of the species Escherichia coli (E. coli) correlated with increased (hs)CRP in 60 healthy adults affected by overweight (33), used bacterial culturing methods to identify bacteria. Plates were incubated in triplicate using selective media for enumeration of total aerobes, anaerobes, coliforms, E. coli, Bifidobacterium, Lactobacillus and Streptococcus thermophilus.

Table 2b – Summary of findings of included studies in this systematic review on gut microbiome measurements in relation to (hs)CRP

<table>
<thead>
<tr>
<th>Study</th>
<th>Summary of finding</th>
</tr>
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<tbody>
<tr>
<td>Le Chatelier E et al. (26)</td>
<td>The phenotype of low gene count people was associated with a more marked inflammatory phenotype with increased hsCRP (q = 0.012; P &lt; 0.001).</td>
</tr>
<tr>
<td>Cotillard A et al. (25)</td>
<td>The low gene count group had a trend towards higher inflammation (hsCRP).</td>
</tr>
<tr>
<td>Tiihonen K et al. (29)</td>
<td>An inverse correlation between the total faecal microbial counts and serum hsCRP (r = -0.51, P = 0.04) was found.</td>
</tr>
<tr>
<td>Dao MC et al. (24)</td>
<td>No difference in hsCRP between high and low Akkermansia group</td>
</tr>
<tr>
<td>Claesson MJ et al. (30)</td>
<td>A reduction in the Oscillibacter co-abundance group and increase in the Bacteroides co-abundance group, coincided with increased levels of CRP.</td>
</tr>
</tbody>
</table>
Negative correlation between the family Ruminococcaceae \((r = -0.59, P = 0.0024)\) with hsCRP were revealed. Within this family, the genera Faecalibacterium and Ruminococcus displayed negative correlations with hsCRP \((r = -0.48, P < 0.05\) and \(r = -0.60, P < 0.01\) respectively).

The strongest associations were found for the amount of the species \(F.\) prausnitzii, which was negatively correlated with serum concentrations of inflammatory circulating markers, hsCRP \((R_s = -0.54, P < 0.01)\).

Subjects with more than 3mg/L hsCRP has significantly lower Lactobacilli, Bifidobacteria, and Streptococcus and higher \(E.\) coli when compared with those who had less than 3mg/L.

The G+C peak values correlated negatively with the higher CRP levels \((r = -0.68; P < 0.02)\).

### Cytokines

Six studies investigated relationships between the abundance of gut microbiome species and pro-inflammatory cytokines. The details and results of these studies are summarized in Table 2c. The total bacterial cell count was positively related to circulating TNF-\( \alpha \) levels in a small Finnish cohort study using qPCR as microbiota analysis method (29). The largest study on the relationship between specific genera, species, and cytokines was mentioned before and included 165 older individuals (30). Lower levels of the Ruminococcus and Prevotella co-abundance group, as well as higher levels of the Oscillibacter co-abundance group coincided with higher interleukin 6 (IL-6) levels. Two small studies mentioned in the hsCRP section, also reported a negative correlation between the family Ruminococcaceae (31) and species \(F.\) prausnitzii (32) with IL-6 levels. The Italian study that included four different age groups, ranging between 25 and 104 years of age (34), showed a negative correlation between IL-6 and species \(Eubacterium hallii, Eubacterium ventriosum, Eubacterium rectale, Clostridium nexile (C. nexile)\) and species in the Clostridium cluster XIVa. A positive correlation with circulating IL-6 was observed for the genera Haemophilus, Pseudomonas, Serratia, Yersinia, Vibrio and Bacillus, and species Eggerthella lenta, Eubacterium cylindroides, \(E.\) coli and Klebsiella pneumoniae. Increased levels of the genera Leminorella, Proteus and Bacillus, and species Alcaligenes faecalis, Eggerthella lenta and Eubacterium cylindroides correlated with higher levels of interleukin 8 (IL-8), while decreased levels of the species \(Eubacterium hallii, Eubacterium ventriosum, Eubacterium rectale, C.\) nexile and species within the Clostridium cluster XIVa related to higher IL-8 levels; this study investigated the gut microbiome by microarray and qPCR analysis (34).

Among all microbiome-interleukin association studies, Schirmer et al. included the largest number of individuals, \(n = 471\), all healthy and mainly young. They investigated cytokine responses \(ex\) \(vivo\) in peripheral blood mononuclear cells (PBMCs) and whole blood stimulated with five different microbial pathogens in relation to microbial taxonomy and functionality, as analyzed by metagenomics (27). For the purpose of this review we have focused on the bacterial taxa and bacteria-derived stimulations: \(E.\) coli-derived LPS, \(Bacteroides (B.)\) fragilis...
representing gram-negative bacteria, and *Staphylococcus (Staph.) aureus* representing gram-positive bacteria. For these stimulations three monocyte-derived cytokines (interleukin 1β (IL-1β), IL-6 and TNF-α) and three lymphocyte-derived cytokines (interferon (IFN)-γ, interleukin 17 (IL-17) and interleukin 22 (IL-22)) were measured. No association was found between bacteria-derived stimulations and IL-1β. On the other hand, IL-6 production after *Staph. aureus* stimulation was negatively related to the species *Lachnospiraceae bacterium 5 1 65FAA*. After stimulating PBMCs with LPS, TNF-α production was negatively related with *Alistipes spp*, *Clostridium spp* and *Bilophila spp* among others. For IL-17, 5 positive associations with genera were identified, including genus *Clostridium*, as well as two negative correlations, with *Faecalibacterium* and *Atopobium*. A differential IFN-γ response was observed for all bacterial stimulations.

**Table 2c - Gut microbiome measurements in relation to cytokines**

<table>
<thead>
<tr>
<th>Study</th>
<th>Summary of finding</th>
</tr>
</thead>
</table>
| **Schirmer et al. (27)** | At genus level: A negative relation was present between *Roseburia* and IL-6 levels (after *B. fragilis* stimulation).  
A negative relation was present between *Faecalibacterium*, *Atopobium* and IL-17 levels (after *Staph. aureus* stimulation).  
A positive relation was present between *Escherichia*, *Anaerotruncus*, *Coprobacillus*, *Clostridium* and *Anaerostipes* and IL-17 levels (after *Staph. aureus* stimulation).  
A negative relation was present between *Oscillibacter* (after LPS stimulation), *Barnesilla* (after LPS and *B. fragilis* stimulation), *Leuconostoc* (after *B. fragilis* stimulation) and IFN-γ levels.  
A positive relation was present between *Megasphaera* (after *B. fragilis* stimulation) and IFN-γ levels.  
A negative relation was present between *Bilophila*, *Odoribacter* and TNF-α levels (after LPS stimulation).  
A positive relation was present between *Methanosphaera* and TNF-α levels (after LPS stimulation).  
At species level: A negative relation was present between *Lachnospiraceae bacterium 5 1 65FAA* and IL-6 levels (after *Staph. aureus* stimulation).  
A negative relation was present between *Parabacteroides johnsonii* and IL-17 levels (after *Staph. aureus* stimulation).  
A positive relation was present between *Escherichia spp*, *B. intestinalis*, *Anaerotruncus spp* and IL-17 levels (after *Staph. aureus* stimulation).  
A negative relation was present between *B. eggerthii*, *Coprococcus comes* and IL-22 levels (after *Staph. aureus* stimulation).  
A positive relation was present between *B. cellulosilyticus* and *R. gnarus* (both after *Staph. aureus* stimulation), *Megasphaera sp.*, *Eubacterium limosum*, *B. nordii* (all after *B. fragilis* stimulation) and IFN-γ levels.  
A negative relation was present between *Lachnospiraceae bacterium 9 1 57FAA* and IFN-γ levels (after *Staph. aureus* stimulation). |
A negative relation was present between *Clostridium leptum*, *Alistipes finegoldii*, *Bilophila spp*, *Bilophila wadsworthia*, *Alistipes onderdonkii*, *Enterococcus faecium*, *Collinsella intestinalis*, *Odoribacter splanchnicus* and TNF-α levels (after LPS stimulation).

A positive relation was present between *Acidaminococcus intestini*, *Methanospaera stadtmanna*, *L. acidophilus* and TNF-α (after LPS stimulation).

**Claesson MJ et al. (30)**
A reduction in abundance of *Ruminococcus* and *Prevotella* and increased abundance of *Oscillibacter* co-abundance groups was accompanied by an increase in IL-6 levels.

**Martínez I et al. (31)**
A negative correlation between the family *Ruminococcaceae* and IL-6 was revealed (P < 0.05).

**Biagi E et al. (34)**
IL-8 correlated positively with *Alcaligenes faecalis et rel*, *Leminorella*, and *Proteus et rel*, *Bacillus*, *Eggertella lenta et rel*, and *Eubacterium cylindroides et rel*.

IL-6 correlated positively with *E. coli et rel*, *Haemoplihus*, *Klebsiella pneumoniae et rel*, *Pseudomonas*, *Serratia*, *Yersinia et rel* and *Vibrio*, *Bacillus*, *Eggertella lenta et rel*, and *Eubacterium cylindroides et rel*.

*Eubacterium halli*, *Eubacterium ventriosum*, *Eubacterium rectale*, *C. nexile*, *Clostridium cluster XIVa* were inversely correlated with IL-6 and IL-8.

**Tiihonen K et al. (29)**
Serum TNF-α was associated with the total number of bacteria (r = 0.42, P = 0.006).

**Furet JP et al. (32)**
The strongest associations were found for the amount of *F. prausnitzii*, which was negatively correlated with serum concentrations of inflammatory circulating markers, IL-6 (Rs -0.65, P < 0.001)

### Circulating LPS

Three studies investigated the relationship between the gut microbiome and circulating LPS levels. The details of these studies and the results are summarized in Table 2d. In a cohort study of 49 individuals with overweight, no relationship was observed for *Akkermansia muciniphila* (*A. muciniphila*) with LPS levels (24). A very small Spanish study of 10 individuals found LPS concentrations to be negatively correlated with the genera *Prevotella* and *Bifidobacterium* (23). Another Mexican study of 60 individuals, analyzed the gut microbiota by qPCR and found that lower levels of the species *E. coli* correlated with lower levels of LPS, however a positive correlation between LPS and *E. coli* was only found in the second tertile (LPS = 1-1.3 EU/mL) presenting fewer *E. coli* compared to the first (LPS < 1 EU/mL) and third (LPS > 1.3 EU/mL) tertiles (35).
Table 2d - Gut microbiome measurements in relation to LPS

<table>
<thead>
<tr>
<th>Study</th>
<th>Summary of finding</th>
</tr>
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<tbody>
<tr>
<td>Dao MC et al. (24)</td>
<td>No difference in LPS between high and low Akkermansia group</td>
</tr>
<tr>
<td>Radilla-Vázquez RB et al. (35)</td>
<td>A low number of E. coli comes along with a greater risk of having high LPS levels (OR = 4.378, P = 0.005). However a positive correlation between LPS and E. coli was only found in the second tertile (LPS = 1-1.3 EU/mL) presenting fewer bacteria compared to the first (LPS &lt; 1 EU/mL) and third (LPS &gt; 1.3 EU/mL) tertiles (P = 0.036).</td>
</tr>
<tr>
<td>Clemente-Postigo M et al. (23)</td>
<td>LPS concentrations correlated negatively with P. intermedia (r = -0.336, P = 0.037) and with the genus Bifidobacterium (r = -0.411; P = 0.009).</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Given the increased prevalence of obesity worldwide, the incidence of ACVD is concomitantly on the rise. Because ACVD events can only be partly explained by traditional cardiovascular risk factors, the gut microbiota is a new area to explore for new diagnostic and therapeutic tools in ACVD. It has already been shown that the gut microbiome contributes to a substantial proportion of the variation in blood lipids (36). Because chronic low-grade inflammation is one of the hallmarks of obesity, this provides another route for the gut microbiota to influence the atherosclerotic process. To our knowledge, this is the first systematic review of human studies investigating relationships between the gut microbiota and markers of chronic low-grade inflammation. Several associations between the gut microbiota and different markers of chronic low-grade inflammation were identified. A low gut microbial diversity has previously been reported in individuals with obesity compared to lean individuals. However, not just the diversity in gut microbiota, but also the presence of specific species has been shown to correlate with chronic low-grade inflammatory markers.

