Unraveling obesity's road to diabetes and cardiovascular disease

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Unraveling Obesity's Road to Diabetes and Cardiovascular Disease:

Contributors to Insulin Resistance, Beta-cell Dysfunction and
Vascular Dysfunction

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Unraveling Obesity's Road to Diabetes and Cardiovascular Disease:

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A black and white photograph of a canyon with layered rock formations. The word "chapter" is written vertically in white, bold, sans-serif font across the center of the image. A semi-transparent rectangular box is positioned on the right side of the image, partially overlapping the rock formations.

chapter

Chapter 1

General introduction

1.1. Introduction

After centuries of predominantly underweight-associated deaths, overweight and obesity now kill more people than underweight.¹ Even though obesity can be prevented by balancing food intake and energy expenditure, its prevalence is still steadily increasing; in the Netherlands, now more than half of the adult population is overweight and 15% is obese.^{1,2} As excess fat mass increases, the risk to develop type 2 diabetes mellitus (T2D), increases in parallel.³⁻⁵ Obese individuals and individuals with T2D are at increased risk to develop cardiovascular disease (CVD); CVD accounts for 52% of deaths in T2D.⁶ When individuals develop T2D, two core processes are usually at play: insulin resistance and beta-cell dysfunction.⁷ Here, we will review both processes, as well as their potential contributors, in more detail. In addition, we address vascular function as a potential mediator of CVD, as well as a surrogate for CVD risk.

1.2. Insulin resistance

Insulin resistance can be regarded as a decreased sensitivity to the metabolic actions of insulin that promote glucose disposal. Typically, skeletal muscle is the primary site of action for insulin-induced glucose disposal, although other tissues like liver, adipose tissue and skin contribute as well.⁸⁻¹⁰ In response to insulin, myocytes and adipocytes translocate glucose transporter 4 (GLUT4) from the cytosol to the plasma membrane to facilitate cellular glucose entry; this rapidly lowers plasma glucose levels.^{11,12} In an insulin resistant state, this response is blunted.¹³⁻¹⁵

The gold standard to measure insulin sensitivity is by means of a euglycemic insulin clamp, during which insulin is infused at a fixed rate while isoglycemia is maintained through a variable infusion of 20% glucose.^{16,17} When steady state is achieved, the rate of glucose perfusion then approximates the whole body glucose disposal (WBGD).^{16,17} This method is described in more detail in *chapter three*.

To link obesity to insulin resistance, several tissue-specific pathways, including visceral and subcutaneous adipose tissue, liver and muscle, have been proposed. These pathways have, however, never been studied simultaneously; hence, whether these pathways act independently of each other has not been elucidated.

1.2.1. Adipose tissue

A large organ with considerable plasticity, adipose tissue is capable of storing massive amounts of energy in the form of triacylglycerol; adipose tissue consists of roughly

$3 \cdot 10^{10}$ adipocytes with an average size of $70 \mu\text{m}$.¹⁸ With excess energy intake and subsequent obesity, adipocyte hyperplasia (increase in cell numbers, up to $9 \cdot 10^{10}$) or hypertrophy (increase in cell size, up to $120 \mu\text{m}$) can occur to various degrees; the former is considered a favorable response and the latter pathological.¹⁹⁻²² In health, subcutaneous adipose tissue (SAT) is the largest compartment and the vast majority of excess energy is stored there. It has been hypothesized that with persistent positive energy balances, the capacity of SAT will be reached and storage of free fatty acids (FFAs) will shift to ectopic sites, predominantly visceral adipose tissue (VAT).^{20,22,23} When SAT fails to store additional FFAs, adipocyte hypertrophy is typically observed; this may result from impaired adipocyte proliferation due to insufficient angiogenesis with ensuing hypoxia (Figure 1.1).^{19,20,24}

Ex vivo, hypertrophic adipocytes are capable of producing interleukin-6 (IL-6), IL-8, leptin, and tumor necrosis factor (TNF);²⁵ it has been suggested that this may lead to massive accumulation of macrophages in adipose tissue.^{19,24,26-29} While under lean conditions most adipose tissue macrophages display an anti-inflammatory or so-called "M2" phenotype, obesity induces polarization toward the inflammatory "M1" state.^{26,30} These M1-macrophages, in turn, produce even more inflammatory cytokines (e.g., TNF); this maintains local inflammation and may result in systemic low-grade inflammation (LGI), which contributes to insulin resistance.^{19,26,29-32}

Although many mechanisms in the adipose tissue of obese individuals have been proposed to trigger macrophage accumulation and polarization (e.g., adipocyte cell death, lipotoxicity by FFAs, increased insulin levels, hyperglycemia, and hypoxia^{19,33}), the exact driver of macrophage accumulation and activation has remained unclear. Recently, several studies in mice demonstrated that natural killer (NK) cells control macrophage accumulation and polarization via the production of interferon gamma (IFN γ), TNF or IL-6 in AT, which was associated with impaired insulin sensitivity and glucose handling.³³⁻³⁵ NK cells, which are innate lymphoid cells specialized in surveilling, recognizing and eliminating virally infected, transformed or stressed cells, are known to produce cytokines to shape an inflammatory environment.³⁶ Moreover, it has been shown that obesity is associated with an activated phenotype of NK cells in human adipose tissue.³⁷ However, neither NK cells' involvement in macrophage accumulation and polarization in adipose tissue, nor their contribution to ensuing insulin resistance has been investigated in humans.

Besides LGI, VAT and SAT may both contribute to insulin resistance by increased lipolysis, as well as adipokine dysregulation.³⁸⁻⁴⁰ Although these mechanisms may be present in SAT as well as VAT, they may contribute to insulin resistance to a different extent. This disparity may, at least in part, arise from SAT's higher ability to expand its capillary network compared to VAT.^{22,41} Hence, compared to SAT, VAT may be more

potent to contribute to insulin resistance through LGL, dysregulation of adipokine release, and lipolysis.^{22,38-42} Interestingly, although SAT is considered to contribute to insulin resistance independent from VAT,³⁸ a fat distribution pattern with high SAT and low VAT volumes is considered a metabolically healthy phenotype.⁴³

Figure 1.1 – Healthy and unhealthy adipose tissue expansion. (A) Healthy adipose tissue (AT) expansion consists of an enlargement of adipose tissue through effective recruitment of adipogenic precursor cells to the adipogenic program, along with an adequate angiogenic response and appropriate remodeling of the extracellular matrix. There are strong individual differences with respect to the potential for adipose tissue expansion. (B) In contrast, pathological adipose tissue expansion consists of massive enlargement of existing adipocytes (i.e., hypertrophy), limited angiogenesis, and ensuing hypoxia. As a result, hypoxia-inducible factor 1- α (HIF-1 α) is induced, which in turn can cause the induction of a fibrotic program. Ultimately, M1-stage macrophages prevail, leading to an inflammatory phenotype that is strongly associated with systemic insulin resistance. Reproduced from Sun et al., with permission.¹⁹

1.2.2. Intrahepatic lipids

The liver is a key regulator in glucose metabolism; it is able to maintain normoglycemia in a fasted state through glycogenolysis and gluconeogenesis, as well as store glucose in the fed state through glycogenesis.¹⁰ With prolonged periods of energy excess, liver biopsies may reveal ectopic fat storage in macrovesicular fat globules in hepatocytes, as well as hepatic injury, illustrated by formation of Mallory's

hyaline bodies in ballooned hepatocytes which may contain microvesicular fat.⁴⁴ The macrovesicular lipid pool in hepatocytes has a variable composition, which may include saturated fatty acids, diacylglycerols and ceramides, which are suggested to exert local lipotoxic effects.⁴⁵⁻⁴⁷ In liver biopsies, non-alcoholic fatty liver disease (NAFLD) is confirmed when $\geq 5\%$ of hepatocytes are fatty, in the absence of excess alcohol consumption or other conditions.⁴⁴ Over time, NAFLD may progress to non-alcoholic steatohepatitis (NASH) and cirrhosis, some will even develop hepatocellular carcinoma.^{48,49} Moreover, incidence of T2D and the risk of CVD appear to be higher in individuals with NAFLD, compared to those without.⁴⁸⁻⁵⁴ Therapies proven to be effective to treat NAFLD are weight loss interventions, pioglitazone, and vitamin E.^{55,56}

The gold standard to quantify IHL and diagnose NAFLD is by liver biopsy.⁴⁴ However, non-invasive imaging techniques like proton magnetic resonance spectroscopy (¹H-MRS) and other magnetic resonance imaging (MRI) sequences (e.g., modified Dixon (mDixon)) have been validated against liver biopsies and proven accurate to quantify IHL and diagnose NAFLD.⁵⁷⁻⁶⁰ ¹H-MRS is even considered to be the gold standard to diagnose NAFLD non-invasively,^{44,49,61} although mDixon is just as, or possibly even more, accurate (Supplementary Table S1.1).^{58,59,62} Of note, other techniques like computed tomography, ultrasound, or enzyme scores are inferior to these MRI-based techniques (Supplementary Table S1.1).^{59,63-66}

The contribution of IHL to insulin resistance is debated; it is unclear whether insulin resistance is a cause or a consequence of IHL. Lipotoxicity of IHL with a detrimental lipid composition, LGL, and stimulation of hypertriglyceridemia by the fatty liver are suggestive of a causative role of IHL in insulin resistance,^{45-47,49,67} whereas adipocyte insulin resistance and subsequent FFA-fluxes towards the liver suggest IHL is a consequence of insulin resistance.^{68,69} In addition, with increased IHL, hepatic glucose production is no longer suppressed (i.e., hepatic insulin resistance), which results in hyperglycemia.^{49,70} Nonetheless, some studies have suggested that IHL, not VAT, is associated with insulin resistance,^{71,72} whereas others argue in favor of mutually independent contributions of IHL and VAT.^{68,73} However, these were cross-sectional studies in some of which individuals were matched on one or more variables (e.g., VAT, IHL).^{71,72} Hence, studies that measure these potential contributors simultaneously in a random sample of the general population are required to determine whether or not they are mutually independent contributors to insulin resistance.

1.2.3. Microcirculation

In our circulation, arteries branch out into the microvasculature, which encompasses blood vessels $< 150 \mu\text{m}$ in diameter and includes arterioles, capillaries and venules.⁷⁴

In skeletal muscle, the primary site of insulin-induced glucose uptake, arterioles ultimately branch out to terminal arterioles, which are oriented perpendicular to muscle fibers and supply them at regular intervals (Figure 2.1). A terminal arteriole and its 12-20 capillaries form the smallest unit of control for capillary perfusion;⁷⁵⁻⁷⁹ venules are oriented similar to the arterioles and are found between two terminal arterioles. As capillaries are embedded in sarcolemma, the surface area for nutrient exchange is further increased.^{80,81} Muscle's vasculature is capable of regulating blood flow and nutrient exchange; at the level of the terminal arterioles, endothelial surface area can be increased through vasodilation and vasomotion. Skeletal muscle's architecture, regulation of vasomotor tone, mechanisms of vasomotion, transendothelial nutrient fluxes, and the effects of exercise and insulin are described in more detail in *chapter two*. Nonetheless, some key intracellular mechanisms and regulatory mechanisms are summarized below.

The inner cellular lining of the vasculature, the endothelium, is a key regulator not only in vasomotor tone, vasomotion and subsequent perfusion, but also in blood cell trafficking, permeability, proliferation, and hemostasis.⁸² The endothelium is vastly heterogeneous along the vascular tree, as well as per organ.^{82,83} In skeletal muscle, the endothelium is continuous and non-fenestrated,⁸² which is why endothelial cells orchestrate nutrient transport to myocytes. In a hyperinsulinemic state, insulin binds to the insulin receptor on the endothelial surface, which results in activation of both vasodilator (through insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase-1 (PDK-1), protein kinase B (Akt), and endothelial nitric oxide synthase (eNOS), which results in nitric oxide (NO) release) and vasoconstrictor pathways (through mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase-1/2 (ERK1/2), which results in endothelin-1 (ET-1) production).⁸⁴⁻⁹² Since the vasodilator pathway predominates in health, a net vasodilation ensues and, hence, hypoperfused capillaries and microvascular units are recruited (i.e., microvascular recruitment (MVR)).^{84,93-99} In obesity, endothelial dysfunction develops; through inflammation (e.g., TNF), FFA, angiotensin-II and oxidative stress, the vasodilator pathway is inhibited, which may blunt insulin-induced vasodilation or even result in paradoxical vasoconstriction (Figure 1.2).^{79,84,85,94,97-110} In addition, insulin is transported across the endothelium in an NO-dependent manner via caveolae, after which myocytes are stimulated by said insulin to transport GLUT4 to the plasma membrane.^{95,111-113} Although its activity in muscle's endothelium has not been studied, the GLUT1 transporter, which has a high capacity for glucose transport even in the absence of excess insulin, is assumed to be involved in transendothelial glucose transport in skeletal muscle, similar to human cardiac tissue.¹¹⁴

Figure 1.2 – Insulin signaling pathways in the endothelium for the production of nitric oxide (NO) and endothelin-1 (ET-1). In healthy individuals, hyperinsulinemia results in a net vasodilation, whereas in obesity, this response is either blunted or paradoxical vasoconstriction may occur. Several circulating and paracrine factors are involved: angiotensin II (AngII), tumor necrosis factor (TNF), and free fatty acid (FFA) inhibit the phosphatidylinositol 3-kinase (PI3K) pathway and stimulate the mitogen-activated protein kinase (MAPK) pathway via a crosstalk at multiple levels. TNF and AngII can inhibit IRS-1 through c-Jun N-terminal kinase (JNK). AngII can bind to the angiotensin type 1 receptor (AT₁R) and inhibit IRS-1 and lead to the production of reactive oxygen species (ROS), which reduces NO bioavailability. FFA can activate protein kinase C theta (PKC-θ), which results in activation of the mitogen-activated protein kinase (MAPK) signaling pathway as well as in inhibition of PI3K. Adiponectin can stimulate 5'-adenosine monophosphate-activated protein kinase (AMPK), which stimulates eNOS and inhibits the MAPK pathway; in obesity, adiponectin levels are decreased. Graphical design by Dr. van Varik.

In addition to MVR, muscle perfusion may also be regulated through vasomotion, i.e., rhythmic vessel diameter oscillations.^{102,115} It has been demonstrated that upon increased metabolic needs, signals originating from the microvasculature travel upstream to larger arterioles and arteries, thereby influencing vessel diameter.¹¹⁶ In addition, stimulation of individual capillaries can trigger vasomotor responses in arterioles.¹¹⁶⁻¹¹⁸ Moreover, signaling from endothelial to smooth muscle cells at myoendothelial junctions can be regulated by NO; the duration of conducted vasodilation is attenuated by reduced NO production.^{116,119} Collectively, these findings suggest that muscle microcirculation can modulate upstream vasodilation and vasomotion. Interestingly, vasomotion may regulate microvascular blood flow

distribution to optimize delivery of nutrients, since continuously altering blood flow delivery to vessels over time increases the number of endothelial cells exposed to plasma for nutrient or hormone exchange¹²⁰ without total flow or cardiac output being changed.¹⁰²

A captivating potential regulator of insulin's vascular actions is perivascular adipose tissue (PVAT) in muscle's microcirculation.¹²¹⁻¹²³ Since adipocytes in PVAT are located in the vicinity of microvessels, adipokines released from PVAT are suggested to have paracrine effects on the microvasculature, including its vasomotor tone.^{103,121,123,124} In rodents, it has been observed that 1) with a high-fat diet, PVAT was infiltrated by bone marrow-derived immune cells and had become inflammatory;¹²⁵ 2) arteries with excess PVAT, in contrast to those with little to none, displayed endothelial dysfunction when on a high-fat diet;¹²⁶ 3) PVAT from obese mice was shown to impair insulin-induced vasodilation in arterioles from lean mice;¹²⁶ 4) PVAT from lean mice could not restore insulin-induced vasodilation in arterioles from obese mice;¹²⁵ and 5) by inhibition of inflammation in obese PVAT, its vasodilator properties were restored, possibly via secretion of adiponectin and stimulation of AMPK α 2, which resulted in NO-production and inhibition of the MAPK-pathway.^{126,127} Limited data is available in humans, although observations in humans are in line with those in rodents.^{122,128} Additional studies are needed to address PVAT's contribution to insulin resistance in humans.

Of note, the contributions of impaired MVR or vasomotion responses to obesity-induced insulin resistance have never been studied in parallel with VAT, SAT, or IHL. Moreover, to the best of our knowledge, no intervention study had evaluated whether MVR can be improved by weight loss, nor assessed the contribution of changes in MVR to changes in insulin resistance.

1.3. Beta-cell function

In the evolution of obesity towards T2D, progressive beta-cell dysfunction is a crucial process. Initially, beta-cell function (BCF) typically adapts to progressive insulin resistance in obesity; when BCF adaptation is inadequate, hyperglycemia and, hence, T2D develop.¹²⁹⁻¹³¹

In glucose-tolerant obese individuals, the presence of insulin resistance induces upregulation of static beta-cell responses such as basal insulin secretion and total stimulated insulin secretion; this can be interpreted as an adjustment of the set-point of BCF by insulin resistance.¹³² With progressive hyperglycemia, static beta-cell

responses may, possibly due to the toxic effect of chronic hyperglycemia, ultimately fail^{132,133} and beta-cell dedifferentiation¹³⁴⁻¹³⁸ and loss of beta-cell mass may develop.^{131,139,140}

Another important aspect of BCF is the ability to appropriately increase the insulin secretion rate (ISR) to cope with acute changes in plasma glucose concentration that results from nutrient intake. The response of beta-cells to hyperglycemia is largely explained by a number of dynamic BCF properties, as described in more detail in *chapters three and six* (e.g., Supplementary Figure S6.1), as well as elsewhere.^{129,132,141-145} Briefly, in response to ingestion of a meal, ISR can be measured and mathematically modeled. From this model, beta-cell glucose sensitivity (β -GS) and its modulation by beta-cell potentiation (the effects of glucose itself and non-glucose secretagogues to magnify the beta-cell response to glucose over time), as well as rapid first phase insulin release (i.e., beta-cell rate sensitivity (β -RS)) can be measured.^{141,142,145}

1.3.1. Beta-cell function in obesity and effects of weight loss

As hyperglycemia normally develops gradually over time, it is conceivable that BCF may already, to some extent, inadequately adapt to the level of insulin resistance in obese non-diabetic individuals. Inadequate obesity-induced BCF alterations, their reversibility, and the drivers thereof are, however, largely unknown.

Inconsistent results have arisen from cross-sectional studies which evaluated dynamic BCF in obese, non-diabetic individuals. In some studies, both β -GS and potentiation factor ratio (PFR; a ratio between late and early potentiation) were decreased in obese individuals with normal¹⁴⁶ or impaired glucose tolerance.^{130,142,146-149} Yet, in other studies in obese individuals with impaired glucose tolerance, an increase in β -GS or PFR, or a tendency thereto, was observed.¹⁴⁸⁻¹⁵¹ These contradictory results may have arisen from the use of an oral glucose tolerance test (OGTT) by some,^{130,142,146-149} and a mixed meal test (MMT) by others.^{145,148,149,151} Nonetheless, in these studies BCF was often not sufficiently adjusted for the level of insulin resistance.

Several non-controlled trials have studied the effects of weight loss, by either low-calorie diet or bariatric surgery, on various aspects of BCF in obese individuals. In (morbidly) obese individuals who underwent bariatric surgery, β -GS and PFR were unchanged two years post-surgery, while β -GS was decreased one year post surgery, although insulin resistance was markedly improved at all time points in all studies.^{150,151} To our knowledge, only one RCT in obese individuals has studied the effects of dietary weight loss, which demonstrated that BCF was unchanged, albeit in the presence of markedly improved insulin resistance.⁵⁵

1.3.2. Intrapancreatic lipids and beta-cell function

Ectopic fat deposition in the pancreas is one of the proposed contributors to beta-cell dysfunction.¹⁵²⁻¹⁵⁴ In postmortem studies, obese individuals had more intrapancreatic lipid (IPL) compared to lean individuals;¹⁵⁵⁻¹⁵⁷ most fat is present in adipocytes that are primarily located in the interlobular and intralobular spaces.¹⁵⁸⁻¹⁶⁰ Alternatively, triglycerides might accumulate in islets and disrupt intracellular processes involved in insulin secretion, as demonstrated in rodents.^{161,162} Nonetheless, IPL is suggested to arise from prolonged elevation in plasma VLDL due to increased hepatic VLDL production in NAFLD, which eventually leads to increased exposure of beta-cells to FFA and impaired BCF.^{49,154,161,163,164} Moreover, NAFLD may induce beta-cell dysfunction via fetuin-A and beta-cell apoptosis via palmitate.¹⁶⁵ Furthermore, an increased number of adipocytes located near Langerhans' islets was associated with an increased number of macrophages within islets,¹⁶⁵ suggestive of a link between local inflammation and BCF.¹⁶⁶ Another potential contributor to obesity-induced beta-cell dysfunction is endothelial dysfunction of pancreas' microvasculature. Interestingly, in rodents, hyperglycemia was able to increase islet blood flow twofold;¹⁶⁷ whether this is insulin-induced and NO-dependent is unknown. Other mechanisms involved in the interaction between endothelial and beta-cells are described into more detail below (Figure 1.3) and elsewhere.¹⁶⁸

Data on the association between IPL and BCF is, however, ambiguous. In obesity, an association between IPL and some, but not all, aspects of BCF was observed, albeit not consistently.^{153,154,169-172} Moreover, in individuals with T2D, no association between IPL and BCF could be detected.¹⁵⁴ The discrepancy in IPL's association with BCF between obese individuals on the one hand and those with T2D on the other, may be due to differences in the localization of intrapancreatic fat depositions, as well as the association between IPL and diabetes duration.^{158,173} Alternatively, IPL may be a more important contributor to early beta-cell dysfunction in obese individuals than with progressive hyperglycemia or T2D.

In non-controlled studies in obese individuals, weight loss was associated with reduced IPL,^{174,175} but not with improved BCF.¹⁷⁵ Data from randomized controlled weight loss trials on both BCF and IPL in obese individuals have, to the best of our knowledge, not been reported.

Figure 1.3 - Diagram summarizing the main factors regulating the islet beta-cell–endothelial cell axis. Islet endothelial cells produce factors that enhance beta-cell proliferation, survival and function under physiological conditions, including hepatocyte growth factor (HGF), fibroblast growth factor (FGF), connective tissue growth factor (CTGF) and thrombospondin-1 (THBS1). Additional islet endothelial cell-secreted factors modulate beta-cell function; for example, endothelin-1 increases insulin release from isolated mouse islets, possibly by triggering calcium influx into beta-cells. Extracellular matrix (ECM) molecules (e.g., fibronectin, collagens, laminins) also affect insulin expression and release from beta-cells, partly via integrins (heterodimeric cell-surface receptors with broad specificity for ECM molecules) and activation of downstream signaling pathways, including focal adhesion kinase (FAK). E-cadherin and connexins govern cell–matrix interaction and cell–cell interactions, respectively, and thus may also mediate islet endothelial–beta-cell crosstalk. Vice versa, beta-cell-secreted factors can also impact islet endothelial cell function. For example, beta-cell-derived insulin binds to the insulin receptor on endothelial cells, resulting in downstream activation of PI3K and Akt and subsequent phosphorylation (p) and activation of eNOS, leading to the production of vasodilatory NO. Furthermore, vascular endothelial growth factor (VEGF), required for maintenance of endothelial fenestration and cell viability, is produced by islet beta-cells and is a major source of VEGF in the islet. Reprinted from Hogan et al., with permission.¹⁶⁸

1.4. Vascular function

As mentioned before, obesity is associated with an increased risk for CVD; moreover, vascular dysfunction has been implicated to contribute to CVD.^{82,176-188} Although microvascular responses like MVR and vasomotion are also (NO-dependent) vascular function tests (*paragraph 1.2.3*), we aim to briefly introduce other commonly used vascular function tests in this paragraph. Their methodology is addressed in *chapters three and seven*.

Since a healthy vasculature encompasses many physiological functions (e.g., NO production, cushioning of blood flow pulsations, barrier function, regulation of hemostasis, prevention of atherosclerosis, and maintenance of vascular structure and capillary density), virtually all of these functions may be affected to some degree by obesity. Over the years, many different tests have been developed to detect impairments in vascular function.

Flow-mediated dilation is a functional marker of endothelial function, as it aims to assess the capacity of the endothelium to induce vasodilation via NO production.¹⁸⁹ Besides its vasodilatory effects, NO also has anti-inflammatory, anti-adhesive and antithrombotic effects.^{94,190} Moreover, it has been demonstrated that impaired flow-mediated dilation is associated with an increased risk of incident CVD.¹⁹¹⁻¹⁹³

Endothelial function can also be assessed by measurements like peripheral arterial tonometry, which measures NO-dependent digital pulse volume amplitude during hyperemia,¹⁹⁴ or plasma biomarkers of endothelial dysfunction.^{195,196} These plasma biomarkers are molecules that are either expressed by endothelial cells and are shed into the circulation in their soluble form via proteolysis (e.g., vascular cell adhesion molecule 1, intercellular adhesion molecule 1, endothelial selectin) or released into the circulation upon injury of the vascular wall (e.g., von Willebrand factor).^{82,83,178-180,197} Increased levels of these plasma biomarkers of endothelial dysfunction are also associated with an increased risk of incident CVD.¹⁸¹⁻¹⁸⁶

Obesity, hyperglycemia, and hypertension may lead to structural remodeling of the arterial wall, for example by replacement of elastin fibers with collagen fibers and cross-linking by advanced glycation end-products,¹⁹⁸⁻²⁰⁰ which results in arterial stiffening. In turn, arterial stiffness results in impaired cushioning function of arteries, increased pressure and flow pulsatility, and transmission of pulsatile load to the microcirculation.²⁰¹⁻²⁰³ On the one hand, this may increase (systolic) blood pressure and decrease coronary perfusion;^{201,203} unsurprisingly, arterial stiffness is associated with incident CVD.^{187,188} On the other hand, the increased pulsatile load toward the microcirculation, like the retinal arterioles, is particularly harmful.²⁰¹⁻²⁰⁴ It has been demonstrated that unfavorable retinal arteriolar and venular calibers, i.e. wider venules and narrower arterioles, were associated with incident CVD.^{205,206}

Importantly, it should be noted that two distinctive objectives to use vascular function measurements can be differentiated in clinical trials: as a surrogate for cardiovascular end points and to evaluate certain properties of the vasculature and identify causal mechanisms. Although we used vascular function for both purposes in this thesis, it should be noted that the use of surrogate outcomes have been frequently criticized.²⁰⁷⁻²¹³ In *Appendix 1.1*, the assumptions, limitations and potential of

surrogate outcomes are further discussed. Nonetheless, investigating the effects of obesity and weight loss may provide valuable insights. Moreover, to our knowledge, the effects of obesity on the vascular function measurements mentioned before and in *chapter three*, have not been assessed in a single study, nor has an RCT on the effects of weight loss on these vascular functions been performed.

1.5. Missing links

Previous studies have investigated whether VAT,^{38,42,68,71-73,214} SAT,^{38,42,215,216} IHL,^{68,71-73,214} or MVR^{98,128,217} contribute to obesity-induced peripheral insulin resistance. Several studies demonstrated that SAT's association with insulin resistance was independent of VAT;^{38,42} some studies argue in favor of mutually independent contributions of VAT and IHL,^{68,73,214} whereas others find that IHL, not VAT, is associated with insulin resistance.^{71,72} For MVR, it has never been shown, neither in muscle nor in skin, that its effect on obesity-induced insulin resistance is independent from other potential contributors. Moreover, although the beneficial effect of weight loss on insulin resistance is well known,^{55,218} it has not been investigated what drives this improvement. Nonetheless, it is crucial to identify whether VAT, SAT, IHL, and MVR act independently to increase obesity-induced insulin resistance, as mutual independence, in contrast to mutual dependence, implies that distinct mechanisms can be targeted to improve insulin resistance.

If VAT were to be an independent contributor to obesity-induced insulin resistance, which is generally accepted in literature,^{22,38-42,68,73} it is valuable to identify the mechanisms through which VAT can exert this effect. Previous studies have demonstrated that VAT can contribute to insulin resistance through increased lipolysis, adipokine dysregulation, and LGI.³⁸⁻⁴⁰ The low-grade inflammatory response in VAT has been intensively studied and signals an important role for macrophage accumulation and inflammatory polarization.^{19,24,26-32} What drives accumulation and inflammatory polarization of macrophages in human VAT is still open for debate. In rodents, infiltration of and cytokine release by NK cells in VAT in response to adipocyte stress have recently been demonstrated to drive macrophage infiltration and polarization.³³⁻³⁵ In humans, however, the role of NK cells in VAT has never been studied.

Beta-cell failure is a *sine qua non* for hyperglycemia and T2D to develop, yet some obese individuals never become hyperglycemic.¹²⁹⁻¹³¹ It may be of great interest to detect early signs of inadequate beta-cell responses to hyperglycemia and identify its drivers, as it could not only lead to identification of individuals at risk for T2D, but also

identify potential targets for treatment. Several studies have investigated whether obesity-induced changes in BCF could be detected^{129,130,142,146-151} and demonstrated that at least some of them were associated with progression to T2D.^{129,130} IPL has been implicated by some to be associated with BCF,^{152-154,163} yet conflicting results have arisen.^{154,169-172} Moreover, the effects of weight loss on BCF and IPL have never been studied concurrently in an RCT, although non-controlled studies found that both BCF^{150,151,175,219} as well as IPL^{152,173-175} were associated with weight loss, possibly in parallel.¹⁷⁵

Finally, it has been generally accepted that a weight loss intervention, either through diet, exercise or bariatric surgery, would be beneficial for vascular function,²²⁰ prevent incident T2D,^{221,222} and curate individuals from T2D.²²³⁻²²⁶ There is substantial evidence to support the latter two hypotheses,²²¹⁻²²⁶ yet only few RCTs have evaluated the effect of weight loss on vascular function.^{227,228} In addition, those studies investigated macrovascular function by means of FMD or cfPWV.^{227,228} Nonetheless, it may be equally valuable to evaluate the effect of a weight loss intervention on microvascular health and function. Not only in the light of insulin resistance, but also since many complications that may develop in T2D arise from and occur in the microcirculation.^{229,230} If weight loss would be able to improve macro- and microvascular function, this might be a mechanism through which incident T2D or its macro- and microvascular complications could be averted.²³¹⁻²³⁵ The effects of a weight loss intervention on microvascular function in particular has, however, not been evaluated in an RCT. Moreover, most RCTs that investigated effects on FMD or cfPWV were performed in participants with obesity-related morbidities.^{227,236,237} Also, physical exercise^{227,236} or weight-loss medication²³⁷ was part of the weight-loss regimens. Hence, the effects of a weight loss intervention by caloric restriction on micro- and most of macrovascular function remains to be demonstrated.

1.6. Outline of this thesis

In *chapter two*, we reviewed the distinctive architecture and properties of muscle's vasculature and described the hierarchical, graded control of muscle perfusion that arises from cooperative interactions among the factors released locally by myocytes, by smooth muscle cells, or by endothelial cells along with systemically and neurally delivered signals. Separately, in *Appendix 2.1*, we present a commentary that addressed capillary recruitment in skin.

In *chapter three*, we assessed the reliability and measurement error of several anthropometric, vascular, metabolic, and inflammatory measurements used

throughout this thesis, as well as describe the methodology of these measurements. The rationale for this study is described in to more detail in the methodological considerations in *Appendix 1.1*.

In *chapter four*, we investigated, in an RCT with a weight loss intervention, the mutually independent contributions of VAT, SAT, IHL and MVR to obesity-associated insulin resistance. Moreover, we investigated whether MVR can be improved by a weight loss intervention and assessed the contributions of weight-loss intervention-induced changes in VAT, SAT, IHL, and MVR on improvements in insulin sensitivity. We used state-of-the-art multiple mediation analyses to provide a deeper understanding of the mutually independence of these variables; the rationale for this statistical method is described in to more detail in the methodological considerations in *Appendix 1.1*.

In *chapter five*, we investigated the presence of NK cells in human VAT, their association with macrophage polarization, and their effects on LGI and insulin resistance in four separate studies. In the first study, NK cells were quantified and linked to macrophage polarization in biopsies from VAT and SAT; moreover, a circulating biomarker for VAT NK cells is presented. In the second study, we investigated whether NK cells from obese individuals directly affect macrophage polarization; hence, NK cells were isolated from peripheral blood and co-cultured with human monocyte-derived macrophages. In the third study, the cross-sectional analyses from the weight loss intervention trial, the associations of VAT NK cells to LGI and subsequent insulin resistance were investigated. A fourth study, the Maastricht Study, was used for validation of these associations in a population-based cohort study.

In *chapter six*, we investigated, in an RCT with a weight loss intervention, the obesity-associated alterations in BCF, and explored IPL as a potential contributor thereof. Moreover, we investigated whether a weight loss intervention could improve BCF and explored whether a decrease in IPL drove improvements in BCF.

In *chapter seven*, we investigated, in an RCT with a weight loss intervention, the obesity-associated alterations in macro- (FMD, cfPWV, peripheral arterial tonometry, and central augmentation index) and microvascular function (retinal vascular diameters), as well as circulating biomarkers of endothelial dysfunction and LGI. In addition, we investigated whether a weight-loss intervention could improve macro- and microvascular function and reduce levels of circulating biomarkers of endothelial dysfunction and LGI.

Finally, in *chapter eight*, we discussed the key findings of this thesis and their clinical implications, highlighted methodological considerations, and identified hypotheses worthwhile to investigate in future research.

