Metabolic consequences of adipose tissue dysfunction

Ilse Marije Schrover

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Metabolic consequences of adipose tissue dysfunction

Metabole gevolgen van disfunctionerend vetweefsel

(met een samenvatting in het Nederlands)

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Ilse Marije Schrover
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Promotor: Prof. dr. F.L.J. Visseren
Copromotor: Dr. W. Spiering

Content

Chapter 1

Introduction

Obesity as the cause of diseases

From diabetes to orthopedic problems, and from sleep apnea to vascular disease; obesity takes its toll on the health of many people. According to the World Health Organisation, one third of the world population is overweight (1.9 billion people) and 650 million people are obese. (1) These numbers have tripled since 1975 and are still rising(1). Not all overweight or obese people develop health problems related to their body mass but have a lower life expectancy(2) and lower quality of life(3).

The risk of obesity related diseases can not be calculated just by measuring the quantity of body mass. More precise measurements of adipose tissue, such as waist circumference or ultrasonography of intra-abdominal and subcutaneous adipose tissue are better related to the risk of obesity-related disorders such as diabetes and vascular diseases.

Adipose tissue is not just a storage depot for fat, resulting from excess caloric intake. Adipose tissue produces various adipokines and inflammatory cytokines and growthfactors inducing or accelerating various disease processes leading to diabetes, vascular disease, thrombosis and cancer(4). Therefore, it is important not to solely focus on adipose tissue quantity but identify patients having metabolic consequences resulting from disturbed adipose tissue function, also referred to as 'adipose tissue dysfunction' - a condition that can be summarized as an imbalanced production of pro- and anti-inflammatory adipokines (derived from adipose tissue) leading to insulin resistance, endothelial dysfunction, sympathetic activation and eventually diabetes and cardiovascular diseases(5). Excess adipose tissue, as seen in overweight and obese individuals, is a major driver of the occurrence of adipose tissue dysfunction, but also lean subjects may have adipose tissue dysfunction and thus run an increased diabetes and vascular risk.

Metabolically healthy obese and normal weight metabolically obese?

The concept of obese, yet metabolically healthy individuals has been postulated(6). Several definitions of metabolically healthy have been proposed, most of them using various combinations of the Metabolic Syndrome criteria, as stated in the ATPIII guidelines (7,8). These individuals show no signs of dyslipidemia, elevated blood pressure or insulin resistance, despite being overweight or obese(6,7,9). The percentage of metabolically healthy obese declines with age, and compared with normal weight metabolically healthy individuals, obese individuals are at increased risk for adverse longterm outcome, even in the absence of metabolic abnormalities(10). This suggests that there is no such thing as metabolically healthy obesity as longterm exposure to obesity results eventually in metabolic changes in the long run in most obese subjects.

On the other hand, there are the so called normal weight, metabolically obese individuals. Despite having a normal BMI (<25kg/m2), these persons fulfill the metabolic syndrome criteria. They have a similar risk for cardiovascular events as metabolically unhealthy obese(11).

Thus, besides obesity generally not being a benign condition in the long run, being at normal weight does not eliminate the risk of a metabolically unhealthy profile. Besides the quantity, the quality of adipose tissue might be very important.

Quantity and quality of adipose tissue

It seemed so simple; the higher the BMI, the higher the risk for diabetes and cardiovascular diseases(12). But the insight that different adipose tissue departments contribute differently to metabolic changes resulted in an extensive explanatory search, trying to find which parameters influence metabolic changes most. Visceral adipose tissue is most strongly associated with an adverse metabolic risk profile, even after adjustment for general adiposity (13), as compared to subcutaneous adipose tissue and general measurements of adiposity, such as BMI and waist circumference. But even in people with similar amounts of (visceral) adipose tissue, differences in metabolic profile exist (14), suggesting that beyond quantity, quality of adipose tissue, also referred to as adipose tissue function, is a major driver of 'obesity' related diseases.

Adipose tissue dysfunction

Adipose tissue dysfunction (ATD) is defined as the imbalance between the production of pro- and anti-inflammatory adipokines by adipose tissue. Overproduction of pro- inflammatory adipokines is the result of inflamed adipose tissue, caused by the infiltration of macrophages into adipose tissue(15,16). The systemic metabolic consequences of ATD include systemic low-grade inflammation, hypercoagulability, elevated blood pressure, dyslipidemia and insulin resistance. Both exogenous factors such as physical inactivity(17,18) and the dietary intake of saturated fat(19,20), as well as endogenous susceptibility such as a low birth-weight(21,22), genetic predisposition (23,24) and an overactive sympathetic nervous system (25,26), may all contribute to the development of ATD.

Objectives of this thesis

With the increase in the prevalence of obesity, it becomes more and more important to identify at an early stage those individuals at highest risk of developing obesity related diseases. Identifying adipose tissue dysfunction rather than only measuring adipose tissue quantity could be an option to select those individuals at high risk. Prevention and early treatment of metabolic disturbances can then be targeted at this group. In the first part of this thesis, we have explored various options for diagnosing adipose tissue dysfunction with imaging techniques (1H-Magnetic Resonance Spectroscopy) and laboratory measurements (adipokines). The second part of this thesis explores pathogenesis and treatment of one of the most prevalent consequence of adipose tissue dysfunction; obesity-related hypertension.

Outline of this thesis

The first part of this thesis describes diagnostic possibilities for diagnosing adipose tissue dysfunction. In **chapter 2,** we provide a review of the different diagnostic modalities for diagnosing adipose tissue dysfunction. In **chapter 3** differences in free fatty acid content, as measured with MR spectroscopy in three adipose tissue depots in obese and lean individuals are described. **Chapter 4** evaluates the effect of an oral fat load on free fatty acid content in different adipose tissue depots in individuals with and without the metabolic syndrome.
In a cohort of patients with cardiovascular disease or at high risk, in whom we have studied the relation between measurements of adiposity, plasma adipokine concentrations and the odds for metabolic syndrome (**chapter 5**).
The second part of the thesis involves research on obesity-related hypertension. In **chapter 6**, using the same SMART cohort, we studied the relation between plasma adipokines and blood pressure. In **chapter 7 and 8** the results are presented of the Target-trial, a randomized controlled trial in medication naive patients with obesity-related hypertension, treated in a cross-over study with aliskiren, hydrochlorothiazide, moxonidine and placebo.
The main findings of this thesis are discussed in **chapter 9**, placing our findings in perspective and identifying the role of adipose tissue dysfunction in (future) clinical practice.

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Chapter 2

Adipose Tissue Dysfunction: Clinical Relevance and Diagnostic Possibilities

Ilse M. Schrover[1], MD; Wilko Spiering[1], MD, PhD;
Tim Leiner[2], MD, PhD; Frank L.J. Visseren[1], MD, PhD

[1] University Medical Center Utrecht, Department of Vascular Medicine
[2] University Medical Center Utrecht, Department of Radiology

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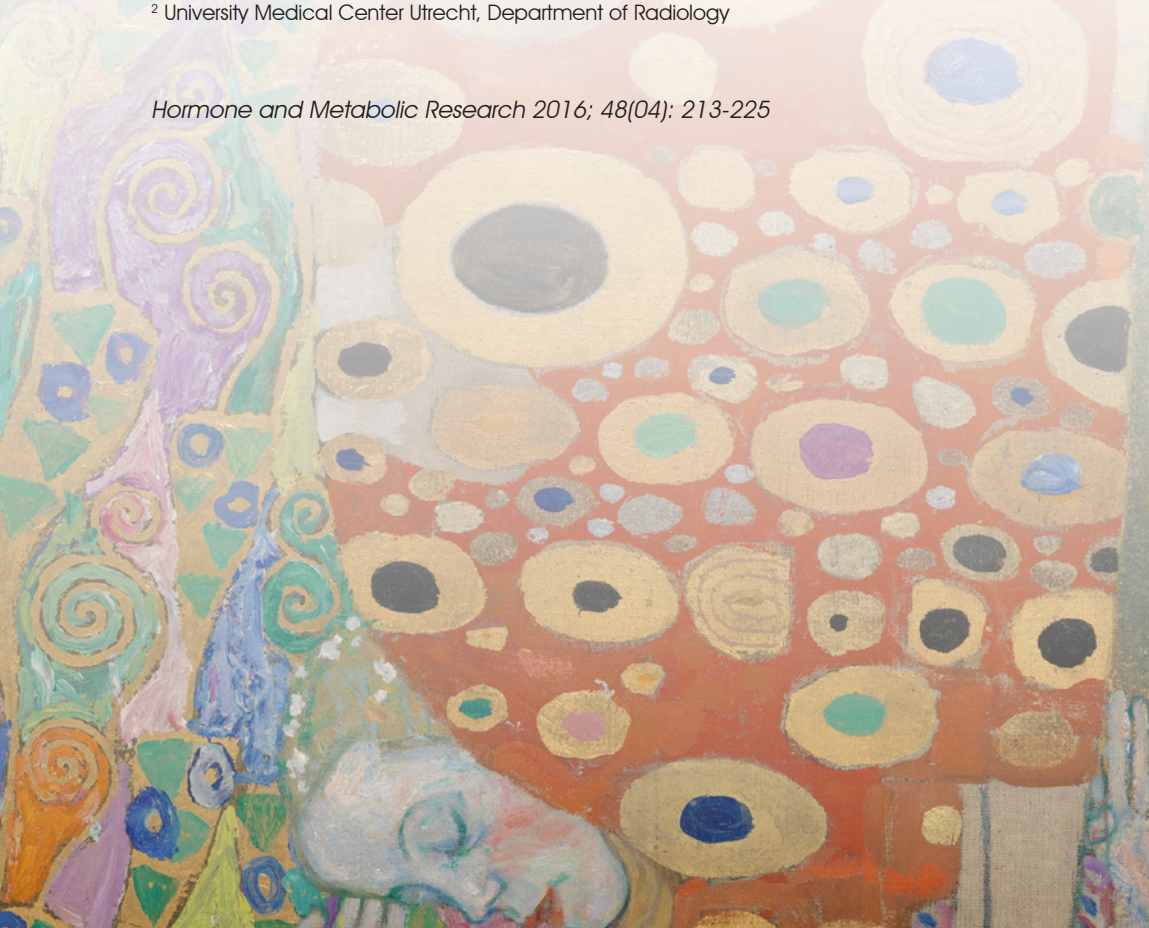

Abstract

Adipose tissue dysfunction is defined as an imbalance between pro- and anti-inflammatory adipokines, causing insulin resistance, systemic low-grade inflammation, hypercoagulability and elevated blood pressure. These can lead to cardiovascular disease and diabetes mellitus type 2. Although quantity of adipose tissue is an important determinant of adipose tissue dysfunction, it can be diagnosed in both obese and lean individuals. This implies that not only quantity of adipose tissue should be used as a measure for adipose tissue dysfunction. Instead, focus should be on measuring quality of adipose tissue, which can be done with diagnostic modalities ranging from anthropometric measurements to tissue biopsies and advanced imaging techniques. In daily clinical practice, high quantity of visceral adipose tissue (reflected in high waist circumference or adipose tissue imaging), insulin resistance or presence of the metabolic syndrome are easy and low-cost diagnostic modalities to evaluate presence or absence of adipose tissue dysfunction.

Abbreviations

BMI	body mass index
DM2	diabetes mellitus type 2
ATD	adipose tissue dysfunction
FFA	free fatty acids
TNF-α	tumour necrosis factor alpha
IL-6	interleukin 6
PAI-1	plasminogen activator inhibitor 1
WC	waist circumference
IL-8	interleukin 8
IL-18	interleukin 18
VAT	visceral adipose tissue
CT	computed tomography
MRI	magnetic resonance imaging
SAT	subcutaneous adipose tissue
RBP-4	retinol binding protein 4
TG	triglycerides
PUFA	poly unsaturated fatty acids
TUFA	total unsaturated fatty acids
HOMA-IR	homeostatic model of assessment – insulin resistance
SFRP-5	secreted frizzled related protein 5
HGF	hepatic growth factor
IP-10	interferon gamma induced protein 10
MCP-1	monocyte chemo attractant protein 1
ELISA	enzyme linked immunosorbent assay

1. Introduction

Worldwide, an estimated 1 billion people are overweight (defined as a body mass index (BMI) >25 kg/m^2). Another 500 million are obese (BMI >30 kg/m^2).[1] A BMI above 25 kg/m^2 is associated with a 30% increase in overall mortality, a 40% increase in vascular mortality and a 120% increase in mortality due to complications of diabetes as compared to a BMI ≤25kg/m.^{2} It is estimated that 1 in every 7 cases of cardiovascular disease is attributable to overweight and 8 in every 10 cases of incident type 2 diabetes mellitus (DM2).[2;3] Moreover, a high BMI is associated with an increased incidence of multiple cancer types.[4;5] On the other hand, BMI is an imperfect measure to estimate the contribution of adiposity to future disease risk and mortality, [6] since not the quantity of adipose tissue itself is the causal factor in the occurrence of cardiovascular diseases, DM2 and cancer, but the metabolic consequences of adiposity as a result of adipose tissue dysfunction (ATD). Insulin resistance, hypertriglyceridemia, low HDL-cholesterol, hypertension, hypercoagulability and low-grade inflammation are metabolic risk factors related to ATD.[7-9] Obese patients who are metabolically healthy do not exhibit unfavourable metabolic changes,[10] are not insulin resistant[11] and have a low risk of developing cardiovascular diseases,[9] indicating that not only adipose tissue quantity matters, but also adipose tissue function. This concept of ATD is signified by patients who are metabolically obese despite a normal weight.[12;13] These patients have an increased risk for DM2 and cardiovascular disease.

The diagnosis or identification of ATD may therefore be of clinical relevance serving as a tool for stratifying risk for cardiovascular diseases, DM2 and even cancer, and may guide preventive treatment with both medication and lifestyle interventions.[14] ATD may even serve as a direct treatment target.[15] This would be in contrast to a more general approach with the focus on adipose tissue quantity reflected by overweight and obesity as measured with BMI.

In this review we evaluate current evidence of different options for diagnosing ATD, ranging from anthropometric measurements to tissue biopsies and advanced imaging techniques. In the absence of the possibility of a direct diagnosis of ATD, we use consequences of ATD as surrogate indication of the presence of ATD.

2. Adipose tissue dysfunction

When total energy intake exceeds energy expenditure, this excess energy is stored in adipose tissue leading to enlargement of adipocytes. As a consequence, hypertrophic adipocytes produce chemotactic adipokines which attract macrophages to adipose tissue.[16;17] Inflamed adipose tissue is able to produce large amounts of free fatty acids (FFA) and pro-inflammatory adipokines, such as tumor necrosis factor alpha (TNF-α), leptin, chemerin and interleukin-6 (IL-6), whereas the production of the protective adipokine adiponectin

is reduced.[18-20] ATD refers to the imbalanced production and release of pro- and anti-inflammatory adipokines. The systemic metabolic consequences of ATD include systemic low-grade inflammation, hypercoagulability, elevated blood pressure, dyslipidemia and insulin resistance. Insulin resistance occurs as a result of interference with the intracellular insulin signaling cascade by TNF-α and FFA in various target organs.[18] Part of these systemic metabolic consequences are clustered in the metabolic syndrome, which is defined as the presence of ≥3 of the following items: waist circumference >102 cm (men) or >88 cm (women), triglycerides ≥1.7 mmol/L, HDL-c <1.03 mmol/L (men) or <1.29 mmol/L (women) or use of lipid-lowering medication, systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg or use of blood pressure-lowering medication, fasting plasma glucose ≥5.6 mmol/L or use of glucose-lowering medication.[21;22]

Although obesity is the most important driver of ATD development, not all, but approximately 80% of obese individuals become insulin resistant.[10] Moreover, 10-40% of non-obese individuals develop insulin resistance,[12;13;23] indicating that other factors are also involved in the development of ATD. Both exogenous factors such as physical inactivity[24;25] and the dietary intake of saturated fat,[26;27] as well as endogenous susceptibility such as a low birth-weight,[28;29] genetic predisposition[30;31] and an overactive sympathetic nervous system,[32;33] may all contribute to the development of ATD.

Dysfunctional adipose tissue contributes to the development of diabetes mellitus by causing insulin resistance[34] and through cytotoxic effects of pro-inflammatory adipokines and free fatty acids on pancreatic beta cells, leading to diminished insulin production (figure 1).[35] There are various pathophysiological mechanisms linking ATD to atherosclerotic vascular diseases, including systemic low-grade inflammation by production of IL-6 by adipose tissue, procoagulant state as a result of plasminogen activator inhibitor-1 (PAI-1) production, direct effects of adipokines on the endothelium, activation of the renin-angiotensin-aldosterone system by adipose tissue production of angiotensinogen and activation of the sympathetic nervous system possibly due to high levels of insulin, leptin and angiotensin II centrally exerting a sympatho-exitatory response.[36-38] Diagnosing ATD may identify patients at high risk for the development of diabetes mellitus and vascular diseases and may guide preventive measures in an early stage. Potential diagnostic tools for identifying patients with ATD are outlined below. The diagnostic value will be evaluated in the context of pathophysiological characteristics (morphologic changes in adipose tissue and plasma adipokine concentrations), as well as to clinical outcome of ATD (metabolic syndrome, DM2 and cardiovascular diseases).

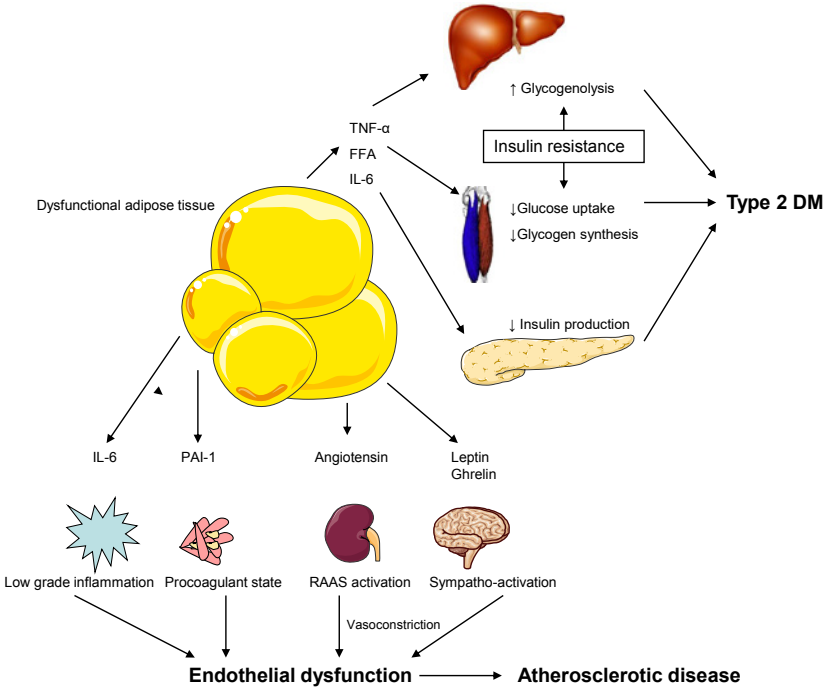

Figure 1. Adipose tissue dysfunction leads to diabetes mellitus and atherosclerotic disease.

Elevated levels of free fatty acids (FFA), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) interfere with the insulin signaling cascade of the liver and muscles (insulin resistance). As a consequence, there is less glucose uptake and glycogen synthesis in the muscle and enhanced glycogenolysis in the liver, causing a raise in serum glucose levels (impaired glucose tolerance). The production of insulin itself is diminished due to toxic effects of IL-6 and FFA on the pancreatic beta-cells. The lack of insulin combined with an impaired glucose tolerance leads to the development of type 2 diabetes mellitus.

Atherosclerotic disease starts with the release of pro-inflammatory (IL-6), procoagulant (plasminogen activator inhibitor 1 (PAI-1)) and hormonal (angiotensin, leptin, ghrelin) factors causing respectively systemic low grade inflammation, a procoagulant state and activation of the renin-angiotensin-aldosterone-system (RAAS) and sympathetic nervous system. The RAAS and sympathetic nervous system activation cause vasoconstriction, in combination with the inflammation and prothrombotic state leading to endothelial dysfunction and eventually atherosclerotic disease.

3. Anthropometric measurements

There is a strong relation between the quantity of adipose tissue and ATD.[39-41] Adipose tissue quantity, as measured with either BMI or waist circumference (WC) is related to plasma concentrations of adipokines, to morphologic characteristics of adipose tissue, and to the development of the metabolic syndrome, DM2 and vascular diseases.[39-41]

Pro-inflammatory adipokines (IL-6, IL-8, IL-18, TNF-α, PAI1 and leptin) are known to be positively correlated with both BMI and WC[42-45], whereas the anti-inflammatory

adiponectin is negatively correlated with BMI and WC.[42-46] In subcutaneous adipose tissue biopsies, IL-6 and IL-8 expression are associated with waist circumference.[47] Moreover, both BMI and WC are associated with the amount of macrophages in both subcutaneous and omental adipose tissue[48], and with adipocyte size.[48]

The relation between adipose tissue quantity, measured with BMI and WC, and metabolic disturbances is illustrated by the fact that only 5% of normal weight individuals (BMI <25 kg/m^2) fulfil the criteria for the metabolic syndrome[11;39;49;50], compared to 20% in subjects with a BMI 25-30 kg/m^2 and 50% in obese (BMI >30 kg/m^2) individuals.[11;39;49;50] Accordingly, the prevalence of insulin resistance, increases when BMI is higher, ranging from 6% in normal weight subjects (BMI <25 kg/m^2) to 60-80% when BMI is >35 kg/m^2.[11;39;51;52]

Waist circumference reflects visceral adipose tissue (VAT) rather than general adiposity[53-55] and has a stronger relation with the metabolic syndrome and insulin resistance, than BMI.[39-41] Within strata of BMI, a high WC (i.e. >88 cm in women and >102 cm in men) doubles the risk of metabolic syndrome compared to persons in the same BMI category with a normal WC.[39] Moreover, the risk for cardiovascular morbidity and mortality is better reflected by WC than by BMI.[40] This is in line with the observation that ATD is most prominently related to the quantity of VAT.[40;41;56]

Clinical recommendation
Based on these facts we recommend the use of waist circumference rather than BMI in the evaluation of possible ATD.

4. Imaging of adipose tissue

As described in paragraph 3, ATD is strongly related to the quantity of adipose tissue and especially to the quantity of VAT.[40;41;55] Therefore, precise quantitative measurement of (visceral) adipose tissue is important in the diagnosis of ATD. Several imaging modalities are capable of measuring adipose tissue depots in different anatomical locations. Ultrasonography, computed tomography (CT) as well magnetic resonance imaging (MRI) have all been used for this purpose.[57-59] In addition, MRI is well-suited to probe adipose tissue (dys)function using MR spectroscopy.[60]

Quantitative measurement of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) with imaging modalities
Ultrasound measurements of adipose tissue are obtained by measuring the distance between the skin and the linea alba in cm (SAT) and the distance between the peritoneum and the

lumbal vertebrae in cm for VAT. [61;62] As such these measurements are a proxy for the total amount of adipose tissue. CT or MRI measurements of adipose tissue are considered the reference standard. Adipose tissue can be measured in a cross-sectionalfashion, e.g. at the level of the L4-L5 vertebrae, where the amount in cm^2 is computed, or as the total volume of adipose tissue in the abdomen using planimetric software.[59;61-63] In most studies quantification relies on manual segmentation, but semi- and fully automated methods are under development.[64;65] Ultrasonographic measurements of adipose tissue are highly correlated to CT- or MRI measurements of adipose tissue, with Pearson correlation coefficients of 0.64-0.81.[61;62]

Perhaps the most interesting and promising family of techniques to quantify the amount of adipose tissue in different body regions are based on multi-echo three-dimensional chemical-shift-encoding water-fat imaging, also known as 'Dixon' methods. These methods encode both spatial position and chemical shift during the acquisition and subsequently estimate the contributions of water and fat to the measured signal in each voxel.[66] Using these methods, adipose tissue and water can be automatically separated per scanned voxel, allowing direct imaged based water and adipose tissue quantitation.[67]

Examples of ultrasound, CT, MRI and Dixon adipose tissue measurements are provided in Figure 2 and 3.

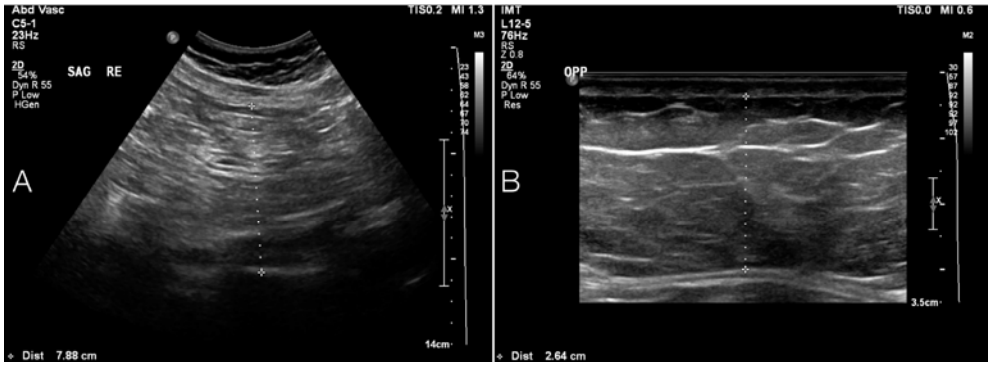

Figure 2. Ultrasonography is well suited for distinguishing subcutaneous and intraabdominal adipose tissue.

Although intra abdominal adipose tissue is not visualized directly with ultrasonography, the anteroposterior distance between the peritoneum behind the rectus musculature and the vertebral colum (panel A) can serve as reasonable proxy measure for the amount of adipose tissue. Measurements are performed with a 5 MHz transducer. Subcutaneous adipose tissue is measured by measuring anteroposterior distance between the skin and the linea alba between the rectus abdominis muscle with a 12.5 MHz transducer (panel B).

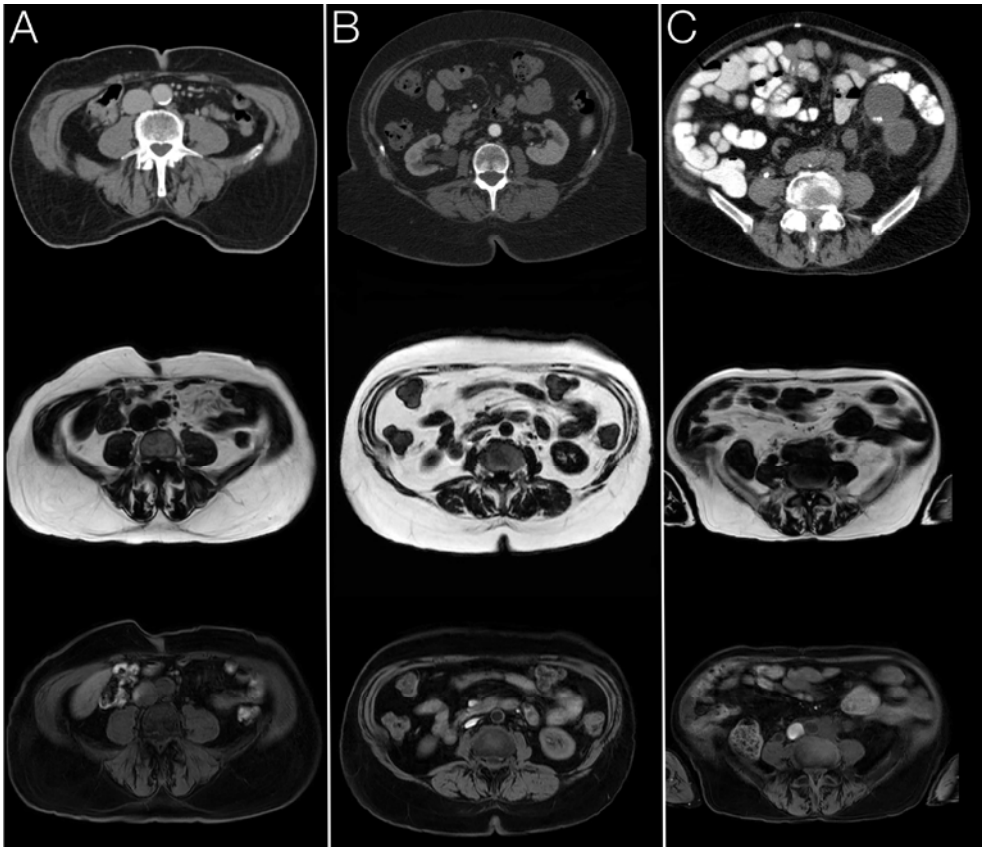

Figure 3. Both CT (top row) and MRI (middle and bottom rows) are highly reliable modalities to map the spatial distribution of abdominal adipose tissue.

The left column (A) shows a subject with relatively little intra abdominal adipose tissue and relatively abundant subcutaneous adipose tissue. In the middle column a subject with abundant intra abdominal as well as subcutaneous adipose tissue is shown, whereas the subject in column (C) has little subcutaneous adipose tissue, but abundant intra-abdominal adipose tissue. MRI images were obtained using the Dixon technique (bottom row) which allows for separation of the signals from water and adipose tissue and reconstruction of separate images showing both components separately, greatly aiding quantification (see text).

The quantity of VAT, as measured with ultrasound, CT or MRI, is positively related to plasma concentrations of the pro-inflammatory adipokines IL-6, TNF-α, leptin and retinol binding protein 4 (RBP-4). [68] [69-72] For SAT, this relation is considerably attenuated or non-existent. [68] [69-72] Adiponectin levels are negatively correlated to both VAT and SAT[69;72-75], although the correlation with VAT is consistently stronger than the correlation with SAT. [69;72;74]

Volumetric CT, MRI and ultrasound measurements of VAT are consistently correlated more strongly to cardiometabolic risk factors (hypertension, impaired fasting glucose) and the development of the metabolic syndrome, DM2 and (subclinical) atherosclerotic disease compared to SAT.[56;76-78;78] About 20% of men and 10% of women have a high amount of VAT (>90th percentile healthy referent sample) at CT-scanning, despite having a normal waist circumference.[79] These persons have a 20% higher risk of developing the metabolic syndrome compared to subjects with an elevated waist circumference, but with a low amount of VAT on CT-scan.[79;80]

Functional imaging of adipose tissue with ^{1}-H-Magnetic Resonance spectroscopy

A promising MR imaging based technique is proton magnetic resonance spectroscopy.

With ^{1}-H-MR-spectroscopy it is possible to quantify localized lipid content in relation to the amount of water. Chemical characteristics of water and fat and their reaction to magnetic forces in the MRI-scanner are used to obtain a visual spectrum of metabolites.[81] ^{1}-H-MR-spectroscopy has been used to quantify free fatty acids, such as triglycerides (TG), poly-unsaturated fatty acids (PUFA), total unsaturated fatty acids (TUFA) and saturated fatty acids (SFA), in the myocardium, liver, breast, muscle, bone marrow and SAT.[82-86] In general, a reduction in saturated fatty acid ingestion reduces the risk of a cardiovascular event, with 17%, probably via reduction of LDL-cholesterol levels. There is no effect of reducing saturated fatty acids on cardiovascular or all-cause mortality. (Cochrane review). Patients with DM2 have more unsaturated fatty acids in the liver compared to patients without DM2, influencing insulin resistance as reflected in higher glucose and HOMA-IR (Homeostatic Model Assessment – Insulin Resistance) levels.[86] Exercise reduces intrahepatic triglyceride content up to 50%, especially in males[87], even in the absence of changes in total body fat or VAT.[88] Low hepatic triglyceride content is related to a lower risk of developing non-alcoholic fatty liver disease, a condition frequently seen in obese subjects.[87]

Hepatic triglyceride content increases from 2.0 to 4.3% in healthy men after a 3-day high fat high energy diet, consisting of 800 ml whipped cream added to a normal diet of about 2100 kcal/day.[85] Contrary, a 3-day low fat low energy diet, consisting of less than 500 kcal/day reduced intrahepatic triglyceride content by 4% in patients with DM2.[89] These observations stress the short-term and flexible reactions of different (non-adipose) tissues to diet and exercise interventions as measured with ^{1}-H-MRS.

Only limited data on lipid composition and reactions to interventions concerning (abdominal) adipose tissue are available. Quantification of triglyceride content of SAT with ^{1}-H-MRS at 1.5 Tesla revealed that there was no correlation with serum lipid concentrations.[82;90] The amount of unsaturated fatty acids in abdominal adipose tissue correlated negatively with the amount of SAT and positively with the amount of VAT.[90] PUFA/TUFA and PUFA/TG ratios, as measured with MR spectroscopy, are higher in persons with the metabolic syndrome, especially in the omental adipose tissue depot, compared to subjects without the metabolic syndrome.[60] MR spectroscopy is a non-invasive technique and a direct

way of measuring metabolic characteristics of abdominal adipose tissue which makes this technique a promising diagnostic tool for the identification of ATD.

Clinical recommendation

Although both CT and MRI measurements of adipose tissue provide detailed information of VAT and SAT, and (in the case of MRS) are feasible in research, implementing these modalities in clinical practice might be difficult due to both costs and logistics. Ultrasound measurements might be a useful alternative.

5. Insulin resistance and metabolic syndrome

Insulin resistance is a condition with decreased sensitivity or responsiveness to the metabolic actions of insulin, caused by interference of the intracellular insulin signaling cascade by TNF-α and FFA.[18] Insulin resistance causes reduced capacity of adipocytes to store FFAs, causing lipid accumulation in muscles, pancreas and liver contributing to insulin resistance. As a result, glucose clearance is diminished and glucose production enhanced in the liver, leading to a hyperinsulinemic state.[91] Insulin resistance or sensitivity can be assessed using several mathematical rules, such as the HOMA-IR or revised Quicki methods. The revised Quicki method correlates better with the reference standard[92] (euglycemic clamp) but necessitates more laboratory values (such as non-esterified fatty acids) than the HOMA-IR (which uses insulin and glucose levels). However, both methods have been used in clinical research regarding adipokines.

Insulin resistance related to adipokines and morphology of adipose tissue

Adiponectin is consistently negatively correlated to insulin resistance [93-97] whereas leptin is consistently positively correlated [96-99] Together, adiponectin and leptin levels explained 38% of HOMA-IR variance in a group of elderly individuals.[97] Resistin[99] and TNF-α[100] were also found to be positively correlated to HOMA-IR. For IL-6 no relation was seen with insulin resistance in non-obese diabetic patients[96], but in patients with a BMI >27kg/m2 a relation between IL-6 and insulin resistance exists[101;102], suggesting a role for the quantity of adipose tissue. In insulin resistant mice, larger adipocytes and more macrophage infiltration were seen than in mice without insulin resistance.[103] In patients undergoing bariatric surgery or cholecystectomy, the presence of foam cells (macrophages loaded with lipids) in VAT was positively correlated to insulin concentrations, whereas there was no correlation between foam cells in the SAT and insulin concentrations.[104] Therefore, measuring insulin resistance is a diagnostic tool for identifying ATD.

Insulin resistance related to metabolic syndrome, DM2 and cardiovascular disease

Yearly, 5-10% of people with insulin resistance develop overt diabetes[105] and the presence of insulin resistance and the metabolic syndrome are highly correlated,[106] with about 60% of patients with insulin resistance also fulfilling the criteria for metabolic syndrome.[107;108] In a large meta-analysis it was shown that there is a 46% increased risk of coronary heart disease per 1 standard deviation increase of HOMA-IR,[109] the risk for all-cause mortality is 64% increased in patients with a HOMA-IR >2.8 as compared to patients with a HOMA-IR <1.4.[110]

Metabolic syndrome related to adipokines and morphology of adipose tissue

Since almost all characteristics of the metabolic syndrome can be regarded as systemic metabolic consequences of ATD, it is not surprising that there are strong associations of the metabolic syndrome with elevated plasma levels of pro-inflammatory adipokines (leptin,TNF-α, IL-6) and lower adiponectin levels. [45;94;98;111;112] Moreover, in SAT biopsies of subjects with metabolic syndrome the macrophage content is higher compared to subjects without the metabolic syndrome, illustrating the relation between morphologic changes in adipose tissue, ATD and clinical features.[113]

Metabolic syndrome related to DM2 and cardiovascular diseases

Since characteristics of the metabolic syndrome are systemic consequences of ATD, consequently, associations between metabolic syndrome and DM2 and cardiovascular diseases originate in the presence of ATD. There is a significant relationship between the metabolic syndrome and the occurrence of incident coronary heart disease, with a 60 to 200% higher risk for subjects with compared to subjects without the metabolic syndrome. [114;115] Also, both cardiovascular (80% higher risk) and overall (40% higher risk) mortality are higher in subjects with the metabolic syndrome compared to subjects without the metabolic syndrome.[115;116] The presence of the metabolic syndrome constitutes an increased risk of 137% of developing type 2 diabetes, independent of glucose levels.[117]

Clinical recommendation

Determining a HOMA level and the presence or absence of metabolic syndrome is recommended.

6. Plasma concentrations of adipokines as a surrogate of ATD

Adipokines are produced by adipose tissue and secreted into the systemic circulation and can be measured in peripheral venous blood samples. The plasma concentrations of various adipokines vary widely between patients and patient groups and can be influenced as a result of interventions such as weight loss and medication.[118-125] Elevated levels of

pro-inflammatory adipokines and decreased levels of anti-inflammatory adipokines are key features of ATD.