**Association of gut microbiome with inflammation**

A lower alpha diversity and gene count of the gut microbiome correlated with higher white blood cell counts and hsCRP levels. Le Chatelier et al. reported that the metabolic phenotype of subjects with an LGC correlated with increased insulin resistance, higher levels of triglycerides and free fatty acids, decreased high density lipoprotein-cholesterol, as well as a marked inflammatory phenotype compared to individuals with high gene count of the gut microbiome. These and other data suggest that individuals with an LGC suffer from metabolic disturbances leading to an increased risk of diabetes mellitus, dyslipoproteinemia and pro-inflammatory status which might ultimately lead to ACVD (24, 37, 38).

The abundance of the genus Bifidobacterium was inversely related to levels of LPS and hsCRP. Bifidobacterium belongs to the Actinobacteria phyla, whose abundance is related to a healthier diet with an increased intake of whole grain cereals and certain vegetables (e.g. black-eyed peas) (39). Several Bifidobacterium taxa are used as probiotics, of which a recent meta-analysis revealed protective effects for cellular immune function (40). Bifidobacterium species are associated with gut barrier functions, a reduction in systemic inflammation and a reduction in the incidence of diabetes in mice (41).
The abundance of the genus *Faecalibacterium* and species *F. prausnitzii* were inversely correlated with hsCRP and IL-17. Besides showing negative correlations with inflammatory markers in individuals with obesity, the proportions of *F. prausnitzii* were lower in patients with a history of stroke, in which the proportion also related with disease severity (42). *Faecalibacterium* is regarded as a next-generation probiotic for its several health-promoting and anti-inflammatory properties (43).

In general, lower levels of *Ruminococcaceae* and *Ruminococcus* are associated with higher levels of hsCRP and IL-6. However, opposite correlations have been observed too; the genus *Ruminococcus* was highly positively correlated with both plasma TMA and TMAO levels, as well as atherosclerotic lesion area in female ApoE/- mice (44). Another large study seems to confirm this relation as they found that the abundance of *Ruminococcus gnavus* (*R. gnavus*) was higher in ACVD patients compared to controls (45). However it’s important to mention that *R. gnavus* species were reassigned to the genus *Blautia* in 2008 on the basis of 16S rRNA gene sequencing and therefore belong to the family *Lachnospiraceae* (46).

The abundance of *Prevotella* was inversely associated with LPS and hsCRP. Furthermore, individuals with obesity have a lower abundance of *Prevotella* species in their gut (47). However large differences have been observed within this genus. Human studies have linked the increased abundance of *Prevotella* species at mucosal sites to localized and systemic disease, including rheumatoid arthritis, metabolic disorders, as well as low-grade systemic inflammation (48). *Prevotella* mediates the inflammatory response via toll-like receptor (TLR)2 activation and Th17 immune response. This *Prevotella*-mediated mucosal inflammation can lead to systemic inflammation. Certain *Prevotella* species are also suggested to play an important role in the pathophysiological relation between periodontitis and ACVD. Patients with ACVD have an enhanced abundance of *Prevotella nigrescens* (*P. nigrescens*) in subgingival plaques (49).

**Gut microbiota in cardiovascular disease**

The number of studies performed in humans to investigate the direct role of the gut microbiota in ACVD is relatively limited. Recently, the largest study in patients with ACVD was conducted by performing a metagenome-wide association study on stools from 218 individuals with ACVD and 187 healthy controls (45). The abundance of *E. coli*, *Klebsiella* spp, *Enterobacter aerogenes*, *R. gnavus*, *Eggerthella lenta*, *Streptococcus* spp, *L. salivarius*, *Solobacterium moorei*, and *Atopobium parvulum* were elevated in patients with ACVD. In contrast, *Roseburia intestinalis*, *F. prausnitzii*, *Bacteroides* spp, *P. copri* and *Alistipes shahii* were relatively depleted in individuals with ACVD. Only smaller cohort studies and studies with methodological limitations have been published previously. In 2015 a study was published in which patients with large-artery atherosclerotic ischemic stroke and transient ischemic accident patients (n = 141) showed increased levels of Proteobacteria and reduced amounts of *Bacteroides*, *Prevotella*, and *Faecalibacterium* compared to 94 non-matched controls by 16S rRNA gene analyses (42). In another small cohort study, the genus *Collinsella* was found to be enriched, while the genera *Roseburia* and *Eubacterium* were depleted in twelve symptomatic atherosclerosis patients compared to thirteen controls (50). The apparently beneficial effects of *Bacteroides*, *Faecalibacterium*, *Roseburia* (*intestinalis*) and *Alistipes* spp have also been found in our review, as they relate to lower levels of inflammatory markers. Next to this,
higher levels of *E. coli*, *Klebsiella spp* and *Eggerthella lenta* associated with higher levels of inflammatory markers.

**Quality and limitations of included studies**

**Gut microbiome in relation to white blood cell count**
Severals studies showed a possible association of the gut microbiome with blood cell counts. The first study by Mikelsaar *et al.* had a number of limitations (28), in particular, focusing only on specific strains of *Lactobacillus* species. The associations could furthermore have been influenced by the inclusion of subjects with osteoarthritis and the lack of adjustment in the analysis for smoking status. Also, a large number of individuals were taking probiotics, which may have influenced the microbiota composition. The study by Schirmer *et al.* included only healthy and mainly young individuals of which the cytokine responses to different stimuli may not be influenced by meta-inflammation (27). The association of gut microbiome with cell counts was also addressed in a large cross-sectional study by our research group (51), where eight different blood cell types were measured and related to microbiome composition, diversity and pathways. However, as we also included a small number of individuals with inflammatory bowel disease, this study was not included in our systemic review. This study identified seventeen bacterial species that related to four different blood cell types and IL-6 levels. A limited number of species/genera were also found in the current review to be related to markers of chronic low-grade inflammation. For example, *Eubacterium ventriosum* correlated negatively to the number of leukocytes and granulocytes, while *R. torques* correlated positively to the number of lymphocytes. We summarized the relationship between bacterial species, blood cell counts, as well as IL-6 as discussed in this study in supplementary material Table 5. After correcting for diet, medication, various physiological and biomedical measures, self-reported diseases and smoking status (in total >200 intrinsic and environmental factors), no significant association of cell counts was observed with bacteria and pathways. This observation may reflect that the reported cell counts are not dependent on the gut microbiota, however it is also likely that taking into account all available phenotypes to the analysis model leads to overcorrection and therefore missing the true-positive results or the association is too small to be detected within the given sample size.

**Gut microbiome in relation to (hs)CRP**
To measure low levels of hsCRP, a high sensitivity analysis needs to be used, however not all studies adopted this method (30, 52). The number of individuals used in the analysis also varied from 12 to 292. The largest study by Le Chatelier *et al.* (26) also used the most robust method to analyse the gut microbiome, quantitative metagenomics. This contrasts the study by Rajkumar *et al.* that cultured and enumerated only six different species (33), or the study by Brignardello *et al.* who used G+C peak content analysis and was unable to find a direct relation between specific gut microbial species and CRP levels (52).

**Gut microbiome in relation to cytokines**
In addition to the large differences in gut microbiome composition, differences in the cytokine measurements exist. The study by Schirmer *et al.* investigated cytokine responses *ex vivo* in PBMCs and whole blood stimulations with five different microbial pathogens (27), while the other studies measured circulating cytokines in blood.
Gut microbiome in relation to LPS
A major limitation of all the three studies investigating the relation to LPS is the small sample size, with a maximum of 49 participants in the study of Dao et al., reducing the power to detect significant associations. LPS levels in the study by Radilla-Vazquez et al. and Clemente-Postigo et al. were assessed by the limulus amebocyte lysate assays, however large differences were observed in the concentrations with a mean level of LPS of 0.16 EU/mL in Spanish cohort (with a mean age of 48 and mean body mass index (BMI) of 28) (23) and a mean LPS level of 1.14 EU/mL in the normal weight and 1.22 EU/mL in the young Mexican adults with obesity (35). It should be noted that measuring circulating LPS is notoriously difficult.

General limitations
After reviewing the literature, we could only include 14 studies that investigated the relationship between gut microbiota and markers of chronic low-grade inflammation in humans. Because the techniques used to analyze the gut microbiome were suboptimal in most studies, several of these studies could only investigate the gut microbiota at a specific taxonomic level. However, contradictive associations can exist in the relationship between different taxonomy levels with clinical markers. As already described before, we found an inverse correlation between the genus Prevotella and inflammatory markers, while increased abundance of certain Prevotella species was associated with low-grade inflammation in systemic diseases, such as rheumatoid arthritis (48). This emphasizes the complex networks among bacterial groups and the large functional differences between species and strains, which should be kept in mind when interpreting the results. Larger studies with state-of-the-art gut microbiome analysis should further investigate this important association. Another important limitation is the heterogeneity between study populations. As mentioned before, the study by Schirmer et al. included only healthy and mainly young individuals of which the cytokine responses to different stimuli may not be influenced by meta-inflammation (27). This contrasts investigations by Claesson et al. where mainly older individuals were included (30).

A major limitation in all studies is the cross-sectional design, which cannot prove causal relationships. Different human intervention studies have been performed with probiotics (53) and even feces transplantation (54). However, none of these studies investigated the specific effects of the microbiome species described here in relation to inflammatory markers. One meta-analysis demonstrated a protective role for several taxa of Bifidobacterium for cellular immune function in humans (40). Another limitation is the diversity in the statistical analysis methods. This depended mainly on the size of the study and the method of analysis of the microbiome. There is some hierarchy in the used statistical methods. The larger studies were able to perform regression analysis adjusting for covariates and multiple comparisons. Other studies used correlation analysis, either pearson’s or spearman’s without corrections. A number of studies only compared differences between subgroups of subjects using the Kruskal Wallis, ANOVA or Mann Whitney U test. In Table 1 we give an overview of the applied statistical methods.
Potential underlying mechanisms

Potential underlying mechanisms are summarized in Figure 2.
Figure 2 – Summary of possible gut microbiota derived mechanisms able to influence the process of chronic low-grade inflammation in ACVD. PAMPs, like peptidoglycans and LPS can stimulate NOD2 or TLR4 respectively and stimulate the production of pro-inflammatory cytokines via NFκB activation as part of the inflammasome. SCFAs can stimulate colonocyte proliferation, but also GPR43 and GPR109A activation which leads to the induction of Treg cells and the production of anti-inflammatory cytokines by Treg and dendritic cells. SCFAs also stimulate the production of gut peptides. GLP-2 production improves tight junctions and mucosal barrier function. Specific species have an influence on these gut permeability mechanisms. Polyunsaturated fatty acids can stimulate the CB1 receptor, which promotes gut permeability. Specific species have shown to enhance Treg cell abundance and induce anti-inflammatory molecules. On the other hand segmented filamentous bacteria can activate TGF-β and thereby promote the development of Th17 cells. Primary and secondary bile acids can inhibit NFκB-dependent transcription of pro-inflammatory cytokines from macrophages and dendritic cells via the TGR5 receptor (88). Taurine increases the production of anti-inflammatory cytokines via NLRP6 inflammasome. TMAO has shown to activate TXNIP-NLRP3 inflammasome and increase the production of pro-inflammatory cytokines. PAMP pathogen associated molecular pattern; SCFA short chain fatty acid, NOD2 nucleotide-oligomerization domain-containing protein 2; NFκB nuclear factor kappa B; LPS lipopolysaccharide; TLR4 toll-like receptor 4; GPR G protein-coupled receptor; Treg regulatory T cell; GLP-2R glucagon like protein-2 receptor; GLP-2 glucagon like peptide-2; GHS-R Growth hormone secretagogue receptor; CB1 cannabinoid receptor 1; TGF-β transforming growth factor beta; TGR5 Takeda G-protein-coupled receptor 5.

Our review focused on human studies, as humans and mice differ significantly in their gut microbiome composition and the pathophysiology of cardiovascular disease. Therefore it remains to be determined in the future whether data gathered in mice can be directly extrapolated to humans. However the human studies are mainly observational and therefore provide limited insight in the underlying mechanisms. Because the majority of mechanistic studies are performed using animal models, we included these data in this part of the discussion on potential underlying mechanisms.