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Supplementary material

Table S1.1 – Comparison of various modalities to diagnose NAFLD and quantify IHL

	Biopsy validated	¹ H-MRS validated	Continuous variable	Different. NASH	Small clinical trials	Large (cohort) studies	Post- hoc	Burden	Costs
Liver biopsy	N/A	N/A	+	++	+/-	-	-	++	€€€€
¹ H-MRS	++	N/A	+	-	++	+/-	-	+/-	€€€
mDixon MRI	++	++	+	-	++	+	-	+/-	€€
CT	+/-	+/-	+/-	-	-	-	-	+	€€
Ultrasound	++	++	+/-	+/-	+/-	+	-	-	€/€€
Enzyme score	-	+	+	-	+/-	++	+	-	€

Techniques are rated whether they are validated against liver biopsies and ¹H-MRS, express IHL as a continuous variable, can differentiate NASH from NAFLD, are suited for small clinical trials and large (cohort) studies and can be assessed after the study is completed (i.e., post-hoc), as well as the burden for study participants and the costs. ¹H-MRS ($r = 0.70 - 0.93$, $p < 0.001$, Cohen's kappa $0.66 - 0.75$; sensitivity 80%, specificity 80%; derived from⁵⁷⁻⁶⁰), mDixon ($r = 0.94$, $p < 0.001$; sensitivity 77%, specificity 87%; derived from^{58,59}) and ultrasound (sensitivity 85%, which decreased with increased BMI, specificity 94%, ROC 0.93, $p < 0.001$; as published in⁶³) are well validated against liver biopsies; CT was validated but performed rather poorly (sensitivity 11-50%, specificity 77-100%, accuracy decreased with increased BMI; from^{59,64,65}) and enzyme scores were not biopsy validated.⁶⁶ MDixon ($r = 0.90-0.99$; ICC = $0.82-0.98$; Cohen's kappa $0.79-0.90$; $p < 0.001$; derived from^{58,62,238}) and ultrasound^{59,63} are well validated against ¹H-MRS; the enzyme score was validated in a single cohort only (sensitivity 86%, specificity 71%, ROC 0.86; from⁶⁶), whereas CT was validated but performed rather poorly.⁵⁹ Liver biopsies, ¹H-MRS, mDixon and enzyme score express IHL as a continuous variable, whereas CT (Hounsfield units) and ultrasound (e.g., liver-to-kidney contrast) can be reported as a continuous variable that serves as a proxy for IHL. Only liver biopsies can reliably diagnose NASH, yet elastography (e.g., transient elastography; $r = 0.74$, $p < 0.001$; ROC 0.62-0.71 for presence of NASH; from²³⁹) can provide some information on NASH status as it measures the stiffness of liver parenchyma as a proxy for fibrosis. For small clinical trials, ¹H-MRS or mDixon are preferred to assess IHL, although sometimes liver biopsies could be warranted to provide additional information on presence or severity of NASH; CT, ultrasound and enzyme scores are deemed inferior to MRI-based techniques. For large (cohort) studies, costs, burden and time consumption often outweigh accuracy. Hence, enzyme scores, ultrasound and mDixon are preferred over biopsies and ¹H-MRS (which can still be a valid option depending on a facility's infrastructure). We consider CT unfit for large (cohort) studies due to its inaccuracy, radiation exposure and costs. The enzyme score is the only test that can be performed upon completion of a study as it only requires stored serum. The burden for participants is highest for a liver biopsy, still rather high for CT because of radiation exposure, and doubtful for MRI-based techniques because of potential claustrophobic responses and noise. Costs of each technique may vary per institution, yet often biopsies are rather expensive, ¹H-MRS is more expensive than mDixon due to longer acquisition time, and CT and ultrasound are still more expensive than laboratory tests on serum. N/A is noted when not applicable.

Appendix 1.1 – Methodological considerations

Renown and recently deceased Dr. Altman once argued that it is, unfortunately, common in research to, either willfully or in ignorance, use the wrong techniques, use the right techniques wrongly, misinterpret their results, report results selectively, cite the literature selectively, and draw unjustified conclusions.²⁴⁰⁻²⁴³ He went on to state that huge amounts of money are wasted on research that is seriously flawed through the use of inappropriate designs, unrepresentative samples, small samples, incorrect methods of analysis, and faulty interpretation;²⁴⁰ the tremendous amount of money wasted was later even quantified by others.²⁴⁴ Moreover, Dr. Altman and others argued that although many journals lack statistical reviewers, they still advance to publish misleading research.^{240,241} In spite of this grim assessment of medical science, it would indeed be valuable to have quality assurance built in at the beginning of research, not the end.²⁴¹

Several quality assessments, including analysis of reliability and responsiveness of measurements, can reduce bias and be performed before a clinical trial is conducted. Moreover, more advanced statistical methods, like multiple mediation analyses, can be used to address multiple competing hypothesis in a single model. Such quality assessments and statistical analysis, as briefly introduced below, are unfortunately also infrequently reported, reported in the wrong way, or disregarded altogether (*chapter three*).²⁴⁵⁻²⁵⁰ Finally, it is valuable to recognize that many outcomes in clinical studies are in fact surrogate outcomes; this has implications for the interpretation of data and treatment strategies. Here, we address reliability and responsiveness of measurements, multiple mediation analysis, and surrogate outcomes.

Reliability

An essential requirement of all measurements in patient care and research is that they are reliable. Reliability is defined as ‘the extent to which scores for patients who have not changed are the same for repeated measurement under several conditions, e.g. over time, on different occasions by the same observer (intra-observer), or on the same occasion by different observers (inter-observer).’^{246,251} The domain reliability, as defined in the COSMIN taxonomy, is divided into the measurement properties measurement error and, again, reliability.^{246,251} It is crucial to understand the difference between measurement error and reliability.

Parameters of measurement error relate to the concept agreement, i.e. the absolute difference between repeated measurements, and can be expressed as standard error of the measurement (SEM) and the limits of agreement (Bland and Altman method), as described in to more detail in *chapter three* and elsewhere.^{246,251,252} In brief, the

SEM can be interpreted as the standard deviation around a single measurement (e.g., when BMI is assessed 100 consecutive times, the SD of these measurements equals the SEM).²⁴⁶ For the limits of agreement, often visualized in Bland-Altman plots,²⁵² the difference between repeated measurements is calculated and a normal distribution of the difference scores is assumed. The limits of agreement represent the mean difference between measurements ± 1.96 SD; hence, 95% of the difference scores will fall between the limits of agreement.^{246,252}

Reliability parameters on the other hand, like intraclass correlation coefficients (ICC), which range in value from 0 (totally unreliable) to 1 (perfect reliability), relate the random error variance to the variance among subjects in a population.^{246,253-256} Consequently, a parameter of reliability is in fact only valid for the population it is measured in and the observers it is measured by.

Responsiveness

To evaluate changes in health status over time, measurement instruments should be able to detect these changes; i.e., the measurement instrument should be responsive.^{246,251} To evaluate responsiveness of a measurement instrument, a study design should be chosen in which it is reasonable to expect that (a proportion of the) individuals would change on the construct under investigation. Otherwise, if the measurement does not change over time, it would be problematic to decide whether the subjects did not change, or whether the measurement instrument was not responsive.²⁴⁶ Clearly, responsiveness is only relevant for measurement instruments used in evaluative applications; if an instrument is only used for discriminating between patients at one point in time, responsiveness is not an issue.^{246,251}

When a gold standard for change is available, the changes in the measurement instrument under investigation can be compared to changes in scores of the gold standard.²⁴⁶ If no gold standard is available, hypotheses are tested to assess responsiveness. For example, it could be hypothesized that when a measurement instrument is responsive, changes in the measurement instrument would be correlated to changes in scores on other instruments known to have adequate responsiveness.²⁴⁶

Multiple mediation

The use of multiple mediation analyses, compared to multivariate regression, can provide a superior reflection of the causal chain one tries to investigate. This helps unravel contributions to disease etiology and to develop targeted, effective therapies.²⁵⁷ Specifically, the advantage of testing the mediating effect of multiple

variables simultaneously is that one disentangles whether or not mediation is independent of the effect of the other mediators, which offers the possibility to compare competing theories against one another within a single model.²⁵⁸⁻²⁶⁰

In addition, particularly when potential mediators have little random error (i.e., the measurement is precise and reflects the underlying mechanism well), multiple mediation analyses can be used to estimate the proportion of the total effect that independent mediators account for. A mediated proportion is essentially the product of two effects: the regression coefficient of the association between determinant and mediator on one hand, and the regression coefficient of the association between mediator and outcome as assessed by multivariate regression analysis (i.e., with all mediators and the determinant in a single regression model) on the other.²⁶⁰ As multiple mediator models are in fact the integrated product of two multivariate regression models, they have a clear advantage over the conventional approach of analyzing each effect with separate regression analyses.

Over time, several modified multiple mediation analyses have been developed, like serial multiple mediation, repeated measures multiple mediation, and a combination of both.²⁵⁹⁻²⁶¹ The serial multiple mediator model is able to assess mediation when it is assumed that one mediator affects another.²⁶⁰ This way, pathways in which each mediator is presumed to affect other mediators causally downstream, can be investigated as well as the proportion of each pathway of the total effect quantified.^{260,261} Repeated measures multiple mediation can be used when data from repeated measurement of the same people on variables in the mediation process is obtained, as in an RCT.²⁶¹ This is considered a valuable addition to regular multiple mediation analyses where determinant, mediator, and outcome are measured at the same moment in time. Recently, a statistical method that combines serial and repeated measures multiple mediation has been developed;²⁶¹ this allows for the assessment of multiple serial pathways in an RCT, for example.

Surrogate outcomes

Although frequently criticized,²⁰⁷⁻²¹³ surrogate outcomes are widely used in research²⁶²⁻²⁶⁶ and new drugs are even commonly approved based on their effect on a surrogate outcome, in the absence of an effect on relevant end points.^{207,211,213,267} Moreover, as end points show substantially smaller responses to interventions than surrogate outcomes,²¹² many of the widely accepted strategies (e.g., for T2D) may be based on artificially inflated expectations.²⁰⁷ In addition, the use and limitations of surrogate outcomes are greatly underreported in RCTs.²¹⁰

The acceptance of surrogate outcomes for clinical end points like CVD or mortality is, at least in part, due to the high costs, requisition of a large sample size, and increased follow-up duration of RCTs that investigate relevant cardiovascular end points; surrogate outcomes are, on the other hand, cheaper and easier to measure.²⁰⁷ In addition, it has been argued that innovation is delayed or obstructed if surrogate outcomes were to be disregarded. Nonetheless, surrogate outcomes should, preferably strongly, be associated with the incidence of relevant end points, as well as be involved in the causal pathway of the relevant end point. More importantly, surrogate outcomes are not intrinsically flawed; they can be helpful in treatment as well as in risk stratification, when interpreted appropriately.²⁰⁷

chapter

2

Chapter 2

Muscle microvasculature's structural and functional specializations facilitate muscle metabolism

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Abstract

We review the evolving findings from studies that examine the relationship between the structural and functional properties of skeletal muscle's vasculature and muscle metabolism. Unique aspects of the organization of the muscle microvasculature are highlighted. We discuss the role of vasomotion at the microscopic level and of flowmotion at the tissue level as modulators of perfusion distribution in muscle. We then consider in some detail how insulin and exercise each modulate muscle perfusion at both the microvascular and whole tissue level. The central role of the vascular endothelial cell in modulating both perfusion and transendothelial insulin and nutrient transport is also reviewed. The relationship between muscle metabolic insulin resistance and the vascular action of insulin in muscle continues to indicate an important role for the microvasculature as a target for insulin action and that impairing insulin's microvascular action significantly affects body glucose metabolism.

Introduction

Muscle's microvasculature is the final interface through which circulating nutrients, hormones, gases, and electrolytes must pass in journeying to and from the systemic circulation. It has evolved multiple structural and functional adaptations to flexibly and efficiently fulfill its role in optimizing muscle function. Here, we examine how the minute-to-minute metabolic activity of skeletal muscle is coupled to the function of muscle's vasculature. We will discuss how bulk blood flow and flow distribution can regulate nutrient delivery to muscle microvasculature as well as how transendothelial transport processes can modulate the exchange of hormones and metabolites between plasma and the myocytes. We focus on acute regulatory relationships and defer discussion of chronic vascular or myocyte adaptation to environmental, nutritional, and most pathological processes.

Muscle blood flow has been measured in numerous classical limb balance and metabolic tracer studies of the acute effects of fasting, feeding, exercise, and hormonal manipulation on muscle nutrient exchange. These methods, however, treat muscle's vasculature as a "black box." Here, we consider muscle microvasculature's specialized architecture and how that architecture might impact nutrient fluxes into and out of the muscle. We will discuss the role of vasomotion and flowmotion in the regulation of microvascular perfusion. We recognize that factors acting on the endothelial cell (EC), the vascular smooth muscle cell (SMC), or pericytes may affect muscle perfusion. We will consider in more detail the effect insulin and exercise have on the endothelium and directly or indirectly on the vascular SMC to influence nutrient delivery.

Beyond muscle perfusion, we will consider the transit/transport of fatty acids, glucose, and insulin across the vascular endothelium of muscle, as these processes offer another pathway whereby the vasculature may regulate myocyte metabolic function. In aggregate, this review will delineate how vascular regulation of blood flow, flow distribution, and endothelial function coordinate to optimally serve myocyte function.

Skeletal muscle architecture

Pioneering work by Spalteholz¹ and Krogh^{2,3} has demonstrated muscle's highly organized vasculature. Arterioles branch off primary arteries, down to terminal arterioles, oriented perpendicular to muscle fibers, and supplying them at regular intervals (1 mm). Each terminal arteriole supplies 15 - 20 capillaries running parallel to

fibers, with many anastomoses, to form a rich network around muscle fibers. Each terminal arteriole with its capillaries forms the smallest unit of control for capillary perfusion (referred to as a microvascular unit).⁴ Venules are arrayed as arterioles and found between two terminal arterioles. Lymph vessels originate within muscle and can ascend with arterioles or venules (Figure 2.1).^{5,6}

Figure 2.1 – Schematic representation of muscle's vascular architecture. **Left:** Arterioles branch off primary arteries, down to terminal arterioles, oriented perpendicular to muscle fibers. **Top right:** Two muscle fibers and their microvascular units, with terminal arterioles as smallest unit of control and lymph vessels (green) ascending along arterioles and venules. In resting muscle (top fiber), the microvasculature is intermittently and not equally perfused, whereas in insulin or exercise stimulated muscle (bottom fiber), vasodilation occurs at the level of the terminal arterioles and control of perfusion shifts upstream. **Bottom right:** Magnification of the capillary embedding in sarcolemma, thereby expanding the contact area between myocytes and endothelial cells. Mitochondria in myocytes cluster along the embedded capillary, in that way reducing the distance oxygen and nutrients need to diffuse.

The proximity between muscle fibers and capillaries is underscored by recent findings showing capillaries embedded in grooves indenting the sarcolemma and expanding the contact area between myocytes and ECs. Moreover, muscle fiber mitochondria appear to cluster along these grooves, thereby reducing the distance oxygen and fatty acids need to diffuse.⁷ Recent elegant imaging studies have also shown that muscle's "mitochondrial reticulum" is further specialized with respiratory complexes that

generate membrane potential (e.g., complexes III and IV) enriched in the perivascular region of the mitochondrial matrix, whereas components using the proton-motive force for ATP synthesis are distributed more within the fiber. This arrangement minimizes oxygen diffusion distances and may allow conductive distribution of proton-motive force.⁸

Although arterioles and venules are organized similarly in most mammalian skeletal muscles, some differences in microvascular architecture exist between aerobic and anaerobic muscles. Aerobic muscles typically have more capillaries, larger capillary length per fiber, and more capillary branches per fiber,⁹ and microvessels may be embedded deeper in sarcolemma and have more perivascular mitochondria.⁷ These are all adaptations that could facilitate gas and nutrient exchange. Muscle's vascular architecture down to the level of terminal arterioles allows blood flow and nutrient exchange to be regulated at several levels. Total skeletal muscle blood flow can increase with rising arterial pressure and maintained muscle resistance artery tone or by vasodilation of resistance arterioles with maintained pressure. Terminal arterioles can increase the endothelial surface area by increasing the fraction of perfused capillaries through vasodilation and SMC-driven vasomotion, thus enhancing nutrient exchange.

Muscle perfusion and nutrient flux with exercise and insulin

Perfusion delivers oxygen, free fatty acids (FFA), glucose, triglycerides, and amino acids to and removes metabolites (amino acids, lactate, carbon dioxide, and ammonia) from muscle. Muscle is a major site of carbohydrate, protein, and fat storage during feeding and a major supplier of nutrients during periods of deprivation. The regulation of fuel fluxes in and out of muscle has been studied intensely *in vivo* in animals and humans using the limb balance method, which is at times supplemented with metabolite tracer measurements. Here, we will consider the relationship between muscle blood flow and nutrient supply to muscle in response to exercise or insulin stimulation, which has been studied intensively. In particular, we will emphasize data from *in vivo* studies in humans. We will consider both intense and very modest stimulation to provide the reader with a sense of the hierarchical relationship between muscle perfusion and metabolic function.

Total limb flow can be assessed in different ways (e.g., plethysmography, dye or thermal dilution, and Doppler ultrasound). The advantages and limitations of these methods have been reviewed elsewhere.¹⁰ Although flow measurement methods have been compared with one another, none can be considered to be the gold

standard to measure skeletal muscle blood flow.¹⁰⁻¹² Of note is that each measures blood flow directed to all tissues of the limb, not just muscle. In overnight-fasted humans, resting limb blood flow averages $\sim 2-5 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ ml}^{-1}$ of limb¹³⁻¹⁵ in both men and women,¹⁶ using the arm or leg.¹⁷⁻¹⁹ Because resting skin and subcutaneous adipose blood flow (on a per 100 ml of tissue basis) are similar to that of muscle,²⁰⁻²² these resting limb flow values are assumed to reflect average blood flow to muscle, i.e., the methods treat the limb as a homogenous tissue represented by muscle.

Vasomotion, the rhythmic vessel diameter oscillations first described by Jones²³ in the bat wing in 1852, is more prevalent in small arteries and arterioles than in conduit arteries.²⁴ These oscillations modify blood flow and are one contribution to periodic flow fluctuations known as flowmotion.^{24,25} In addition to vasomotion, heart rate, respiration, and autonomic factors influence flowmotion. The several components of flowmotion are distinguished by their frequencies; heart rate and respiratory contributions arise at 1 and 0.3 Hz, respectively, whereas low-frequency signals associated with vasomotion arise at 0.1 Hz for myogenic activity, 0.04 Hz for neurogenic activity, and 0.01 Hz for endothelial activity.²⁶⁻²⁸

Vasomotion originates from an oscillator component in the SMCs and synchronizing mechanisms, which are modified by ECs.²⁴ Upon increased metabolic needs, signals originating from the microvasculature travel upstream to larger arterioles and arteries, possibly via gap junctions in the SMCs,²⁹ thereby influencing vessel diameter. In addition, vasomotor responses in arterioles can be triggered by stimulation of individual capillaries, and changes in membrane potential appear to be responsible for these observations.²⁹⁻³¹ Moreover, NO can regulate EC-to-SMC signaling at myoendothelial junctions, and reduced NO production attenuates the duration of conducted vasodilation.^{29,32} These findings suggest that muscle microcirculation modulates upstream vasodilation and vasomotion.

In vivo, vasomotion may regulate microvascular flow distribution to optimize delivery of nutrients and regulate local hydraulic resistance.³³ In addition to regulating blood flow distribution, vasomotion of terminal arterioles also affects lymph flow, thus influencing water transport in muscle interstitium and tissue homeostasis.⁶ Vasomotion, by continuously altering flow delivery to vessels over time, increases the number of ECs exposed to plasma for nutrient or hormone exchange³⁴ without total flow or cardiac output being changed. When perfusion demand exceeds flowmotion's compensatory capacity, blood flow control shifts upstream, enabling increases in total flow.^{24,35}

Studying muscle vasomotion requires invasive direct blood vessel visualization (e.g., intravital microscopy),³⁶⁻³⁹ and it is affected by anesthetic agents and a host of

experimental variables.³⁷ Its invasive nature limits the study of vasomotion in humans. By contrast, cutaneous flowmotion can be studied using laser-Doppler flowmetry (LDF) and has become popular in clinical studies.⁴⁰⁻⁴³ To quantify flowmotion, Fourier or wavelet analyses methods are used to determine the relative contribution of each frequency to the observed LDF signal.^{26,37,42} A drawback of LDF measurements is that they are affected by nearby vessels and blood pressure fluctuations, and therefore, they may not necessarily reflect only the rhythmic activities of blood vessels themselves.^{24,44,45} Several disorders influence vasomotion and flowmotion; hemorrhage increases vasomotion, whereas obesity and diabetes reduce flowmotion and vasomotion.^{24,42} Taken together, vasomotion provides a local mechanism for vascular adaptation to altered metabolic needs, and its regulation impacts muscle perfusion.

Exercise and muscle perfusion

Myocyte and related whole body metabolic changes with acute and chronic exercise were reviewed recently.⁴⁶ Oxygen and nutrient delivery to muscle during intense exercise and the accompanying blood flow changes have been studied extensively. The increased bulk blood flow during exercise arises both from local factors [e.g., potassium, adenosine, phosphate, lactate, hydrogen ion, nitric oxide (NO), and prostaglandins] released by the muscle and by neurovascular changes that relax muscle vasculature. With very intense whole body exercise, blood flow and oxygen delivery to the tissue become limiting secondary to limitations of respiratory and cardiac function.^{47,48} By contrast, with intense exercise of isolated muscle groups, neither cardiorespiratory function nor hemodynamic factors limit muscle performance. Thus, blood flow to the human quadriceps can increase >50-fold during brief, intense exercise.⁴⁷ Increasing the arterial oxygen content does not further increase quadriceps O₂ extraction or peak work rate by either trained or untrained muscle.⁴⁹ Muscle's remarkable flow reserve can meet even extreme muscle O₂ demands. In addition to oxygen, glucose and fatty acids are important fuels during exercise. Carbohydrates (both circulating glucose and muscle glycogen) are the preferred fuel for intense, brief-duration muscle exercise. With intense muscle exercise, glucose extraction can increase more than sixfold and leg blood flow 10-fold, whereas plasma glucose changes little.⁵⁰ Interestingly, whereas glucose concentrations in resting muscle cytosol are extremely low, intense exercise dramatically increases glucose both in muscle interstitium⁵¹ and within muscle cells in humans⁵⁰ and rodents.⁵² The > 30-fold increase in leg glucose uptake seen with intense exercise indicates that both the delivery of glucose to muscle

microvasculature and transcapillary glucose transfer have capacity sufficient to meet glucose metabolic needs.

Vascular responses to light exercise (operationally, we consider $\leq 25\%$ max as “light”) have been studied much less, and their link to muscle performance is less clear. However, studies of light exercise provide insight into the hierarchical regulation of muscle perfusion. With light exercise, vasomotion within the terminal arterioles appears to cease, and the number of perfused capillary increases without significant changes in total muscle blood flow.^{34,53,54} Honig et al.,³⁴ by flash-freezing and sectioning muscle, showed that low-frequency electrical stimulation was nearly as effective as near-tetanic stimulation for recruiting hypoperfused microvasculature in canine gracilis muscle. Further increasing contractile activity increases total blood flow, as regulation shifts upstream to progressively larger arterioles.³⁵ Factors driving microvascular perfusion changes include venous Po_2 , which, by enhancing venular EC NO production and erythrocyte NO release,^{35,55-57} promotes dilation of nearby arterioles. Hemodynamic forces, i.e., shear stress-induced NO production, further modulate hyperemic responses.³⁵ These are aided by prostaglandins and endothelium-derived hyperpolarizing factors to increase perfusion with exercise.⁴⁸ It appears that inhibition of one system (e.g., NO synthase) can be compensated by the remaining factors, since skeletal muscle blood flow is preserved in such circumstances.⁵⁸ If, however, two pathways are inhibited simultaneously, the increase in skeletal muscle blood flow is reduced.^{48,59} These effectors act as sympatholytics to counteract the exercise-induced vasoconstriction of distal arterioles and feed arteries by sympathetic nerves.⁴⁸

Insulin and muscle perfusion

The effects of insulin on limb metabolism have been studied extensively using limb balance methods combined with euglycemic insulin infusion. Andres et al.⁶⁰ first reported insulin-stimulated forearm glucose uptake and noted an accompanying increase in forearm blood flow. Baron⁶¹ and Laakso et al.⁶² first reported that insulin-induced muscle glucose uptake paralleled insulin's ability to increase total leg blood flow. In subsequent work, obesity, type 1 and type 2 diabetes, and increased plasma free fatty acid concentrations⁶²⁻⁶⁵ each impaired insulin-stimulated limb blood flow and glucose uptake in parallel. These studies suggested that 1) insulin stimulates limb blood flow in both humans and animals; 2) during a high-dose insulin clamp, increased limb flow correlates with muscle glucose uptake; 3) acute and chronic metabolic insulin resistance is associated with impaired insulin-stimulated increases of limb blood flow; and 4) insulin enhances limb blood flow by increasing nitric oxide

production.^{62,65-67} This led investigators to suggest that impaired arteriolar relaxation in response to insulin contributes significantly to metabolic dysfunction in insulin-resistant muscle.

Insulin's vasodilatory effects arise from its binding to the insulin receptor on ECs. This stimulates the phosphatidylinositol 3-kinase/Akt pathway to activate endothelial NO synthase (eNOS)^{68,69} and induces NO-mediated vasodilation.⁷⁰ Insulin also stimulates the MAPK pathway and enhances production of the vasoconstrictor endothelin-1.^{66,67,71} In health, insulin's vasodilatory effect dominates. With endothelial dysfunction, as occurs with obesity and type 2 diabetes, the PI3K pathway appears to be selectively inhibited by low-grade inflammation, FFA, oxidative stress, and reduced perivascular adiponectin release.⁷²⁻⁷⁶ In these circumstances, a reduced vasodilatory response or even paradoxical vasoconstriction can be seen with hyperinsulinemia.^{72,77}

Insulin at high physiological concentrations relaxes resistance arterioles and increases limb blood flow in humans. In addition, there is convincing evidence that this response is blunted in insulin-resistant individuals with obesity⁷⁸ and type 1⁷⁹ or type 2 diabetes.⁸⁰ This effect of insulin on blood flow is most evident when insulin is infused continuously at a rate of $3 \text{ mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ to achieve marked steady-state hyperinsulinemia for $\geq 2 \text{ h}$. It has been more difficult to reproduce this effect infusing insulin at a lower rate or for shorter duration.⁸¹ This has led to questioning of the physiological importance of this response. In addition, in insulin-resistant subjects, pharmacologically increasing blood flow often does not improve insulin's metabolic action measured as limb glucose uptake.⁸² However, in the latter circumstance, it remains likely that while perfusion is improved insulin resistance in the myocyte persists, and short-term correction of perfusion is insufficient to overcome that metabolic defect.

Over the last 15 years, research on insulin's action on muscle perfusion has added focus on the microvasculature. There, analogous to the effect of low-frequency stimulation reported by Honig et al.,^{34,53} relaxation of terminal arterioles could recruit hypoperfused capillaries and expand the EC surface available for nutrient exchange⁸³ without changing total flow. However, there were no noninvasive methods available to quantify the microvascular volume perfused in intact muscle in animals or humans. In 1997, a technique was introduced to estimate the endothelial surface available for nutrient exchange based on the single-pass conversion of 1-methylxanthine (1-MX) to 1-methylurate (1-MU) by xanthine oxidase, which is present on capillary endothelium. Increased endothelial exposure to substrate (i.e., capillary recruitment) would increase 1-MX to 1-MU conversion.^{84,85} Later, contrast-enhanced ultrasound (CEUS) was used to investigate microvascular perfused volume regulation.^{86,87} Using either method, we found that in healthy animals and humans, physiological doses of insulin

and carbohydrate-containing meals each increased microvascular perfusion volume even in the absence of increases in total limb blood flow.^{77,86,88} Using CEUS, we found that, from the start of an euglycemic insulin clamp, microvascular blood volume increases within 15 – 30 min, and this microvascular change precedes and correlates with the rise in forearm glucose uptake.⁸⁹⁻⁹¹ Moreover, chronic (obesity) and acutely induced (lipid infusion) insulin resistance are associated with an impaired microvascular response to insulin or meal ingestion.^{77,92-94} Using CEUS,^{54,88} we also confirmed the original finding by Honig et al.⁵³ that light exercise increased microvascular perfused volume ~3-fold with minimal increases in total limb blood flow (Figure 2.2). However, with more intense exercise, both microvascular blood volume and total limb blood flow increase.^{54,88,95} Interestingly, cardiac muscle behaves similarly to skeletal muscle during hyperinsulinemia⁹⁶ and feeding,⁹⁷ and its increases in microvascular blood volume are blunted by insulin resistance as well.⁹⁸

Figure 2.2. Top: contrast-enhanced ultrasound images of rat thigh muscle as a function of time after a high-energy ultrasound pulse with the muscle at rest or given a 1-Hz electrical stimulation. **Bottom:** greater plateau video intensity (red) corresponding to a larger number of microbubbles in the imaged volume of the stimulated muscle.

Insulin is not the only hormone that can “recruit” muscle microvasculature. Both glucagon-like peptide-1 (GLP-1)^{99,100} and adiponectin,¹⁰¹ like insulin, increase microvascular volume, albeit each acting by different biochemical pathways. By contrast, epinephrine can increase muscle blood flow without increasing perfused

microvascular volume.¹⁰² The responses to physiological hyperinsulinemia and low-intensity exercise suggest a hierarchical response by muscle vasculature whereby microvascular units respond to these stimuli, whereas with more intense stimulation (high physiological insulin concentrations or heavy exercise) more proximal arterial elements also respond, leading to the flow increases seen in those settings. Whether this graded response involves antidromic signals through the vascular network or again is a response to multiple locally generated metabolic signals is not known.

Nutrient and hormone transfer across the endothelium

Here we very briefly review evolving evidence that the vascular endothelium in tissues like muscle and adipose may actively mediate the transendothelial transport (TET) of substrates (e.g., glucose or FFA) and hormones, including insulin. As would be expected, such a role for the endothelium is of consequence principally in tissues with a continuous endothelium, like muscle and adipose, but less consequential in liver and other splanchnic tissues.¹⁰³

We consider first the endothelium's role in FFA transport, as FFAs are a major muscle fuel. It has long been known that lipoprotein lipase (LPL) is secreted by skeletal myocytes and adipocytes but resides on the luminal surface of the vascular EC in muscle and fat, where it cleaves fatty acid monomers from triglycerides bound to circulating lipoproteins.¹⁰⁴ This process can be regulated by insulin, which increases LPL expression. Recently, it was found that the EC possesses a receptor for LPL [glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1)]¹⁰⁵ and that when LPL binds to GPIHBP1 a vesicular transport system mediates LPL's TET to the luminal membrane, where it encounters circulating triglycerides.¹⁰⁶ Mutations in either GPIHBP1 or LPL that interfere with this pathway can cause severe hypertriglyceridemia in humans.¹⁰⁷

In addition to this pathway for LPL transport that provides triglyceride-derived fatty acids to the EC, the EC possesses membrane-associated fatty acid transport proteins (FATPs) as well as fatty acid-binding proteins (FABPs). The former take up FFA derived either from triglyceride hydrolysis or circulating as FFA associated with albumin or other plasma proteins. FATP3 and FATP4 are expressed by ECs along with the scavenger receptor CD36. Each appears to be involved in movement of fatty acids across the EC luminal plasma membrane. Expression of both FATP3 and FATP4 is increased by VEGF β secreted by myocytes and adipocytes acting via VEGFR1. Muscle expression of VEGF β is increased by the transcriptional coactivator PGC-1 α , which is a central player in the regulation of mitochondrial oxidative metabolism within the

myocyte. In the context of increasing muscle oxidative capacity, muscle signaling to adjacent ECs to enhance FFA uptake may be part of an adaptive response. Expression of CD36 by ECs is upregulated by peroxisome proliferator-activated receptor- γ , which enhances fat mass and adipogenesis. Interestingly, inhibition of VEGF β in diabetic animal models decreases muscle ectopic fat and improves glucose metabolism.¹⁰⁸ By contrast, VEGFA knockout in muscle increases susceptibility to high-fat diet induced insulin resistance, which is due at least in part to decreased microvascular perfusion.¹⁰⁹ The transport of FFA across the ECs is presumably bidirectional, particularly in adipose tissue, although this has not been clarified in detail.

In contrast to the complex regulatory system involved in the movement of FFA across the EC, glucose transport from the vascular lumen into and across the endothelium is mediated by the constitutive activity of the GLUT1 transporter. The system has a high capacity in the absence of exogenous insulin, as indicated by the marked increases in glucose transport that occur with exercise (see above). The GLUT1 transporter is expressed in both vascular smooth muscle and endothelial cells. In the former, the transporter activity is downregulated by high ambient glucose concentrations.¹¹⁰ This adaptive response is absent in aortic ECs. The function of this transporter is important to the metabolic activity of the EC, which receives a significant fraction of its energy from glycolytic ATP production.¹¹¹ The specific transport capacity in brain microvascular ECs has been studied extensively, as ECs perform a critical function in assuring adequate glucose delivery to the central nervous system. Cardiac muscle microvascular glucose transport capacity has been investigated and utilizes the same high-capacity GLUT1 transporter reported for other endothelia.¹¹¹ To our knowledge, GLUT1 transporter function specific to skeletal muscle microvasculature has not been studied. As noted previously, the observation that muscle interstitial and myocyte glucose concentrations rise during intense exercise suggests that glucose transport through the vascular endothelium is not limiting even under conditions of high substrate demand. Indeed, the high capacity of the system may in part be responsible for damage that occurs to the microvascular endothelium as a result of hyperglycemia in the diabetic state.¹¹² The EC does not appear to defend itself from high levels of intracellular glucose, which via several pathways can generate reactive oxygen species.^{112,113} When this is unchecked, as occurs in persistent hyperglycemic states, vascular injury occurs.

