Adipose tissue as an immunological organ

Implications for childhood obesity

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Adipose tissue as an immunological organ

Implications for childhood obesity

Vetweefsel als een immunologisch orgaan

Implicaties voor kinderen met obesitas

(met een samenvatting in het Nederlands)

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PREFACE

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CHILDHOOD OBESITY IN THE 21ST CENTURY

CHAPTER 1

General introduction

THE OBESITY EPIDEMIC

The world is caught in an obesity epidemic. Over the last three decades, the mean body-mass index (BMI) increased with 1.2 kg/m^2 worldwide, affecting both developed and developing countries (1). Translated to fat mass, the world population gained approximately 1540 kg in weight during the minute you were reading these first sentences[1]. In the Netherlands for instance, 5% of the adults were obese (BMI>30kg/m^2) in 1981, while 11% of the adults were obese in 2004 (2). The prevalence of obesity is projected to rise further over the coming decades despite signs of stabilisation in some populations. In the United States (US), which have the highest prevalence of obesity in the developed world, 30-35% of the adults were obese in 2008, while 45-51% of the adults are projected to be obese in 2030, with far-reaching consequences for population health (3). After all, the obesity epidemic coincides with an explosion of obesity-related health problems, including type 2 diabetes, fatty liver disease and cardiovascular disease (4-6).

In line with the increasing prevalence of obesity in adults, childhood obesity is also on the rise. As for the adult population, the increased caloric intake and sedentary lifestyle partly account for the childhood obesity epidemic (7). Childhood obesity is often defined as a BMI higher than 2.5 standard deviations for age and gender (7, 8). In 1980, 0.3% of the boys and 0.5% of the girls in the Netherlands were obese according to this definition, compared to 1.8% of the boys and 2.2% of the girls in 2009 (9). In the US the prevalence of childhood obesity is even higher, with 16.9% obese children in 2009 (10). The high and increasing prevalence of childhood obesity is worrying because of its detrimental health effects. Childhood obesity is associated with psychosocial complications, orthopaedic complaints, and an increased risk of type 2 diabetes, cardiovascular disease and premature death later in life (4, 7, 11). Moreover, weight loss turns out to be difficult. According to a recent Cochrane review, only combined behavioural programs and lifestyle interventions aimed at a change in diet and increased physical activity provide a clinically meaningful decrease in the weight of obese children (12). For surgical interventions, long-term prospective studies are needed to establish whether the resulting weight loss outweighs risks of surgical complications and life-long nutritional deficiencies (7).

ADIPOSE TISSUE CONTROLS GLUCOSE AND LIPID HOMEOSTASIS

Adipose tissue (AT) was long considered a neutral lipid storage organ, evolved for energy storage. Research over the past decades however challenged that traditional concept, when revealing that AT plays a pivotal role in controlling whole-body glucose and lipid homeostasis (13, 14). Both a high BMI, as in obesity, and physical wasting with weight loss, coinciding with disorders like rheumatoid arthritis, are associated with deranged glucose and lipid homeostasis (13, 15). Here, we will discuss four

[1] Assumptions: mean height 1.70m, world population 7.000.000.000, *ceteris paribus*.

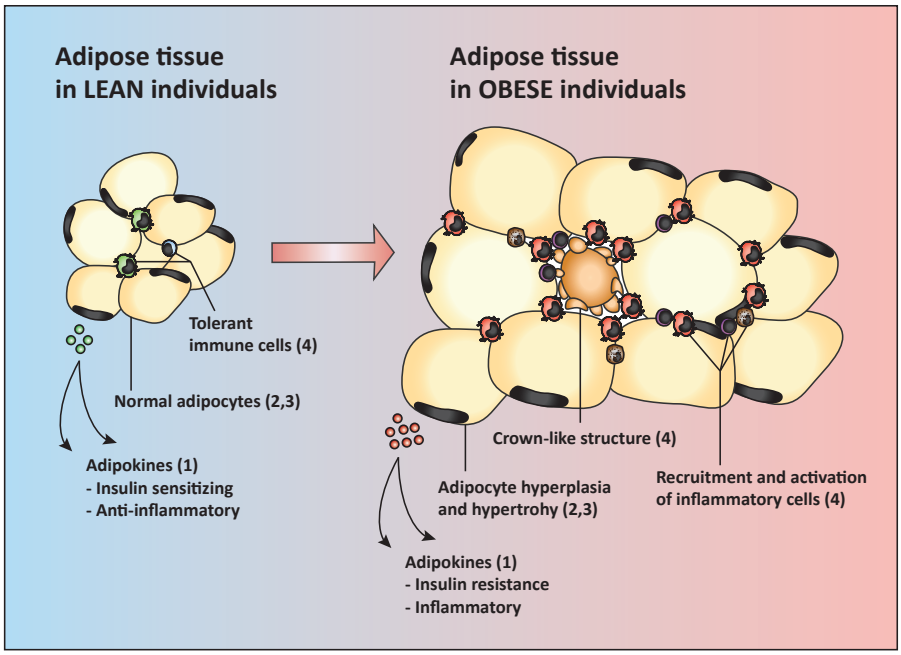

Figure 1 Four milestones in adipose tissue research
Schematic representation of adipose tissue in lean (left) and obese (right) individuals. Numbers represent the four milestones in adipose tissue research, as discussed in the text. Through these four interconnected mechanisms, adipose tissue controls whole-body glucose and lipid metabolism.

1. Adipokines. While adipose tissue (AT) of lean individuals secretes insulin sensitizing and anti-inflammatory adipokines, AT of obese individuals secretes inflammatory adipokines that promote insulin resistance.
2. Adipocyte numbers. The adipocyte hyperplasia in obesity leads to hyperleptinaemia and leptin resistance, which is associated with increased appetite and reduced energy expenditure.
3. Adipocyte hypertrophy. Obesity coincides with adipocyte hypertrophy, which activates a plethora of inflammatory cascades.
4. AT-resident immune cells. Whereas AT of lean individuals harbours tolerant immune cells, obesity leads to the recruitment and activation of inflammatory immune cells and the formation of so-called crown-like structures, which propagates AT inflammation and insulin resistance.

milestones in AT research that uncovered the key role of AT in glucose and lipid homeostasis (Figure 1).