Pathogen-associated molecular patterns
A possible mechanism for the pro-inflammatory cytokines is the appearance of pathogen-associated molecular patterns (PAMPs) derived from microbiota in the gut and the circulation. Such PAMPs can be sensed by PRR. After activating a PRR, the inflammasome, an important part of our innate immune system that responds to danger signals (55), is activated with an enhanced production of the transcription factor nuclear factor kappa B (NF-κB). Different bacterial components have been demonstrated to be a PAMP and thereby initiate the inflammasome pathway with NF-κB production. LPS is believed to confer its deleterious effect on the cardiovascular system mainly via upregulation of pro-inflammatory cytokines, a process that is mediated via TLR4. Importantly, LPS derived from different gut microbial species are not equally toxigenic and can differently induce TLR4 signaling (56). LPS forms a complex with LPS-binding protein which then binds to CD14 (mainly from macrophages, but
also neutrophils, monocytes and hepatocytes) or nucleotide-binding oligomerization domain (NOD)1, to initiate an acute immune response mainly via TLRs and NF-κB (57). Humans with obesity exhibit elevated levels of LPS-binding protein (58), possibly contributing to the explanation of increased LPS levels in these individuals. Endotoxins derived from gut bacteria are normally detoxified in the liver. However when the influx increases, it may exceed the capacity of Kupffer cells and thereby enter the systemic circulation (59). The importance of LPS in ACVD has been demonstrated for the first time by the Bruneck study (60). Subjects with levels exceeding 50 pg/mL faced a 3-fold higher risk of developing incident atherosclerosis. Subclinical endotoxemia was shown in mice to accelerate atherosclerosis by programming monocytes into a non-resolving inflammatory state (61). High fat diet (HFD)-fed mice treated with antibiotics showed an increase in Prevotella species and a decrease in endotoxemia (62). This observation is in accordance with the findings by Clemente et al. and Claesson et al., which describe an inverse relationship between Prevotella species, LPS and IL-6 levels (23, 30).

However, no causal relationships has been shown between the abundance of Prevotella species and levels of inflammatory markers. Another important PAMP is the peptidoglycan that can active the NOD receptors. NOD receptors can recognize bacterial determinants once they are phagocytosed by macrophages or dendritic cells. HFD-fed mice deficient in NOD2 show increased bacterial translocation and insulin resistance (63). A role for NOD2 in atherosclerosis has also been demonstrated in human genetic and mouse knockout studies (64, 65).

Short Chain Fatty Acids (SCFAs)
SCFAs are produced by the microbiome through degradation of dietary fibers; the most important SCFAs are butyrate, acetate and propionate (66). An important function of these SCFAs is to promote colonocyte proliferation, which can improve insulin resistance in mice via promotion of energy expenditure and induction of mitochondria function (67, 68). SCFAs are also important in the regulation of appetite via gut hormones (66). Activation of different G protein coupled receptors (GPR), mainly GPR41 and GPR43 have been shown to suppress inflammation, partly via epithelial survival and integrity, but also via induction of regulatory T (Treg) cells. Activation of GPR109A by butyrate has been shown to affect colonic macrophages and dendritic cell maturation and function, which stimulates the induction of transforming growth factor (TGF)-β to promote the induction of Treg cells and IL-10 producing T cells (69). Besides this, butyrate and acetate act as histone deacetylase (HDAC) inhibitors in dendritic cells and T cells with modulation of gene expression and contributing to epigenetic modulation (70). This induces extrathymic generation of Treg cells from naïve T cells and limits the secretion of pro-inflammatory cytokines (71). F. prausnitzi, Eubacterium rectale, Eubacterium hallii and R. bromii appear to be responsible for the majority of butyrate production (72), this makes sense given the inverse correlation of these species with hsCRP and some pro-inflammatory cytokines (27, 30-32, 34).
Gut peptides
Different gut hormones have been shown to support epithelial barrier formation and anti-inflammatory properties. Ghrelin levels are decreased in response to HFD and can promote lymphocyte development in the primary lymphoid organs (73). Cani et al. reported that higher endogenous glucagon-like peptide 2 (GLP-2) production is associated with an improvement of the mucosal barrier function, improved tight junctions, as well as decreased plasma LPS concentrations. This was associated with an increased abundance of Bifidobacterium species (74) and might be one of the reasons for the inverse observation between Bifidobacterium species and inflammatory markers (23, 33). Obesity can also enhance the secretion of the pro-inflammatory serotonin. Indigenous spore-forming bacteria, mainly Clostridium species can promote serotonin production from colonic enterochromaffin cells (75). In mouse models it was shown that serotonin could recruit T cells and stimulate the production of pro-inflammatory cytokines (76, 77).

Gut permeability & endocannabinoid system
HFD negatively influences gut barrier function by inducing a number of changes in the gut microbiome and the endocannabinoid (eCB) system. The eCBs are derivatives of polyunsaturated fatty acids that can activate the G-coupled receptors CB1 and CB2. When the CB1-receptor is activated, an increase in the intestinal epithelial barrier permeability is induced, which can contribute to circulating LPS concentrations. HFD increases the expression of the colonic CB1 receptor of the eCB system (78). Treatment with prebiotics leading to a change in gut microbiome was associated with a reduction in metabolic endotoxemia and improvement of gut barrier function via reduction of the CB1 receptor (79). Activation of CB2 receptor has been shown to improve glucose tolerance in rats (80). CB2 receptor expression can mainly be increased by Lactobacillus supplement, specifically L. acidophilus, and decreased by Clostridium supplement (80, 81). Administration of A. muciniphila in mice increased the levels of eCBs in the small intestine, which via activation of the CB2 receptor contribute to the anti-inflammatory effects and improved gut barrier function (82). Besides its effects on gut permeability, the eCB system has also been shown to control food intake and energy expenditure both centrally and peripherally (83). Furthermore, the microbiome can influence gut barrier function and inflammation directly via specific species like Bifidobacterium species as well as via the production of SCFA (74, 84, 85). However, it should be noted that even if relations exist between the composition of the gut microbiota and gut permeability, the direct involvement of specific gut microbes and/or metabolites needs further elucidation.

Bile acids
The gut microbiota can also influence the inflammatory state of the host through an extensive involvement in bile acid homeostasis (86). Primary bile acids are derived from the oxidation of cholesterol in the liver and are secreted into the intestine to solubilize lipids for absorption.
Microbial bile salt hydrolase, mainly produced by gut bacteria from the genera *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *Bacteroides* can deconjugate primary bile acids to secondary bile acids (86). Free taurine, an amino acid to conjugate bile acids, can enhance the activation of the NOD-like receptor family pyrin domain containing 6 (NLRP6) inflammasome and thereby increase production of IL-18 by the intestinal epithelium, which supports epithelial barrier function (87). Primary and secondary bile acids can inhibit NF-κB-dependent transcription of pro-inflammatory cytokines from monocytes, macrophages, dendritic cells and kupffer cells via the farnesoid X and Takeda G-protein-coupled receptor (TGR5) receptor (88).

**Development/differentiation of innate immune cells**

An important role for the gut microbiome exists in the development/differentiation of immune cells. In germ-free mice fed a diet with low LPS content, the mesenteric lymph nodes and Peyer’s patches contained fewer CD4+ T lymphocytes, mainly due to fewer amounts of CD4+ FoxP3+ Treg lymphocytes (89). Species belonging to the *Clostridium cluster IV* can enhance Treg cell abundance and induce anti-inflammatory molecules (90). This is in accordance with the seemingly protective role of *Faecalibacterium* and *Ruminococcus* species (30, 31). Furthermore, intestinal Th17 cell development is dependent on the microbiome. Dendritic cells and macrophages in the lamina propria sense segmented filamentous bacteria, such as Clostridia related bacteria, and produce IL-6 and integrin molecules that contribute to the activation of TGF-β and thereby promote Th17 cell development in the intestine (91, 92). It has been shown that jejunal T cell inflammation in human obesity correlates with decreased enterocyte insulin signaling (93). High fat feeding has also been shown to influence the immune cell populations in the digestive tract. In mice, after 12 weeks of HFD the proportion of Th1 cells and CD8+ T cells increased in the small bowel and colon, while the proportion of Treg cells decreased (94).

**Trimethylamine-N-oxide (TMAO)**

The most convincing link between the gut microbiota and ACVD is trimethylamine-N-oxide (TMAO) (95), a metabolite derived from dietary choline or carnitine through the action of gut bacteria. Bacteria from the phylum Proteobacteria and Firmicutes (96). TMA is converted in the liver to TMAO via flavin monooxygenase 3 (FMO3) (97). TMAO has shown to promote recruitment of activated leukocytes to human endothelial cells (98). This was supported by another *in vitro* study in which TMAO activated TXNIP-NLRP3 inflammasome and IL-1β and IL-18 were released in a dose- and time-dependent manner, while endothelial nitric oxide synthase (eNOS) and production of nitric oxide (NO) were inhibited (99). The same research group later found that TMAO up-regulated vascular cell adhesion molecule-1 (VCAM-1) expression and promoted monocyte adherence (100). LDLR-deficient mice fed a choline diet showed elevated inflammatory gene expression compared with controls (98). ApoE-deficient mice treated with antibiotics had lower TMAO levels and a reduced atheroma size, while
supplementing the diet with 1% choline increased foam cell formation, which could be prevented by antibiotics (101). Fecal transplantation from high TMAO-producing mice to ApoE-deficient mice accelerates atherosclerosis (102). Plasma TMAO levels are an independent prognostic marker for ACVD in patients undergoing elective coronary angiography (95).

Table 3 – Summary of findings and possible mechanisms. Correlations between gut microbial genera, species, and markers of low-grade inflammation that have been found in two independent cohorts. Furthermore, the possible mechanisms via which these gut bacteria could contribute to low-grade inflammation are shown. LPS Lipopolysaccharide level; WBC white blood cell count; (hs)CRP (high sensitivity)C-reactive protein level; TNF-α tumor necrosis factor alpha; IL Interleukin

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<thead>
<tr>
<th>Inflammatory marker</th>
<th>Possible mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial diversity and taxonomy</strong></td>
<td></td>
</tr>
<tr>
<td>↓ Alpha diversity or gene count</td>
<td>↑                                  ↑</td>
</tr>
<tr>
<td>↓ genus <em>Bifidobacterium</em></td>
<td>↑                                  ↑</td>
</tr>
<tr>
<td>↓ genus <em>Faecalibacterium</em> (and species <em>F. prausnitzii</em>)</td>
<td>↑                                  ↑</td>
</tr>
<tr>
<td>↓ genus <em>Ruminococcus</em> (and family <em>Ruminococcaceae</em>)</td>
<td>↑                                  ↑</td>
</tr>
<tr>
<td>↓ genus <em>Prevotella</em></td>
<td>↑                                  ↑</td>
</tr>
</tbody>
</table>

As discussed above, the gut microbiome can influence low-grade inflammation via several mechanisms. Because low-grade inflammation is a hallmark of obesity that is linked to ACVD, the gut microbiota composition could influence the risk for atherosclerosis and harbour possible diagnostic and therapeutic options. One human study demonstrated a positive result for fecal transplantation with regard to obesity-induced insulin resistance (54). Eighteen insulin resistant men who were randomized to duodenal infusion of microbiota from either a heterologous lean donor or an autologous fecal microbiota showed improvement in insulin sensitivity 6 weeks after infusion of microbiota from lean donors. The improvement in insulin sensitivity in recipient patients correlated with an increase in the number of butyrate-
producing bacteria, pointing towards a regulatory role for butyrate derived from gut microbial metabolism leading to improved insulin sensitivity. Multiple studies are currently being undertaken to investigate the possibilities of fecal transplantation as treatment option for obesity and obesity-related complications. Indirect evidence for the influence of the gut microbiota on ACVD comes from animal studies. *ApoE*-deficient mice reared under germ-free conditions fed a low cholesterol diet exhibited atherosclerotic plaques in the aorta, in contrast to conventionally reared *ApoE*-deficient mice fed a low cholesterol diet who did not develop atherosclerotic aortic plaques (103). Germ-free mice receiving a fecal transfer from genetically or diet-induced obesity mice with obesity developed greater adiposity on a HFD than did recipients of microbiota from lean mice (18, 104, 105). Similarly, fecal transfer from humans into germ-free mice mirrored the adiposity of its donor (106). Not only the adiposity status, but also its complications, like insulin resistance and non-alcoholic fatty liver disease, were demonstrated to be transmissible via the gut microbiota (104, 107). This has been shown to be driven (at least partly) by pro-inflammatory microbial products (62, 108).