The EC in tissues with a continuous endothelium also constitutes a barrier to insulin movement into the tissue. Indeed, estimates of muscle interstitial insulin concentrations using either lymphatic sampling¹¹⁴ or microdialysis¹¹⁵ suggest that insulin TET could be rate limiting for insulin's action on muscle. This led us to examine the cellular pathway of insulin TET. Most ECs express insulin, IGF-I, and the insulin/IGF-I hybrid receptor.¹¹⁶ In aortic ECs and preadipocytes,¹¹⁷ the insulin receptor

associates with a specialized lipid raft domain, i.e., caveolae. Caveolae can be internalized and mediate TET of a variety of proteins (e.g., albumin in pulmonary microvascular ECs).¹¹⁸ In a series of experiments, we found that caveolae are required for insulin uptake by and transport across aortic ECs.¹¹⁹ In these ECs, preserved insulin signaling through phosphatidylinositol 3-kinase to Akt and eNOS is necessary for insulin TET.¹²⁰ Interestingly, inflammatory cytokines or several days of high-fat diet diminish EC insulin transport.¹²⁰ If muscle microvascular ECs behave similarly, then impaired EC insulin signaling and TET could contribute to the impaired muscle insulin action of insulin resistance. As we had seen for microvascular recruitment, for insulin TET, NO appears to be a critical regulatory factor. When phosphatidylinositol 3-kinase or NO synthase is inhibited, insulin transport declines. Giving sodium nitroprusside (3-30 μ M) can restore insulin uptake¹²¹ in aortic ECs. Surprisingly, an EC-specific insulin receptor knockout mouse (with Cre-recombinase driven by Tie-2) did not display any metabolic phenotype.¹²² However, more recently, another EC-specific insulin receptor knockout (with Cre-recombinase driven by a vascular endothelial-cadherin promoter) displayed glucose intolerance and delayed insulin action on insulin target tissues (Konishi M, Sakaguchi M, Cai W, Rask-Madsen C, and Kahn CR, unpublished observations). Likewise, the EC-specific IRS-2 knockout mouse is glucose intolerant and has impaired insulin-induced microvascular perfusion and diminished muscle insulin delivery.¹²³ These genetic models underscore the role of EC insulin signaling to whole body metabolic functioning.

Recent work using adipose microvascular ECs has suggested a similar vesicle-mediated insulin TET process, and initial studies implicate clathrin-coated vesicles, not caveolae.¹²⁴ However, there is a lack of data clarifying whether insulin regulates that transport or whether a transendothelial insulin concentration gradient is present in adipose tissue and whether insulin TET limits insulin action in adipose tissue.¹²⁵

Conclusion

In summary, a hierarchical, graded control of muscle perfusion arises from cooperative interactions among the factors released locally by myocytes, by SMCs, or by ECs along with systemically and neurally delivered signals. Very modest contractile activity or physiological hyperinsulinemia promptly increases microvascular perfusion volume even in the absence of changes in limb blood flow. In both cases, EC exchange surface for nutrient or hormone delivery expands without requiring increased cardiac work. Greater contractile activity or hyperinsulinemia triggers signals that may ascend the vascular network and provoke relaxation of progressively larger vessels, increasing total flow to the expanded microvascular network. Beyond insulin and exercise, other

humoral factors (e.g., GLP-1, angiotensin II, and adiponectin) similarly enhance muscle microvascular perfusion. However, the role of these factors in normal muscle physiology is still being unraveled. Evolving evidence indicates that (at least for insulin and triglycerides/FFA), beyond altered perfusion, the microvasculature, particularly the EC, actively regulates the delivery of insulin and FFA to the muscle via regulation of specialized transport mechanisms. Thus, the EC of muscle vasculature is increasingly recognized as a key regulator of normal muscle physiology and metabolic health. Much remains to be learned regarding the normal regulation of the integrated functioning of the skeletal muscle myocytes and its vasculature and how this regulation is altered by disease or dysfunction.

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Appendix 2.1

Capillary recruitment: lessons from skin microvasculature

Yvo H.A.M. Kusters, Coen D.A. Stehouwer

Crosstalk Series: De novo capillary recruitment in healthy muscle is/is not necessary to explain physiological outcomes

J Physiol 2015.

In this crosstalk both discussants raise important issues regarding current methodological limitations while assessing the concept of capillary recruitment. On the one hand, contrast-enhanced ultrasound is unable to visualize individual capillaries,¹ whereas intravital microscopy in surgically prepared skeletal muscle is likely to disturb baseline observations.² Because of these limitations, it is not possible to disprove either one of the models.

We do, however, believe that earlier observations in skin can add to this discussion. Although skin as a model for muscle also has limitations, the human data with skin videomicroscopy show that insulin can in fact cause capillary recruitment in at least one organ system. Our skin data show that the cutaneous microvascular response to insulin (i.e. capillary recruitment) behaves, and can be manipulated, in a way that is entirely consistent with the model described by Dr. Barrett et al., in which microcirculation and muscle behave as a functional unit with respect to insulin sensitivity and muscle glucose uptake.¹⁻⁵

Hence, we do not concur with Dr. Poole that the Kroghian model of capillary recruitment can be dismissed. Yet, progress with regard to the validity of these models requires that the methodological issues discussed above be resolved.

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Journal

Chapter 3

Reliability, measurement error and methodology of anthropometric, vascular, metabolic and inflammatory variables

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In preparation

Abstract

Background

The measurement of variables is a vital part of clinical practice and biomedical research. However, the reliability of measurements is frequently misused, incompletely considered, or not at all assessed in biomedical research. We investigated the reliability, which relates the random error variance to the variance among subjects in a population, and measurement error, which is related to the concept agreement, of several commonly used anthropometric, vascular, metabolic and inflammatory variables.

Methods

We evaluated visceral (VAT) and subcutaneous adipose tissue (SAT), intrahepatic (IHL) and intrapancreatic lipid (IPL), muscle microvascular recruitment (MVR), capillary microscopy (CM), flowmotion, sublingual microvascular glycocalyx, flow-mediated dilation (FMD), reactive hyperemia index (RHI), carotid-femoral pulse wave velocity (cfPWV), augmentation index (Aix), carotid distensibility, distensibility coefficient (DC), compliance coefficient (CC) and intima-media thickness of the common carotid artery (cIMT), whole body glucose disposal (WBGD), beta-cell glucose sensitivity (b-GS), beta-cell rate sensitivity (b-RS), potentiation factor ratio (PFR), CD11B surface expression of circulating NK cells (NK-CD11B), and a z-score of plasma biomarkers of low-grade inflammation (z-LGI). We evaluated reliability and measurement error for intra- and inter-observer comparisons as well as over time for the execution of the measurements. Similar comparisons were made for the analyses of the measurements. When available, measurements were also compared to their gold standard.

Results

Most measurements were highly reliable and had acceptable measurement error, yet sublingual microvascular glycocalyx and some indices derived from CM and flowmotion were not reliable and had a considerable measurement error. Analyses of all measurements was reliable and had acceptable measurement error. VAT, SAT and IHL were compared to their gold standard and considered valid.

Conclusion

Here, we established the reliability and measurement error of commonly used anthropometric, vascular, metabolic and inflammatory variables. This detailed understanding of sources of measurement error and the reliability of variables in a certain study population allows for the adequate selection of outcome measurements in observational and intervention studies, as well as provide data for more accurate sample size calculations for such studies.

Introduction

The measurement of variables is a vital part of clinical practice and biomedical research. Measurements are, however, seldom perfect.¹ To assess the quality of the measurement of a variable, the COSMIN statement is generally used; it defines a good measurement as reliable, responsive, valid, and interpretable (Supplementary Figure 3.1).² However, the reliability of measurements is frequently misused, incompletely considered, or not at all assessed in biomedical research.^{3,4} Nonetheless, an appropriate assessment and interpretation of reliability is essential for correct study designs and power calculations, as well as to develop strategies to improve reliability in the measurement of variables.

Potentially confusingly, the domain reliability in the COSMIN taxonomy is divided into the measurement properties measurement error and, again, reliability (also known as reproducibility, repeatability, or test-retest performance; Supplementary Figure 3.1).^{1,2} It is important to understand the difference between measurement error and reliability. Parameters of measurement error relate to the concept agreement, i.e., the absolute difference between repeated measurements. Two commonly reported, and related, parameters of measurement error are the standard error of the measurement (SEM) and the limits of agreement (Bland and Altman method). The SEM can be interpreted as the standard deviation around a single measurement (e.g., when waist circumference is assessed 100 consecutive times, the SD of these measurements equals the SEM).¹ A different parameter of measurement error are the limits of agreement.⁵ In Bland-Altman plots, the difference between repeated measurements is plotted against the average of those measurements. This allows for the visualization of systematic differences, as well as the limits of agreement. If one assumes a normal distribution of difference scores, 95% of the difference scores will fall between the limits of agreement.^{1,5}

Reliability parameters on the other hand, like intraclass correlation coefficients (ICC), which range in value from 0 (totally unreliable) to 1 (perfect reliability), relate the random error variance to the variance among subjects in a population.^{1,6,7} Consequently, a parameter of reliability is in fact only valid for the population it is measured in and the observers it is measured by. If, for example, reliability were to be determined in a population where variance between subjects is very high (e.g., determining insulin sensitivity in a population with trained athletes and individuals with type 2 diabetes are included), reliability is generally high. When this reliability is then assumed for the measurement itself, regardless of the population (e.g., when studying only obese individuals without type 2 diabetes), the reliability of that measurement is grossly overestimated.

Although the COSMIN Statement was primarily designed for patient reported outcomes,² most of its criteria can be applied to observer-derived measurements as well. As observers are then, possibly, involved twice in the process, i.e., the conduction of the measurement as well as the (offline) analyses of the data, the measured variable can entail measurement error derived from both.

In this thesis, a multitude of anthropometric, vascular, metabolic and inflammatory measurements have been used. Nevertheless, the reliability of some, like muscle microvascular recruitment (MVR) by means of contrast enhanced ultrasound (CEUS), has never been reported at all. Other measurements, like nailfold capillary microscopy for example, are known for their relatively high inter-observer variance.⁸ As a result, researchers are commonly subjected to an extensive training program before they are deemed capable to perform or analyze such measurements. Moreover, it is important to identify where sources of error arise, as strategies to improve a measurement's reliability can be developed and deployed from this insight.

It is, therefore, important to 1) determine whether measurement error arises predominantly from the acquisition of data or its analyses; 2) to assess to what extent measurements differ within one observer, as well as between observers; and 3) determine to what extent a measurement varies over time. To our knowledge, this is the first study to evaluate the sources of measurement error for the measurements in this thesis and provide substantiated recommendations on the improvement of reliability.

We assessed reliability and measurement error several ways. First, we evaluated reliability and measurement error for intra- and inter-observer reliability and reliability over time, for the execution of the measurements. Then, we evaluated the reliability and measurement error for intra- and inter-observer reliability for the (offline) analyses of the measurements. Finally, we compared measurements to their gold standard, when available. For each of these assessments we provided the ICC, SEM, and Bland-Altman plot with limits of agreement.

Materials and methods

Study designs

In this manuscript on reliability, data from several separate studies are reported in order to provide as much information on reliability and measurement error as possible in a single report. Reliability data of MVR were collected in a dedicated CEUS Reproducibility Study in which individuals were measured on two occasions one week

apart with handgrip exercises as stimulus for MVR. On each study day, CEUS was performed twice with 60 minutes in between. CEUS measurements were conducted in a temperature-controlled room ($T = 24^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) after a 12-hour overnight fast and with the individuals in the supine position for at least 30 minutes prior to the measurements. Individuals were instructed to refrain from alcoholic beverages for a period of 72 hours prior to each study day and strenuous physical exercise 24 hours prior to each study day.

From a weight loss intervention (WLI) trial, which is described in more detail elsewhere,^{9,10} we derived data on 1) the reliability over time of abdominal visceral adipose tissue volume (VAT), abdominal subcutaneous tissue volume (SAT), intrahepatic lipid content (IHL), intrapancreatic lipid content (IPL), MVR, sublingual microvascular perfused boundary region (PBR), capillary microscopy, vasomotion, flow-mediated dilation (FMD), reactive hyperemia index (RHI) and whole body glucose disposal (WBGD); 2) the reliability of VAT, SAT, IHL, IPL, MVR, vasomotion, and FMD analyses; and 3) the comparison of VAT, SAT and IHL measurements to their respective gold standard. The data on reliability over time was derived from the weight stable control group that did not alter their diet or exercise pattern for eight weeks. The comparison of our VAT, SAT, and IHL measurements to their gold standard were performed in a random subsample of lean and abdominally obese individuals.

The reliability data of carotid-femoral pulse wave velocity (cfPWV), augmentation index (Aix), carotid distensibility, distensibility coefficient (DC) of the common carotid artery (CCA), compliance coefficient (CC) of the CCA, and carotid IMT (cIMT) measurements were investigated in a subsample of the Maastricht Study.¹¹ In this subset of 12 individuals, two investigators performed these measurements consecutively on the first day. One week later, one investigator repeated these measurements. In this study, participants were asked to refrain from smoking and drinking coffee, tea or alcoholic beverages ≤ 3 hours prior to the study. Participants were allowed to have a light meal (breakfast and/or lunch). All measurements were performed after 10 minutes of rest and talking or sleeping was not allowed during the examination.¹² The reliability of VAT and SAT analyses were determined in a random subset of 29 participants in the Maastricht study. Two observers (YK and an experienced abdominal radiologist (StH)) analyzed all images in parallel and in a masked fashion, unaware of each other's analyses.

The reliability of capillary microscopy analyses were assessed in a standardized training set of representative images chosen from several studies.^{11,13} This training set has been used by previous and current researchers in order to standardize scoring criteria for (continuously) perfused capillaries. Observers were unaware of each other's analyses.

Data on the reliability of FMD measurements have been published before,¹⁴ yet are included in this manuscript to illustrate the difference in reliability of several common vascular function measurements as well as their analyses. In this Acute Hyperglycemia Study, which aimed to normalize FMD to local wall shear stress, participants were studied on two separate days with a week in between. On each study day, participants underwent repeated FMD measurements with ten minutes in between; assessors of FMD videos were not masked. Individuals were instructed to refrain from exercise, smoking, vitamin supplement use, caffeine consumption, and alcohol consumption. Measurements were performed after a 12-hour overnight fast in supine position in a climate-controlled room.

Study populations

In the CEUS Reproducibility study, 5 women and 3 men were recruited by advertisements and enrolled at the Maastricht University Medical Center, The Netherlands, from September, 2012 to April, 2013. Participants were aged 18 - 55 years, non-smokers, free of cardiovascular disease, and had a BMI below 25 kg/m². Individuals were excluded when office blood pressure was > 140/90 mmHg, when they used medication affecting blood pressure, serum lipids or glucose metabolism, when they were pregnant or lactating, when they were allergic to the contrast agent used, and/or when they had known cardiac dysrhythmias or a family history of sudden cardiac death. The study was approved by the Maastricht University Medical Center ethics committee, performed in accordance with the Declaration of Helsinki, and registered at the Dutch research database ToetsingOnline (NL37229.068.11). All participants gave written informed consent.

The details on the individuals enrolled in the randomized controlled weight loss trial with blinded analyses are described elsewhere.^{9,10} Briefly, 53 abdominally obese and 25 lean men were recruited by advertisements and enrolled at the Maastricht University Medical Center in Maastricht, The Netherlands, from August, 2012 to May, 2014. Participants were aged 18 - 65 years, non-smokers, nondiabetic, free of CVD, had a waist circumference below 94 cm (lean) or between 102 - 110 cm (abdominally obese), and a stable body weight for at least 3 months. Men were excluded when fasting plasma glucose was > 7.0 mmol/l, when HbA1c was > 6.5%, when serum total cholesterol was > 8.0 mmol/l, when serum triacylglycerol was > 4.5 mmol/l, when they had a contra-indication for magnetic resonance imaging (MRI), and/or when they used medication affecting blood pressure, serum lipid or glucose metabolism. The study was approved by the Maastricht University Medical Center ethics committee, performed in accordance with the Declaration of Helsinki, and registered at clinicaltrials.gov (NCT01675401). All participants gave written informed consent.

The rationale and methodology of the Maastricht Study, as well as its population characteristics, have been described previously.^{11,12} To reiterate from,¹² the study focuses on the etiology, pathophysiology, complications and comorbidities of T2DM and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Individuals were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known T2DM status, with an oversampling of individuals with T2DM, for reasons of efficiency. Clinical characteristics of the individuals included in the Maastricht Study are summarized elsewhere.^{12,15} Reliability was assessed in a subset of 12 individuals (6 men; 60.8 ± 6.8 years; 6 T2DM). The study is registered at the Dutch research database ToetsingOnline (NL31329.068.10), has been approved by the Minister of Health, Welfare and Sports of the Netherlands, on the basis of the Health Council's opinion (Permit 131088-105234-PG), and performed in accordance with the Declaration of Helsinki. All participants gave written informed consent.

As described previously, the Acute Hyperglycemia Study, in which reliability of FMD measurements is already mentioned, consisted of 22 men.¹⁴ All individuals were non-diabetic, free of cardiovascular disease, and did not use any medication. The study was approved by the Maastricht University Medical Center ethics committee, performed in accordance with the Declaration of Helsinki, and registered at the Dutch research database ToetsingOnline (NL33485.068.10). All participants gave written informed consent.

Anthropometric measurements

Visceral and subcutaneous adipose tissue volumes

In the WLI trial, VAT and SAT volumes were obtained by MRI through two-dimensional (2D) T1-weighted turbo spin echo (TSE) imaging on a 3.0T Philips Achieva MRI scanner with a dedicated sixteen-element torso coil (XL Torso coil, Philips Healthcare, The Netherlands) as described previously.⁹ Nine 5 mm thick transverse slices with 10 mm gaps centered at the top of the L4 vertebral body were acquired using the following scan parameters: repetition time (TR) = 526 ms, echo time (TE) = 10 ms, turbo spin echo factor = 4, number of signal averages (NSA) = 1, field of view (FOV) = 400 x 322 mm, acquired matrix size = 308x164, acquired in-plane voxel size = 1.3 x 1.96 mm, reconstructed matrix size = 512 x 412, and a reconstructed voxel size of 0.78 x 0.78 mm. Images were analyzed offline after completion of the trial with dedicated software (Hippo Fat, IFC CNR, Pisa, Italy) to provide VAT and SAT volumes.¹⁶

In the Maastricht Study, VAT and SAT area were acquired using a 3T MRI system (MAGNETOM Prismafit, Siemens Healthineers, Erlangen, Germany) with body matrix and supine radiofrequency coils. Subjects were positioned supine. After an initial scout view, a single-slice transversal image at the top level of the L4 vertebral body was obtained during breath-hold to assess areas of the visceral and subcutaneous adipose tissue compartments using a T1-weighted TSE pulse sequence with the following parameters: acquired and reconstructed in-plane voxel size $2 \times 2 \text{ mm}^3$, slice thickness 8 mm, TR 550 ms, NSA = 1, parallel imaging (GRAPPA) factor = 2, turbo factor = 7. Depending on the size of the patient, the FOV was $384 \times 312 \text{ mm}^2$ with a TE of 36 ms or $500 \times 406 \text{ mm}^2$ with a TE of 38 ms. Images were analyzed offline after completion of the trial with dedicated software (Hippo Fat, IFC CNR, Pisa, Italy) to provide VAT and SAT volumes.¹⁶

The reliability over time was derived from the weight stable control group of the WLI trial, consisting of 26 abdominally obese men. Intra- and inter-observer reliability of the analyses was assessed in a subset of 29 scans from the Maastricht study. For intra-observer reliability this subset was analyzed twice with two weeks in between. During that same period, this subset was analyzed by an experienced and certified abdominal radiologist to determine the inter-observer reliability. In addition, this subset of 29 scans was also analyzed by determining SAT and VAT pixel by pixel (MRIcron version 6.6.2013, Chris Rorden, CRNL, USA), which could be considered to be the gold standard for VAT and SAT analyses.¹⁶

Intrahepatic lipid content

With the same MRI scanner and coil, IHL was assessed through mDixon imaging as described before.⁹ Two 6 mm thick transverse slices through the liver were acquired using a 2D three-point T1-fast field echo (T1-FFE) mDixon pulse sequence, to correct for T2* relaxation. The scan parameters were as follows: TR = 150 ms, TE1 = 0.85 ms, $\Delta\text{TE} = 0.7 \text{ ms}$, flip angle (FA) = 10° , NSA = 1, FOV = $375 \times 310 \text{ mm}$, acquired matrix size = 92×77 , acquired in-plane resolution = $4.08 \times 4.02 \text{ mm}$, reconstructed matrix size = $384 \times 320 \text{ mm}$, and a reconstructed voxel size of $0.98 \times 0.97 \text{ mm}$. The intrahepatic fat percentage was calculated in three regions of interest (ROI) within the liver parenchyma, carefully avoiding blood vessels. The fat content was expressed as the weighted mean fat signal, divided by the sum of the weighted mean water and fat signal.

The reliability over time was derived from the weight stable control group of the WLI trial consisting of 26 abdominally obese men. Intra-observer reliability of the analyses was assessed by analyzing a random subset of 10 scans derived from lean and obese individuals from the WLI trial twice, with one year in between. In parallel with the

second analysis by the first observer (YK), another subset of 15 scans was analyzed by an experienced and certified abdominal radiologist to determine the inter-observer reliability. In addition, intra-slice, inter-slice and inter-ROI reliability was assessed in the baseline scans of the WLI trial (n = 74).

Moreover, we compared IHL by means of the mDixon sequence to proton resonance spectroscopy (^1H -MRS), the gold standard for non-invasive quantification of IHL, in a random subset of 45 measurements in lean and abdominally obese individuals in the WLI trial. The decision whether additional ^1H -MRS was performed was dependent on the availability of trained personnel. The ^1H -MRS scan was performed using the same MRI scanner and torso coil and the spectra were obtained from a 30 x 30 x 30 mm voxel placed centrally in the right lobe of the liver by using point-resolved spectroscopy volume selection (PRESS) with the following scan parameters: TR = 4000 ms, TE = 32.5 ms, 2048 sample points. A total of 32 spectra with NSA of 2 were acquired. A water signal was acquired as a reference (NSA = 2 x 8). To keep the TR constant, the subject was instructed to breathe exactly at the 4s-rhythm of the sequence. Shimming was performed by using second-order FASTMAP-based shimming.

Intrapancreatic lipid content

Information on IPL was obtained through mDixon imaging with the same MRI scanner and coil. A six mm thick transverse slice through the pancreas was acquired using a 2D three-point T1-fast field echo mDixon pulse sequence, to correct for T2* relaxation. The scan parameters were as follows: TR = 150 ms, TE1 = 0.85 ms, ΔTE = 0.7 ms, FA = 10°, NSA = 1, FOV = 375 x 310 mm, acquired matrix size = 92 x 77, acquired in-plane resolution = 4.08 x 4.02 mm, reconstructed matrix size = 384 x 320 mm, and a reconstructed voxel size of 0.98 x 0.97 mm. IPL was calculated in one ROI within the pancreas parenchyma, preferably encompassing the head and body of the pancreas. In the WLI trial, the ROI was matched between baseline and follow-up measurements. IPL was expressed as the mean fat signal divided by the sum of the mean water and fat signal. The reliability over time was derived from the weight stable control group of the WLI trial consisting of 26 abdominally obese men. Intra-observer reliability of the analyses was assessed by analyzing a random subset of 15 scans derived from lean and obese individuals from the WLI trial twice, with one year in between. In parallel with the second analysis by the first observer (YK), the subset was analyzed by an experienced and certified abdominal radiologist to determine the inter-observer reliability. In contrast to IHL, ^1H -MRS is not considered to be superior to mDixon for IPL quantification, as mDixon allows for image-based determination of the volume of interest, which reduces the risk of erroneously including non-pancreatic tissue into the volume of interest.^{17,18}

Microvascular measurements

Muscle microvascular recruitment

CEUS was used to assess MVR in skeletal muscle. For these measurements, flexor muscles of the right arm were visualized with a Toshiba Aplio XG ultrasound system (Supplementary Figure 3.2A; Toshiba, Japan). On a transparent overhead projector sheet covering the display, characteristic morphological features were copied in order to visualize the exact same ROI with each measurement. The microvascular blood volume (MBV) of forearm skeletal muscle was determined with contrast harmonic imaging. Meanwhile, sulfur hexafluoride gas-filled microbubbles (SonoVue, Bracco diagnostics, The Netherlands) were intravenously and continuously infused. In the CEUS Reproducibility Study, microbubbles were infused during 10 minutes. After three minutes, when steady state microbubble concentration is typically achieved, five real-time replenishment curves of 25 seconds were obtained after microbubble disruption by a high mechanical index ultrasound pulse. Individuals were then asked to perform dynamic handgrip exercises at 80% of their maximal hand grip strength. During the first two minutes individuals contracted once every 5 seconds, followed by one contraction every 30 seconds for another 2.5 minutes, adding up to a total exercise time of 4.5 minutes. During the last 2.5 minutes, five replenishment curves of 25 seconds each were acquired in between muscle contractions. In the WLI study, microbubbles were infused for 6 minutes both in a fasted state, as well as during hyperinsulinemia. After three minutes, five real-time replenishment curves of 25 seconds were acquired after microbubble disruption by a high mechanical index ultrasound pulse. Replenishment curves were stored and analyzed offline upon completion of the study using the CHI-Q software (Toshiba, Japan). One region of interest was drawn in the deep flexor muscles, carefully avoiding blood vessels, and was matched between all measurements. The replenishment curves were fitted to the exponential function $y = A(1 - e^{-\beta t})$ and averaged as described before.^{9,19} Briefly, MBV was calculated by subtracting the value of the fitted curve after one second (y_1) from the plateau phase (A-value); herewith, signal from rapid filling arterioles and venules are discarded. MVR was calculated as the increase in MBV induced by either dynamic handgrip exercises or hyperinsulinemia.

Intra-observer reliability of the measurements were determined in the CEUS Reproducibility Study by measuring microvascular recruitment induced by hand grip exercises twice on each study day ($n = 8$). The reliability over time was determined in two study populations. First, in the CEUS Reproducibility Study where MVR induced by hand grip exercises was assessed on two separate days, one week apart ($n = 7$). Second, from the weight stable control group of the WLI trial where insulin-induced MVR was assessed in 26 abdominally obese men on two separate days, eight weeks

apart. Intra-observer reliability of the analyses was assessed by analyzing a subset of 21 CEUS recordings in lean and abdominally obese individuals that participated in the WLI trial. The subset consisted of 10 random recordings and 11 recordings of relatively poor image quality. The second assessment took place one week after the first MVR analyses were completed.

Capillary microscopy

Nailfold capillaries were visualized by means of intravital videomicroscopy, which allowed for direct visualization of nutritive skin capillaries (Capiscope, KK Technology, Devon, England). Nailfold capillaries of the dorsal skin of the third and fourth finger of the right hand were visualized with a total system magnification of 100 x and recorded at 25 frames per second. Each frame measures 640 x 480 pixels with 256 grey levels per pixel. During the measurements, the investigated finger was immobilized in a mass of clay and paraffin oil was applied to the imaged area of the nailfold to improve skin translucency and reduce skin reflections as described previously (Supplementary Figure 3.2B).²⁰ Capillaries 3 to 5 mm proximal to the terminal row of capillaries in the middle of the nailfold, with the exact location dependent of the image quality and perpendicularity of the visualized capillaries, were analyzed. The ROI size was 1 mm². On a transparent overhead projector sheet covering the monitor, characteristic morphological features were copied in order to visualize the exact same ROI during each measurement, as well as between study days. In case a specific ROI could not be retrieved on the second study day, a different ROI that met the quality standards was recorded. Yet, only ROIs that could be matched between both visits were used to assess the data on reliability over time reported in this manuscript. For each finger, continuously (≥ 15 seconds) perfused capillaries in the rested state, perfused capillaries during hyperemia, capillary recruitment by hyperemia and capillary density during venous occlusion were recorded in a fasted state and during an insulin clamp. Hyperemia was induced by inflation of a miniature cuff (Digit cuff, Hokanson Inc., Bellevue, USA) that was positioned around the base of the finger to suprasystolic pressure (240 mmHg) for four minutes, after which the cuff was rapidly deflated. During the period of post-occlusive reactive hyperemia, all capillaries that were perfused at any moment during a 15-second period were counted. Capillary recruitment during post-occlusive hyperemia (Δ HE) was defined as the difference between number of perfused capillaries during hyperemia and the number of continuously perfused capillaries in the rested condition and is considered a functional capillary reserve capacity. Five minutes after the suprasystolic pressure was released from the cuff, the cuff was re-inflated to 50 mmHg (i.e., subdiastolic pressure) for two minutes in order to induce venous congestion. After one minute, the number of capillaries that were erythrocyte-filled at any moment during a 15-second period were counted; this method is considered to visualize the maximal

number of skin capillaries. Capillary recruitment by hyperinsulinemia (Δ Ins) was defined as difference in terms of percentage between continuously perfused capillaries during hyperinsulinemia and continuously perfused capillaries in the fasted condition. All recorded videos were stored, masked and subsequently analyzed offline by a single investigator (PN). For these offline analyses, a semi-automatic image analysis application (CapiAna, Ed Gronenschild and Alfons Houben, Maastricht, The Netherlands) was used as described into more detail elsewhere.⁸ To exclude a learning effect or a shift in judgment during the analyses of the WLI trial, the recordings were divided into three batches and between group effects and treatment effects were determined for each batch; nonetheless, no difference in either comparison was found between batches.

The reliability over time was derived from the weight stable control group of the WLI trial, which consists of 26 abdominally obese men, of whom 24 men had at least one ROI that was identical between both visits. The intra-observer reliability was determined in a training set of six individuals selected from the Maastricht Study and the inter-observer reliability of the analyses was determined in a second training set of five individuals selected from several previous studies^{11,13} and analyzed by two investigators more than one year apart.

Flowmotion

The periodic blood flow fluctuations, known as flowmotion, which in part arise from rhythmic vessel diameter oscillations known as vasomotion,²¹ were non-invasively assessed in skin by means of laser Doppler flowmetry (LDF; PeriFlux 5000, PeriMed AB, Järfälla, Sweden) as described previously.²⁰ In summary, LDF is based on the emission of a beam of 780 nm wavelength laser light carried by a fiber-optic probe. As the laser light penetrates the skin, a fraction of the light is backscattered by moving erythrocytes, which leads to a shift in frequency proportional to the velocity of erythrocyte movement (i.e., the Doppler principle). The magnitude and frequency distribution of these changes in wavelength are directly related to the number (i.e., volume) and velocity of the blood cells in the sampled volume. This information is detected and transported by a returning optical fiber and converted to an electronic signal. Typically, the measuring depth is 0.5-1 mm, yet as the exact volume that is measured is unknown, the blood flow is expressed in arbitrary units. Subsequently, flowmotion can be analyzed by either Fourier or wavelet analyses, which quantifies the relative contribution of each frequency to the observed LDF signal. Low frequency signals associated with vasomotion arise from endothelial (~ 0.01 Hz), neurogenic (~ 0.04 Hz), and myogenic activity (~ 0.1 Hz).²¹⁻²⁷ The LDF probe was positioned at the level of the left wrist (Supplementary Figure 3.2C), which renders less movement artifacts than the fingers. All recordings were stored, masked, and analyzed offline.

Here, we used Fast Fourier transformation of the LDF signal with the build-in software (PSW 2.5.5, PeriMed AB, Järfälla, Sweden) as it is the method used in previous papers by our research group.

The reliability over time was derived from a subset of 14 abdominally obese men randomized to the weight stable control group of the WLI trial. The subset was based on the enrollment date, i.e., the 14 individuals included in these analyses are the first weight stable controls that participated. The intra-observer reliability of the analyses was determined in a training set of six individuals selected from the Maastricht Study and analyzed more than four months apart. The inter-observer reliability of the analyses was determined in a random subset of 35 lean and abdominally obese men that participated in the WLI trial.

Sublingual microvascular perfused boundary region (Glycocalyx)

The glycocalyx was assessed in terms of the sublingual microvascular perfused boundary region (PBR), which reflects the part of the glycocalyx that can be penetrated by moving erythrocytes, and measured with a dedicated and automated on-line quantification system (GlycoCheck™, Maastricht, The Netherlands).²⁸ The sublingual microcirculation was visualized by placement of a sidestream dark field handheld imaging device under the tongue. Here, the imaging software automatically controlled the quality of the videorecordings and only those of sufficient quality (1 - 2 minutes in total) were recorded. Upon completion of the recordings, the automated software recognizes the microvessels, divides them in over 3000 vascular segments, and calculates in them the PBR as a measure of glycocalyx barrier properties.²⁸ The PBR is reported for microvessels from 5 to 25 μm , where lower PBR-values reflect a healthier glycocalyx barrier function. The reliability over time was derived from the weight stable control group of the WLI trial consisting of 26 abdominally obese men. In contrast to the measurements, the analyses of PBR are automated and therefore observer independent.

Macrovascular measurements

Flow-mediated dilation

Details on flow-mediated dilation (FMD) measurements and part of its reliability have been published elsewhere.¹⁴ Briefly, with the individual in supine position for at least 20 minutes, the right shoulder and arm were positioned on soft supports for optimal comfort and stability. A well-accessible segment of the brachial artery was visualized with a 7.5 MHz, 40 mm linear array ultrasound system (MyLab™ 70, Esaote, Maastricht, The Netherlands) in Duplex-mode to record simultaneously artery diameter and center-line blood flow sonogram. The transducer position was fixed

using a custom-made stereotactic probe holder. The electronically steered pulsed-Doppler beam was set at an angle of 65 - 70 degrees with respect to the vessel long axis. The length of the pulsed-Doppler sample volume was set at approximately half the vessel diameter to ensure consistent registration of the center-line velocity despite possible movements over the recording period. A sphygmomanometer cuff (Hokanson TD 312, D.E. Hokanson Inc., Bellevue, USA) was positioned on the right lower arm and rapidly inflated to at least 200 mmHg (or higher when simultaneous EndoPAT™ measurements indicated incomplete arterial occlusion) for 5 minutes to elicit upon release the hyperemic response required for brachial artery flow-mediated dilation measurement. For each FMD measurement, duplex images were recorded continuously on DVD over a total of 15 minutes: a 5 minutes reference period (i.e., before cuff-inflation), 5 minutes during lower arm cuff-inflation, and 5 minutes during hyperemia following rapid cuff-release. Recorded videos were analyzed offline using proprietary wall detection and Doppler velocity tracing software (MyFMD; Prof. A.P. Hoeks, Department of Biomedical Engineering, MUMC+, Maastricht, The Netherlands) implemented in Matlab (The Mathworks, Natick, USA). In the weight loss study, recordings were masked prior to analysis.