1. Adipokines

Instead of being a passive bystander, AT can secrete bio-active proteins with profound effects on glucose and lipid homeostasis. The AT-secreted proteins are collectively referred to as adipokines, partly because of their structural and functional resemblance with inflammatory cytokines (16). The first adipokine identified was adipsin, functionally known as complement factor D. After its identification in mice in 1987 (17), it was also found in human adipose tissue (18). Adipsin was implicated

in glucose and lipid homeostasis as it is secreted by adipocytes, present in high levels in the circulation, and decreased in obesity (19). The exact role of adipsin in glucose and lipid homeostasis still needs clarification, though. Its discovery opened the way for the identification of other AT-secreted factors. In 1993, tumor necrosis factor (TNF) was identified as an inflammatory product of AT, upregulated in obesity (20). Importantly, AT-secreted TNF was shown to propagate AT inflammation and insulin resistance, and provided the first evidence for a functional link between obesity and inflammation (21). Subsequently, the adipokines leptin and adiponectin were identified in 1994 and 1995, respectively (22-24). Both adipokines are implicated in metabolic control and immune modulation, albeit with opposite functions. Whereas leptin expression is upregulated in obese AT and promotes insulin resistance and inflammation (25), adiponectin levels are downregulated in obese AT. Differential tissue expression of the adiponectin receptors AdipoR1, AdipoR2 and T-cadherin mediates anti-inflammatory effects of adiponectin on target tissues ranging from adipose tissue, liver, muscle and endothelium to circulating immune cells (26, 27). Taken together, AT controls glucose and lipid homeostasis via the secretion of adipokines. In obesity, the secretion of adipokines is skewed towards pro-inflammatory adipokines such as TNF and leptin, which promote AT inflammation and insulin resistance. Of note, adipokines take centre stage in this thesis, and a few important adipokines are listed in Table 1 below.

2. Adipocyte numbers

The second milestone is that adipocyte numbers correlate with obesity, food intake and energy expenditure. Though it may now seem self-evident, Spalding *et al* showed in a landmark study in 2008 that adipocyte numbers are significantly enhanced in obese individuals compared to lean counterparts (44). Furthermore, they showed that one's adipocyte numbers are set during childhood, and hardly change in lean and obese adults, even after marked weight loss (44). Thereby, childhood was presented as an important window of opportunity for the prevention of adulthood obesity. Moreover, the work of Spalding *et al* provided an intriguing explanation for the frequently observed 'yo-yo' body weight patterns observed after anti-obesity therapy. Adipocytes, predominantly in subcutaneous adipose tissue depots, produce the adipokine leptin, which decreases appetite and increases energy expenditure (16). Leptin levels correlate with adipocyte numbers, and are thus enhanced in obese people. Interestingly, prolonged exposure to high leptin levels in obesity induces leptin resistance, which partly explains the increased appetite and food intake in obesity (35). Upon weight loss, obese people show a relative leptin deficiency, which further increases appetite and decreases energy expenditure. Thereby, obese people that have lost weight show a tendency to return to their baseline weight, the so-called 'yo-yo' body weight curve (45). In patients with anorexia nervosa, which have hypoleptinemia, the reverse pattern is observed. Refeeding in anorexia patients is followed by hyperleptinemia, which decreases appetite and increases energy expenditure (46). Thus, anorexia patients that gain weight also show a tendency to

Table 1 Adipokines

Adipokine	Function	Thesis chapter	References
Adiponectin	Adiponectin binds to the AdipoR1 and AdipoR2 receptors, which activate AMPK and PPARα, and exhibit ceramidase activity. Effects: - Insulin sensitivity ↑ - Inflammation ↓ - During fasting, adiponectin promotes food intake in an AdipoR1 and AMPK-dependent manner. Adiponectin levels are decreased in obesity.	2-6,8	(28, 29)
Cathepsin S	Protease with elastolytic capacity, involved in ECM degradation. Cathepsin S promotes adipogenesis. Systemically, cathepsin S contributes to atherogenesis, possibly through elastin degradation in the vascular wall. Cathepsin S levels are increased in obesity.	4-7	(30, 31)
Chemerin	Ligand for CMKLR1, CCRL2, GPR1. Chemoattractant function, promotes inflammation, adipogenesis and angiogenesis. Chemerin levels are increased in obesity, and specifically during hyperinsulinemia.	4-7	(32, 33)
EGF	EGF signaling through the EGF receptor (EGFR) reduces insulin sensitivity in obese mice. EGFR inhibition decreases AT inflammation. Mechanism currently unknown. EGF levels are increased in childhood obesity.	6	(34)
Leptin	Leptin decreases appetite, and exerts pro-inflammatory effects, e.g. increased production of TNF-α and IL-6. Obesity coincides with high leptin levels and leptin resistance, which promotes insulin resistance.	2-8	(16, 35)
MCP-1/CCL2	Chemokine involved in the recruitment of monocytes and T lymphocytes. Implicated in the recruitment of monocytes and T cells to AT in obesity. MCP-1 expression is increased in obese AT.	2,3,5	(33, 36)
PAI-1	Antifibrinolytic and procoagulant activity. High levels of PAI-1 are associated with the development of type 2 diabetes, and an increased risk of ischemic cardiovascular disease. PAI-1 impairs adipocyte differentiation and insulin resistance *in vitro*. PAI-1 expression is decreased by adiponectin, and enhanced by oxidative stress and inflammation. PAI-1 levels are increased in obesity.	5	(37-39)
RBP-4	Transports retinol (vitamin A) from the liver to peripheral tissues. Transgenic overexpression of RBP-4 in mice leads to insulin resistance. Conversely, increased renal clearance of RBP-4 ameliorates insulin sensitivity. RBP-4 effects on retinoic acid signaling and RBP-4 binding to specific cell surface receptors such as STRA6 may partly explain the association between RBP-4 and insulin resistance. RBP-4 levels are increased in obesity.	5,7	(40, 41)
TIMP-1	Inhibitor of matrix metalloproteinases, with a pivotal role in ECM remodelling. TIMP-1 seems to inhibit adipogenesis and angiogenesis, possibly through its effects on the ECM. TIMP-1 levels are increased in obesity.	5,6	(42, 43)

return to their 'baseline' weight. Taken together, adipocyte numbers are set during childhood, and reflect a metabolic setpoint that is difficult to alter later in life.