Pro-inflammatory adipokines

The adipokine leptin is the product of the *obese*-gene and is known for its inhibitory effect on the sense of appetite.[126;127] The production of leptin by adipose tissue is stimulated by pro-inflammatory cytokines as TNF-α and by lipopolysaccharide.[16] At a certain point the brain may become desensitized for the inhibitory effects on the food intake, a state called leptin resistance, creating a vicious circle of overeating, gaining weight and developing insulin resistance.[128;129]

Retinol-binding protein-4 (RBP-4) is an adipokine involved in the transport of retinol (vitamin A) throughout the body.[130] It is secreted by hepatocytes, adipocytes and macrophages[131;132] and is important in regulating glucose homeostasis. Expression of RBP-4 is inversely related to the cellular expression of glucose transporter type 4.[133] High plasma concentrations of RBP-4 are related to decreased insulin sensitivity and to features of the metabolic syndrome.[134;135]

Produced by monocytes, macrophages and adipocytes, TNF-α is a pro-inflammatory cytokine that plays an important role in the development of insulin resistance by inducing apoptosis of adipocytes[91] and by interfering with the intracellular insulin signaling pathway downstream from the insulin receptor.[133]

Anti-inflammatory adipokines

Adiponectin was discovered in 1996[136] and is the most intensely studied adipokine. Adiponectin has anti-inflammatory and anti-atherogenic properties, and is positively correlated with insulin sensitivity.[137] Infusion of adiponectin in rats increases insulin sensitivity.[138] There is a strong relation between adiponectin plasma levels and the amount of VAT,[139] whether this is an independent effect regardless the size of other adipose tissue depots is subject of debate.[74;139-141]

Recently, secreted frizzled related protein 5 (Sfrp5) is identified as a novel adipokine with anti-inflammatory characteristics, being an antagonist of the inflammatory protein WNT5a, preventing WNT5a from binding to its receptor. In Sfrp5 deficient mice, a high calorie diet induced severe glucose intolerance and an accumulation of macrophages in their adipose tissue, both diminished after administration of Sfrp5.[142] Sfrp5 is downregulated in obese individuals, causing high levels of WNT5a, possibly leading to inflammation and insulin resistance.[133] No studies have been performed yet towards the association between Sfrp5 and cardiovascular diseases or DM2.

Adipokine concentrations related to morphology of adipose tissue

In non-obese individuals, adipose tissue mainly consists of adipocytes, small amounts of pre-adipocytes, lymphocytes, macrophages, fibroblasts and vascular cells.[143] Two phenotypes of macrophages are abundant in adipose tissue; the M1 macrophages produce pro-inflammatory cytokines[16;144] (IL-6, TNF-α) and stimulate adipocytes to secrete pro-inflammatory adipokines (leptin, resistin, RBP-4).[133] M2 macrophages downregulate the synthesis of pro-inflammatory adipokines by adipocytes and upregulate secretion of anti-inflammatory adipokines (SFRP-5, adiponectin).[16;133] In obese individuals, enlarged adipocytes produce chemotactic cytokines that mainly attract M1 macrophages causing an imbalance in pro- and anti-inflammatory adipokines, a condition referred to as ATD.[17;145] Plasma levels of adipokines relate to morphologic characteristics of both subcutaneous and VAT.[146-148] Plasma progranulin,[148] adiponectin,[147] HGF,[147] IP-10[147] and MCP-1[146] are strongly correlated with the number of infiltrated macrophages and adipocyte size in adipose tissue biopsies. These observations show that plasma levels of adipokines adequately reflect the inflammatory (and dysfunctional) state of the adipose tissue and can therefore be used in diagnosing ATD. Table 1 shows an overview of frequently studied adipokines and their characteristics.

Adipokine concentrations related to metabolic syndrome, DM2 and cardiovascular diseases

Elevated concentrations of plasma adipokines are associated with the development of the metabolic syndrome. High levels of (pro-inflammatory) leptin, RBP-4, PAI-1 and visfatin and a low level of the protective adiponectin are seen in patients with insulin resistance, metabolic syndrome and DM2.[45;94;98;111;134;149-162] Interestingly, for these adipokines, associations with metabolic syndrome hold even after adjusting for BMI.[45;111;154;158;159;162].

For atherosclerotic disease, the association of serum adipokine levels and the development of disease is less distinct. Although a 44% risk reduction for myocardial infarction was observed in patients with the highest levels of adiponectin[114], later studies and meta-analyses[163-167] showed no relation between levels of adiponectin, leptin, adipsin, resistin and PAI-1 and the development of atherosclerotic disease (after adjustment for risk factors). The association between ATD and atherosclerotic disease might be mediated via risk factors of the metabolic syndrome, by development of insulin resistance and inflammation or by direct effects of adipokines on the vessel wall.[168]

Reliability of adipokine measurement in plasma

Plasma adipokine levels are fairly stable over time within individuals and a random peripheral blood sample therefore is a reliable representation of the mean level.[169] Adipokines can be measured by enzyme linked immunosorbent assay (ELISA),[170] and with a multiplex immuno-assay.[171] A fairly good correlation is seen between measurements with multiplex

Table 1. Overview of characteristics of some of the most important and well known adipokines

Adipocytokine	Characteristics				
	Anti-inflammatory	Pro-inflammatory	Metabolic function	Chemotaxis	Other
Adiponectin	+	-	↑	-	
Adipsin	+	-	↑	-	
Apelin	-	-	↓	-	
Adipolin	+	-	↑	-	
Chemerin	-				
C-reactive protein	-	+	-	-	
Ghrelin	-	-	↓	-	
Granulocyte colony stimulating factor	-	-	-	+	
Hepatic Growth Factor (HGF)	-	-	-	-	Angiogenesis
Interleukin 1 beta (IL1-β)	-	+	-	-	
Interleukin 6 (IL-6)	+ (via inhibition IL1)	+	-	-	
Interleukin 8 (IL-8)	-	+	-	-	
Interleukin 17beta (IL-17β)	-	+	-	-	
Interleukin 21 (IL-21)	-	+	-	-	
Interferon gamma induced protein 10 (IP10)	-	+	-	+	
Leptin	+	+	↓↑		
Lipocalin					
Monocyte chemo attractant protein 1 (MCP1)	+	-	-	+	
Macrophage migration inhibitory factor (MIF)	-	+	-	+	
Nerve Growth Factor (NGF	-	-	-	-	Angiogenesis
Omentin	+	-	↑	-	
Plasminogen activator inhibitor 1 (PAI-1)	-	-	-	-	Prothrombotic agent
Retinol binding protein 4 (RBP-4)	-	+	↓	-	
Resistin	-	+	↓	-	
Serpin	-	+	-	-	Prothrombotic agent
Serum Amyloid A protein 1 (SAA1)	-	+	-	-	
Secreted Frizzled related Protein (SFrP5)	+	-	-	-	
Tumor Necrosis Factor alpha (TNF-α)	-	+	-	?	
Thrombopoietin (TPO)	-	-	-	-	Prothrombotic agent
Transformation Growth Factor beta (TGF-β)	+	-	-	-	Pro-apoptotic
Visfatin	-	-	↑	-	
Vaspin	-	-	↑	-	
Vascular Cell Adhesion Molecule 1	-	+	-	?	

+ ; characteristic present -; characteristic absent ↑; enhances metabolic function/insulin sensitivity ↓; attenuates metabolic function/insulin sensitivity ? not entirely known

assay and ELISA, next to little cross-reactivity between the antibodies of the different adipokines.[171] This makes multiplex immuno-assay a suitable technique for adipokine profiling in patients or cohorts as, in contrast to ELISA, multiple adipokines can be measured in a single measurement.

Clinical recommendation
Measurement of adipokines is not routinely available in most laboratories and there are no reference values yet for adipokine plasma concentrations, making interpretation of adipokine levels on an individual level difficult and therefore these measurements are not yet useful in daily clinical practice.

7. Visceral adiposity index

The visceral adiposity index (VAI) was developed to estimate visceral adiposity dysfunction. It is a sex-specific index based on WC, BMI, triglycerides and HDL-cholesterol. [172] This index is correlated to all factors of the metabolic syndrome and also to the occurrence of cardiovascular events. The association with the metabolic syndrome is not so surprising since three factors of the metabolic syndrome are also used in the VAI. The association with cardiovascular events however, is interesting, since other surrogates for ATD do not show this association. Moreover, VAI is associated with many adipokines, and showed better correlations then WC or BMI.Specific measurements of VAT or SAT were not shown in this study . [173]However, if triglycerides are >3,15 mmol/L or if WC is large, the VAI is unreliable. [174] Moreover, the VAI is developed and validated in a Caucasian cohort and it is uncertain how VAI would perform in other populations.

Clinical recommendation
The VAI could be a reliable method for determining ATD, taking limitations into account.

8. Adipose tissue biopsies to measure ATD

Adipose tissue biopsies are potentially the most direct way to evaluate ATD although this is a morphological evaluation and not a functional evaluation. The clinical usefulness of adipose tissue biopsies might be limited, especially with regard to VAT biopsies, as they can only be obtained during abdominal surgery. A needle biopsy of SAT however could be performed more easily however and could be used in clinical practice. Great advantage of taking biopsies from SAT or VAT is that cellular structures of adipocytes, macrophage infiltration, and *ex vivo* production of adipokines can be investigated. A key feature of ATD is infiltration of macrophages in adipose tissue[17] and polarization of these macrophages

predominantly to the M1-phenotype.[175] Elevated *ex vivo* production of pro-inflammatory adipokines by adipose tissue biopsies and diminished production of anti-inflammatory adipokines[18-20] reflect a state of ATD. In adipose tissue biopsies from subjects with either insulin resistance, metabolic syndrome or DM2 all features of ATD are seen.[113;141;176-180] There is enhanced macrophage infiltration,[113;179] higher expression of pro-inflammatory adipokines[141;178] and lower expression of adiponectin[73;176;177;180] as compared to biopsies of overweight, yet metabolically healthy controls. Figure 4 shows In pericoronary adipose tissue biopsies obtained during cardiac surgery, macrophage infiltration and polarization towards the pro-inflammatory M1-type are more pronounced in patients with coronary atherosclerosis than in those without.[181;182] Also, there is a negative association between adiponectin concentrations and macrophage infiltration in adipose tissue in patients undergoing abdominal aortic surgery.[147]

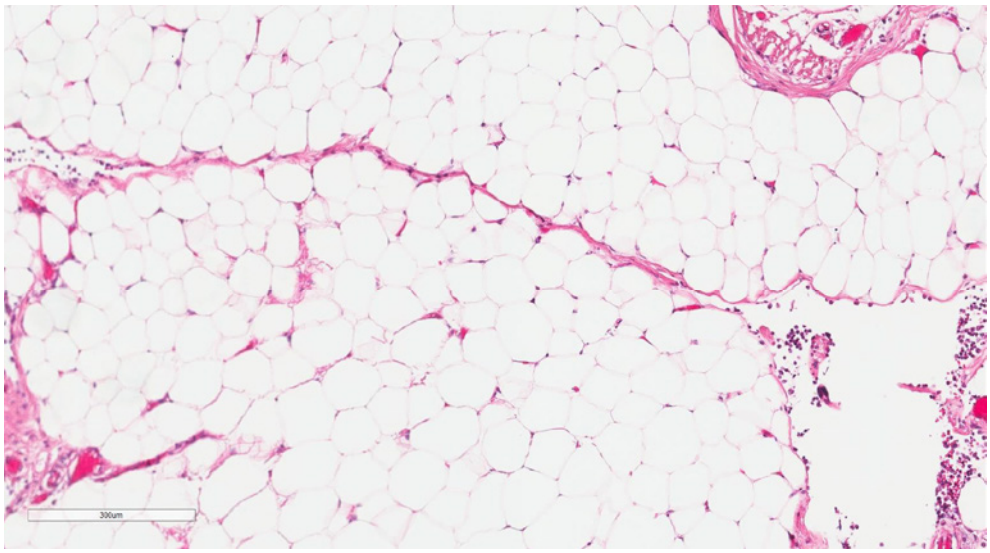

Figure 4. Adipose tissue biopsy from a person with metabolic syndrome; showing adipocytes and signs of inflammation.

After weight loss, either due to a (very) low calorie diet or bariatric surgery, significant improvements in metabolic parameters, such as insulin sensitivity, are seen.[183-185] These effects are measurable directly after the intervention and linger when the weight loss is sustained. Improvements in characteristics of ATD in adipose tissue biopsies develop simultaneously with the metabolic improvements. Reduction of both macrophage infiltration, adipocyte size and inflammatory adipokine concentrations are seen after weight loss due to bariatric surgery and very low calorie diets.[186-188] These effects were seen both shortly (5 days) and 1-3 months after the weight loss intervention, when participants

had approximately lost 15% of their body weight.[186-188] Although VAT is generally believed to be more pathogenic, morphologic changes were seen in both subcutaneous and visceral biopsies.[186-188] However, no direct comparison between morphologic changes in different depots has been studied.

Clinical recommendation

In daily clinical practice, adipose tissue biopsies merely for diagnostic purposes will not be performed, and therefore studying adipose tissue biopsies will remain primarily a research area.

9. Conclusion

Adipose tissue dysfunction is an imbalance in the production of pro- and anti-inflammatory adipokines leading to insulin resistance, endothelial dysfunction and eventually to DM2 and vascular diseases. Thus, diagnosing ATD is of clinical relevance and may even be considered a future treatment target. ATD can be diagnosed in both lean and obese individuals. Adipose tissue biopsy is considered to be the reference standard for the diagnosis of ATD, as most features of ATD can be directly assessed. Other means are measurement of adipokine plasma levels in peripheral blood samples, although this is not implementable at an individual level due to large intra-individual variations and lack of standardization of the measurements.

'Currently, we consider waist circumference, insulin resistance and the presence of the metabolic syndrome to be the main options to be used in daily clinical practice for estimating ATD. Clearly, it would be a great advantage when more direct diagnostic tools could be used. Of the diagnostic options mentioned, measuring plasma adipokines in blood is, to our opinion, most promising, since this is relatively non-invasive and cheap compared to other options such as imaging and biopsies (especially abdominal adipose tissue biopsies). Possibly, a panel of several pro- and anti-inflammatory adipokines could be compiled, giving clinicians an 'adipokine-score' indicative of the level of ATD.'

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Chapter 3

Feasibility and reproducibility of free fatty acid profiling in abdominal adipose tissue with 1H-magnetic resonance spectroscopy at 3 T: differences between lean and obese individuals

Ilse M. Schrover[1], MD; Tim Leiner[2], MD, PhD; Dennis W.J. Klomp[2], PhD; Jannie P. Wijnen[2], PhD; Cuno S.P.M Uiterwaal[3], MD, PhD; Wilko Spiering[1], MD, PhD; Frank L.J. Visseren[1], MD, PhD

Departments of Vascular Medicine[1], Radiology[2] and Julius Center for Health Sciences and Primary Care[3], University Medical Center Utrecht, The Netherlands.

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Abstract

Purpose: To evaluate feasibility and reproducibility of free fatty acid (FFA) measurement, for diagnosing adipose tissue dysfunction, by ^{1}H-Magnetic Resonance Spectroscopy (^{1}H-MRS) in different abdominal adipose tissue depots in healthy obese and lean subjects.

Materials and Methods: Poly-unsaturated fatty acids (PUFA), total unsaturated fatty acids (TUFA),triglycerides (TG) and their ratios were determined in three adipose tissue depots of 12 obese and 13 lean subjects. Subjects underwent two separate examinations to assess reproducibility.

Results: In lean subjects, 44% of measurements failed due to inclusion of non-adipose tissue in the spectroscopy voxel, as opposed to 23% in obese subjects. Reproducibility of PUFA, TUFA and TG was moderate to good in obese subjects (Intraclass Correlation Coefficients (ICCs) 0.18-0.75), and poor to moderate in lean subjects (ICCs -1.04-0.55) in subcutaneous and omental adipose tissue. In the perirenal adipose tissue, ICCs were poor in both lean and obese subjects (-0.794-0.013). PUFA/TUFA and PUFA/TG were higher in omental adipose tissue in obese vs. lean subjects ($35*10^{-3}$ vs. $0.16*10^{-3}$, p=0.01 and $2.05*10^{-3}$ vs. $0.01*10^{-3}$, p=0.02 respectively).

Conclusion: ^{1}H-MRS is a feasible and reproducible method for FFA profiling in abdominal adipose tissue in abdominally obese individuals. ^{1}H-MRS has potential as diagnostic tool for non-invasive identification of adipose tissue dysfunction.

Key words: spectroscopy, proton, adipose tissue, metabolic syndrome, fatty acid

Introduction

Adipose tissue, in particular abdominal adipose tissue, is a metabolically active organ that produces pro- and anti-inflammatory hormones and cytokines (adipocytokines) influencing systemic metabolism, leading to insulin resistance, low-grade inflammation and hypercoagulability(1). Abdominal adipose tissue is considered dysfunctional when there is an imbalance in pro- and anti-inflammatory adipocytokine production; favouring pro-inflammatory adipocytokine production (2;3). Dysfunctional adipose tissue is causally related to the development of insulin resistance, the metabolic syndrome, cardiovascular diseases, type 2 diabetes mellitus (T2DM) and cancer(4;5).

In patients with a history of cardiovascular disease, the presence of metabolic syndrome leads to a 50% increased risk of a subsequent cardiovascular events and a 45% increased mortality risk(6). There is an increased risk of common cancers in patients with the metabolic syndrome, especially breast cancer and colorectal cancer(7). Identifying adipose tissue dysfunction (ATD) may identify subjects at risk of developing these diseases(1). Estimation of adipose tissue (dys)function can be done indirectly by measuring plasma concentrations of adipocytokines, by determining the presence of the metabolic syndrome or by measuring insulin resistance(8;9). A direct, but invasive method to diagnose ATD is performing a subcutaneous adipose tissue biopsy(10;11). However, the link between subcutaneous adipose tissue and development of adipose tissue dysfunction is much weaker compared to intra-abdominal adipose tissue(12). A reliable non-invasive estimation of abdominal adipose tissue function could be used in determining the risk for cardiovascular diseases, T2DM and cancer and may also play a role in evaluating the effects of interventions.

With ^{1}H-Magnetic Resonance Spectroscopy (^{1}H-MRS) metabolic imaging of tissues can be performed to study their chemical composition non-invasively. In both liver and myocardial tissue, free fatty acids (FFA) have been identified and quantified by MRS (13;14). These measurements have been validated with gas chromatography, and have been shown to adequately discriminate triglycerides (TG), (poly)unsaturated, and saturated fatty acids (PUFA resp. TUFA)(13;14). When subcutaneous and intra-abdominal adipose tissue biopsies are compared with respect to their FFA profile, intra-abdominal adipose tissue has a significantly higher proportion of saturated fatty acids and a significantly lower proportion of mono-unsaturated fatty acids[15]. Moreover, BMI and visceral fat area were negatively correlated to ω-3 fatty acids (poly unsaturated fatty acids) (15), which are believed to be cardioprotective (16-18). Since the amount of intra-abdominal adipose tissue is an important determinant of ATD and its metabolic consequences (12), this implies that differences in FFA could be indicative for ATD(15). With ^{1}H-MRS at 1.5 Tesla, the triglyceride content of adipose tissue can be quantified, but (poly)unsaturated fatty acids can only be identified (19). ^{1}H-MRS at higher field strength (3.0 Tesla) increases the chemical shift dispersion thereby improving spectral resolution and increases signal-to-noise ratio (SNR), thus enabling better identification and quantification of free fatty acids.

The main objective of our study was to investigate the feasibility and reproducibility of quantifying triglycerides and (poly)unsaturated fatty acids in different abdominal adipose tissue depots with ^{1}H-MRS at 3.0 Tesla in abdominally obese and lean subjects. In addition, we aimed to determine the association between free fatty acids and clinical markers of ATD, such as (characteristics of) the metabolic syndrome and insulin resistance.

MATERIALS AND METHODS

Study Design And Participants

A single centre, cross-sectional study was performed at a university medical center. The study protocol was approved by the local Ethics Committee and conducted in accordance with the principles of the Declaration of Helsinki. All participants gave written informed consent prior to enrolment after full explanation of study procedures.

Between May 2011 and April 2012healthy men and women, (n=25, mean age 48 (range 25-65) were included. Participants were not included if they had a medical history of cardiovascular, liver, renal or pulmonary disease or if they used any medication, except for acetaminophen, antacids, topical creams, inhalation medication, nasal sprays, and eye drops. Women who were pregnant or lactating and persons with MRI-incompatible devices were not included.

Half of the participants were abdominally obese, as defined by a waist circumference of >102 cm (men) or >88 cm (women). They were age- and gender matched to participants with a waist circumference of ≤102 cm (men) or ≤88 cm (women). With an interval of 1 week, all subjects underwent 2 ^{1}H-MRS-examinations. Participants were asked to fast overnight prior to both MR examinations. On the day of the first examination, fasting blood samples and anthropometric measurements were obtained.

Laboratory And Anthropometric Measurements

Fasting venous blood samples were collected at the day of the first ^{1}H-MRS examination for measurement of glucose, insulin, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol. Waist circumference was measured halfway between the lower rib and the iliac crest in full expiration. Body mass index (BMI) was calculated as weight (kg) divided by height^{2} (m). Blood pressure was measured in both arms with an automatic device (Omron™, 705 IT Intellisense, Hoofddorp, The Netherlands); the highest blood pressure was used for data analysis.

Participants fulfilled the ATPIII criteria for the metabolic syndrome when at least 3 of the following characteristics were present: waist circumference >102 cm (men) or >88 cm (women), HDL <1.04 mmol/L (men) or <1.29 mmol/L (women), blood pressure ≥130 mmHg systolic and/or ≥85 mmHg diastolic, fasting glucose ≥5.6 mmol/L or triglycerides ≥1.7 mmol/L (20).

^{1}H-Magnetic Resonance Spectroscopy (MRS)

All measurements were performed on a 3.0 Tesla Philip (Achieva) MR scanner (Philips Healthcare, Best, the Netherlands) using a cardiac RF coil. Subjects underwent transversal and coronal T2-weighted Turbo Spin Echo (T2 TSE) sequences (slice thickness 4mm/gap 0.4mm), the images obtained with these sequences were used for placement of the voxels, combined with a reference scan for localisation in the sagittal plane. Voxels were placed in three adipose tissue depots : perirenal (retroperitoneal) adipose tissue depot (both left and right), subcutaneous adipose tissue depot (both left and right), and the omental adipose tissue depot. Voxels in the perirenal adipose tissue were placed next to or just below the lower pole region of the left and right kidney. With a margin of 10 cm to both the cranial and caudal side, the position of the perirenal adipose tissue voxel was used as point of reference for placement of the subcutaneous adipose tissue voxels (Figure1A). The intra-abdominal adipose tissue voxel was placed at the omentum (Figure 1B).

Voxel size was chosen depending on the amount of adipose tissue present at the different locations and could vary between subjects. The voxel was placed in the adipose tissue, avoiding inclusion of other tissues such as the kidney, bowels or muscles, guided by transversal, coronal and sagittal images. For the omental spectra, respiratory triggering was used. MR spectra were acquired with a STEAM sequence with TE/TR = 8.9/2000 ms and 32 acquisitions. Second order shim gradients were used, with shimming based on the voxel. No water suppression or REST slabs were used.

Room time, including taking subjects in and out of the scanner, positioning of the subjects in the scanner, acquisition of localizers, and T2 weighted images for voxel planning and performing the ^{1}H-MRS, was approximately 35 minutes.

The MR spectra were evaluated using jMRUI software, using AMARES for quantification (21), calculating the area under the curve (AUC) of the metabolites in the MR spectrum.

The methylene (triglyceride) resonance at 1.3 ppm, the PUFA resonance at 2.75 ppm, the water resonance at 4.7 ppm and the TUFA resonance at 5.4 ppm were quantified (Figure 2). MR spectra with a water content >20% ([TG] / [TG] + [H_2O]) <0.8) were not included in the analysis, as this is susceptible to assessing tissues other than adipose tissue (e.g. bowel or kidney). The 20% cut-off value was chosen to balance maximal sensitivity of the spectral measurement and minimal data-exclusion. In the spectra where a PUFA resonance could be clearly distinguished by sight, but AMARES failed to attribute an AUC to the resonance, the AUC was not declared missing, but the lowest value of the PUFA resonance in all subjects in the specific adipose tissue depot was attributed and used for analyses. A sensitivity analysis was performed to test the robustness of our findings when only attributed PUFA resonances by AMARES were included.

Comparison of AUCs of 'single' metabolite resonances is only possible after correction for the distance between the cardiac coil and the voxel, since signal is lost with increasing distance due to the sensitivity profile of the coil, which is the case in more obese subjects. Therefore, we calculated ratios of the different metabolite resonances, providing an internal

correction and making comparison possible. TUFA/TG, PUFA/TG, and PUFA/TUFA ratios were calculated for all spectral measurements.

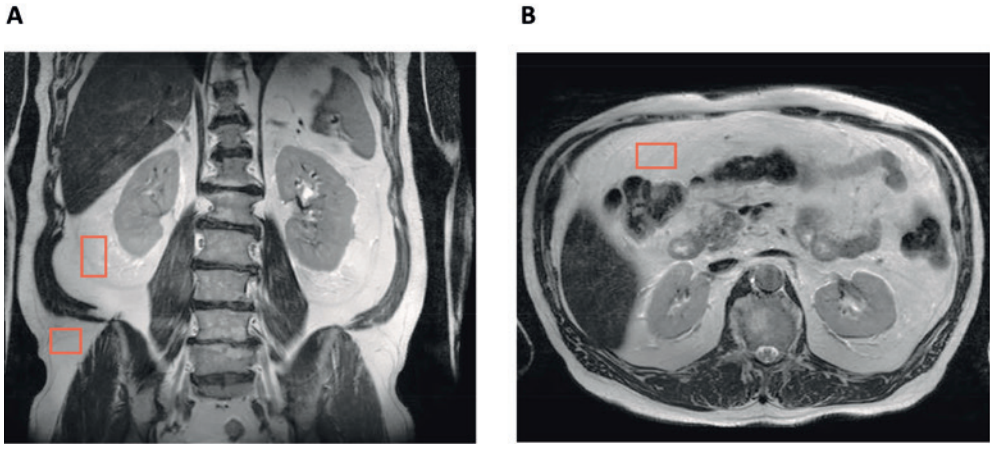

Figure 1. A: MRI image with voxel placement in subcutaneous and perirenal adipose tissue. B: MRI image with voxel placement in omental adipose tissue)

Data Analyses

Baseline characteristics of subjects are presented as means with standard deviations or as medians with interquartile ranges (IQR) when data were not normally distributed.

Reproducibility of measurements was assessed by means of Bland-Altman plots(22), with differences between similar measurements at 2 time points (2 examinations) plotted against the average of both measurements. For each ratio in each adipose tissue depot a Bland-Altman plot was made. Additionally, intraclass correlation coefficients (ICCs) for paired measurements were calculated for TG, TUFA and PUFA measurements in the different depots.

Differences in free fatty acid ratios between the lean and abdominally obese subjects, subjects with and subjects without the metabolic syndrome and those subjects with ≥2 vs. <2 characteristics were assessed with Mann-Whitney-U tests. Linear regression analysis was used to evaluate relations between metabolic and anthropometric parameters as independent variables and the free fatty acid ratios as separate dependent variables. For presentation purposes, ratios were multiplied by 1000. Triglyceride (serum) data were log-transformed due to skewed distribution. P-values <0.05 were considered statistically significant. All analyses were performed with SPSS 15.0 (SPSS Inc, Chicago, IL).

RESULTS

Subjects

Twenty-five subjects were included, 12 with and 13 without abdominal obesity (Table 1). Average age in the 2 groups was 48±13 years and 40% of the subjects were male. In the abdominally obese group the prevalence of metabolic syndrome was 42% and 11 of the 12 abdominally obese subjects had at least 2 characteristics of the metabolic syndrome, as compared to only 2 of the 13 subjects in the lean group (Table 1).

Table 1. Baseline characteristics

	Total (n=25)	Lean (n=13)	Abdominally obese (n= 12)
Male (%)	40	39	42
Age (years)	48 ± 13	48 ± 13	48 ± 13
Height (cm)	174 ± 8	174 ± 9	173 ± 8
Weight (kg)	75.0 ± 16.7	64.8 ± 12.4	86.1 ± 13.5
BMI (kg/m²)	24.7 ± 4.6	21.2 ± 2.3	28.6 ± 3.0
WC (cm)			
Men	97 ± 11	88 ± 7	106 ± 4
Women	84 ± 13	73 ± 6	97 ± 6
Systolic BP (mmHg)	140 ± 18	130 ± 11	151 ± 17
Diastolic BP (mmHg)	82 ± 8	79 ± 9	86 ± 6
Glucose (mmol/L)	5.0 ± 0.6	4.6 ± 0.3	5.3 ± 0.7
Insulin (mIU/L)	6 (4-11)	6 (4-7)	10 (5-12)
HOMA-IR	1.52 (0.82-2.42)	1.25 (0.66-1.52)	2.26 (1.46-2.61)
Cholesterol (mmol/L)	5.5 ± 1.2	5.3 ± 1.1	5.8 ± 1.3
HDL-cholesterol (mmol/L)			
Men	1.31 ± 0.32	1.38 ± 0.43	1.25 ± 0.20
Women	1.60 ± 0.40	1.69 ± 0.37	1.49 ± 0.44
LDL-cholesterol (mmol/L)	3.6 ± 1.0	3.4 ± 0.9	3.8 ± 1.1
Triglycerides (mmol/L)	0.8 (0.5-1.7)	0.7(0.5-1.0)	1.5 (0.6-2.0)
Metabolic syndrome (n (%))	6 (24)	1 (8)	5 (42)
Metabolic syndrome components (n (%))			
0	5 (20)	5 (39)	0 (0)
1	7 (28)	6 (46)	1 (8)
2	7 (28)	1 (8)	6 (50)
3	4 (16)	1 (8)	3 (25)
4	2 (8)	0 (0)	2 (17)

Data are presented as means + standard deviations or as medians with interquartile ranges
n= number of subjects. BMI: body mass index. WC: waist circumference. BP: blood pressure. HOMA-IR: Homeostatic Model of Assessment – Insulin Resistance. HDL: High Density Lipoprotein LDL: Low Density Lipoprotein

Feasibility And Reproducibility Of PUFA, TUFA And TG Measurement In Adipose Tissue Depots By 1H-MRS

In total, 250 spectroscopic measurements in the perirenal (n=100), subcutaneous (n=100) and intra-abdominal adipose tissue (n=50) were performed. In 7 (3%) of these measurements it was not possible to determine the PUFA, TUFA or TG resonance, due to movement of the participant, causing scanning of non-adipose tissue (reflected in a large water signal and very small TG signal). In all other measurements, after post processing with jMRUI software, metabolic components could be clearly discriminated. In Figure 2, a representative spectrum of adipose tissue of an obese participant is shown, whereas in figure 3 representative spectra of all adipose tissue depots in both obese and lean participants are shown on a smaller scale, avoiding predominance of the TG resonance and making quantitative comparison possible. Overall, 81 out of 243 (33%) of measurements contained >20% water, indicating that other tissues than adipose tissue were also included in the voxel. This was more prominent in the omental (49%) than in the subcutaneous adipose tissue (13%). Also, marked differences between abdominally obese and lean subjects were observed as 44% of the measurements in the lean subjects contained >20% water compared to 23% in the measurements in abdominally obese subjects. The AUC of the PUFA resonance was not attributed by the software in 38% (obese subjects) and 57% (lean subjects) of measurements, although the resonance was visually detectable. There were small, non-significant differences between the measurements at the left and right side in the perirenal and subcutaneous adipose tissue depots.

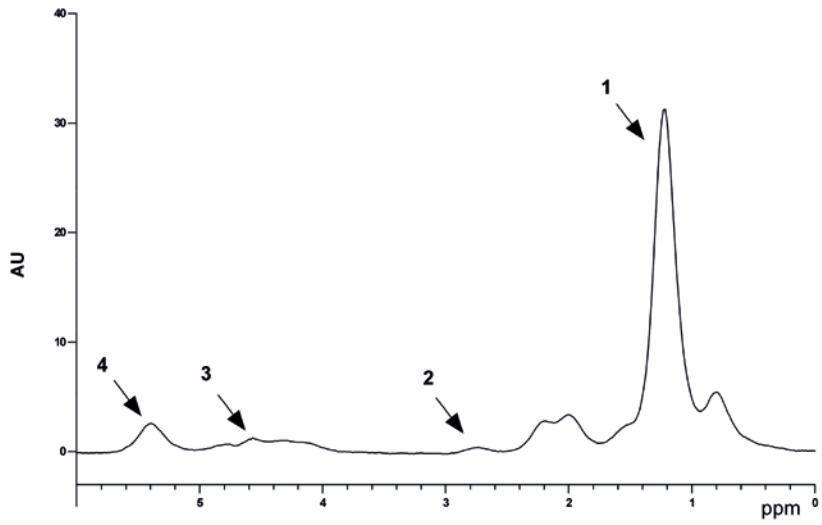

Figure 2. Obtained spectrum after processing with jMRUI showing the different free fatty acids resonances.

1= triglycerides; 2=Poly Unsaturated Fatty Acids (PUFA); 3= water; 4= Total Unsaturated Fatty Acids (TUFA)

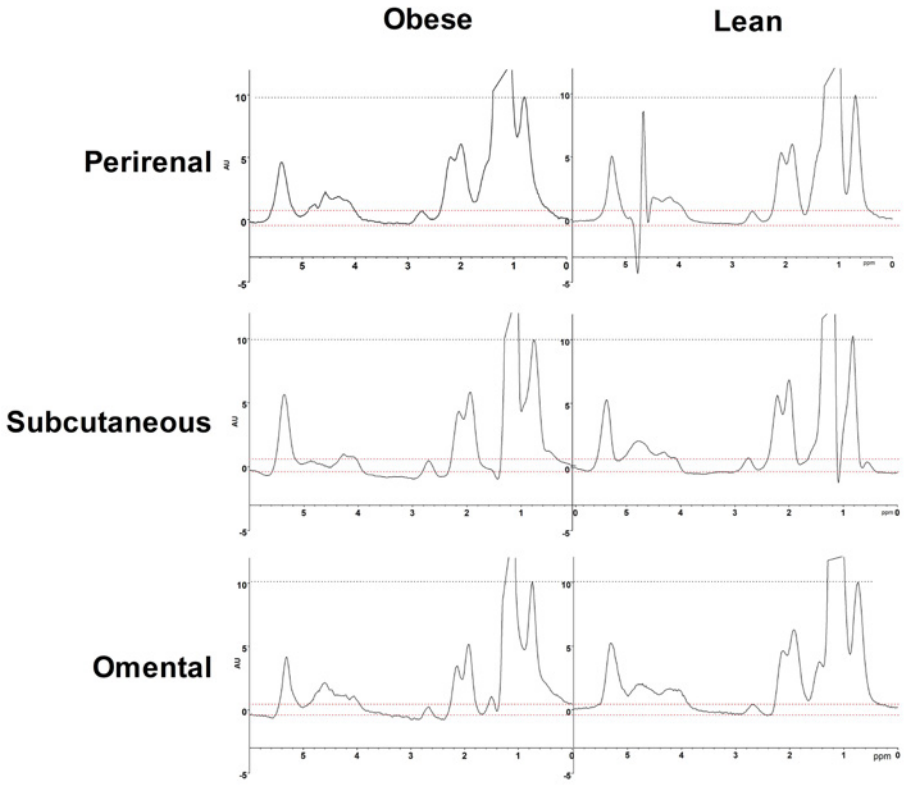

Figure 3. Spectra from three adipose tissue depots in obese and lean participants. Spectra were normalized at the 0.9ppm peak.

Red lines provide the quantitative comparison of PUFA resonances between obese and lean participants.

Bland-Altman plots for TUFA/TG, PUFA/TG, PUFA/TUFA in the different adipose tissue depots are shown in Figure 4. In these plots, abdominally obese and lean subjects are depicted by respectively black and white dots. ICCs were low or negative in the perirenal adipose tissue depot in both the obese and lean subjects, ranging from -0.794 to 0.013. In the subcutaneous adipose tissue depot, ICCs were substantially higher in the obese subjects (PUFA: 0.67, TUFA:0.70, TG: 0.60) than in the lean subjects (PUFA: -0.03, TUFA: 0.15, TG: 0.55). A similar difference was seen in the omental adipose tissue depot; ICCs were -0.00 (PUFA), -1.04 (TUFA) and -0.28 (TG) in lean subjects and 0.75 (PUFA), 0.18 (TUFA) and 0.47 (TG) in obese subjects.

TUFA/TG, PUFA/TG And PUFA/TUFA In Different Abdominal Adipose Tissue Depots

Median and interquartile ranges for TUFA/TG, PUFA/TG and PUFA/TUFA in the perirenal, subcutaneous and omental adipose tissue depots are shown in Table 2. Differences between depots were not statistically significant for any of the ratios.