Intra-observer reliability and reliability over time were assessed in the FMD in Acute Hyperglycemia Study.¹⁴ For intra-observer reliability, individuals were measured twice in a fasted state with ten minutes in between on each study day. The second study day was repeated one week later, from which reliability over time was obtained. In addition, reliability over time was also derived from the weight stable control group of the WLI trial, where 23 FMD measurements were available for analysis.¹⁰ Both intra- and inter-observer reliability of the offline analyses were assessed in a random subset of 25 masked FMD measurements in lean and abdominally obese individuals. One observer (JoR) repeated the analyses three days apart. In that same period, a second observer (PJ) analyzed the same subset of FMD measurements.

Reactive hyperemia index

The reactive hyperemia index (RHI) was measured during FMD measurements with a dedicated device (EndoPAT 2000, Itamar Medical Ltd, Caesarea, Israel) as described previously.¹⁰ Briefly, on the index finger of both hands a pneumatic probe that measures arterial pulsatile volume changes (dubbed peripheral arterial tone (PAT) by the manufacturer) is placed. The RHI was quantified as the postocclusion to preocclusion PAT-signal in the occluded hand, normalized to the values in the control hand, then further corrected for baseline vascular tone.²⁹ Reliability over time was determined in a subset of 14 individuals in the weight stable control group of the WLI Trial. The first 14 individuals to complete the study were included in this subset.

Carotid-femoral pulse wave velocity and augmentation index

Carotid-femoral pulse wave velocity (cfPWV), the ratio of travelled distance divided by transit time, was determined according to recent guidelines with applanation tonometry (SphygmoCor, Atcor Medical, Australia) as described previously.^{12,30} Briefly, pressure waveforms were determined at the right common carotid and right common femoral arteries. Difference in the time of pulse arrival from the R-wave of the electrocardiogram between the two sites (transit time) was determined with the maximum upstroke algorithm. The pulse wave travel distance was calculated as the total direct straight distance (measured with an infantometer) between the two arterial sites. The augmentation index (Aix) was determined by radial applanation tonometry and computed by built-in software (SphygmoCor v9, Atcor Medical, Sidney, Australia).^{31,32} Intra- and inter-observer reliability, and reliability over time were assessed in 12 individuals that participated in the Maastricht study and visited the research facility for a second time one week after their first visit. On their first visit, one observer repeated cfPWV and Aix measurements, directly thereafter a second observer repeated the measurements.

Distensibility, distensibility coefficient, compliance coefficient, and carotid intima-media thickness

After completion of cfPWV and Aix measurements, the left common carotid artery (CCA) was visualized directly proximal from the bulbous (0 - 10 mm) in fast brightness mode with a 7.5 MHz, 40 mm linear array ultrasound system (MyLab™ 70, Esaote, Maastricht, The Netherlands) equipped with an ARTLAB system (Esaote, Maastricht, The Netherlands). Ultrasound data and 3-lead ECG signal were simultaneously recorded during 3 consecutive 6 seconds measurements. Herewith, measurements of diameter, distensibility, and carotid intima-media thickness (cIMT) were simultaneously acquired. The cumulative radiofrequency signals were digitized and stored and analyzed offline by custom-build software (Dist13; Prof. A.P. Hoeks, Department of Biomedical Engineering, MUMC+, Maastricht, The Netherlands). Herewith, the change in diameter as a function of time (distensibility) was estimated, diastolic diameter was calculated as the difference in position between the anterior and posterior wall markers, and the cIMT was calculated as the distance from the leading edge interface between lumen and intima to the leading edge interface between media and adventitia of the posterior wall. Subsequently, distensibility coefficient (DC), which represents arterial stiffness, and compliance coefficient, which represents arterial buffering capacity, were calculated using the following formulas³³⁻³⁵:

$$DC = (2\Delta D \times D + \Delta D^2) / (PP \times D^2) \text{ (in } 10^{-3}/kPa)$$

$$CC = \pi \times (2D \times \Delta D + \Delta D^2) / (4 \times P)P \text{ (in } mm^2/kPa)$$

Intra- and inter-observer reliability and reliability over time were assessed in 10 individuals that participated in the Maastricht study and visited the research facility for a second time one week after their first visit. On their first visit, one observer repeatedly measured cIMT and distensibility, directly thereafter a second observer repeated the measurements.

Metabolic measurements

Whole body glucose disposal

It is generally accepted that a euglycemic insulin clamp, as developed by Dr. Andres' group in the 70's,^{36,37} is the best available method to determine peripheral or whole body glucose disposal (WBGD).³⁸⁻⁴¹ With this technique, exogenous insulin is administered to achieve and maintain high physiological insulin levels, usually for 2 hours or longer. Here, insulin was infused in a primed continuous manner; insulin (Novorapid, Novo Nordisk, Bagsvaerd, Denmark) was first infused at 120 mU/kg/min for 10 minutes, after which its infusion rate was lowered to 60 mU/kg/min for 170 minutes. As insulin adsorbs to infusion equipment, 3 ml of whole blood was added per 60 ml syringe to reduce adsorption.⁴² In order to maintain isoglycemia, a variable rate 20% glucose infusion is administered throughout the clamp. Ideally, one aims to maintain arterial glucose at a constant level, yet cannulation of the brachial artery is considered a rather invasive procedure. Instead, a hand is often heated to "arterialize" venous blood.^{37,43,44} This, however, alters blood flow in the heated and contralateral arms, as well as increase norepinephrine levels and thereby influence (micro)vascular and metabolic measurements.⁴³ Therefore, we used the method by Clerk et al., which recognizes that when arterial glucose is maintained at a constant level during an insulin clamp, venous glucose concentrations decline progressively with time, and the arteriovenous glucose difference increases in proportion to insulin sensitivity.¹⁹ In this model, the target for venous glucose to decline is 0.27 mmol/l for every 1 mg/kg/min increase in glucose infusion rate above the basal hepatic glucose production (estimated at 2 mg/kg/min), the rationale for which is based on previous studies and provided into more detail elsewhere.¹⁹ Whole body glucose disposal was estimated from the steady-state glucose infusion rate (90 - 150 min of the clamp). Plasma glucose concentrations during the clamp were measured by first centrifuging 0.5 ml of whole blood at 13,000 x g for 30 seconds and then analyzed with an on-site YSI2300 glucose analyzer (YSI, Yellow Springs, USA).

Beta-cell function

As beta-cell function (BCF) cannot be described by a single measure, we used three dynamic BCF measures derived from mathematically modeling mixed meal test (MMT) data, i.e., beta-cell glucose sensitivity (β -GS), beta-cell rate sensitivity (β -RS) and potentiation factor ratio (PFR).⁴⁵⁻⁴⁷ The parameter β -GS is the slope of the glucose-insulin secretion dose-response function, which reflects the dependence of insulin secretion on absolute glucose concentration at any time during the MMT.⁴⁵⁻⁴⁷ The dose-response relationship is modulated by beta-cell potentiation, which accounts for higher insulin secretion during the descending phase of hyperglycemia than during the ascending phase of an OGTT or MMT for the same glucose concentration. Beta-cell potentiation is set as a positive function of time and averages 1 during the MMT. Therefore, it represents the relative potentiation of the insulin secretion response to glucose. The beta-cell potentiation parameter used in the present analysis represents the ratio of the beta-cell potentiation factor at the end of the MMT relative to the beta-cell potentiation factor at the start. Beta-cell rate sensitivity is a marker of early phase insulin release, and represents the dynamic dependence of insulin secretion on the rate of change in glucose concentration.⁴⁵⁻⁴⁷ The test meal of the MMT consisted of two muffins (containing 56.6 g fat) and 300 ml low-fat milk (0% fat milk; Friesland-Campina, Woerden, The Netherlands). The mixed meal had an energy content of 4598 kJ (fat / carbohydrate / protein: 28 En% / 59 En% / 13 En%) and provided 26.5 g protein, 121.0 g carbohydrates and 56.6 g fat (fatty acid composition: 33.9 g saturated fatty acids, 2.2 g trans fatty acids, 14.5 g monounsaturated fatty acids, and 2.7 g polyunsaturated fatty acids). One batch of muffins was prepared for the entire study by a research nurse; muffins were baked at 180°C in a fan-assisted oven for 20 minutes, packaged per portion, and frozen at -20°C. Reliability of BCF measurements over time was determined in 26 individuals in the weight stable control group of the WLI Trial.

Inflammatory measurements

CD11B surface expression

In *chapter five* of this thesis, we found that CD11B surface expression, measured as mean fluorescence intensity on total circulating NK cells (from here on referred to as NK-CD11B), was closely associated with NK cell accumulation in VAT. CD11B has been described as a maturation marker of NK cells.^{48,49} This integrin can mediate cell adhesion, but is also part of the complement receptor 3 complex, which is involved in cytotoxic activation of NK cells.⁵⁰ Reliability and measurement error of NK-CD11B was determined in a subset of individuals in the weight stable control group of the WLI Trial; the last 22 individuals to complete the study were included in this subset. Blood was drawn after an overnight fast and a 30-minute acclimatization period and frozen

for flow cytometry. Upon completion of the WLI trial, blood was thawed and stained for flow cytometry using CD3-fitc (BD 561807), CD19-fitc (BD 555412), CD66b-fitc (BD 555724), CD56-PE (BD 345810), HLA-DR-V500 (BD 561224), CD14-APC-H7 (BD 641394), CD16-PerCP (BD 560717), CD11B-BV421 (BD 562632), CD11c-PE-Cy7 (BD 561356), and CX3CR1-APC (Biolegend 341609). The gating strategy (Supplementary Figure 3.3) was as follows. From single blood cells, live cells were gated based on forward and side scatters. From live cells, NK cells were gated as negative for CD3 (T cells), CD19 (B cells), CD66b (granulocytes), HLA-DR (monocytes, dendritic cells) and positive for CD56. Singlets were gated based on both forward and side scatters. Live cells were defined as negative for live-death staining. NK cells were CD3- and CD56+, which were subdivided according to CD56 and CD16 surface expression. Each panel was optimized using fluorescence minus one (FMO) controls and isotype controls, yielding similar measures as auto-fluorescent samples. For each experiment, an auto-fluorescence control was performed. Samples were measured with a FACS-Canto II (BD Biosciences, San Jose, CA, USA). Results were analyzed with FACSdiva software (BD Biosciences, San Jose, CA, USA).

Plasma biomarkers of low-grade inflammation

Biomarkers of low-grade inflammation (C-reactive protein, serum amyloid A, interleukin-6, interleukin-8, soluble intercellular adhesion molecule-1, and tumor necrosis factor) were assessed by a multiarray detection system based on electrochemiluminescence technology (SECTOR Imager 2400, MesoScaleDiscovery, Gaithersburg, Maryland, USA) as described previously.⁵¹ This system uses multi-array plates fitted with multielectrodes per well with each electrode being coated with a different catching antibody. The assay procedure then follows that of a classic sandwich ELISA with any of the analytes of interest captured on the relevant electrode. These captured analytes were then in turn detected by a secondary analyte-specific ruthenium-conjugated antibody, which is capable of emitting light after electrochemical stimulation. A particular advantage of this system is the ability to measure different biomarkers of low-grade inflammation and/or endothelial dysfunction simultaneously in relatively small (25 or 50 μ l) serum samples. For reasons of statistical efficiency and to reduce the influence of biological variability,⁵² overall biomarker scores were calculated for endothelial dysfunction and low-grade inflammation as follows.⁵³ First, each individual biomarker was log-transformed if warranted. Then, a z-score was calculated according to the formula (individual value – population mean)/population standard deviation (SD); the population mean and SD were determined from the baseline samples only. Finally, the resulting individual biomarker z-scores were averaged into an overall biomarker score of low-grade inflammation.

Statistical analyses

Intraclass correlation coefficients

All variables in this paper are continuous, hence, intraclass correlation coefficients (ICC) were calculated to provide a parameter of reliability. For all reliability analyses, i.e., inter- and intra-observer reliability and reliability over time, ICC_{agreement} was chosen as systematic differences between observers are included in the error variance, in contrast to ICC_{consistency}.^{1,6,7} For all reliability analyses, except comparison with a gold standard, a two-way mixed model, where people effects are random and the item effects are fixed, was used. This model is chosen when each measurement is rated by the same observers, who are the only observers of interest.⁶ For the comparisons with a gold standard, a one-way random model was used.¹

Limits of agreement and Bland-Altman plots

Measurement error can be found in the limits of agreement, and Bland and Altman have designed a plot in which systematic errors and limits of agreement are presented.⁵ In these plots, the mean of two measurements is plotted on the x-axis, whereas the difference between the measurements is plotted on the y-axis. Every comparison in measurement is added as a dot and, typically, four (horizontal) lines are added. One represents the line of perfect agreement ($y = 0$), one line is added to represent the mean difference between measurements (or observers when inter-observer variance is assessed), and two lines to represent the upper and lower limit of agreement. The limits of agreement are calculated as mean difference $\pm 1.96 \times SD_{\text{difference}}$ and, hence, 95% of the dots will fall between these lines. When differences between repeated measurements or between observers are large, the lines representing the limits of agreement will be further away from the mean difference. Paired T-tests were conducted to assess statistical significance of mean differences. These Bland-Altman plots were made for each comparison.

Standard error of the measurement

The SEM is a parameter of measurement error. It shows how far apart the outcomes of repeated measures are, i.e., it is the standard deviation around a single measurement.¹ A SEM may (SEM_{agreement}) or may not (SEM_{consistency}) include the systematic error (e.g., between observers). SEM_{consistency} equals $\sqrt{S^2_{\text{error}}}$, where residual error can be found in ANOVA or variance component analyses. Yet, it can also be calculated from the limits of agreement as SEM_{consistency} = $SD_{\text{difference}} / \sqrt{2}$. Of note, in case no systemic error is present, SEM_{agreement} equals SEM_{consistency}. Here, we have reported only SEM_{consistency} unless noted otherwise.¹

Linear regression

To analyze the association between IHL measured by means of mDixon and $^1\text{H-MRS}$, linear regression was used. As two techniques were compared, with subjects and observers being identical, no confounders were added to the model.

Software and data expression

Analyses were performed using SPSS version 20. Where appropriate, data are expressed as ICC, SEM, means $\pm$ SD, median [IQR], or count. Two-tailed p-values of <0.05 were considered statistically significant.

Results

Anthropometric measurements

For reliability over time of VAT, SAT, IHL and IPL measurements, data on 26 individuals from the weight stable control group were used. The ICC_{time} of each was > 0.89 (Table 3.1; $p < 0.001$) and the SEM_{time} was 0.17 L for VAT, 0.12 L for SAT, 1.10%-point for IHL (by means of mDixon), and 1.14%-point for IPL. The Bland-Altman plots of the reliability over time with the limits of agreement are presented below (Figures 3.1A, 3.2A, 3.3A and 3.4A).

For intra-observer reliability of analyses, data of 29 (VAT, SAT) or 10 (IHL, IPL) scans were used. The $\text{ICC}_{\text{intra}}$ for each was > 0.95 (Table 3.2; $p < 0.001$) and the $\text{SEM}_{\text{intra}}$ was 7.9 cm^2 per slice for VAT, 4.4 cm^2 per slice for SAT, 0.56%-point for IHL, and 0.66%-point for IPL. The Bland-Altman plots of the intra-observer reliability of the analyses with the limits of agreement are presented below (Figures 3.1B, 3.2B, 3.3B and 3.4B). In addition, we assessed for IHL the ICC for intra-slice (0.976; $n = 74$), inter-slice (0.968; $n = 73$) and overall inter-ROI reliability (0.966; $p < 0.001$ for each; $n = 73$), as well as the $\text{SEM}_{\text{intra-slice}}$ (0.79%-point) and $\text{SEM}_{\text{inter-slice}}$ (0.84%-point).

Inter-observer reliability of analyses was assessed in 29 (VAT, SAT), 15 (IHL) or 14 scans (IPL; one excluded due to technical difficulties). The $\text{ICC}_{\text{inter}}$ was > 0.97 for VAT, SAT and IHL, and 0.88 for IPL ($p < 0.001$ for each). The $\text{SEM}_{\text{inter}}$ was 11.4 cm^2 per slice for VAT, 6.8 cm^2 per slice for SAT, 0.33% for IHL, and 1.72% for IPL. The Bland-Altman plots of the inter-observer reliability of the analyses with the limits of agreement are presented below (Figures 3.1C, 3.2C, 3.3C and 3.4C).

Table 3.1 – Intraclass correlation coefficients for measurements.

	Intra-observer	Inter-observer	Over Time
Anthropometric variables			
Visceral Adipose Tissue	N/A	N/A	0.974 ^{***a}
Subcutaneous Adipose Tissue	N/A	N/A	0.988 ^{***a}
Intrahepatic Lipid	N/A	N/A	0.972 ^{***a}
Intrapancreatic Lipid	N/A	N/A	0.897 ^{***a}
Microvascular variables			
Muscle Microvascular Recruitment	0.886 ^{*b}	N/A	0.828 ^{*c} , 0.613 ^{*a}
Capillary Microscopy (continuously perfused)	N/A	N/A	0.660 ^{**d}
Capillary Microscopy (Δ Insulin)	N/A	N/A	0.049 ^d
Capillary Microscopy (HE)	N/A	N/A	0.815 ^{***d}
Capillary Microscopy (Δ HE)	N/A	N/A	0.670 ^{**d}
Capillary Microscopy (VO)	N/A	N/A	0.811 ^{***d}
Flowmotion (endothelial component)	N/A	N/A	0.628 ^e
Flowmotion (neurogenic component)	N/A	N/A	0.696 ^{*e}
Flowmotion (myogenic component)	N/A	N/A	0.000 ^{e§}
Sublingual microvascular perfused boundary region	N/A	N/A	0.000 ^{a§}
Macrovascular variables			
Flow-mediated Dilation	0.677 ^{*f}	N/A	0.526 ^{*f} , 0.533 ^{**g}
Reactive Hyperemia Index (EndoPAT™)	N/A	N/A	0.579 th
Carotid-Femoral Pulse Wave Velocity	0.984 ^{***i}	0.815 ^{**i}	0.931 ^{***j}
Augmentation Index (@75bpm)	0.610 ^{**i}	0.597 ^{*i}	0.853 ^{**j}
Carotid Distensibility	0.906 ^{***i}	0.750 ^{*i}	0.902 ^{***k}
Distensibility coefficient CCA	N/A	0.846 ^{**i}	0.916 ^{***l}
Compliance coefficient CCA	N/A	0.840 ^{**i}	0.974 ^{***l}
Intima-Media Thickness CCA	0.840 ^{***k}	0.265 ⁱ	0.867 ^{**k}
Metabolic variables			
Whole Body Glucose Disposal	N/A	N/A	0.881 ^{***a}
Beta-cell Glucose Sensitivity	N/A	N/A	0.461 ^{*a}
Beta-cell Rate Sensitivity	N/A	N/A	0.447 ^{*a}
Potential Factor Ratio	N/A	N/A	0.418 ^{*a}
Inflammatory variables			
CD11B Expression on Circulating Natural Killer Cells	N/A	N/A	0.738 ^{**m}
Z-Score Low-Grade Inflammation Plasma Biomarkers	N/A	N/A	0.949 ^{***a}

Data presented as intraclass correlation coefficient (ICC) with absolute agreement in a two-way mixed effects model. Intra-observer, inter-observer ICCs and ICC over time are presented. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Analyzed in ^a $n = 26$; ^b $n = 7$; ^c $n = 6$; ^d $n = 24$; ^e $n = 13$; ^f $n = 22$, as reported in Van Bussel et al. – with permission¹⁴, ^g $n = 23$; ^h $n = 14$; ⁱ $n = 12$; ^j $n = 11$; ^k $n = 10$; ^l $n = 9$; ^m $n = 22$. [§] Negative ICC values were reported as zero, as described before. Δ Insulin = capillary recruitment by hyperinsulinemia, HE = hyperemia, Δ HE = capillary recruitment by hyperemia, VO = venous occlusion, CCA = common carotid artery, N/A was noted when data was not available or not applicable.

For VAT and SAT analyses, pixel-by-pixel quantification had been considered the gold standard.¹⁶ Hence, the VAT and SAT surface per slice obtained by the Hippo Fat software was compared to pixel-by-pixel analyses. The ICC for both was > 0.96 (Table 3.3; $p < 0.001$ for each; $n = 29$) and the SEM was 7.9 cm^2 per slice for VAT and 4.4 cm^2

per slice for SAT. Both VAT and SAT tended to be slightly underestimated when analyzed with the Hippo Fat software. The companion Bland-Altman plots are presented below (Figures 3.1D and 3.2D).

Figure 3.1 – Bland-Altman plots of abdominal visceral adipose tissue (VAT) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two VAT volume measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of VAT area in a single slice; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of VAT area in a single slice; the mean difference was statistically significant (-12.5 cm^2 ; $p < 0.05$). **D.** Agreement between pixel-by-pixel quantification and Hippo Fat software of VAT area, the mean difference was statistically significant (13.8 cm^2 ; $p < 0.05$). Visually, a trend towards greater increase in measurement error with increasing mean VAT area was seen, yet was not statistically significant ($\beta = 0.005$; $p = 0.86$). Nonetheless, these limits of agreement should be interpreted with care.

As for IHL analyses, the mDixon sequence was compared to ^1H -MRS with the latter considered to be the gold standard. The ICC between both techniques was 0.82 ($p < 0.001$; $n = 45$), whereas the SEM was not calculated due to structural overestimation in individuals with low IHL and underestimation in individuals with high IHL, as illustrated by the Bland-Altman plot (Figure 3.3D). By means of linear regression, we found that IHL by means of mDixon was associated with IHL by means of ^1H -MRS (Figure 3.3E; $\beta = 0.432$; $p < 0.001$). Moreover, the presence of NAFLD could

be differentiated nearly as well with mDixon as compared to ^1H -MRS (Cohen's kappa 0.90).

Figure 3.2 – Bland-Altman plots of abdominal subcutaneous adipose tissue (SAT) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two SAT volume measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of SAT area in a single slice; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of SAT area in a single slice; the mean difference was statistically significant (-5.2 cm^2 ; $p < 0.05$). **D.** Agreement between pixel-by-pixel quantification and Hippo Fat software of SAT area; the mean difference was statistically significant (16.1 cm^2 ; $p < 0.05$).

Figure 3.3 – In panel A-D, Bland-Altman plots of intrahepatic lipid (IHL; in %-points) measurements and analyses are presented, where the green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two IHL measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of IHL; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of IHL; the mean difference was not statistically significant. **D.** Agreement between IHL quantification by means of mDixon and ^1H -MRS. As the assumptions of the Bland-Altman analyses are violated, the mean difference and limits of agreement are useless. **E.** Scatterplot between IHL by means of ^1H -MRS and mDixon. The green line represents perfect agreement, whereas the orange line represents the regression equation fitted to the data.

Figure 3.4 – Bland-Altman plots of intrapancreatic lipid (IPL; in %-points) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two IPL measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of IPL; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of IPL; the mean difference was not statistically significant.

Table 3.2 – Intraclass correlation coefficients for analyses.

	Intra-observer	Inter-observer
Anthropometric variables		
Subcutaneous Adipose Tissue	0.998 ^{***a}	0.996 ^{***a}
Visceral Adipose Tissue	0.992 ^{***a}	0.971 ^{***a}
Intrahepatic Lipid	0.995 ^{***b}	0.992 ^{***c}
Intrapancreatic Lipid	0.955 ^{***b}	0.882 ^{***d}
Microvascular variables		
Muscle Microvascular Recruitment	0.977 ^{***e}	N/A
Capillary Microscopy (baseline)	0.940 ^{**f}	0.989 ^{***g}
Capillary Microscopy (HE)	0.995 ^{**f}	0.967 ^{***g}
Capillary Microscopy (Δ HE)	0.881 ^{**f}	0.492 ^{*g}
Capillary Microscopy (VO)	0.956 ^{**f}	0.987 ^{***g}
Flowmotion (endothelial component)	1.000 ^{***g}	0.882 ^{***h}
Flowmotion (neurogenic component)	0.987 ^{***g}	0.957 ^{***h}
Flowmotion (myogenic component)	0.988 ^{***g}	0.847 ^{***h}
Macrovascular variables		
Flow-mediated Dilation	0.881 ^{***i}	0.836 ^{***i}

Data presented as intraclass correlation coefficient (ICC) with absolute agreement in a two-way mixed effects model. Intra- and inter-observer ICCs are presented. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Analyzed in ^a $n = 29$; ^b $n = 10$; ^c $n = 15$; ^d $n = 14$; ^e $n = 21$; ^f $n = 6$; ^g $n = 5$; ^h $n = 35$; ⁱ $n = 25$. N/A is noted when data was not available.

Table 3.3 – Intraclass correlation coefficients between current method and gold standard.

Visceral Adipose Tissue	0.967 ^{***a}
Subcutaneous Adipose Tissue	0.987 ^{***a}
Intrahepatic Lipid	0.823 ^{***b}

Data presented as one-way intraclass correlation coefficient (ICC). ICC SAT and VAT compares analyses with Hippo Fat software to pixel-by-pixel analyses. ICC IHL compares mDixon sequence to 1H-MRS.

*** $p < 0.001$. Analyzed in ^a $n = 29$, ^b $n = 45$.

Microvascular measurements

Muscle microvascular recruitment

In the CEUS Reproducibility Study, the intra-observer reliability of MVR measurement by means of CEUS was assessed in 7 individuals (one individual lacked one MVR measurement due to technical issues) and in 6 individuals over time (two individuals dropped out because of personal issues). ICC_{intra} and ICC_{time} were 0.89 and 0.83, respectively (Table 3.1; $p < 0.05$ for each), whereas SEM_{intra} was 0.17 and SEM_{time} was 0.43 (videointensity). The companion Bland-Altman plots are presented below (Figures 3.5A and 3.5B). The overall intra-observer reliability of MVR measurements was 0.86 ($ICC_{overall}$; $p < 0.001$). In addition, the reliability of MVR measurements over time was also assessed in the weight stable control group of the WLI trial ($n = 26$), where the ICC_{time} was 0.61 ($p < 0.05$), SEM_{time} was 20%, and its Bland-Altman plot is depicted below (Figure 3.5C).

As for the analyses of MVR, ICC_{intra} was 0.98 ($p < 0.001$) and SEM_{intra} was 9.0% ($n = 21$). The Bland-Altman plot of the intra-observer reliability of the analyses with the limits of agreement is presented below (Figure 3.5D). Of note, no adverse events related to CEUS were observed in 143 measurements in 82 unique individuals.

Capillary microscopy

For reliability over time of capillary microscopy measurements, data on 24 individuals from the weight stable control group of the WLI Trial were used as two of the 26 controls (i.e., 8%) had no matching ROI between baseline and follow-up measurements. The ICC_{time} of continuously perfused capillaries, insulin-induced capillary recruitment, perfused capillaries during post-occlusive hyperemia, capillary recruitment by hyperemia and erythrocyte-filled capillaries during venous occlusion were 0.66, 0.05, 0.82, 0.67, and 0.81 respectively (Table 3.1). The SEM_{time} was 11.3 continuously perfused capillaries, 18%-point insulin-induced capillary recruitment, 9.6 capillaries during hyperemia, 8.5 recruited capillaries, and 9.8 capillaries during venous occlusion. The respective Bland-Altman plots are presented below (Figures 3.6A, 3.7, 3.8A, 3.9A and 3.10A). The intra- and inter-observer reliability of capillary

microscopy analyses were assessed in 6 and 5 individuals, respectively. ICC_{intra} for analyses of continuously perfused capillaries, perfused capillaries during post-occlusive hyperemia, capillary recruitment by hyperemia and erythrocyte-filled capillaries during venous occlusion were all >0.88 ($p < 0.01$ for each), as their respective SEM_{intra} was 2.9, 1.2, 3.9 and 4.4 capillaries. ICC_{inter} was >0.96 for continuously perfused capillaries, perfused capillaries during post-occlusive hyperemia and erythrocyte-filled capillaries during venous occlusion, yet for capillary recruitment by hyperemia was markedly lower (0.49; $p < 0.01$ for each ICC; Table 3.2). SEM_{inter} was 1.4 continuously perfused capillaries, 3.4 capillaries during hyperemia, 3.2 recruited capillaries, and 3.5 capillaries during venous occlusion. The Bland-Altman plots of intra-observer (Figures 3.6B, 3.8B and 3.9B) and inter-observer agreement (Figures 3.6C, 3.8C, 3.9C and 3.10C) are presented below.

Figure 3.5 – Bland-Altman plots of measurements and analyses of microvascular recruitment (MVR; in %-points) in forearm skeletal muscle. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Intra-observer agreement between two MVR measurements performed on the same day (1 hour in between); the mean difference was not statistically significant. **B.** Agreement over time between two MVR measurements with 1 week in between; the mean difference was not statistically significant. **C.** Agreement over time between two MVR measurements with 8 weeks in between; the mean difference was not statistically significant. **D.** Intra-observer agreement in analyses of MVR; the mean difference was not statistically significant.

Figure 3.6 – Bland-Altman plots of continuously perfused nailfold capillaries at baseline (BL; i.e., fasted) by means of nailfold capillary videomicroscopy measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two capillary videomicroscopy measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of capillary videomicroscopy; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of capillary videomicroscopy; the mean difference was statistically significant (-3.2 capillaries; $p < 0.05$).

Flowmotion

Data from 13 individuals from the weight stable control group of the WLI Trial were used to assess the reliability of flowmotion analyses over time. For the endothelial (ICC_{time} 0.63; $p = 0.06$) and neurogenic component of flowmotion (ICC_{time} 0.70; $p < 0.05$) appeared to be reliable over time to some extent, whereas the myogenic component was not (Table 3.1; ICC_{time} -0.03 and thus converted to 0.00; $p = 0.52$).^{54,55} The latter could, however, be attributed to one measurement that contained several artifacts; when this measurement was excluded, the reliability increased substantially (ICC_{time} 0.67; $p < 0.05$; $n = 12$). The SEM_{time} was 0.55 arbitrary units (AU) for the endothelial, 0.88 AU for the neurogenic, and 1.57 AU for the myogenic component. If the measurement with artifacts was excluded, SEM_{time} for the myogenic component dropped to 0.77 AU. The companion Bland-Altman plots are presented below (Figures 3.11A, 3.12A, 3.13A and 3.13B).

Figure 3.7 – Bland-Altman plot of agreement over time in insulin-induced increase in continuously perfused nailfold capillaries (Δ Ins; in %-points) by means of nailfold capillary videomicroscopy measurements; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Figure 3.8 – Bland-Altman plots of perfused nailfold capillaries during post-occlusive hyperemia (HE) by means of nailfold capillary videomicroscopy measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two capillary videomicroscopy measurements with 8 weeks in between; the mean difference was statistically significant (-7.4 capillaries; $p < 0.05$). **B.** Intra-observer agreement in analyses of capillary videomicroscopy; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of capillary videomicroscopy; the mean difference was not statistically significant.

Figure 3.9 – Bland-Altman plots of capillary recruitment by hyperemia (Δ HE) by means of nailfold capillary videomicroscopy measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two capillary videomicroscopy measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of capillary videomicroscopy; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of capillary videomicroscopy; the mean difference was statistically significant (7.4 capillaries; $p < 0.05$).

Intra-observer reliability of flowmotion analyses was assessed in a subset of participants of the Maastricht Study ($n = 5$), whereas the inter-observer reliability ($n = 35$) of flowmotion analyses was evaluated in a masked subset of the WLI trial. ICC_{intra} was 1.00 for the endothelial, 0.99 for the neurogenic and 0.99 for the myogenic component (Table 3.2; $p < 0.001$ for each), as the SEM_{intra} of the respective components were 0.02, 0.26 and 0.27 AU. The ICC_{inter} was lower for all components, yet was still > 0.84 (Table 3.2; $p < 0.001$) for each. The SEM_{inter} was 0.30 arbitrary units (AU) for the endothelial, 0.32 AU for the neurogenic, and 0.62 AU for the myogenic component. The Bland-Altman plots of intra-observer (Figures 3.11B, 3.12B and 3.13C) and inter-observer agreement (Figures 3.11C, 3.12C and 3.13D) are presented below.

Figure 3.10 – Bland-Altman plots of erythrocyte-filled capillaries during venous occlusion (VO) by means of nailfold capillary videomicroscopy measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two capillary videomicroscopy measurements with 8 weeks in between; the mean difference was statistically significant (-6.7 capillaries; $p < 0.05$). **B.** Intra-observer agreement in analyses of capillary videomicroscopy; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of capillary videomicroscopy; the mean difference was not statistically significant.

Sublingual microvascular perfused boundary region (Glycocalyx)

Reliability over time of PBR was assessed in 26 individuals from the weight stable control group of the WLI Trial. ICC_{time} was -0.15 and was thus converted to 0.00 ($p = 0.76$;⁵⁴) and SEM_{time} was 0.20 μm . The Bland-Altman plot is presented below (Figure 3.14).

Figure 3.11 – Bland-Altman plots of the endothelial-derived flowmotion spectrum (FM_{EC}) by means of laser Doppler flowmetry (LDF) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two LDF measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of FM_{EC}; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of FM_{EC}; the mean difference was not statistically significant.

Figure 3.12 – Bland-Altman plots of the neurogenic flowmotion spectrum (FM_{NC}) by means of laser Doppler flowmetry (LDF) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two LDF measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Intra-observer agreement in analyses of FM_{NC}; the mean difference was not statistically significant. **C.** Inter-observer agreement in analyses of FM_{NC}; the mean difference was not statistically significant.

Figure 3.13 – Bland-Altman plots of the myogenic flowmotion spectrum (FM_{MC}) by means of laser Doppler flowmetry (LDF) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Agreement over time between two LDF measurements with 8 weeks in between; the mean difference was not statistically significant. **B.** Agreement over time between two LDF measurements with 8 weeks in between, one outlier was excluded to illustrate its effect on agreement; the mean difference was not statistically significant. **C.** Intra-observer agreement in analyses of FM_{MC} ; the mean difference was not statistically significant. **D.** Inter-observer agreement in analyses of FM_{MC} ; the mean difference was not statistically significant.