3. Adipocyte hypertrophy

Next to increased adipocyte numbers, obesity is also associated with large adipocytes, so-called adipocyte hypertrophy (47). Adipocytes have an enormous capacity to store triglycerides, and can quickly reduce circulating lipid levels after high caloric feeding. Upon prolonged high caloric feeding though, hypertrophic adipocytes show reduced triglyceride storage and increased lipolysis (13). In other words, hypertrophic adipocytes become dysfunctional. Cellular stress seems to underlie this phenomenon, as adipocyte hypertrophy induces ER stress, inflammasome activation and eventually apoptosis (48-50). Apoptotic hypertrophic adipocytes form the center of so-called crown-like structures (CLS), which are extensively discussed in chapter 2. Importantly, hypertrophic adipocytes activate a plethora of inflammatory cascades, among which the release of inflammatory lipids and adipokines (13). Thereby, hypertrophic adipocytes seem one of the most important initiators of the AT inflammation that characterizes obesity, and leads to insulin resistance, i.e. the development of type 2 diabetes (13, 48).

4. AT-resident immune cells

Lastly, adipocytes exert metabolic control in concert with AT-resident immune cells. The crucial role of AT-resident immune cells was first proposed in 2003, when Weisberg and Xu and co-workers discovered high numbers of macrophages in obese AT (51, 52). Furthermore, AT-resident macrophages (ATMs) were shown to polarize towards an inflammatory phenotype in obesity (53, 54). Blocking the recruitment of ATMs or the inflammatory polarization of ATMs inhibits AT inflammation and the development of insulin resistance in diet-induced obese mice (55, 56). Since 2003, multiple AT-resident immune cell populations have been identified, including inflammatory mast cells, B-2 cells, $CD8^+$ T cells and IFN-γ^+ T helper 1 (Th1) cells, and immune modulatory regulatory T cells (Tregs) and IL-4 producing eosinophils (57-62). The interactions between adipocytes and AT-resident immune cells are concentrated in CLS, consisting of apoptotic hypertrophic adipocytes encircled by AT macrophages (ATMs), mast cells and probably also other immune cells (63). In obese AT, the number of CLS increases together with the number of hypertrophic adipocytes.

◀ **Table 1.** In this thesis, adipokine profiles of several patient groups are investigated (obese children, children with type 1 diabetes, obese adults). The most important adipokines in the context of these patient groups are listed in table 1. Thesis chapters refer to the chapter(s) discussing the adipokines. Abbreviations: EGF, epidermal growth factor; MCP-1, monocyte chemoattractant protein 1; CCL2, chemokine (C-C motif) ligand 2; PAI-1, plasminogen activator inhibitor 1; RBP-4, retinol binding protein 4; TIMP-1, tissue inhibitor of metalloproteinases 1; AMPK, AMP-activated protein kinase; PPARα, peroxisome proliferator-activated receptor alpha; ECM, extracellular matrix; CMKLR1, chemokine-like receptor 1; CCRL2, chemokine (C-C motif) receptor-like 2; GRP1, G protein-coupled receptor 1; EGFR, epidermal growth factor receptor; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; STRA6, stimulated by retinoic acid gene homolog 6.

Taken together, hypertrophic adipocytes propagate AT inflammation and insulin resistance via their interaction with AT-resident immune cells, next to the effects of adipocyte hypertrophy on the secretion of inflammatory mediators described above. The role of AT-resident immune cells is reviewed in detail in chapter 2.

ADIPOSE TISSUE AS AN IMMUNOLOGICAL ORGAN

As discussed above, AT regulates whole-body glucose and lipid homeostasis via the secretion of adipokines, through adipocyte cell numbers that reflect an indivual's metabolic setpoint, via adipocyte hypertrophy which leads to cellular stress and inflammation, and finally in concert with AT-resident immune cells. Importantly, three of these four milestones in AT-research have eminent immunological aspects: adipokines, adipocyte hypertrophy and AT-resident immune cells. In fact, these three milestones illustrate that inflammatory mechanisms partly underlie AT control of glucose and lipid homeostasis. In other words, AT is an immunological organ, which has important repercussions for whole body glucose and lipid homeostasis.

In addition to their immunological role, recent studies suggest that some AT depots may even function as a lymphoid organ. Lymphoid organs are commonly classified as primary, secondary and tertiary lymphoid organs. In thymus and bone marrow, which are the primary lymphoid organs, immature progenitor cells transit, proliferate, maturate and differentiate into mature lymphocytes (64). Secondary lymphoid organs, including the spleen, lymph nodes, tonsils, Peyer's patches and other mucosa associated lymphoid tissues (MALT), drain a specific body area and facilitate a localized immune response to antigens entering that particular region (64). Tertiary or ectopic lymphoid organs structurally resemble secondary lymphoid organs with organized B cell compartments, germinal zones and T cell compartments with antigen presenting cells (APCs), but only form at sites of chronic inflammation (65). Currently, two AT depots are known as lymphoid organs. First, the mouse and human omentum is considered a unique secondary lymphoid organ. It contains uncapsulated leukocyte clusters, so-called milky spots, that open directly to the peritoneal cavity and host effective B cell maturation zones and B and T cell responses to peritoneal antigens (66). Second, similar lymphoid structures were identified in mouse and human mesenteric AT, which is another AT depot in the peritoneal cavity. Mesenterial AT-associated lymphoid clusters were shown to produce large amounts of T helper 2 cell (Th2) cytokines such as IL-5, IL-6 and IL-13, and specifically promote helminth expulsion (65, 67). Though it is not yet known when the mesenterial lymphoid clusters form, and whether these clusters should be classified as secondary or tertiary lymphoid organs, their explicit antiparasitic function seems to distinguish them from omental milky spots. Taken together, omental and mesenterial AT are considered unique lymphoid organs, with specific roles in the peritoneal immune defense.

It is tempting to speculate on the presence of lymphoid structures in other AT depots, especially in obesity. As will be discussed in chapter 2, AT inflammation in obesity appears to be propagated and sustained by autoreactive T cells, together with

inflammatory macrophages. In other inflammatory disorders such as type 1 diabetes, tertiary lymphoid structures play a pivotal role in the activation and proliferation of autoreactive T cells (68, 69). Intriguingly, blocking of the formation of tertiary lymphoid structures prevents the pancreatic activity of autoreactive T cells and development of type 1 diabetes (65, 68, 70). However, the formation of tertiary lymphoid-like structures in obese AT has not been proven yet (59, 61, 62). Moreover, the presence of lymphoid structures in omental and mesenterial AT may be explained by their location in the peritoneal cavity, with its continuous flow of nutrients, particulates, immune cells and frequently also pathogens (71). Whether similar lymphoid structures are present in AT depots that play less of a role in the defense against pathogens, such as subcutaneous AT (SCAT) or visceral AT (VAT) surrounding retro-peritoneal organs, is questionable. In this thesis, we will leave the lymphoid functions of AT aside, and focus on the immune cells and inflammatory mediators that function as effectors in obesity-associated AT inflammation.