**Perspective**

While the gut microbiota is increasingly recognized as a determinant of obesity, its influence on the development of low-grade inflammation and ACVD remains largely unexplored. Future studies should investigate the underlying pathophysiology, with a focus on mechanisms leading to low-grade inflammation. This is especially important because the CANTOS trial has already shown that inhibiting a pro-inflammatory pathway reduced ACVD related mortality (10). The gut microbiota could serve as a diagnostic marker by which a more pro-inflammatory state could be detected in an early stage and could predict the risk to develop certain ACVD states. For these diagnostic options, first the gut microbiota leading to low-grade inflammation in humans needs to be established in more detail. Well-designed clinical studies with state-of-the-art analysis of the gut microbiota, like shot gun metagenomics are required. By using metagenomics analysis, not only the taxonomy of the gut microbiota, but also the functionality can be investigated and can possibly contribute to new diagnostic strategies. Future studies should include subjects at risk for ACVD, such as individuals affected by obesity. Future studies should also focus on prospective and intervention studies. Potentially, the use of prebiotics or probiotic strains or fecal microbiota transplantation via capsules could become a promising therapeutic option to prevent low-grade inflammation and ACVD in the future.

**Conclusions**

In this review, we have provided an overview of the relationships between the gut microbiota and markers of low-grade inflammation in humans and discussed the possible mechanisms. These data reinforce the importance of human research into the gut microbiota in relation to the innate and adaptive immune system to prevent and treat atherosclerotic cardiovascular disease.
REFERENCES


### SUPPLEMENTAL MATERIAL

**Supplemental Table 1 - Extended version of characteristics of studies included in this systematic review.**

| Identification | Design Description | Country | n | Baseline characteristics | Study population | Inflammatory outcome parameter | Method | Microbial analysis method | Assessment | Analysed by qPCR |
|----------------|-------------------|---------|---|--------------------------|------------------|--------------------------------|--------|--------------------------|------------|----------------|---|
| Schirmer et al - 2016 | Observational study | The Netherlands | 47 | BMI 23 ± 3 Age 29 ± 14 Comorbidities - | 471 Healthy adults | IL-1β, IL-6, IL-17, IL-22, IFN-γ and TNF-α | ELISA (R&D Systems & Sanquin) | Quantitative metagenomics | Microbial taxonomy, alpha and beta diversities using metagenomic shotgun sequencing (Illumina HiSeq). | |
| Dao MC et al - 2016 | Dietary intervention study | France | 49 | MI 49 ± 1 and 28 ± 1 Age 44 ± 2 and 46 ± 3 Comorbidities - | 38 Obese and 11 overweight adults | hsCRP, IL-6 and LPS | ELISA (Quantikine US, R&D System Europe Ltd., Abingdon, UK) | Quantitative metagenomics | Microbial taxonomy, alpha and beta diversities using metagenomics shotgun sequencing (SOLiD). Microbial composition by group- | A. muciniphila | |

**qPCR** quantitative polymerase chain reaction; **BMI** body mass index; **IL** interleukin; **IFN-γ** interferon gamma; **TNF-α** tumor necrosis factor alfa; **ELISA** Enzyme Linked Immunosorbent Assay; **hsCRP** high sensitivity C-reactive protein; **LPS** lipopolysaccharide; **WBC** white blood cell; **16S rRNA** 16S ribosomal ribonucleic acid.
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Country</th>
<th>Participants</th>
<th>Metabolic Measures</th>
<th>Microbial Analysis</th>
<th>Other Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radilla-Vázquez RB et al - 2016</td>
<td>Observational study</td>
<td>Mexico</td>
<td>BMI 21 and 35 Age 21 ± 2 and 21 ± 2</td>
<td>Comorbidities -</td>
<td>32 Obese and 32 lean young adults</td>
<td>LPS</td>
</tr>
<tr>
<td>Rajkumar H et al - 2014</td>
<td>RCT with probiotics</td>
<td>India</td>
<td>BMI 29 Age 49</td>
<td>Comorbidities -</td>
<td>60 Overweight, healthy middle-aged adults</td>
<td>hsCRP, IL-1β, IL-6 and TNF-α</td>
</tr>
<tr>
<td>Le Chatelier E et al - 2013</td>
<td>Observational study</td>
<td>Denmark</td>
<td>BMI 30 Age 57</td>
<td>Comorbidities -</td>
<td>169 Non-obese and 123 obese adults</td>
<td>WBC count, hsCRP and IL-6, TNF-α and</td>
</tr>
<tr>
<td>Study Authors</td>
<td>Study Design</td>
<td>Duration</td>
<td>Region</td>
<td>BMI</td>
<td>Age</td>
<td>Comorbidities</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
<td>----------</td>
<td>--------</td>
<td>-----</td>
<td>-----</td>
<td>--------------</td>
</tr>
<tr>
<td>Cotillard A et al - 2013</td>
<td>Dietary intervention study</td>
<td>Twelve weeks, six week energy-restricted high-protein diet followed by a six week weight-maintenance diet</td>
<td>France</td>
<td>49</td>
<td>38</td>
<td>Obese and 11 overweight adults</td>
</tr>
<tr>
<td>Clemente-Postigo M et al - 2013</td>
<td>Intervention study</td>
<td>Three consecutive periods of 20 days; only red, de-alcoholized red wine or gin</td>
<td>Spain</td>
<td>10</td>
<td>10</td>
<td>Healthy, middle-aged adults</td>
</tr>
</tbody>
</table>

Phyla: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria; Genera: *Bacteroides*, *Prevotella*, *Lactobacillus*, *C. cluster IV*, *C. histolyticum*, *Blautia coccoides-Eubacterium rectale group*, *Enterococcus* and *Bifidobacteria*; Species: *Parabacteroides distasonis*, *E. coli*.
| **Martínez I et al - 2013** | Randomized cross-over trial with dietary intervention | Twelve weeks, four-week treatments with daily dose of 60 g of whole-grain barley, brown rice, or an equal mixture of the two. | USA | 28 | BMI 25 ± 5  
Age 26 ± 6  
Comorbidities -  
Healthy young adults | hsCRP and IL-6 | hsCRP by ELISA  
(Symansis, Timaru, New Zealand).  
Cytokines by ELISA  
(R&D Systems, Minneapolis, MN, USA). | 16s rRNA analysis by next generation sequencing | Microbial taxonomy, alpha and beta diversity using 16S rRNA gene metabarcoding (454 GS FLX). |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Claesson MJ et al - 2012** | Observational study | Ireland | 17 | 8 | BMI 27 ± 5  
Age 78 ± 8 and 36 ± 6  
Comorbidities: some have DM and hypertension  
165 Aged and 13 middle-aged adults | CRP, IL-6, IL-8 and TNF-α | CRP and cytokines by commercial multi-spot microplates (Meso Scale Diagnostics) | 16s rRNA analysis by next generation sequencing | Microbial taxonomy, alpha and beta diversity using 16S rRNA gene metabarcoding (454 GS FLX). |
| **Brignardello J et al - 2010** | Observational study | Chile | 24 | BMI 36 ± 5  
and 24 ± 2  
Age 34 ± 12 and 30 ± 8  
Comorbidities -  
Obese and non-obese young - middle aged adults | CRP | ? | G+C peak content analysis | Microbial composition by G+C peak content analysis | C. perfringens, E. coli, Micrococcus lysodeikticus |
<table>
<thead>
<tr>
<th>Mikelsaar M et al - 2010</th>
<th>Observational study</th>
<th>Estonia</th>
<th>38</th>
<th>BMI 27 ± 4 Age 72 ± 5 Comorbidities: scheduled for elective orthopedic surgery</th>
<th>38 Healthy older adults</th>
<th>WBC count</th>
<th>Standard laboratory methods using certified assays</th>
<th>qPCR</th>
<th>Microbial composition by group-specific PCR (qPCR)</th>
<th>Nine different Lactobacillus species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furet JP et al - 2010</td>
<td>Bariatric surgery intervention study</td>
<td>France</td>
<td>43</td>
<td>BMI 48 ± 2 and 22 ± 0 Age 44 ± 2 and 36 ± 3 Comorbidities: some had hypertension, DM2, dyslipidemia, OSAS</td>
<td>30 Obese individuals and 13 non-obese adults</td>
<td>hSCRP, orosomucoid</td>
<td>ELISA (R&amp;D Systems, Minneapolis, MN)</td>
<td>qPCR</td>
<td>Microbial composition by group-specific PCR (qPCR)</td>
<td>Bacteroides, Prevotella, Bifidobacterium, Lactobacillus, Leuconostoc, Pediococcus, C. leptum, C. coccoides, E. Coli, F. prausnitzii</td>
</tr>
<tr>
<td>Biagi E et al - 2010</td>
<td>Observational study</td>
<td>Italy</td>
<td>84</td>
<td>BMI Age 25-104 Comorbidities: some have DM and hypertension</td>
<td>20 Young and 64 older adults</td>
<td>WBC count and IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, IFN-γ and TNF-γ</td>
<td>White blood cell counts via monoclonal antibodies. Cytokines via multiplex sandwich ELISA technology.</td>
<td>Microarray analyses, qPCR</td>
<td>Microbial composition by PCR (qPCR) and HITCHip microarray.</td>
<td>C. leptum group, Bifidobacterium genus, Methanobrevibacter genus, A. muciniphila, F. prausnitzii</td>
</tr>
<tr>
<td>Tiihonen K et al - 2010</td>
<td>Observational study</td>
<td>Finland</td>
<td>40</td>
<td>BMI 33 ± 2 and 23 ± 2 Age 33 ± 2 and 23 ± 2 Comorbidities: ?</td>
<td>20 Obese and 20 non-obese adults</td>
<td>hSCRP, IL-6 and TNFα</td>
<td>Cytokines by ELISA (Quantikinew, R&amp;D Systems Inc., Minneapolis, MN, USA). HsCRP by turbidimetry (Hitachi 912, Roche, GmbH, Germany).</td>
<td>qPCR</td>
<td>Microbial composition by group-specific PCR (qPCR)</td>
<td>Total Bifidobacteria, Lactobacillia, C. group XIVa, C. perfringens, Bacteroides, Sulphate-reducing bacteria</td>
</tr>
</tbody>
</table>
Supplemental Table 2 – Quality assessment of observational studies included in this systematic review using a modified version of the Newcastle-Ottawa scale for quality assessment of cohort and case-control studies (NOS scale).

<table>
<thead>
<tr>
<th>Study</th>
<th>Selection (max 4 stars)</th>
<th>Comparability (max 2 stars)</th>
<th>Outcome (max 2 stars)</th>
<th>Score</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Representativeness of sample</td>
<td>Sample size</td>
<td>Non-respondent</td>
<td>Ascertainment of exposure</td>
</tr>
<tr>
<td>Schirmer et al.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Radilla-Vazquez et al.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Le Chatelier et al.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Claesson et al.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Brignardello et al.</td>
<td>x</td>
<td></td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Mikelsaar et al.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Biagi et al.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Tiihonen et al.</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
Table S3 – *Quality Assessment of non-randomized intervention studies included in this systematic review using the Newcastle-Ottawa scale for quality assessment of cohort studies (NOS scale)*. NA not applicable.