Figure 3.14 – Bland-Altman plot of agreement over time in sublingual microvascular perfused boundary region (PBR) measurements with 8 weeks in between; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Macrovascular measurements

Flow-mediated dilation

As published before, intra-observer reliability and reliability over time of FMD measurements was determined in a group of 22 individuals from the Acute Hyperglycemia Study.¹⁴ In this study, ICC_{intra} was 0.68, ICC_{time} was 0.53 (Table 3.1; $p < 0.05$ for each), SEM_{intra} was 1.3%-point and SEM_{time} was 1.9%-point; the Bland-Altman plots are presented below (Figures 3.15A and 3.15B). Additionally, we also evaluated reliability over time in 23 individuals from the weight stable control group of the WLI Trial (3 of 26 controls were excluded due to technical issue), which yielded similar results for ICC_{time} (0.53; $p < 0.01$; Table 3.1) and SEM_{time} (1.8%-point).

Intra- and inter-observer reliability of FMD analyses were assessed in a subset of 25 lean and abdominally obese individuals that participated in the WLI trial. ICC_{intra} was 0.88, ICC_{inter} was 0.84 (Table 3.2; $p < 0.001$ for each), SEM_{intra} was 0.3%-point, and SEM_{inter} was 0.7%-point. The Bland-Altman plots of intra-observer and inter-observer agreement of the analyses are presented below (Figures 3.15C and 3.15D).

Reactive Hyperemia Index

Reliability of RHI measurements over time was assessed in a subset of 14 individuals from the weight stable control group of the WLI trial (i.e., the control subjects that were enrolled when 60% of the trial was completed). In this subset, ICC_{time} was 0.58 (Table 3.1; $p < 0.001$) and SEM_{time} was 0.32%-point; the Bland-Altman plot is presented below (Figure 3.16).

Carotid-femoral pulse wave velocity and augmentation index

The intra- and inter-observer reliability ($n = 12$), as well as the reliability over time of cfPWV and Aix measurements ($n = 11$) were determined in a subset of individuals from the Maastricht Study. For cfPWV all ICCs were > 0.81 (Table 3.1; $p < 0.01$ for each), whereas for Aix ICC_{intra} (0.61) and ICC_{inter} (0.60; Table 3.1; $p < 0.05$ for each) were considerably lower. For cfPWV, SEM_{intra} was 0.3 m/s, SEM_{inter} was 1.0 m/s and SEM_{time} was 0.9 m/s. As for Aix, SEM_{intra} , SEM_{inter} and SEM_{time} were 1.8, 4.3 and 3.1%-point, respectively. The companion Bland-Altman plots are presented below (Figures 3.17A-C and 3.18A-C).

Figure 3.15 – Bland-Altman plots of Flow-mediated Dilation (FMD; in %-points) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Intra-observer agreement of FMD measurements; the mean difference was not statistically significant. **B.** Agreement over time between two FMD measurements with 1 week in between; the mean difference was not statistically significant. **C.** Intra-observer agreement over time between two FMD measurements with 8 weeks in between; the mean difference was not statistically significant. **D.** Intra-observer agreement in FMD analyses; the mean difference was statistically significant (-0.2%-point; $p < 0.05$). **E.** Inter-observer agreement in FMD analyses; the mean difference was not statistically significant.

Figure 3.16 – Bland-Altman plot of agreement over time in Reactive Hyperemia Index (RHI) measurements with 8 weeks in between; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Figure 3.17 – Bland-Altman plots of carotid-femoral pulse wave velocity (cfPWV) measurements. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Intra-observer agreement of cfPWV measurements; the mean difference was not statistically significant. **B.** Inter-observer agreement of cfPWV measurements; the mean difference was not statistically significant. **C.** Agreement over time between two cfPWV measurements with 1 week in between; the mean difference was not statistically significant.

Figure 3.18 – Bland-Altman plots of augmentation index (at 75 heartbeats per minute; Aix @75bpm; in %-points) measurements and analyses. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Intra-observer agreement of Aix @75bpm measurements; the mean difference was not statistically significant. **B.** Inter-observer agreement of Aix @75bpm measurements; the mean difference was not statistically significant. **C.** Agreement over time between two Aix @75bpm measurements with 1 week in between; the mean difference was not statistically significant.

Carotid distensibility, distensibility coefficient, compliance coefficient, and intima-media thickness

The intra-observer reliability of carotid distensibility ($n = 12$) and cIMT ($n = 11$), the inter-observer reliability of carotid distensibility, DC, CC and cIMT ($n = 12$ for each), and reliability over time of carotid distensibility ($n = 11$), DC ($n = 9$), CC ($n = 9$) and cIMT ($n = 11$) were determined in a subset of individuals from the Maastricht Study. For carotid distensibility, all ICCs were > 0.75 (Table 3.1; $p < 0.05$ for each) as SEM_{intra} , SEM_{inter} and SEM_{time} were 0.4, 0.8 and 0.5%-point, respectively. For both DC and CC, ICC_{inter} and ICC_{time} were > 0.84 (Table 3.1; $p < 0.01$ for each). For DC SEM_{inter} and SEM_{time} were 1.1 and $2.5 \cdot 10^{-3}$ /kPa, respectively, whereas for CC, SEM_{inter} was 0.14 and SEM_{time} was $0.06 \text{ mm}^2/\text{kPa}$. As for cIMT, both ICC_{intra} and ICC_{time} were > 0.84 ($p < 0.01$

for each), whereas ICC_{inter} was remarkably low (0.27; Table 3.1); SEM_{intra} , SEM_{inter} and SEM_{time} were estimated at 31, 93 and 49 μm , respectively. The companion Bland-Altman plots are presented below (Figures 3.19-22).

Figure 3.19 – Bland-Altman plots of carotid distensibility (Dist.; in %-points) measurements. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Intra-observer agreement of carotid distensibility measurements; the mean difference was not statistically significant. **B.** Inter-observer agreement of carotid distensibility measurements; the mean difference was not statistically significant. **C.** Agreement over time between two carotid distensibility measurements with 1 week in between; the mean difference was not statistically significant.

Figure 3.20 – Bland-Altman plots of carotid distensibility coefficient (DC) measurements. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Inter-observer agreement DC measurements; the mean difference was not statistically significant. **B.** Agreement over time between two DC measurements with 1 week in between; the mean difference was statistically significant (-2.2 /MPa; $p < 0.05$).

Figure 3.21 – Bland-Altman plots of carotid compliance coefficient (CC) measurements. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Inter-observer agreement CC measurements; the mean difference was not statistically significant. **B.** Agreement over time between two CC measurements with 1 week in between; the mean difference was statistically significant (-0.08 mm²/kPa; $p < 0.05$).

Figure 3.22 – Bland-Altman plots of carotid intima-media thickness (cIMT) measurements. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement. **A.** Intra-observer agreement of cIMT measurements; the mean difference was statistically significant (42 μm ; $p < 0.05$). **B.** Inter-observer agreement of cIMT measurements; the mean difference was not statistically significant. **C.** Agreement over time between two cIMT measurements with 1 week in between; the mean difference was not statistically significant.

Metabolic measurements

Whole body glucose disposal

The reliability over time of WBGD measurement by means of a euglycemic insulin clamp was assessed in the weight stable control group of the WLI trial ($n = 26$). In this study population, ICC_{time} was 0.88 (Table 3.1; $p < 0.001$) and SEM_{time} was 0.61 mg/kg/min; the Bland-Altman plot is presented below (Figure 3.23). No adverse events (e.g., hypoglycemia) occurred in 129 clamps in 79 unique individuals.

Beta-cell function

The reliability over time of BCF measurements were assessed in the weight stable control group of the WLI trial ($n = 26$). The ICC_{time} for $\beta\text{-GS}$, $\beta\text{-RS}$ and PFR were 0.46, 0.45, and 0.42 respectively ($p < 0.05$ for each). The SEM_{time} was 11.9 pmol/min/ m^2/mM

for β -GS, 203 $\text{pmol}/\text{m}^2/\text{mM}$ for β -RS, and 0.16 for PFR. The respective Bland-Altman plots are presented below (Figures 3.24-26).

Figure 3.23 – Bland-Altman plot of agreement over time in whole body glucose disposal (WBGD) measurements by means of a euglycemic insulin clamp with 8 weeks in between; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Figure 3.24 – Bland-Altman plot of agreement over time in beta-cell glucose sensitivity (β -GS) measurements by means of mathematically modeling mixed meal test data with 8 weeks in between; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Figure 3.25 – Bland-Altman plot of agreement over time in beta-cell rate sensitivity (β -RS) measurements by means of mathematically modeling mixed meal test data with 8 weeks in between; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Figure 3.26 – Bland-Altman plot of agreement over time in potentiation factor ratio (PFR) measurements by means of mathematically modeling mixed meal test data with 8 weeks in between; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Inflammatory measurements

CD11B surface expression

Data from 22 individuals from the weight stable control group of the WLI Trial were used to assess the reliability of NK-CD11B over time. ICC_{time} was 0.74 ($p < 0.01$) and SEM_{time} was 2231 arbitrary units. The respective Bland-Altman plot is presented below (Figure 3.27).

Plasma biomarkers of low-grade inflammation

The reliability over time of z-LGI was assessed in the weight stable control group of the WLI trial ($n = 26$); ICC_{time} was 0.949 ($p < 0.001$). Since z-LGI is dependent of the population it is measured in, neither SEM_{time} was calculated, nor was a Bland-Altman plot created.

Figure 3.27 – Bland-Altman plot of agreement over time CD11B expression on circulating NK cells (NK-CD11B) measurements by means of flow cytometry with 8 weeks in between; the mean difference was not statistically significant. The green line represents perfect agreement, the orange line represents the mean difference, and the red lines represent the limits of agreement.

Discussion

In this report we established the reliability and measurement error of the measurement and analyses of several anthropometric, vascular, metabolic, and inflammatory variables. To our knowledge, it is the first study that A) reported the reliability and measurement error on these variables within a single research group, B) reported the reliability and measurement error of the measurement and (offline) analyses of these variables separately, and C) reported reliability and measurement error of MVR by means of CEUS. We will address the reliability and measurement error of the gamut of variables below.

Anthropometric variables

The measurements and the analyses of both VAT and SAT volumes were very reliable and had little measurement error. The reliability presented here is in line with intra- and inter-observer reliability of VAT and SAT quantification by means of computed

tomography (CT) analyses in the Framingham Study (ICC = 0.99 for each).^{56,57} Only when we compare VAT quantification methods, was there a non-significant trend towards increasing differences between both methods with increasing VAT volume. This difference may have arisen from the limitations of pixel-by-pixel analyses, in which an observer often either does or does not count a pixel at the edges between VAT and surrounding tissue, whereas software like Hippo Fat uses thresholding of grey scales to quantify VAT. Hence, although pixel-by-pixel analyses are often considered to be the gold standard, this may be open for debate. As both VAT and SAT volumes are very reliable within and between trained observers, there are no objections to letting multiple observers analyze such data, though it would be advisable to adjust for systematic differences between observers.

As for IHL and IPL, measurements and analyses were reliable, although both tended to have a considerable measurement error over a time course of 8 weeks. This may, at least in part, be due to the expected biological variance in a control group of a WLI Trial in which some controls are likely to better their life style whereas others may loosen the reins. In many weight loss intervention studies a control group is, however, lacking; as a result data on biological variance of IHL and IPL are scarce. As the SEM_{time} of IHL and IPL were comparable, this indicates more variance over time in IPL values than IHL. In addition, since regional differences in the lipid content of both liver and pancreas are common, it would be recommended to standardize the location of measurement, in particular in repeated measurements, as well as to determine IHL and IPL in multiple volume of interest, if possible.^{17,58}

With regard to the comparison of mDixon to ¹H-MRS to assess IHL, there was a high level of agreement and NAFLD could be differentiated well. Yet, there was some overestimation of IHL at low levels and an underestimation at higher IHL-levels, which would make mDixon prone to type 2 errors. As the signal intensity in water and fat images of mDixon can only adopt positive values, noise is erroneously counted as IHL, particularly at low IHL levels. Thus, this appears to be intrinsic to the mDixon sequence. In a recent study comparing mDixon to ¹H-MRS, the ICC between both methods was substantially higher (0.98) and the SEM was very low (0.01%-point, calculated from⁵⁸). This difference might be due to incorporation of bile ducts and blood vessels in the volume of interest, which ¹H-MRS does as well, as well as due to different scan parameters. Therefore, we chose not to recalculate IHL values based on the available data. It is worthwhile to compare our current mDixon settings to those used in different studies to evaluate whether reliability and measurement error can be further improved and type 2 errors avoided.

Microvascular variables

Here, we first present the reliability and measurement error of MVR by means of CEUS. We consider the CEUS-based measurements of MVR to be safe, reliable, and to have a low intra-observer measurement error (i.e., within-day) and a moderate measurement error over time (20%-point MVR by hyperinsulinemia, 8 weeks between visits). When the time interval was shorter and handgrip exercises were used to induce MVR instead of hyperinsulinemia, the reliability of MVR measurements obviously increase. Nonetheless, the overall reliability and measurement error of MVR measurements, either induced by exercise or hyperinsulinemia, would endorse the use of CEUS even in relatively small study populations. Of note, when the expected effect of an intervention is estimated to be lower than the measurement error of this technique, the sample size needed to demonstrate an effect on MVR will increase considerably.¹

With regard to MVR analyses, the intra-observer reliability of a single, well-trained observer is considered to be excellent, whereas inter-observer reliability was not assessed at this time. Likely, the intra-observer reliability reported here is even underestimated, as measurements of substandard image quality were oversampled. Because inter-observer reliability is lacking here, we cannot advise whether MVR can be measured or analyzed by multiple observers without introducing considerable error. Nonetheless, no benefit is to be expected from letting multiple observers analyze the same data to improve reliability. Instead, stakeholders should invest in adequate training of study staff to minimize error in the acquisition and analyses of MVR data, as well as limit the number of observers trained to perform CEUS and its analyses to ensure sufficient expertise.

In contrast to MVR by means of CEUS, we found that reliability was lower for other microvascular variables. Although the reliability of continuously perfused capillaries, perfused capillaries during post-occlusive hyperemia and erythrocyte-filled capillaries during venous occlusion for both measurements as well as analyses were reasonably good, the reliability of capillary recruitment by hyperemia or hyperinsulinemia was either considerably lower or absent. This allows for several conclusions. First, this illustrates the importance of measurement error, as measurement error was comparable between all videomicroscopy measures, whereas mainly the between-subject variance differs between these variables and, by extension, oft provides a false sense of reliability when other variables than capillary recruitment are reported. Second, the reliability of nailfold capillary recruitment by hyperemia is comparable to that of FMD, a generally accepted vascular function measurement. Third, insulin-induced capillary recruitment has a comparable measurement error as MVR, yet has substantially less between-subject variance. Finally, inter-observer variance is

particularly bothersome in nailfold videomicroscopy. As capillary videomicroscopy analyses are laborious, we advise to adjust for the systematic error between observers and to limit the number of observers; to analyze data by more than one observer to increase reliability is far from realistic for most studies.

As for flowmotion, reliability over time for the endothelial and neurogenic component were reasonable, whereas no reliability was found for the myogenic component, although the latter could be attributed to a measurement that contained multiple artifacts. This underscores the importance of efforts to reduce the influence of recorded artifacts, either by using different analyses (e.g., wavelet analyses), by improving the quality of the recordings, or by extending the duration of the measurements to allow the selection of an artifact-free time frame.

As it is impossible to measure flowmotion at the exact same recording site twice,^{59,60} it could be considered to measure flowmotion at several standardized sites and then average the results of those recordings. One could justify the latter by the assumption that the microvascular dysfunction one strives to quantify would affect the skin microvasculature as a whole, not just focally. Although inter-observer reliability of flowmotion analyses was good, the difference between observers is most obvious in recordings of poor quality. Hence, reliability of analyses would profit by using different analyses (e.g., wavelet analyses), by improving the quality and number of the recordings, by limiting the number of observers to one observer, or – in contrast – by increasing the number of observers. To differentiate whether just one experienced observer or multiple observers should analyze flowmotion data, one should study the raw data to identify the origin of error. For example, does one observer handle data, perhaps those of poor quality, in a wrong way or just differently? Or are the differences due to the selection of the analyzed timeframe in the recordings? This should be further investigated in future reliability studies.

Lastly, reliability over time of PBR (i.e., glyocalyx) in the sublingual vascular bed was absent in this study population. In statistical terms this means that the between-subject variance was very low, whereas the measurement error was large, which is visible in the Bland-Altman plot and the SEM. The absence of reliability is in contrast to the whitepaper by the manufacturer, which states that PBR is “highly reproducible”, although data on reliability was not provided, nor cited.²⁸ We, therefore, advise against the use of this technique for research questions where the estimated effect size is low (e.g., below the SEM), like in the subset of abdominally obese individuals without T2DM used in our analyses. Conversely, in situations with large between-subject variance, like a septic shock, PBR might be more reliable. This is illustrated by a recent study that assessed inter-observer reliability of PBR in a combined population of patients in the emergency room or intensive care unit, where

reliability was markedly higher (ICC = 0.75) as a result of larger between-subject variance (i.e., PBR ranged from ~1.8 to ~3.2.⁶¹ Interestingly, the SEM was comparable to our data (SEM 0.18 μ m, calculated from⁶¹). Hence, as we stated before, reliability data should only be judged in the population and clinical setting it is measured in.

Macrovascular variables

As published before, the intra-observer reliability of FMD measurements as well as the reliability over time, were reasonable.¹⁴ The reliability over time of FMD measurements in obese individuals randomized to the weight stable control group was comparable to data from our¹⁴ and other research groups.⁶²⁻⁶⁶ As for the analyses of FMD, intra-observer reliability was excellent, whereas inter-observer reliability was slightly lower, mainly in measurements of substandard image quality. We should, however, not neglect that the measurement error of FMD measurements is substantial, which should significantly impact power calculations. If we, however, compare FMD to other endothelial-dependent and nitric-oxide mediated vascular measurements like MVR, nailfold capillary recruitment and the endothelial component of flowmotion, the reliability of FMD appeared to be slightly lower, although not statistically significantly. The reliability of RHI over time was acceptable and comparable to the ICC and SEM in adult volunteers reported elsewhere (ICCs 0.56-0.73; SEM_{time} 0.38;^{67,68}). In our study population, reliability of RHI and FMD were comparable, whereas often FMD is considered to be somewhat more reliable when measured simultaneously.⁶⁷ As FMD and RHI measurements had a SEM comparable to other studies,^{14,62,65-67} either FMD had less between-subject variance, or RHI had more between-subject variance than expected.

Our data on the inter-observer and intra-observer reliability and measurement error of cfPWV and Aix were excellent and are slightly better than reported in individuals with type 2 diabetes.⁶⁹ In a different study, where both healthy individuals and individuals with end-stage renal disease were included, reliability was obviously higher, yet SEM or limits of agreement were not presented.⁷⁰

As for carotid distensibility, DC and CC, reliability and measurement error were excellent and were comparable to those reported by others.⁷¹⁻⁷³ As these measurements are already usually repeated three times, little improvement in reliability is to be expected from increasing the number of measurements or observers. Unfortunately, no data was available to determine the reliability and measurement error of the analyses of these variables. It would be advisable to determine these in the near future, though, since variables like these are used in large study samples (e.g., the Maastricht Study), where having a single observer for analyses is often not feasible.

The intra-observer reliability and reliability over time of cIMT, as well as their measurement error were excellent and comparable to those reported by others.⁷¹ The inter-observer reliability was, however, considerably lower, without any evidence for a structural difference between the two observers. Although inter-observer reliability was poor in this study population, its SEM was actually comparable to studies that reported good inter-observer reliability.⁷¹ Here, again, the crux is the lack of between-subject variance, as all cIMT measurements in our population were between 0.75 and 1 mm. The appreciation of cIMT measurements should therefore, as with every measurement, be related on the population and situation it is determined in. In addition, it is known that cIMT measurements have a substantial measurement error that depends, for example, on the system used, the anatomical location measured and the observer that performs the measurement.⁷⁴ Thus, standardization of cIMT measurements is essential to reduce measurement error. From our data, it would also be sensible to either have each individual measured by multiple observers or to have one observer measure all individuals.

Metabolic variables

The reliability of WBGD over time in our population was excellent. We can compare the reliability and measurement error of WBGD in our study population to the first study on the euglycemic insulin clamp, in which arterial blood glucose was drawn and repeated measures were available in four individuals. In that study,³⁷ the ICC_{time} was 0.97 and the SEM_{time} was 0.32 mg/kg/min. That study most likely had a superior reliability compared to ours due to 1) the use of arterial blood glucose levels, 2) less biological variance over time, because there was less time in between measurements (1 week vs. 8 weeks), and 3) a greater between-subject variance of WBGD. As for dynamic BCF measurements on the other hand, reliability was modest and SEM_{time} was reasonable. Since obese men were studied, between-subject variance may have been relatively small; this may have reduced the reliability of the BCF measurements.

Inflammatory measurements

To our knowledge, the reliability of NK-CD11B over time had not been described previously, yet it proved to be very good. For z-LGI, reliability over time was downright excellent, which illustrates the statistical effectiveness of this approach, as argued before.^{51,52}

Our study has several strengths. First, we simultaneously presented various commonly used anthropometric, vascular, metabolic and inflammatory variables, many of which are reported in this thesis. In addition, we assessed not only reliability, but also measurement error in order to gain a deeper understanding of the performance of

each technique regardless from the between-subject variance in the study population. Moreover, we assessed reliability and measurement error for both the measurement of a variable as well as its analyses, which allows for a more detailed understanding of sources of error.

Several limitations of this reliability study should also be noted. First, for many variables the sample size was relatively small to obtain a robust estimate of reliability. Second, reliability data were currently not available for all possible comparisons; future studies should fill in the gaps. Third, we often used the weight stable control group of the WLI trial which consisted of abdominally obese men only that were partaking in a life style trial. It is known that participation in a trial in itself leads to changes in behavior that influence outcome variables, which by extension reduces reliability. As this held true for many variables, their reliability can be effectively compared to one another. Fourth, ICCs are only relevant in the population it is measured in and (when applicable) to the stimulus used (e.g., handgrip exercises or hyperinsulinemia for MVR). Finally, reliability and measurement error are dependent on the observer(s) who performed the measurements or analyses; they may differ substantially when other observers are evaluated.

Conclusion

Here, we established the reliability and measurement error of MVR by means of CEUS for the first time, as well as determine reliability and measurement error of other commonly used anthropometric, vascular, metabolic and inflammatory variables. This detailed understanding of sources of measurement error and the reliability of variables in a certain study population allows for the adequate selection of outcome measurements in observational and intervention studies, as well as provide data for more accurate sample size calculations for such studies.

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Supplementary material

Table S3.1 – Study population characteristics CEUS Reproducibility Study.

Age [Yr]	26.4 [20.3 - 29.4]
Sex [M/F]	3 / 5
yWeight [kg]	68.3 [54.4 - 78.9]
BMI [kg/m ²]	20.9 [20.3 - 24.0]
Office Systolic Blood Pressure [mmHg]	118 [113 - 128]
Office Diastolic Blood Pressure [mmHg]	70 [66 - 78]
Maximum handgrip power [kg]	21 [17 - 31]

Data presented as median [IQR] or count.

Figure S3.1 – Domains of health measurement instruments and relationships of measurement properties according to the COSMIN taxonomy. The domain reliability refers to the extent to which scores for individuals who have not changed are the same for repeated measurement under several conditions. The domain reliability consists of three measurement properties. Internal consistency is specific to patient reported outcomes and is not applicable to the variables we investigated in this paper. The measurement property reliability refers to the proportion of the total variance in the measurements which is due to the “true” differences between individuals, as described in the classical test theory.^{2,75} Measurement error refers to the systematic and random error of a patients’ score that is not attributed to true changes in the construct to be measured.² Both reliability and measurement error can be further subdivided in intra-observer (repeated measurements by one observer), inter-observer (repeated measurements on the same occasion by different observers), or over time (test-retest with relevant amount of time in between). For further explanation of the other domains, we refer to the original COSMIN statement.² COSMIN, Consensus-based Standards for the selection of health Measurement INstruments; HR-PRO, health related-patient reported outcome. Reproduced from: Mokkink et al. 2010, with permission.²

A **B** **C**

Figure S3.2 – Position of individuals during several microvascular measurements. **A.** Position of the transducer to image the right forearm flexor muscles by means of contrast enhanced ultrasound. **B.** Capillary videomicroscopy of the right middle finger. **C.** Flowmotion measurement (laser Doppler flowmetry) located at the left hand, just distal of the wrist. Photography by Dr. J.L.J.M. Scheijen, with permission.

Figure S3.3 – Gating strategy for flow cytometry in the weight loss intervention trial, which is described into more detail in the main text.

A black and white photograph of a desert landscape featuring large, undulating sand dunes. The dunes are characterized by distinct horizontal ripples and shadows that emphasize their curves and ridges. The lighting creates a high-contrast scene, with bright highlights on the crests and deep shadows in the valleys. Overlaid on the lower half of the image is the text 'chapter 4' in a large, bold, sans-serif font. The word 'chapter' is white, while the number '4' is a dark gray, matching the tones of the dunes.

chapter 4

Chapter 4

Independent tissue contributors to obesity-associated insulin resistance

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J Clin Invest Insight. 2017 Jul 6;2(13)

Abstract

Background

Induction of insulin resistance is a key pathway through which obesity increases risk of type 2 diabetes, hypertension, dyslipidemia, and cardiovascular events. Although the detrimental effects of obesity on insulin sensitivity are incompletely understood, accumulation of visceral, subcutaneous and liver fat, and impairment of insulin-induced muscle microvascular recruitment (MVR) may be involved. As these phenotypic changes often coincide in obesity, we aimed to unravel whether they independently contribute to insulin resistance and thus constitute *separate* targets for intervention.

Methods

We measured visceral (VAT) and subcutaneous adipose tissue (SAT) volumes and intrahepatic lipid content (IHL) by MRI, and whole body glucose disposal (WBGD) and MVR (using contrast-enhanced ultrasound) responses to a euglycemic insulin clamp in lean ($n = 25$) and abdominally obese men ($n = 52$). Abdominally obese men were randomized to dietary weight loss intervention or habitual diet.

Results

Obesity-associated increases in VAT, SAT and IHL, along with the decrease in MVR, contributed independently to insulin resistance. Moreover, a dietary weight loss intervention reduced insulin resistance and mediation analyses showed that decreased IHL and insulin-induced MVR, but not decreased VAT or SAT volumes, independently contributed to improved insulin resistance seen with weight loss.

Conclusion

Quantifying the mutually independent contributions of visceral and subcutaneous adipose tissue, intrahepatic lipid, and insulin-induced muscle microvascular recruitment reveals distinct targets for treating obesity-associated insulin resistance.

Introduction

Insulin resistance, i.e. reduced insulin-induced whole body glucose disposal (WBGD), is common in obesity and plays a key role in obesity-associated type 2 diabetes, hypertension, dyslipidemia and cardiovascular disease.^{1,2} Several tissue-specific pathways, including adipose tissue, liver and muscle, have been proposed to link obesity to insulin resistance; however, whether these pathways act independently of each other has not been elucidated.

Excess visceral adipose tissue (VAT) in obesity causes insulin resistance through increased lipolysis, dysregulation of adipokine release, and induction of low-grade inflammation.²⁻⁴ However, additional mechanisms that explain insulin resistance in obesity have been postulated, notably increased subcutaneous adipose tissue (SAT),² increased intrahepatic lipid (IHL) accumulation,⁵ and impairment of insulin-induced microvascular recruitment (MVR) in muscle.⁶

SAT's effect on insulin resistance is controversial; the increased lipolysis and adipokine dysregulation in SAT is considered to contribute to insulin resistance,² yet a fat distribution pattern with high SAT and low VAT volumes is considered a metabolically healthy phenotype.⁷ The involvement of IHL on the other hand is also debated, as IHL has been postulated as both a cause and consequence of insulin resistance. Lipotoxicity of IHL rich in saturated fatty acids, diacylglycerols and ceramides; secretion of inflammatory cytokines; and increased triglyceride synthesis by the fatty liver are suggestive of the former,⁸⁻¹⁰ whereas adipocyte insulin resistance with increased NEFA fluxes towards the liver suggest the latter.^{11,12} In turn, skeletal muscle's vasculature can contribute to insulin resistance when physiological insulin-induced, nitric oxide-mediated MVR is impaired.¹³

A key question is whether VAT, SAT, IHL, and MVR act independently to increase insulin resistance. Mutual independence, in contrast to mutual dependence, implies that distinct mechanisms can be targeted to improve insulin resistance.

We addressed this issue in two ways. First, we determined the independent contributions of VAT, SAT, IHL, and MVR to insulin resistance in 25 lean and 52 abdominally obese men (flow diagram in Figure 4.1). Next, we assessed their contributions to weight loss-induced improvement of insulin resistance by randomizing the abdominally obese men to either an 8-week dietary weight loss program or habitual diet (control group).

Figure 4.1 – A flow diagram of participants throughout the study. After screening, 25 lean men were eligible for participation and all completed the study. Baseline measurements were performed in 54 abdominally obese men; one dropped out for personal reasons before completing the measurements. For the cross-sectional analyses, all lean men and 52 abdominally obese men were included, one was excluded from analyses due to protocol violations (use of anti-hypertensive medication during participation). One lean individual did not complete the MR imaging due to claustrophobia. A total of 53 abdominally obese men were randomized to weight-loss intervention or weight-stable control groups. One individual discontinued control treatment because of illness, one dropped out because of noncompliance with the program to lose weight and another individual dropped out for personal reasons after assignment to the weight-loss treatment. One individual completed the trial but was excluded from all analyses because of protocol violations. In agreement with the protocol, the study was stopped after 50 completed follow-up measurements. Few harms were observed during this study; one individual had an incidental finding on MRI which required further analysis and another individual developed thrombophlebitis after the follow-up measurements.

Methods

Study population

In this randomized controlled trial with blinded analyses, 53 abdominally obese and 25 lean men were recruited by advertisements and enrolled at the Maastricht University Medical Center in Maastricht, The Netherlands, from August, 2012 to May, 2014. Participants were aged 18 - 65 years, non-smokers, nondiabetic, free of CVD, had a waist circumference below 94 cm (lean) or between 102 - 110 cm (abdominally obese), and a stable body weight for at least 3 months. Men were excluded when fasting plasma glucose was > 7.0 mmol/l, when HbA_{1c} was $> 6.5\%$, when serum total cholesterol was > 8.0 mmol/l, when serum triacylglycerol was > 4.5 mmol/l, when they had a contra-indication for MR imaging, and/or when they used medication affecting blood pressure, serum lipid or glucose metabolism.

Study design

Abdominally obese men were randomly assigned in a 1:1 ratio to either an 8-week weight loss program or maintaining their habitual diet for 8 weeks. Abdominally obese men were studied at baseline and after completion of their 8-week program. Lean men were studied at baseline only and thus not randomized. Randomization was performed by an independent investigator using block randomization with variable block sizes and stratifying for ages below and above 50 years. The allocation was revealed by the independent investigator to the participant and research team upon completion of all baseline measurements. Study data were blinded to the assessor prior to analysis and de-blinded upon completion of all analyses by an independent investigator.

The weight loss program consisted of 4-5 weeks of a very low calorie diet providing 2.1 MJ/day (Modifast, Novartis Nutrition, Breda, The Netherlands), 1 - 2 weeks of energy-restricted diet providing 4.2 MJ/day, a weight stable phase of 2 weeks to maintain the newly achieved weight, and weekly dietary counseling. During their 8-week habitual diet, the control group was monitored as well in order to avert fluctuations in weight. Both groups were instructed not to alter their exercise pattern throughout the study. Three individuals dropped out and one individual was excluded from analyses due to protocol violations (Figure 4.1). A sample size of 25 individuals per group was calculated to detect a mean difference in MVR, the least precise of the techniques used, of 20% with a power ($1-\beta$) of 0.80 with $\alpha = 0.05$. In agreement with our protocol, the study was stopped after 50 completed follow-up measurements.

Measurements were conducted in a temperature-controlled room ($T = 24^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) after a 12-hour overnight fast with the individuals in the supine position. Individuals were instructed to refrain from alcohol and meals rich in lipids for a period of 24 hours prior to each study day, and strenuous physical exercise for a period of 48 hours prior to each study day. After insertion of two intravenous catheters and a 30-minute acclimatization period, baseline vascular measurements were performed.

Hyperinsulinemic euglycemic clamp

Metabolic insulin sensitivity was assessed by a 1 mU/kg/min euglycemic insulin clamp as described previously.²⁹ Briefly, insulin (Novorapid, Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed continuous manner for 180 minutes. Meanwhile, isoglycemia was maintained with a variable rate 20% glucose infusion. Metabolic insulin sensitivity was estimated from the steady-state glucose infusion rate (90 - 150 min of the clamp).

Contrast enhanced ultrasound

Microvascular blood volume of forearm skeletal muscle was measured with a Toshiba Aplio XG ultrasound system (Toshiba, Otawara, Japan) during continuous infusion of sulfur hexafluoride gas-filled microbubbles (SonoVue, Bracco diagnostics, Amsterdam, The Netherlands). After steady state microbubble concentration was achieved (3 minutes), five real-time replenishment curves of 30" were obtained after microbubble disruption by a high mechanical index ultrasound pulse. These replenishment curves were stored and analyzed offline in a blinded fashion upon completion of the trial using the CHI-Q software (Toshiba, Otawara, Japan). The replenishment curves were fitted to the exponential function $y = A(1 - e^{-\beta t})$ and averaged as described before²⁶ providing reliable estimations of MVR ($\text{ICC}_{\text{intra-observer}} = 0.86$; $p < 0.001$). MVR was calculated as the relative increase in microvascular blood volume induced by the insulin clamp.