In conclusion, AT can be considered an immunologically active organ, and some AT depots even exert lymphoid functions. The immunological actions of AT have important implications. First, AT exerts control of glucose and lipid homeostasis through various immunological mechanisms, as discussed above. In order to understand AT metabolic control, further insight in the underlying immunological mechanisms is required. Second, the inflammatory actions of AT link obesity to its metabolic and cardiovascular complications. Especially inflammatory adipokines, which propagate and sustain the low-grade systemic inflammation observed in obesity, have been implicated in metabolic and cardiovascular complications. Thereby, we have come to the core of this thesis: *Adipose tissue as an immunological organ; Implications for childhood obesity.*

OUTLINE OF THE THESIS

The first part of this thesis (chapter 2 and 3) focuses on inflammatory mechanisms in AT, with special attention for the role of AT-resident immune cells. In chapter 2, the role of AT-resident immune cells in lean and healthy AT versus obese and dysfunctional AT will be discussed. In chapter 3, we report our discovery of CD1d-restricted natural killer T (iNKT) cells in AT. Though their presence in AT may come as no surprise given the abundance of lipid antigens in AT, which pre-eminently suits the lipid antigen-reactive iNKT cells, we will show that AT-resident iNKT cells fulfill a key role in metabolic homeostasis and the prevention of AT inflammation, partly through their interaction with CD1d-proficient adipocytes.

The second part of this thesis (chapter 4-6) focuses on the systemic effects of AT inflammation, and specifically on the role of adipokines. In chapter 4, we report the development and validation of a novel adipokine multiplex immunoassay, which allows for rapid and high-throughput measurement of 25 adipokines in only 50 µl of various biological samples. In chapter 5, our first patient study is documented. Interestingly, obesity is not the only disorder associated with enhanced levels of

circulating inflammatory adipokines. In chapter 5, we elaborate on the role of AT in auto-inflammatory disorders such as childhood type 1 diabetes (T1DM). We show that circulating inflammatory adipokine levels are significantly enhanced in childhood T1DM, and plasma of children with T1DM contains adipogenic factors that may well contribute to the AT dysfunction observed in T1DM. In chapter 6, a cross-sectional study of circulating inflammatory mediators in obese children versus healthy non-obese controls is presented. Next to circulating adipokines, we study the involvement of $CD14^{+}CD16^{++}$ and $CD14^{++}$ monocyte populations in the low-grade systemic inflammation coinciding with childhood obesity. As we will show, systemic inflammation is associated with lower insulin sensitivity, and provides a link between childhood obesity and its metabolic and cardiovascular complications.

The final part of this thesis (chapter 7 and 8) focuses on the clinical implications of AT inflammation for childhood obesity. In chapter 7, we investigate the role of vitamin D in childhood obesity. Vitamin D deficiency is endemic in childhood obesity, and associated with a lower insulin sensitivity. Here, we report evidence suggesting that vitamin D suppresses systemic inflammation, and thereby ameliorates insulin sensitivity. Supplementation of vitamin D in childhood obesity may thus improve insulin sensitivity. In chapter 8, potential immune modulatory interventions for childhood obesity will be discussed, and a future perspective on the treatment of childhood obesity will be provided. Next to vitamin D supplementation, salicylate derivates and dietary interventions that propagate immune modulatory microbiota may help to prevent systemic inflammation and its metabolic and cardiovascular complications in childhood obesity. However, their safety, cost-effectiveness and implementation in the integrative treatment of childhood obesity require careful consideration.

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PART I

INFLAMMATORY MECHANISMS IN ADIPOSE TISSUE

CHAPTER 2

Adipose tissue-resident immune cells: key players in immunometabolism

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ABSTRACT

Adipose tissue (AT) plays a pivotal role in whole-body lipid and glucose homeostasis. AT exerts metabolic control through various immunological mechanisms that instigated a new research field termed immunometabolism. Here, we review AT-resident immune cells and their role as key players in immunometabolism. In lean subjects, AT-resident immune cells have housekeeping functions ranging from apoptotic cell clearance to extracellular matrix remodeling and angiogenesis. However, obesity provides bacterial and metabolic danger signals that mimic bacterial infection, and drives a shift in immune-cell phenotypes and numbers, classified as a prototypic T helper 1 (Th1) inflammatory response. The resulting AT inflammation and insulin resistance link obesity to its metabolic sequel, and suggests that targeted immunomodulatory interventions may be beneficial for obese patients.

IMMUNOMETABOLISM

The worldwide explosion of obesity-related health problems has challenged the traditional concept of AT as a neutral lipid storage organ, and research over the past several years established AT as a *bona fide* endocrine organ that plays a pivotal role in controlling whole-body glucose and lipid homeostasis (1). Moreover, immunological mechanisms were described that underlie AT metabolic control, and have instigated a new field of research termed immunometabolism (2).

Multicellular organisms depend on two central mechanisms for their survival: the ability to store energy to prevent starvation and the ability to fight infection. With these highly conserved metabolic and immune pathways, a need to balance these pathways accurately has evolved: an immune response is highly energy-demanding and shifts energy away from nonessential functions (3). Conversely, infection and sepsis often result in metabolic disruptions such as insulin resistance (4). Four lines of evidence underscore the amalgamation between metabolism and immunology in AT. First, AT dysfunction and metabolic derangements in obesity are associated with low-grade systemic inflammation (2). Second, adipocytes are far more than lipid-storage cells. For example, the fat body in *Drosophila melanogaster* comprises AT, liver, and immunological moieties in one functional unit (5), and adipocytes in higher organisms including mice and humans are reminiscent of the integrated functionality seen in lower organisms. Through the expression of Toll-like receptors (TLRs), adipocytes sense microbial ligands and host