<table>
<thead>
<tr>
<th></th>
<th>Selection (max 4 stars)</th>
<th>Comparability (max 2 stars)</th>
<th>Outcome (max 2 stars)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Representativeness of sample</td>
<td>Non exposed cohort</td>
<td>Ascertainment of exposure</td>
<td>Outcome of interest not present at start</td>
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<tr>
<td>Dao et al.</td>
<td>x</td>
<td>NA</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Cotillard et al.</td>
<td>x</td>
<td>NA</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Furet et al.</td>
<td>x</td>
<td>NA</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
Table S4 – *Quality Assessment for randomized Intervention studies using the Cochrane risk of bias 2.0 guidelines.*

<table>
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<th>Author</th>
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<th>Allocation Concealment</th>
<th>Missing outcome data</th>
<th>Measurement of outcome</th>
<th>Selective Reporting</th>
<th>Overall bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clemente-Postigo <em>et al.</em></td>
<td>Some concerns</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Some concerns</td>
</tr>
<tr>
<td>Rajkumar <em>et al.</em></td>
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<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
<td>Low risk</td>
</tr>
<tr>
<td>Martínez <em>et al.</em></td>
<td>Some concerns</td>
<td>Some concerns</td>
<td>Low risk</td>
<td>Some concerns</td>
<td>Some concerns</td>
<td>High risk</td>
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</table>
Supplemental Table 5 – Findings from large cross-sectional study by Zhernakova et al. Relation between bacterial species, blood cell counts, and IL-6 in a cross-sectional study performed in 1,135 individuals.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Species</th>
<th>Coefficient</th>
<th>N</th>
<th># of non-zero</th>
<th>P-value</th>
<th>Q-value</th>
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</thead>
<tbody>
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<td>IL-6 level</td>
<td>d__Bacteria</td>
<td>p__Bacteroidetes</td>
<td>c__Bacteroidia</td>
<td>o__Bacteroidales</td>
<td>f__Porphyromonadaceae</td>
<td>g__Parabacteroides</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Bacilli</td>
<td>o__Lactobacillales</td>
<td>f__Streptococcaceae</td>
<td>g__Lactococcus</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Eubacteriaceae</td>
<td>g__Eubacterium</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Eubacteriaceae</td>
<td>g__Eubacterium</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Lachnospiraceae</td>
<td>g__Coprococcus</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Lachnospiraceae</td>
<td>g__Coprococcus</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Lachnospiraceae</td>
<td>g__Coprococcus</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Ruminococcaceae</td>
<td>g__Anaerotruncus</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Ruminococcaceae</td>
<td>g__Anaerotruncus</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Ruminococcaceae</td>
<td>g__Anaerotruncus</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
<td>f__Ruminococcaceae</td>
<td>g__Anaerotruncus</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>d__Bacteria</td>
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<td>c__Clostridia</td>
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<td>f__Ruminococcaceae</td>
<td>g__Anaerotruncus</td>
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<td>Leukocytes</td>
<td>d__Bacteria</td>
<td>p__Firmicutes</td>
<td>c__Clostridia</td>
<td>o__Clostridiales</td>
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</tr>
<tr>
<td>Cell Type</td>
<td>Phylum</td>
<td>Class</td>
<td>Order</td>
<td>Family</td>
<td>Genus</td>
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</tr>
<tr>
<td>----------------</td>
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<tr>
<td>Leukocytes</td>
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<td>Clostridia</td>
<td>Clostridales</td>
<td>Ruminococcaceae</td>
<td>Subdoligranulum</td>
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<td>Lachnospiraceae</td>
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<tr>
<td>Lymphocytes</td>
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<td>Clostridiales</td>
<td>Ruminococcaceae</td>
<td>Subdoligranulum</td>
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<td>Monocytes</td>
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<td>Clostridia</td>
<td>Clostridiales</td>
<td>Eubacteriaceae</td>
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<td>Monocytes</td>
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<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
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</tbody>
</table>
CHAPTER 8

Summary, discussion and future perspectives
Introduction

CVD is one of the most important causes of death worldwide. A major contributing factor is the still increasing prevalence of obesity. To tackle this epidemic of the cardiovascular morbidity and mortality, the first goal should be to prevent people to become obese. This has proven to be a very challenging goal. Firstly, because food is available everywhere in high income countries and unhealthy food is generally even cheaper (1). Secondly, because modern society is characterized by a more sedentary life style. Therefore, to reduce the incidence of obesity the primary goal for governments worldwide should be to shift the dietary pattern to more healthy foods and to promote physical activity.

The worldwide increase in obesity has led to an increased prevalence of cardiovascular risk factors, labeled the metabolic syndrome. Interestingly however, not all individuals with obesity develop the metabolic syndrome and resulting CVD. Metabolically healthy individuals with obesity have a substantially lower risk for CVD. The central hypothesis in our studies is that differences in inflammatory status partly explain the interindividual differences in cardiovascular risk within individuals with overweight and obesity. The overall aim of this thesis is to obtain more insight in factors influencing obesity-associated low-grade inflammation in relation to atherosclerosis. A better understanding of these factors and the role of low-grade inflammation in atherosclerosis might offer novel diagnostic tools and eventually treatment strategies to prevent or treat CVD.

The first factor that I investigated is the distribution of adipose tissue. Men generally store more fat around and in their abdomen, while women have more fat at the hip and thigh region. Numerous studies have shown that VAT is related to a higher risk for cardiovascular events (2, 3). Conflicting results have been shown in the relation between SAT and cardiovascular risk. However, subcutaneous fat is not a homogeneous compartment. Furthermore, it is unknown whether differences in fat distribution can differentiate between metabolically healthy and unhealthy overweight subjects. Besides this, the question remains whether different adipose tissue distributions in men versus women are associated with a different inflammatory phenotype.

The second factor that influences CVD risk is sex. Important sex differences have been observed in the pathophysiology of atherosclerotic CVD. Sex hormones and adipokines are shown to have a sex-specific effect (4). While it is implied that the same mechanisms control inflammation in women and men with regard to CVD, this has never been systematically investigated.

The third factor is the chronic low-grade inflammation itself. Although obesity is a well-known risk factor for atherosclerotic cardiovascular diseases, a sizeable number of individuals with obesity do not develop these complications. Obesity leads to a state of chronic low-grade inflammation, which is a key regulatory process in the development of atherosclerosis. Low-grade inflammation, especially the IL-1 family of circulating inflammatory proteins, has been
shown to play a tremendous role in patients with CVD. It is unknown whether inter-individual variation of chronic inflammation explain the differences in atherosclerotic risk in the obese population.

The last factor is the gut microbiota. Approximately 15 years ago, the first study was published that showed a relation between the gut microbiome and obesity. Nowadays, different strains and pathways have been related to cardiometabolic risk factors and diseases. The exact mechanism by which the gut microbiome influences the cardiovascular risk is not clear.

Summary

Role of fat distribution in relation to metabolic dysregulation
In chapter 3 we investigated the sex-specific relations between the different adipose tissue compartments with hepatic fat content and the factors that define a person as metabolically healthy or unhealthy. We found clear sex-specific differences in the abdominal adipose tissue distribution. While women and men had similar BMI levels, women had significantly lower volumes of VAT, but higher volumes of dSAT and almost twice as high volumes of sSAT compared to men. In individuals with the metabolic syndrome, the hepatic fat content was significantly higher in women and men. Also, men with the metabolic syndrome had significantly more dSAT. After adjustment for other factors, only in men the dSAT was inversely associated with HDL-c. The sSAT volume showed no association with the metabolic syndrome traits. However, only in women with obesity we found an inverse association between the sSAT volume and hepatic fat content. When focusing on the SAT, it seems that dSAT has an intermediate metabolic phenotype between the VAT and sSAT. These different adipose tissue compartments should therefore be taken into account when assessing an individual’s cardiovascular risk. Next to that sex-specific analyses have been proven to be essential with regard to the contribution of fat distribution to metabolic dysregulation.

Role of fat distribution in obesity-associated low-grade inflammation
In chapter 4 we show that sex-specific differences in fat distribution influence it’s relation to chronic low-grade inflammation. In men VAT is positively associated to circulating leukocyte number, circulating IL-6, hsCRP and IL-18BP, while in women SAT is the main contributing adipose tissue compartment to their pro-inflammatory state. Next to this, we found in women a positive association between superficial SAT and IL-6 and IL-1Ra production of stimulating PBMCs with different stimuli. To investigate whether the origin of this inflammatory association is within the adipose tissue itself, we analyzed the adipose tissue and found in men a positive association between the adipocyte cell size, VAT and SAT. In women, the gene expression level of different pro-inflammatory genes was related to mainly the superficial SAT. This highlights the importance of sex-specific analyses for possible cardiovascular management.
Role of sex in immunometabolic regulation

In chapter 5, we investigated the impact of sex on the relation between inflammation and metabolic syndrome in overweight women and men. We found that metabolic syndrome in women is mainly characterized by a deficiency of the anti-inflammatory mediator adiponectin, while in men metabolic syndrome presents with higher levels of proinflammatory mediators such as IL-6 and leptin. The metabolic syndrome traits might not have an equal contribution to the sex-specific effects and we therefore evaluated these factors separately. The triglyceride level appeared to be the most important parameter in explaining the sex-specific effects of metabolic syndrome for both IL-6 and adiponectin. Both in women and men, we found increased number of leukocytes, lymphocytes and monocytes in metabolically unhealthy individuals. Furthermore, we found only in men with the metabolic syndrome an enhanced cytokine production capacity.

We were able to exclude various circulating metabolites and lipoproteins to be responsible for these differences. These findings suggest that the mechanisms by which inflammation and the immune system can cause adverse cardiometabolic consequences of obesity are different in women and men. We therefore argue for a sex-specific approach with regard to inflammation as therapeutic targets to prevent CVD in individuals with obesity.

The contribution of cytokines to CVD risk, a crucial role for the IL-18 pathway

To investigate whether differences in inflammation, especially from the IL-1 family of cytokines, contribute to the individual risk for atherosclerotic disease in individuals with obesity, we performed a systemic analysis of this relation in chapter 6. A few circulating cytokines were related to carotid atherosclerosis, however after additional adjustment for BMI, only IL-18BP concentrations remained positively associated with maximum plaque thickness. IL-18BP is known as an inhibitor of IL-18, which has actually been related to CVD. In order to investigate the underlying mechanism, we analyzed the relation with lipoprotein composition. IL-18BP was strongly associated with the VLDL particles, mainly the small- to large VLDL particles. This relation was validated in a younger, healthier cohort. In addition, in our 300-OB cohort we found that IL-18BP is a marker of adipose tissue inflammation. In a large clinical cohort from Boston we found that IL-18BP levels were positively related to cardiovascular events in patients with a history of a myocardial infarction. IL-18BP might therefore have diagnostic value and possible therapeutic consequences.

The role of gut microbiota

We performed a large metagenome-based study to assess the association between the gut microbiome, plasma metabolome and cardiovascular risk and describe the results in chapter 7. We found specific microbial associations with the lipoprotein composition. Carotid atherosclerosis was associated with the bacterial pathway L-methionine and the Ruminococcus species with the hepatic fat content. The gut microbiome-derived TMAO levels in the plasma were positively associated with VAT. We also found significant associations between bacterial pathways and circulating levels of IL-6, IL-18BP, adiponectin and...
glycoprotein N-acetylglucosamine (GlcNAc). These data support further research in microbiome-targeted diagnostic and therapeutic approaches for CVD.

The exact mechanisms by which the gut microbiome could influence the obesity-associated low-grade inflammation and atherosclerosis are not fully known. In Chapter 8, we describe the results of a systematic review of human studies on the relation between the gut microbiome composition and markers of low-grade inflammation. Lower gut microbial diversity was associated with higher white blood cell counts and high sensitivity C-reactive protein (hsCRP) levels. We also found that hsCRP and IL-6 were related to the abundance of Bifidobacterium, Faecalibacterium, Ruminococcus and Prevotella species. In addition, this review speculates on possible gut microbiota associated mechanisms, such as the inflammasome, innate immune system, bile acids and gut permeability. These data also support additional human research to evaluate the role of the gut microbiota as potential diagnostic and therapeutic strategies for CVD.

Discussion
In this part, we reflect on our aggregated main findings in the broader context of the literature.