Visceral and subcutaneous fat volumes, and intrahepatic lipid

Information on visceral and subcutaneous adipose tissue volumes was obtained through two-dimensional (2D) T1-weighted turbo spin echo (TSE) imaging on a 3.0T Philips Achieva MRI scanner with a dedicated sixteen-element torso coil (XL Torso coil, Philips Healthcare, Best, The Netherlands). Nine 5 mm thick transverse slices with 10 mm gaps centered at the top of the L4 vertebral body were acquired using the following scan parameters: repetition time (TR) = 526 ms, echo time (TE) = 10 ms, turbo spin echo factor = 4, number of signal averages (NSA) = 1, field of view (FOV) = 400 x 322mm, acquired matrix size = 308 x 164, acquired in-plane voxel size = 1.3 x 1.96 mm, reconstructed matrix size = 512 x 412, and a reconstructed voxel

size of 0.78 x 0.78 mm. Images were analyzed offline after completion of the trial with dedicated software (Hippo Fat, IFC CNR, Pisa, Italy) to provide subcutaneous and visceral fat volumes.

The same MRI scanner and coil were used to assess intrahepatic lipid content through mDixon imaging. Two 6 mm thick transverse slices through the liver were acquired using a 2D three-point T1-fast field echo (T1-FFE) mDixon pulse sequence, to correct for T2* relaxation. The scan parameters were as follows: TR = 150 ms, TE1 = 0.85 ms, $\Delta TE = 0.7$ ms, flip angle (FA) = 10°, NSA = 1, FOV = 375 x 310 mm, acquired matrix size = 92 x 77, acquired in-plane resolution = 4.08 x 4.02 mm, reconstructed matrix size = 384 x 320 mm, and a reconstructed voxel size of 0.98 x 0.97 mm. The intrahepatic fat percentage was calculated in three regions of interest within the liver parenchyma, carefully avoiding blood vessels. The fat content was expressed as the weighted mean fat signal, divided by the sum of the weighted mean water and fat signal. The mDixon sequence was validated against ^1H -MRS in a random subset of participants and yielded comparable results ($n = 45$; ICC = 0.82; $p < 0.001$). The ^1H -MRS scan was performed using the same MRI scanner and torso coil and the spectra were obtained from a 30 x 30 x 30 mm voxel placed centrally in the right lobe of the liver by using point-resolved spectroscopy volume selection (PRESS) with the following scan parameters: TR = 4000 ms, TE = 32.5 ms, 2048 sample points. A total of 32 spectra with NSA of 2 were acquired. A water signal was acquired as a reference (NSA = 2 x 8). To keep the TR constant, the subject was instructed to breathe exactly at the 4s-rhythm of the sequence. Shimming was performed by using second-order FASTMAP-based shimming.

Plasma measurements

Plasma glucose was determined with a YSI2300 glucose analyzer (YSI, Yellow Springs, OH, USA). Samples were analyzed for total cholesterol (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), HDL cholesterol (precipitation method; Roche Diagnostics, Mannheim, Germany), triacylglycerol with correction for free glycerol (GPO Trinder; Sigma-Aldrich, St. Louis, MO, USA), HbA1c (Bio-Rad, Hercules, CA, USA), and insulin (RIA; Millipore, Billerica, MA, USA).

Statistics

Data are expressed as means $\pm$ SD or median [25th - 75th percentile]. Two-tailed independent Student's t-tests or Mann-Whitney U tests were used to assess differences between groups at baseline. We used one-factor ANCOVA with the baseline value as a covariate to detect differences between groups over time. Analyses were performed using SPSS version 20. Multiple mediation analyses were

carried out with the PROGRESS plug-in for SPSS version 2.13 (A.F. Hayes, Ohio State University, OH, USA). In the cross-sectional mediation model, complete mediation was found. Therefore, the independent variable BMI was omitted from the model to estimate the contributions of each mediator³⁰ and Monte Carlo confidence intervals were calculated with R statistical software (version 3.2.2). For sensitivity analyses we also used multiple mediation analyses with a repeated measures design, which were carried out with the MEMORE plug-in for SPSS. All regression and mediation models were adjusted for the potential confounder age. Two-tailed p-values of <0.05 were considered statistically significant.

Study approval

The study was approved by the Maastricht University Medical Center ethics committee, performed in accordance with the Declaration of Helsinki, and registered at clinicaltrials.gov (NCT01675401). All participants gave written informed consent.

Results

Demographic and metabolic features of the study population

After a screening procedure, 25 lean and 52 abdominally obese individuals were enrolled in this study (Figure 4.1). Compared with lean men, abdominally obese men had higher BMI, VAT, SAT and IHL, and lower MVR and WBGD during an insulin clamp (Figure 4.2; $p < 0.001$ for each). In addition, abdominally obese men had higher baseline HOMA-IR, fasting plasma glucose, and fasting insulin levels compared to lean men (Table 4.1; $p < 0.001$ for each). At baseline, abdominally obese individuals randomized to the weight loss program and those randomized to the habitual diet were comparable (Table 4.1).

Contributors to obesity-associated insulin resistance

We observed that VAT ($r = -0.615$; $p < 0.001$), SAT ($r = -0.564$; $p < 0.001$), IHL ($r = -0.603$; $p < 0.001$), and MVR ($r = 0.567$; $p < 0.001$) were each associated with WBGD (Figure 4.3). As expected, VAT, SAT, IHL, and MVR were all associated with one another, which can be inferred from the attenuation in the individual regression coefficients when the age-adjusted associations were additionally adjusted for the other variables (Table 4.2). Yet, a multivariate regression model including VAT, SAT, IHL, and MVR as determinants of WBGD shows that each was independently associated with WBGD (Table 4.2). Of note, no statistically significant interaction between VAT and SAT was found ($p = 0.223$).

Figure 4.2 – Levels of (A) body mass index (BMI), (B) whole body glucose disposal (WBGD), (C) abdominal visceral adipose tissue volume (VAT), (D) abdominal subcutaneous adipose tissue volume (SAT), (E) intrahepatic lipid (IHL), and (F) insulin-induced muscle microvascular recruitment (MVR). Results for lean men (n = 25), abdominally obese men (n = 52), the control group at baseline (Ctrl_{pre}; n = 26), and the intervention group at baseline (Int_{pre}; n = 23) and after follow-up (Ctrl_{post}; n = 26, Int_{post}; n = 23) are shown. One lean individual lacked MRI data due to claustrophobia; analyses on VAT, SAT, and IHL are thus reported for 24 lean men. Box plots are as follows: black line, median; box edges, 1st and 3rd quartiles; whiskers, minimum and maximum of all data. Cross-sectional differences were assessed by means of independent Student's t-tests (lean vs. obese; * p < 0.001), differences over time by means of paired t-test (baseline vs. follow-up; ### p < 0.001), and differences between groups over time by means of one-factor ANCOVA with baseline value as covariate (control Group vs. intervention group; \$\$\$ p < 0.001).**

Table 4.1 – Study population characteristics.

Parameter	Lean (n=25)	Obese (n=52)	Weight Stable Baseline (n=26)	Weight Loss Baseline (n=23)
Age [Yr]	53.7 [25.0-61.6]	51.8 [45.7-60.7]	52.0 [45.4-61.1]	52.4 [46.8-61.7]
Weight [kg]	74.9 ± 8.3	96.9 ± 8.4 ***	95.9 ± 8.9	98.2 ± 8.1
Fat Free Mass [kg]	60.8 ± 6.4	69.6 ± 6.1 ***	69.2 ± 6.6	69.7 ± 5.9
Fat Mass [kg]	14.1 ± 4.8	27.2 ± 4.6 ***	26.7 ± 4.6	28.5 ± 4.2
BMI [kg/m ²]	23.3 ± 1.8	30.1 ± 2.1 ***	29.9 ± 2.5	30.2 ± 1.5
Waist Circumference [cm]	84.9 ± 6.3	106.5 ± 3.6 ***	106.2 ± 3.8	106.8 ± 3.4
Hip Circumference [cm]	96.6 ± 4.3	107.5 ± 5.2 ***	107.2 ± 6.0	108.1 ± 4.4
Visceral Fat [L] ^a	0.890 ± 0.417	2.341 ± 0.720 ***	2.526 ± 0.752	2.167 ± 0.643
Subcutaneous Fat [L] ^a	1.452 ± 0.513	3.089 ± 0.780 ***	2.915 ± 0.811	3.226 ± 0.642
Intrahepatic Lipid [%] ^a	3.43 [3.13-3.78]	4.96 [3.90-7.86] ***	5.34 [4.24-9.23]	4.21 [3.59-6.63]
Microvascular Recruitment [%]	44.4 ± 41.3	-3.5 ± 27.3 **	0.7 ± 27.6	-5.0 ± 26.6
Whole Body Glucose Disposal [mg/kg/min]	6.76 ± 1.79	4.08 ± 1.30 ***	4.03 ± 1.38	4.14 ± 1.25
HOMA-IR	1.65 ± 0.46	2.84 ± 1.38 ***	2.90 ± 1.40	2.64 ± 1.21
Fasting Plasma Glucose [mmol/l]	5.35 ± 0.29	5.64 ± 0.48 **	5.75 ± 0.53	5.49 ± 0.37
Fasting Plasma Insulin [mIU/l]	7.1 ± 1.9	11.6 ± 5.4 ***	11.5 ± 5.6	11.2 ± 5.0
Clamped Plasma Insulin [mIU/l]	71.0 ± 18.0	100.0 ± 33.3 ***	91.3 ± 30.3	112.3 ± 34.7
HbA1c [%]	5.18 ± 0.37	5.30 ± 0.37	5.33 ± 0.39	5.24 ± 0.35
Total Cholesterol [mmol/l]	4.55 ± 0.78	5.56 ± 0.97 ***	5.63 ± 0.86	5.60 ± 1.09
LDL Cholesterol [mmol/l] ^b	2.82 ± 0.70	3.68 ± 0.89 ***	3.71 ± 0.85	3.70 ± 0.96
HDL Cholesterol [mmol/l]	1.26 ± 0.26	1.11 ± 0.21 **	1.08 ± 0.23	1.15 ± 0.17
Triacylglycerol [mmol/l]	0.95 [0.67-1.11]	1.66 [1.17-2.19] ***	1.75 [1.20-2.38]	1.43 [0.94-2.02]
24-h Systolic Blood Pressure [mmHg]	117.5 ± 8.8	123.4 ± 8.7 **	125.6 ± 7.6	120.2 ± 9.2
24-h Diastolic Blood Pressure [mmHg]	72.5 ± 9.4	80.4 ± 7.3 ***	82.0 ± 6.7	78.3 ± 7.6

Data presented as mean ± SD or median [25-75th percentile]. Baseline differences between obese and lean men were assessed by means of independent Student's T-test or Mann-Whitney U test where appropriate;

* p < 0.05, ** p < 0.01, *** p < 0.001 compared to baseline values of the lean men. Analyzed in ^a 24 lean and 52 obese men; ^b 25 lean and 50 obese men.

In addition, VAT (20.7%; 2.4 to 46.6%), SAT (34.6%; 7.6 to 53.5%), IHL (26.5%; 11.8 to 44.7%), and MVR (18.3%; 5.8 to 34.0%) were independent mediators of the association between BMI and WBGD (Figure 4.4A). Since the confidence intervals of the four mediators overlap, we cannot conclude that any one mediator is more important than any other.

Effects of weight loss

In abdominally obese men randomized to the control group, BMI, VAT, SAT, IHL, MVR, and WBGD were unchanged over time (Figure 4.2). Conversely, in abdominally obese men randomized to the weight loss intervention, BMI (-3.04 ± 0.77 kg/m²), VAT (-0.723 ± 0.371 L), SAT (-0.782 ± 0.261 L), and IHL ($-1.69 \pm 2.05\%$) decreased, whereas MVR ($39.5 \pm 49.0\%$) and WBGD (1.31 ± 1.22 mg/kg/min) increased (Figure 4.2;

$p < 0.001$ for each vs. baseline; $p < 0.001$ for each vs. control group). After weight loss, IHL and MVR were similar to the mean values in lean men while VAT and SAT remained increased and WBGD remained impaired. In addition, HOMA-IR (0.06 ± 0.84), fasting plasma glucose (0.00 ± 0.28 mmol/l), and fasting insulin levels (0.2 ± 3.0 mIU/l) did not change in the control group, whereas clamped insulin levels increased (15.2 ± 28.7 mIU/l; $p = 0.012$). In abdominally obese men randomized to the weight loss intervention on the other hand, HOMA-IR (-0.97 ± 0.92 ; $p < 0.001$), fasting plasma glucose (-0.21 ± 0.33 mmol/l; $p < 0.05$), fasting insulin levels (-3.8 ± 3.5 mIU/l; $p < 0.001$), and clamped insulin levels (-26.1 ± 35.6 mIU/l; $p = 0.002$) decreased ($p < 0.05$ for each vs. control group).

Figure 4.3 – Cross-sectional bivariate associations between abdominal visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), intrahepatic lipid (IHL) and insulin-induced muscle microvascular recruitment (MVR), and whole body glucose disposal (WBGD). (A) Association between VAT and WBGD in lean ($n = 24$) and abdominally obese men ($n = 52$). (B) Association between SAT and WBGD in lean ($n = 24$) and abdominally obese men ($n = 52$). (C) Association between IHL and WBGD in lean ($n = 24$) and abdominally obese men ($n = 52$). (D) Association between MVR and WBGD in lean ($n = 25$) and abdominally obese men ($n = 52$). Data of lean (○ open circles) and abdominally obese men (● closed circles) are shown. Pearson's correlation coefficients and their p-values are reported for all bivariate associations.

Table 4.2 – Cross-sectional multivariate regression models with WBGD as dependent variable

	β (Age-adjusted)	p-value	β (Fully Adjusted)	p-value
VAT [L]	-1.525	<0.001	-0.509	0.032
SAT [L]	-1.070	<0.001	-0.444	0.011
Ln IHL [%]	-2.539	<0.001	-1.498	<0.001
MVR [%]	0.0279	<0.001	0.0128	0.003

The associations between abdominal visceral adipose tissue (VAT), abdominal subcutaneous adipose tissue (SAT), log-transformed intrahepatic lipid content (Ln IHL) or insulin-induced muscle microvascular recruitment (MVR), and whole body glucose disposal (WBGD) were assessed by means of linear regression. In all models, age-adjusted regression coefficients and regression coefficients adjusted for age and each of the other variables are reported, as well as their respective p-values (n = 76 for all models).

Association with improved insulin resistance

Changes in VAT (r = -0.551; p < 0.001), SAT (r = -0.528; p < 0.001), IHL (r = -0.501; p < 0.001), and MVR (r = 0.499; p < 0.001) were associated with changes in WBGD (Figure 4.5). Changes in VAT, SAT, IHL, and MVR were associated with one another, which was reflected by the attenuation in the individual regression coefficients when the age-adjusted associations were additionally adjusted for the other variables (Table 4.3). In a multivariate regression model, changes in IHL and changes in MVR, but not changes in VAT or SAT, were independently associated with changes in WBGD (Table 4.3). Consequently, the association between weight loss intervention and change in WBGD was independently mediated by changes in IHL (26.6%; 4.4 to 69.6%) and changes in MVR (26.3%; 2.4 to 59.9%), but not statistically significantly by changes in VAT or SAT (Figure 4.4B).

Table 4.3 – Multivariate regression models in the RCT with Δ WBGD as dependent variable.

	β (Age-adjusted)	p-value	β (Fully Adjusted)	p-value
Δ VAT [L]	-1.331	<0.001	-0.516	0.190
Δ SAT [L]	-1.366	<0.001	-0.245	0.567
Δ IHL [%]	-0.295	<0.001	-0.170	0.031
Δ MVR [%]	0.0146	<0.001	0.0089	0.017

The associations between change in abdominal visceral adipose tissue (Δ VAT), change in abdominal subcutaneous adipose tissue (Δ SAT), change in intrahepatic lipid content (Δ IHL) or change in insulin-induced muscle microvascular recruitment (Δ MVR), and change in whole body glucose disposal (Δ WBGD) were investigated by means of linear regression. In all models, age-adjusted regression coefficients and regression coefficients adjusted for age and all of the other variables are reported, as well as p-values for both models (n = 49).

A

B

Figure 4.4 – Multiple mediator models. (A) Multiple mediator model with VAT, SAT, Ln IHL, and MVR as statistically significant mediators (percentage of total effect mediated (β of mediated effect (95%-CI using the Monte Carlo method)) of the association between BMI and WBGD ($n = 76$, age-adjusted). The confidence intervals of the mediators overlap, which implies that no mediator contributes more than the other. (B) Multiple mediator model with Δ IHL and Δ MVR as statistically significant mediators (percentage of total effect mediated (β of mediated effect (bootstrapped 95%-CI))) of the association between the weight loss intervention and Δ WBGD ($n = 49$, age-adjusted). The variables Δ VAT and Δ SAT were not statistically significant mediators of this association. The confidence intervals of the mediators overlap, which implies that no mediator contributes more than the other.

Sensitivity analyses

We conducted sensitivity analyses by including the individual left out for protocol violations in the analyses, as well as using waist circumference or total fat mass in our model instead of BMI to reflect obesity, which did not materially change the results (data not shown). Moreover, we used WBGD with glucose space correction and WBGD with adjustment for insulin levels during the clamp as outcome in our models, which did not materially change the results (data not shown). Additionally, we performed multiple mediation analyses in the intervention group only, using a repeated measures design. These analyses confirmed that changes in IHL and changes in MVR were statistically significant mediators, whereas changes in VAT or SAT were not (data not shown).

Figure 4.5 – Bivariate associations between change in abdominal visceral adipose tissue volume (Δ VAT), change in abdominal subcutaneous adipose tissue volume (Δ SAT), change in intrahepatic lipid content (Δ IHL) and change in insulin-induced muscle microvascular recruitment (Δ MVR), and change in whole body glucose disposal (Δ WBGD; $n = 49$). (A) Association between Δ VAT and Δ WBGD. (B) Association between Δ SAT and Δ WBGD. (C) Association between Δ IHL and Δ WBGD. (D) Association between Δ MVR and Δ WBGD. Data of abdominally obese controls ($\blacklozenge$ closed diamonds, $n = 26$) and abdominally obese men undergoing the weight loss intervention (∇ open triangles, $n = 23$) are shown. Pearson's correlation coefficients and their p -values are reported for all bivariate associations.

Discussion

In this report we identified VAT, SAT, IHL, and MVR as mutually independent contributors to obesity-associated insulin resistance, which therefore constitute separate targets for intervention. To our knowledge, no studies have simultaneously determined multiple tissue contributors to insulin resistance using state of the art techniques in humans, or assessed their mutual independence, either in the basal steady state or after a minimal weight loss intervention. Studies on the contributions of VAT, SAT, and IHL to insulin resistance in the basal state have been ambiguous. Some argue in favor of mutually independent contributions of VAT and IHL,^{11,14} whereas others find that IHL, not VAT, associates with insulin resistance.^{5,15} Similarly, the SAT contribution to insulin resistance, independent of VAT, is both supported² and disputed.^{16,17} In addition, the contribution of MVR to insulin resistance has never been studied in parallel with VAT, SAT, or IHL. By measuring VAT, SAT, IHL, and MVR simultaneously, our data clarify that, at least in abdominally obese men, these variables contribute to insulin resistance independently and to a similar extent.

By decreasing obesity and insulin resistance with a weight loss intervention, we show that changes in IHL and MVR contribute to improved insulin sensitivity, which supports their tight connection with insulin resistance. Our data are insufficiently precise to exclude that changes in VAT and SAT contribute to improved insulin resistance with weight loss. At least for changes in VAT, involvement seems probable as it is estimated to mediate, albeit not statistically significantly, ~30% of the change in WBGD, an effect size consistent with its contribution to WBGD in the basal state. As we observed significant bivariate but not multivariate associations between changes in VAT and SAT on the one hand and WBGD on the other, the degree of VAT and SAT reduction achieved here may have been insufficient to improve insulin resistance in an independent way. This observation is in line with studies using interventions such as omentectomy (0.3 to 1.0 kg VAT loss)^{18,19} or liposuction (0.4 to 1.7 kg SAT loss)²⁰ which have limited effects on insulin resistance.

In addition to establishing relationships between VAT, SAT, IHL, and MVR on the one hand and WBGD on the other, we aimed to provide a deeper understanding of the processes that contribute to obesity-associated insulin resistance. Multiple mediation analyses, as compared to multivariate regression, can provide a superior reflection of the causal chain one tries to investigate. This helps unravel contributions to disease etiology and to develop targeted, effective therapies.²¹ Specifically, the advantage of testing the mediating effect of multiple variables simultaneously is that one learns whether or not mediation is independent of the effect of the other mediators, which offers the possibility to compare competing theories against one another within a single model.^{22,23} In addition, particularly when potential mediators have little random

error (i.e. the measurement is precise and reflects the underlying mechanism well), multiple mediation analyses can be used to estimate the proportion of the total effect that independent mediators account for. A mediated proportion is essentially the product of two effects; in our analyses, the effect of obesity on each mediator is the first, and the independent effect of each mediator on insulin resistance is the second effect. Such multiple mediator models are therefore the integrated product of two multivariate regression models, which has clear advantages over the conventional approach of analyzing each effect with separate regression analyses. By using these multiple mediation analyses in this report we were able to provide an estimate of the magnitude of the independent contributions of VAT, SAT, IHL, and MVR to obesity-associated insulin resistance.

Of note, the fact that we found complete statistical mediation (i.e., a sufficient set of potential component causes)²⁴ in the cross-sectional model provides no information about the existence of other possible contributors that were not included in our statistical model. For example, determinants and consequences of the mediators identified could contribute if included in the analyses. We therefore do not claim to have identified all tissue contributors to obesity-associated insulin resistance.

Our study has some limitations. First, no additional measurements were conducted between baseline and follow-up, so we could not determine a temporal hierarchy of improvements for various contributors to insulin resistance. We emphasize that the aim of this study was not to provide an accurate time course for the exerted effects. Rather we used a weight loss intervention, an instrument to modify insulin resistance, to probe the contributions of changes in VAT, SAT, IHL, and MVR to improvement of insulin resistance. Also, this study was carried out in men only. Nevertheless, studies have shown similar (bivariate) associations between VAT, SAT, IHL, MVR, and insulin resistance in women, as well as improvements in these variables with weight loss.^{2,25-28} Amounts of IHL and VAT are, however, typically greater in men, whereas the amount of SAT is greater in women;^{2,10} this might affect the relative contribution of each mediator in women in the multiple mediator models. Whilst we identified the relative contribution of each mediator in men in this study, their proportional contributions in women remain to be explored.

Here we have established for the first time the mutually independent contributions of VAT, SAT, IHL, and MVR to insulin resistance. This finding implies that, compared to interventions with a single target tissue, interventions aimed at multiple contributors to insulin resistance will yield superior effectiveness. Hence, therapies aimed at multiple therapeutic targets for insulin resistance will be most effective in reducing the global burden of type 2 diabetes and cardiovascular diseases.

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chapter

5

Chapter 5

NK cells in human visceral adipose tissue contribute to obesity-associated insulin resistance through macrophage polarization and low-grade inflammation

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Under review

Abstract

Obesity-associated insulin resistance is a major risk factor for type 2 diabetes. Experimental evidence suggests that natural killer (NK) cell accumulation in visceral adipose tissue (VAT) is a primary event causing insulin resistance. Data on the role of NK cells in insulin resistance in humans are, however, absent.

We show that NK cell accumulation in VAT is associated with inflammatory adipose tissue macrophages and is reflected by CD11B surface expression on circulating NK cells. In addition, CD11B on circulating NK cells was associated with VAT volume, systemic low-grade inflammation (LGI), and insulin resistance. Moreover, CD11B surface expression on circulating NK cells partially explained the pathway between VAT, LGI, and insulin resistance. NK cells from obese donors produced higher levels of TNF and were able to drive inflammatory polarization of macrophages. We confirmed NK cells' involvement in obesity-associated insulin resistance in a population-based cohort of 839 individuals. Taken together, these data show that NK cell accumulation in VAT contributes to LGI and subsequent insulin resistance in humans.

Introduction

The global prevalence of obesity is rising at an alarming rate. Obesity is a major driver of insulin resistance, which increases the risk of developing type 2 diabetes, hypertension, dyslipidemia, and cardiovascular events.¹ Ever since Hotamisligil et al. discovered in 1993 that tumor necrosis factor (TNF) is a determinant of insulin resistance and is produced by hypertrophic adipose tissue (AT),² insights have been emerging that chronic low-grade inflammation (LGI) in AT caused by massive macrophage infiltration in AT is important for the development of insulin resistance.³⁻⁶ Not only quantitative, but also qualitative shifts in macrophage phenotype take place in expanding AT. While under lean conditions most macrophages display an anti-inflammatory or so-called “M2” phenotype, obesity induces the polarization of AT macrophages toward the inflammatory “M1” state.⁷ Phenotypically, these AT macrophage (ATM) subsets could be distinguished by the surface expression of the integrin CD11C.⁷ The accumulation of inflammatory CD11C⁺ ATMs correlates with insulin resistance in humans.⁸ The inflammatory cytokines that are secreted from CD11C⁺ ATMs are thought to be major contributors to the low-grade chronic inflammatory state of AT that drives systemic insulin resistance.⁹ Many factors in AT of obese individuals have been proposed to trigger macrophage accumulation and polarization, such as adipocyte cell death, lipotoxicity by fatty acids, increased insulin levels, hyperglycemia, and hypoxia.⁹ However, the exact driver of macrophage accumulation and activation remains unclear. Recently, several studies showed, using experimental mouse models, that natural killer (NK) cells control macrophage accumulation and polarization via the production of interferon gamma (IFN γ), TNF or IL-6 in AT, resulting in impaired insulin sensitivity and glucose handling.⁹⁻¹¹ These important findings may provide essential insight in the origin of inflammatory macrophages and could offer new pathways to target inflammation in the context of insulin resistance development. NK cells are innate lymphoid cells that are specialized in surveilling, recognizing and eliminating virally infected, transformed or stressed cells. In addition, NK cells also produce cytokines to shape an inflammatory environment.¹² Moreover, human obesity is associated with an activated phenotype of NK cells in AT.¹³ However, no data is available showing whether NK cells contribute to AT macrophage accumulation and polarization and consequent insulin resistance in humans. Therefore, the aim of this study is to evaluate whether NK cells accumulate in human AT and, through LGI, contribute to insulin resistance and the development of type 2 diabetes in humans.

We addressed this by first conducting two cross-sectional studies using biopsies from visceral and subcutaneous adipose tissue, followed by an in-depth metabolic phenotyping study in a larger study population. Finally, we validated our findings in a large population-based cohort study.

Results & discussion

VAT NK cells accumulate in obesity and are associated with inflammatory macrophage polarization

To investigate whether NK cells accumulate in human AT, we analyzed stromal vascular fractions of VAT and subcutaneous adipose tissue (SAT) in lean and abdominally obese men using flow cytometry (baseline characteristics, SupplementaryTable S5.1).¹⁴ Obese men had a higher waist circumference (124.0 [121.3-130.3] vs. 89.0 [86.0-93.0] cm; $p < 0.0001$) and were more insulin resistant as reflected by higher HOMA-IR (5.63 [3.54-7.16] vs. 1.73 [1.27-3.30]; $p < 0.0001$) compared to lean men. Moreover, obese men displayed increased levels of NK cells in their VAT (Figure 5.1A), but not in SAT (3.77 ± 1.84 vs. 3.52 ± 2.10 %; $p = 0.758$; Figure 5.2A) or blood (0.28 [0.15-0.44] vs. 0.20 [0.14-0.41] $\times 10^9$ /l; $p = 0.401$; Table S5.1). These data are in line with previous studies in mice showing that VAT is the predominant AT depot displaying NK cell accumulation.^{9-11,15} In a second study, using adipose tissue biopsies from female controls and female obese women (BMI 28.1 ± 3.1 vs. 42.6 ± 1.6 kg/m²; $p = 0.001$), we found that the fraction of CD56^{dim}CD16^{bright} NK cells in VAT was increased in obese individuals ($50.1 \pm 7.3\%$ vs. $87.4 \pm 4.5\%$; $p = 0.0021$) while the fraction of CD56^{bright}CD16⁻ cells was decreased ($43.5 \pm 10.8\%$ vs. $2.9 \pm 1.2\%$; $p < 0.0001$) compared to controls. In SAT of these individuals, a significant decrease of CD56^{bright}CD16⁻ NK cells ($12.6 \pm 4.1\%$ vs. $1.4 \pm 0.9\%$; $p = 0.0018$), but no increase in CD56^{dim}CD16^{bright} NK cells ($85.1 \pm 3.3\%$ vs. $91.7 \pm 3.3\%$; $p = 0.285$) was observed. These data are in concordance with a study showing that, despite total NK cells in VAT being equal, there was a shift towards more CD56^{dim} NK cells and less CD56^{bright} NK cells in VAT of obese individuals.¹⁶ It was previously shown that VAT, but not SAT, from humans contained ligands of the activating receptor NKp46.¹¹ We now report a trend towards increased surface expression of NKp46 in the CD56^{dim}CD16^{bright} NK cell subset in VAT (635 ± 96 vs. 397 ± 45 mean fluorescence intensity (MFI), $p = 0.06$; Figure 5.3A), albeit not statistically significantly. No difference was found in surface expression level of NKG2D (2704 ± 181 vs. 2970 ± 497 MFI; $p = 0.772$), another activating receptor of VAT NK cells. In SAT, NKp46 surface expression was not different. Interestingly, in contrast to blood NK cell subsets, neither VAT nor SAT NK cell subsets displayed increased CD11B expression (data not shown). The aforementioned experimental mouse studies showed that NK cell infiltration in VAT leads to inflammatory macrophage polarization, which is hallmarked by an accumulation of “M1” polarized CD11C⁺ macrophages compared to resident “M2” CD11C⁻ polarized macrophages. We next investigated whether increased levels of NK cells in VAT is associated with macrophage polarization and found that an increase in VAT-resident NK cells was associated with a higher inflammatory macrophage ratio in VAT (M1/M2 ratio; $r = 0.511$; $p = 0.003$; Figure 5.1B). Additional adjustment for the

potential confounder age did not materially change this association. In SAT, NK cells did not coincide with macrophage polarization ($r = 0.109$; $p = 0.597$; Figure 5.2B-C). These findings support the involvement of VAT-resident NK cells in M1 macrophage polarization, as previously described in rodents.⁹⁻¹¹ In contrast, while NK cells are present in SAT, they do not seem to exert the same effects as in VAT. This observation is in line with the findings of O'Rourke et al., showing that compared to SAT, VAT is particularly enriched in IFN γ -producing NK cells.¹⁶ Hence, SAT's contribution to insulin resistance appears to be mediated by different pathways compared to VAT, which may involve adipocyte lipolysis and adipokine dysregulation.¹⁷

Figure 5.1 – Biopsy Study, visceral adipose tissue. (A) NK cells in visceral adipose tissue (VAT) in lean and obese individuals ($n = 31$; $* p < 0.05$). (B) Association between NK cells in VAT and M1-to-M2 ratio in VAT ($n = 31$). (C) CD11B expression on circulating NK cells (NK-CD11B) in lean and obese individuals ($n = 31$; $*** p < 0.001$). (D) Association between NK cells in VAT and NK-CD11B ($n = 29$). Differences between groups were assessed by means of independent Student's t-tests (panels A, C). Box plots are as follows: black line, median; box edges, 1st and 3rd quartiles; whiskers, minimum and maximum of all data. Pearson's correlation coefficients, regression coefficients, and their p-values are reported for all bivariate associations (panels B, D). Data of lean (o open circles) and obese men (● closed circles) are shown.

Figure 5.2 – Biopsy Study, subcutaneous adipose tissue. (A) NK cells in subcutaneous adipose tissue (SAT) in lean and obese individuals ($n = 26$). (B) M1 macrophage polarization (M1-to-M2 ratio) in SAT in lean and obese individuals ($n = 31$). (C) Association between NK cells in SAT and M1-to-M2 ratio in SAT ($n = 26$). (D) Association between NK cells in SAT and CD11B expression on circulating NK cells (NK-CD11B; $n = 24$). Differences between groups were assessed by means of independent Student's *t*-tests (panels A-B). Box plots are as follows: black line, median; box edges, 1st and 3rd quartiles; whiskers, minimum and maximum of all data. Pearson's correlation coefficients, regression coefficients, and their *p*-values are reported for bivariate associations (panels C-D). Data of lean (○ open circles) and obese men (● closed circles) are shown.

VAT NK cells are reflected by CD11B on circulating NK cells

Since biopsies are burdensome for study participants and are virtually impossible to implement in large studies, we searched for a proxy for NK cell accumulation in VAT. We primarily focused on NK cells in peripheral blood. Interestingly, NK cell numbers in blood were not associated with NK cell numbers in VAT ($r = 0.058$, $p = 0.759$). We did, however, find a suitable candidate in CD11B expression on these circulating NK cells. CD11B has been described as a maturation marker of NK cells.^{18,19} This integrin can mediate cell adhesion, but is also part of the complement receptor 3 complex, which is involved in cytotoxic activation of NK cells.²⁰ We found that CD11B surface expression, measured as MFI, on total circulating NK cells (from here on referred to as NK-CD11B) is higher in obese compared to lean men (Figure 5.1C). Moreover, NK-

CD11B was closely associated with NK cell accumulation in VAT ($r = 0.561$, $p = 0.002$; Figure 5.1D). Additional adjustment for the potential confounder age did not materially change this association. NK cells in SAT on the other hand were not associated with NK-CD11B ($r = 0.091$; $p = 0.671$; Figure 5.2D). Since VAT NK cells were associated with an increase in the M1/M2 ratio, we confirmed that the M1/M2 ratio was associated with NK-CD11B as well ($r = 0.364$; $p = 0.048$). In conclusion, NK cells accumulate in VAT of obese individuals and are associated with inflammatory macrophage polarization. Moreover, CD11B surface expression on circulating NK cells can be used as a surrogate marker for VAT NK cell accumulation in studies where VAT biopsies are neither available nor feasible.