Box 1 Lipids as inflammatory mediators in obesity

In conditions ranging from congenital generalized lipodystrophy to diet-induced obesity and aging, toxic effects of lipid spillover on pancreas, muscle, liver and AT have been observed, and led Unger and others to propose the 'lipotoxicity' hypothesis (68). In addition to the lipid-induced insulin resistance that has primarily been attributed to diacylglycerol accumulation (69), lipid spillover activates several inflammatory pathways. First, free FAs can induce endoplasmic reticulum (ER) stress, leading to the activation of c-Jun N-terminal kinases (JNKs) and nuclear factor κB (NF-κB), two major inflammatory pathways (70). In obesity, AT shows several signs of ER stress, including increased phosphorylation of the two ER stress-sensor proteins – PRKR-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1α (IRE-1α) – and enhanced JNK activity and glucose-regulated protein 78 (GRP78) expression downstream of these sensor proteins (71). Interestingly, reduction of ER stress by administration of chemical chaperones was shown to alleviate AT insulin resistance (72). Second, saturated FAs were shown to activate NF-κB in a TLR-4-mediated fashion. Although other TLRs may also contribute (4,73,74), the role of TLR-4 is currently best-studied: mice lacking TLR-4 or with a loss-of-function mutation in TLR-4 showed protection against AT inflammation and insulin resistance upon HFD feeding or lipid infusion (75,76). Direct binding of saturated FA to TLR-4 is currently debated, and alternative explanations include effects of saturated FAs on membrane partitioning and ceramide synthesis (77,78). Furthermore, the effects of saturated FA on ceramide synthesis seem to depend on TLR-4 because a loss-of-function mutation in TLR-4 blocks ceramide accrual in lipid-infused mice (79). Third, lipid spillover has recently been associated with inflammasome activation and subsequent caspase-1-mediated IL-1β release. HFD was shown to activate the NLRP3 inflammasome, whereas knockout of the NLRP3 inflammasome protected HFD-fed mice from AT inflammation, adiposity and insulin resistance (80,81). It is tempting to speculate on a central role for the inflammasome in lipid-induced inflammation because TLR-4 stimulation and ER stress can both drive inflammasome activation (82,83).

In conclusion, lipid spillover in obesity, and particularly the overflow of saturated FAs, activates ER stress, TLR-4 and inflammasome-mediated inflammatory pathways. Although some lipid components such as omega-3 FAs exhibit anti-inflammatory and insulin-sensitizing effects (84), the total effects of lipid spillover in obesity are profoundly inflammatory.

products released upon tissue damage, the so-called danger signals (4). Furthermore, adipocytes ubiquitously express TNF-α receptors (6). In response to inflammatory signals such as the increased expression of TNF-α in obese AT, insulin action in adipocytes is inhibited, and adipocytes secrete a wide range of inflammatory cytokine mediators, termed adipokines (7). Third, lipids are far more than a source of energy. As elaborated in Box 1, lipid spillover seen in obesity activates a plethora of AT inflammatory cascades via TLRs, endoplasmic reticulum (ER)-stress mediators, and NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome-mediated pathways. Finally, immune cells are colocalized with adipocytes in AT. This review focuses on the role of visceral AT (VAT)-resident immune cells that have recently been shown to integrate metabolic and immunological functions, and comprise a pivotal pathogenic link between obesity and its metabolic sequelae.

The high immune-cell numbers in VAT suggest immunological control of the VAT energy reserve, such as observed in lymph nodes surrounding perinodal AT (PAT). Although PAT can modulate lymphocyte proliferation and activation by the release of fatty acids (FAs) and adipokines, activated lymphocytes induce lipolysis in PAT, presumably to fuel the immune response (8). In analogy with this, Chawla and coworkers proposed a model explaining the link between metabolism and immunology in VAT and other AT depots as an adaptive strategy (9). Their 'energy-on-demand model', as we will call it, assumes that immune-cell populations in VAT have evolved to fulfill the energetic demands for an

Box 2 The energy-on-demand model

AT-resident immune cells exert a wide range of functions, but can roughly be divided into two groups: on the one hand immune cells that drive AT inflammation and insulin resistance, and on the other hand immune cells that protect against these pathologies (Figure 1). The first group consists of M1 macrophages, mast cells, B-2 cells, $CD8^+$ T cells, and IFN-γ^+ $CD4^+$ T cells. Strikingly, these cells all produce TNF-α or IFN-γ, or induce the polarization of inflammatory M1 macrophages. Thereby, the AT-resident inflammatory immune cells drive what is commonly referred to as a T helper 1 (Th1) cell response, pivotal for an efficient immune response against bacteria. The second group comprises M2 macrophages, eosinophils, and regulatory T cells, which all produce IL-10, IL-4, or IL-13, and drive what is commonly referred to as a T helper 2 (Th2) response, that is instrumental for an efficient immune response against parasites. Taken together, a prototypic anti-bacterial Th1 response in AT is associated with AT inflammation and insulin resistance, whereas a Th2-skewed immune-cell response, such as seen in parasite-infested organisms, protects against these pathologies (Figure 2).

Chawla and coworkers recently proposed a model explaining Th1 and Th2 immune-cell responses in AT from a bioenergetic perspective (9). Bacterial infections create an acute bioenergetic demand because macrophages and T cells utilize circulating nutrients for an effective Th1 response and bacterial clearance (85,86). The Th1 response in AT may serve to fuel the activated immune system by promoting inflammation and insulin resistance, leading to the mobilization of nutrients via gluconeogenesis, hyperglycemia, and lipolysis. For most parasitic infections, however, deprivation of circulating nutrients is required for blocking parasite consumption of host nutrients, and slow down parasite growth (9). Accordingly, the Th2 response in AT prevents inflammation and insulin resistance, and may serve to sequester nutrients for the host. Taken together, the energy-on-demand model presents the Th1 and Th2 responses in AT as an adaptive strategy enabling a tailored immune response against bacteria and parasites.

Although the energy-on-demand model has its shortcomings – for instance, the full spectrum of immune responses against bacteria and parasites is not taken into account (87) – it helps to explain the AT inflammation observed in obesity. Obesity provides bacterial danger signals, inflammatory lipids, and other metabolic danger signals that mimic bacterial infection (Box 1, Box 3), and thereby drive an inflammatory Th1 response in AT, with deleterious effects for the host (Figure 2).

effective immune response against pathogens diverging from bacterial species to parasitic worms (Box 2). According to their model, and in the case of bacterial infections, AT inflammation and insulin resistance would be induced to raise circulating nutrient levels, and allow a quick and effective anti-bacterial response. In cases of parasitic infestations, however, sequestration of nutrients is preferred as a means to slow down parasite growth. Accordingly, the various AT-resident immune-cell populations would serve to drive AT inflammation and insulin resistance during bacterial infections, and sequester nutrients during parasitic invasions. The energy-on-demand model is a hypothesis that helps explain the role of AT-resident immune cells in obesity, and is discussed in Box 2.