Table 2. TUFA/TG, PUFA/TG en PUFA/TUFA in the different adipose tissue depots

	TUFA/TG	PUFA/TG	PUFA/TUFA
Perirenal	$80.4*10^{-3}$ ($70.5*10^{-3}$ – $100.1*10^{-3}$)	$0.11*10^{-3}$ ($0.03*10^{-3}$ – $0.93*10^{-3}$)	$1.31*10^{-3}$ ($0.28*10^{-3}$ – $12.1*10^{-3}$)
Subcutaneous	$92.8*10^{-3}$ ($82.7*10^{-3}$ – $122.3*10^{-3}$)	$0.03*10^{-3}$ ($0.00*10^{-3}$ – $0.44*10^{-3}$)	$0.52*10^{-3}$ ($0.08*10^{-3}$ – $5.2*10^{-3}$)
Omental	$83.0*10^{-3}$ ($67.2*10^{-3}$ – $92.4*10^{-3}$)	$0.65*10^{-3}$ ($0.03*10^{-3}$ – $2.76*10^{-3}$)	$15.34*10^{-3}$ ($0.17*10^{-3}$ – $47.0*10^{-3}$)

Data are presented as medians and interquartile ranges

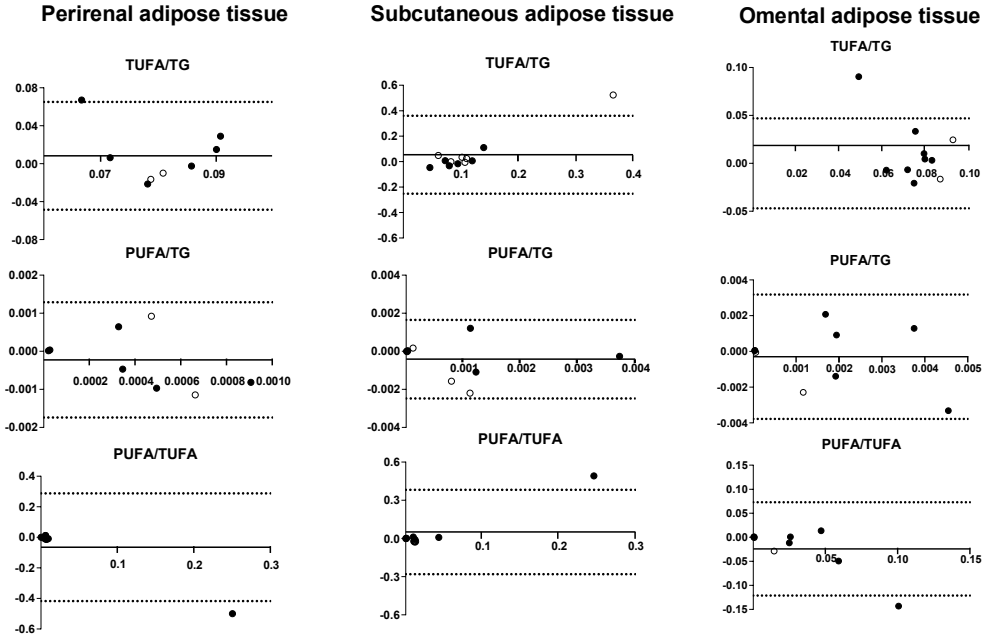

Figure 4. Only data from voxels with ≤20% water content were used.

Averages of 2 measurements (2 examinations, X) are plotted against the differences between the 2 measurements (Y) Continuous lines represent the mean of the differences, dotted lines represent 1.96 SD.
○ lean individuals
● abdominally obese individuals

TUFA/TG, PUFA/TG And PUFA/TUFA In Lean Compared To Abdominally Obese Subjects

In omental adipose tissue, both PUFA/TG and PUFA/TUFA were significantly higher in abdominally obese subjects compared to lean subjects ($2.05*10^{-3}$ (IQR $0.49*10^{-3}$ – $5.08*10^{-3}$) vs. $0.01*10^{-3}$ (IQR $0.01*10^{-3}$ – $0.64*10^{-3}$), p=0.02 and $35.47*10^{-3}$ (IQR $10.36*10^{-3}$ – $35.47*10^{3}$) vs. $0.16*10^{-3}$(IQR $0.07*10^{-3}$ – $7.99*10^{-3}$), p=0.01, respectively) (Figure 5). In the other abdominal adipose tissue depots, no differences between either one of the ratios between lean and abdominally obese subjects were observed.

TUFA/TG, PUFA/TG And PUFA/TUFA In Subjects With Or Without Metabolic Syndrome

Six subjects fulfilled the criteria for the metabolic syndrome, 19 did not. There were no differences in either PUFA/TG or TUFA/TG in the adipose tissue depots between subjects with and subjects without the metabolic syndrome. The PUFA/TUFA ratio in omental adipose tissue was significantly higher in subjects with compared to subjects without the metabolic syndrome ($67.62*10^{-3}$ (IQR $20.15*10^{-3}$ – $127.95*10^{-3}$) vs. $1.07*10^{-3}$ (IQR $0.11*10^{-3}$ – $27.04*10^{-3}$) p= 0.03)(Figure 5). No differences in ratios were observed in the perirenal or subcutaneous adipose tissue depots.

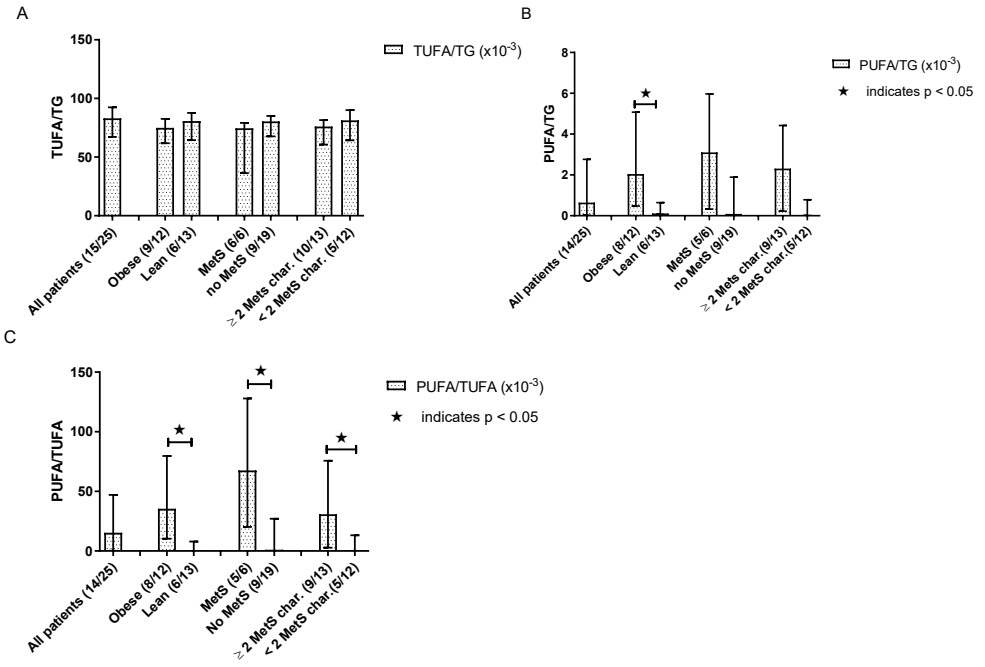

Figure 5. A: TUFA/TG ratio. B: PUFA/TG ratio. C: PUFA/TUFA ratio

Data are presented as medians with interquartile ranges. Obese: abdominally obese;
TG: Triglycerides; PUFA: Poly Unsaturated Fatty Acids; TUFA: Total Unsaturated Fatty Acids;
MetS: Metabolic Syndrome; Char: characteristic
(x/y) where x=number of subjects with valid measurements and y=total number of subjects in category

TUFA/TG, PUFA/TG And PUFA/TUFA In Subjects With <2 Or ≥2 Characteristics Of The Metabolic Syndrome

12 Subjects had <2 characteristics of the metabolic syndrome, 13 had ≥2 characteristics. In the omental adipose tissue depot, subjects with ≥2 characteristics had a statistically significantly higher PUFA/TUFA than the subjects with <2 characteristics ($30.81*10^{-3}$ (IQR $2.78*10^{-3}$ – $75.68*10^{-3}$) vs. $0.15*10^{-3}$ (IQR $0.07*10^{-3}$ – $13.19*10^{-3}$) , p=0.02) (Figure 5). In all

other abdominal adipose tissue depots no differences were observed between the 2 groups in FFA ratios.

Sensitivity Analysis

Using only the values of the PUFA resonance attributed with AMARES resulted in marginal differences between the studied subgroups, without changing the conclusion. No differences between subgroups were found in the perirenal and subcutaneous adipose tissue depots in FFA ratios, as in the primary analyses. In the omental adipose tissue depot, statistical significance was maintained in the comparison between participants with and without the metabolic for the PUFA/TUFA ratio, (p=0.008) and lost in the comparison between obese vs lean (p=0.10) and participants with ≥2 vs <2 characteristics of the metabolic syndrome (p=0.07). For the PUFA/TG ratio in the omental adipose tissue depot, the difference between obese and lean participants lost statistical significance (p=0.22), but the difference between participants with and without the metabolic syndrome reached statistical significance (p=0.04).

Relation Between Clinical Parameters And PUFA/TG, TUFA/TG and PUFA/TUFA

A single SD increase in BMI resulted in a decrease of 25.6 (95%CI -46.2- -5.1) in TUFA/TG in the perirenal (Table 3a) and a 0.6 increase (95%CI 0.3- 1.0) in PUFA/TG in the subcutaneous adipose tissue depot (Table 3b). A single SD increase in WC also resulted in a decrease of TUFA/TG in the perirenal adipose tissue depot (-27.9 (95%CI -48.6- -7.1)) (Table 3a). In the omental adipose tissue depot a single SD increase in LDL-c was related to an increase in PUFA/TG (1.1 (95%CI 0.1- 2.1)) and PUFA/TUFA (13.6 (95%CI 2.4- 24.8)) (Table 3c).

Table 3a. Relation between clinical parameters and change in FFA ratio's in the perirenal adipose tissue depot

Clinical parameter	TUFA/TG	PUFA/TG	PUFA/TUFA
BMI (kg/m²) SD: 4.6	**-25.6 (-46.2- -5.1)***	**0.6 (0.0- 0.8)***	5.4 (-0.4- 11.1)
WC (cm) SD: 13.8	**-27.9 (-48.6- -7.1)***	0.2 (-0.3- 0.8)	2.4 (-4.7- 9.5)
Systolic BP (mmHg) SD: 17.6	-8.9 (-33.1- 15.2)	0.3 (-0.2- 0.7)	3.3 (-2.4- 9.0)
HDL-c (mmol/L) SD: 0.39	13.9 (-3.8- 31.6)	-0.2 (-0.5- 0.2)	-2.0 (-6.9- 2.8)
TG† (mmol/L) SD: 0.65	-19.6 (-42.7- 3.6)	0.3 (-0.2- 0.8)	3.5 (-2.8- 9.8)
LDL-c (mmol/L) SD: 1.0	15.3 (-4.3- 35.0)	-0.2 (-0.6- 0.2)	-2.7 (-8.0- 2.5)
HOMA-IR SD: 1.13	-7.3 (-25.9- 11.2)	0.3 (-0.0- 0.6)	3.1 (-1.0- 7.3)

Data are presented as regression coefficients (β) and 95% confidence intervals (95%CI), showing that (for example) a difference in BMI of 1SD (4.6 kg/m²) decreases the TUFA/TG ratio in perirenal adipose tissue with 25.6.

BMI: body mass index. WC: waist circumference. BP: blood pressure. HOMA-IR: Homeostatic Model of Assessment – Insulin Resistance. HDL-c: High Density Lipoprotein cholesterol LDL-c: Low Density Lipoprotein cholesterol *p<0.05, †: log transformed data

Table 3b. Relation between clinical parameters and change in FFA ratios in the subcutaneous adipose tissue depot

Clinical parameter	TUFA/TG	PUFA/TG	PUFA/TUFA
BMI (kg/m²) SD: 4.6	-38.0 (-100.0- 24.0)	**0.6 (0.3- 1.0)****	1.9 (-57.9- 61.7)
WC (cm) SD: 13.8	-45.6 (-110.8- 19.6)	0.4 (-0.1- 0.8)	9.5 (-54.0- 73.0)
Systolic BP (mmHg) SD: 17.6	-21.7 (-78.8- 35.4)	0.2 (-0.2- 0.7)	15.1 (-41.9- 72.0)
HDL-c (mmol/L) SD: 0.39	-27.5 (-84.1- 29.1)	-0.2 (-0.6- 0.2)	**48.9 (0.7-97.2)***
TG† (mmol/L) SD: 0.65	-24.8 (-83.7- 34.1)	0.2 (-0.2- 0.7)	-32.1 (-87.9- 23.7)
LDL-c (mmol/L) SD: 1.0	-3.1 (-68.6- 62.3)	0.2 (-0.3- 0.7)	-22.8 (-85.0- 39.3)
HOMA-IR SD: 1.13	-31.1 (-89.9- 27.7)	**0.5 (0.2- 0.9)****	-17.33 (-72.6- 38.0)

Data are presented as regression coefficients (β) and 95% confidence intervals (95%CI), showing that (for example) a difference in BMI of 1SD (4.6 kg/m²) decreases the TUFA/TG ratio in perirenal adipose tissue with 38.0
BMI: body mass index. WC: waist circumference. BP: blood pressure. HOMA-IR: Homeostatic Model of Assessment – Insulin Resistance. HDL-c: High Density Lipoprotein cholesterol LDL-c: Low Density Lipoprotein cholesterol *p<0.05 **p<0.01 †: log transformed data

Table 3c. Relation between clinical parameters and change in FFA ratio's in the omental adipose tissue depot

Clinical parameter	TUFA/TG	PUFA/TG	PUFA/TUFA
BMI (kg/m²) SD: 4.6	0.0 (-16.9- 16.9)	1.2 (-0.5- 2.8)	13.6 (-5.9- 33.1)
WC (cm) SD: 13.8	-2.8 (-22.8- 17.2)	1.1 (-1.0- 3.2)	13.1 (-11.3- 37.4)
Systolic BP (mmHg) SD: 17.6	-5.0 (-15.7- 5.6)	0.6 (-0.6- 1.7)	7.5(-6.3- 21.3)
HDL-c (mmol/L) SD: 0.39	-4.6 (-13.6- 4.4)	-0.4 (-1.4- 0.7)	-4.2 (-16.6- 8.2)
TG† (mmol/L) SD: 0.65	8.8 (-3.1- 20.6)	1.1 (-0.1- 2.4)	13.4 (-1.3- 28.1)
LDL-c (mmol/L) SD: 1.0	5.8 (-5.1- 16.8)	**1.1 (0.1- 2.1)***	**13.6 (2.4- 24.8)***
HOMA-IR SD: 1.13	2.4 (-19.8- 15.1)	0.7 (-1.2- 2.6)	8.5 (-13.7- 30.6)

Data are presented as regression coefficients (β) and 95% confidence intervals (95%CI), showing that (for example) a difference in BMI of 1SD (4.6 kg/m²) increases the TUFA/TG ratio in perirenal adipose tissue with 0.002.
BMI: body mass index. WC: waist circumference. BP: blood pressure. HOMA-IR: Homeostatic Model of Assessment – Insulin Resistance. HDL-c: High Density Lipoprotein cholesterol LDL-c: Low Density Lipoprotein cholesterol *p<0.05, †: log transformed data

An increase of 1 SD in HOMA-IR resulted in an increase of PUFA/TG (0.5 (95%CI 0.2- 0.9)) and a 1 SD increase of HDL-c resulted in an increase of PUFA/TUFA (48.9 (95%CI 0.7- 97.2)) (Table 3b) in the subcutaneous adipose tissue depot.

DISCUSSION

In the present study we found that with ^{1}H-MRS at 3.0 Tesla it is feasible to measure free fatty acid profiles in perirenal, subcutaneous and omental adipose tissue in abdominally obese individuals. In lean subjects, however, 44% of measurements had to be excluded from analyses due to inclusion of substantial amounts of non-adipose tissue. Therefore ^{1}H-MRS requires further refinement before becoming feasible in a higher fraction of lean individuals. PUFA/TUFA and PUFA/TG ratios are higher in abdominally obese subjects and in subjects with characteristics of the metabolic syndrome, implying a relation with adipose tissue dysfunction.

Free fatty acid composition in adipose tissue has been probed with ^{1}H-MRS at various locations, such as in breast tissue (23), subcutaneous adipose tissue of the lower extremities (24), muscular tissue (25), and bone marrow (24). In patients with elevated liver fat (>5%, detected with ^{1}H-MRS) and healthy volunteers, TUFA's, PUFA's and triglycerides could be detected with ^{1}H-MRS at 1.5 Tesla in both subcutaneous(19;26) and perirenal adipose tissue(26). In the current study on 3T, we were able to obtain comparable free fatty acid spectra in approximately half the time needed on 1.5 Tesla, since acquisition time could be shortened due to higher signal to noise ratio.. Moreover, in contrast to earlier research (19) we were able to quantify fatty acid and triglyceride resonances. However, there are still technical issues that limit accuracy of the measurements and applicability as a diagnostic tool, especially for the lean individuals. A substantial proportion of the perirenal and omental voxels showed >20% water content, indicating that other tissues than adipose tissue, such as kidney or bowel structures, were also in the voxel. This occurred most often in lean subjects, where the amount of adipose tissue was less. Although voxels were placed exclusively in the adipose tissue based on T2-TSE MR images, respiratory and bowel motion has likely caused substantial partial volume effects in the voxel. This assumption is supported by the limited amount of water seen in the spectroscopic measurements of the subcutaneous adipose tissue, which exhibited very limited shifts due to respiration and peristaltic movements. To avoid this problem, respiratory triggering, breath hold commands, navigation techniques or higher spatial resolution scanning are probably needed to decrease partial volume effects during measurement of the spectra. Quantification of PUFA resonance was challenging, which is in line with what has already been described by others (19). In the present study, quantification of the PUFA resonance was mainly problematic when a small voxel size was used, even further reducing the area under the curve of this already relatively small resonance. Despite implementing prior knowledge in the AMARES quantification software, the PUFA resonance could not be fitted in a substantial amount of measurements, especially when small voxels were used in lean subjects. Attribution of the lowest value of other PUFA measurements in the same adipose tissue depot clearly introduces bias. However, to our judgement, declaring measurements invalid although the PUFA resonance was visually detectable, would mean loss of information. These reasons might explain the wide variation

in PUFA/TG and PUFA/TUFA we observed. The results of the sensitivity analyses, only marginally changing results and not changing the conclusions, validate the attribution of lowest values for PUFA resonances not attributed by AMARES. To increase SNR of PUFA, it could be considered to use a voxel size of at least 15x15x15 mm or to increase the number of signal averages (NSA) although this also implies longer scanning time, or to use a more efficient MRS technique like the PRESS sequence(19;26) or the recently proposed sLASER pulse sequence which can improve SNR by a factor of two compared to the STEAM pulse sequence used in this study(27) In this study, the STEAM sequence was chosen over PRESS sequences (too large chemical shift displacement artefacts due to low B1 strength) and sLASER sequences (not routinely available at our scanner).
As can be seen in the Bland-Altman plots and ICCs, reproducibility was fair for the spectroscopic measurements in the abdominally obese subjects in the subcutaneous and omental adipose tissue depot, although several outliers were seen, probably due to the technical issues described above. In lean subjects, reproducibility was poor, mainly caused by the high percentage of measurements containing >20% non-adipose tissue in the sampled voxels. The fair reproducibility implies that the free fatty acid profile measurements in abdominally obese subjects are stable when conducted after an overnight fast.

Subjects with a metabolically unhealthy profile, i.e. those with obesity and/or with ≥2 characteristics of the metabolic syndrome have higher PUFA/TUFA and PUFA/TG ratios compared to lean and non-metabolic syndrome subjects, especially in omental adipose tissue, suggesting that FFA ratios might be of value in diagnosing ATD. PUFAs are polyunsaturated fatty acids containing at least 2 double bonds in their chain, of which the ω-3 fatty acids are generally believed to be cardioprotective (16-18), whereas ω-6 fatty acids are thought to have pro-inflammatory actions and cause insulin-resistance(28;29). The Western diet contains far more ω-6 than ω-3 fatty acids with a ratio of approximately 10-20:1(30;31). Hypothetically, a high level of PUFA might therefore reflect a more pro-inflammatory status of adipose tissue, which is equivalent to ATD. The pro-inflammatory character of PUFA is further underlined by the positive associations between anthropometric and metabolic parameters with PUFA/TUFA and PUFA/TUFA in the different adipose tissue depots, implying a relation of FFA ratios with ATD. The positive association between HDL-cholesterol and PUFA/TUFA in the subcutaneous adipose tissue depot is dissimilar from the other findings in our study, since an increase in HDL is usually associated with a decreased risk of (the consequences of) adipose tissue dysfunction(32;33)
Strengths of our study include the careful selection of subjects avoiding influence of medication and enabling discrimination between abdominally obese and lean subjects, the performance of ^{1}H-MRS at 3.0 Tesla and the reproducibility measurements.
Limitations of our study need to be considered, most importantly the considerable amount of measurements which contained >20% water which were therefore deemed not valid for analyses (despite the use of respiratory triggering) and the difficulties with the quantification

of the PUFA resonance, particularly in lean subjects. 'In principle, it might be that repeated measurements at more than 2 occasions would have changed results, although we currently have no reasons to believe that the main findings, particularly between lean and obese individuals would change materially'.Before ^{1}H-MRS can be considered as a diagnostic tool in lean subjects, it is of vital importance that the percentage of valid measurements increases by enhancing the quality of the measurement technique. Direct post-processing of data after measuring provides the opportunity to instantly implement changes in the scanning protocol and is highly advisable.

In conclusion, measurement of free fatty acid profiles in subcutaneous and abdominal adipose tissue depots with ^{1}H-MRS is feasible with adequate reproducibility in abdominally obese individuals. Although limited by low reproducibility in the lean subjects we show for the first time that FFA ratios are different between metabolically healthy and unhealthy subjects. When the limitations encountered in this study can be overcome, ^{1}H-MRS at 3.0 Tesla may be a non-invasive diagnostic tool for identifying early stages of adipose tissue dysfunction

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Chapter 4

The effect of an oral fatload on perirenal, omental and subcutaneous adipose tissue composition as measured with 1H-MR Spectroscopy Different reactions to oral fat challenge in individuals with and without metabolic syndrome.

Ilse M Schrover[1], Wilko Spiering[1], Jannie Wijnen[2], Dennis W.J. Klomp[2], Tim Leiner[2], Frank L.J. Visseren[1]

Departments of Vascular Medicine[1] and Radiology[2]

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Abstract

Introduction: Adipose tissue dysfunction plays a role in the development of diabetes and cardiovascular diseases. The composition of free fatty acids (FFA) in plasma and tissues is different in individuals with and without the metabolic syndrome in fasting state. We aimed to investigate the change of free fatty acids content in perirenal, omental and subcutaneous adipose tissue after an oral fatload, in persons with and without the metabolic syndrome.

Patients and methods: Twelve subjects with metabolic syndrome and 12 without metabolic syndrome were age- and gender-matched. Before and after an oral fat challenge a MRI scan of visceral, renal and subcutaneous adipose tissue was performed, measuring FFA with 1H-MR Spectroscopy. Differences in ratios of poly unsaturated fatty acids (PUFA), total unsaturated fatty acids (TUFA) and triglycerides (TG) before and after an oral fatload and between patients with and without metabolic syndrome were calculated and statistically evaluated with t-tests.

Results: Delta PUFA/TUFA ratio (before – after oral fat load) was statistically significantly different between participants with and without the metabolic syndrome in omental adipose tissue (+2.15% ± 4.98% versus $-89.6*10^{-3} \pm 75.7*10^{-3}$) (p=0.022), not in the subcutaneous and renaladipose tissue. Delta PUFA/TG ratio was in omental adipose tissue significantly different between patients with and without metabolic syndrome ($0.7*10^{-3} \pm 4.8*10^{-3}$ versus $-8.7*10^{-3} \pm 7.2*10^{-3}$ (p=0.041), not in the other departments.

Conclusion: After an oral fatload the content of FFA and TG in (omental) adipose tissue changes more in subjects with the metabolic syndrome than in subjects without the metabolic syndrome. These results indicate unfavorable adipose tissue function in metabolic syndrome patients that might reflect differences in adipose tissue function and which may contribute to the development of diabetes mellitus and cardiovascular diseases.

Introduction

Generally, it is thought that the quantity of adipose tissue is the most important driver of adipose tissue dysfunction.(1) Besides quantity there is mounting evidence that 'quality' of adipose tissue, reflected by plasma levels of adipokines, is strongly related to the development of diabetes and cardiovascular disease (REF review Hajer). Apparently, there are obese persons without metabolic changes, also referred to as 'metabolically healthy obese'(5) and vice versa normal weight persons with metabolic changes that are usually seen in obese subjects, referred to as 'metabolically obese, normal weight individuals' (6). Adipose tissue dysfunction (ATD) could be defined as the imbalanced production of pro- and anti-inflammatory adipokines and interleukins by adipose tissue(2). Adipose tissue dysfunction is associated with an increased risk of diabetes mellitus and cardiovascular diseases, in many cases ATD precedes the development of the metabolic syndrome(3,4). Especially abdominal adipose tissue is related to ATD, as opposed to subcutaneous adipose tissue(1,7). With the measurements BMI or waist circumference, used in clinical practice, the quantity of these adipose tissue locations cannot be established precisely and therefore these measurements do not provide an adequate estimate of ATD(8). Identifying ATD may help to identify individuals at risk for diabetes mellitus type 2 and cardiovascular disease, and may direct preventive therapy. Nowadays, the focus on prevention of vascular diseases is mainly aimed at presumed unhealthy obese individuals while lean individuals with ATD, and thus at risk, are usually not screened and counselled. Therefore, methods of diagnosing ATD could be of use in clinical practice. One of these new methods could be ^{1}H-Magnetic Resonance Spectroscopy (^{1}H-MRS). With ^{1}H-MRS metabolic imaging of tissues can be performed to study their chemical composition non-invasively. In both liver and myocardial tissue, free fatty acids (FFA) have been identified and quantified by MRS (9,10). These measurements have been validated with gas chromatography(9,10), and have been shown to adequately discriminate triglycerides (TG), polyunsaturated fatty acids (PUFA), total unsaturated fatty acids (TUFA), and saturated fatty acids (SFA). When subcutaneous and intra-abdominal adipose tissue biopsies are compared with respect to their FFA profile, intra-abdominal adipose tissue has a significantly higher proportion of SFA acids and a significantly lower proportion of mono-unsaturated fatty acids(11). Moreover, BMI and visceral fat area were negatively correlated to ω-3 fatty acids (11)(poly unsaturated fatty acids), which are believed to be cardioprotective(12–14). Since the amount of intra-abdominal adipose tissue is an important determinant of ATD and its metabolic consequences, this implies that differences in FFA content could be indicative for ATD and that changes in FFA content may even induce ATD. In an earlier study, we found that FFA ratios are different between metabolically healthy and unhealthy subjects in fasting state(15). Since modern human beings are the most part of the day in a non-fasting state, and as FFA may affect ATD, we aimed to investigate the effect of an oral fatload on the content of free fatty acids and their ratio's in various adipose tissue depots in persons with and without the metabolic syndrome.

Materials and Methods

Study Design and Participants

A single centre, cross-sectional study was performed at the University Medical Center Utrecht. The study protocol was approved by the local Ethics Committee and conducted in accordance with the principles of the Declaration of Helsinki. All participants gave written informed consent prior to enrolment after full explanation of study procedures.

Between December 2012 and July 2015 24 healthy men and women were included. Exclusion criteria were a medical history of cardiovascular, liver, renal or pulmonary disease or use of any medication, except for acetaminophen, antacids, topical creams, inhalation medication, nasal sprays, and eye drops. Women who were pregnant or lactating and persons with MRI-incompatible devices were not included. Only participants with a BMI > 25 kg/m2 were included, since determining fatty acids in individuals with a lower BMI had proven difficult in our earlier study.

Participants were age- and gender-matched on the presence or absence of metabolic syndrome, as defined by the ATP III criteria. Participants were screened in fasting condition, determining whether or not they had the metabolic syndrome. The first 12 eligible participants were included, independent of their metabolic syndrome status. The next 12 participants were matched to the first 12 and invited to the study visit 2-3 weeks after the screening visit. Participants were asked to follow a low-fat diet the 3 days prior to the study visit, instructions were provided by the study team. After an overnight fast, participants came to the research unit, were anthropometric measurements were obtained and blood was drawn. ^{1}H-MRS-examination was performed afterwards, followed by the oral fat challenge. Six hours after the fat challenge, blood was drawn again and the post fatload ^{1}H-MRS-examination was performed. During that day, participants were only allowed to drink water or tea without sugar.

Laboratory and Anthropometric Measurements

Fasting venous blood samples were collected before the first (fasting) and second (6 hours after oral fat challenge) ^{1}H-MRS examination for measurement of glucose, insulin, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol. Waist circumference was measured halfway between the lower rib and the iliac crest in full expiration. Body mass index (BMI) was calculated as weight (kg) divided by height^{2} (m). Blood pressure was measured in both arms with an automatic device (Omron™, 705 IT Intellisense, Hoofddorp, The Netherlands); the highest blood pressure was used for data analysis.

Participants fulfilled the ATPIII criteria for the metabolic syndrome when at least 3 of the following characteristics were present: waist circumference >102 cm (men) or >88 cm (women), HDL <1.04 mmol/L (men) or <1.29 mmol/L (women), blood pressure ≥130 mmHg systolic and/or ≥85 mmHg diastolic, fasting glucose ≥5.6 mmol/L or triglycerides ≥1.7 mmol/L. (16)

Oral fatload

For the fat challenge, a fresh cream was used with a 40% fat emulsion, a poly-unsaturated/ saturated fat ratio of 0.10, containing 0.001% cholesterol and 3% carbohydrates representing a total energy content of 3700 Kcal/L. The volume of the fat load was adjusted for body surface (100 g fat and 7.5 g glucose per m2 body surface) with a maximum of 500 mL. Participants were asked to ingest the fat challenge within 30 minutes.

^{1}H-Magnetic Resonance Spectroscopy (MRS)

All measurements were performed on a 3.0 Tesla Philip (Achieva) MR scanner (Philips Healthcare, Best, the Netherlands) using a cardiac RF coil. Subjects underwent transversal and coronal T2-weighted Turbo Spin Echo (T2 TSE) sequences (slice thickness 4mm/gap 0.4mm), the images obtained with these sequences were used for placement of the voxels, combined with a reference scan for localisation in the sagittal plane. Voxels were placed in the liver and three adipose tissue depots: perirenal (retroperitoneal) adipose tissue depot (both left and right), subcutaneous adipose tissue depot (both left and right), and the omental adipose tissue depot. Voxels in the liver were placed in the right liver lobe. Voxels in the perirenal adipose tissue were placed next to or just below the lower pole region of the left and right kidney. With a margin of 10 cm to both the cranial and caudal side, the position of the perirenal adipose tissue voxel was used as point of reference for placement of the subcutaneous adipose tissue voxels. The intra-abdominal adipose tissue voxel was placed at the omentum.

Voxel size was chosen depending on the amount of adipose tissue present at the different locations and could vary between subjects (range of voxel volume was between 1 and 8 cm^3). The voxel was placed in the adipose tissue, avoiding inclusion of other tissues such as the kidney, bowels or muscles, guided by transversal, coronal and sagittal images. For the omental spectra, respiratory triggering was used. MR spectra were acquired with a STEAM sequence with TE/TR = 8.9/2000 ms and 32 acquisitions. Second order shim gradients were used, with shimming based on the voxel. No water suppression or REST slabs were used.

Room time, including taking subjects in and out of the scanner, positioning of the subjects in the scanner, acquisition of localizers, and T2 weighted images for voxel planning and performing the ^{1}H-MRS, was approximately 35 minutes. The MR spectra were evaluated using jMRUI software, using AMARES for quantification, calculating the area under the curve (AUC) of the metabolites in the MR spectrum. The methylene (triglyceride) resonance at 1.3 ppm, the PUFA resonance at 2.75 ppm, the water resonance at 4.7 ppm and the TUFA resonance at 5.4 ppm were quantified (Figure 1). MR spectra with a water content >20% ([TG] / [TG] + [H_2O]) <0.8) were not included in the analysis, as this is susceptible to assessing tissues other than adipose tissue (e.g. bowel or kidney). The 20% cut-off value was chosen to balance maximal sensitivity of the spectral measurement and minimal data-exclusion. In the spectra where a PUFA resonance could be clearly distinguished by sight, but AMARES failed to attribute an AUC to the resonance, the AUC was not declared missing,

but the lowest value of the PUFA resonance in all subjects in the specific adipose tissue depot was attributed and used for analyses.

Comparison of AUCs of 'single' metabolite resonances is only possible after correction for the distance between the cardiac coil and the voxel, since signal is lost with increasing distance due to the sensitivity profile of the coil, which is the case in more obese subjects. Therefore, we calculated ratios of the different metabolite resonances, providing an internal correction and making comparison possible. PUFA/TG, and PUFA/TUFA ratios were calculated for all spectral measurements. For spectral measurements in the liver, TG/water ratios were calculated.

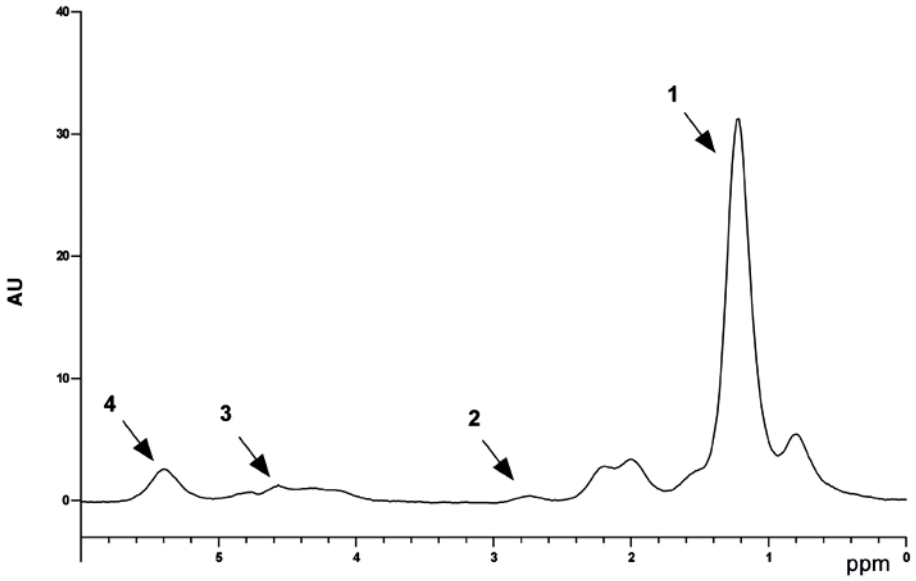

Figure 1. Obtained spectrum after processing with jMRUI showing the different free fatty acids resonances.

1= triglycerides; 2=Poly Unsaturated Fatty Acids (PUFA); 3= water; 4= Total Unsaturated Fatty Acids (TUFA)

Data Analyses

Baseline characteristics of subjects are presented as means with standard deviations or as medians with interquartile ranges (IQR) when data were not normally distributed. Differences in free fatty acid ratios between the participants with and without the metabolic were assessed with t-tests. Triglyceride (serum) data were log-transformed due to skewed distribution. P-values <0.05 were considered statistically significant. All analyses were performed with SPSS 24.0 (SPSS Inc, Chicago, IL).

Results

Baseline characteristics

12 age and sex-matched subjects with and 12 without metabolic syndrome were included in the study. Their baseline characteristics are shown in Table 1. Patients with metabolic syndrome had higher BMI (30.7 ± 2.8kg/m2 vs 26.1 ± 0.9 kg/m2), systolic blood pressure (144 ± 9 mmHg vs 137 ± 15 mmHg), triglycerides (1.9 (IQR 1.2-2.5) mmol/L vs 1.0 (IQR 0.8-1.5) mmol/L) and HOMA-IR (homeostatic measurement of insulin resistance, 2.5 (IQR 2.2-3.2) vs 1.6 (IQR 1.4-2.4)).

Table 1. Baseline characteristics

	Metabolic syndrome (n=12)	No metabolic syndrome (n=12)
Male (n)	7	7
Age (years)	57 ± 14	53 ± 13
Height (cm)	175 ± 8	176 ± 7
Weight (kg)	94 ± 12	80 ± 8
BMI (kg/m²)	30.7 ± 2.8	26.1 ± 0.9
WC (cm)		
Men	111 ± 7	98 ± 6
Women	108 ± 6	92 ± 6
Systolic BP (mmHg)	144 ± 9	137 ± 15
Diastolic BP (mmHg)	87 ± 5	81 ± 8
Glucose (mmol/L)	5.9 ± 1.5	5.1 ± 0.5
Insulin (mIU/L)	9 (9 – 14)	7 (6 – 9)
HOMA-IR	2.5 (2.2 – 3.2)	1.6 (1.4 – 2.4)
Cholesterol (mmol/L)	5.6 ± 0.9	5.5 ± 0.5
HDL-cholesterol (mmol/L)		
Men	1.14 ± 0.13	1.28 ± 0.13
Women	1.53 ± 0.36	1.56 ± 0.06
LDL-cholesterol (mmol/L)	3.3 ± 0.9	3.5 ± 1.2
Triglycerides (mmol/L)	1.9 (1.2 – 2.5)	1.0 (0.8 – 1.5)

Data are presented as means + standard deviations or as medians with interquartile ranges

n= number of subjects. BMI: body mass index. WC: waist circumference. BP: blood pressure. HOMA-IR: Homeostatic Model of Assessment – Insulin Resistance. HDL: High Density Lipoprotein LDL: Low Density Lipoprotein

Blood and liver triglycerides (delta) before and after oral fat challenge

Serum triglyceride levels changed from 1.4 (IQR 0.8-1.8) mmol/L to 2.7 (IQR 1.9-3.6) mmol/L (p=0.02) in the participants without metabolic syndrome after the oral fat challenge and from 2.7 (IQR 1.5-3.3) mmol/L to 3.4 (IQR 3.2-6.6) mmol/L (p= 0.08) in the participants with metabolic syndrome. There were no significant differences in the change of triglyceride

levels after the oral fat challenge between patient with and without metabolic syndrome (1.7 vs. 1.4, p=0.18) presented in Figure 2.

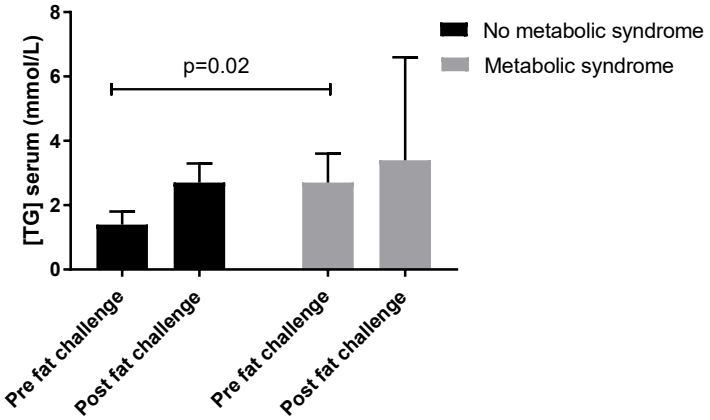

Figure 2. **Change in serum triglycerides before and after the oral fat challenge.**

Triglyceride/water ratio in the liver decreased after the oral fat challenge from $119.1*10^{3}$ (IQR $36.0*10^{-3}$ – $120.0*10^{-3}$) to $19.2*10^{-3}$ (IQR $0.1*10^{-3}$ – $159.8*10^{-3}$) (p 0.20) in the participants without the metabolic syndrome. In the participants with metabolic syndrome liver triglyceride content decreased from $244.1*10^{-3}$ (IQR $47.3*10^{-3}$ – $301.3*10^{-3}$) to $147.9*10^{3}$ (IQR $33.8*10^{-3}$ – $256.5*10^{-3}$) (p 0.45). No statistical significant differences were observed in change of triglyceride liver content before and after fatload between patients with and without metabolic syndrome (Figure 3).