Figure 5.3 – NK cell function. (A) Surface expression of NKp46 on VAT NK cells from lean ($n = 3$) and obese individuals ($n = 6$). (B) Blood-derived NK cells from lean ($n = 6$; white bars) and obese individuals ($n = 8$; black bars) were isolated and co-cultured with either human monocyte derived macrophages where after surface expression of M1 markers (CCR7, CD86 and CD64), or incubated with monocytes that were first polarized to an M2 phenotype where after loss of M2 markers (loss of CD209 and CD206; i.e., polarization towards M1) was assessed. An aggregate total effect of polarization towards an M1 state was calculated (i.e., a z-score composed of -CD209, -CD206, CCR7, CD86, and CD64); compared to lean individuals, obese individuals displayed more polarization towards the M1 phenotype (mean difference 1.08 (95%-CI 0.06-2.10; * $p < 0.05$)). (C) Intracellular TNF levels were measured in blood-derived NK cells of lean ($n = 4$) and obese individuals ($n = 6$) using flow cytometry. (D) Association between CD11B surface expression and intracellular TNF levels of blood-derived NK cells ($n = 8$).

Figure 5.4 – Metabolic phenotyping study. (A) CD11B expression on circulating NK cells (NK-CD11B) in lean and abdominally obese individuals ($n = 64$; ** $p < 0.01$). (B) Association between visceral adipose tissue volume (VAT) and NK-CD11B in lean and abdominally obese individuals ($n = 63$). (C) Association between NK-CD11B and low-grade inflammation (z-LGI; composite z-score of CRP, SAA, IL-6, IL-8, sICAM-1, and TNF) in lean and abdominally obese individuals ($n = 64$). (D) Association between NK-CD11B and whole-body glucose disposal (WBGD) during a hyperinsulinemic, euglycemic clamp in lean and abdominally obese individuals ($n = 64$). Difference between groups was assessed by means of independent Student's t-tests (panel A). Box plots are as follows: black line, median; box edges, 1st and 3rd quartiles; whiskers, minimum and maximum of all data. Pearson's correlation coefficients, regression coefficients, and their p-values are reported for all bivariate associations (panels B-D). Data of lean (o open circles) and obese men (● closed circles) are shown.

NK cells from obese individuals produce enhanced levels of TNF and induce inflammatory polarization of macrophages

In the second study, we investigated whether NK cells from obese individuals directly affect macrophage polarization. Hence, NK cells were isolated from peripheral blood and co-cultured with human monocyte-derived macrophages. We investigated the capacity of NK cells to either promote the expression of M1 surface markers (CCR7, CD86 and CD64) in resting macrophages or to inhibit M2 surface marker expression (CD206 and CD209) in M2-polarized macrophages, as a model for resident adipose tissue macrophages.⁷ Compared to NK cells from lean individuals, NK cells from obese donors promoted M1 polarization in monocyte-derived macrophages ($p = 0.040$;

Figure 5.3B). This observation that NK cells can induce M1 polarization in obesity is in line with the association between VAT NK cell numbers and M1/M2 ratio demonstrated in the first study (Figure 5.1B).

Furthermore, compared to lean donors, peripheral blood NK cells from obese subjects displayed higher intracellular TNF levels (124 ± 22 vs. 184 ± 19 MFI, $p = 0.002$; Figure 5.3C). These data are in accordance with the report of Lee et al. showing that epididymal adipose tissue NK cells from obese mice produce enhanced levels of TNF.⁹ Although IFN γ also has been implicated in the causal contribution of mouse adipose tissue inflammation and concomitant insulin resistance,¹¹ we did not observe elevated IFN γ levels in the NK cells of obese individuals (data not shown). Finally, CD11B surface expression on NK cells was closely associated with TNF production in these cells ($r = 0.806$, $p = 0.016$; Figure 5.3D). Additional adjustment for the potential confounder age did not materially change this association. The expression of CD11B on NK cells may thus be a reflection of their activation level and their capacity to polarize macrophages towards a more inflammatory state. However, whether CD11B has a role in either the migration of NK cells into VAT or in macrophage polarization, or is a mere reflection of a closely related mechanism should be elucidated by future studies. Collectively, these data show that NK cells from obese individuals promote inflammatory polarization of macrophages, possibly via the increased production of TNF.

NK cells are inversely associated with insulin resistance and partially mediate the association between VAT volume and insulin resistance

To determine the relation between VAT NK cells and insulin resistance, we performed analyses in a third study including 25 lean (waist circumference <94 cm) and 53 abdominally obese men (waist circumference 102-110 cm).²¹ We used state-of-the-art in-depth metabolic and inflammatory phenotyping to provide precise estimations of VAT and SAT volumes and insulin resistance. FACS data were available in 22 lean and 42 abdominally obese men who were consecutively enrolled. In this study, VAT and SAT volumes were determined by magnetic resonance imaging (MRI), plasma cytokine levels with the MSD system,²² and insulin sensitivity by means of a 1 mU/kg/min euglycemic insulin clamp. By design, obese men had an increased waist circumference compared to lean men (106.6 [103.5 - 109.1] vs. 85.8 [80.6 - 89.8] cm; $p < 0.001$), as well as higher VAT (2.384 ± 0.751 vs. 0.872 ± 0.434 L; $p < 0.001$) and SAT volumes (3.067 ± 0.798 vs. 1.437 ± 0.527 L; $p < 0.001$), and a decreased whole-body glucose disposal (WBGD; 4.04 ± 1.23 vs. 6.85 ± 1.85 mg/kg/min; $p < 0.001$; Table S5.2; STAR Methods). NK-CD11B was significantly higher in obese compared to lean men (Figure 5.3A), reflecting increased VAT NK cell accumulation. As expected, increased VAT volume was inversely associated with WBGD ($r = -0.640$, $p < 0.001$). Moreover,

VAT volume was associated with higher NK-CD11B ($r = 0.302$; $p = 0.016$; Figure 5.3B), confirming the relationship between NK-CD11B and VAT expansion. In addition, NK-CD11B was associated with a LGI z-score (i.e., composite score of CRP, SAA, IL-6, IL-8, sICAM-1, and TNF; $r = 0.491$; $p < 0.001$; Figure 5.3C), and WBGD ($r = -0.309$; $p = 0.013$; Figure 5.3D). Additional adjustment for age did not materially change any of these associations.

We next explored whether VAT NK cells, by means of NK-CD11B, and consecutive systemic LGI contribute to the association between VAT volume and insulin resistance. We investigated the contribution of this pathway to insulin resistance by means of multiple mediation analyses. VAT NK cells, by means of NK-CD11B, and subsequent LGI partially explained the association between VAT and insulin resistance ($\beta -0.075$ (-0.233 to -0.011); Figure S5.1). As a result, 53.9% of the contribution of LGI to VAT-associated insulin resistance appears to be explained by NK-CD11B. This is indicative that, in humans, NK cell accumulation in VAT leads to systemic LGI and subsequent induction of insulin resistance and thereby constitutes a separate target for intervention.

Finally, as recent literature in mice suggests that the inflammatory cytokines TNF and IL-6 in particular may be responsible for the association between VAT NK cells and insulin resistance,^{9,10} we explored whether VAT NK cells contribute to insulin resistance via these cytokines in humans as well. We found that NK-CD11B was associated with both TNF ($r = 0.349$; $p = 0.005$) and IL-6 ($r = 0.274$; $p = 0.029$). In additional pathway analyses we found that VAT NK cells contribute to VAT-associated insulin resistance mainly through TNF ($\beta -0.048$ (-0.170 to -0.006)), but not statistically significantly via IL-6 ($\beta -0.018$ (-0.077 to 0.043)).

Collectively, our data thus show that, in humans, NK cell accumulation in VAT is associated with inflammatory macrophage polarization, systemic LGI, and insulin resistance. Interestingly, our multiple mediation analyses argue for a robust involvement of NK cells and LGI in obesity-associated insulin resistance. These data are in line with previously published data showing that more TNF-producing NK cells accumulate in the AT of obese mice.⁹ Intriguingly, in mice, obesity regulated NK cell numbers in VAT by enhanced recruitment and local proliferation.^{9,11} Here we show a close association between NK-CD11B and NK cell accumulation in human VAT. Moreover, NK-CD11B mediated the association between VAT volume, LGI, and insulin resistance. These results are at least suggestive that in humans, recruitment of NK cells from the circulation plays an important quantitative role in the development of VAT inflammation and systemic LGI. This does, however, not exclude the possibility that either recruited or resident NK cells may proliferate in AT during the development of obesity. Nevertheless, it is tempting to speculate that increased

integrin expression on NK cell surface may lead to enhanced recruitment of NK cells in VAT, although blocking CD11B on NK cells failed to attenuate *in vitro* adhesion to endothelial cells.²³ Since CD11B has been linked to NK cell activation,²⁰ circulating NK cells of obese people were found to display an activated, but exhausted, phenotype, rendering these cells less functional.²⁴ It would be of great interest to evaluate whether CD11B also mechanistically contributes to VAT NK cell accumulation, or merely reflects NK cell activation in VAT. In the former case, CD11B would be an interesting target to prevent NK cell accumulation in human VAT to reduce insulin resistance, as was shown in mice.^{9,11,15}

Validation of findings in external cohort

We aimed to validate our findings in a population-based cohort study. Hereto, we used an observational population-based cohort study enriched with type 2 diabetes. In this study, individuals underwent VAT and SAT quantification by means of MRI; NK cells were determined in blood by flow cytometry; insulin sensitivity was quantified by means of the Matsuda Index; and glucose metabolism status was assessed through fasting plasma glucose and an oral glucose tolerance test as described elsewhere.²⁵ First, we reconfirm that VAT is associated with LGI (standardized β 0.252; $p < 0.001$; $n = 696$; fully adjusted model) and insulin resistance by means of the Matsuda Index (standardized β -0.462; $p < 0.001$; $n = 397$; fully adjusted model). More importantly, VAT volume was associated with NK cell accumulation in VAT as assessed by NK-CD11B ($p = 0.006$; Table 5.1). There was no interaction with sex or diabetes status. When diabetic individuals or individuals that used blood-pressure-lowering, lipid-lowering, glucose-lowering, or anti-inflammatory medication were excluded from the analysis, the results did not materially change. Adjusting the model for SAT volume did not alter the results (data not shown). In addition, NK-CD11B was positively associated with the z-score of LGI, plasma TNF levels, and plasma IL-6 levels ($p < 0.05$ for each; Table S5.4). Finally, we found that NK-CD11B, as a measure of VAT NK cells, was associated with insulin resistance ($p = 0.013$; Table 5.2). There was again no interaction with sex or diabetes status, nor did the exclusion of diabetic individuals or individuals that used blood-pressure-lowering, lipid-lowering, glucose-lowering, or anti-inflammatory medication materially change the results (data not shown). We can therefore conclude that the findings of the first three independent studies were validated in a cohort study that was obtained from the general population.

Table 5.1 – Associations between VAT and NK-CD11B (n = 696).

	Model	NK-CD11B [AU]	
		β (95%-CI)	p-value
VAT [cm ²]	1	3.90 (1.84; 5.97)	<0.001
	2	3.21 (0.94; 5.48)	0.006

Model 1: Adjusted for age, sex, and glucose metabolism status. Model 2: Fully adjusted model (model 1 + systolic blood pressure, total-to-HDL cholesterol ratio, smoking status, prior cardiovascular disease, and use of antihypertensive, anti-inflammatory, glucose-lowering or lipid-lowering medication). Multiple linear regression analyses in individuals with normal glucose metabolism (n = 405), prediabetes (n = 93), and type 2 diabetes (n = 198). The beta of each model is presented along with its respective 95%-CI and p-value.

Table 5.2 – Associations between NK-CD11B and the Matsuda Index (n=540).

	Model	Matsuda Index	
		β (95%-CI)	p-value
NK-CD11B	1	2337 (609; 4064)	0.008
	2	2212 (440; 3783)	0.013

Model 1: Adjusted for age, sex, and glucose metabolism status. Model 2: Fully adjusted model (model 1 + systolic blood pressure, total-to-HDL cholesterol ratio, smoking status, prior cardiovascular disease, and use of antihypertensive, anti-inflammatory, glucose-lowering or lipid-lowering medication). Multiple linear regression analyses in individuals with normal glucose metabolism (n = 313), prediabetes (n = 71), and type 2 diabetes (n = 156). The beta of each model is presented along with its respective 95%-CI and p-value. NK-CD11B was transformed to $1 / \text{NK-CD11B}$ and the Matsuda Index to SQRT Matsuda Index to fulfill the assumptions of the regression model and are presented as such.

In conclusion, we show for the first time that NK cells in VAT contribute to LGI and subsequent insulin resistance in humans. Since experimental studies suggest that NK cell accumulation is an early event in the development of VAT inflammation, targeting these cells may hold great promise to inhibit this pathway leading to insulin resistance. These findings pave the way for novel therapeutic strategies to target the inflammatory component of insulin resistance development, which has until now been proven rather unsatisfactory.²⁶ Moreover, we identified CD11B surface expression on circulating NK cells to be a reflection of NK cell accumulation in VAT. Aside from the obvious benefit of this minimally invasive measurement as a marker of VAT inflammation, future studies should address whether CD11B on NK cells contributes to their accumulation and can be used as therapeutic target.

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Supplementary material

Figure S5.1 – Pathway analysis. Serial multiple mediator model in which CD11B expression on circulating NK cells (NK-CD11B) statistically significantly contributes through low-grade inflammation (z-LGI; composite z-score of CRP, SAA, IL-6, IL-8, sICAM-1, and TNF) to the association between abdominal visceral adipose tissue (VAT) and insulin resistance by means of whole-body glucose disposal (WBGD; $n = 63$; age-adjusted). Data presented as β of mediated effect (bootstrapped 95%-CI). Significant associations are represented by black lines, whereas grey lines represent associations that are not statistically significant.

Table S5.1 – Biopsy study population characteristics.

	Lean (n = 15)	Obese (n = 17)	p-value
Age [Yr]	52.1 ± 6.1	49.5 ± 5.8	0.215
Weight [kg]	76.0 [73.0 - 81.0]	115.0 [111.2 - 133.8]	<0.001
Fat Free Mass [kg]	58.2 ± 5.8	76.2 ± 7.9	<0.001
Fat Mass [kg]	16.2 [14.7 - 20.5]	41.8 [36.9 - 48.9]	<0.001
BMI [kg/m ²]	23.6 [22.7 - 24.1]	37.1 [35.1 - 38.7]	<0.001
Waist Circumference [cm]	89.0 [86.0 - 93.0]	124.0 [121.3 - 130.3]	<0.001
Hip Circumference [cm] ^a	92.5 [89.5 - 94.5]	114.8 [111.6 - 118.8]	<0.001
HOMA-IR	1.73 [1.27 - 3.30]	5.63 [3.54 - 7.16]	<0.001
Fasting Plasma Glucose [mmol/l]	5.44 ± 0.93	6.08 ± 0.97	0.069
HbA1c [%]	5.2 [5.1 - 5.5]	5.6 [5.5 - 6.1]	<0.001
Diabetes Status [Y/N]	0 / 15	3 / 14	N/A
Glucose lowering Therapy [Y/N]	0 / 15	3 / 14	N/A
Systolic Blood Pressure [mmHg] ^b	132.3 ± 15.1	145.6 ± 18.1	0.053
Diastolic Blood Pressure [mmHg] ^b	83.6 ± 9.2	83.8 ± 8.4	0.956
White Blood Cell Count, Blood [x10 ⁹ /L]	7.81 ± 2.27	6.63 ± 1.39	0.094
Neutrophilic Granulocyte Count, Blood [x10 ⁹ /l]	4.62 [3.61 - 6.18]	3.70 [3.11 - 4.70]	0.235
Eosinophilic Granulocyte Count, Blood [x10 ⁹ /l]	0.22 [0.20 - 0.38]	0.15 [0.11 - 0.26]	0.040
Basophilic Granulocyte Count, Blood [x10 ⁹ /l]	0.05 ± 0.02	0.04 ± 0.02	0.105
Lymphocyte Count, Blood [x10 ⁹ /l]	1.99 ± 0.50	1.78 ± 0.69	0.333
Monocyte Count, Blood [x10 ⁹ /l]	0.46 [0.37 - 0.69]	0.54 [0.41 - 0.57]	0.761
NK Cell Count, Blood [x10 ⁹ /l] ^a	0.20 [0.14 - 0.41]	0.28 [0.15 - 0.44]	0.401
NK-CD11B MFI, Blood [AU] ^c	4953 ± 1083	7126 ± 1342	<0.001
SAT NK Cell Count [% of Live] ^d	3.52 ± 2.10	3.77 ± 1.84	0.758
VAT NK Cell Count [% of Live] ^e	2.70 [1.64 - 4.09]	3.79 [2.66 - 6.02]	0.025
VAT M1/M2 Ratio	0.78 [0.62 - 1.09]	1.05 [0.85 - 2.55]	0.074
VAT TNF Expression [Fold of Control] ^f	0.66 [0.48 - 1.33]	1.28 [0.71 - 2.17]	0.062

Data presented as mean ± SD or median [IQR]. Baseline differences between lean and obese men were assessed by means of independent Student's T-test or Mann-Whitney U test, where appropriate. ^a Analyzed in 15 lean and 16 obese men; ^b analyzed in 11 lean and 17 obese men; ^c analyzed in 14 lean and 16 obese men; ^d analyzed in 12 lean and 14 obese men; ^e analyzed in 15 lean and 16 obese men; ^f analyzed in 15 lean and 16 obese men.

Table S5.2 – Metabolic phenotyping study population characteristics.

	Lean (n = 22)	Obese (n = 42)	p-value
Age [Yr]	55.0 [23.2 - 61.2]	52.4 [46.5 - 61.4]	0.661
Weight [kg]	75.8 ± 8.2	96.4 ± 8.8	<0.001
Fat Free Mass [kg]	62.8 ± 5.6	70.6 ± 6.0	<0.001
Fat Mass [kg]	13.0 ± 4.6	25.9 ± 4.8	<0.001
BMI [kg/m ²]	23.4 ± 1.9	30.1 ± 2.0	<0.001
Waist Circumference [cm]	85.8 [80.6 - 89.8]	106.6 [103.5 - 109.1]	<0.001
Hip Circumference [cm]	96.8 ± 4.4	107.3 ± 5.5	<0.001
Visceral Adipose Tissue Volume [l] ^a	0.872 ± 0.434	2.384 ± 0.751	<0.001
Subcutaneous Adipose Tissue Volume [l] ^a	1.437 ± 0.527	3.067 ± 0.798	<0.001
Whole-Body Glucose Disposal [mg/kg/min]	6.85 ± 1.85	4.04 ± 1.23	<0.001
HOMA-IR	1.7 ± 0.5	2.9 ± 1.3	<0.001
Fasting Plasma Glucose [mmol/l]	5.36 ± 0.28	5.66 ± 0.46	0.002
HbA1c [%] ^b	5.15 ± 0.38	5.28 ± 0.39	0.211
Systolic Blood Pressure [mmHg]	116.5 ± 8.1	122.1 ± 8.4	0.012
Diastolic Blood Pressure [mmHg]	71.1 ± 8.6	79.7 ± 6.9	<0.001
White Blood Cell Count, Blood [x10 ⁹ /l] ^c	4.89 ± 1.32	5.67 ± 1.25	0.027
Neutrophilic Granulocyte Count, Blood [x10 ⁹ /l] ^d	2.59 ± 0.88	3.16 ± 1.01	0.032
Eosinophilic Granulocyte Count, Blood [x10 ⁹ /l] ^d	0.22 ± 0.13	0.21 ± 0.13	0.789
Basophilic Granulocyte Count, Blood [x10 ⁹ /l] ^d	0.03 [0.00 - 0.05]	0.03 [0.00 - 0.06]	0.894
Lymphocyte Count, Blood [x10 ⁹ /l] ^d	1.60 ± 0.37	1.77 ± 0.46	0.151
Monocyte Count, Blood [x10 ⁹ /l] ^d	0.46 ± 0.14	0.52 ± 0.15	0.128
NK Cell Count, Blood [x10 ⁹ /l] ^d	0.10 [0.08 - 0.15]	0.14 [0.10 - 0.19]	0.037
NK-CD11B MFI, Blood [AU]	10999 ± 2445	12865 ± 2664	0.008
Low-grade Inflammation z-score	-0.21 ± 0.73	0.11 ± 0.60	0.059
Plasma TNF [pg/ml]	1.85 [1.50 - 2.05]	1.96 [1.62 - 2.31]	0.161
Plasma IL-6 [pg/ml]	0.58 [0.32 - 0.97]	0.64 [0.47 - 0.90]	0.344

Data presented as mean ± SD or median [IQR]. Baseline differences between lean and obese men were assessed by means of independent Student's T-test or Mann-Whitney U test where appropriate. ^a Analyzed in 21 lean and 42 obese men; ^b analyzed in 22 lean and 41 obese men; ^c analyzed in 21 lean and 39 obese men; ^d analyzed in 21 control and 38 obese men.

Table S5.3 – Validation study population characteristics – The Maastricht Study.

	NGT (n = 480)	Prediabetes (n = 109)	T2DM (n = 250)	p-value
Age [Yr]	58.5 ± 8.5	60.3 ± 8.5 ***	63.7 ± 8.2 ###	<0.001
Sex [M/F]	199/281	49/60	160/90 ###	<0.001
Smoking Status [never/former/ current]	207/218/55	35/56/18	78/130/42 ##	0.010
Weight [kg]	74.1 ± 13.2	78.1 ± 12.9 **	87.5 ± 15.9 ###	<0.001
Fat Free Mass [kg]	47.4 [40.9 - 57.7]	49.7 [41.3 - 57.0]	55.8 [46.6 - 62.3] ###	<0.001
Fat Mass [kg]	23.9 [20.0 - 28.8]	26.8 [22.0 - 34.8] ***	30.9 [24.4 - 39.4] ###	<0.001
BMI [kg/m ²]	25.5 ± 3.5	27.5 ± 4.4 ***	29.9 ± 5.1 ###	<0.001
Waist Circumference [cm]	89.4 ± 10.9	96.7 ± 11.2 ***	105.1 ± 13.1 ###	<0.001
Hip Circumference [cm]	99.2 [95.0 - 104.0]	100.5 [95.8 - 107.1] **	103.6 [99.0 - 111.3] ###	<0.001
Visceral Adipose Tissue [cm ²] ^a	112.8 [69.4 - 172.9]	160.8 [117.0 - 220.7] ***	248.5 [171.0 - 317.3] ###	<0.001
Fasting Plasma Glucose [mmol/l]	5.20 ± 0.41	5.85 ± 0.61 ***	7.61 ± 1.51 ###	<0.001
HOMA-IR ^b	1.20 [0.88 - 1.65]	1.71 [1.21 - 2.70] ***	2.27 [1.30 - 3.19] ###	<0.001
Matsuda Index ^c	4.27 [3.10 - 6.18]	2.07 [1.34 - 3.44] ***	1.79 [1.10 - 2.97] ###	<0.001
HbA1c [%]	5.3 [5.1 - 5.5]	5.6 [5.3 - 5.9] ***	6.6 [6.1 - 7.0] ###	<0.001
Systolic Blood Pressure [mmHg]	130.2 ± 17.0	133.8 ± 16.0 *	138.4 ± 16.9 ###	<0.001
Diastolic Blood Pressure [mmHg]	77.4 ± 9.9	76.3 ± 9.6	75.5 ± 9.5	0.125
Glucose lowering Therapy [%]	0	0	75.6	N/A
Blood Pressure Lowering Therapy [%]	20.8	44.0 ***	69.2 ###	<0.001
Lipid Lowering Therapy [%]	14.2	39.4 ***	72.0 ###	<0.001
Anti-inflammatory Therapy [%]	12.7	16.5	13.2	0.572
History of Cardiovascular Disease [%]	11.9	17.4	25.6 ###	<0.001
White Blood Cell Count, Blood [x10 ⁹ /l]	5.05 [4.30 - 5.90]	5.60 [4.70 - 6.80] ***	6.00 [5.10 - 7.30] ###	<0.001
Neutrophilic Granulocyte Count, Blood [x10 ⁹ /l]	2.76 [2.24 - 3.35]	3.23 [2.47 - 4.14] ***	3.46 [2.85 - 4.19] ###	<0.001
Eosinophilic Granulocyte Count, Blood [x10 ⁹ /l]	0.14 [0.09 - 0.20]	0.17 [0.11 - 0.22] *	0.17 [0.11 - 0.24] ###	<0.001
Lymphocyte Count, Blood [x10 ⁹ /l]	1.63 [1.37 - 1.98]	1.72 [1.43 - 2.07]	1.84 [1.52 - 2.24] ###	<0.001
Monocyte Count, Blood [x10 ⁹ /l]	0.42 [0.34 - 0.50]	0.46 [0.36 - 0.58] ***	0.48 [0.40 - 0.61] ###	<0.001
NK Cell Count, Blood [x10 ⁹ /l]	0.19 [0.13 - 0.27]	0.21 [0.14 - 0.27]	0.22 [0.14 - 0.31] #	0.121
NK-CD11B MFI, Blood [AU]	8866 ± 2188	9140 ± 2200	9419 ± 2291 ###	0.006
Low-grade Inflammation Z-score	-0.34 ± 0.92	0.27 ± 0.94 ***	0.53 ± 0.91 ###	<0.001
Plasma TNF [pg/ml]	2.06 [1.75 - 2.40]	2.17 [1.93 - 2.62] ***	2.46 [2.15 - 2.82] ###	<0.001
Plasma IL-6 [pg/ml]	0.47 [0.30 - 0.71]	0.59 [0.42 - 0.90] ***	0.81 [0.56 - 1.10] ###	<0.001

Data presented as mean ± SD or median [IQR]. Baseline differences between individuals with prediabetes and normal glucose metabolism (NGM), and type 2 diabetes (T2DM) and NGM were assessed by means of independent samples Student's T-test, Mann-Whitney U test, or chi-square test where appropriate. P-value prediabetes compared to NGM; * p < 0.05, ** p < 0.01, *** p < 0.001. P-value T2DM compared to NGM; # p < 0.05, ## p < 0.01, ### p < 0.001. Differences between all groups were assessed by means of one-way ANOVA, Kruskal-Wallis test, or chi-square test where appropriate; respective p-values reported in separate column. ^a Analyzed in 405 NGM, 93 prediabetes, and 198 T2DM; ^b analyzed in 310 NGM, 73 prediabetes, and 179 T2DM; ^c analyzed in 313 NGM, 71 prediabetes, and 156 T2DM.

Table S5.4 – Associations between NK-CD11B and z-LGI, plasma TNF or plasma IL-6 (n=839).

	Model	z-LGI	
		β (95%-CI)	p-value
NK-CD11B (x1000)	1	0.039 (0.010; 0.067)	0.007
	2	0.028 (0.000; 0.055)	0.048
	Model	z-TNF	
		β (95%-CI)	p-value
NK-CD11B (x1000)	1	0.040 (0.011; 0.070)	0.007
	2	0.034 (0.005; 0.064)	0.023
	Model	z-IL-6	
		β (95%-CI)	p-value
NK-CD11B (x1000)	1	0.073 (0.044; 0.102)	<0.001
	2	0.042 (0.014; 0.071)	0.004

Model 1: Adjusted for age, sex, and glucose metabolism status. Model 2: Fully adjusted model (model 1 + systolic blood pressure, total-to-HDL cholesterol ratio, smoking status, prior cardiovascular disease, and use of antihypertensive, anti-inflammatory, glucose-lowering or lipid-lowering medication). Multiple linear regression analyses in individuals with normal glucose metabolism (n = 480), prediabetes (n = 109), and type 2 diabetes (n = 250). The beta of each model is presented along with its respective 95%-CI and p-value.

Star*methods

Experimental model and subject details

Biopsy studies

Age-matched men who were scheduled to undergo abdominal or bariatric surgery were recruited. The decision to undergo surgery was made independently of this research protocol, between patient and physician. The control group consisted of 15 lean men. The obese group was composed of 17 men. Within obese individuals there were three patients with type 2 diabetes, each was on glucose-lowering medication. Six obese individuals were on lipid-lowering medication and 10 used antihypertensive medication. None of the lean individuals used any medication. Lean individuals were included due to surgery of inguinal hernia, except for one person undergoing laparoscopic cholecystectomy. Clinical characteristics of the individuals included in the study are summarized in Supplementary Table S5.1. Major exclusion criteria were presence of a history of heart, lung or kidney disease, and/or presence of endocrine anomalies. The study protocol was approved by the Medical Ethical Committee Jessa hospital and Hasselt University, Hasselt, Belgium, in accordance with the Declaration of Helsinki, and all individuals gave their written informed consent before participating in the study. Body weight, height, waist/hip circumference, and blood pressure were determined at the morning of surgery. Fat and lean body mass were estimated by bio-electrical impedance analyses (Bodystat 1500; Bodystat Ltd., Isle of Man, U.K.). Fasting venous blood samples were collected after an overnight fast for measurement of plasma glucose, serum insulin, and glycated hemoglobin. Plasma glucose was measured by the glucose oxidase method using an AU2700 analyzer (Beckman Coulter, Brea, CA, USA). Serum insulin was assessed by immunoassay (ADVIA Centaur Insulin IRI; Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). Glycated hemoglobin was assessed by high performance liquid chromatography using a HA-8160 Hi-Auto A1C analyzer (Menarini, Zaventem, Belgium). Blood cell counts were done with ADVIA 2120 Hematology System (Siemens). After an overnight fast, blood was drawn and adipose tissue biopsies were taken from the abdominal subcutaneous adipose tissue depot (SAT) and the omentum majus (VAT). For each adipose tissue depot, approximately 1-3 g adipose tissue was collected. The tissue was immediately brought to the laboratory for further processing. Stromal vascular fraction (SVF) cells were obtained by collagenase digestion of adipose tissue fragments in Dulbecco's Modified Eagle's Medium (DMEM)-Ham's F12 with Collagenase 11 (Sigma Aldrich C7657, 0.0875 mg/ml), collagenase 1 (Sigma Aldrich C0130, 1.216 mg/ml) and DNase 1 (Sigma Aldrich DN25, 0.09 mg/ml) under gentle shaking (60 cycles/min) at 37°C. The resulting suspension was filtered through a 200

μm filter and fat cells were removed as floating fraction after centrifugation (5 min, 1250 rpm). SVF cells were washed once with DMEM-Ham's F12 to eliminate collagenase, filtered through a 70 μm filter and stained for flow cytometry. In an additional study, we collected adipose tissue biopsies from female controls and female obese women ($\text{BMI} = 28.1 \pm 3.1$ vs 42.6 ± 1.6 ; $p = 0.001$) for assessment of adipose tissue NK cell subsets.

Metabolic phenotyping study

In this cross-sectional study with blinded analyses, 53 abdominally obese and 25 lean men were recruited by advertisements and enrolled at the Maastricht University Medical Center in Maastricht, The Netherlands, from August, 2012 to May, 2014 as described previously.²¹ Participants were aged 18-65 years, non-smokers, nondiabetic, free of CVD, had a waist circumference below 94 cm (lean) or between 102 - 110 cm (abdominally obese), and a stable body weight for at least 3 months. Men were excluded when fasting plasma glucose was > 7.0 mmol/l, when HbA1c was $> 6.5\%$, when serum total cholesterol was > 8.0 mmol/l, when serum triacylglycerol was > 4.5 mmol/l, when they had a contra-indication for MRI, and/or when they used medication affecting blood pressure, or serum lipid or glucose metabolism. Clinical characteristics of the individuals included in the study are summarized in Supplementary Table S5.2. All participants gave written informed consent. The study was approved by the institutional medical ethical committee (NL41397.068.12) and performed in accordance with the Declaration of Helsinki.

Metabolic and vascular measurements were conducted in a temperature-controlled room ($T = 24^\circ\text{C} \pm 0.5^\circ\text{C}$) after a 12-hour overnight fast with the individuals in the supine position. Individuals were instructed to refrain from alcohol and meals rich in lipids for a period of 24 hours prior to each study day, and strenuous physical exercise for a period of 48 hours prior to each study day. After insertion of two intravenous catheters and a 30-minute acclimatization period, baseline vascular measurements were performed and blood was taken and frozen for flow cytometry analysis. Metabolic insulin sensitivity was assessed by a 1 mU/kg/min euglycemic insulin clamp as described previously.²⁷ Briefly, insulin (Novorapid, Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed continuous manner for 180 minutes. Meanwhile, isoglycemia was maintained with a variable rate 20% glucose infusion. Metabolic insulin sensitivity was estimated from the steady-state glucose infusion rate (90 - 150 min of the clamp). Information on visceral and subcutaneous adipose tissue volumes was obtained through two-dimensional T1-weighted turbo spin echo (TSE) imaging on a 3.0T Philips Achieva MRI scanner with a dedicated sixteen-element torso coil (XL Torso coil, Philips Healthcare, Best, The Netherlands). Nine 5 mm thick transverse slices with 10 mm gaps centered at the top of the L4 vertebral body were

acquired using the following scan parameters: repetition time (TR) = 526 ms, echo time = 10 ms, turbo spin echo factor = 4, number of signal averages (NSA) = 1, field of view (FOV) = 400 x 322 mm, acquired matrix size = 308 x 164, acquired in-plane voxel size = 1.3 x 1.96 mm, reconstructed matrix size = 512 x 412, and a reconstructed voxel size of 0.78 x 0.78 mm. Images were analyzed offline after completion of the trial with dedicated software (Hippo Fat, IFC CNR, Pisa, Italy) to provide subcutaneous and visceral fat volumes.

Validation study (The Maastricht Study)

For this validation dataset we used data from the Maastricht Study, a population-based cohort study. The rationale and methodology have been described previously (Schram et al., 2014). In brief, the study focuses on the etiology, pathophysiology, complications and comorbidities of T2DM and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Individuals were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known T2DM status, with an oversampling of individuals with T2DM, for reasons of efficiency. Clinical characteristics of the individuals included in the study are summarized in Supplementary Table S5.3. For this study, fresh whole blood of 1104 participants of the Maastricht Study consecutively enrolled between January and November 2013 were used for flow cytometry. The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All individuals gave written informed consent.