The various innate, bridging, and adaptive AT-resident immune cells discussed below exert distinct functions (Figures 1 and 2). As will be reviewed, obesity provides bacterial and metabolic danger signals that drive inflammatory AT-resident immune-cell responses (Box 3). Thereby, AT-resident immune cells link the metabolic derangements in obesity to AT inflammation and insulin resistance.

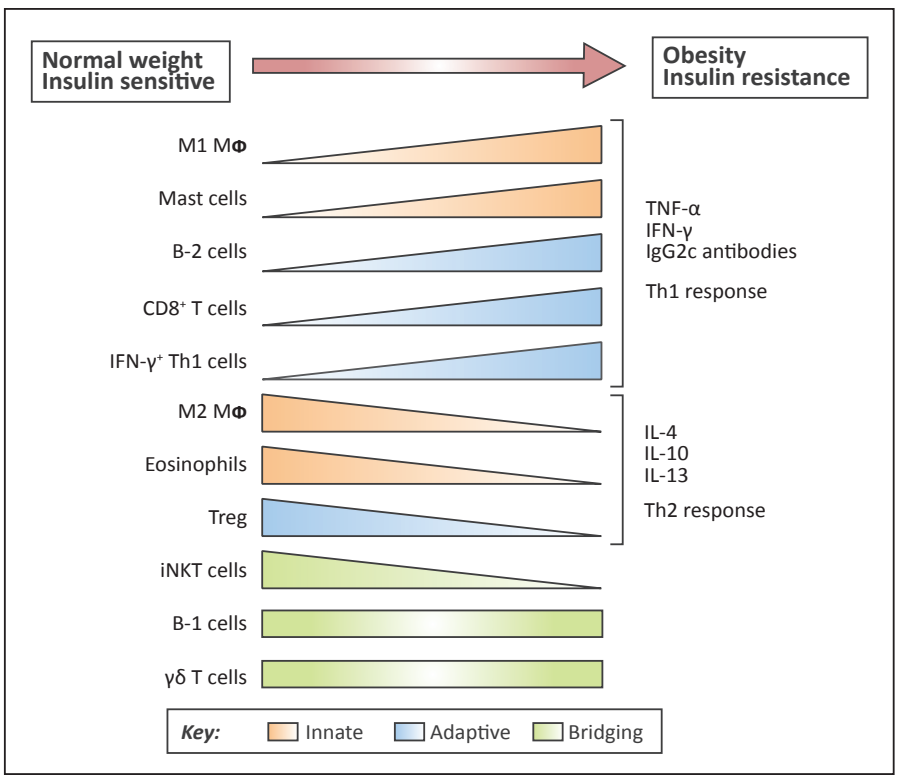

Figure 1 Immune-cell populations in adipose tissue
Schematic representation of the different immune-cell populations (innate, bridging and adaptive) in adipose tissue and obesity-associated alterations in their numbers. Cells producing TNF-α, IFN-γ and IgG2c antibodies initiate a Th1 response, whereas cells producing IL-4, IL-10 and IL-13 start a Th2 response. Mϕ, macrophages.

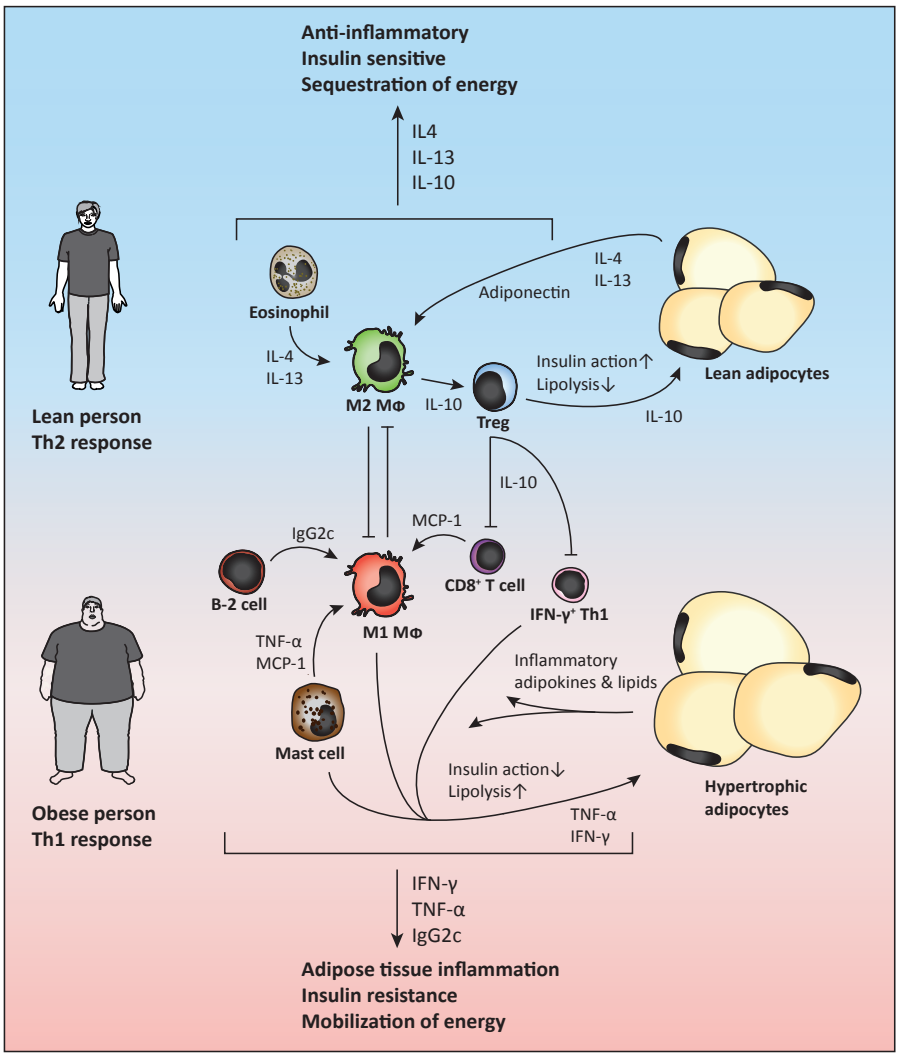

Figure 2 Obesity drives a Th1 response in adipose tissue
Schematic representation of adipocyte immune-cell interactions and the soluble mediators involved in adipose tissue of lean versus obese individuals. In lean individuals, multiple interactions between adipocytes and AT-resident immune cells (eosinophils, M2 macrophages, Treg) help to maintain an anti-inflammatory environment. Obesity provides bacterial and metabolic danger signals that mimic bacterial infection, and drive a shift in immune-cell phenotypes and numbers (B-2 cells, mast cells, M2 macrophages, $CD8^+$ T cells, IFN-γ^+ Th1 cells), classified as a prototypic T helper 1 (Th1) inflammatory response. The disruption of the delicate balance between adipocytes and AT-resident immune cells in obesity contributes importantly to the development of AT inflammation, insulin resistance, and energy mobilzation. Mϕ, macrophages.