Fat distribution
Important sex-specific differences have been found in the distribution of adipose tissue. However, almost no study has investigated the separate contribution of the different layers of the SAT, quantified by MRI or CT on metabolic dysregulation or low-grade inflammation in individuals with obesity. We found that men had relatively more dSAT than sSAT and significantly higher volumes of VAT compared to women despite similar BMI levels. We found that only in men dSAT has an intermediate phenotype between the VAT and sSAT with regard to the traits of the metabolic syndrome. Previous studies found that the adipocytes from dSAT have higher lipolytic activity of adipocytes from dSAT compared to sSAT, which contribute substantially to FFA levels in the circulation (5). A potentially protective effect of sSAT in the development of hepatic steatosis in women is its high level of adiponectin, compared to dSAT (6). The expression of genes involved in inflammatory pathways, such as interleukin-6 and MCP1, were also higher in dSAT compared with sSAT (6, 7).

A possible explanation for the difference between women and men in fat distribution and also possibly for the metabolic association could be the hormonal status. Hyperandrogenism in normal weight PCOS women has previously been associated with preferential visceral fat deposition and moreover an increased proportion of small subcutaneous abdominal adipocytes, which could limit subcutaneous fat storage (8). Moreover, six months of testosterone therapy in aging men with a low normal bioavailable testosterone decreased subcutaneous fat on the abdomen and lower extremities, while visceral fat remained unchanged (9).
Not only did we find significant sex specific differences in the contribution of fat distribution to metabolic dysregulation, we also observed important sex differences in the relation with systemic low-grade inflammation. We identified in women that mainly SAT is related to white blood cell count, circulating levels of IL-6 and IL-18BP and production of pro-inflammatory cytokines after ex vivo stimulation. In contrast, VAT showed the strongest association with leukocytes and comparable associations with circulating cytokines as SAT in men. This suggests that the hypothesis about a beneficial effect of SAT is at least not applicable for women. Most previous studies did not perform sex-specific analyses or used a general population as cohort. Our study focused on individuals with overweight and obesity as they are at higher risk for CVD. Within this population the contribution of adipose tissue to low-grade inflammation appears to be sex-specific. One hypothesis relates to the adipose tissue itself as we found a significant positive association between the amount of SAT with an upregulation of multiple pro-inflammatory genes. This could explain the possible detrimental effect of SAT in women compared to men. The other hypothesis relates to the differential status in sex hormones. Estradiol for example has been shown to upregulate the mRNA expression of both estrogen receptors the SAT in women, and one receptor in the SAT and VAT adipocytes in men (10). These findings call for a sex-specific approach with regard to interpretation of adipose tissue distribution and its contribution to low-grade inflammation.

This emphasizes the need for future research on the role of fat distribution to distinguish the dSAT and sSAT and perform sex-specific analyses. By deciphering the immunometabolic effects of these different fat compartments, a better insight of its role in CVD is gained. This could eventually lead to new sex-specific diagnostic tools and possibly therapeutic options. In our study, we had the advantage of an MRI to evaluate the fat distribution. Most studies made use of ultrasound techniques to detect the amount of different adipose tissue compartment, however the accuracy in obesity is rather limited. When monitoring possible interventions, more sophisticated techniques as an MRI or CT scan are highly recommended. A limitation is the cross-sectional design of our study. Further prospective studies need to investigate the relation between the adipose tissue compartments and immunometabolic risk factors over time to identify the causal pathway.

**Sex differences**

Important sex-specific differences have been observed in the pathophysiology of atherosclerotic CVD. However, up till now sex is usually not taken into account with regard to diagnostic and therapeutic strategies for CVD. We have demonstrated that the immunometabolic relation with various adipose tissue compartment differs between women and men.

In addition, we found a significant impact of sex on the relation between inflammation and metabolic syndrome in overweight women and men. Metabolic syndrome in women is mainly characterized by a deficiency of the anti-inflammatory mediator adiponectin, while in men
metabolic syndrome presents with higher levels of proinflammatory mediators such as IL-6 and leptin, and also an enhanced cytokine production capacity. Previous studies have shown higher pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines in the presence of metabolic syndrome (11, 12). However, no direct comparison has been made between women and men in larger studies. The circulating triglyceride concentration appears to be the most important condition influencing the distinct changes of the inflammatory parameters in women and men when they develop the metabolic syndrome. We did not find that sex-hormones are the causal factor, however we could not measure all sex hormones. This might still be of interest as colleagues found in vitro that progesterone for example can prevent the development of a pro-inflammatory phenotype of the macrophage by stimulation with oxidized low-density lipoprotein particles (unpublished data). Future research should investigate this possible role of the sex-hormones in obesity-associated low-grade inflammation.

These findings suggest that the mechanisms by which inflammation can cause adverse cardiometabolic consequences of obesity are different in women and men. Future research on the diagnostic and therapeutic perspectives of low-grade inflammation should include sex-specific analysis. Besides this, we argue for a sex-specific approach with regard to inflammation as a therapeutic target to prevent CVD in individuals with obesity.

**Obesity-associated low-grade inflammation and atherosclerosis**

As shown in this thesis, fat distribution, sex and gut microbiota have an influence on obesity-associated low-grade inflammation and atherosclerosis. We also investigated the impact of (mainly) the IL-1 family of cytokines and its mediators on atherosclerotic CVD in individuals with overweight and obesity. We identified that IL-18BP showed a stronger relation to the presence of carotid atherosclerosis than currently used markers of inflammation (hsCRP, IL-6). We found that the strong relation between hsCRP and IL-6 with atherosclerosis was largely mediated by BMI. This was recently also seen in a meta-analysis between hsCRP and all-cause mortality (13). We were initially surprised by the finding that IL-18BP had a stronger relation with carotid atherosclerosis, as IL-18BP down-regulates Th1 responses by binding IL-18 and thus reducing the bio-activity of IL-18. Multiple lines of evidence have shown that IL-18 is prospectively and independently associated with cardiovascular events in both women and men (14-17). We also found a significant association between IL-18 and IL-18BP, however within our 300-OB cohort after additional adjustment for BMI, only IL-18BP remained associated with the maximum plaque thickness. We could confirm this relation in a large clinical cohort; IL-18BP was associated with cardiovascular events, death and overall mortality among a population of patients undergoing coronary angiography. IL-18BP levels even improved cardiovascular risk prediction beyond the traditional risk factors (manuscript submitted). The recent data from the CANTOS trial support the importance of IL-18, as after IL-1β inhibition with canakinumab, there remains substantial residual inflammatory risk that is related to both IL-18 and IL-6 (18). This emphasizes the need for future research to unravel
the role of the IL-18 pathway in the atherosclerotic process. Further mechanistic insights need to be gained before therapeutic opportunities can be developed.

We have a number of hypotheses on the pathophysiological role of IL-18BP on atherosclerosis. First, as we observed a strong relation between IL-18BP and lipoproteins, especially VLDL lipoproteins, we hypothesize that IL-18BP upregulates the VLDL production by the liver. We found in animals lacking IL-18 a decreased expression of genes known to be involved in hepatic lipid metabolism including PPP1R3G and ENHO. One possible explanation could be related to free IL-18. Previous studies have shown the importance of the free IL-18, as this is the bioactive form (19, 20). As we calculated the free IL-18 but did not observe a relation between the free IL-18 and atherosclerosis markers, we suggest that it is not the free IL-18 itself that explains the influence of the IL-18 pathway on atherosclerosis.

Another hypothesis for our finding is that IL-18 actually has a protective effect on the atherosclerotic process. We and others have shown results in line with this observation with increased atherosclerosis in the absence of IL-18 in mice (21, 22). This is in contrast to several lines of evidence that have implicated IL-18 in the development of obesity, type 2 diabetes and atherosclerosis. However, colleagues have previously demonstrated that the increased IL-18 concentrations in patients with obesity are most likely a compensatory mechanism that increases IL-18 production due to “IL-18 resistance” at the level of cellular receptors (23). Next to this, recent evidence has shown that different types of inflammasomes produce different phenotypic action of IL-18. A recent study demonstrated that IL-18 production from the NLRP1 inflammasome prevents obesity and metabolic syndrome (24). The cell type in which NLRP1 functions to cleave IL-18 remains however unclear. In line with this, mice deficient in NLRP3 or NLRP6 are also more vulnerable to colitis due to a lack of sufficient IL-18 (25, 26).

Another possibility is that the increase in IL-18BP in metabolic syndrome is a compensatory mechanism for the increase in the IL-18 level equivalently to the rise in IL-1ra with increasing IL-1β (27). IL-18BP is present in the serum of healthy humans at a 20-fold molar excess compared to IL-18 (28). IL-18BP was a better predictor than IL-18 for carotid atherosclerosis and even future cardiovascular events. Differences in half-life might explain why IL-18BP is a better marker than IL-18 for activation of the IL-18/IL-18BP pathway, which is linked to IL-1β production. The prognostic properties of IL-18BP should be investigated in large studies.

Besides binding IL-18, IL-18BP can also bind IL-37 and in doing so, enhances the ability of IL-18BP to inhibit the induction of IFN-γ by IL-18 (29). IL-37 binds to the IL-18 receptor α with a very low affinity but in mice expressing human IL-37, a profound anti-inflammatory effect is observed (30). We and others have previously shown protective effects against obesity-induced inflammation and insulin resistance by IL-37 (31, 32). By binding IL-37 the anti-inflammatory effect could be diminished. Measurement of human plasma IL-37 levels is
currently not possible. Hopefully future work will enable us to further explore the role of IL-18BP and IL-37 in the atherosclerotic process.

**Gut microbiota**

Several studies suggest that the gut microbiota contributes to chronic low-grade inflammation and metabolic dysregulation observed in individuals with obesity. A large Danish study, found that a low gene count from the faeces was associated with increased insulin resistance, higher levels of triglycerides, decreased HDL-c as well as a more pro-inflammatory phenotype (33). Most studies on the role of the gut microbiota have been performed in mice up till now. However, the gut microbiota from mice is significantly different compared to human gut microbiota. In our systematic review we identified many associations on the relation between gut microbial species and markers of chronic low-grade inflammation.

**Gut microbial species**

First of all, a low-gene count was associated with higher white blood cell counts and a higher level of hsCRP. The hypothesis is that a lower gene count is associated with a lower diversity of the gut microbiota, which is important for the digestion of food and the production of short chain fatty acids (SCFA). Secondly, possible protective features of the genus *Bifidobacterium* and *Faecalibacterium* were found. This could be due to higher levels of the gut hormone ghrelin like peptide 2, which is related to a healthier mucosal barrier. The *Bifidobacterium* species are frequently used in probiotics nowadays. The *Faecalibacterium* has been related to the production of the SCFA butyrate, which can induce the production of regulatory T cells and limit the secretion of pro-inflammatory cytokines (34). Next to that, butyrate is an important energy source for colonic epithelial cells. A low level of *Faecalibacterium* species have earlier also been observed in individuals with a cerebral atherosclerotic event (35). We also identified in the LifeLines Deep study that the *Faecalibacterium* species were associated with a lower risk of CVD based on the metabolite composition. Metabolites are intermediate or end products of metabolism and have been related to CVD risk (36). They are a possible way by which the gut microbiota could influence the immune system and CVD risk. In our 300-OB study we identified that the *Ruminococcus* species sp_6_39_BFAA related to VLDL lipoprotein composition and to hepatic fat content. Levels of members of the *Ruminococcaceae* family have been differentially related to pro-inflammatory cytokines and metabolites. In *ApoE/-/-* mice, the genus *Ruminococcus* was highly positively correlated to the metabolite TMAO levels as well as atherosclerotic lesion area (37). Also, after correction for TMAO levels, we found a significant relation of the *Ruminococcus* species sp_6_39_BFAA with VLDL lipoprotein composition and hepatic fat content.

**Gut microbial pathways**

Aside from specific species, also bacterial pathways have been demonstrated to relate to cardiovascular risk, e.g. via lipoproteinemia (38). We found that the gut microbial L-methionine pathway was related to maximum plaque thickness of the carotid artery. Not just
one microbial species was responsible for this L-methionine pathway activity, but many different species contributed in small proportions. The L-methionine pathway and its metabolic products have previously been associated with CVD. It is hypothesised that L-methionine induces atherosclerosis by increasing plasma homocysteine levels. Hyperhomocysteinemia has also been related to CVD development in a meta-analysis and lowering homocysteine levels by folic acid supplementation found a 10% reduction in stroke risk and 4% reduction in CVD risk (39). Recently in mice, a high methionine diet in Apo-/ mice leading to hyperhomocysteinemia induced a pro-inflammatory status via NLRP3 inflammasome activation. It would be very interesting to investigate the gut microbial composition of individuals with homocysteinuria. Next to this, further research should reveal the possible role of the gut microbiota when interventions as folic acid are given.