To determine glucose metabolism, all individuals (except those who used insulin or those with a fasting glucose level above 11.0 mmol/l) underwent a standardized seven-point oral glucose tolerance test after an overnight fast as previously described.²⁵ Diabetes mellitus and prediabetes were defined according to the WHO 2006 criteria, the latter defined as presence of either impaired fasting glucose or impaired glucose tolerance. People with diabetes mellitus and people who used glucose-lowering medication were considered as having type 2 diabetes if they had no self-reported type 1 or other specific type of diabetes. Fasting blood samples were collected from all individuals and HbA1c, and total, LDL and HDL cholesterol were determined as described elsewhere.²⁵ Office blood pressure was determined according to a standardized protocol. Smoking status was assessed by questionnaire, and history of CVD and medication use during an interview. The magnetic resonance images were acquired using a 3T MRI system (MAGNETOM Prisma^{fit}, Siemens

Healthineers, Erlangen, Germany) with body matrix and supine radiofrequency coils. Subjects were positioned supine. After an initial scout view, a single-slice transversal image at the top level of the L4 vertebral body was obtained during breath-hold to assess areas of the visceral and subcutaneous adipose tissue compartments using a T1-weighted TSE pulse sequence with the following parameters: acquired and reconstructed in-plane voxel size $2 \times 2 \text{ mm}^3$, slice thickness 8 mm, TR 550 ms, NSA = 1, parallel imaging (GRAPPA) factor = 2, turbo factor = 7. Depending on the size of the patient, the FOV was $384 \times 312 \text{ mm}^2$ with a TE of 36 ms or $500 \times 406 \text{ mm}^2$ with a TE of 38 ms.

Method details

Flow cytometry

Fresh heparinized whole blood (50 μl) (study 1 and 3) or thawed whole blood (study 2) was used for staining for flow cytometry using CD3-fitc (BD 561807), CD19-fitc (BD 555412), CD66b-fitc (BD 555724), CD56-PE (BD 345810), HLA-DR-V500 (BD 561224), CD14-APC-H7 (BD 641394), CD16-PerCP (BD 560717), CD11B-BV421 (BD 562632), CD11c-PE-Cy7 (BD 561356), and CX3CR1-APC (Biolegend 341609). The complete gating strategy was published elsewhere (Wouters¹⁴ and Supplemental Figure S3.3 in *chapter three*). Briefly, from single blood cells, live cells were gated based on forward and side scatters. From live cells, NK cells were gated as negative for CD3 (T cells), CD19 (B cells), CD66b (granulocytes), HLA-DR (monocytes, dendritic cells) and positive for CD56. For samples from female donors, we used PBMCs and stained them with CD16-FITC, CD56-PerCP-Cy5.5, NKP46-PE, NKG2D -APC, and CD11B-Horizon V450. Singlets were gated based on both forward and side scatters. Live cells were defined as negative for live-death staining. NK cells were CD3- and CD56+, which were subdivided according to CD56 and CD16 surface expression.

Isolated SVF cells were stained with CD45-PE-Cy7 (BD 557748), CD3-fitc (BD 561807), CD19-fitc (BD 555412), CD56-fitc (BD 562794), CD66b-fitc (BD 555724), CD11B-BV421 (Biolegend 301324), and CD11c-APC-Cy7 (Biolegend 337218). Cocktail 2 included CD45-PE-Cy7 (BD 557748), CD3-V500 (BD 561416), CD4-PerCP (Biolegend 300528), CD8 APC-H7 (BD 641400), CD19-BV421 (Biolegend 302234), and CD56-APC (Biolegend 318310). SVF cells from VAT and SAT were gated for live single cells based on forward and side scatters. Immune cells were selected based on CD45 expression. Lymphocyte populations in AT were identified based on CD19 for B-cells and CD3 for T-cells. T-cells were further subdivided in CD8^+ Tc cells and CD4^+ Th cells. Non-B- and T-cells were gated for CD56 to identify NK cells (cocktail 2). From CD45^+ cells B-cells (CD19), T-cells (CD3), NK cells (CD56) and granulocytes (CD66b) were excluded for the identification

of macrophages (defined as CD11B^{hi}), which were subdivided in CD11B⁺CD11C⁻ and CD11B⁺CD11C⁺ macrophages (cocktail 1). The adipose tissues from female donors were stained with CD16-FITC (BD 555406), NKp46-PE (BD 557991), CD56-PerCP-Vio (Miltenyi 130-100-681), NKG2D-APC (Miltenyi 130-096-610), CD3-APC-Vio770 (Miltenyi 130-096-610), CD11B-V421 (562632), and a fixable viability stain V510 (BD 564406). SVF single cells were identified based on scatter parameters, and live cells were gated based on live death marker. NK cells were identified as CD11B⁺CD3⁻ cells, which were further subdivided according to CD56 and CD16 expression. Samples were measured with a FACS-Canto II (BD Biosciences). Results were analyzed with FACSdiva software (BD Biosciences). Each panel was optimized using fluorescence minus one (FMO) controls and isotype controls, yielding similar measures as auto-fluorescent samples. For each experiment, an auto-fluorescence control was performed.

NK cell isolation and measurements

PBMCs were isolated from fresh blood using density gradient centrifugation (Histopaque, Sigma-Aldrich H889-500ML). For flow cytometry of surface markers, 250 000 PBMCs were stained with Live/death fixable viability stain (BD 564406), CD3-APC-Vio770 (Miltenyi 130-096-610), CD56 PerCP-Vio700 (Miltenyi 130-100-681), CD11B BV421 (BD 562632), CD16 FITC (BD 555406), NKp46/CD335 (Miltenyi 130-092-607), NKG2D (Miltenyi 130-092-673), and measured with with a FACS-Canto II (BD Biosciences). Results were analyzed with FACSdiva software (BD Biosciences). Singlets were identified based on scatter plots. Live cells were gated using the viability stain, after which NK cells were identified as CD3⁻CD56⁺ cells.

For intracellular staining assays and for co-culture experiments, PBMCs were used for NK cell isolation using magnetic bead separation assays (Human NK isolation kit, Miltenyi 130-092-657). Per well, 50,000 NK cells were plated in 96 round-bottom plates. Monensim (BD # 554724) and Brefeldin (BD # 51-2301) were added according to manufacturer's instructions for 15 hours. Hereafter, cells were stained with viability stain (BD 564406), CD3-APC-Vio770 (Miltenyi 130-096-610), CD56 PerCP-Vio700 (miltenyi 130-100-681), CD11B BV421 (BD 562632). Hereafter Fixation and permeabilization solutions were added (Invitrogen 00-5123-43, 00-5223-56, 00-8333-56). After incubation, TNF-PE (Biolegend 502909) and IFN γ -V450 (BD # 560371) antibodies were added, and measured with with a FACS-Canto II (BD Biosciences). Results were analyzed with FACSdiva software (BD Biosciences). Singlets were identified based on scatter plots. Live cells were gated using the viability stain, after which NK cells were identified as CD3⁻CD56⁺ cells. All experiments included isotype controls and auto-fluorescent controls. For co-culturing experiments, human peripheral blood cells from a healthy donor were differentiated into human monocyte-derived macrophages as described previously.²⁸ In short, 5 x 10⁶ PBMCs

were plated in 24-well Primaria plates (Corning 353847). After 2h, non-adherent cells were discarded and culture medium was added (RPMI1640-Glutamax, Gibco # 61870-010; Pen/Streptomycin 5000 U, Gibco # 15070; Sodium Pyruvate 100mMol (100x) Gibco # 11360-039; NEN-AminoAcids (100x) Gibco # 11140-035) supplemented with 10% Inactivated Human AB serum; Milan Anal. # 000084; lot# 00625. Cells were differentiated for 7 days before further use. M2-polarized macrophages were obtained by incubation with 20 ng/ml IL-4 (Miltenyi 130-093-924) and 100 µg/ml IL-10 (ThermoFisher PHC0105) for 16 hours. Next, 50,000 isolated NK cells were added on confluent macrophages for 24h. Hereafter, cells were harvested for flow cytometry analysis using Live/death fixable viability stain (BD 564406) and CD64-BV421 (Biolegend 305019), CCR7-FITC (Biolegend 353216), CD86-APC or CD206-BV421 (BD 564062) and CD209-FITC (Biolegend 330103) for flow cytometry.

Plasma biomarkers of low-grade inflammation

The assessment of biomarkers of low-grade inflammation (C-reactive protein, serum amyloid A, interleukin 6, interleukin 8, soluble intercellular adhesion molecule 1, and tumor necrosis factor) were assessed by a multiarray detection system based on electro-chemiluminescence technology (MesoScaleDiscovery, SECTOR Imager 2400, Gaithersburg, Maryland, USA) as described previously.²⁹ Briefly, this system uses multiarray plates fitted with multielectrodes per well with each electrode being coated with a different catching antibody. The assay procedure then follows that of a classic sandwich ELISA with any of the analytes of interest captured on the relevant electrode. These captured analytes were then in turn detected by a secondary analyte-specific ruthenium-conjugated antibody, which is capable of emitting light after electrochemical stimulation. A particular advantage of this system is the ability to measure different biomarkers of low-grade inflammation and/or endothelial dysfunction simultaneously in relatively small (25 or 50 µl) serum samples.

Quantification and statistical analysis

For each analyses, the number of individuals, data expression, and statistical test used are indicated in each figure and table legend. Throughout the results section, data are expressed as mean $\pm$ SD or median [IQR]. Differences between groups were assessed by means of two-tailed independent Student's t-tests or Mann-Whitney U tests where appropriate. We used Pearson's correlation coefficients and linear regression analyses to investigate the associations between VAT, VAT NK cells, CD11b surface expression, z-scores of LGI, plasma TNF levels, and insulin resistance where appropriate. Log transformation, square root transformation, or reciprocal transformation of variables were performed if warranted to not violate the assumptions of the statistical tests.

Analyses were performed using SPSS version 20. Multiple mediation analyses were carried out with the PROGRESS plug-in for SPSS version 2.13 (A.F. Hayes, Ohio State University, OH, USA). All regression and mediation models were adjusted for potential confounders and specified per analysis. Two-tailed p-values of < 0.05 were considered statistically significant.

Additional resources

The first biopsy study (ClinicalTrial.gov, NCT02598544) and the metabolic phenotyping study have been registered (ClinicalTrial.gov, NCT01675401). The validation study is registered at the Dutch research database ToetsingOnline (NL31329.068.10) and has been approved by the Minister of Health, Welfare and Sports of the Netherlands, on the basis of the Health Council's opinion (Permit 131088-105234-PG).

chapter

Chapter 6

Obesity-associated alterations in beta-cell function can be reversed by weight loss and are partially explained by pancreatic fat: a randomized controlled trial

EMBARGOED

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chapter

Chapter 7

Diet-induced weight loss improves not only cardiometabolic risk markers but also markers of vascular function in a randomized controlled trial in abdominally obese men

EMBARGOED

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chapter

Chapter 8

General discussion

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Nederlandstalige samenvatting

Wereldwijd lijden steeds meer mensen aan overgewicht en obesitas, waardoor er tevens meer mensen ziektes als diabetes (type 2) en hart- en vaatziekten ontwikkelen. Deze ziekten leiden op hun beurt weer tot veel ziektelast, ziektekosten en overmatige sterfte. Eerdere onderzoeken hebben getoond dat er een aanzienlijke overlap is tussen de risicofactoren voor diabetes en hart- en vaatziekten.

Wanneer iemand diabetes ontwikkelt, zijn er vaak twee cruciale veranderingen in het lichaam. Enerzijds raakt het lichaam ongevoelig voor de werking van het hormoon insuline, hetgeen men insulineresistentie noemt. Anderzijds kunnen de bètacellen in de alvleesklier onvoldoende insuline aanmaken om de glucosewaarde in het bloed (de “bloedsuiker”) onder controle te houden.

Deze twee processen houden nauw verband met elkaar. Insuline is immers nodig om voedingsstoffen als glucose te verplaatsen van het bloed naar andere organen, zoals het hart, spieren en vetweefsel. In het geval van insulineresistentie is er meer insuline nodig om dit effect te bereiken, waardoor de bètacellen in de alvleesklier meer insuline moeten aanmaken. Als de bètacellen echter onvoldoende aan die verhoogde vraag kunnen voldoen, zal diabetes ontstaan. Het onvoldoende aanmaken van insuline door bètacellen (hetgeen men bètaceldisfunctie kan noemen) is echter niet zwart-wit, het ontstaat waarschijnlijk geleidelijk. Het is echter onduidelijk of bij mensen met obesitas er reeds sprake is van enige vorm van bètaceldisfunctie. Bijkomend probleem is dat het moeilijk is om bètaceldisfunctie te onderscheiden van de noodzakelijke compensatie voor insulineresistentie.

Bij de ontwikkeling van hart- en vaatziekten zijn er een aantal welbekende risicofactoren als hoge bloeddruk (hypertensie), hoog cholesterol (hypercholesterolemie), roken en oudere leeftijd. Bij de algemene bevolking zijn enkele risicofactoren minder bekend die wel belangrijk zijn, zoals insulineresistentie en verslechterde functie van de bloedvaten (vaatdisfunctie). Op zijn beurt kan vaatdisfunctie zich op verschillende niveaus manifesteren. Zo kan de werking van de binnenste laag cellen van de bloedvaten, het endotheel, verminderd zijn (endotheeldisfunctie), maar ook de elasticiteit van de grote slagaders kan afnemen (vaatverstijving). Endotheeldisfunctie kan ook in de kleinste bloedvaten (de microcirculatie) van spieren voorkomen en leiden tot insulineresistentie. Dit mechanisme, alsook andere functies van de microcirculatie in de spieren, worden in hoofdstuk twee verder beschreven en hieronder beknopt besproken.

Microcirculatie van de spier

In de skeletspieren lopen de allerkleinste bloedvaatjes (de capillairen) parallel aan de spiervezels, waardoor deze optimaal voedingsstoffen (zoals glucose, aminozuren en vetzuren) en zuurstof aan spiercellen kunnen aanbieden en afvalstoffen kunnen afvoeren. In rust is slechts een deel van de bloedvaatjes van de spier doorbloed. Er is dus een grote reservecapaciteit die benut kan worden, zoals bijvoorbeeld tijdens sporten. Ook een stijging in insuline – zoals na het eten van een maaltijd – leidt tot doorbloeding van meer bloedvaatjes. Hierdoor wordt het contactoppervlak voor (glucose)transport sterk vergroot. Dit is mogelijk doordat het endotheel van de allerkleinste aanvoerende bloedvaten, de terminale arteriolen, stikstofoxide produceren als de insulinespiegel in het bloed stijgt. Stikstofoxide zorgt voor vaatverwijding van de terminale arteriolen, waardoor er meer capillairen doorbloed worden; dit wordt microvasculaire rekrutering genoemd (grafisch weergegeven in figuur 2.1). Enerzijds wordt door microvasculaire rekrutering een normale glucosespiegel in het bloed hersteld en anderzijds wordt er energie opgeslagen in de spieren voor toekomstige inspanningen. Bij mensen met obesitas is er vaak sprake van onvoldoende microvasculaire rekrutering als gevolg van endotheeldisfunctie: er wordt minder stikstofoxide geproduceerd en er ontstaat weinig tot geen vaatverwijding. Hierdoor kan het lichaam voedingsstoffen als glucose niet goed opslaan, stijgt de glucosespiegel in het bloed tot hogere waarden en blijft deze glucosespiegel ook gedurende langere tijd verhoogd. Mede hierdoor kan op den duur onherstelbare schade ontstaan, bijvoorbeeld aan slagaders in het hele lichaam en de microcirculatie van bijvoorbeeld ogen en nieren.

Doelstellingen van dit proefschrift

Het onderzoek beschreven in dit proefschrift had diverse doelstellingen, te weten:

1. Welke mechanismen leiden, onafhankelijk van elkaar, tot insulineresistentie? (hoofdstukken vier en vijf)
2. Is er reeds sprake van veranderde bètacelfunctie bij mensen met obesitas en draagt vervetting van de alveesklier hieraan bij? (hoofdstuk zes)
3. Leidt gewichtsverlies tot verbeterde insulinegevoeligheid, bètacelfunctie, vaatfunctie en een gunstiger cardiovasculair risico? (hoofdstukken vier, zes en zeven)
4. Hoe betrouwbaar en nauwkeurig zijn de meetmethodes die we hebben gebruikt om bovenstaande te onderzoeken (hoofdstuk drie)?

Een verschil in aanpak ten opzichte van voorgaande onderzoeken is dat wij gebruik hebben gemaakt van een statistische methode genaamd meervoudige mediatie-analyse. Hiermee konden meerdere alternatieve hypothesen in één statistisch model getoetst worden, met onder andere als doel om de onafhankelijkheid van bepaalde

mechanismen te onderzoeken. Onafhankelijkheid is essentieel, als er immers verschillende manieren zijn om ziek te worden, zijn er ook verschillende behandelingen mogelijk én noodzakelijk. In het vervolg van deze Nederlandstalige samenvatting worden de belangrijkste bevindingen van dit proefschrift beschreven.

Belangrijkste bevindingen van dit proefschrift

Onderliggende mechanismen van insulineresistentie

Als obesitas ontstaat, kunnen er diverse veranderingen in het lichaam optreden. Zowel onderhuids (subcutaan) vet als vet in de buikholte (visceraal vet) nemen toe, er kan leververvetting optreden en er kan sprake zijn van verminderde microvasculaire rekrutering in de spieren. Ieder van deze veranderingen zou kunnen leiden tot insulineresistentie. Echter, of dit onafhankelijke bijdragen zijn, was niet eerder onderzocht. In hoofdstuk vier hebben wij laten zien, mede met behulp van meervoudige mediatie-analyses, dat subcutaan en visceraal vet, leververvetting en microvasculaire rekrutering – onafhankelijk van elkaar – een deel van de insulineresistentie in obesitas kan verklaren. Dit betekent dus dat ieder een separaat behandeldoel vormt in de preventie en behandeling van insulineresistentie. Bovendien hebben we, door een deel van de mensen met obesitas af te laten vallen, onderzocht of veranderingen in subcutaan en visceraal vet, leververvetting en microvasculaire rekrutering leiden tot minder insulineresistentie. Het bleek dat veranderingen in leververvetting en microvasculaire rekrutering onafhankelijk van elkaar de verbetering in insulineresistentie kunnen verklaren. Hoewel gewichtsreductie ook tot vermindering van subcutaan en visceraal vet leidde, droegen zij niet statistisch significant bij aan de vermindering van insulineresistentie.

Als we verder inzoomen naar de wijze waarop een overmaat aan visceraal vet kan leiden tot insulineresistentie, dan blijkt dit deels verklaard te worden door een laaggradige ontstekingsreactie (inflammatie) die ontstaat in het viscerale vetcompartiment. In het viscerale vet zijn macrofagen (een type witte bloedcel) verantwoordelijk voor de productie van inflammatoire signaalmoleculen (cytokines), zoals tumor necrosis factor. Wat ertoe leidt dat macrofagen aanwezig zijn in visceraal vet en deze ontstekingsreactie op gang brengen, is onduidelijk in mensen. In hoofdstuk vijf hebben wij in vier onafhankelijke studies onderzocht of een toename van natural killer cellen (NK-cellen; een ander type witte bloedcel) in visceraal vet kan leiden tot een toename van inflammatoire macrofagen in visceraal vet, laaggradige inflammatie en insulineresistentie.

Onze volgende bevindingen ondersteunen de betrokkenheid van NK-cellen:

1. mensen met obesitas hadden meer NK-cellen in hun visceraal vet;
2. de hoeveelheid NK-cellen in visceraal vet was geassocieerd met inflammatoire veranderingen van macrofagen;
3. als NK-cellen en macrofagen buiten het lichaam samengebracht worden, zorgen NK-cellen afkomstig van mensen met obesitas ervoor dat macrofagen inflammatoire veranderingen ondergaan;
4. de mate van CD11B (een eiwit aan het celoppervlak) expressie op NK-cellen in het bloed was geassocieerd met de hoeveelheid NK-cellen in visceraal vet;
5. CD11B expressie was geassocieerd met tumor necrosis factor productie door NK-cellen, bloedspiegels van inflammatoire cytokines en insulineresistentie;
6. meervoudige mediatie-analyses ondersteunen de hypothese dat meer NK-cellen in visceraal vet leiden tot laaggradige inflammatie en insulineresistentie;
7. al deze observaties waren specifiek voor visceraal vet en werden niet gevonden in subcutaan vet;
8. de associatie tussen CD11B expressie, laaggradige inflammatie en insulineresistentie werd bevestigd in een groot onderzoek uitgevoerd bij bewoners van de regio Maastricht (de Maastricht Studie).

In eerdere onderzoeken in muizen werd het mechanisme hoe NK-cellen macrofagen beïnvloeden en tot inflammatie en insulineresistentie leiden reeds gedetailleerd beschreven. Derhalve zijn wij van mening dat onze onderzoeksbevindingen een sterke aanwijzing zijn dat NK-cellen ook in mensen betrokken zijn bij het ontstaan van insulineresistentie.

Onderliggende mechanismen van bètaceldisfunctie

Als insulineresistentie ontstaat, produceren de bètacellen in de alvleesklier meer insuline als reactie op de stijgende glucosespiegel in het bloed. Bij mensen met diabetes is er sprake van een gestoorde bètacelfunctie: de insulineproductie kan de insulinebehoefte niet meer bijbenen. Het is plausibel dat er reeds in een vroeger stadium, bijvoorbeeld bij mensen met overgewicht of obesitas, een zekere mate van maladaptie van bètacellen is.

Tevens kan er bij mensen met obesitas vervetting van de alvleesklier ontstaan. Een dergelijke vervetting van de alvleesklier zou de werking van de bètacellen kunnen belemmeren; wellicht dat zo een subtiele vorm van bètaceldisfunctie kan ontstaan. In hoofdstuk zes hebben wij onderzocht of er – rekening houdend met de mate van insulineresistentie – aanwijzingen zijn voor bètaceldisfunctie bij mensen met obesitas. Een van de eigenschappen van bètacelfunctie (de zg. potentiatie factor) bleek te zijn veranderd bij mensen met obesitas. Door gewichtsreductie bij mensen met obesitas

herstelde deze bètacelfunctie zich tot het niveau van slanke mensen. Bovendien kon, met behulp van meervoudige mediatie-analyses, een mogelijke betrokkenheid van alvleeskliervervetting meer aannemelijk gemaakt worden. In onze studie kon de exacte locatie van de vetstapeling in de alvleesklier niet vastgesteld worden. Naar aanleiding van deze studieresultaten en eerdere observaties van de microcirculatie in de spier hebben wij de hypothese geformuleerd dat vetcellen die zich in de nabijheid van de kleine bloedvaten van de alvleesklier bevinden, aldaar via endotheeldisfunctie leiden tot afname van vasodilatatie en bètaceldisfunctie.

Effecten van gewichtsverlies

In de meerderheid van de hoofdstukken in dit proefschrift zijn de effecten van gewichtsreductie bij mensen met obesitas onderzocht. In hoofdstuk vier hebben wij laten zien dat gewichtsreductie leidt tot een verbeterde microvasculaire rekrutering in spier en een afname van subcutaan vet, visceraal vet, leververvetting en insulineresistentie. Vervolgens werd in hoofdstuk zes beschreven dat gewichtsreductie leidt tot een herstel van bètacelfunctie, mogelijk door afname van alvleeskliervervetting. Tot slot laten wij in hoofdstuk zeven zien dat traditionele risicofactoren voor hart- en vaatziekten, zoals hypertensie en hypercholesterolemie, verbeteren door gewichtsreductie. Anderzijds werden diverse aspecten van vaatfunctie onderzocht, hetgeen wisselende resultaten opleverde. Door gewichtsreductie verbeterde de microcirculatie van het netvlies (de retina): de verhouding tussen de diameters van retinale arteriolen en venulen werd genormaliseerd tot het niveau van slanke mensen. Bovendien daalde de bloedspiegel van sE-selectine, een maat voor endotheeldisfunctie. Echter, een andere veelgebruikte uitkomstmaat voor endotheeldisfunctie (flow-mediated dilation) verschilde in onze studie niet tussen mensen met of zonder obesitas en verbeterde ook niet door gewichtsreductie. Tevens zorgde gewichtsreductie niet voor een statistisch significante verbetering in vaatstijfheid van de grote lichaamsslagaders. Dit is ook niet te verwachten, aangezien de verstijving van bloedvaten een langdurig proces is, waarvan de reversibiliteit door een kortdurende interventiestudie (een gewichtsreductieprogramma van acht weken in ons geval) waarschijnlijk zeer beperkt is.

Samenvattend toont onze interventiestudie dat gewichtsreductie bij mensen met obesitas enerzijds leidt tot een verbetering van de processen die leiden tot diabetes (insulineresistentie en bètaceldisfunctie), alsook verbeteringen van traditionele risicofactoren voor hart- en vaatziekten (hypertensie en hypercholesterolemie). Anderzijds zagen wij dat gewichtsreductie leidde tot een verbetering van enkele vaatfunctiemetingen (bijv. microvasculaire rekrutering, vaatdiameters in de retina, sE-selectine), doch niet alle (bijv. flow-mediated dilation en vaatstijfheid). Hoewel het

reeds duidelijk was dat gewichtsreductie bij mensen met obesitas leidt tot minder diabetes, blijft het onduidelijk of het ook leidt tot minder hart- en vaatziekten.

Conclusie

Samenvattend laat dit proefschrift zien dat:

1. visceraal vet, subcutaan vet, leververvetting en microvasculaire rekrutering in de spier onafhankelijk van elkaar bijdragen aan insulineresistentie en verbeteren door gewichtsreductie bij mensen met obesitas;
2. NK-cellen in visceraal vet geassocieerd zijn met inflammatoire veranderingen van macrofagen, laaggradige inflammatie en insulineresistentie;
3. er veranderingen in bètacelfunctie zijn bij mensen met obesitas, welke herstellen na gewichtsreductie en mogelijk ten dele verklaard worden door veranderingen in alvleeskliervervetting;
4. gewichtsreductie leidt tot verbetering van traditionele risicofactoren voor hart- en vaatziekten en enkele, doch niet alle, vaatfunctiemetingen; en
5. de in dit proefschrift gebruikte meetmethodes betrouwbaar en nauwkeurig waren.

De bevindingen beschreven in dit proefschrift kunnen enerzijds leiden tot het formuleren en testen van gerichte preventie- en behandelstrategieën en anderzijds aanleiding geven tot gericht vervolgonderzoek naar de rol van bovengenoemde processen. Tot slot illustreert dit proefschrift de meerwaarde van intensieve samenwerking tussen diverse disciplines, alsmede het simultaan onderzoeken van diverse hypothesen.

Valorization addendum

Besides education and research, valorization is a main task of universities; the impact of research to society is hereby maximized. Here, the valorization potential of this thesis' main findings will be discussed. We will address the relevance and innovations of this thesis, the framework for future studies on insulin resistance and beta-cell dysfunction, the value of reliability data, and the need for in-depth phenotyping studies.

Relevance of the findings

In order to develop successful strategies to prevent or treat obesity-associated conditions like type 2 diabetes and cardiovascular disease, a thorough understanding of the pathophysiological processes involved is vital. As a substantial proportion of the general population is obese, advances in this area could substantially impact obesity-related morbidity, loss of quality of life, mortality, and health care costs. In this thesis, we identified independent contributors to insulin resistance and beta-cell dysfunction, the two core processes involved in the development of type 2 diabetes. First, we showed that visceral and subcutaneous adipose tissue, intrahepatic lipid and muscle microvascular recruitment are independent contributors to insulin resistance. This means that they constitute separate targets for intervention. Second, we identified NK cells in visceral adipose tissue to induce local and systemic inflammation and thereby contribute to insulin resistance. Third, we identified, in abdominally obese individuals, early alterations in beta-cell function independent from compensatory changes due to insulin resistance, as well as demonstrated that a weight loss intervention can restore these alterations. Fourth, we showed that a part of these obesity-induced changes in beta-cell function could be explained by intrapancreatic lipid. Finally, we demonstrated that a weight loss intervention improved traditional cardiovascular risk factors (e.g., blood pressure, serum cholesterol levels, insulin resistance), as well as some, but not all, vascular function measurements.

Likely, the identification of the independent contributors to insulin resistance and beta-cell function will have a societal impact. The novel findings mentioned above could eventually lead to therapies aimed to intervene with these pathways to prevent, delay the development, or treat type 2 diabetes and cardiovascular disease. Moreover, these findings could lead to the identification of distinct subtypes of obesity, each of which may require a different treatment strategy. Nonetheless, several future studies should be undertaken before these contributors can be considered a true – and safe – therapeutic target. Hence, several ideas for future studies, as well as how their results may impact prevention and treatment strategies will be further discussed below.

Future studies on insulin resistance

Besides the identification of independent tissue contributors to insulin resistance, our novel finding that NK cells in visceral adipose tissue appear to be involved in local and systemic low-grade inflammation and subsequent insulin resistance is particularly valuable. Even though research in rodents demonstrated NK cell accumulation in visceral adipose tissue is an early event into the development of low-grade inflammation and insulin resistance, the cross-sectional design of our studies in humans precludes any conclusions about a temporal association of NK cell accumulation to induction of inflammation and insulin resistance. To provide further evidence for NK cell's involvement in insulin resistance in humans, valuable information can be obtained by studying individuals in whom either NK cells are depleted or who have a loss-of-function mutation. First, those individuals could be evaluated for inflammatory changes in visceral adipose tissue and insulin resistance. Second, an intervention study subjecting NK cell depleted individuals as well as individuals with normal NK cell numbers and function, to short term overfeeding. Ideally, such a study could involve repeated visceral adipose tissue biopsies along with the assessment of insulin resistance. Third, repeated visceral adipose tissue biopsies obtained in intervention studies in obese individuals who are scheduled to undergo repeated abdominal surgery (e.g., bariatric vs. non-bariatric surgery) may provide valuable insights on NK cell's contribution to insulin resistance, as well as the reversibility thereof. Finally, Mendelian randomization techniques may be used to evaluate NK cell involvement in obesity-associated insulin resistance, type 2 diabetes, and cardiovascular disease. Currently, to the best of our knowledge, neither of those study ideas are being investigated. As the data on NK cells in this thesis was the result of a remarkable cooperation between departments, universities and hospitals in this region, the infrastructure to successfully execute studies as those outlined above is already in place.

Future studies on beta-cell function

Whilst the contributors to beta-cell dysfunction are incompletely understood, endothelial dysfunction and lipid accumulation in the pancreas may be involved. To evaluate endothelial dysfunction, the vasodilatory potential of pancreatic arterioles could be determined by means of imaging techniques or ex vivo pressure myography. In humans, pancreatic arterioles are difficult to obtain and the pancreatic vasculature is difficult to assess with current imaging techniques. To probe the concept of pancreatic endothelial dysfunction, studies in animals could be considered to gain arguments for its involvement in beta-cell dysfunction. In addition, the involvement and localization of intrapancreatic lipid can be evaluated by means of electron microscopy images of distinct proportions of human pancreas. Herewith, one can assess whether beta-cells of obese individuals contain intracellular lipid depositions,

as well as identify adipocytes in the direct vicinity of pancreatic (micro)vasculature. As electron microscopy images of relatively large proportions of human pancreas have been collected in another Dutch research project (Nanoscopy project), it should be feasible to demonstrate whether intracellular lipid depositions in beta-cells of obese individuals exist.

The value of reliability

The reliability of many measurements used in research or clinical practice appears to be misused, incompletely considered, or ignored altogether. Nonetheless, by properly assessing and interpreting the reliability of measurements, study designs, power calculations and decision making in clinical practice could benefit substantially. In this thesis, the reliability of a multitude of anthropometric, vascular, metabolic and inflammatory measurements has been assessed. Reliability data, as presented in this thesis, has been combined with information on a measurement's responsiveness to a dietary weight loss intervention. As a result, the study design, power calculation, and selection of measurements has already impacted several studies at our department. Moreover, insight into sources of error have been identified and addressed accordingly. Collectively, this reduces the amount of money wasted on studies with a high probability of a type 2 error (e.g., due to overestimation of a measurement's reliability), as well as the time and money spent on measurements that are unsuited for a certain study design or population.

The potential of in-depth phenotyping in intervention studies

Perhaps above all, this thesis illustrates the potential of combining in-depth phenotyping, accurate and state-of-the art measurements, and multiple mediation analyses. It allowed for the identification of mutually independent contributors to insulin resistance and beta-cell dysfunction. In contrast to the majority of research papers in this field, we probed competing theories within a single study. By performing multiple mediation analysis in a randomized controlled trial, we could probe whether changes in certain variables contributed to changes in an outcome variable. Hence, this provided additional arguments for the involvement of certain pathways. If this approach (i.e., the simultaneous measurement of potential contributing variables to an outcome, ideally with little measurement error) were implemented more often in clinical trials, competing theories could be tested. Such an insight would lead to more targeted therapies, as well as reduce the number of studies needed to be conducted in a field of research.

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Curriculum Vitae

Yvo Hubert Anna Maria Kusters was born on June 9, 1986 in Heerlen, the Netherlands; he grew up in Simpelveld. He graduated from secondary school in 2004 (VWO, Bernardinus College, Heerlen, cum laude). In the same year, he started medical school at Maastricht University, and in January 2011 he obtained his medical degree (cum laude). Whilst studying medicine, he completed the Honors program Research in Medicine, was awarded the top 3% student award, and was a (semi-)professional cyclist. In March 2011, he started his PhD research under supervision of Prof. Coen Stehouwer, Prof. Casper Schalkwijk and Dr. Alfons Houben at the Department of Internal Medicine of the Maastricht University Medical Centre, within the CARIM School for Cardiovascular Diseases. In 2015, he obtained a Master's degree of Science in Epidemiology at Maastricht University (cum laude). During his PhD project, he worked at the University of Virginia in the United States of America for nine months, under supervision of Dr. Eugene Barrett. In March 2016, he started his residency in Internal Medicine at Maastricht University Medical Center. In June 2019, he started his fellowships in Nephrology and Intensive Care Medicine.