Box 3 Bacterial and metabolic danger signals drive M1 polarization of ATMs in obesity

The energy-on-demand model assumes that bacterial antigens can evoke Th1 immune-cell responses in AT to mobilize energy (Box 2). Interestingly, obesity also provides bacterial danger signals that drive a Th1 response. Obese mice show increased LPS translocation from the gut, resulting in increased circulating levels of LPS (88). When similar LPS levels were induced in lean mice by subcutaneous infusion of LPS, lean mice showed a metabolic phenotype resembling obesity, including enhanced ATM numbers, AT inflammation, and insulin resistance (88). In humans, circulating levels of LPS were also shown to correlate with AT inflammation and insulin resistance (89). Furthermore, hematopoietic lineage-restricted TLR-4$^{-/-}$ mice exhibited reduced ATM numbers and M1 polarization on a HFD (90), indicating that TLR-4 ligands such as LPS indeed contribute to the M1 polarization of ATMs in obesity.

In addition to bacterial danger signals (e.g. LPS), obesity provides metabolic danger signals that can drive M1 polarization of ATMs. As discussed in Box 1, there is ample evidence showing that saturated FAs such as palmitate induce AT inflammation in a TLR-4-dependent manner. Saturated FAs, in conjunction with LPS, may thus account for the increased M1 polarization observed in wildtype mice on a HFD, which is absent in hematopoietcic TLR-4$^{-/-}$ animals (90). Indeed, saturated FAs were shown to drive inflammatory macrophage polarization in a TLR-4-mediated fashion *in vitro* (91). It should be noted that FA-independent mechanisms may also contribute to TLR-4 activation in obesity, because TLR ligands such as heat-shock proteins (HSP60 and HSP70), high mobility group box 1 (HMGB-I), and hyaluronan are elevated in obese patients with type 2 diabetes (92), whereas TLR antagonists such as eicosapentaenoic acid (93) and C1q/TNF-related protein 3 (CTRP3; below) (94,95) are decreased in obesity. Moreover, because lipid spillover activates macrophage inflammatory cascades via ER stress and NLRP3 inflammasome activation (Box 1), these pathways were implicated in M1 polarization of ATMs in obesity (81,96). Finally, adipocyte dysfunction in obesity seems to drive M1 polarization of ATMs in several ways. First, adipocytes can modulate macrophage polarization via the secretion of adipokines such as the well-known M2-polarizing adipokine adiponectin. Adiponectin secretion decreases in obesity, resulting in M1 polarization of ATMs (97). The recently identified adiponectin-related molecules belonging to the CTRP family (98), and in particular CTRP3, may play similar roles (95). Second, adipocyte lipid homeostasis may influence ATM function because a reduction of lipolysis was shown to decrease ATM numbers (99). Third, obesity is associated with increased numbers of CLS in AT, especially in mice. Although adipocyte necrosis in CLS appears to be independent of the encircling macrophages, the phagocytosis of adipocyte-derived fragments may well drive M1 polarization of ATMs (9,100).

Taken together, obesity provides both bacterial and metabolic danger signals that drive M1 polarization of ATMs, and thereby an inflammatory Th1 response in AT.

INNATE IMMUNITY

Innate immune-cell responses are evoked by general danger signals associated with invading pathogens, for instance via pattern-recognition receptors such as TLRs. Innate immune cells including neutrophils, dendritic cells (DCs), macrophages, mast cells, and eosinophils differentiate from a common myeloid precursor, and have all been identified in AT (10).

Upon infection, neutrophils are generally among the first immune cells to arrive at the site of inflammation. In obesity, the role of neutrophils has been studied only poorly. However, a few reports in humans and mice suggest systemic neutrophil activation in obesity, and transient infiltration of AT by neutrophils, at the onset of obesity-induced AT inflammation (11,12). As is the case for neutrophils, AT-resident DCs have not been well studied. Although antigen-presenting DCs have been implicated in lymphocyte maturation in omental milky spots (13), most of the antigen-presenting cells (APCs)

in AT were classified as macrophages based on their F4/80 (mouse) or EMR1 (human) expression (9).

Macrophages, unlike the short-lived neutrophils, can have a lifespan of years, and reside in many tissues where they fulfill housekeeping functions (10). In AT, macrophages were observed to encircle and phagocytose necrotic adipocytes in so-called crown-like structures (CLS) (14). Furthermore, AT macrophages (ATMs) have been implicated in extracellular matrix (ECM) remodeling, angiogenesis, and the proliferation and differentiation of adipocyte precursors in AT (15,16). Finally, ATMs can secrete interleukin-10 (IL-10), which preserves adipocyte insulin sensitivity via the inhibition of inflammatory mediators such as TNF-α, among many other functions (17,18). In obesity, however, ATM numbers increase and ATM function alters. Whereas ATMs in lean mice comprise around 10–15% of all cells in visceral AT, obese mice show up to 50% ATMs (19,20). In humans, ATM numbers are lower but also increase with obesity (from 4% in visceral AT of lean subjects to approximately 12% in obese patients) (21). The increased ATM numbers in mice and men are the result of monocyte recruitment, mediated via the chemokine receptor pathways CCR2/CCL2, CCR1/CCL5 and others (22–24).