 Diagnostic possibilities of the gut microbiota
The gut microbiota could possibly serve as a diagnostic tool to identify individuals at risk for CVD. We identified metabolites that are associated with the gut microbiota and relate to CVD risk. The gut microbial metabolites are secreted into the portal vein. Taking blood from this specific region in high risk individuals could further identify interesting metabolites. Further investigation into these metabolites and their mechanisms are needed. These can be performed first by adding such metabolites to in vitro cell cultures and then via ex vivo models. TMAO is a metabolite that has been shown to have prognostic value for CVD events. A meta-analysis showed that higher circulating TMAO may independently predict the risk of subsequent cardiovascular events and mortality (40). In our 300-OB study, we identified a relation with the volume of the visceral adipose tissue compartment. Further research incorporating TMAO and possible other prognostic metabolites in risk stratification need to be performed.

 Therapeutic possibilities of the gut microbiota
Given the associations found between gut microbiota and its metabolites with metabolic syndrome and cardiovascular disease, interventions aimed at modifying the gut microbiome could possibly reduce CVD morbidity and mortality. It would for example be very interesting to see whether individuals with a higher risk for CVD, and possibly especially those individuals with low-grade inflammation, would benefit from supplementation of *Bifidobacterium* and *Faecalibacterium* species. A few studies with probiotics containing these species have been performed (41), however all are limited by the fact that a mix of different species are used in the probiotic treatment. Not only supplementation with specific species via probiotics have been investigated as possible therapeutic interventions; one faeces transplantation study has been completed with regard to CVD. In 2012 Vrieze and colleagues were the first to identify that infusion of gut microbiota from lean individuals into individuals with metabolic syndrome could improve peripheral insulin sensitivity (42). However, further examination of the individual responses elucidated patients who responded the faeces transplantation, but also patients who did not respond. New studies have suggested that the success of colonization
with specific strains appears to increase if this bacterial species already exists in the recipient (43). Individuals with high gut microbial gene richness at baseline showed no metabolic improvement (44), which suggests a complex contribution of different gut microbial strains to health. Further research should investigate whether a more personalized approach by analysing the gut microbiota to select the intervention could contribute to improved outcomes. Next to these host factors, also so-called superdonors have been identified (45). Superdonors are donors whose stool results in significantly more successful fecal microbiota transplantation outcomes than the stool of other donors. Nevertheless, whether these superdonors are good for all recipients and every disease type remains an open question (44). Furthermore, it is likely that the efficacy of one species depends on the network of other species that are present in the gut. The interpretation of gut microbiota modulating interventions are therefore quite complex and duplication of existing studies could reveal real interesting gut microbial species and pathways when consistent results are found. Further research is needed to get more insight into the complex networks of the gut microbiota in order to develop diagnostic and therapeutic opportunities of the gut microbiota on obesity related low-grade inflammation and eventually on CVD.

**Future perspectives**

The insights that we obtained in this thesis with regard to factors influencing the low-grade inflammatory status in individuals with overweight and obesity contribute to a better understanding of the development of CVD in patients with overweight and obesity. We have shown a sex-specific role of different abdominal adipose tissue compartments in the contribution to metabolic dysregulation and low-grade inflammation. We also found sex-specific pathways that influence inflammation in obesity. Next to that, we found a possible new biomarker for atherosclerosis in subjects with overweight and obesity, IL-18BP, which might be related to increased hepatic production of lipoproteins. Lastly, we identified several bacterial species and pathways that were related to metabolites, hepatic steatosis and carotid atherosclerosis. Altogether, the results of our studies may add towards a better understanding of the pathophysiology of obesity-associated low-grade inflammation and its role in atherosclerotic CVD. Next to that, they highlight several factors that should be taken into account in future studies. First of all, we highlight the need for sex-specific analysis and the division of the SAT into dSAT and sSAT when investigating the role of fat distribution in CVD risk. Future studies should investigate further the sex-specific mechanisms leading to inflammatory dysregulation in obesity. As women with the metabolic syndrome were characterized by defective anti-inflammatory mechanisms, whereas men had higher concentrations of pro-inflammatory mediators, this would propose that women and men might benefit from a differential anti-inflammatory pharmacological intervention to prevent the adverse cardiometabolic effect of obesity. Sex-specific analyses are crucial to diminish the burden from CVD in both women and men. Also the role of IL-18BP should be further investigated. Not only should future studies investigate the additional prediction capacities of IL-18BP in different cohorts, they should
also focus on possible underlying mechanisms. This would hopefully lead to more personalized diagnostic and possibly even therapeutic tools. Finally, our identified CVD related gut microbial species and pathways should be replicated in future studies. Further research is needed to confirm whether these gut microbial species and pathways can be used as diagnostic and therapeutic options in CVD risk management.

In conclusion, by identifying these different factors and mechanisms that contribute to metabolic dysregulation and chronic low-grade inflammation, we hope to improve the diagnostics tools to identify individuals at risk for CVD at an earlier stage and to personalize CVD treatment strategies.
References

CHAPTER 9

Nederlandse samenvatting
**Hart- en vaatziekten en aderverkalking**

Hart- en vaatziekten blijven één van de belangrijkste oorzaken van sterfte wereldwijd. Het belangrijkste onderliggende mechanisme om hart- en vaatziekten te krijgen is aderverkalking, in medische termen ook wel atherosclerose genoemd. Aderverkalking is een chronisch ontstekingsproces van de slagaders. Witte bloedcellen eten het cholesterol op, waardoor ze aan de binnenzijde van het bloedvat blijven plakken en aldaar ontstekingsstoffen worden geproduceerd. Dit trekt andere ontstekingscellen aan, waardoor een ophoping aan de binnenzijde van het bloedvat optreedt, dit wordt een plaque genoemd. Wanneer deze plaque, of een afgescheurd deel hiervan, het bloedvat volledig afsluit, krijgt het achterliggende weefsel geen bloed en daarmee geen zuurstof meer. Wanneer dit in de kransslagaders optreedt, ontstaat een hartinfarct, wanneer dit in de hersenen vast komt te zitten ontstaat een herseninfarct.

![Illustratie van de verschillende fasen van aderverkalking](image)

Figuur 1 – overzicht van de verschillende fases van aderverkalking


Er zijn verschillende onderzoeken geweest die gekeken hebben welke ontstekingscellen of ontstekingsstoffen meehelpen in het proces van aderverkalking. We kennen twee mechanismen ten aanzien van de ontsteking: ‘gewone’ ontstekingscellen maar ook cellen die juist de ontsteking tegen willen werken, de anti-ontstekingscellen. Recent onderzoek heeft aangetoond dat het remmen van het ontstekingsstofje IL-1β ervoor zorgt dat patiënten minder hart- en vaatziekten ontwikkelen.

**Mijn hypothese en onderzoek bij de proefpersonen**

Dit heb ik gedaan door in totaal 300 proefpersonen te onderzoeken die allemaal overgewicht hadden. Deze personen hebben verschillende vaatmetingen ondergaan om te kijken of er aderverkalking zichtbaar was, daarnaast hebben we de klassieke risicofactoren zoals de bloeddruk en het cholesterol gemeten. Aangezien de ontsteking ook in het vetweefsel aanwezig is en hier nog niet zoveel van bekend is, heb ik bij iedereen een klein beetje vet afgenomen net onder de huid in een buikplooi. Om de ontstekingscellen en -stoffen beter in kaart te brengen, hebben we bloed afgenomen waarop allerlei experimenten zijn verricht. Alle proefpersonen hebben een klein beetje ontlasting opgevangen, waarna wij de darmbacteriën hebben kunnen meten. Alle proefpersonen hebben daarnaast een MRI ondergaan, hiermee kon ik de vetverdeling binnen het lichaam bekijken en de samenstelling van de plaque in de halsslagaders analyseren.

**Vetweefsel**
Ons vetweefsel is een belangrijk orgaan dat vele hormonen produceert en nodig is voor de opslag van energie. Het grootste deel van het vetweefsel zit opgeslagen in de buik en bij de benen. Vet dat tussen de organen in de buik zit, het viscerale vet genaamd, is geassocieerd met een hoger risico op het ontstaan van hart- en vaatziekten. Dit kennen we als de appelvorm die met name bij mannelijke personen wordt gezien. Van het vetweefsel dat onder de huid zit, deels bij de heupen, het subcutane vetweefsel genaamd, zijn tegenstrijdige gegevens te vinden: we weten niet goed of dit een risicofactor is of juist mogelijk een beschermend effect heeft op het ontstaan van hart- en vaatziekten. We kennen dit met name als de peervorm bij de vrouwen.

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Figuur 2 – Links de hoogte waar het MRI plaatje rechts gemaakt is. Rechts: vet op de verschillende plaatsen: vet tussen de organen, vet in de diepe vetlaag onder de huid en vet oppervlakkig om de huid.

Ongeveer tien jaar geleden werd ontdekt dat het vetweefsel dat onder de huid zit, onderverdeeld kan worden in een diepe en een oppervlakkige laag. Daar dit alleen met geavanceerde technieken als een CT-scan of een MRI-scan goed in beeld kan worden gebracht, zijn er nog maar enkele studies die hebben gekeken of deze vetweefsellagen mogelijk een verschillende bijdrage kunnen leveren aan aderverkalking. Daar het jaren duurt voordat aderverkalking ontstaat en het zich langzaam uitbreidt, probeert men in wetenschappelijk onderzoek vaak te kijken of er een relatie bestaat met risicofactoren voor aderverkalking. Wij hebben daarom de volumes van de verschillende vetlagen: vet tussen de organen, het diepe vet en het oppervlakkige vet net onder de huid gerelateerd aan onze gemeten ontstekingscellen en -stoffen, de ontsteking die in het vetweefsel zit, maar ook aan de klassieke risicofactoren die tot nog toe bekend zijn. Hierin hebben we interessante nieuwe bevindingen gedaan die ook verschillend bleken te zijn voor mannen en vrouwen.

Het vet dat tussen de organen zat bleek bij mannen en vrouwen slecht te zijn, terwijl het vet dat dieper onder de huid ligt alleen bij mannen slecht bleek te zijn. Bij vrouwen met obesitas (een ergere variant van overgewicht) bleek het oppervlakkige vet onder de huid juist beschermend te zijn hiervoor.

Ten aanzien van de relatie met de ontstekingswaarden zagen we ook een opvallend man-vrouw verschil. Bij mannen draagt het vet dat tussen de organen zit het sterkste bij aan meer ontstekingscellen en -stoffen in het lichaam, terwijl bij vrouwen juist het vet dat onder de huid zit, het oppervlakkige en het diepe, vooral gerelateerd is aan meer ontstekingscellen en -stoffen. Een deel van de verklaring zit in het vetweefsel, aangezien de vrouwen daar meer ontstekingsstoffen produceren. Hierdoor lijkt het ook dat de klassieke risicofactoren en de chronische milde ontsteking echt een ander mechanisme hebben waarmee ze hart- en vaatziekten verklaren.
Man-vrouw verschillen
Zoals hierboven beschreven, zijn er reeds duidelijke verschillen aanwezig in de verdeling van het vetweefsel en de bijdrage van dit vetweefsel aan risicofactoren voor het ontstaan van aderverkalking. In het wetenschappelijk onderzoek worden vrouwen vaak als semi-man gezien, veel medicijnstudies zijn bijvoorbeeld alleen op mannen onderzocht, terwijl er op vele vlakken grote verschillen zijn. We hebben daarom gekeken of mannen en vrouwen met de klassieke risicofactoren ook een ander profiel van hun ontstekingscellen en -stoffen hadden, en dat bleek zo te zijn! Terwijl bij mannen met de klassieke risicofactoren de gewone ontstekingscellen werden aangezet, bleken vrouwen met de klassieke risicofactoren juist een verlaging van de cellen te hebben die de ontstekingscellen tegenwerken, zij hadden dus minder anti-ontstekingscellen. Dit heeft ook consequenties voor mogelijke behandelingen die gericht zijn op ontsteking bij hart- en vaatziekten. Bij mannen zouden de interventies zich moeten richten op de ‘gewone ontstekingscellen’ zoals ontstekingsremmende medicatie, terwijl bij vrouwen mogelijk juist meer anti-ontstekingscellen gegeven of aangezet moeten worden. Hopelijk draagt dit bij aan een nieuwe stap binnen de geneeskunde en zullen de komende onderzoeken mannen en vrouwen apart analyseren en uiteindelijk zelfs apart behandelen.