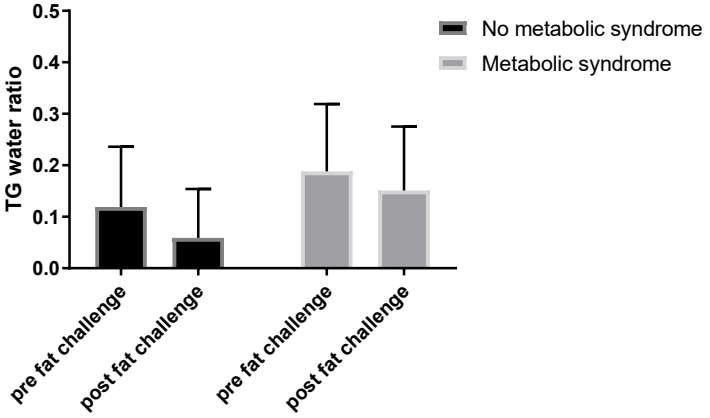

Figure 3. Change in liver triglycerides before and after the oral fat challenge.

Ppm: parts per million

PUFA/TUFA ratios in renal, omental and subcutaneous adipose tissue

Figure 4 shows the PUFA/TUFA ratios before and after the oral fat challenge in renal, subcutaneous and omental adipose tissue. The PUFA/TUFA ratio before the oral fat challenge was only significantly different between participants with and without the metabolic syndrome in omental adipose tissue. In participants without the metabolic syndrome, PUFA/TUFA ratio was $19.1*10^{-3} \pm 21.9*10^{-3}$ versus $101.9*10^{-3} \pm 87.3*10^{-3}$ in the participants with metabolic syndrome (p=0.046). After the oral fat challenge, the ratio was different only in subcutaneous adipose tissue, being $39.6*10^{-3} \pm 33.4*10^{-3}$ versus $8.30*10^{-3} \pm 10.4*10^{-3}$ in the participants with and without the metabolic syndrome respectively (p=0.046). The delta PUFA/TUFA ratio was only statistically significantly different between participants with and without the metabolic syndrome in omental adipose tissue ($21.5*10^{-3} \pm 49.8*10^{-3}$ versus $-89.6*10^{-3} \pm 75.7*10^{-3}$) (p 0.022).

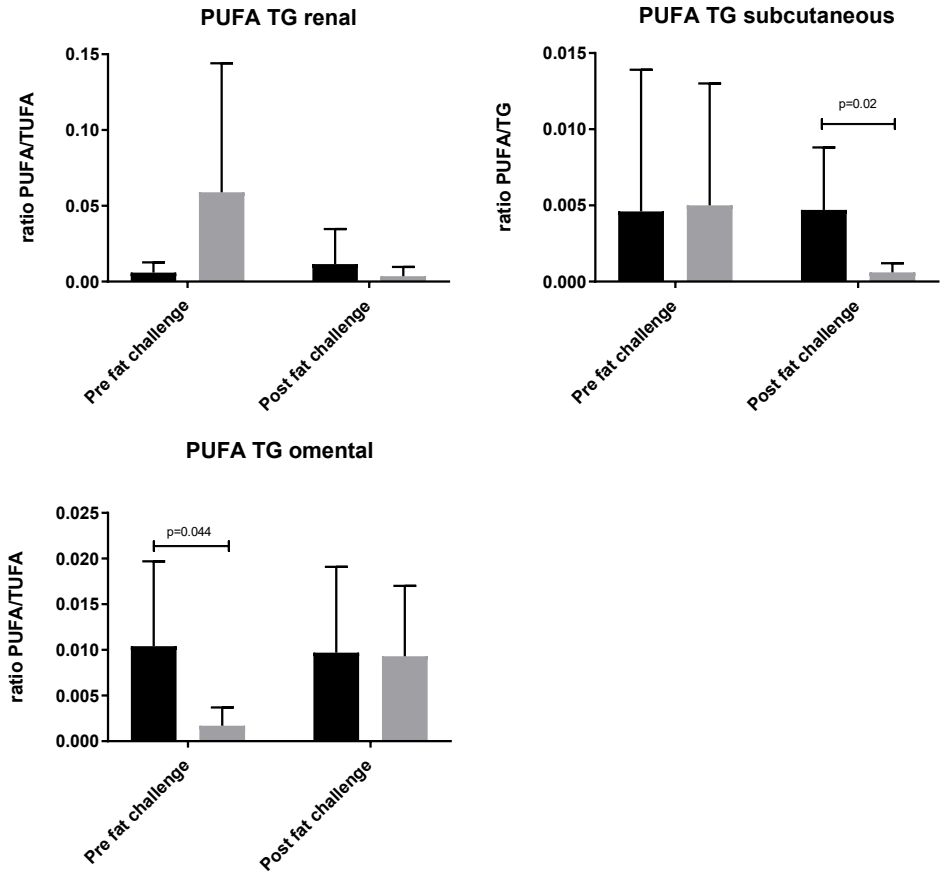

Figure 4. Ratios of PUFA/TG in three adipose tissue depots before and after oral fat challenge; in individuals with and without metabolic syndrome.

Significant differences are shown in the graph.

PUFA/TG ratios in different renal, omental and subcutaneous adipose tissue

Figure 5 shows the PUFA/TG ratios before and after the oral fat challenge in the renal, subcutaneous and omental adipose tissue. The PUFA/TG ratio before the oral fat challenge was only significantly different between participants with and without the metabolic syndrome in omental adipose tissue. In participants without the metabolic syndrome, PUFA/TG ratio was $1.7*10^{-3} \pm 2.0*10^{-3}$ versus $10.5*10^{-3} \pm 9.3*10^{-3}$ (p 0.044) in the participants with metabolic syndrome. After the oral fat challenge, the ratio differed only in subcutaneous adipose tissue between participants with and without metabolic syndrome, being $4.7*10^{-3} \pm 4.1*10^{-3}$ versus $0.6*10^{-3} \pm 0.6*10^{-3}$ (p 0.02). The change in PUFA/TG ratio was in omental adipose tissue significantly different between patients with and without metabolic syndrome ($0.7*10^{-3} \pm 4.8*10^{-3}$ versus $-8.7*10^{-3} \pm 7.2*10^{-3}$ (p 0.041).

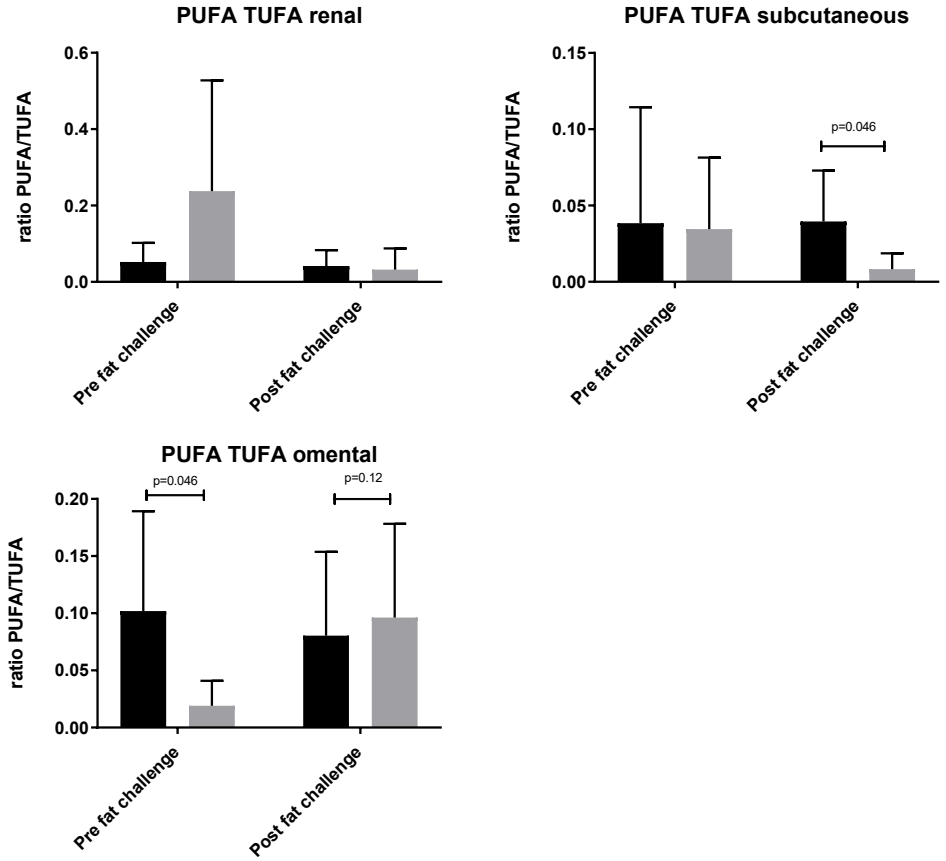

Figure 5. Ratios of PUFA/TUFA in three adipose tissue depots before and after oral fat challenge; in individuals with and without metabolic syndrome.

Significant differences are shown in the graph.

Discussion

In the present study it is shown that PUFA/TG and PUFA/TUFA ratios in omental adipose tissue are in the fasting state higher in persons with the metabolic syndrome compared to subjects without metabolic syndrome. After an oral fat challenge, the change in PUFA/TUFA and PUFA/TG ratio is higher in persons without the metabolic syndrome than in those with the metabolic syndrome. These results indicate that in persons without the metabolic syndrome, uptake of fatty acids and triglycerides within the adipose tissue occurs faster than in persons with metabolic syndrome.

As expected, the level of triglycerides in serum rose after the oral fat challenge. Participants with the metabolic syndrome had higher baseline of triglycerides, the change in triglycerides was not different. From previous studies, it is known that after approximately 4 hours, there is a triglyceride peak in the blood(17). We therefore decided to perform the ^{1}H-MRS 6 hours after the oral fat challenge, when supposedly the adipose tissue handling of the fat challenge was in process. Since we did not perform a blood sample 4 hours after the fat challenge, we do not know for sure whether we actually performed the MRI at or just after the serum triglyceride peak.

The liver triglyceride levels decreased after the oral fat challenge in both patients with and without metabolic syndrome, which might be looked upon as a surprise. After ingestion of a fat rich meal the triglycerides from the meal will be packed into chylomicrons, which are metabolized into FA at the tissue level. During this metabolization, not all FA will be used by the tissue as there is a so-called spill-over. These FA are picked up by the liver and transformed into liver TG, this process occurs quickly after the ingestion. Also, the chylomicron-remnants are taken up by the liver, contributing to liver TG but with a little delay(17). This might imply that the peak of liver TG content is later than 6 hours post fatload, which means that in the present study we timed the ^{1}H-MRS after the fat challenge to early, therefore not showing the peak of liver TG. On the other hand, there are also arguments that the liver TG peak is earlier than 6 hours after an oral fatload as the spillover peak may be most important. A recent study showed that there was an increase in triglyceride content in the liver as measured with 1H-MRS 3 hours after ingestion of a fat rich meal, and no further increase 5 hours after this meal (18). This would suggest that we timed the MRI too late.

Both the PUFA/TUFA and the PUFA/TG ratio are higher in the participants with metabolic syndrome than in those without metabolic syndrome, but only in the omental adipose tissue department. This is similar to observations in our earlier study, where we compared lean and obese persons(15). PUFA consist (mainly) of omega-6 and omega-3, according to the location of the double bond(19). The modern diet is high in omega-6 and low in omega-3 PUFA (20), with a ratio of 10:1, where a ratio of 1:1 was probably present during human development. Although both omega-6 and omega-3 PUFA lead to the formation of eicosanoids, eicosanoids derived from omega-6 are pro-inflammatory and those derived from omega-3 are anti-inflammatory(21,22). Epidemiological data show elevated risks

of auto-immune diseases, cancer and cardiovascular disease with unbalanced PUFA(23). Hence, higher PUFA/TUFA and PUFA/TG ratios probably reflect higher omega-6 fatty acids in patients with metabolic syndrome.

There were no differences between participants with and without the metabolic syndrome in the renal and omental adipose tissue department after the oral fat challenge. In the subcutaneous adipose tissue department both the PUFA/TUFA and PUFA/TG ratios were higher in the participants with the metabolic syndrome. This might be seen as a surprise, since the subcutaneous adipose tissue department is considered to be the least active(1,24). However, there is evidence that subcutaneous adipose tissue function, may change and is relevant, as lower CT attenuation (measured with Houndsfield Units) of both VAT and SAT is associated with adverse cardiometabolic risk beyond total adipose tissue mass.(25) The baseline values of PUFA/TUFA and PUFA/TG were not taken into account, although those differed substantially. Therefore, from these analyses it can only be concluded that shortly after a oral fat load, the result in renal and omental adipose is similar.

In the omental adipose tissue compartment, the change in PUFA/TUFA and PUFA/TG ratios after an oral fatload are significantly larger in participants without the metabolic syndrome. This suggest differences in lipid catabolism in the postprandial state. It has been postulated that in persons with metabolix syndrome, the intestine is synthesizing more TG de novo for export in chylomicrons in response to a fat load than persons without the metabolic syndrome(26). The net result in adipose tissue after a certain amount of time could therefore be similar in both persons with and without the metabolic syndrome, but the effect of the fat load in the metabolic syndrome prolonged. The oral fat load, containing a high omega-6 to omega-3 PUFA ratio, increases the cholesterol efflux and production of cholesterol esters, eventually leading to the formation of foam cells (and in the long term atherosclerosis)(22). In this case, the endothelial walls of persons with metabolic syndrome are exposed to a greater deal to the foam cells. Strengths and limitations of our study need to be considered. This is a small, mechanistic and hypothesis generating study with highly standardized measurements. The small number of participants leaves little room for elaborate statistical analyses.

In conclusion, after an oral fatload the content of FFA and TG in (visceral) adipose tissue depots changes more in subjects with the metabolic syndrome than in subjects without the metabolic syndrome. These results indicate unfavorable adipose tissue function in metabolic syndrome patients that might contribute to the development of diabetes mellitus and cardiovascular diseases.

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Chapter 5

The relation between body fat distribution, plasma concentrations of adipokines and the metabolic syndrome in patients with clinical manifest vascular disease

I.M.Schrover[1], MD; Y. van der Graaf[2], MD, PhD;
W.Spiering[1], MD, PhD; F.L.J.Visseren[1], MD, PhD

Department of Vascular Medicine[1] and the Julius Center for Health Sciences and Primary Care[2]
University Medical Center Utrecht, The Netherlands

On behalf of the SMART study group (individual members are mentioned at the end of the manuscript)

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Abstract

Introduction: We evaluated the relation between adipokines plasma concentrations and body fat distribution and metabolic syndrome.

Methods: In a cohort of 1215 patients with clinical manifest vascular disease the relations between subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), waist circumference (WC), body mass index (BMI) and plasma concentrations of adipsin, chemerin, monocyte chemoattractive protein-1 (MCP-1), migration inhibitory factor (MIF), nerve growth factor (NGF), resistin, plasma amyloid A1 (SAA1), adiponectin, leptin, plasminogen activator inhibitor-1 (PAI-1) and hepatic growth factor (HGF) were cross-sectionally assessed with linear regression and adjusted for age and gender. The relation between adipokines and metabolic syndrome was cross-sectionally evaluated using logistic regression. An adipokine profile was developed to measure the effect of combined rather than single adipokines.

Results: Adiposity was related with higher NGF, HGF, MIF, leptin and adipsin and with lower chemerin, PAI-1, resistin, SAA1 and adiponectin. Strongest positive relations were between BMI and adipsin (β0.247; 95CI%0.137-0.356) and leptin (β0.266; 95%CI0.207-0.324), strongest negative relations were between BMI and SAA1 (β -0.266; 95%CI-0.386- -0.146) and VAT and adiponectin (β-0.168; 95%CI-0.226- -0.111). There was no relation between SAT and adipokines. Odds for metabolic syndrome was higher with each 1SD higher HGF (OR1.21; 95%CI1.06-1.38) and leptin (OR1.26; 95%CI 1.10-1.45) and lower with each 1SD higher adiponectin (OR0.73; 95%CI0.64-0.83) and resistin (OR0.85; 95%CI0.74-0.97). The adipokine profile was related to the metabolic syndrome (OR1.03; 95%CI1.00-1.06).

Conclusion: Plasma concentrations of adipokines are related to obesity and body fat distribution. The relations between adipokine concentrations and metabolic syndrome are independent of VAT.

Introduction

Quantity of adipose tissue is a major driver of adipose tissue dysfunction (ATD) leading to metabolic derangements such as insulin resistance, low-grade inflammation, dyslipidemia and elevated blood pressure(1). Although 10-40% of the obese population is metabolically healthy, they have a higher risk of subclinical and clinical atherosclerosis than normal weight metabolically healthy individuals.(2,3) On the other hand, 5-8% of the healthy weight population is 'metabolically obese'(4,5).This can be explained by the fact that ATD may also be present in the absence of obesity(4,5). Apparently, ATD in a subject that is considered to be at healthy weight may also lead to metabolic changes ultimately leading to the development of metabolic syndrome, diabetes mellitus type 2 (DM2) and cardiovascular disease(4,5). Adipose tissue dysfunction is characterized by an imbalance between the production of pro- and anti- inflammatory adipokines. These adipokines can either be produced by adipocytes or by the vascular stromal fraction in adipose tissue, containing macrophages(6), contributing to insulin resistance and endothelial dysfunction(7). As a consequence, metabolic risk factors such as hypertriglyceridemia, low HDL-cholesterol and chronic low-grade inflammation occur, being causal factors in the development of cardiovascular disease and type 2 diabetes(8,9). Pathophysiological mechanisms that underlie the development of ATD in the absence of obesity are exogenous factors such as physical inactivity(10,11) and the dietary intake of saturated fat(12,13), as well as endogenous susceptibility such as a low birth-weight(14), genetic predisposition(15) and sympathetic nervous system activity(16).
Since ATD exists most abundantly, but not exclusively in patients with obesity, the production of adipokines is not likely to be totally dependent on adipose tissue quantity. However, plasma levels of many pro-inflammatory adipokines such as chemerin(17), retinol binding protein-4 (18), leptin(19), lipocalin-2 and monocyte chemo-attractant protein-1(20) and the anti-inflammatory adipokine adiponectin(18,21,22) are related to general adiposity as well as to intra-abdominal fat. Upper body subcutaneous fat could be a new adipose tissue depot of interest, since this is related to arterial stiffness(23). Higher concentrations of chemerin(24) and leptin (25,26), and lower concentrations of adiponectin (27) are associated with the presence of metabolic syndrome and cardiovascular disease.
In the present study we evaluated the relation between plasma concentrations of 11 adipokines and different measures of general adiposity (BMI, waist circumference) and body fat distribution (subcutaneous adipose tissue; SAT, and visceral adipose tissue; VAT) and metabolic syndrome in a cohort of patients with clinical manifest vascular disease.

Methods and procedures

Study design and patients

The study cohort consisted of patients participating in the Second Manifestations of ARTerial disease-Magnetic Resonance (SMART-MR) study, a prospective study in patients with clinical

manifest vascular disease. Details of the design and participants have been described elsewhere(28,29). In brief, between May 2001 and December 2005, patients newly referred to the University Medical Center Utrecht with recent (i.e. within 6 months prior to inclusion) manifest vascular disease coronary artery disease (CAD), cerebrovascular disease (CVD), peripheral arterial disease (PAD) or an aortic abdominal aneurysm (AAA)) were invited to participate. CAD was defined as a recent diagnosis of angina pectoris, myocardial infarction or coronary revascularization. CVD was defined as a recent diagnosis of ischemic stroke, transient ischemic attack or amaurosis fugax. PAD was defined as a recent clinical diagnosis of PAD (Fontaine stage 2, 3, or 4). For each patient, the presence or absence of the metabolic syndrome was determined. We defined the metabolic syndrome according to the NCEP criteria(30) as the presence of three or more of the following characteristics: waist circumference >102cm (men) or >88cm (women); triglycerides ≥1.7mmol/L; high density lipoprotein <1.29mmol/L (women) or <1.00mmol/L(men); blood pressure ≥130/85mmHg; fasting glucose ≥5.6mmol/L.

AAA was defined as an abdominal aortic aneurysm of ≥3.0 cm or recent abdominal aortic surgery. Patients with terminal malignant disease, those not independent in daily activities and not sufficiently fluent in Dutch were not included. The Medical Ethics Committee approved the study and all patients gave written informed consent. After inclusion, patients underwent a standardized vascular screening including measurements of the vascular risk factors, non-invasive measurement of sub-clinical atherosclerosis and MRI of the brain. Risk factors, medical history and functioning were assessed with questionnaires that the patients completed prior to their visit. A total of 1309 patients with clinical manifest vascular disease were included in the SMART-MR study, in 1215 of which adipokine concentrations and adiposity measurements were available and eligible for cross-sectional analyses.

Adiposity measurements

Body mass index (BMI), waist circumference (WC), subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were measured in this cohort. BMI was calculated by weight (kg)/height (m) ^{2}. Waist circumference was measured halfway the lower rib and the iliac crest in standing position. B-mode ultrasound of the abdomen was obtained to measure visceral and subcutaneous adipose tissue and performed by well-trained registered vascular technologists in a certified vascular laboratory. Ultrasonographic measurements were performed in supine position using an ATL HDI 3000 ultrasound device (Philips Medical Systems, Eindhoven, The Netherlands) with a C 4-2 transducer. There was no bowel preparation performed before the ultrasound measurement. Visceral adipose tissue was ultrasonographically measured as the distance between the peritoneum and the lumbar spine using electronic callipers. A strict protocol was used, including the position of the transducer and pressure applied on the transducer. The transducer was placed in a straight line drawn between the left and right midpoints of the lower rib and the iliac crest. Measurements were performed at the end of a quiet expiration, applying minimal pressure

without displacement or compression of the abdominal cavity. The distance was measured three times at slightly different positions; the visceral adipose tissue was calculated as the mean value of these three measurements. Previously, the ultrasound protocol for measuring visceral adipose tissue was validated with computed tomography (CT) at our center(31). Ultrasonographic measurements were strongly associated with CT measurements of visceral adipose tissue; Pearson's correlation coefficient was 0.81 ($p < 0.001$). Also, an interobserver coefficient of variation of 5.4% was found for ultrasound measurements of visceral adipose tissue, indicating good reproducibility. For determining subcutaneous fat, the distance between the linea alba and the skin was measured. Abdominal muscles were thus excluded for intra-abdominal and subcutaneous fat measurements.

Measurement of plasma concentrations of adipokines

Adipokines were measured in plasma which had been stored at -80° Celsius within hours after collection. The plasma concentrations of the adipocytokines Monocyte Chemotactic Protein 1 (MCP-1), Nerve Growth Factor (NGF), Hepatic Growth Factor (HGF), Migration Inhibitory Factor (MIF), Adiponectin, Adipsin, Chemerin, Resistin, Plasma Amyloid A1 (SAA1), Plasminogen Activator Inhibitor 1 (PAI1), Interferon gamma inducible Protein-10 (IP-10) and Leptin were measured with a microbead-based Luminex multiplex immuno assay (Bio-Rad, Munich, Germany). These specific adipokines were chosen for their effects on different (patho)physiological mechanisms; inflammation, coagulation and glucose metabolism.. Fluorophore-labeled microbeads were coated with specific monoclonal antibodies against the aforementioned proteins. The beads coated with capture antibodies were used together with secondary biotinylated antibodies. Measurements and data analysis of all assays were performed on the Bioplex system in combination with Bioplex manager software (Biorad) The multiplex immunoassay results correlated significantly with ELISA measurements. All adipokine concentrations were reported in ng/L. The analysis protocol as well as the characteristics of the assay have been published previously(32). Plasma high sensitivity CRP (hsCRP) was measured by immunonephelometry (Nephelometer Analyzer BN II, Dade-Behring, Marburg, Germany) with a lower detection limit of the test of 0.2 mg/l. As high CRP concentrations may have a different pathophysiological origin than low-grade inflammation as seen in obesity and vascular diseases, subjects with CRP concentrations >15 mg/L (n=53) were excluded from the analyses. Insulin sensitivity was calculated with the homeostasis model assessment of insulin resistance (HOMA-IR) with the formula: [fasting insulin (mU/l) * fasting glucose (mmol/l)]/22.5. HOMA-IR was not calculated in patients using exogenous insulin.

Data analyses

Mean and standard deviation (SD) are reported if continuous variables were normally distributed and median and interquartile range if distributions were skewed. Categorical variables are expressed as percentages (numbers). Concentrations of adipokines were log-transformed, due to a skewed distribution. The relations between measures of adiposity

and adipokine concentrations were quantified with linear regression analyses. Since IP-10 showed a skewed distribution even after log-transformation, this adipokine was only used in the 'adipokine profile'. Results are expressed as beta regression coefficients (β) and 95% confidence intervals (95%CI) denoting the change in adipokine concentration per standard deviation (SD) increase in adiposity measurements. Three models were used to estimate this relation. Firstly, a model was made without adjustments for the potential confounders. Secondly, a model was made with adjustments for the potential confounders gender and age. In the third model, additional adjustments were made for current smoking, lipid lowering therapy, history of DM2 and hsCRP.

The relations between adipokines and (characteristics of) the metabolic syndrome were assessed with logistic regression analysis. Since we hypothesize that metabolic syndrome is the consequence of the combination of various pro- and anti-inflammatory adipokines, rather than the effect of single adipokines, we developed an 'adipokine profile'. The adipokine profile was constructed weighing all adipokines equally and not taking into account their correlations, not aiming on predicting individual risk, but being hypothesis generating. All adipokines were divided into quartiles, patients in the most favorable quartile regarding cardiovascular risk received 1 point, patients in the least favorable quartile received 4 points. As this was performed for all patients with all 11 adipokines, a profile from 11 through 44 was possible. Also, the relations between single adipokines and (characteristics of) the metabolic syndrome were assessed. Results are expressed as odds ratios (OR) with 95%CI. For these analyses, only data from patients without DM2 were used, since DM2 can be regarded as an ultimate consequence of the metabolic syndrome and would therefore influence results. Two models were used, an unadjusted model and a model adjusted for age and gender. No other additional adjustments were performed, since possible confounders (such as hsCRP) can also be seen as intermediates in the relation between adipokines and metabolic syndrome. To reduce bias and to improve statistical efficiency, missing values for visceral fat thickness (n=8), subcutaneous fat thickness (n=21), BMI (n=1), waist circumference (n=52), smoking status (n=9) and eGFR (n=14) were completed in the dataset by single regression imputation.

Results

Baseline characteristics

Baseline characteristics in tertiles of waist circumference are presented in Table 1. Data are gender pooled since waist circumference cut off points are different for men and women. The population was predominantly male (80%) and the mean age was 59±10 years. In concordance with an increase in waist circumference over tertiles, a rise in BMI, VAT and SAT was seen, as well as an increase in the prevalence of metabolic syndrome, hypertension, elevated triglycerides and elevated fasting glucose concentrations.

Table 1. Baseline characteristics according to tertiles of waist circumference

	Tertile 1 (n=394)	Tertile 2 (n=393)	Tertile 3 (n=367)
Waist circumference cm (mean - range)			
Men*	87 (70-92)	97 (93-101)	109 (102-133)
Women*	76 (59-84)	89 (85-93)	102 (94-127)
Male gender (%)	80	82	80
Age (years)*	58 ± 10	59 ± 10	60 ± 9
Current smoking (%)	37	31	40
Medical history			
Peripheral artery disease (%)	23	25	19
AAA (%)	8	8	11
Cerebral artery disease (%)	27	22	22
Coronary artery disease (%)	57	65	70
Diabetes mellitus type 2 (%)	9	13	24
BMI (kg/m2)*	24.0 ± 2.5	26.4 ± 2.1	30.5 ± 3.5
Subcutaneous adipose tissue (cm)*	2.2 ± 1.2	2.5 ± 1.3	2.8 ± 1.5
Visceral adipose tissue (cm)*	7.7 ± 1.8	9.6 ± 2.1	11.3 ± 2.4
Systolic blood pressure (mmHg)*	140 ± 21	143 ± 23	145 ± 22
Diastolic blood pressure (mmHg)*	81 ± 11	82 ± 12	84 ± 12
Metabolic syndrome (%)	6	19	57
Total cholesterol (mmol/L)*	4.8 ± 1.0	4.9 ± 1.0	5.0 ± 1.0
Triglycerides (mmol/L)#	1.2 (0.9 – 1.7)	1.5 (1.2 – 2.1)	1.7 (1.3 – 2.4)
HDL-cholesterol (mmol/L) – men*	1.32 ± 0.36	1.20 ± 0.32	1.14 ± 0.32
HDL-cholesterol (mmol/L) – women*	1.67 ± 0.40	1.45 ± 0.40	1.34 ± 0.41
LDL-cholesterol (mmol/L)*	2.8 ± 0.9	2.8 ± 0.9	2.9 ± 0.9
eGFR (ml/min/1.73m^2)*	78 ± 16	77 ± 17	79 ± 18
Insulin (mIU/L)#	7 (5 – 9)	10 (8 – 14)	13 (10 – 19)
HOMA-IR#	1.80 (1.20 – 2.49)	2.63 (1.97 – 3.73)	3.64 (2.61 – 6.14)
hs-CRP (mg/L)#	1.3 (0.7 – 3.0)	1.7 (0.8 – 3.4)	2.7 (1.5 – 4.9)
Glucose (mmol/L)*	5.8 ± 1.3	6.2 ± 1.5	6.8 ± 2.0
Medication			
Antihypertensive medication (%)	67	75	85
Glucose lowering medication (%)	7	10	17
Lipid lowering medication (%)	69	72	71
Platelet aggregation inhibition (%)	79	77	77

Data are presented as mean ± SD (*) or as median + interquartile range (#), unless otherwise indicated. Waist circumference tertiles were separately determined for men and women and thereafter pooled to provide an equal sex-distribution over the tertiles.

AAA: abdominal aorta aneurysm; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; eGFR: Glomerular Filtration Rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation; HOMA-IR: homeostasis model of assessment – insulin resistance; hs-CRP: high sensitivity C-reactive protein.

Relation between total adiposity and fat distribution and plasma concentrations of adipokines

Higher BMI, higher waist circumference and higher VAT were all related to higher plasma concentrations of the adipokines NGF, HGF, MIF, leptin and adipsin (Figure 1 and Table S1) after adjustment for the potential confounding factors age and gender. The strongest positive relations were seen between BMI and adipsin (β 0.247; 95CI% 0.137-0.356) and between BMI and leptin (β 0.266; 95%CI 0.207-0.324). There were no or very weak relations between SAT and plasma concentrations of adipokines (Figure 1 and Table S1). Adiposity was negatively related with chemerin, PAI-1, resistin, SAA1 and adiponectin concentrations. The strongest negative relations were seen between BMI and SAA1 (β -0.266; 95%CI -0.386 - -0.146) and between VAT and adiponectin (β -0.168; 95%CI -0.226 - -0.111). Additional adjustment for the confounding factors smoking, use of lipid-lowering medication, history of diabetes and hsCRP only marginally attenuated relations between adiposity measurements and adipokine concentrations (Figure 1 and Table S1). There was no statistical interaction of type 2 diabetes mellitus in the relations between adiposity measurements and adipokine concentrations (p-values for interaction >0.05).

Relation between adipokine profile and metabolic syndrome

The adipokine profile ranged from 17-44, with a median score of 30 (IQR 27-33). A 1 point higher adipokine profile was related to a higher risk of metabolic syndrome (OR 1.03, 95% CI 1.00-1.06) adjusted for age and gender. Additional adjustment for visceral adipose tissue, changed this relationship to a small extent (OR 1.02, 95% CI 0.99-1.05). A sensitivity analysis including only the male patients did not change the results.

Relation between adipokine plasma concentrations and presence of metabolic syndrome

A 1 SD higher plasma concentration of HGF was related to a 21% higher risk of metabolic syndrome (OR 1.21; 95%CI 1.06-1.38) adjusted for age and gender, and a 26% higher risk for metabolic syndrome was seen with each 1 SD higher leptin concentration (OR 1.26; 95%CI 1.10-1.45), in the analyses adjusted for age and gender (Table 2). A 1 SD higher concentration of adiponectin was related with a 27% lower risk of metabolic syndrome (OR 0.73; 95%CI 0.64-0.83) and a 15% lower risk for metabolic syndrome was seen with each 1 SD higher resistin concentration (OR 0.85; 95%CI 0.74-0.97).

No interaction was observed of VAT in the relation between adipokines and presence of the metabolic syndrome. In table 3 the relation between 1 SD increase in adipokines and metabolic syndrome stratified into quartiles of VAT is shown, adjusted for age and gender. Additional adjustment for BMI, a measure of general adiposity, did not change the results.

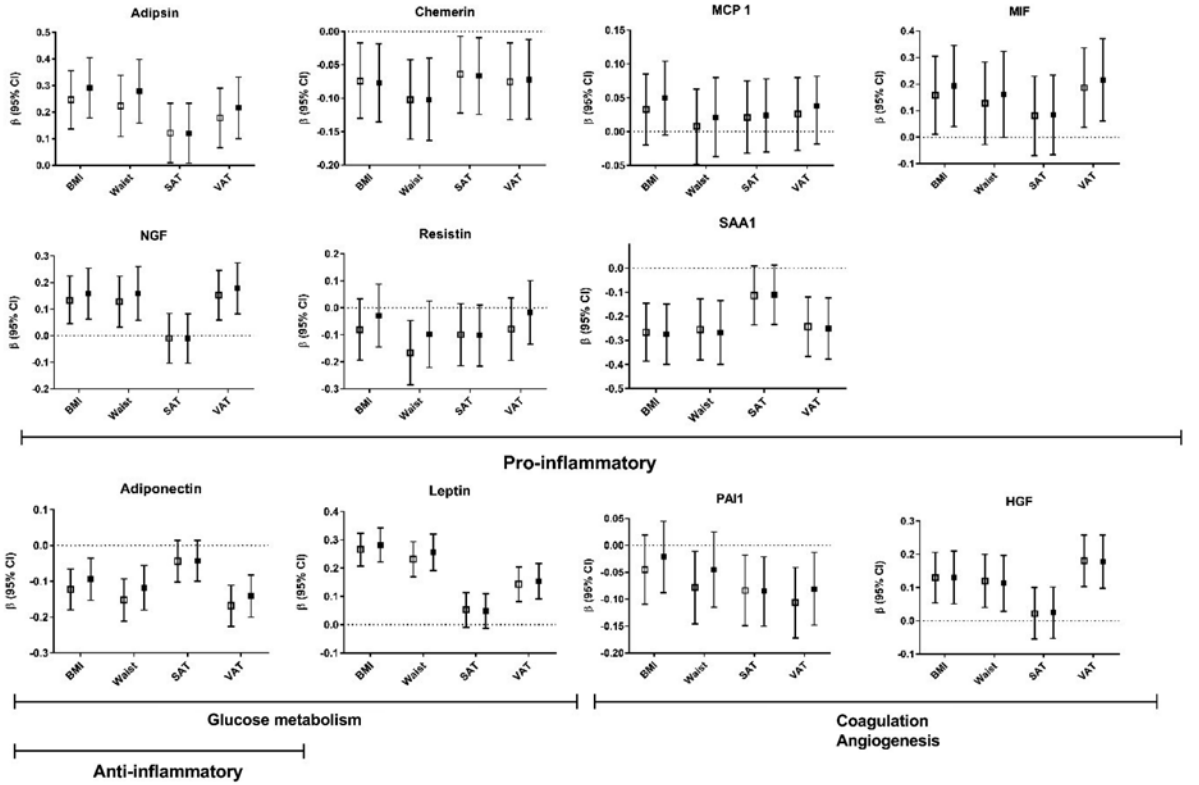

Figure 1. Relations between adiposity measurements and adipokines

Results are presented as regression coefficients (β) and 95% confidence interval (95%CI) per 1 SD increase in adiposity measurements. Model II (open squares) is adjusted for age and gender, Model III (closed squares) is additionally adjusted for current smoking, lipid lowering therapy, history of diabetes type 2 and hsCRP

BMI: body mass index, WC: waist circumference, SAT: subcutaneous adipose tissue VAT: visceral adipose tissue, MCP1: monocyte chemo-attractant protein 1, NGF: nerve growth factor, HGF: hepatic growth factor, PAI1: plasminogen activator inhibitor 1, MIF: migration inhibitory factor, SAA1: serum amyloid A1.