Ontstekingsstoffen
Zoals eerder aangegeven weten we eigenlijk nog niet volledig welke ontstekingscellen en -stoffen het belangrijkste zijn bij aderverkalking. Uit vele onderzoeken blijkt de IL-1 familie belangrijk te zijn, waar onder andere IL-1β en IL-18 toe behoren. Om meer inzicht te krijgen in deze familie, hebben we verschillende stofjes die tot deze familie behoren óf die invloed hebben op stofjes binnen deze familie in het bloed geanalyseerd. Van deze ontstekingsstofjes hebben we vervolgens gekeken of ze een relatie tonen met de klassieke risicofactoren en met aderverkalking. Hierbij deden we een verrassende bevinding. We hadden een nieuw stofje ontdekt dat een betere relatie vertoonde met aderverkalking dan de ontstekingsstoffen die tot nog toe gebruikt werden. Deze stof heet IL-18BP, dit was uiteraard reuze interessant! Toevallig hadden collega’s reeds muisenzonderzoek verricht waarin o.a. de rol van dit stofje onderzocht werd en een mogelijke rol bevestigd kon worden. Door een samenwerking met een groot cohort aan patiënten met hart- en vaatziekten in Boston hebben we vervolgens ook aan kunnen tonen dat IL-18BP een betere voorspelling voor een nieuw hartinfarct kan doen bovenop de klassieke risicofactoren. We hebben verscheidene hypotheses waarom IL-18BP een betere maat is voor hart- en vaatziekten en deze dienen we in de toekomst goed te gaan onderzoeken. Daarnaast dient de prognostische waarde van IL-18BP in toekomstige onderzoeken bevestigd te worden alvorens het in de praktijk toegepast zou kunnen worden. Het zou prachtig zijn als we op deze manier een bijdrage kunnen leveren aan het beter voorspellen van hart- en vaatziekten en daarmee de kans daarop kunnen verkleinen.
Darmbacteriën
Het laatste onderdeel dat ik in mijn thesis onderzocht heb, is de relatie van de darmbacteriën met de aan overgewicht veroorzaakte chronische milde ontsteking en de relatie met aderverkalking. Dit is echt een nieuw onderdeel in de geneeskunde, waarvan pas een aantal jaren geleden duidelijk is geworden dat het een rol kan spelen bij ziekte en gezondheid. De eerste studies toonden aan dat personen met overgewicht minder diversiteit in hun darmflora hadden wat invloed had op de vertering van hun voedsel. Vervolgonderzoeken hebben in kleine groepen aangetoond dat bepaalde darmbacteriën vaker voorkomen bij personen met hart- en vaatziekten dan bij gezonde personen. Onze hypothese is dat de darmbacteriën stoffen produceren waardoor ons afweersysteem wordt aangezet en daarmee een invloed heeft op aderverkalking. We hebben daarom in onze 300 personen met nieuwe geavanceerdere technieken de samenstelling van de darmbacteriën onderzocht en vele stofjes onderzocht die een mogelijke rol konden spelen. Hierbij vonden we inderdaad een rol voor enkele specifieke darmbacteriën en een paar specifieke stofjes. Deze dienen nu in nieuwe onderzoeken bestudeerd te worden. Mogelijk dat het onderzoeken van de ontlasting bij kan dragen aan het eerder ontdekken van een verhoogd risico op hart- en vaatziekten en kan het herstellen van de darmflora bijdragen aan het voorkomen van hart- en vaatziekten.

Het toekomstplaatje
Uiteindelijk is het natuurlijk de droom om personen met een verhoogd risico op hart- en vaatziekten in een vroeger stadium op te sporen door verder te kijken dan alleen de klassieke risicofactoren. Hopelijk kun je die personen vervolgens op een persoonsgerichte wijze gaan behandelen. Dat betekent mogelijk dat de ene persoon behandeld dient de worden met cholesterolverlagers, terwijl de andere persoon onstekingsremmers moet krijgen of juist anti-ontstekingsstoffen, en weer een ander mogelijk een herstel van de darmflora nodig heeft. Hopelijk kunnen we door middel van onze onderzoeken uiteindelijk een echte bijdrage leveren om hart- en vaatziekten eerder op te sporen en beter te kunnen behandelen.
APPENDICES

Dankwoord
List of Publications
PhD Portfolio
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Curriculum Vitae
**DANKWOORD**

Zo. En dan is het precies zeven jaar later dat ik de eerste woorden van mijn dankwoord typ. Waarschijnlijk zal dit het meest gelezen hoofdstuk zijn van mijn thesis, ik hoop daarom dat ik niemand vergeet!

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Lieve Mark, Krissie en Noud, dank dat we zo’n fijne band hebben en elkaar weten te vinden als het nodig is. Gelukkig heb je je nooit geïrriteerd dat je kleine zusje ook mee op stap ging. Ik geniet enorm van jouw droge humor, de etentjes samen bij pap en mam en de altijd zeer gezellige weekendjes weg. Daarnaast vind ik het prachtig om te zien hoe Saar en Noud samen opgroeien, dat zijn nu al dikke maatjes!

Lieve pap en mam, bedankt voor de onvoorwaardelijke steun en het vertrouwen dat jullie me gegeven hebben. Ik ben blij dat jullie me altijd hebben gestimuleerd om te doen wat ik leuk vind. Dank daarnaast voor alle aanmoedigingen tijdens het sporten, de fijne wandelingen en onze onvergetelijke reizen. Jullie hebben een heerlijke veilige thuishaven gecreëerd en me altijd van goed advies voorzien. Ik houd zeer veel van jullie en ben enorm trots op jullie!

Lieve lieve Jeroen, waar te beginnen. Bedankt dat je er elke keer opnieuw voor me bent! Dank ook voor je prachtige impulsieve ideeën, de overheerlijke gerechten die jij als geen ander kunt koken en voor onze wielrenritjes of wandelingen samen door het bos. Ik vind het heerlijk om zulke diepgaande gesprekken te kunnen voeren en jouw kijk op de wereld met jouw enthousiasme en passie te mogen delen. Ik kijk uit naar de toekomst waarin we samen kunnen genieten met Saar en ons tweede kindje straks. Ik houd van jou!

Lieve kleine Saar, wat ben ik enorm trots op jou, ik houd enorm veel van jou!
LIST OF PUBLICATIONS


2. van den Munckhof ICL*, Frishberg A*, Ter Horst R, Schraa K, Joosten LAB, Joost Rutten JHW, Iancu IM, Dregoesc IM, Tigua BA, Netea MGN, Riksen NP, Gat-Viks I. An integrative model of cardiometabolic traits identifies two types of metabolic syndrome. Submitted


* Contributed equally
**PHD PORTFOLIO**

**Name PhD candidate:** I.C.L. van den Munckhof  
**Department:** Internal Medicine  
**Graduate School:** Radboud Institute for Health Sciences  
**PhD period:** 14-10-2013 – 1-2-2016 and 1-2-2017 – 1-2-2018 and 12-8-2019 – 11-11-2019  
**Promotors:** Prof. dr. J. de Graaf, prof. Dr. N.P. Riksen, prof. Dr. L.A.B. Joosten  
**Co-promotor:** Dr. J.H.W. Rutten

<table>
<thead>
<tr>
<th><strong>TRAINING ACTIVITIES</strong></th>
<th><strong>Year(s)</strong></th>
<th><strong>ECTS</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Courses &amp; Workshops</td>
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<tr>
<td>- NCEBP Science day</td>
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<td>- BROK course</td>
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<tr>
<td>- PhD workshop poster design and presentation</td>
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<tr>
<td>- MRI course</td>
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<tr>
<td>- Workshop vascular ultrasound (EACPT focus meeting)</td>
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<tr>
<td>- Internal medicine science day</td>
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<tr>
<td>- PhD course Statistics</td>
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<td>- PhD course Scientific writing</td>
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<td>b) Seminars &amp; lectures</td>
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<tr>
<td>- Masterclass gut microbiome by prof. dr. Martin Blaser (oral)</td>
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<tr>
<td>c) Symposia &amp; congresses</td>
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<td>- New Frontiers in IL-1 and diabetes</td>
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<td>- EACPT Focus Meeting</td>
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<td>- Cardiovascular conference (poster)</td>
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<td>- Puls – Dutch Heart Foundation</td>
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<td>- International Atherosclerosis Society congress (Amsterdam, The Netherlands) (poster)</td>
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<td>- Belgian hypertension meeting (Antwerpen, Belgium) (oral)</td>
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<td>- American Heart Association congress (Orlando, Florida) (poster)</td>
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<td>Summer Frontiers – Systems Biology of Innate Immunity</td>
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<td>Translation Cardiovascular Research Meeting – Dutch Heart Foundation</td>
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<td>Radboud New Frontiers in the Microbiome (poster and poster prize)</td>
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**d) Other**

- Cytokine meeting
- Vascular damage theme meeting
- Vasculometabolic meetings

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<tr>
<td>Vascular damage theme meeting</td>
<td>2014 and 2017</td>
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<tr>
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<td>2014 and 2017</td>
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**TEACHING ACTIVITIES**

**e) Lecturing**

- Teacher in 5HKC1 Atherosclerose – Promotie-onderzoek; Niet-invasieve vaatmetingen
- Instructor vascular ultrasound course PhD candidates

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<th>Time (h)</th>
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<tr>
<td>Instructor vascular ultrasound course PhD candidates</td>
<td>2019</td>
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**f) Supervision of internships / other**

- Supervision of a student – T. Brand – Master Medicine (Radboudumc, Nijmegen)
- Supervision of a student – M. Simonds – Master Biomedical Sciences (Radboudumc Nijmegen)
- Supervision of a student – A. Bhadai – Master Medical Biology (Radboudumc Nijmegen)

<table>
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<th>Time (h)</th>
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<td>Supervision of a student – T. Brand – Master Medicine (Radboudumc, Nijmegen)</td>
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<tr>
<td>Supervision of a student – A. Bhadai – Master Medical Biology (Radboudumc Nijmegen)</td>
<td>2020</td>
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**TOTAL** 34.3
RESEARCH DATAMANAGEMENT

The data obtained in this thesis are archived according to the Findable, Accessible, interoperable and Resuable (FAIR) principles (Wilkinson, 2016). Raw and processed data were stored digitally on a local server of the department of Internal Medicine, which is backed up daily on the Radboudumc server, and on paper in the form of CRF documents and lab journals. The paper versions of our CRF data are separately locked from the questionnaires in file cabinets. The databases only contain anonymous information via coding. The vascular ultrasound images are stored at a separate internal drive for which only authorized individuals have access. The MRI images are stored at another internal drive at the department of Radiology which is also locked. Besides this the gut microbiome data is stored at an internal drive at the Department of Genetics and can be requested via the European Genome Phenome Archive (EGAD00001005083).

All human studies described in this thesis were conducted according to the principles of the declaration of Helsinki and were approved by the Medical Ethics Committee of the Radboudumc. All participants gave written informed consent before participation. All animal experiments in this study were approved by the Ethics Committee on Animal Experiments of the Radboudumc. Data generated in this thesis are part of published articles and files are available upon reasonable request.
CURRICULUM VITAE

Inge van den Munckhof werd geboren op 12 januari 1989 te Venlo en groeide op met haar ouders en broers in Melderslo. In 2007 voltooide zij de middelbare school aan het Dendron College in Horst en datzelfde jaar begon zij de studie Geneeskunde aan de Radboud Universiteit in Nijmegen.


Inge woont samen met haar vriend Jeroen Verbruggen, ze hebben een dochter Saar en verwachten in april hun tweede kindje.