Table 2. Relation between adipokines and presence of metabolic syndrome

Model	Adipsin	Chemerin	MCP1	MIF	NGF	Resistin	SAA1	Adiponectin	Leptin	PAI1	HGF
I	0.96 (0.85-1.09)	0.91 (0.80-1.04)	1.08 (0.95-1.23)	1.05 (0.92-1.19)	1.12 (0.98-1.27)	**0.85* (0.75-0.97)**	0.98 (0.86-1.11)	**0.72* (0.63-0.83)**	**1.21* (1.06-1.38)**	0.94 (0.83-1.08)	**1.20* (1.05-1.38)**
II	0.96 (0.85-1.09)	0.91 (0.80-1.03)	1.08 (0.95-1.23)	1.06 (0.93-1.21)	1.12 (0.99-1.28)	**0.85* (0.74-0.97)**	0.98 (0.86-1.12)	**0.73* (0.64-0.83)**	**1.26* (1.10-1.45)**	0.94 (0.82-1.07)	**1.21* (1.06-1.38)**
III	**0.85* (0.74-0.98)**	0.99 (0.86-1.14)	1.06 (0.92-1.23)	0.98 (0.85-1.13)	1.04 (0.90-1.19)	**0.85* (0.73-0.98)**	1.11 (0.96-1.29)	**0.82* (0.71-0.96)**	1.14 (0.98-1.34)	1.04 (0.90-1.20)	1.08 (0.93-1.25)

Data are presented as odds ratios (OR) with 95% Confidence Intervals (95% CI) per 1SD increase in adipokine concentrations. *p <0.05

MCP1: monocyte chemo-attractant protein 1, NGF: nerve growth factor, HGF: hepatic growth factor, PAI1: plasminogen activator inhibitor 1, MIF: migration inhibitory factor, SAA1: serum amyloid A1. Model I: crude model, Model II: adjusted for age and gender, Model III: additionally adjusted for visceral adipose tissue

Table 3. Association between adipokines and the metabolic syndrome, stratified to quartiles of visceral adipose tissue.

Adipokine	Q1 (3.4 – 7.5cm) OR (95%CI)	Q2 (7.6-9.2cm) OR (95%CI)	Q3 (9.3-11.1cm) OR (95%CI)	Q4 (11.2-18.9cm) OR (95%CI)	P for interaction
MCP 1	0.76 (0.53-1.08)	0.97 (0.68-1.37)	1.03 (0.74-1.44)	0.84 (0.62-1.14)	0.49
NGF	0.81 (0.54-1.21)	1.15 (0.79-1.67)	1.25 (0.90-1.72)	0.94 (0.70-1.28)	0.36
HGF	0.94 (0.65-1.35)	0.92 (0.62-1.37)	1.05 (0.78-1.40)	0.93 (0.66-1.32)	0.84
PAI1	0.96 (0.57-1.61)	1.39 (0.93-2.06)	0.93 (0.68-1.28)	0.78 (0.59-1.03)	0.94
MIF	1.18 (0.75-1.85)	1.07 (0.71-1.62)	1.09 (0.80-1.47)	1.09 (0.77-1.54)	0.70
Adipsin	0.99 (0.65-1.50)	1.04 (0.71-1.52)	0.85 (0.63-1.15)	0.84 (0.63-1.12)	0.15
Adiponectin	0.58 (0.35-0.97)	0.68 (0.45-1.03)	0.64 (0.45-0.91)	0.76 (0.57-1.01)	0.51
Resistin	0.62 (0.38-1.00)	0.95 (0.64-1.40)	0.84 (0.63-1.12)	0.88 (0.68-1.16)	0.21
Chemerin	0.91 (0.59-1.41)	1.02 (0.70-1.49)	0.83 (0.59-1.15)	0.86 (0.64-1.16)	0.18
SAA1	0.84 (0.56-1.27)	1.27 (0.83-1.93)	1.01 (0.72-1.40)	0.79 (0.60-1.05)	0.40
Leptin	0.88 (0.56-1.40)	1.43 (0.97-2.11)	1.00 (0.70-1.43)	0.90 (0.66-1.23)	0.71

Results are presented as odds ratios (OR) with 95% confidence intervals (95%CI) for the presence of the metabolic syndrome with a 1SD increase in adipokines, stratified into quartiles of visceral adipose tissue (VAT, in cm)). P-value for interaction is the interaction of continuous measured VAT in the relation between adipokines and the metabolic syndrome. MCP1: monocyte chemo-attractant protein 1, NGF: nerve growth factor, HGF: hepatic growth factor, PAI1: plasminogen activator inhibitor 1, MIF: migration inhibitory factor, SAA1: serum amyloid A

Discussion

In this study, it is demonstrated that general and visceral adiposity, but not subcutaneous adiposity, is related to plasma concentrations of adipokines in patients with clinical manifest vascular disease. The newly developed adipokine profile and several single adipokine plasma concentrations are related to the presence of the metabolic syndrome. This relation partly sustained even after adjustment for adiposity, indicating that adipose tissue (dys)function (as reflected by the adipokine profile) is more important than quantity of adipose tissue in the relation to the metabolic syndrome.

Relations between adiposity and adipokine concentrations

The pro-inflammatory adipokines NGF, MIF and adipsin were related to general and abdominal adiposity, which was shown in various earlier studies (17–19,21,22). This is in line with the concept that an increase in adipose tissue quantity causes a shift in the balance between production of pro- and anti-inflammatory adipokines, resulting in an excess of pro-inflammatory adipokines. However, some relations between adiposity measurements and adipokines in our study were unexpected in the light of this line of reasoning. This was the case for the negative relations of adiposity and the adipokines SAA1, chemerin and resistin, who are believed to be pro-inflammatory(33,34). Index-event bias could explain this phenomenon. In the present study patients were all diagnosed with clinical evident atherosclerotic disease, in contrast to the patients included in previous studies. This inverse relationship has been shown before. Chemerin was negatively related to visceral adipose tissue in patients with hepatic steatosis , suggesting a modulating function of chemerin in the link between insulin resistance and steatosis(35). A similar function of chemerin in atherosclerotic patients could be present, explaining the negative relation between VAT and chemerin. Also, in a general population with approximately 10% DM2, an inverse association between anthropometric measurements and resistin plasma levels was found (36). Since SAA1 is a lipoprotein, the extensive use of lipid-lowering therapy in our cohort could (although we corrected for use of lipid-lowering medication) influence results. Index-event bias could also explain the negative relation between adiposity and the pro-coagulant PAI-1 we found, contrary to results in earlier literature. These unexpected results limit the generalizability of our study and emphasize the possibility of different associations in patients with already diagnosed cardiovascular disease.

Also shown in earlier studies(21,37) we found general and abdominal adiposity to be related to the procoagulant adipokine HGF, the glucose metabolism influencing adipokine leptin and, negatively, to the anti-inflammatory adipokine adiponectin. No relations between the subcutaneous adipose tissue and adipokines were found, indicative of the more important role of visceral adipose tissue in the development of type 2 diabetes and atherosclerotic disease.

Adipokine profile and metabolic syndrome

Since the metabolic syndrome consists of different pathophysiological components and is the result of various metabolic derangements potentially caused by different adipokines, we decided to combine the individual effects of adipokines in an adipokine profile. There was a significant relation between this profile and the presence of the metabolic syndrome, even after adjustment for visceral adiposity. This indicates an effect of adipose tissue (dys) function beyond quantity. Apparently, the function of adipose tissue is more important than the quantity with respect to the metabolic consequences leading to disease such as DM2 and vascular diseases.

Single adipokines and metabolic syndrome

With respect to the relations between individual adipokines and the metabolic syndrome, we found leptin and HGF to be positively related to the metabolic syndrome. The positive relation between plasma concentrations of leptin and the metabolic syndrome has been demonstrated in patients with metabolic syndrome(38).Elevated leptin concentrations are related to both waist circumference(39) [35]and insulin resistance, even in euglycemic patients(40). The relation between HGF and metabolic syndrome was previously also shown in 1474 healthy subjects(41).Of the metabolic syndrome characteristics, HGF is most likely to influence blood pressure, since it mediates remodeling of the vascular wall(42). An association between HGF and insulin resistance has also been reported(41).

As expected, higher concentrations of adiponectin decreased the odds for presence of the metabolic syndrome. As shown in various studies(38,43), higher adiponectin is associated with lower pro-inflammatory adipokine concentrations, and consequently, the absence of adipose tissue dysfunction. Interestingly, recent Mendelian randomization studies show a counterintuitive association between higher adiponectin levels and cardiovascular mortality(44) but also lower adiponectin levels being associated with increased risk of metabolic syndrome and hyperglycemia.(45)

Surprisingly, higher concentrations of the pro-inflammatory resistin also decreased the odds for metabolic syndrome. In healthy individuals, resistin is positively associated with insulin sensitivity(36). Paradoxically, in patients with low-grade inflammation or patients with atherosclerosis the resistin plasma level is elevated(34,46). Since our cohort comprised patients with cardiovascular disease, we expected resistin concentrations to relate positively to the metabolic syndrome. There is some suggestion in literature that certain classes of anti-hypertensive drugs could decrease resistin concentrations(47), which could explain our results.

The absence of interaction of VAT in the relation between adipokines and the metabolic syndrome again emphasizes the role of adipose tissue function rather than quantity in the development of metabolic derangements, despite losing statistical significance when correcting for VAT in the relation between adipokines and metabolic syndrome (table 2) However, it should be noted that changes in plasma concentrations of adipokines could also be the result of metabolic changes.

Limitations and strengths

Strenghts of our study include the large cohort and the number of adipokines that were measured. Moreover, our cohort provides real world data of a clinically relevant group of patients. Also, some study limitations should be considered. Limitations include that the cohort comprised of patients with clinical manifest vascular disease, which may limit generalizability to patients without vascular disease. No data were available of physical activity or dietary intake of patients, which could influence results, since little activity and high intake of saturated fatty acids can cause metabolic syndrome, even in lean individuals. (48)Also, since measurement of plasmatic adipokines is not current practice, there is no current implementation of this study in general practice. Moreover, although we adjusted for several potential confounders in the multivariable analyses, there may be residual confounding. As this is a cross-sectional study one should be cautious in making causal inferences.

Conclusion

In conclusion, general adiposity and visceral adiposity are related to plasma concentrations of adipokines that reflect adipose tissue (dys)function. Adipokine concentrations are related to the presence of metabolic syndrome, independent of the amount of VAT. Therefore, adipose tissue dysfunction and not adiposity per se are likely to be the causal link between (abdominal) adiposity and metabolic changes causing the metabolic syndrome, eventually leading to DM2 and vascular disease.

Competing interest

The authors declare no competing interests

Members of the SMART study group

A. Algra, MD, PhD; Y. van der Graaf, MD, PhD; D. E. Grobbee, MD, PhD; G. E. H. M. Rutten, MD, PhD, Julius Center for Health Sciences and Primary Care; F. L. J. Visseren, MD, PhD, Department of Internal Medicine; G.J. de Borst, MD, PhD, Department of Vascular Surgery; L. J. Kappelle, MD, PhD, Department of Neurology; T. Leiner, MD, PhD, Department of Radiology; H.M. Nathoe, MD, PhD, Department of Cardiology

Author contributions

IS, FV and WS contributed to the conception or design of the work. YG contributed to the acquisition, analysis, or interpretation of data for the work. IS drafted the manuscript. FV, WS and YG critically revised the manuscript. All gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Table S1. Relation between adiposity measurements and adipokine concentrations.

	Model	MCP 1	NGF	HGF	PAI1	MIF	Adipsin	Adiponectin	Resistin	Chemerin	SAA1	Leptin
BMI 3.8kg/m^2	II	0,033 (-0,020 - 0,085)	**0,133* (0,045 - 0,225)**	**0,130* (0,054 - 0,206)**	-0,045 (-0.109 - 0.019)	**0,158* (0,011 - 0,305)**	**0,247* (0,137 - 0,356)**	**-0,123* (-0,180 - -0,066)**	-0,081 (-0,194 - 0,033)	**-0,074* (-0,130 - -0,017)**	**-0,266* (-0,386 - -0,146)**	**0,266* (0,207 - 0,324)**
	III	0,050 (-0,005 - 0,104)	**0,158* (0,063 - 0,254)**	**0,131* (0,051 - 0,210)**	-0,021 (-0,088 - 0,045)	**0,193* (0,040 - 0,346)**	**0,292* (0,178 - 0,405)**	**-0,094* (-0,153 - -0,036)**	-0,028 (-0,145 - 0,088)	**-0,077* (-0,135 - -0,018)**	**-0,274* (-0,399 - -0,149)**	**0,282* (0,222 - 0,343)**
WC 11.0cm	II	0.008 (-0.048 - 0.063)	**0,128* (0,032 - 0,224)**	**0.120* (0.040 - 0.200)**	**-0,078* (-0,146 - -0,011)**	0,128 (-0,027 - 0,283)	**0,224* (0,109 - 0,339)**	**-0.152* (-0.212 - -0.093)**	**-0.166* (-0.285 - -0.047)**	**-0.102* (-0.161 - -0.042)**	**-0,255* (-0,381 - -0,128)**	**0,232* (0,170 - 0,294)**
	III	0.021 (-0.037 - 0.080)	**0,159* (0,058 - 0,260)**	**0.113* (0.029 - 0.197)**	-0,045 (-0,115 - 0,025)	0,161 (-0,001 - 0,323)	**0,279* (0,159 - 0,399)**	**-0.118* (-0.180 - -0.056)**	-0.098 (-0.221 - 0.025)	**-0.102* (-0.163 - -0.040)**	**-0,267* (-0,399 - -0,134)**	**0,257* (0,192 - 0,321)**
SAT 1.4cm	II	0,021 (-0,032 - 0,075)	-0,024 (-0,118 - 0,070)	0,022 (-0,055 - 0,100)	**-0.084* (-0.149 - -0.018)**	0,081 (-0,069 - 0,230)	**0,122* (0,010 - 0,233)**	-0,044 (-0,102 - 0,014)	-0.100 (-0.215 - 0.015)	**-0,064* (-0,122 - -0,007)**	-0.114 (-0.236 - 0.009)	0.053 (-0.009 - 0.114)
	III	0,024 (-0,030 - 0,078)	-0,024 (-0,118 - 0,070)	0,025 (-0,053 - 0,102)	**-0,085* (-0.150 - -0.021)**	0,084 (-0,066 - 0,234)	**0,121* (0,009 - 0,233)**	-0,043 (-0,100 - 0,014)	-0.102 (-0.216 - 0.011)	**-0,066* (-0,124 - -0,009)**	-0.111 (-0.234 - 0.012)	0.049 (-0.013 - 0.110)
VAT 2.6cm	II	0,026 (-0,028 - 0,080)	**0,153* (0,059 - 0,246)**	**0,181* (0,103 - 0,258)**	**-0.106* (-0.172 - -0.041)**	**0,187* (0,037 - 0,337)**	**0,178* (0,066 - 0,290)**	**-0,168* (-0,226 - -0,111)**	-0,079 (-0,195 - 0,037)	**-0,075* (-0,132 - -0,017)**	**-0,243* (-0,366 - -0,120)**	**0.143* (0.082 - 0.204)**
	III	0,038 (-0,018 - 0,094)	**0,178* (0,082 - 0,274)**	**0,178* (0,098 - 0,258)**	**-0.081* (-0.148 - -0.013)**	**0,216* (0,061 - 0,371)**	**0,217* (0,101 - 0,332)**	**-0,141* (-0,200 - -0,082)**	-0,017 (-0,135 - 0,101)	**-0,072* (-0,131 - -0,012)**	**-0.250* (-0.377 - -0.123)**	**0.154* (0.091 - 0.217)**

Results are presented as regression coefficients (β) and 95% confidence interval (95%CI) per 1 SD increase in adiposity measurements . * p<0.05

Model II is adjusted for age and gender, Model III is additionally adjusted for current smoking, lipid lowering therapy, history of diabetes type 2 and hsCRP

BMI: body mass index, WC: waist circumference, SAT: subcutaneous adipose tissue VAT: visceral adipose tissue, MCP1: monocyte chemo-attractant protein 1, NGF: nerve growth factor, HGF: hepatic growth factor, PAI1: plasminogen activator inhibitor 1, MIF: migration inhibitory factor, SAA1: serum amyloid A1.

Chapter 6

Adipokines are related to blood pressure via multiple pathophysiological mechanisms, independent of adiposity measurements

Ilse M. Schrover[1], MD; Frank L. J. Visseren[1], MD, PhD, Wilko Spiering[1], MD, PhD

Department of Vascular Medicine[1] University Medical Center Utrecht, The Netherlands

On behalf of the SMART study group (individual members are mentioned at the end of the manuscript)

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Abstract

Introduction: The relation between obesity and hypertension might be mediated through adipokines. Adipokines influence sympathetic nervous system activity, renin-angiotensin-aldosterone system (RAAS)-hormones and endothelial inflammation, which all lead to higher blood pressure.

Methods: In a cohort of 1211 patients from the Second Manifestations of ARTerial disease Magnetic Resonance (SMART-MR) study, the relation between plasma concentrations of 12 adipokines (adiponectin, adipsin, chemerin, resistin, plasminogen activator inhibitor 1 (PAI-1), hepatic growth factor (HGF), nerve growth factor (NGF), leptin, serum amyloid A1 (SAA1), migration inhibitory factor (MIF), monocyte chemo attractant protein 1 (MCP-1), and interferon gamma induced protein 10 (IP-10)) and blood pressure was assessed using linear regression, adjusted for age and gender. Additional regression analyses were performed adjusting for insulin resistance, heart rate, inflammation and adiposity measurements. An adipokine profile was developed to measure the effect of combined rather than single adipokines.

Results: The adipokine score ranged from 17-44, with a median score of 30 (IQR 27-33). A 1 point higher adipokine score was related to a small, but significant increase in blood pressure (β 0.068 mmHg, 95%CI 0.067-0.581) adjusted for age and gender. Each standard deviation (SD) difference in NGF (2.2 mmHg (95%CI 0.9-3.4), HGF (1.5 mmHg (95%CI 0.3-2.8), MIF (1.6 mmHg (95%CI 0.4-2.9), and leptin (1.6 mmHg (95%CI 0.2-2.9) was associated with a higher SBP. A SD difference in resistin was associated with a lower SBP (-1.3 mmHg (95%CI -2.6-0.0). Additional adjustments for insulin resistance, heart rate and inflammation attenuated the associations, although to a small extent. Relations between adipokines and blood pressure did not change after adjustment for subcutaneous adiposity and were only mildly attenuated after adjustment for general and abdominal adiposity measurements.

Conclusion: Adipokines are related to blood pressure, partly independent of general and abdominal adiposity. Dysfunctional adipose tissue and not adiposity per se is likely to be the causal link between (abdominal) adiposity and hypertension.

Introduction

The rationale behind the successful treatment of hypertension in overweight or obese individuals with either renin-angiotensin-aldosterone system (RAAS) inhibitors(1,2) or sympathico-blocking agents(3) can be found in the upregulation of the RAAS and activation of the sympathetic nervous system (SNS) in overweight or obese patients(4). Presumably, these mechanisms are activated via secretion of adipokines and hormones from the adipose tissue(5). The adipokines leptin(6) and resistin(7) are known to enhance SNS activity, and inflammatory adipokines (such as interferon gamma induced protein 10 (IP-10), chemerin and monocyte chemo-attractant protein 1(MCP-1)) enhance the inflammatory process in the endothelial cells(8–10). Endothelial dysfunction, resulting from the inflammatory process in the endothelial cell, is related to the development of hypertension(11) via increased vascular resistance. Both enhanced sympathetic activity and RAAS activation increase sodium reabsorption and consequently cause volume overload(4). Moreover, sympathetic activity increases insulin resistance, and insulin resistance stimulates sympathetic outflow, establishing a vicious circle(12,13) in the pathogenesis of hypertension. Local adipose tissue RAAS activation occurs independent from systemic blood pressure and blood pressure feedback loops in response to hypertrophy of the adipocytes(14–16). Figure 1 provides an overview of the pathophysiological pathways involved in the relation between adipokines and blood pressure(4,6–8,17–25).

Quantity of adipose tissue, combined with factors as physical inactivity(26), dietary intake of saturated fat(27) and genetic predisposition(28,29), causes the enhanced and imbalanced secretion of adipokines and hormones from the adipose tissue, an effect called adipose tissue dysfunction, resulting in endothelial dysfunction and insulin resistance(30).

In this study, we evaluated the relation between adipokines and blood pressure in a population of patients with prior vascular disease. Furthermore, we evaluated the contribution of the quantity of adipose tissue in this relation.

Methods and procedures

Study design and patients

The study cohort consisted of patients participating in the Second Manifestations of ARTerial disease-Magnetic Resonance (SMART-MR) study, a prospective magnetic resonance imaging (MRI) study in patients with clinical manifest vascular disease. Details of the design and participants have been described elsewhere(31,32). In brief, between May 2001 and December 2005, patients newly referred to the University Medical Center Utrecht with manifest coronary artery disease (CAD), cerebrovascular disease (CVD), peripheral arterial disease (PAD) or an aortic abdominal aneurysm (AAA) were invited to participate. CAD was defined as a recent diagnosis of angina pectoris, myocardial infarction or coronary

revascularization. CVD was defined as a recent diagnosis of ischemic stroke, transient ischemic attack or amaurosis fugax. PAD consisted of those with a recent clinical diagnosis of PAD (Fontaine stage 2, 3, or 4). AAA was defined as an abdominal aortic aneurysm of ≥3.0 cm or recent abdominal aortic surgery. Patients with terminal malignant disease, those not independent in daily activities and not sufficiently fluent in Dutch were excluded. After inclusion, patients underwent a standardized vascular screening including measurements of the vascular risk factors, non-invasive measurement of sub-clinical atherosclerosis and MRI of the brain. Risk factors, medical history and functioning were assessed with questionnaires that the patients completed prior to their visit. A total of 1309 patients with clinical manifest vascular disease were included in the SMART-MR study, in 1211 of which adipokine concentrations and blood pressure measurements were available and eligible for cross-sectional analyses.

Data acquisition

Adipocytokines were measured in serum which had been stored at -80° Celsius within hours after collection. The serum concentrations of the adipocytokines Monocyte Chemotactic Protein 1 (MCP-1), Interferon gamma induced protein 10 (IP-10), Nerve Growth Factor (NGF), Hepatic Growth Factor (HGF), Migration Inhibitory Factor (MIF), adiponectin, adipsin, chemerin, resistin, Serum Amyloid A1 (SAA1), Plasminogen Activator Inhibitor 1 (PAI-1) and leptin were measured with a microbead-based Luminex multiplex immuno assay (Bio-Rad, Munich, Germany). Fluorophore-labeled microbeads were coated with specific monoclonal antibodies against the aforementioned proteins. The beads coated with capture antibodies were used together with secondary biotinylated antibodies. Measurements and data analysis of all assays were performed on the Bioplex system in combination with Bioplex manager software (Biorad). The analysis protocol as well as the characteristics of the assay have been published previously(33). Serum high sensitivity CRP (hsCRP) was measured by immunonephelometry (Nephelometer Analyzer BN II, Dade-Behring, Marburg, Germany) with a lower detection limit of the test of 0.2 mg/L. As high CRP levels may have a different pathophysiological origin than low-grade inflammation as seen in obesity and vascular diseases, subjects with hsCRP levels >15 mg/L (n=53) were excluded from the analyses. Insulin sensitivity was calculated with the homeostasis model assessment of insulin resistance (HOMA-IR) with the formula: [fasting insulin (mU/L) * fasting glucose (mmol/L)]/22.5. HOMA-IR was not calculated in patients using exogenous insulin.

Blood pressure was measured two times in sitting position at the right and left upper arm using an appropriate cuff size in obese subjects. Blood pressure level was subsequently defined as the highest mean of the measurements on one arm(32). Body mass index (BMI) was calculated as the weight (kg) / height^2 (cm). Waist circumference (WC) was measured halfway between the lower rib and the iliac crest and was taken in standing position. The amount of visceral adipose tissue (VAT) was measured with an abdominal ultrasound, performed by well-trained registered vascular technologists in a certified vascular laboratory.

Ultrasonographic measurements were performed in supine position using an ATL HDI 3000 ultrasound device (Philips Medical Systems, Eindhoven, The Netherlands) with a C 4-2 transducer. There was no bowel preparation performed before the ultrasound measurement. Visceral adipose tissue was ultrasonographically measured as the distance between the peritoneum and the lumbar spine using electronic callipers. The distance was measured three times at slightly different positions. The visceral adipose tissue was calculated as the mean value of these three measurements. For determining subcutaneous adipose tissue (SAT), the distance between the linea alba and the skin was measured. Abdominal muscles were thus excluded for both intra-abdominal and subcutaneous fat measurements.

Data analyses

Mean and standard deviation (SD) are reported if continuous variables were normally distributed and median and interquartile range if distributions were skewed. Categorical variables are expressed as percentages (numbers). Concentrations of adipokines were logarithmically logtransformed, due to a skewed distribution in the study population. For IP-10, distribution was still skewed after logarithmically transformation. Since IP-10 activates RAAS locally(8) and might therefore be important in the pathogenesis of hypertension, we decided to analyze the association between IP-10 and hypertension in quartiles of IP-10. For each patient, the presence or absence of the metabolic syndrome was determined. We defined the metabolic syndrome according to the NCEP criteria (34) as the presence of three or more of the following characteristics: waist circumference >102 cm (men) or >88 cm (women); triglycerides ≥1.7 mmol/L; high density lipoprotein cholesterol <1.29 mmol/L (women) or <1.00 mmol/L (men); blood pressure ≥130/85 mmHg; fasting glucose ≥5.6 mmol/L.

Observed blood pressure is subject to the effect of treatment. Without correction, this can lead to substantial shrinkage of the true effect size and a marked reduction in statistical power(35,36). The most appropriate method to take antihypertensive treatment into account is adding a sensible constant to the observed blood pressure in treated subjects(36). Therefore, a constant value of 10/5 mmHg was added to the observed blood pressure of individuals using one class of blood pressure-lowering medication, because this constant equals the average effect produced by the main types of blood pressure-lowering drugs(37). In addition, 5/2.5 mmHg was added to the observed blood pressure for the use of every subsequent class of blood pressure-lowering medication. The following classes of blood pressure-lowering medication were distinguished: beta-blockers, diuretics, ACE-inhibitors, calcium-channel blockers, alpha-1-receptor antagonists, and angiotensin 2 type 1-receptor antagonists.

The relationship between adipokine concentrations and blood pressure was quantified with linear regression analysis. Results are expressed as beta regression coefficients (β) and 95% confidence intervals (95%CI) denoting the change in blood pressure per standard deviation (SD) increase in adipokine concentrations measurements. Analyses were adjusted

for age and gender. To account for possible effects of antihypertensive medication other than lowering blood pressure, an exploratory analysis was performed adjusting for antihypertensive medication.

We hypothesize that the effect on blood pressure is the consequence of the combination of various pro- and anti-inflammatory adipokines, rather than the effect of single adipokines, therefore we developed an 'adipokine score'. All adipokines were divided into quartiles, patients in the most favorable quartile regarding cardiovascular risk received 1 point, patients in the least favorable quartile received 4 points. As this was performed for all patients with all 12 adipokines, a score from 12 through 48 was possible. Since activation of the sympathetic nervous system, insulin resistance, low-grade inflammation and adiposity were thought to be intermediates in the relation between adipokines and blood pressure(38,39), we adjusted the analyses separately in exploratory models additionally for heart rate, HOMA-IR, hsCRP and the adiposity measurements BMI, WC, SAT and VAT. In the analyses additionally adjusted for heart rate, patients using beta-blocking agents were excluded, leaving 591 patients. In the analyses additionally adjusted for insulin resistance, patients with DM2 were excluded, leaving 986 patients. Statistical analyses were performed with SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

Results

Table 1 summarizes the characteristics of the patients in the study. Antihypertensive medication was used by 73% of patients, with beta-blockers and ACE-inhibitors being the most prescribed drugs. The observed blood pressure was on average 143 ± 22 / 83 ± 12 mmHg, with a heart rate of 63 ± 13 beats/min. Half of the patients fulfilled the diagnostic criteria of the metabolic syndrome.

Relation between adipokines and adjusted blood pressure

The relation between standardized differences of adipokine concentrations and adjusted blood pressure is shown in Table 2. Each standard deviation (SD) difference in NGF (2.2 mmHg (95%CI 0.9-3.4), HGF (1.5 mmHg (95%CI 0.3-2.8), MIF (1.6 mmHg (95%CI 0.4-2.9), and leptin (1.6 mmHg (95%CI 0.2-2.9) was associated with a significant positive SBP difference. A SD difference in resistin was associated with a significant negative SBP difference (-1.3 mmHg (95%CI -2.6-0.0). Adjustment for use of medication (all classes of antihypertensives) attenuated the results to a small extent (Table 2).

IP-10, which was analyzed in quartiles due to its skewed distribution, showed no association with SBP (Supplemental Data S1).

Table 1. Baseline characteristics of patients

	All patients (n=1215)
Male (%)	80
Age	59 ± 10
Current smoking (%)	36
Cerebrovascular disease (%)	23
Cardiovascular disease (%)	61
AAA (%)	8
Peripheral artery disease (%)	21
Systolic BP (mmHg)	143 ± 22
Diastolic BP (mmHg)	83 ± 12
Heart rate (bpm)	63 ± 13
BMI (kg/m^2)	26.8 ± 3.8
WC (cm)	95 ± 11
VAT (cm)	9.4 ± 2.6
SAT (cm)	2.5 ± 1.3
Glucose (mmol/L)	6.2 ± 1.7
Insulin (mIU/L)	10 (7-14)
Cholesterol (mmol/L)	4.9 ± 1.0
TG (mmol/L)	1.5 (1.1-2.1)
HDL-c (mmol/L)	1.29 ± 0.39
LDL-c (mmol/L)	2.8 ± 0.9
TSH (mU/L)	1.8 (1.2-2.5)
eGFR (ml/min/1.73m^2)	78 ± 17
hsCRP (mg/mL)	1.8 (0.9-3.7)
Metabolic syndrome (%)	51
Diabetes (%)	15
Antihypertensive medication (%)	73
Number of antihypertensives	1 (0-2)
Betablockers (%)	51
Diuretics (%)	15
ACE-inhibitors (%)	24
AT1-antagonists (%)	9
Calcium antagonists (%)	21
Alpha-blockers (%)	1
Lipid-lowering medication (%)	69
Platelet aggregation (%)	76
Glucose-lowering medication (%)	11
Adipokine concentrations	
MCP-1 (pg/ml)	256.7 (154.7-377.4)
IP-10 (pg/ml)	122.3 (71.9-192.8)
NGF (pg/ml)	15.4 (8.0-23.9)

Table 1. Continued

	All patients (n=1215)
HGF (pg/ml)	560.3 (336.4-892.6)
MIF (pg/ml)	611.0 (298.0-1154.4)
Adiponectin (ug/ml)	19.7 (12.6 – 33.0)
Adipsin (ug/ml)	2.4 (0.6 – 6.8)
Chemerin (ng/ml)	26.6 (1.7 – 7.2)
PAI-1 (ug/ml)	3.8 (1.2 – 6.2)
Resistin (ng/ml)	39.3 (7.3 – 12.9)
SAA1 (mg/dl)	0.4 (0.1 – 1.2)
Leptin (ng/L)	37.5 (21.8 – 72.4)

AAA: abdominal aorta aneurysm; BP: blood pressure; BMI: body mass index; WC: waist circumference; VAT: visceral adipose tissue; SAT: subcutaneous adipose tissue; TG: triglycerides; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; TSH: thyroid stimulating hormone; eGFR: estimated glomerular filtration rate, calculated with the Modification of Diet in Renal Disease (MDRD) formula; hsCRP: high-sensitivity C-reactive protein; MCP-1; monocyte chemo-attractant protein 1; IP-10: interferon gamma induced protein 10; NGF: Nerve Growth Factor; HGF: Hepatic Growth Factor; MIF: Migration Inhibitory Factor; SAA1: Serum Amyloid A1; PAI-1: Plasminogen Activator Inhibitor 1

Table 2. Association between adipokines and blood pressure

	Model 2	p-value	Model 3	p-value
Adiponectin	-0.93 (-2.24-0.37)	0.161	-0.76 (-2.00-0.49)	0.233
Chemerin	-1.14 (-2.40-0.13)	0.079	-1.10 (-2.30-0.10)	0.072
MCP-1	1.22 (-0.07-2.50)	0.064	0.62 (-0.60-1.83)	0.319
MIF	1.63 (0.37-2.90)	**0.012**	1.23 (0.03-2.42)	**0.044**
SAA1	-0.51 (-1.80-0.79)	0.441	-0.05 (-1.28-1.17)	0.935
PAI-1	-0.14 (-1.42-1.14)	0.829	-0.18 (-1.40-1.03)	0.771
HGF	1.52 (0.25-2.79)	**0.019**	1.07 (-0.12-2.27)	0.079
Resistin	-1.29 (-2.57- -0.01)	**0.049**	-1.29 (-2.51- -0.07)	**0.039**
Leptin	1.58 (0.24-2.91)	**0.021**	0.91 (-0.36-2.19)	0.159
NGF	2.17 (0.90-3.44)	**0.001**	1.90 (0.70-3.10)	**0.002**

Data are presented as regression coefficients (β) and 95% confidence intervals (95% CI) per 1 standard deviation (SD) increase in adipokine concentrations.

Model 2: adjusted for age and gender

Model 3: model 2 additionally adjusted for classes of antihypertensive medications

MCP-1: Monocyte chemo-attractant protein 1; IP-10: Interferon gamma induced protein 10; NGF: Nerve Growth Factor; HGF: Hepatic Growth Factor; MIF: Migration Inhibitory Factor; SAA1: Serum Amyloid A1; PAI-1: Plasminogen Activator Inhibitor 1

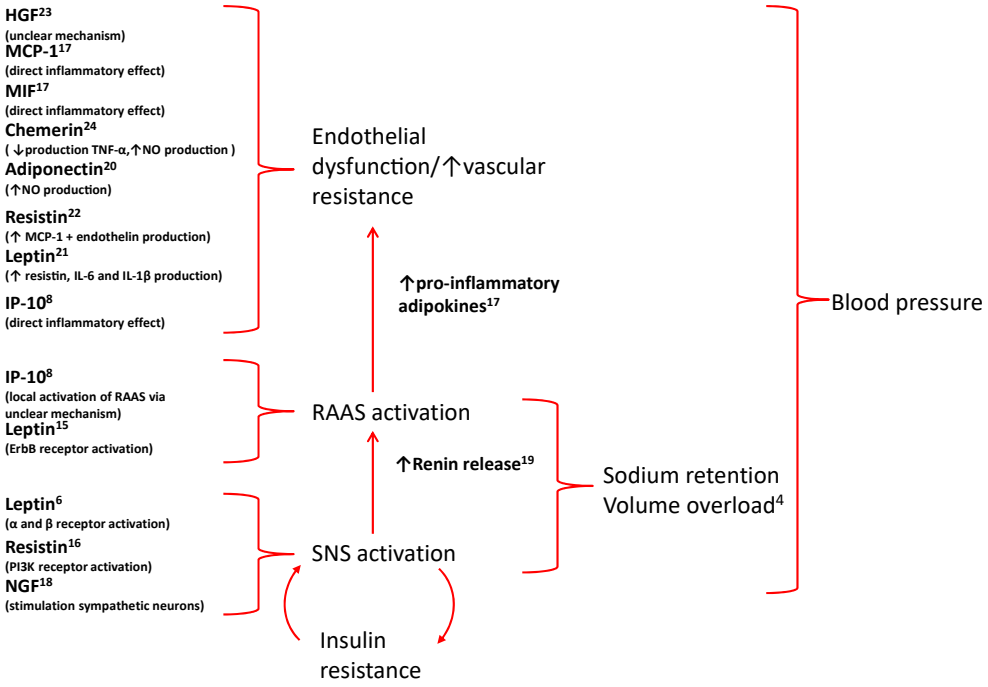

Figure 1. Schematic overview of pathophysiological link between adipokines and blood pressure

Upregulation of HGF, MCP-1, MIF, chemerin, resistin, leptin and IP-10 and downregulation of adiponectin occurs in reaction to obesity. These adipokines all cause endothelial dysfunction, giving rise to higher vascular resistance and consequently higher blood pressure. RAAS activation in the adipose tissue occurs under influence of IP-10 and leptin. Leptin, resistin and NGF enhance SNS activation. Both RAAS activation and SNS activation lead to sodium retention and volume overload, which raise blood pressure. Moreover, SNS activation causes insulin resistance, which in its turn activates the SNS, creating a vicious circle. Finally, renin is upregulated in response to an activated SNS, activating the RAAS system. In response to angiotensin II, part of the activated RAAS system, pro-inflammatory adipokines are upregulated, resulting in higher production of MCP-1 and MIF, enhancing the process of endothelial dysfunction.

RAAS: renin-angiotensin-aldosterone system; SNS: sympathetic nervous system; MCP-1: Monocyte chemo-attractant protein 1; IP-10: Interferon gamma induced protein 10; NGF: Nerve Growth Factor; HGF: Hepatic Growth Factor; MIF: Migration Inhibitory Factor; SAA1: Serum Amyloid A1; PAI-1: Plasminogen Activator Inhibitor 1

Relation between adipokine concentrations and blood pressure, adjusted additionally for low-grade inflammation, insulin resistance and sympathetic nervous system activity.

Figure 2 shows the relation between adipokine concentrations and blood pressure adjusted for age and gender and additionally adjusted for hsCRP (Figure 2a), HOMA-IR (Figure 2b) and heart rate (Figure 2c). Additional adjustment for hsCRP attenuated the relations between adipokines and blood pressure to a very small extent. None of the significant relations were lost. Adjustment for insulin resistance, an analysis only performed in the group of patients

without DM2, led to loss of significance of the relation between leptin and blood pressure, corrected for age and gender (1.8 mmHg (95%CI 0.0-3.5). After additional adjustment for HOMA-IR, the relation between leptin and blood pressure in this group was 1.6 mmHg (95% -0.1-3.4). All other adipokines were not related to blood pressure in this group, and no major effects were seen after adjustment for HOMA-IR. Additional adjustment for heart rate was performed in the group of patients not using beta-blockers. There were no significant relations beforehand in this subgroup of patients (n=595), and adjustment led to small attenuations of these relations.

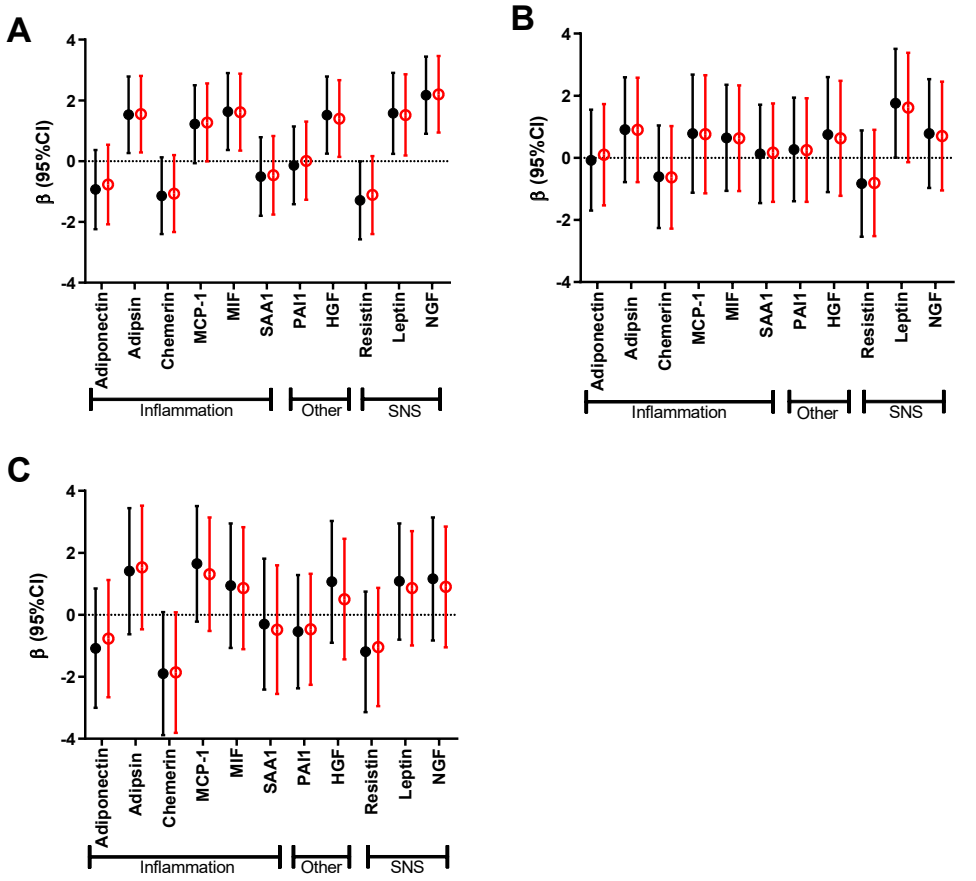

Figure 2. Association between adipokines and blood pressure adjusted for possible intermediate factors

Results are presented as regression coefficients (β) and 95% confidence intervals (95%CI) per 1 SD increase in adipokine concentrations. Black squares represent model 2 (adjusted for age and gender), red squares represent model 3 (model 2 additionally adjusted for A) hsCRP B) HOMA-IR and C) heart rate). HsCRP: high sensitivity CRP; MCP-1: Monocyte chemo-attractant protein 1; IP-10: Interferon gamma induced protein 10; NGF: Nerve Growth Factor; HGF: Hepatic Growth Factor; MIF: Migration Inhibitory Factor; SAA1: Serum Amyloid A1; PAI-1: Plasminogen Activator Inhibitor 1

Relation between adipokine concentrations and blood pressure, adjusted additionally for adiposity measurements.

Figure 3 shows the relation between adipokines and blood pressure after additional adjustments for measures of adiposity (BMI (Figure 3a), WC (Figure 3b), SAT (Figure 3c) and VAT (Figure 3d). Relations between adipokines and blood pressure were most attenuated by BMI and VAT. The significant relation between leptin and blood pressure after adjustment for age and gender was attenuated after adjustment for BMI (0.8 mmHg (95%CI -0.6-2.2) and VAT (1.2 mmHg (95%CI -0.2-2.5)). For adipsin and blood pressure, the significant relation present after adjustment for age and gender was lost after further adjustment for BMI (1.1 mmHg (95%CI-0.1-2.4) and VAT (1.3 mmHg (95%CI 0.0-2.5)).

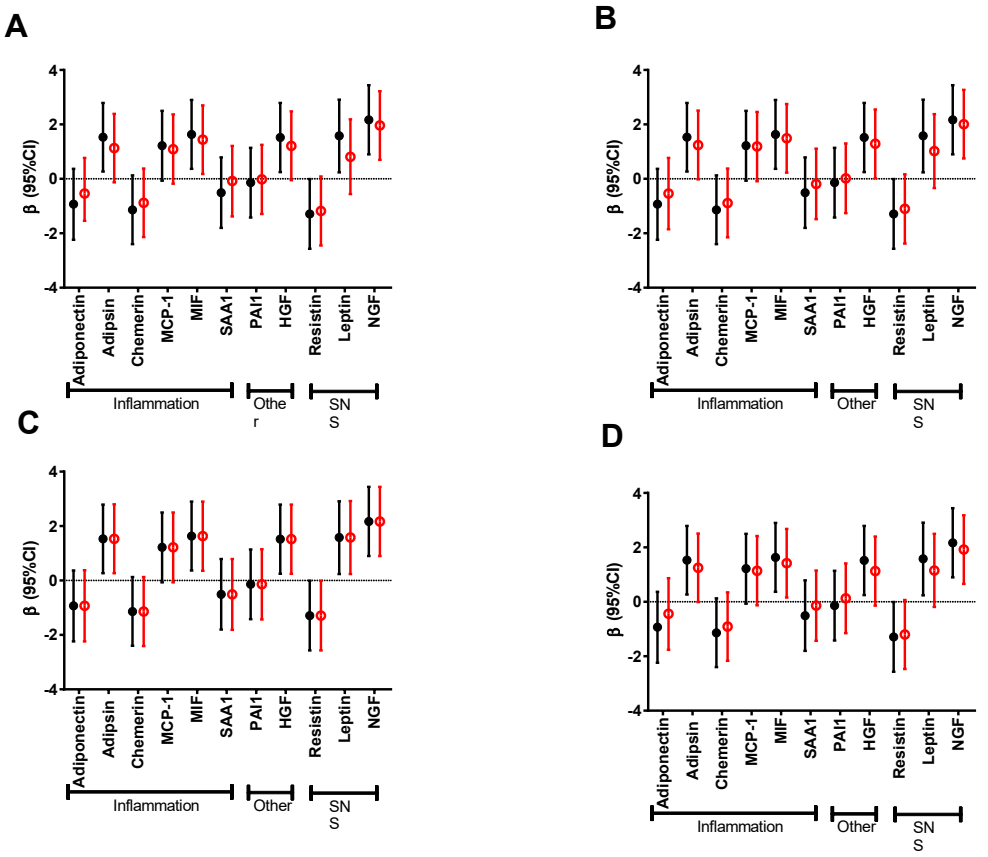

Figure 3. Association between adipokines and blood pressure adjusted for adiposity measurements.

Results are presented as regression coefficients (β) and 95% confidence intervals (95%CI) per 1SD increase in adipokine concentrations. Black squares represent model 2 (adjusted for age and gender), red squares represent model 3 (model 2 additionally adjusted for A) BMI, B) WC, C) SAT, and D) VAT). HsCRP: high sensitivity CRP; MCP-1: Monocyte chemo-attractant protein 1; IP-10: Interferon gamma induced protein 10; NGF: Nerve Growth Factor; HGF: Hepatic Growth Factor; MIF: Migration Inhibitory Factor; SAA1: Serum Amyloid A1; PAI-1: Plasminogen Activator Inhibitor 1

Finally, the relation between resistin and blood pressure was attenuated after adjustment for BMI to -1.2 mmHg (95%CI -2.5-0.1) and after adjustment for VAT to -1.2 mmHg (95%CI -2.5-0.1). The amount of SAT did not change the relation between adipokines and blood pressure (data not shown).

Relation between adipokine score and blood pressure

The adipokine score ranged from 17-44, with a median score of 30 (IQR 27-33). A 1 point higher adipokine score was related to a small but significant increase in blood pressure (β 0.068 mmHg, 95%CI 0.067 – 0.581), adjusted for age and gender. Additional adjustment for visceral adipose tissue, did not change this relationship (β 0.056, 95%CI 0.009 – 0.524).

Discussion

In this study we found that the adipokines leptin, NGF, HGF, MIF and adipsin to be positively, and resistin negatively associated with blood pressure levels. We also found that an adipokine score, combining effects of adipokines, was positively associated with blood pressure levels. Mostly, adjustment for general and abdominal adiposity did not lead to loss of significant relations between adipokines and blood pressure. Neither did exploratory adjustment for inflammation (as an intermediate in the relation between adipokines and blood pressure).
Several pathophysiological mechanisms come into play with the development of hypertension in the obese. The most important are activation of the sympathetic nervous system, enhancement of inflammation leading to endothelial dysfunction and local RAAS activation.

Activation of sympathetic nervous system

Leptin, resistin and NGF are adipokines known to activate the sympathetic nervous system(6,7,22). Activation of the SNS causes vasoconstriction(22), sodium retention(4) and activation of the RAAS-system(23), all increasing blood pressure. NGF stimulates sympathetic neurons(18), leptin activates the α- and β-receptors(6) and resistin activates the PI3K(7) pathway, which is a catalyzing process in the sympathetic nerve activity induced by an excess of insulin(40). Our data show an increase of blood pressure when concentrations of leptin and NGF are higher, consistent with this theory of increased sympathetic activity. Resistin however, was negatively related to blood pressure in our study. Possibly, the extensive use of antihypertensive medication in our cohort could explain this. RAAS inhibitors(41) have shown to elevate resistin levels, whereas amlodipine lowers resistin levels(42). Since 31% of patients use a RAAS-inhibitor and 21% of patients a calcium channel blocker this might very well have influenced our results.

Enhancement of inflammation

HGF, MCP1, MIF, chemerin, adiponectin, resistin, leptin and IP-10 enhance inflammation, resulting in endothelial dysfunction, higher vascular resistance and ultimately a rise in blood pressure(30). Of these, MCP1(21), MIF(21) (17) and IP-10(8) have a direct inflammatory effect on the endothelial cell. Resistin stimulates the production of MCP-1 and endothelin(17). Leptin on its turn stimulates the production of resistin, IL-6 and IL-1β(25). The angiogenic factor HGF also enhances endothelial inflammation, via a yet unknown mechanism(18). Besides these pro-inflammatory adipokines, chemerin and adiponectin are known to diminish inflammation, both enhancing the production of nitric oxide (NO) (24). Moreover, chemerin release also attenuates the production of TNF-alfa, a cytokine involved in (systemic) inflammation (19). Our data show an increase of blood pressure when concentrations of MIF, HGF, and leptin are higher, in line with earlier studies. The negative relation between resistin and blood pressure is hard to explain via the inflammatory pathway, possibly the use of different classes of antihypertensive medication could be an explanation, as discussed above. Surprisingly, contrary to extensive literature, adiponectin showed no relation with blood pressure in our data. Most of these studies are performed in patients without vascular disease, this selection bias might account for the differences. Notably, the vasorelaxing effect of NO in vascular patients, suffering from a severe degree of atherosclerosis, is substantially less than in patients without atherosclerosis(43).

RAAS activation

Finally, IP-10(8) and leptin(20) activate the RAAS, leading to higher blood pressure levels. IP-10 exerts an unknown local effect, leptin activates the erbB receptor(20), a tyrosine kinase receptor which enhances the production of epidermal growth factor. This eventually leads to augmented renal sodium handling via the RAAS. This leads to fluid retention and higher blood pressure. Leptin also enhances the production of aldosterone, leading to endothelial dysfunction and hypertension(15,16). Indeed, our data show higher blood pressure with higher leptin levels, in line with these studies. IP-10 showed no relation with blood pressure in our cohort. Although we corrected for use of antihypertensives agents in our analyses, there is a synergistic effect of using RAAS inhibitors with statin therapy on reducing inflammatory markers such as IP-10(44). In our cohort, ~70% of patients used lipid-lowering therapy, mostly statins, possibly influencing results.

Adipokine score

We developed the adipokine score since we hypothesize that the development of high blood pressure_consists of different pathophysiological components and is the result of various metabolic derangements potentially caused by different adipokines. Therefore, we decided to combine the individual effects of adipokines in an adipokine sum score. We did see a small, but significant effect of higher adipokine scores on blood pressure. Since the adipokine score is non-weighted, no direct comparison between the single adipokines and

their relations to blood pressure and the adipokine score and its relation to blood pressure can be made. In one of our previous studies, we saw the adipokine score to be related to the development of metabolic syndrome.(45) A more sophisticated adipokine score, taking effect sizes into account, could be a more precise measurement to estimate the relation with blood pressure, but this simple method could be more useful in clinical practice.

Adjustment for intermediates in the presumed pathophysiological process of developing high blood pressure

The sympathetic nervous system, insulin resistance and inflammation are thought to be intermediates in the relation between adipokines and blood pressure. Adjustment for heart rate (SNS), HOMA-IR (insulin resistance) and hsCRP (inflammation) might reveal which of these mechanisms is most important in the pathophysiology of hypertension. Attenuation of the effect between adipokines on blood pressure after adjustment for one of these factors might indicate that adipokines exert their effect on blood pressure via either SNS-activation, inflammation or insulin resistance. Surprisingly, none of the factors largely attenuated the effect of adipokines on blood pressure. The relations between resistin, leptin and NGF, the adipokines known to influence sympathetic activity, did not attenuate after additional adjustment for heart rate as compared to adjustment for age and gender. This suggests that sympathetic activity is not an intermediate in the relation between the adipokines and blood pressure. Although heart rate is not the perfect marker of general sympathetic activity, it correlates to other measures of sympathetic activity, such as muscle sympathetic nerve activity (MSNA) and plasma norepinephrine (46). HsCRP did also not influence the relation between inflammatory adipokines and blood pressure. This in contrast to a study in over 1000 community dwelling persons, where a synergistic relation between adiponectin and hsCRP was related to the development of the metabolic syndrome(47), besides both being independently related to the metabolic syndrome. Possibly, the use of medication, especially lipid-lowering therapy, influenced adipokine and hsCRP concentrations in our cohort. During therapy with statins and fibrates, adiponectin concentrations increase and leptin and resistin concentrations decrease(48,49). In large randomized trials, average dose statin therapy does not lower hsCRP concentrations(50). This discrepancy in effect of medication on adipokines and hsCRP could alter the influence of hsCRP on the pathophysiology of hypertension in patients treated with lipid-lowering drugs. Finally, adjustment for insulin resistance (HOMA-IR) did not alter the relation between adipokines and blood pressure to a great extent. Insulin resistance is directly interrelated to hypertension, via increased sodium reabsorption(51), increased SNS activity(52) and vascular smooth muscle growth(53). Although these mechanisms are influenced by adipokines, in our study, insulin resistance was not found to be an intermediate factor in the relation between adipokines and blood pressure. These results suggest that neither sympathetic activity, nor insulin resistance or inflammation, are very important in the pathophysiology of the development of hypertension. It is presumably more likely that there is an intensive interplay between all adipokines released from the

adipose tissue and their combined actions on SNS, inflammation and insulin resistance, rather than one dominating pathophysiological pathway.

Adjustment for adiposity measurements

The relation between NGF, HGF and MIF and blood pressure was found to be independent from adiposity measurements. This emphasizes the importance of other factors than adiposity in the development of adipose tissue dysfunction and eventually hypertension, such as genetic influences, dietary factors and physical activity. The relation between leptin and blood pressure is largely explained by general or abdominal, but not by subcutaneous adiposity. Prior research shows similar results(54), but an adiposity independent relation between leptin and blood pressure has also been reported(55). Subcutaneous adipose tissue did not attenuate any of the relations between adipokines and blood pressure, subscribing that body fat distribution is important in the pathogenesis of blood pressure, with general and abdominal adiposity being most pathogenic.

Adjustment for antihypertensive medication

Adjustment for the use of antihypertensive medication led to attenuation of the association between adipokines and blood pressure. However, it is debatable whether all antihypertensive medications should be seen as confounders in the relation between adipokines and blood pressure. Treatment with RAAS inhibitors shows an increase of adiponectin and resistin in patients with hypertension(41,56,57), whereas treatment with beta-blockers and calcium channel blockers does not change adiponectin concentrations(57,58) and lowers resistin concentrations(42). In hypertensive patients treated with thiazide diuretics, a decrease in adiponectin concentrations is found(58). Leptin concentrations decrease when patients are treated with beta-blockers(59) and remain unchanged during treatment with RAAS inhibitors and calcium channel blockers(56,59). These observations underline the differential influences of antihypertensive medication on adipokines. The extensive use of antihypertensive medication of all classes in our study, combined with a considerable percentage of patients using more than one antihypertensive medication, warrant cautious interpretation of the results adjusted for use of medication.

Strengths and limitations

Strengths of our study include the large cohort and the range of adipokines that were measured. Also, some limitations should be considered. The cohort comprised of patients with a recent history of clinical vascular disease, which may limit generalizability to patients without vascular disease. Moreover, although we adjusted for several potential confounders, there may be residual confounding. We performed numerous analyses, but decided not to correct for multiple testing, since the adipokines were selected beforehand on their possible associations with blood pressure on pathophysiological grounds. As this is a cross-sectional study one should be cautious in making causal inferences.

In conclusion, adipokine concentrations are related to blood pressure, partly independent of general and abdominal adiposity. A complicated and intensive interplay between adipokines and their actions on several pathophysiological mechanisms influence blood pressure and the development of hypertension. Dysfunctional adipose tissue and not adiposity per se is likely to be the causal link between (abdominal) adiposity and hypertension.

Members of the SMART study group

A. Algra, MD, PhD; Y. van der Graaf, MD, PhD; D. E. Grobbee, MD, PhD; G. E. H. M. Rutten, MD, PhD, Julius Center for Health Sciences and Primary Care; F. L. J. Visseren, MD, PhD, Department of Internal Medicine; G.J. de Borst, MD, PhD, Department of Vascular Surgery; L. J. Kappelle, MD, PhD, Department of Neurology; T. Leiner, MD, PhD, Department of Radiology; H.M. Nathoe, MD, PhD, Department of Cardiology

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Supplemental Data S1. Association between IP-10 (quartiles) and systolic blood pressure

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Model 2	0.08 (-0.24 - 1.42)	0.05 (-7.12 – 21.29)	-0.10 (-34.47 – 0.96)	-0.04 (-7.08 – 3.55)
Model 3	0.07 (-0.22 – 1.34)	0.04 (-7.92 – 18.46)	-0.09 (-31.22 – 2.07)	-0.06 (-7.93 – 2.38)

Data are presented as regression coefficients (β) and 95% confidence intervals (95% CI) per 1 increase in logarithmically transformed IP-10 concentration.

Model 2: adjusted for age and gender

Model 3: model 2 additionally adjusted for classes of antihypertensive medications

Chapter 7

Differential effects of renin-angiotensin-aldosterone system inhibition, sympathoinhibition and diuretic therapy on endothelial function and blood pressure in obesity-related hypertension: a double-blind, placebo-controlled cross-over trial

J.A.N. Dorresteijn[a], I.M. Schrover[a], F.L.J. Visseren[a], P.G. Scheffer[b], P.L. Oey[c] , A.H. Danser[d] , W. Spiering[a]

a. Department of Vascular Medicine, University Medical Center Utrecht, Utrecht, The Netherlands.
b. Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands.
c. Department of Nephrology, University Medical Center Utrecht, Utrecht, The Netherlands.
d. Division of Pharmacology and Vascular Medicine, Department of Internal Medicine,Erasmus Medical Center, Rotterdam, The Netherlands.

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Structured abstract

Objectives: To determine the effects of renin-angiotensin-aldosterone system inhibition, sympathoinhibition and diuretic therapy on endothelial function and blood pressure in obesity-related hypertension

Methods: A randomized, four-way, double-blind, crossover study in 31 adults with previously untreated obesity-related hypertension, in which the effects of 8 weeks inhibition of the renin-angiotensin-aldosterone system (using aliskiren 300mg), sympathoinhibition (using moxonidine 0.4mg), diuretic therapy (using hydrochlorothiazide 25mg) or placebo on flow-mediated dilation and 24-hour blood pressure were compared.

Results: The median flow-mediated dilation during placebo was 4.0% [IQR 2.9-5.5%], and was increased by aliskiren (0.81%, 95%CI 0.02%-1.79%), but not by moxonidine (0.20%, 95%CI -0.46%-1.03%) or hydrochlorothiazide (0.39%, 95%CI -0.31%-1.26%). Similarly, compared to placebo mean 24-hour blood pressure was most reduced by aliskiren (-9.8/-6.3mmHg) and to a lesser degree by hydrochlorothiazide (-5.9/-2.6mmHg). Moxonidine did not significantly affect blood pressure despite reduction of muscle sympathetic nerve activity. Insulin sensitivity deteriorated during hydrochlorothiazide treatment and was unaffected by aliskiren or moxonidine. Unlike aliskiren and moxonidine, hydrochlorothiazide reduced urinary 8-iso-prostaglandin F2α-VI, a marker of oxidative stress. Vascular stiffness, systemic inflammation, leptin, adiponectin, and other oxidative stress markers (plasma malondialdehyde, myeloperoxidase activity, and urinary 8-hydroxydeoxyguanosine) were unaffected by treatment.

Conclusions: Renin-inhibition, but not sympathoinhibition or diuretic therapy, improves endothelial function and results in larger reductions of 24-hour, office, and central blood pressure in obesity-related hypertension. This adds weight to the hypothesis that inhibition of the renin-angiotensin-aldosterone system is an effective first step in the treatment of obesity-related hypertension.

Keywords: Hypertension, Obesity, Endothelial function, Renin angiotensin system, Sympathetic nervous system

Introduction

Obesity-related hypertension is increasingly recognized as a distinct hypertensive phenotype requiring a customized approach to diagnosis and management[1, 2]. Multiple maladaptive mechanisms that could explain the relation between obesity and hypertension have been proposed, but activation of the renin-angiotensin-aldosterone system (RAAS) and sympathetic overdrive are believed to be key mediators[1]. Both processes cause impairment of vascular function and thereby contribute to the development of hypertension. Still, because few clinical trials have been performed in obese hypertensive patients, it is unknown whether direct intervention in either one of these mechanisms is more effective for normalizing vascular function and blood pressure than other types of blood pressure lowering treatment[2]. As a result, guidelines do not provide specific recommendations for antihypertensive drug therapy in obese patients[2-4]. In the absence of such preference, thiazide-type diuretics are often advised as the preferred initial treatment in all hypertensive patients[3]. Yet, for obese hypertensive patients this recommendation may not be so logical, because thiazide-type diuretics are associated with increased risk for developing diabetes mellitus and do not counteract the maladaptive processes linking obesity to hypertension.
In this cross-over study of patients with obesity-related hypertension, we aimed to determine how inhibition of the RAAS, inhibition of the sympathetic nervous system (SNS), and treatment with a thiazide-type diuretic affect vascular function, blood pressure and the mechanisms that are involved in the pathophysiology of obesity-related hypertension. These mechanisms include secretion of adipocytokines leptin and adiponectin by adipose tissue, systemic inflammation, oxidative stress, RAAS-hormone production, and sympathetic outflow to the heart and tissues. For this purpose, we examined the effects of treatment with once daily standard maintenance doses of aliskiren (a potent renin-inhibitor), moxonidine (a centrally acting I_1-imidazolinereceptor agonist, inhibiting SNS-activity), and hydrochlorothiazide (HCTZ) in patients with obesity-related hypertension.

Methods

A randomized, four-way, double-blind, single-center, crossover study was performed in 31 adult Caucasian patients with previously untreated obesity-related hypertension (**Figure 1**). Screening was followed by a 40 weeks study period in which patients received each of four once daily monotherapies sequentially in random order: aliskiren 300mg, moxonidine 0.4mg, HCTZ 25mg, and matching placebo. Each treatment period began with a two-week titration phase during which halved doses were used. Thereafter, patients were force-titrated to full medication doses. The efficacy and tolerability of each treatment was assessed after eight weeks, followed by a one-week tapering period and a one-week washout period. Therefore, each treatment cycle lasted ten weeks in total. Patients subsequently crossed over to the

next treatment period until they had completed each of the four treatments. The study was conducted in accordance with the principles of the Declaration of Helsinki as adopted by the 59th WMA general assembly, Seoul 2008. The institutional review board of the University Medical Center Utrecht approved the study and all participants gave their written informed consent. This study was registered on http://www.clinicaltrials.gov; unique identifier: NCT01138423.

Participants were men and post-menopausal women, 30-70 years of age, who fulfilled the criteria for abdominal obesity (waist circumference >102 cm [men] or >88 cm [women]), (pre)hypertension, and the metabolic syndrome[5]. (Pre)hypertension was defined as office systolic blood pressure (SBP) >130 mmHg and/or office diastolic blood pressure (DBP) >85 mmHg during two screening visits on separate days. Blood pressure was measured two times on both arms in sitting position after the subject had been seated for some minutes using an appropriately sized arm-cuff and an automated oscillometric blood pressure device[6]. Blood pressure level was defined as the highest mean of the measurements on one arm during the first visit, and as the mean of the measurements on that same arm during all subsequent visits. Main exclusion criteria were previously treated hypertension, secondary hypertension, current smoking or cessation <3 months or type 2 diabetes mellitus. For a complete list of all eligibility criteria, please refer to the **Supplemental Digital Content**.

The primary endpoint was endothelial function measured by Flow-Mediated Dilation (FMD) after eight weeks treatment with aliskiren, moxonidine or HCTZ as compared to endothelial function after placebo treatment. Secondary endpoints were 24-hour blood pressure, office blood pressure, vascular stiffness (i.e. carotid-radial Pulse Wave Velocity, PWV; Pulse Wave Analysis, PWA; augmentation index, Aix; and aortic pressure), sympathetic activity (i.e. Muscle Sympathetic Nerve Activity, MSNA; Heart Rate Variability, HRV; and heart rate), RAAS-activity (i.e. the plasma concentrations of prorenin, renin, and aldosterone), inflammation (i.e. high-sensitivity C-reactive protein, hs-CRP), oxidative stress (i.e. malondialdehyde, MDA; 8-iso-prostaglandin F2α-VI, iPF2α-VI; 8-hydroxy-2-deoxyguanosine, 8-OHdG; and myeloperoxidase activity, MPO), adipose tissue function (i.e. leptin and adiponectin), insulin resistance (i.e fasting glucose and Homeostatic Model Assessment for Insulin Resistance, HOMA-IR[7]), lipid metabolism (i.e. total cholesterol; high-density lipoprotein cholesterol, HDLc; low-density lipoprotein cholesterol, LDLc; triglycerides; apolipoprotein A1, apoA1; and apolipoprotein B, apoB), and renal fractional sodium excretion. Safety parameters were estimated glomerular filtration rate (eGFR; based on the Modification of Diet in Renal Disease formula), serum potassium and sodium.

The order in which patients underwent the four blinded treatments was randomized. A concealed list on which allocation numbers were connected to drug sequences (balanced Latin square) was composed and kept by the trial pharmacist prior to study initiation and each eligible patient was allocated to the first available treatment sequence on this list in ascending order. Blinding of participants, outcome assessors, and care providers was maintained by uniform package of moxonidine, HCTZ, and placebo into capsules and

aliskiren and placebo into tablets. During each treatment period, patients received both capsules and tablets following a double-dummy design.
Patients were instructed to refrain from smoking or using >14 units of alcohol per week during the entire course of the study. A liberal salt diet was allowed, but patients were educated about the importance of avoiding excess salt consumption. Patients were contacted by telephone twice during each treatment period to reinforce these lifestyle restrictions, monitor compliance, and register the occurrence of adverse events.
After eight weeks, all outcome measurements were performed during a single morning time visit between 2 and 5 hours after drug intake, with the patients having fasted for at least 13 hours and abstained from vigorous exercise and drinking beverages containing caffeine during the morning of the study. During the 24 hours following each visit, ambulatory blood pressure measurement and simultaneous 24-hour urine collection were performed. Patients were asked to return any unused study medication. Compliance was assessed by pill counting and defined as a patient taking ≥80% of the daily dose and no overdose. Please refer to the **Supplemental Digital Content** for a detailed description of the outcome measurements.

Data analyses
To obtain 90% power of detecting 1% difference of FMD between active treatment and placebo with 95% confidence (two-sided), 22 complete cases would be needed, assuming that the standard deviation of within-person FMD-changes is 1%. Because MSNA is time-consuming and invasive, it was performed in a subpopulation of 15 participants who were invited in consecutive order until this number was reached.
Changes in endothelial function between active treatment and placebo were assessed using a linear mixed effect model. An intention-to-treat analysis was performed based on the total number of subjects that completed each individual intervention. Thus, follow up data were included in the analyses even when patients did not complete all four treatments. The model was adjusted for time since randomization to account for possible time trends that may occur due to changes in lifestyle or weight during the course of the study. No carry-over effects were observed. The 95% confidence intervals (95%CI) were approximated on the basis of the coefficients' standard errors. Similar methods were used for analyses of secondary and safety outcomes. For each outcome the normality assumption was tested using the Shapiro-Wilk statistic and skewed outcome-variables were natural log-transformed. The mean outcome levels during placebo- and active treatment were then calculated in natural log-units and exponentiated back to obtain estimates of the effect of treatment and accompanying 95%CI on a linear scale. Analyses of FMD and 24-hour mean blood pressure were performed with and without adjustment for 24-hour sodium excretion to reduce residual error caused by variations of sodium intake.
Statistical analyses were performed with the open source statistical software package R, version 2.11.1 (R Foundation for Statistical Computing, www.R-project.org).

Results

Between September 2010 and May 2011 65 patients were screened. Of these, 33 did not meet the eligibility criteria and one declined after screening (**Figure 1**). Reasons for exclusion were no metabolic syndrome (n=16), no (pre)hypertension (n=6), restricted medication use (n=3), probable obstructive sleep apnea syndrome (n=2), newly diagnosed type 2 diabetes mellitus (n=2), one or more out-of-range laboratory values (n=2), recent history of malignancy (n=1), and stage 3 hypertension (n=1). Baseline characteristics of the remaining 31 patients successfully enrolled are shown in **Table 1**. Of these, 28 completed the trial per protocol and three patients left the study before completion due to withdrawal of consent (n=2) or initiation of restricted medication (n=1). As a result, follow-up data were obtained from 30 patients while on placebo, from 28 patients while on aliskiren, from 30 patients while on moxonidine, and from 29 patients while on HCTZ (**Figure 1**). Counting of returned study medication did not reveal incompliance of any study participants. Furthermore, randomization resulted in balanced distribution of the interventions over treatment periods one to four (**Appendix table 1**, please refer to the Supplemental Digital Content**)**.

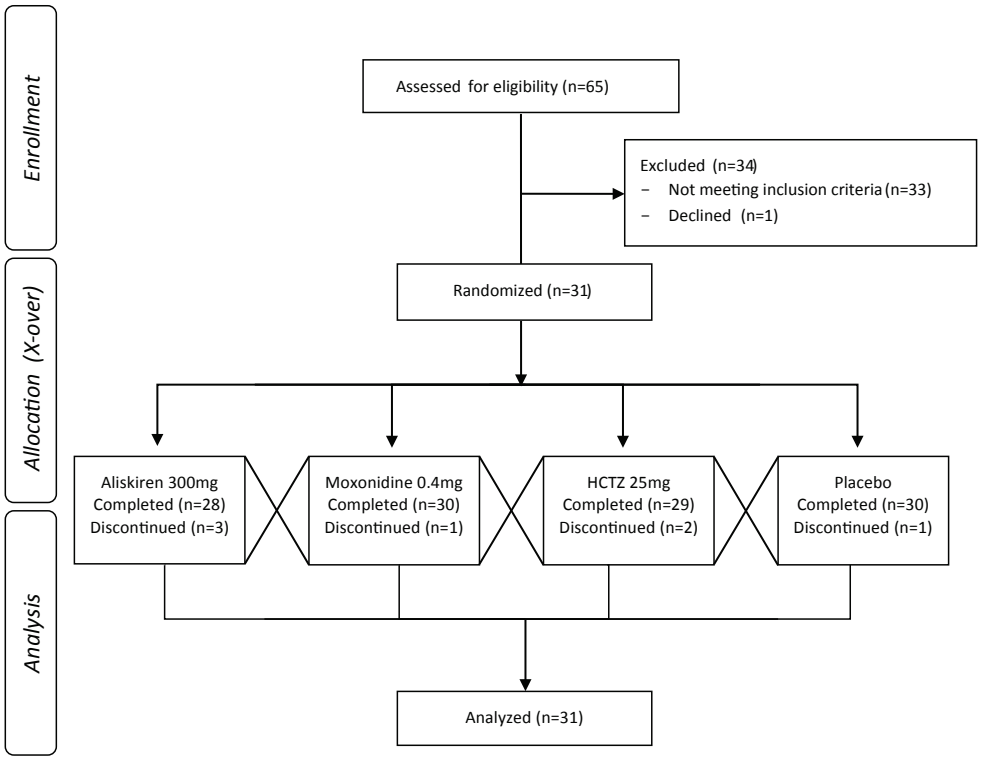

Figure 1. Flow diagram (adapted from CONSORT 2010 template for parallel group RCTs)[32].

Table 1. Baseline characteristics.

Characteristic		N=31
Gender	Males, No. (%)	23 (74%)
	Females, No. (%)	8 (26%)
Age [years]		60 [55-63]
BMI* [kg/m^2]		31 [28-32]
Waist circumference [cm]	Males	111 [107-116]
	Females	98 [95-99]
Office blood pressure [mmHg] 1st screening visit	Systolic	153 [145-161]
	Diastolic	90 [86-98]
Office blood pressure [mmHg] 2nd screening visit	Systolic	153 [145-167]
	Diastolic	88 [84-96]
Resting heart rate [beats/minute]		69 [61-80]
Fasting glucose [mmol/L]		5.6 [5.0-6.0]
Total cholesterol [mmol/L]		5.7 [5.1-6.2]
HDL-cholesterol † [mmol/L]	Males	1.02 [0.94-1.22]
	Females	1.31 [1.28-1.76]
Triglycerides [mmol/L]		1.90 [1.40-2.30]
eGFR ‡ [mL/min/1.73m^2]		79 [73-86]

Data are presented as median [interquartile range] unless otherwise specified. * BMI = Body Mass Index, † HDL-cholesterol = high density lipoprotein cholesterol, ‡ eGFR = Glomerular Filtration Rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation

Endothelial function and blood pressure

The median FMD during placebo was 4.0% [interquartile range (IQR) 2.9%-5.5%]. **Figure 2** demonstrates that FMD increased significantly during aliskiren by 0.81% (95%CI 0.02%-1.79%; p<0.05), but not during moxonidine (0.20%, 95%CI -0.46-1.03; p=0.58) or HCTZ (0.39%, 95%CI -0.31%-1.26%; p=0.31). There were no significant FMD-differences between aliskiren versus HCTZ (p=0.31), aliskiren versus moxonidine (p=0.14) or HCTZ versus moxonidine (p=0.63). Adjustment for 24-hour sodium excretion did not alter the above conclusions.

Also shown in **Figure 2**, is that the largest reduction of 24-hour blood pressure (median level during placebo: 131/80 mmHg) was observed during aliskiren (mean reduction -9.8/-6.3 mmHg). Aliskiren compared to moxonidine and HCTZ had a larger effect on 24-hour SBP (p<0.001 and p=0.03) and 24-hour DBP (p<0.001 and p<0.001). HCTZ, but not moxonidine, also resulted in significant reduction of 24-hour blood pressure (mean reduction -5.9/-2.6 mmHg). The effects of moxonidine and HCTZ were not significantly different (p for SBP=0.13 and p for DBP=0.57). Adjustment for 24-hour sodium excretion did not alter the above conclusions. Median office blood pressure during placebo was 149/90 mmHg. Again, the largest treatment effect was seen during aliskiren (mean reduction -12.1/-5.5 mmHg).

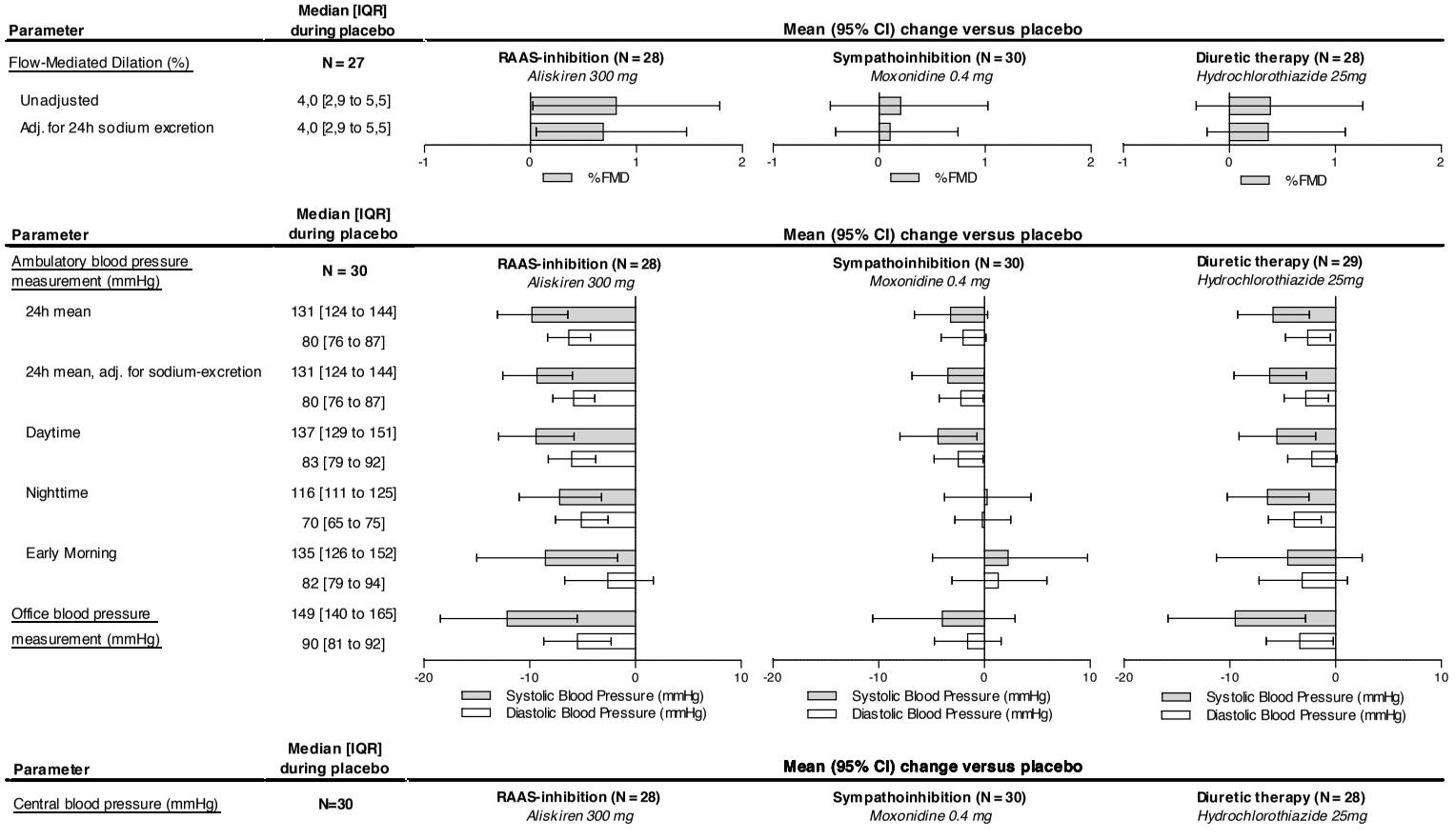

Figure 2. Effects of RAAS-inhibition, sympathoinhibition, and diuretic therapy versus placebo on Flow-Mediated Dilation and blood pressure.

Vascular stiffness

PWV was not affected by 8 weeks treatment with aliskiren, moxonidine or HCTZ (**Table 2**). Yet, the PWA Aix, which indicates the speed of pulse wave reflection from the peripheral vascular bed, was lowered by aliskiren (-3.1%, 95%CI -6.3-0.0%) and HCTZ treatment (-3.4%, 95%CI -6.5--0.3%). As a result, reductions of central SBP and central pulse pressure were larger than reductions in brachial pressure during treatment with aliskiren or HCTZ (**Figure 2**). Pulse wave reflection and central blood pressure were not affected by moxonidine.

Sympathetic nerve activity and heart rate variability

Of the 60 scheduled MSNA-registrations in 15 study subjects, 14 were declared missing, leaving 46 successful registration for the analyses. Reasons for missing MSNA data were preterm study withdrawal of the patient (n=4), practical limitations (n=4) or insufficient technical quality (n=6). **Table 2** shows that, compared to placebo, MSNA decreased after 8 weeks treatment with moxonidine by -5.9 bursts/minute and -7.0 bursts/100 heart beats. This was accompanied by non-significant reductions in resting and average 24-hour heart rate, but the HRV LF/HF-ratio remained unchanged. Aliskiren and HCTZ treatment did not result in alteration of MSNA. After aliskiren treatment, however, reduction of the HRV LF/HF-ratio was observed, suggesting decreased sympathetic outflow to the sinoatrial-node.

Renin-Angiotensin-Aldosterone System

Both aliskiren and HCTZ treatment resulted in a rise in renin and prorenin concentrations (**Table 2**). Yet, only during HCTZ-treatment this was accompanied by a marked increase in plasma aldosterone. Moxonidine did not affect renin or prorenin, but led to a borderline significant increase in plasma aldosterone.

Inflammation and oxidative stress

Median hs-CRP during placebo-treatment was 1.8 mg/L [IQR 1.3-2.9 mg/L] and was not affected by treatment (**Table 2**). Moreover, no changes were observed in MDA, MPO or urinary 8-OHdG. Yet, urinary iPF2α-VI was significantly reduced by HCTZ, although not by aliskiren or moxonidine (**Table 2**).

Metabolic effects

HOMA-IR index was increased during HCTZ treatment, meaning that HCTZ reduced insulin sensitivity in these metabolic syndrome patients. Yet, no concomitant rise in fasting glucose was observed (**Table 3**). Aliskiren and moxonidine did not affect glucose metabolism. Moxonidine resulted in a reduction of all lipid particles, whereas during aliskiren- and HCTZ-treatment no lipid changes were observed compared to placebo. No changes in leptin and adiponectin concentrations were observed during any treatment compared to placebo (**Table 3**).

Table 2. Effects of RAAS-inhibition, sympathoinhibition, and diuretic therapy versus placebo on vascular stiffness, sympathetic activity, plasma RAAS hormone concentrations, inflammation and oxidative stress.

		Placebo		RAAS-inhibition (Aliskiren 300mg)		Sympathoinhibition (Moxonidine 0.4mg)		Diuretic therapy (Hydrochlorothiazide 25mg)
Parameter	**N**	**Median [IQR] during placebo**	**N**	**Mean (95% CI) Δ vs. placebo**	**N**	**Mean (95% CI) Δ vs. placebo**	**N**	**Mean (95% CI) Δ vs. placebo**
Vascular stiffness								
Pulse Wave Velocity (m/s)	29	8.4 [7.6-9.2]	27	0.1 (-0.5-0.6)	29	0.4 (-0.2-0.9)	27	0.4 (-0.2-1.0)
Augmentation Index (%)	30	32.0 [26.3-38.8]	28	-3.1 (-6.3-0.0)	30	-0.3 (-3.4-2.8)	28	-3.4 (-6.5--0.3)*
Sympathetic activity								
MSNA (Bursts / minute)	14	46 [40-50]	10	2.3 (-3.6-8.2)	11	-5.9 (-11.7--0.1)	11	-0.9 (-6.6-4.8)
MSNA (Bursts / 100 heart beats)	14	73 [66-81]	10	6.3 (-2.8-15.5)	11	-7.0 (-16.0-2.1)	11	1.0 (-7.9-9.8)
MSNA (Burst area / minute)	14	4,476 [2,966-5,993]	10	894 (-1,045-2,832)	11	-1,677 (-3,585-231)	11	-437 (-2,315-1,441)
MSNA (Burst area / 100 heart beats)	14	6,998 [4,619-10,607]	10	1,544 (-1,481-4,570)	11	-2,451 (-5,429-527)	11	465 (-2,470-3,400)
Heart Rate Variability LF/HF-ratio	30	1.29 [0.80-2.74]	28	-0.8 (-1.1--0.3)**	30	-0.2 (-0.7-0.5)	28	-0.5 (-0.9-0.1)
Average 24-hour heart rate (per minute)	30	71 [69-79]	28	1.5 (-0.5-3.6)	30	-1.3 (-3.4-0.7)	29	1.6 (-0.4-3.6)
Resting heart rate (per minute)	30	61 [58-67]	28	1.1 (-1.1-3.4)	30	-0.9 (-3.0-1.3)	28	0.2 (-1.9-2.5)
RAAS-hormones								
Renin (ng/L)	30	5.6 [4.2-8.8]	28	32.5 (22.8-45.3)***	30	-0.1 (-1.6-1.9)	29	6.2 (3.2-10.3)***
Prorenin (ng/L)	30	54 [41-73]	28	18.1 (10.6-25.5)***	30	2.6 (-4.7-10.0)	29	26.4 (19.1-33.8)***
Aldosterone (ng/L)	30	53 [33-74]	28	1.6 (-9.4-12.6)	30	10.2 (-0.7-21.0)	29	34.0 (23.1-44.9)***
Inflammation and oxidative stress								
Hs-CRP (mg/L)	28	1.8 [1.3-2.9]	28	0.49 (-0.02-1.15)	29	0.13 (-0.30-0.68)	28	0.45 (-0.06-1.09)
Malondialdehyde (µmol/L)	30	4.5 [4.1-4.9]	28	-0.05 (-0.23-0.14)	30	0.05 (-0.13-0.24)	29	-0.02 (-0.20-0.17)
8-iso-prostaglandin F2α-VI (pmol/mmol creatinin)	29	266 [233-337]	28	-12 (-35-13)	30	-11 (-34-13)	28	-45 (-65--23)***
8-hydroxy-2-deoxyguanosine (nmol/mmol creatinin)	30	1.27 [1.09-1.45]	28	-0.03 (-0.11-0.06)	30	0.00 (-0.08-0.09)	28	-0.02 (-0.10-0.07)
Myeloperoxidase activity (U/L)	27	55.8 [44.0-80.5]	24	-4.3 [-17.2-8.5]	28	-4.2 [-16.5-8.2]	27	4.1 [-8.3-16.6]

* indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001.
RAAS = Renin-Angiotensin-Aldosterone System, MSNA = Muscle Sympathetic Nerve Activity, LF = Low Frequency, HF = High Frequency, Hs-CRP = high sensitivity C-reactive protein

Table 3. Effects of RAAS-inhibition, SNS-inhibition, and diuretic therapy versus placebo on glucose and lipid metabolism, adipose tissue function, renal function and serum electrolytes.

	Placebo		RAAS-inhibition (Aliskiren 300mg)		Sympathoinhibition (Moxonidine 0.4mg)		Diuretic therapy (Hydrochlorothiazide 25mg)	
Parameter	**N**	**Median [IQR] during placebo**	**N**	**Mean (95% CI) Δ vs. placebo**	**N**	**Mean (95% CI) Δ vs. placebo**	**N**	**Mean (95% CI) Δ vs. placebo**
Metabolic parameters								
HOMA-IR	30	1.8 [1.2-2.7]	28	0.22 (-0.07-0.56)	30	0.07 (-0.20-0.39)	29	0.31 (0.01-0.66)*
Fasting glucose (mmol/L)	30	5.1 [4.9-5.5]	28	-0.07 (-0.27-0.13)	30	0.24 (0.03-0.45)*	29	0.08 (-0.12-0.28)
Total cholesterol (mmol/L)	30	5.5 [4.9-6.2]	28	0.00 (-0.20-0.20)	30	-0.25 (-0.44--0.06)*	29	0.12 (-0.08-0.32)
LDL-cholesterol (mmol/L)	30	3.7 [3.1-4.1]	28	-0.02 (-0.19-0.15)	30	-0.14 (-0.30-0.03)	29	0.08 (-0.08-0.26)
HDL-cholesterol (mmol/L)	30	1.10 [0.99-1.32]	28	0.02 (-0.03-0.06)	30	-0.04 (-0.08-0.00)	29	0.00 (-0.04-0.04)
Triglycerides (mmol/L)	30	1.50 [1.20-2.18]	28	0.05 (-0.14-0.26)	30	-0.13 (-0.29-0.06)	29	0.12 (-0.08-0.33)
Apolipoprotein A1 (g/L)	30	1.42 [1.28-1.62]	28	0.03 (-0.02-0.08)	30	-0.04 (-0.08-0.01)	29	0.01 (-0.03-0.06)
Apolipoprotein B (g/L)	30	1.03 [0.91-1.18]	28	-0.01 (-0.05-0.04)	30	-0.06 (-0.10--0.02)*	29	0.04 (-0.01-0.08)
Adipokines								
Leptin (ng/mL)	30	16.4 [9.5-32.8]	28	-1.09 (-3.14-1.25)	30	-1.70 (-3.65-0.52)	28	-0.76 (-2.85-1.62)
Adiponectin (µg/mL)	30	6.9 [5.2-10.4]	28	-0.03 (-1.18-1.27)	30	-0.50 (-1.57-0.72)	28	-0.20 (-1.32-1.08)
Renal function and electrolytes								
eGRF (mL/min/1.73m^2)	30	86 [78-98]	28	0.8 (-2.0-3.6)	30	2.6 (-0.1-5.4)	29	-2.1 (-4.9-0.6)
Fractional sodium excretion	30	0.69 [0.48-0.85]	28	-0.15 (-0.29-0.02)	30	-0.11 (-0.25-0.06)	29	0.10 (-0.09-0.32)
Serum sodium (mmol/L)	30	139 [137-140]	28	-0.3 (-0.9-0.3)	30	-0.2 (-0.8-0.4)	29	-0.3 (-0.9-0.3)
Serum potassium (mmol/L)	30	4.1 [4.0-4.5]	28	0.09 (-0.02-0.20)	30	0.04 (-0.07-0.14)	29	-0.28 (-0.39--0.18)***

* indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001.
RAAS = Renin-Angiotensin-Aldosterone System, HOMA-IR = homeastasis model assessment for insulin resistance, LDL = low-density lipoprotein, HDL = high-density lipoprotein, eGFR = Glomerular Filtration Rate as estimated by the Modification of Diet in Renal Disease (MDRD) formula

Safety

Kidney function in terms of eGFR was not affected by treatment and neither were serum sodium levels. Serum potassium was mildly decreased during HCTZ treatment and mildly increased during aliskiren treatment. No serious adverse events occurred. The number of non-serious adverse events was 22 during placebo, 32 during moxonidine treatment, 21 during aliskiren-treatment, and 27 during HCTZ treatment (**Appendix table 2**, please refer to the Supplemental Digital Content). Moxonidine compared to placebo more often led to complaints of a dry mouth (n=5 vs. n=1 during placebo), fatigue (n=3 versus n=1 during placebo), and muscle ache (n=3 versus n=1 during placebo), but was generally well-tolerated.

Discussion

This cross-over trial shows that RAAS-inhibition (using aliskiren) as opposed to sympathoinhibition (using moxonidine) or diuretic therapy (using HCTZ) improves endothelial function in obesity-related hypertension. Endothelial function was quantified in terms of FMD, which is often regarded as a direct preclinical marker of atherosclerosis. A meta-analysis shows that every 1% improvement in FMD is associated with 13% reduction of cardiovascular events[8]. Standard maintenance doses of aliskiren compared to moxonidine or HCTZ also led to larger reductions in 24-hour and office blood pressure. Because both aliskiren and HCTZ also improved the PWA Aix, reductions in central blood pressure were even more pronounced than reductions in brachial blood pressure. Notably, central blood pressure is an important determinant of target organ damage, cardiovascular events, and mortality[9]. Yet, only during treatment with aliskiren, these reductions of central and brachial blood pressure were accompanied by improvement of FMD. Previous studies have demonstrated a modest inverse association between blood pressure and FMD[10]. Due to the limited number of observations, however, we were unable to assess a possible correlation between the decrease in blood pressure and FMD in patients treated with aliskiren.

This study adds to an increasing body of evidence that RAAS-inhibition is an effective and well-tolerated first pharmacologic treatment step in obesity-related hypertension. In a previous parallel-group trial for example, 12-weeks treatment with aliskiren 300mg compared to HCTZ 25mg also led to greater blood pressure reductions in obese patients with hypertension[11]. This difference, favoring aliskiren, was maintained until 52 weeks, even after add-on of amlodipine in both groups. Yet, the present study is the first randomized placebo-controlled trial to our knowledge evaluating the effect of RAAS-inhibitors on endothelial function in obesity-related hypertension. Previously, endothelium-dependent relaxation in normal weight hypertensives was shown to be improved by aliskiren, although not by ramipril[12]. Similarly, in hypertensive patients with impaired glucose tolerance, FMD improvement was demonstrated with telmisartan, although not with losartan[13]. A third study in 63 predominantly lean hypertensives showed improvements of FMD with HCTZ, irbesartan or

quinapril[14]. Finally, in a study in 60 patients with primary hypertension whose mean BMI was in the overweight range, no improvements of endothelium-dependent vasodilation were observed with valsartan or HCTZ[15]. Yet, in all these studies, endothelial function was compared to baseline, not to placebo.

In the present study, renin-inhibition with aliskiren was used to inhibit RAAS-activity, but RAAS-inhibition can also be established by interference at other levels of the RAAS cascade, such as angiotensin-converting enzyme inhibition or angiotensin II receptor blockade. In general, different types of RAAS-inhibitors show similar effectiveness for reducing blood pressure[12, 16, 17]. Importantly, however, aliskiren has the ability to attain high concentrations in adipose and skeletal muscle tissue, allowing inhibition of local RAAS-activity[18]. This could explain findings from a previous study, demonstrating that aliskiren compared to irbesartan more effectively lowered blood pressure in patients with obesity-related hypertension[19]. The capacity of aliskiren to suppress angiotensin II formation is supported by an approximately 6-fold increase of renin in the present study. Circulating aldosterone, however, equaled its level at baseline after chronic treatment. This is in agreement with findings from previous studies[20, 21] and does not exclude that the release of adipocyte-derived aldosterone remained suppressed[22].

An important causal role in the pathogenesis of obesity-related hypertension has also been attributed to sympathoactivation[1]. Promising new treatment modalities, such as renal denervation and baroreflex activation therapy are based on this theory[23]. Therefore, drugs that inhibit SNS-activity, including moxonidine, receive renewed attention and their reintroduction in clinical practice is seriously considered[23]. Yet, binding of brain stem I_1-imidazolinereceptors by moxonidine did not result in marked improvement in blood pressure or endothelial function in the present study. This finding cannot be explained by a lack of adherence: moxonidine was generally well tolerated and pill counting did not indicate noncompliance. Moreover, MSNA measurements prove that sympathetic activity was reduced by moxonidine, yet not followed by improvements in blood pressure and endothelial function in these patients with obesity-related hypertension. Confusingly, in a previous study in a similar patient population marked improvements in blood pressure were observed after 6 months moxonidine-treatment[24]. However, that study was not placebo-controlled. Even so, larger blood pressure reductions were seen in the amlodipine-treated arm of that study, although the difference was not significant due to the small sample size. Another study in patients with obesity-related hypertension, demonstrated improvements in FMD during moxonidine-treatment, but that study was non-randomized and open-label[25]. Previous studies have shown that RAAS-inhibition with candesartan or aliskiren may also reduce MSNA[26, 27]. This was not reproduced in the present study, although we did observe a reduction of the HRV LF/HF-ratio during aliskiren-treatment. The finding that HCTZ does not affect SNS-activity on the other hand is consistent with previous knowledge[26].

Thirdly, oxidative stress has been hypothesized to contribute to the development of hypertension in obese patients[1]. In the present study, however, markers of oxidative stress were not influenced by RAAS-inhibition or sympathoinhibition, suggesting that other mechanisms may be responsible for the improvements in endothelial function and blood pressure. Interestingly, HCTZ reduced systemic oxidative stress as evidenced by lower creatinin-adjusted concentration of urinary iPF2α-VI during HCTZ treatment. A putative protective effect of thiazide diuretics against oxidative stress has been reported before[28]. Moreover, in a previous study in patients with obesity-related hypertension urinary iPF2α-VI was reduced by 8 weeks treatment with a combination of aliskiren 300mg and HCTZ 25mg compared to ramipril 10mg[29]. HCTZ, however, did not influence the level of urinary 8-OHdG, a sensitive and stable biomarker of DNA oxidative damage, and no changes were observed in plasma MPO, a leukocyte derived NO-scavenger, or MDA, a lipid peroxide that is a less specific marker of oxidative stress.

Although systemic inflammation, and serum leptin and adiponectin concentrations are thought to be importantly implicated in the pathogenesis of hypertension and metabolic sequelae resulting from obesity[1], the present study shows that RAAS-inhibition, sympathoinhibition, and thiazide-type diuretics do not affect the mechanisms involved. Vascular stiffness was, furthermore, not affected during 8 weeks follow-up in this study, but changes in vascular stiffness may well take more time to develop. On the other hand, the detrimental effect of HCTZ on insulin sensitivity, shown in many previous studies[26], was confirmed in the present study. A beneficial effect on insulin sensitivity has previously been attributed to moxonidine[24] and aliskiren[30]. This was not confirmed by our study, but it could be that these beneficial effects take longer than 8 weeks to develop. Finally, a reduction of all lipid-particles was observed during moxonidine-treatment. Such lipid profile changes have been demonstrated before, but the clinical significance is uncertain[31].

A limitation of this study is that the sample size was sufficient to study FMD-differences between active treatment and placebo, but not between mutual active treatments. Still, we found that aliskiren compared to moxonidine and HCTZ resulted in larger blood pressure reduction. Second, although standard maintenance doses of all three drugs were used, evidence of equal potency in normal weight hypertensive patients is not available. Finally, we infer that the effects of other RAAS-inhibitors and SNS-inhibitors may be similar to those of aliskiren and moxonidine. Yet, this needs to be confirmed by further research. Strengths of this study include that it was a randomized, double-blinded, placebo-controlled trial, minimizing the potential for information bias or confounding bias and enabling adjustment of the results for any placebo-effects. Moreover, the four-way comparison is a unique feature of this study. Finally, we performed a wide range of outcome measurements to achieve a better insight in the possible mechanisms of action of the drugs under study.

Conclusions

Renin-inhibition by aliskiren, but not sympathoinhibition by moxonidine or diuretic therapy by hydrochlorothiazide, improves endothelial function and results in larger reductions of 24-hour, office, and central blood pressure in obesity-related hypertension. This adds weight to the hypothesis that inhibiting the RAAS is an effective first step in the treatment of obesity-related hypertension.

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Chapter 8

Identifying treatment response to antihypertensives in patients with obesity-related hypertension

I.M. Schrover[1], MD; J.A.N. Dorresteijn[1], MD, PhD; J.E. Smits[1], MD; A.H. Danser[2], PhD; F.L.J. Visseren[1], MD, PhD, W. Spiering[1], MD, PhD

1. Department of Vascular Medicine, University Medical Center Utrecht, Utrecht, The Netherlands.
2. Division of Pharmacology and Vascular Medicine, Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.

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Abstract

Background: In patients with obesity-related hypertension (ORH), reaction to anti-hypertensive medication is likely influenced by patientcharacteristics.

Materials and Methods: Effects of aliskiren, moxonidine and hydrochlorothiazide on 24-hour blood pressure (BP) were compared to placebo. Linear mixed effect models were used to analyze the effect of patient characteristics on BP levels and treatment response.

Results: Systolic BP response to aliskiren was higher in patients with a BMI>30.7 kg/m2 compared to patients with a BMI≤30.7 kg/m2 (-21mmHg versus -4mmHg). In patients with a hsCRP>1.8 mg/L the systolic BP response to aliskiren was higher than in patients with a low hsCRP (-15mmHg versus -7mmHg). Hydrochlorothiazide (HCTZ) treatment effect on systolic BP was -13mmHg when heart rate >71 beats/min compared to -3mmHg when heart rate was ≤71 beats/min.
Conclusion: In patients with ORH, BP response to aliskiren is positively related to BMI and hsCRP. Systolic BP response to HCTZ is positively related to heart rate and negatively to renin levels.

Trialregistration: Clinicaltrials.gov NCT01138423. Registered June 4th, 2010.

Keywords: hypertension, obesity, treatment, interaction

Introduction

Subjects who are either overweight or obese have a 2 time higher risk of developing hypertension as compared to persons with a normal BMI.(1,2) In patients with this so-called obesity-related hypertension (ORH), both blood pressure level as well as the reaction to anti-hypertensive medication are likely to be influenced by patient characteristics. Age and gender are known to influence blood pressure levels in the general population and are likely to also influence blood pressure in patients with ORH. At age 55, 7% of the Western population is diagnosed with hypertension,(1) which rises to 34% at age 65 and 77% at age 80.(3) Women (72%) are more likely to develop hypertension than men (61%) during their lifetime.(3) Patient characteristics linked to the underlying pathophysiological mechanisms of ORH could also influence blood pressure. The major underlying mechanism linking obesity to hypertension is thought to be adipose tissue dysfunction, defined as an imbalance between the release of pro- and anti-inflammatory adipokines, resulting in activation of the renin-angiotensin-aldosterone system (RAAS), sympathetic overdrive, low-grade inflammation and oxidative stress.(4–7) Eventually, this leads to endothelial dysfunction, vascular hypertrophy and impaired natriuresis.(4,8) In a previous study, we showed that in subjects with ORH the blood pressure lowering effect was greatest during treatment with direct renin inhibitor aliskiren.(9) Knowledge of patient characteristics that influence the blood pressure response to antihypertensive treatment in patients with ORH may help to identify the most effective blood pressure lowering treatment in individual patients. Response to anti-hypertensive medication varies significantly in patients with previously untreated primary hypertension(10–12) and is therefore presumably dependent on patient-specific characteristics such as gender, age, BMI, salt intake and level of RAAS hormones.(12–18)

In the present study we evaluated which patient characteristics influence the blood pressure-lowering effect of the direct renin inhibitor aliskiren, the sympathicolytic agent moxonidine and the diuretic hydrochlorothiazide in patients with ORH.

Methods

We used data from a cross-over trial in which 31 previously untreated patients with ORH were successively treated with aliskiren, moxonidine, hydrochlorothiazide (HCTZ) and matching placebo, in random order, each for 8 weeks. A detailed description of the methods has been published earlier.(9) In summary, a four-way, double-blind, single-center, crossover study was performed in 31 adult Caucasian patients (men and post menopausal women) with previously untreated ORH from September 2010 until March 2012. ORH was defined as a blood pressure >130 mmHg systolic and/or >85 mmHg diastolic and abdominal obesity (waist circumference >102 cm (men) or >88 cm (women)). This is in accordance with the metabolic syndrome criteria, which all patients fulfilled(19)

(Pre)hypertension was defined as office systolic blood pressure (SBP) >130 mmHg and/or office diastolic blood pressure (DBP) >85 mmHg during two screening visits on separate days. Blood pressure was measured two times on both arms in sitting position after the subject had been seated for some minutes using an appropriately sized arm-cuff and an automated oscillometric blood pressure device.(20) Blood pressure level was defined as the highest mean of the measurements on one arm during the first visit, and as the mean of the measurements on that same arm during all subsequent visits.. When subjects were eligible to participate in the study they entered a 40-weeks study-period in which they received each of four subsequent once daily monotherapies sequentially: aliskiren 300 mg, moxonidine 0.4 mg, HCTZ 25 mg, and matching placebo. The efficacy of each treatment on ambulatory (24-hr) blood pressure was assessed after eight weeks. The study was conducted in accordance with the principles of the Declaration of Helsinki as adopted by the 59th WMA general assembly, Seoul 2008. The institutional review committee of the University Medical Center Utrecht approved the study and all patients gave their written informed consent.

Data analyses

A linear mixed effect model was used to determine which characteristics modified the relationship between treatment (aliskiren, moxonidine or HCTZ) and 24-hr systolic and diastolic blood pressure (DBP), adjusted for time since randomization, age and gender. Patient characteristics considered were gender, BMI, waist, age, fasting glucose, mean 24-hr heart rate, aldosterone, renin, muscle sympathetic nerve activity (MSNA), 24-hr urine sodium excretion, hsCRP, adiponectin and leptin. These characteristics were measured during placebo treatment (in fasting condition between 7 and 9 am), except BMI and waist circumference, which were only measured at study baseline. MSNA was only performed in a subgroup of 15 patients due to the invasiveness and time-consuming nature of the measurement. Adiponectin, leptin and hsCRP levels were logtransformed due to their skewed distribution. All available follow-up data were included in the analyses even when patients did not complete all four treatments. The 95% confidence intervals (95%CI) were approximated on the basis of the coefficients' standard errors.

In the analysis determining characteristics influencing reaction to medication, scale variables were dichotomized at the median level and treatment effect was presented accordingly, combined with p-values for interaction. Analyses were performed with the statistical package R, version 2.11.1 (R Foundation for Statistical Computing, www.R-project.org).

Results

Baseline characteristics

Patients consisted of 23 men and 8 women with a median age of 60 years (IQR 55-63 years), a median BMI of 30.7 kg/m^2 (IQR 27.7-32.2 kg/m^2) and a median office blood pressure of 153/88 mmHg (IQR 145-167 mmHg systolic and 84-96 mmHg diastolic; Table 1).

Table 1. Patient characteristics during treatment with placebo

Characteristic		N = 31
Sex	Men (n, %)	23 (74%)
	Women (n, %)	8 (26%)
Age (years)		60 (55-63)
BMI (kg/m²)#		30.7 (27.7-32.2)
Waist circumference (cm)#	Men	111 (107-116)
	Women	98 (95-99)
Office blood pressure (mmHg)	Systolic	153 (145-167)
24-hr blood pressure (mmHg)	Diastolic Systolic	88 (84-96) 131 (124-144)
Heart rate (beats/minute)		71 (69-79)
MSNA (bursts/minute)		46 (39-50)
Sodium intake (mmol/day)		87 (58-118)
Fasting glucose (mmol/L)		5.6 (5.0-6.0)
Total cholesterol (mmol/L)		5.7 (5.1-6.2)
HDL-cholesterol (mmol/L)	Men	1.02 (0.94-1.22)
	Women	1.31 (1.28-1.76)
Triglycerides (mmol/L)		1.90 (1.40-2.30)
hsCRP (mg/L)		1.8 (1.3-3.3)
eGFR (ml/min/1.73m²)		79 (73-86)
Renin (pg/ml)		57.2 (42.4-79.4)
Aldosterone (pg/ml)		53.4 (33.2-76.4)
Adiponectin (µg/ml)		68.9 (51.9-104.1)
Leptin (ng/ml)		16.4 (9.5-33.2)

Data are presented as medians and interquartile ranges unless otherwise indicated.
BMI, body mass index; HDL-cholesterol, high density lipoprotein cholesterol;
eGFR, Glomerular Filtration Rate, as estimated by the Modification of Diet in
Renal Disease (MDRD) equation; ABPM, ambulatory blood pressure measurement; MSNA, muscle sympathetic nerve activity; hsCRP, high sensitivity C reactive protein.
#BMI and waist circumference were only measured at baseline

Determinants of blood pressure response to aliskiren

Table 2a shows the relation between patient characteristics and 24-hr SBP in response to treatment with the RAAS-inhibitor aliskiren.

SBP response to aliskiren was significantly related to BMI. SBP decreased with 21 mmHg (95%CI -27 to -14 mmHg) in patients with a BMI above the median of 30.7 kg/m² versus a decrease of 4 mmHg (95%CI -9 to 1 mmHg) in those with a BMI below the median, p for interaction=0.01).

SBP response was also significantly related to hsCRP in patients with >1.8 mg/L (-15 mmHg, 95%CI -20 to -10 mmHg) compared with patients with ≤1.8 mg/L (-7 mmHg, 95%CI -12 to -2 mmHg, p for interaction=0.03). None of the other patient characteristics was an effect modifier in the relation between treatment with aliskiren and blood pressure reduction.

Table 2a. Effect of RAAS inhibition (aliskiren 300 mg) on 24-hr SBP

		24-hr SBP	P for interaction
Sex	**Men**	-13 (-18 to -8)	0.75
	Women	-4 (-7 to -2)	
Age	**>60 years**	-7 (-12 to -2)	0.88
	≤60 years	-11 (-15 to -6)	
BMI	**>30.7 kg/m2**	-21 (-27 to -14)	**0.01**
	≤30.7 kg/m2	-4 (-9 to 1)	
Waist (sexpooled)	**Men >111 cm Women >98 cm**	-17 (-25 to -9)	0.39
	Men ≤111 cm Women ≤98 cm	-10 (-14 to -5)	
Heart rate$	**>71/min**	-14 (-20 to -7)	0.71
	≤71/min	-9 (-14 to -4)	
MSNA	**>46 bpm**	-9 (-17 to 1)	0.16
	≤46 bpm	-9 (-15 to -3)	
hsCRP†	**>1.8 mg/L**	-15 (-20 to -10)	**0.03**
	≤1.8 mg/L	-7 (-12 to -2)	
Renin	**>57.2 pg/ml**	-16 (-22 to -9)	0.73
	≤57.2 pg/ml	-9 (-14 to -3)	
Aldosterone	**>53.4 pg/ml**	-14 (-21 to -7)	0.75
	≤53.4 pg/ml	-11 (-16 to -6)	
Sodium intake#	**>87 mmol/day**	-14 (-20 to -7)	0.48
	≤87 mmol/day	-9 (-14 to -4)	
Glucose	**>5.1 mmol/L**	-20 (-26 to -13)	0.13
	≤5.1 mmol/L	-8 (-13 to -3)	
Adiponectin†	**>68.9 µg/ml**	-11 (-16 to -6)	0.56
	≤68.9 µg/ml	-13.3 (-20 to -7)	
Leptin†	**>16.4 ng/ml**	-16 (-21 to -10)	0.81
	≤16.4 ng/ml	-8 (-13 to -2)	

BP change (95% confidence interval) in mmHg from placebo in subgroups of patients corrected for age and gender. Patient characteristics were dichotomized on the median level and p's for interaction were determined accordingly. SBP, systolic blood pressure; BMI, body mass index; MSNA, muscle sympathetic nerve activity; hsCRP, high sensitivity C-reactive protein; bpm, beats per minute

estimated on the basis of 24-hr sodium excretion in urine; $ measured with ABPM; † log transformed

Determinants of blood pressure response to hydrochlorothiazide

Table 2b shows the relation between patients characteristics on 24-hr SBP response to diuretic therapy with hydrochlorothiazide. During treatment with HCTZ, blood pressure response was significantly related to resting heart rate. When heart rate was >71 beats/min there was a decrease of 13 mmHg (95%CI -19 to -7 mmHg) in SBP as opposed to a decrease of 3 mmHg (95%CI -7 to 2 mmHg) in SBP when heart rate was ≤71 beats/min (p for interaction=0.03). Renin level was also an effect modifier in the relation between treatment with HCTZ and blood pressure reduction. Renin levels below the median of 57.2 pg/ml were

related to larger SBP reductions (-8 mmHg, 95% -13 to -3 mmHg) than renin levels above the median (-6 mmHg, 95%CI -12 to 1 mmHg; p for interaction=0.04). None of the other patient characteristics was an effect modifier in the relation between treatment with HCTZ and blood pressure reduction.

Table 2b. Effect of diuretic therapy (HCTZ 25 mg) on 24-hr SBP

		24-hr SBP	P for interaction
Sex	**Men**	-7 (-12 to -2)	0.35
	Women	-4 (-7 to -2)	
Age	**>60 years**	-4 (-8 to 1)	0.74
	≤60 years	-7 (-12 to -3)	
BMI	**>30.7 kg/m2**	-11 (-18 to -5)	0.99
	≤30.7 kg/m2	-4 (-9 to 1)	
Waist (sexpooled)	**Men >111 cm Women >98 cm**	-8 (-16 to 1)	0.79
	Men ≤111 cm Women ≤98 cm	-6 (-11 to -1)	
Heart rate$	**>71/min**	-13 (-19 to -7)	**0.03**
	≤71/min	-3 (-7 to 2)	
MSNA	**>46 bpm**	-6 (-14 to 3)	0.17
	≤46 bpm	-8 (-14 to -1)	
hs-CRP†	**>1.8 mg/L**	-8 (-14 to -3)	0.70
	≤1.8 mg/L	-5 (-10 to 0)	
Renin	**>57.2 pg/ml**	-6 (-12 to 1)	**0.04**
	≤57.2 pg/ml	-8 (-13 to -3)	
Aldosterone	**>53.4 pg/ml**	-8 (-15 to -1)	0.54
	≤53.4 pg/ml	-7 (-12 to -2)	
Sodium intake#	**>87 mmol/day**	-7 (-13 to 0)	0.42
	≤87 mmol/day	-8 (-12 to -3)	
Glucose	**>5.1 mmol/L**	-8 (-15 to -2)	0.74
	≤5.1 mmol/L	-6 (-11 to -1)	
Adiponectin†	**>68.9 µg/ml**	-8 (-13 to -3)	0.94
	≤68.9 µg/ml	-6 (-13 to 1)	
Leptin†	**>16.4 ng/ml**	-10 (-16 to -4)	0.29
	≤16.4 ng/ml	-4 (-10 to 1)	

BP change (95% confidence interval) in mmHg from placebo in subgroups of patients corrected for age and gender. Patient characteristics were dichotomized on the median level and p's for interaction were determined accordingly. SBP, systolic blood pressure; BMI, body mass index; MSNA, muscle sympathetic nerve activity; hsCRP, high sensitivity C-reactive protein; bpm, beats per minute

estimated on the basis of 24-hr sodium excretion in urine; $ measured with ABPM; † log transformed

Determinants of blood pressure response to moxonidine

Table 2c shows the relation between participant characteristics and change in 24-hr SBP in response to sympatholytic therapy with moxonidine.

During moxonidine use, women (-4 mmHg SBP, 95%CI -7 to -1 mmHg) tended to have larger blood pressure reductions than men (-3 mmHg SBP, 95%CI -8 to 3 mmHg; p for interaction=0.06).

Table 2c. Effect of sympatho-inhibition (moxonidine 0.4 mg) on 24-hr SBP

		24h SBP	P for interaction
Sex	**Men**	-3 (-8 to 3)	0.06
	Women	-4 (-7 to -1)	
Age	**>60 years**	-2 (-7 to 3)	0.09
	≤60 years	-4 (-9 to 1)	
BMI	**>30.7 kg/m2**	-4 (-11 to 3)	0.91
	≤30.7 kg/m2	-3 (-8 to 2)	
Waist (sexpooled)	**Men >111cm Women >98cm**	-2 (-10 to 7)	0.84
	Men ≤111cm Women ≤98cm	-4 (-9 to 1)	
Heart rate$	**>71/min**	-8 (-15 to - 2)	0.53
	≤71/min	-1 (-5 to 4)	
MSNA	**>46 bpm**	0 (-9 to 9)	0.15
	≤46 bpm	-7 (-14 to 0)	
hs-CRP†	**>1.8 mg/L**	-5 (-10 to 1)	0.46
	≤1.8 mg/L	-2 (-7 to 3)	
Renin	**>57.2 pg/ml**	-2 (-9 to 5)	0.07
	≤57.2 pg/ml	-5 (-11 to 0)	
Aldosterone	**>53.4 pg/ml**	-4 (-12 to 3)	0.84
	≤53.4 pg/ml	-4 (-9 to 1)	
Sodium intake#	**>87 mmol/day**	-4 (-11 to 4)	0.25
	≤87 mmol/day	-4 (-9 to 1)	
Glucose	**>5.1 mmol/L**	-5 (-12 to 2)	0.81
	≤5.1 mmol/L	-3 (-8 to 2)	
Adiponectin†	**>68.9 µg/ml**	-5 (-10 to 1)	0.31
	≤68.9 µg/ml	-4 (-11 to 3)	
Leptin†	**>16.4 ng/ml**	-6 (-11 to 1)	0.45
	≤16.4 ng/ml	-2 (-8 to 4)	

BP change (95% confidence interval) in mmHg from placebo in subgroups of patients corrected for age and gender. Patient characteristics were dichotomized on the median level and p's for interaction were determined accordingly. SBP, systolic blood pressure; BMI, body mass index; MSNA, muscle sympathetic nerve activity; hsCRP, high sensitivity C-reactive protein; bpm, beats per minute

estimated on the basis of 24-hr sodium excretion in urine; $ measured with ABPM; † log transformed

Also, participants ≤60 years (-4 mmHg, 95%CI -9 to 1 mmHg) compared with participants >60 years (-2 mmHg, 95%CI -7 to 3 mmHg; p for interaction=0.09) tended to have larger reductions in SBP.

None of the other patient characteristics was an effect modifier in the relation between treatment with moxonidine and blood pressure reduction.

Sensitivity analyses
In patients with a screening blood pressure of >140/90mmHg, results were not significantly different from those in the patients with a screening blood pressure >130/85mmHg.

Discussion

In patients with obesity-related hypertension the characteristics BMI and hsCRP influence the blood pressure lowering effect of the RAAS inhibitor aliskiren. Heart rate and renin levels influence the effect of the diuretic HCTZ and age and gender influence the effect of the sympatho-inhibitor moxonidine.

The positive association between adipose tissue and blood pressure is mainly driven by visceral adipose tissue in males and by adipose tissue deposited elsewhere in females.(16) As a reaction to accumulation of visceral adipose tissue there is enhanced sympathoactivation, driven partly by testosterone levels. Therefore, blocking sympathoactivation might be more beneficial in reducing blood pressure in men than in women. Although women have lower sympathoactivation than men at all ages, women show a greater increase in sympathetic activity with increasing age,(21,22) followed by a more marked increase in mean blood pressure, suggesting that sympathetic activity has a greater influence on blood pressure in women than in men.(21) MSNA and, to a lesser extent, heart rate are indicative for sympathetic activation. Unexpectedly, we did see the best reaction to moxonidine on SBP in the younger patients. Although a standard maintenance dose of moxonidine (0.4 mg once daily) was given, this might not have blocked all sympathetic activity, especially in those patients with high baseline sympathetic activity. This might also explain why MSNA did not modify the relationship between moxonidine and 24-hr SBP, in combination with the relatively small number of patients (15) undergoing MSNA. Unexpectedly, heart rate was found to influence SBP when patients were treated with HCTZ, which does not exhibit sympatho-inhibitory effects.(23) To our knowledge, this has not been reported previously.

One of the other mechanisms underlying ORH is an inappropriately normal or even elevated RAAS activity. An increasing BMI is correlated to an augmented activity of the RAAS system (24,25) as reflected in higher levels of angiotensinogen, renin, angiotensin I and angiotensin

converting enzyme (ACE) in obese persons as compared to lean persons.(24–26) Independent adipose tissue production of angiotensinogen(27) and factors stimulating the adrenal gland in producing aldosterone(28) are presumably responsible for this enhanced activity in obese individuals. The synthesis and secretion of angiotensinogen in adipose tissue does not only contribute to elevated local angiotensin II concentrations, causing oxidative stress and local inflammation, but also lead to higher systemic RAAS activity.(7)

Renin and aldosterone levels drop significantly with weight loss, even when the BMI remains >25 kg/m^2, suggesting that there is a linear association between BMI and RAAS-activation. Inhibiting RAAS could therefore be more effective in terms of blood pressure reduction in individuals with the highest BMI.(13) Aliskiren, being a direct renin-inhibitor, is known to reduce RAAS activity at both the systemic level(29) and also penetrates the adipose tissue at levels sufficient to reduce tissue RAAS activity.(30) Since enhanced inflammation is, at least partly, a reflection of locally enhanced RAAS activation(31), this may explain the higher blood pressure reductions in patients with the highest hsCRP plasma concentrations. Unexpectedly, blood pressure lowering response to aliskiren was not influenced by either renin or aldosterone plasma concentrations, nor was there an influence of renin and aldosterone plasma concentrations on blood pressure level, despite the known inhibitory effect of aliskiren on both systemic and adipose tissue RAAS activity.(29,30) There is limited evidence supporting a role for measuring RAAS hormones as guidance for anti-hypertensive therapy. Small retrospective studies show conflicting results concerning the modifying effects of plasma renin activity (PRA) on the blood pressure lowering effect of RAAS inhibitors.(32,33) A small prospective study shows PRA to correlate with blood pressure reduction in patients treated with angiotensin receptor blockers.(34) In our study we did not measure PRA but plasma renin concentration (PRC), but both parameters are highly correlated in untreated individuals.(35)

In ORH, due to impaired pressure natriuresis, volume overload can be an underlying pathophysiological phenomenon.(4) Both higher age and low renin levels are associated with a high intravascular volume status. Thus, older patients and those with low renin levels have a larger blood pressure reduction in response to diuretic therapy.(12,14,17)
In this study, patients with the lowest plasma renin concentrations, an indication for high volume status, responded most to HCTZ therapy with respect to systolic blood pressure lowering. This is in line with observations in overweight hypertensive patients.(14) Contrary to our expectations, no influence on blood pressure response of sodium intake in either of the treatment arms was seen. For patients treated with aliskiren, 1 earlier study has shown that those on a low salt diet (<5 gr NaCl/day) experience the greatest antihypertensive effect(18) compared to patients on a normal to high salt diet (>10 gr NaCl/day), with a blood pressure difference of 9.4 mmHg. The same beneficial effect of a low sodium diet has been reported for ACE inhibitors and angiotensin receptor blockers.(15) A less prominent effect

of low sodium diet is seen when patients are treated with other classes of antihypertensive drugs (calciumblockers, betablockers and diuretics), approximately 4 mmHg blood pressure difference between a low and high sodium diet.(15) In both studies, a large difference in sodium intake was created, either by using a cross-over design with one period of low and one period of high sodium intake or by using a 'normal' sodium intake control group opposed to a group instructed to adhere to a low sodium diet. In our study, no such contrast was created and since patients were asked to adhere to a normal salt diet, a relatively low sodium intake (5.1 gr NaCl/day) was observed, which might explain why we did not find sodium intake to influence blood pressure response. Moreover, those studies did not exclusively study patients with ORH, although the mean BMI in both studies was >25 kg/m2.

Strengths and limitations of our study need to be considered. The influence on blood pressure response of possible effect modifiers could be analyzed during treatment with three different classes of antihypertensive medication, aimed at different pathophysiological mechanisms. Blood pressure and heart rate were measured very accurately with 24-hr ambulant blood pressure measurements. An important limitation of this study is the relatively small sample size, especially the amount of women participating in the trial. In this study, participants were regarded hypertensive with a relatively low blood pressure of >130mmHg systolic or >85mmHg diastolic. In this patient category, there is no proof of better outcome with lowering blood pressure. However, the aim of our study was not to investigate the change in outcome of lowering blood pressure, but to study the pathophysiological reactions in patients with obesity and hypertension to different antihypertensive drugs. Moreover, not treating patients with a higher blood pressure and concomitant risk (metabolic syndrome) is unethical, and would undermine the careful design with a placebo period in this study.
Since many determinants were measured, some of the significant results might be false-positive due to multiple testing. However, determinants were not chosen and analyzed in a random way, but were chosen on their pathophysiological link with hypertension.

Conclusions

The blood pressure-lowering response of antihypertensive medication is for aliskiren influenced by BMI and hsCRP, for HCTZ by heart rate and renin levels and for moxonidine by gender.
This emphasizes the multi-factorial mechanism of development of ORH, with activation of the RAAS system, sympathetic activation and volume overload being causative factors. Patient characteristics may guide choice of blood pressure-lowering medication in patients with ORH.

Abbreviations:

BMI: body mass index
HCTZ: hydrochlorothiazide

hsCRP: high-sensitivity C-reactive protein
MSNA: muscle sympathetic nerve activity
ORH: obesity-related hypertension
RAAS: renin-angiotensin-aldosterone system
SAT: subcutaneous adipose tissue
SBP: systolic blood pressure
VAT: visceral adipose tissue

Declarations

Ethics approval and consent to participate: All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This was judged by the Ethics Committee of the Universal Medical Center Utrecht, the Netherlands (reference number: 10-215/G-E).

Consent for publication: not applicable.
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Authors' contributions: IS and JD (Dorresteijn) drafted the manuscript; IS, JD (Dorresteijn), FV and WS were involved in analyzing and interpreting the data, JD (Danser) analyzed the renin and aldosteron samples and advised on the impact of their results. All authors read and approved the final manuscript.

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Chapter 9

General discussion

Diagnosing adipose tissue dysfunction, rather than identifying individuals with obesity

Obesity is not a benign disorder, but the concept and diagnosis of adipose tissue dysfunction (ATD) in both obese and normal weight individuals might lead to better risk stratification and contribute to better prevention of cardiovascular disease and diabetes. Not all overweight or obese patients are at the same risk for cardiovascular events and diabetes. The metabolic syndrome is currently the best clinical tool to approximate ATD, as explained in Chapter 2. Prevalence of the metabolic syndrome rises from 31% in individuals with a BMI <30 kg/m2 to 69% in those with a BMI >40 kg/m2(1). The presence or absence of the metabolic syndrome in these individuals depends partly on the amount of visceral adipose tissue (VAT). A 1 SD increase of VAT was associated with an OR of 2.78 (95%CI 1.78 – 4.3) for having metabolic syndrome in normal weight individuals, an OR of 1.63 (95%CI 1.21 – 2.01) in overweight and 1.43 (95%CI 0.99 – 1.64) in obese individuals(2). This emphasizes the need for other adipose tissue measures, other than BMI, to better identify those patients at increased risk for developing metabolic syndrome and ultimately vascular diseases, diabetes and malignancies(3). On a pathophysiological level, ATD causes endothelial dysfunction, inflammation and insulin resistance (chapter 2). Eventually, these lead to, among others, hypertension and elevated glucose levels, which are components of the metabolic syndrome. Therefore, we hypothesize that ATD precedes the metabolic syndrome.

Once the metabolic syndrome is present, the risk of developing diabetes type 2 is elevated in normal weight (hazard ratio (HR) 4.7 (95%CI 2.8 – 7.8)), overweight (HR 8.5 (95%CI 5.5 – 13.4)) and obese (HR 16.3 (95%CI 10.4 – 25.6)) individuals compared to normal weight individuals without metabolic syndrome(4). The risk of developing a myocardial infarction (HR 1.95 (95%CI 1.65 – 2.31) in men and 3.17 (95%CI 2.53 – 3.97) in women) or other vascular event (HR 2.63 (95%CI 1.32 – 4.72)) is also increased in individuals with the metabolic syndrome, irrespective of BMI (5,6). But even within the group of patients with metabolic syndrome there is a range of risks for developing vascular events. Apparently not all metabolic syndrome patients are at the same high risk.

This observation leads to the question whether differences in ATD might explain the differences in risk for vascular events

Treatment of patients with the metabolic syndrome consists primarily of lifestyle changes, with a focus on weight reduction and physical exercise(7). Lifestyle programs have limited capacity, are costly and many patients are not very motivated to reduce weight(7). Insight in adipose tissue function and the subsequent short-term metabolic consequences may motivate patients to stick to a healthy lifestyle program including weight reduction. Our hypothesis claiming ATD to precede the metabolic syndrome, in combination with obesity eventually leading to metabolic derangements presumes that patients with ATD are at risk for cardiovascular events and diabetes at short term, and patients with obesity, but not having ATD yet are also at risk at long term. Therefore, diagnosing ATD could trigger

more intensified follow-up and early treatment of hypertension and dyslipidemia could be initiated. Commonly used risk prediction tools most likely underestimate CV risk in patients with ATD(8), 75% of patients with metabolic syndrome are at low risk according to the Framingham risk score. Young age, the absence of insulin resistance in prediction tools and limited role of BMI presumably explain this. In general, weight reduction in overweight or obese patients is beneficial, but weight reduction in overweight or obese patients with ATD is more urgent. However, no definite measurement or method is available for the diagnosis of ATD. This thesis has explored different options to approximate ATD, and offers opportunities and considerations for diagnosing ATD in clinical practice.

Using plasma adipokine concentrations for diagnosing ATD

The pathophysiological process in adipose tissue, leading to ATD most likely precedes the development of metabolic syndrome and most likely most patients with metabolic syndrome have various levels of ATD. Plasma levels of leptin (9) and adiponectin (10), two of the most extensively studied adipokines, show moderate sensitivity and specificity for determining the presence or absence of cardiometabolic abnormalities (hypertension, hypertriglyceridemia). Although these studies are conducted in unselected populations, the limited sensitivity and specificity (65%-76%) make these reference values not useful in daily practice.

Adding adipokine measurements to other risk factors for ATD shows interesting results. Measurement of adiponectin and BMI combined predicts intima-media thickness (IMT) better than BMI or adiponectin alone(11). Pro-inflammatory adipokines such as monocyte chemo attractant protein 1 (MCP-1), interleukin 6 (IL-6) and resistin are positively associated with insulin resistance, regardless of BMI (12). Both plasma concentrations of adiponectin and leptin were found to increase the risk of developing diabetes type 2. Adiponectin explained one third of the risk for diabetes mellitus (32.1 % (95% CI 16.8 -49)) in asymptomatic individuals in a 7 year follow-up period (13). RR for developing diabetes for a 1 logtransformed unit increment in leptin levels was 1.37 (95% CI, 1.13-1.66) for men and 0.96 (95% CI, 0.90-1.03) for women(14). Using adipokine measurements could diagnose ATD before the metabolic syndrome is present, although reference values are much needed. Once the diagnosis of ATD is made, early intervention could be performed with a lifestyle program or medical treatment. Life style interventions improve plasma adipokine concentrations, and decrease endothelial dysfunction and insulin resistance, suggesting that ATD at a certain stage can be influenced(15,16). Metformin treatment increases plasma adiponectin concentration (17), decreases the plasma concentrations of pro-inflammatory adipokines(18) and, in insulin resistant individuals, lowers the absolute risk for developing diabetes type 2 with 25%(19). Treatment with a cannabinoid receptor ligand (Rimonabant) in obese individuals with the metabolic syndrome improved their lipid and carbohydrate

metabolism and endothelial function(20), increased their adiponectin concentration and lowered MCP-1 plasma concentrations(21). However serious side-effects (mainly psychiatric) have led to the termination of further development of this drug. Selective cannabinoid receptor ligands are under investigation, which do not exert psychiatric side-effects but have similar beneficial metabolic effects.

In primary hypertensive subjects, current guidelines suggest not to treat with antihypertensive drugs in patients <50 years of age, who do not exhibit any other risk factors for cardiovascular diseases(22). However, it has been shown that higher plasma resistin levels are associated with renal function impairment in hypertensive individuals of all ages, as reflected by decreased eGFR(23). Moreover, the independent association of resistin with eGFR suggests involvement of resistin in the progression of kidney damage in the early stages of hypertension(23). In these individuals, elevated resistin levels might be a trigger in the decision whether or not antihypertensive treatment should be initiated. Obviously, this hypothesis first needs to be evaluated in an interventional study.

Although current evidence usually links one adipokine to (a feature of) the metabolic syndrome(15,16,23), we believe that combining adipokine concentrations could be a better reflection of adipose tissue function and thus in determining ATD since the development of ATD is not dependent on a single adipokine (Chapter 5 and 6). The adipokine profile we studied correlates with the presence of metabolic syndrome and hypertension in individuals with cardiovascular disease, whereas single adipokines differed in their association with the metabolic syndrome or hypertension. Although it is known that metabolic syndrome confers an increased cardiovascular and diabetes risk, a next step to establish the relevance of plasma adipokine concentrations would be to evaluate the relation between plasma adipokine concentrations and actual vascular and diabetes risk in large cohort studies in various groups of patients.

Using imaging in the diagnosis of ATD

Ultrasound, CT- and MR-scans can be used to estimate ATD as there is a clear relation between adipose tissue mass and ATD. Ultrasound is a reliable method to measure VAT(24), which correlates to the presence of the metabolic syndrome more than BMI and waist circumference(25).

Different tissue characteristics on CT-scans are expressed with level of Hounsfield Units (HU), and making use of HU is often mentioned as imaging modality to evaluate adipose tissue characteristics potentially related to ATD. In physics, zero Hounsfield Units is the radiodensity of distilled water at a standard pressure and temperature. A change in 1 Hounsfield Unit represents a change of 0.1% of the attenuation coefficient of water. Adipose tissue radiodensity detected by CT-scans is hypothesized to be associated with differences in adipose tissue composition(26). In general, adipose tissue quality, estimated with CT

attenuation, is associated with all-cause mortality, non-cardiovascular death and cancer death(27). Focusing on cardiovascular diseases, CT attenuation from both visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) is associated with coronary artery calcification(28). The adipose tissue surrounding the heart is known as epicardial adipose tissue (EAT). If dysfunctional epicardial adipose tissue is present, the local pro-inflammatory status elevates the risk of developing coronary calcification and ischemic heart disease(29). In asymptomatic individuals, a higher volume of EAT and a lower density of EAT are associated with the presence of coronary calcium, a known risk factor for the development of cardiovascular disease(30). The lower density is also related to the pro-inflammatory adipokines PAI-1 and MCP-1 and inversely to the anti-inflammatory adiponectin (30). This was also shown in a study in 140 patients with cardiovascular disease, in which a standard deviation lower density (5HU) showed a 90% increase in odds for being in a higher coronary artery calcification group, independent of EAT volume and BMI(31). Moreover, a decrease in HU of 1 SD was associated with more cardiovascular risk factors(32). However, in individuals suspected for ischemic heart disease, only EAT volume (and not density) is associated with myocardial ischemia and improves the discriminative power for myocardial ischemia prediction over coronary calcification score(33). Finally, MR-spectroscopy measurements can be performed, as we did in Chapter 3 and 4. With these measurements, we were able to show differences in free fatty acid profiles between individuals with and without the metabolic syndrome. These differences were most pronounced in the visceral adipose tissue, both fasting and after an oral fatload. Preferably, a free fatty acid profile can be compared to a 'reference profile' of a normal-weight, metabolically healthy individual. This is an expensive and time-consuming technique, but provides insight in the metabolism of fatty acids in both fasting and post-prandial conditions.

In CT examinations, compared to the spectroscopy measurements, no special measurements are necessary to obtain information, since HU measurements are provided with every CT-scan. Hence, if in an overweight or obese patient without a history of diabetes or cardiovascular disease a CT-scan is performed, the presence of a lower density of abdominal adipose tissue may indicate presence of ATD and thus stratify individuals towards a higher risk for developing diabetes or cardiovascular disease.

Using adipose tissue biopsies in the diagnosis of ATD

Adipose tissue biopsies are potentially the most direct way to evaluate ATD although this is a morphological evaluation and not a functional evaluation. In adipose tissue biopsies macrophage infiltration and the formation of crown like structures can be seen. These are related to inflammation (34) and endothelial dysfunction (35), even early in the development of ATD (34). In middle-aged patients undergoing abdominal surgery, presence of insulin resistance as measured with HOMA-IR was positively associated with the presence of crown

like structures in both VAT and SAT, independent of waist circumference and BMI (36). With respect to differences in adipose tissue depots, SAT contains the lowest amount of macrophages and pro-inflammatory adipokine concentrations(37) and the highest amount of the anti-inflammatory adiponectin(38). No differences in LPL-activity and lipolysis were found between SAT and VAT biopsies(39). Based on these differences, VAT biopsies are considered to be better predictors of metabolic abnormalities than SAT biopsies(38).
The clinical usefulness of adipose tissue biopsies might be limited, especially with regard to VAT biopsies, as they can only be obtained during abdominal surgery and especially when other (less invasive) diagnostics tests for ATD become available. However, when individuals undergo abdominal surgery for non-malignant and non-life threatening disorders (for instance cholecystectomy), a small biopsy can be taken to examine for ATD. If there are signs of ATD in the biopsy, frequent monitoring of metabolic derangements due to ATD and early intervention could be considered. Which individuals should be evaluated for ATD remains subject of debate, and is discussed later on.

Treating obesity-related disorders with tailored medication

Hypertension

As shown in chapter 7 and 8, RAAS inhibitors are the treatment of choice for obese hypertensive patients. Systolic blood pressure decreased most during treatment with the renin-blocker aliskiren in individuals with obesity-related hypertension, as compared to a diuretic agent and a centrally acting agent. This is most likely due to the paracrine signaling from adipose tissue, releasing RAAS hormones locally besides the systemic presence of RAAS hormones(40,41). With a higher concentration of RAAS hormones, inhibitors of this pathway are more likely to exert a beneficial effect. Also, inhibiting sympathetic overdrive, which is more prevalent in obese individuals, with a centrally acting agent (moxonidine) in obese hypertensive patients has been shown to reduce blood pressure to a maximum of 18 mmHg systolic (42). This in contradiction with our findings in which moxonidine did not lower blood pressure to a great extent (-3 mmHg systolic).

Heart failure / Obesity cardiomyopathy

Severe obesity produces hemodynamic alterations that predispose to changes in cardiac morphology and ventricular function, which may lead to the development of heart failure. Obesity-related hypertension, sleep apnea and hypoventilation probably contribute to the development of obesity cardiomyopathy(43). This condition is – at least partly – reversible with weight loss(43), but since this is at best time consuming and often not achieved, pharmacological treatment should also be considered. Several studies found positive effects of mineralocorticoid receptor antagonism on the outcome of obesity-associated cardiomyopathy(44,45). In those patients with abdominal obesity, there was a more

pronounced effect of treatment with eplerenon on cardiovascular death or hospitalization for heart failure. This is different from the regular treatment of heart failure, in which ACE-inhibitors or ARBs combined with diuretics are the cornerstones of treatment. Aldosterone antagonists are usually only used in these patients when hypokalemia complicates the treatment with diuretics, but should be considered as second line treatment in obesity-associated cardiomyopathy.

Dyslipidemia

One of the characteristics of ATD and metabolic syndrome is hypertriglyceridemia, accompanied by low HDL-cholesterol levels. Insulin resistance and insulin deficiency in ATD lead to decreased activity of the enzyme lipoprotein lipase (LPL), which degrades VLDL-particles and chylomicrons. Since those particles contain vast amounts of triglycerides, slow degradation leads to hypertriglyceridemia(46). Excessive intake of saturated fatty acids, leading to even more chylomicrons and alcohol, inhibiting LPL, lead to even higher triglyceride levels(47). Treatment of hypertriglyceridemia in those with and without cardiovascular disease is considered only necessary when triglyceride levels rise above 10 mmol/L, since these levels are related to the development of pancreatitis(48,49). On average, individuals with hypertriglyceridemia based on the presence of ATD have triglycerides plasma concentrations ranging from 2 to 5 mmol/L (Chapter 3, 4, 7, 8) and do not fulfill the conditions for treatment as advocated in current guidelines.

Recently, the PCSK-9 inhibitors (proproteïn convertase subtilisine/kexine type 9) have been introduced in the treatment of dyslipidemia in patients not reaching their LDL-c target levels. Patients with atherosclerotic disease have higher PCSK-9 levels than those without atherosclerotic disease(50). PCSK-9 levels were also positively related to the adipose tissue dysfunction parameters insulin resistance and HbA1c. Hepatic PCSK-9 expression may be regulated by insulin, although no definite prove is available.(50,51). Interestingly, PCSK-9 levels were not associated with body fat distribution(51). Thus, individuals with high levels of PCSK-9 – leading to higher LDL-c levels, since the LDL-c receptor is downregulated by this enzyme – are more likely to have ATD and might benefit from PCSK-9 inhibition. This hypothesis needs to be confirmed in a prospective intervention study.

Future perspectives

Focusing on the diagnosis of adipose tissue dysfunction, in addition to determining overweight or obesity, could be a next step to recognize individuals at risk for developing diabetes type 2 and cardiovascular disease. Identifying individuals with adipose tissue dysfunction remains a challenge. Currently in clinical practice determination of the metabolic syndrome criteria is most suitable. Screening for metabolic syndrome should be considered in those individuals likely to benefit most from treatment as a result of this diagnosis. Hence,

young asymptomatic individuals who are either overweight or obese are a suitable group. The likelihood of determining ATD/metabolic syndrome is large, and motivation for weight loss could be enhanced when already metabolic effects are present. The presence of ATD might be added to the eligibility criteria to perform bariatric surgery. Currently, persons are eligible for bariatric surgery with a BMI >40kg/m^2, and with a BMI >35kg/m^2 if there is comorbidity (like type 2 diabetes or severe hypertension). It could be considered to add presence of ATD as a comorbidity to direct earlier intervention, before diabetes and cardiovascular disease can develop. Also, individuals with normal weight, but presenting with one of the metabolic syndrome criteria (for instance hypertension) could be screened, if other characteristics of ATD (dyslipidemia, insulin resistance) are present as well. In the further future, being able to diagnose ATD before the presence of the metabolic syndrome, might lead to more specific and earlier interventions, although treating too early might be a pitfall, since this might lead to overtreatment and considerable costs. The presence of specific ATD characteristics in individuals with the metabolic syndrome could further stratify the risk of developing diabetes and cardiovascular disease. This leads to a more individualized approach, aiming at intervening in those who are at highest risk. Before implementing parts of the ATD concept in clinical practice, cohort studies are needed to establish reference values of plasma adipokines, to evaluate whether or not there is better risk stratification when adding adipokine concentrations to metabolic syndrome characteristics, to establish whether treatment decisions based on presence or absence of ATD lead to a favorable outcome, and whether or not this is cost-effective.

Key findings of this thesis

- Diagnosing adipose tissue dysfunction in an individual level is currently best done by determining metabolic syndrome criteria. (Chapter 2)
- Individuals with metabolic syndrome differ from those without metabolic syndrome regarding their free fatty acid composition in visceral adipose tissue. (Chapter 3 and 4)
- The handling of an oral fat challenge by the visceral adipose tissue is different between individuals with and without the metabolic syndrome (i.e. adipose tissue dysfunction). (Chapter 4)
- Higher adipokine levels are associated with (components of) the metabolic syndrome and blood pressure. (Chapter 5)
- Patients with obesity-related hypertension are best treated with RAAS-blockers, possibly due to the ability of excess adipose tissue to produce angiotensin II and aldosterone. (Chapter 7 and 8)
- None of the possible three pathophysiological mechanisms (RAAS activation, sympathetic activity and sodium retention behind obesity-related hypertension could be pointed as the most important one. (Chapter 8)

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Chapter 10

Nederlandse samenvatting
(voor niet ingewijden)

Suikerziekte en hart- en vaatziekten zijn een groot gezondheidsprobleem. Een belangrijke risicofactor voor het krijgen van deze ziekten is het hebben van overgewicht (een body mass index (BMI) > 25kg/m2) of obesitas (BMI >30kg/m2). Opvallend is echter dat er mensen zijn met overgewicht of obesitas die niet of pas op erg late leeftijd gezondheidsproblemen krijgen door hun gewicht, én dat er mensen zijn met een normaal gewicht die wel hart- en vaatziekten of suikerziekte krijgen. Ook als je rekening houdt met andere risicofactoren (zoals roken) blijft dit gegeven bestaan. Dit verschil kan mogelijk verklaard worden door niet alleen te kijken naar de hoeveelheid vetweefsel, maar ook naar de kwaliteit van het vetweefsel, ook wel functie genoemd. Vetweefsel scheidt adipokines af, stoffen die verschillende eigenschappen hebben. Als het vetweefsel goed functioneert, scheidt het beschermende adipokines af, die hart- en vaatziekten en suikerziekte helpen voorkomen. Als het vetweefsel disfunctioneert dan scheidt het vetweefsel juist ontstekingsbevorderende adipokines af, waardoor het risico op hart- en vaatziekten en suikerziekte toeneemt. Over dit disfunctionerende vetweefsel en de gevolgen daarvan gaat dit proefschrift.

In **hoofdstuk 1** wordt uitgelegd dat overgewicht en obesitas leiden tot veel gezondheidsproblemen. Vetweefsel is niet alleen een opslagplaats, maar een actief orgaan dat allerlei stoffen produceert die invloed hebben op de ontwikkeling van hart- en vaatziekten en suikerziekte. Vetweefsel kan beoordeeld worden op kwantiteit (hoeveelheid vetweefsel) en op kwaliteit (hoe goed functioneert het vetweefsel), waarbij gedacht wordt dat kwaliteit belangrijker is dan kwantiteit.

Hoofdstuk 2 geeft een overzicht van de verschillende mogelijkheden om de diagnose disfunctionerend vetweefsel te stellen. Dit kan relatief simpel met het meten van de buikomvang, maar dit is geen nauwkeurige methode, omdat er veel mensen zijn met een vergrote buikomvang die gezond vetweefsel hebben. Beter is het om bijvoorbeeld de criteria voor het metabool syndroom te gebruiken. Deze betreffen waarden in het bloed, de bloeddruk en de buikomvang; als er 3 of meer van deze criteria positief zijn is er sprake van het metabool syndroom. Het hebben van metabool syndroom is een teken van disfunctionerend vetweefsel. Ook kan disfunctionerend vetweefsel bepaald worden door bepaalde stoffen die vetweefsel uitscheidt te meten of door met een speciale Magnetische Resonantie (MR)-techniek de verschillende vetzuren in beeld te brengen. Het meten van de adipokines wordt nu alleen nog in onderzoeksverband gebruikt. Ook kan een biopt van vetweefsel laten zien of het vetweefsel goed van kwaliteit is, maar ook dit is in de praktijk moeilijk toepasbaar, omdat het niet wenselijk is om bij iedereen een biopt te nemen.

De beeldvormende techniek (MR – Spectroscopie) uit hoofdstuk 2 hebben we toegepast op vrijwilligers in **hoofdstuk 3 en 4.** Bij deze techniek worden de verschillende vetzuren in het vetweefsel zichtbaar gemaakt. In **hoofdstuk 3** hebben we dit gedaan bij 13 vrijwilligers met een normaal gewicht en 12 vrijwilligers met overgewicht. Omdat deze methode nog maar weinig gebruikt werd in vetweefsel in de buik, moest eerst beoordeeld worden of de techniek betrouwbaar en reproduceerbaar was. Daarom hebben de vrijwilligers het MR onderzoek 2 keer gehad. Er werden slechts kleine verschillen tussen de 2 metingen

gezien, waarmee aangetoond werd dat het een reproduceerbaar onderzoek is. Daarnaast werd gekeken naar verschillen tussen de 2 groepen: hierbij zagen we verschillen in de samenstelling van het vetweefsel tussen deze 2 groepen, waarbij de slanke mensen gezonder vetweefsel hadden. **In hoofdstuk 4** onderzochten we alleen vrijwilligers met overgewicht, waarbij de helft het metabool syndroom had en de andere helft niet. Hierbij zagen we dat de mensen met overgewicht zonder metabool syndroom een andere vetzuursamenstelling hadden dan de mensen met overgewicht én metabool syndroom. Na de eerste MR-scan kregen de vrijwilligers een vetbelasting (slagroom) te drinken, 6 uur laten werd opnieuw middels MR-spectroscopie gekeken hoe zij hadden gereageerd op de vetbelasting. In de mensen met metabool syndroom waren meer veranderingen te zien in het vetweefsel dan bij de mensen zonder metabool syndroom. Dit suggereert dat de mensen met metabool syndroom een mindere kwaliteit vetweefsel hebben, gemeten aan de hoeveelheid vetzuren in het vetweefsel.

Hoofdstuk 5 en 6 gaan over de relatie tussen adipokines en het optreden van het metabool syndroom. Adipokines zijn stoffen die worden gemaakt door vetweefsel en een gunstig of ongunstig effect op het risico op hart- en vaatziekten kunnen hebben. In **hoofdstuk 5** zien we in een grote database van mensen met hart- en/of vaatziekten dat een hogere waarde van een aantal adipokines (hepatic growth factor en leptin) een grote kans geeft op het hebben van het metabool syndroom. Het hebben van een hogere waarde van adiponectin (een beschermende stof uit het vetweefsel) geeft een lagere kans op het hebben van het metabool syndroom. Daarnaast hebben we alle adipokines gecombineerd in een 'adipokine profiel', waarbij een 'slechte' waarde van elke adipokine punten opleverde. Hoe hoger de score op het adipokine profiel, hoe groter de kans op het hebben van het metabool syndroom. Dit was onafhankelijk van de hoeveelheid vetweefsel, hetgeen betekent dat het hebben van niet goed functionerend vetweefsel belangrijker is dan de absolute hoeveelheid vetweefsel. In **Hoofdstuk 6** is dezelfde onderzoeksmethode gebruikt om te kijken naar de kans op het hebben van hoge bloeddruk. Hierbij zagen we ongeveer dezelfde resultaten, waarbij ook de absolute hoeveelheid vetweefsel minder belangrijk was dan de functie van het vetweefsel op het krijgen van een hoge bloeddruk.

Hoofdstuk 7 en 8 gaan over het behandelen van een hoge bloeddruk die veroorzaakt wordt door overgewicht/niet goed functionerend vetweefsel. In **hoofdstuk 7** worden de resultaten van de Target-studie gepresenteerd. In deze studie zijn 31 patiënten met hoge bloeddruk door overgewicht 4 periodes van 8 weken behandeld met verschillende medicijnen tegen hoge bloeddruk. Aan het eind van elke periode werd 24 uur lang de bloeddruk gemeten en werd de vaatfunctie gemeten door middel van zogenaamde flow-mediated dilation (FMD). Dit is een maat voor hoe goed de vaten kunnen ontspannen; ontspannen vaten zijn gezonder. Het bleek dat het medicijn aliskiren het beste werkt in deze groep patiënten: dit verbeterde de vaatfunctie en gaf de grootste daling in bloeddruk. Deze daling komt mogelijk doordat vanuit het vetweefsel bepaalde bloeddrukverhogende stoffen worden uitgescheiden die door aliskiren geremd worden. Deze stoffen worden niet geremd door

de andere medicijnen die in deze studie gebruikt werden. **Hoofdstuk 8** gaat verder in op deze gedachte. Hierbij is gekeken welke factoren binnen de groep van mensen met obesitas gerelateerde hoge bloeddruk zorgen voor de grootste daling in bloeddruk. Hierbij blijkt dat bij de patiënten met de hoogste body mass index (kg/m2) aliskiren het beste werkt. Dit zou kunnen komen doordat meer vetweefsel leidt tot meer afgifte van de stoffen die de bloeddruk verhogen.

In **hoofdstuk 9** ten slotte wordt een reflectie gegeven op de voorgaande hoofdstukken en een algemene discussie over de metabole gevolgen van niet goed functionerend vetweefsel gevoerd. Hierbij komt vooral naar voren dat het belangrijker is om mensen met niet goed functionerend vetweefsel op te sporen dan mensen met overgewicht of obesitas. Deze mensen hebben misschien wel te veel, maar kunnen goed functionerend vetweefsel hebben. Dit kan op verschillende manieren gedaan worden; op dit moment kan dit met name door de metabool syndroom criteria te gebruiken. Mogelijk kunnen in de toekomst de in dit proefschrift beschreven methoden, zoals het gebruik van de adipokines of beeldvormende technieken een rol spelen bij de diagnostiek hiervan. Daarnaast moet meer gekeken worden naar de mogelijkheden om mensen met niet goed functionerend vetweefsel te behandelen met de medicatie die hiervoor het meest geschikt is. Voorbeelden hiervan zijn gegeven in hoofdstukken 7 en 8.

Kortom, het aloude concept van het hebben van overgewicht als risicofactor voor het krijgen van hart- en vaatziekten en/of suikerziekte is achterhaald, nuttiger is het om te kijken naar het functioneren van het aanwezige vetweefsel.

Chapter 11

Dankwoord

Curriculum vitae

Dankwoord

Acht jaar na het starten van mijn promotietraject ga ik het afronden. Ouder, maar vooral ook veel wijzer. In die acht jaar hebben velen mij terzijde gestaan; inhoudelijk, mentaal, tijdens de successen, op moeilijke momenten, in en buiten het ziekenhuis. Zonder jullie was het niet gelukt!

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Curriculum Vitae

Ilse-Marije Schrover was born on the 13th of May 1982 in Alkmaar, The Netherlands.
After graduating from the 'Gymnasium Felisenum' in Velsen-Zuid in 2000, she studied Medicine at Utrecht University. During her study, she was first engaged in research at the department of Gastro-enterology of the Antonius Hospital Nieuwegein (under supervision of Dr. Timmer and Prof. Dr. Weusten), where she studied the outcome of patients after endoscopic drainage of pancreatic infections.
In August 2006, she obtained her medical degree and started working at the Department of Internal Medicine of the Gelre Hospitals Apeldoorn. In May 2007, she started her residency Internal Medicine in the same hospital under supervision of Dr. C.G. Schaar. In May 2009, she continued her residency Internal Medicine at the University Medical Center Utrecht, under supervision of Prof. Dr. D.W.Biesma. In September 2010, she started the work described in this thesis at the Department of Vascular Medicine, University Medical Center Utrecht, under supervision of Prof. Dr. F.L.J. Visseren.
In September 2013 she continued her training in Internal Medicine at the University Medical Center Utrecht, under supervision of Prof. Dr. M.M.E. Schneider and Prof. Dr. H.A.H Kaasjager. In May 2014 she started her training in Vascular Medicine at the same hospital under supervision of Prof. Dr. F.L.J.Visseren, which she finished in 2017. She currently works as internist-vascular medicine at the Medisch Centrum Leeuwarden.
Ilse-Marije cohabitates with Mathijs Rodenburg, together they have 2 children, Mare (2014) and Hidde (2016),