In addition to increased numbers, ATMs show an altered phenotype and function in obesity. From an anti-inflammatory (M2) phenotype characterized by IL-10 secretion, arginase 1 and CD206 expression in lean mice and humans, in obesity ATMs polarize towards an inflammatory (M1) phenotype characterized by TNF-α secretion, and expression of nitric oxide synthase 2 and CD11c (14,25). In addition to M1-polarized macrophages, mixed M1/M2 phenotypes have also been reported in mouse and human obese AT, reminiscent of the mixed M1/M2 phenotypes observed in other diseases (26,27). Importantly, the inflammatory ATM polarization in obesity towards an M1 or mixed M1/M2 profile plays a pivotal role in the development of AT inflammation in obesity (9). Several mechanisms by which inflammatory ATMs induce AT inflammation and insulin resistance have been described. First, M1-polarized $CD11c^+$ macrophages block insulin action in adipocytes via TNF-α-mediated downregulation of the glucose transporter GLUT4 and inhibition of insulin signaling (28). Ablation of $CD11c^+$ macrophages was shown to increase AT expression of IL-10, to reduce AT expression of pro-inflammatory cytokines, and to improve insulin sensitivity (29). Second, inflammatory ATMs contribute to disproportionate accumulation of collagen and other ECM components, resulting in AT fibrosis, stress and inflammation (15,30). Third, inflammatory ATMs drive the recruitment and activation of other immune cells in AT by the secretion of chemokines, the presentation of antigens on MHC class I and II molecules, and costimulatory signals such as the CD40/CD40L dyad (9,29). Inflammatory polarization of ATMs is driven by two classes of danger signals. In addition to bacterial danger signals such as the TLR-4 ligand lipopolysaccharide (LPS), obesity-associated metabolic danger signals play an important role in ATM polarization (Box 3). Thereby, ATMs present as the sentinels of AT immune homeostasis, driving chronic AT inflammation and insulin resistance upon prolonged exposure to metabolic danger signals in obesity.

For modulation of local immune responses, tissue-resident macrophages are often assisted by other innate immune cells, such as TLR-proficient mast cells (31). Upon high-fat diet (HFD) feeding, liver and AT-resident mast cell numbers increase proportionally with fat mass, ATM numbers and insulin resistance (32). On the contrary, in mast cell-deficient mice, fat mass decreases together with ATM numbers and insulin resistance. Considering these findings, it was hypothesized that mast cells appear in obese AT before the more numerous macrophages, and may even regulate the influx of macrophages, for instance via MCP-1 secretion (32). Alternatively, the decreased fat mass and increased resting metabolic rate in mast cell-deficient mice may indicate a role for mast cells in balancing oxidative metabolism in AT and liver. AT-resident mast cells produce TNF-α and IFN-γ, known as modulators of lipolysis, insulin resistance, and oxidative metabolism (32–35). Finally, *in vitro* experiments have shown that mast cells can induce protease expression by adipocytes, which promotes microvessel growth, in an IL-6- and IFN-γ-dependent fashion (32). Therefore, mast cells have been implicated in AT angiogenesis and subsequent AT expansion in obesity. Taken together, mast cells appear capable of regulating adipocyte lipid and glucose metabolism, ECM remodeling and angiogenesis, and present as capable assistants of ATM-mediated AT inflammation in obesity. Notably, liver-resident mast cell numbers are also increased in obesity (32), and may regulate insulin sensitivity in parallel to the AT-resident mast cells.

Eosinophils represent a third innate immune-cell type in AT. Similarly to mast cells, eosinophil cell numbers in AT are lower than ATM numbers. In contrast to mast cells, however, eosinophils prevent AT inflammation and insulin resistance, and AT-resident eosinophil numbers decrease on a HFD. In fact, AT-resident eosinophils were identified as the major IL-4-expressing cells in AT. Making use of IL-4 reporter mouse models, approximately 90% of the IL-4-producing cells in AT were identified as eosinophils (36). Because IL-4 and IL-13 play a key role in M2 polarization of ATMs (9), it was hypothesized that AT-resident eosinophils drive M2 polarization. Indeed, making use of eosinophil knockout mice and hyper-eosinophilic mouse models, it was shown that AT-resident eosinophil numbers are positively correlated with M2 ATM numbers. Furthermore, adoptive transfer of wild type and IL-4/IL-13-deficient eosinophils revealed that eosinophils drive M2 polarization of ATMs in an IL-4/IL-13-mediated fashion (36). Thus, succumbing AT-resident eosinophil numbers may underlie the M1 polarization of ATMs in obesity. Taken together, AT-resident eosinophils counterbalance the effects of mast cells, in part by driving M2 polarization of ATMs. Finally, the low AT mass in hyper-eosinophilic mice, and high AT mass in eosinophil-deficient mice, are not easily explained by mere effects of eosinophils on ATMs (36), and suggest that eosinophils also regulate metabolism, AT inflammation, and insulin resistance at another level, possibly beyond AT.

ADAPTIVE IMMUNITY

While innate immune-cell responses are evoked by danger signals and play a key role in the initiation of inflammation, B-2 and T lymphocytes exert adaptive

immune functions crucial for a specific and decisive immune response, and for the development of immunological memory (37). Consistent with their wide-ranging antigen receptor repertoire, B-2 and T lymphocytes are not only involved in the defense against pathogens but also in sterile inflammation and autoimmune disorders (38,39). Their discovery in AT, as the largest group of immune cells after ATMs (40), started an intriguing search for the antigens involved, and for their interplay with other AT-resident immune cells.

B-2 cells comprise the majority of B lymphocytes in immune organs, such as spleen and lymph nodes, but also in AT, and accumulate in AT upon HFD feeding. B-2 cell-derived high-affinity IgG2c antibodies are elevated in obese mice and have emerged as mediators of insulin resistance. Upon transfer of IgG2c antibodies, lean mice showed AT inflammation and insulin resistance (40). Interestingly, the preferred localization of IgG antibodies in regions of CLS suggest that part of the IgG targets in AT are CLS-associated (40). It was hypothesized that IgG2c antibodies influence macrophage polarization because the influx of AT-resident B cell numbers precedes M1 polarization of ATMs (41), ATMs show reduced M1 polarization in B cell deficient mice, and IgG2c antibodies induce TNF-α production by macrophages *in vitro* (40). In obese humans, however, more than 100 IgG targets associated with insulin resistance were identified, predominantly intracellular proteins ubiquitously expressed in AT and other tissues (40). The IgG targets outside AT suggest that B-2 cells also exert effects on insulin resistance outside AT.

Similarly to B-2 cells, T-lymphocyte numbers are also increased in obese AT (42,43). Three T-lymphocyte subsets were identified, with distinct roles in AT immune homeostasis: $CD8^+$ T cells, IFN-γ^+ $CD4^+$ T (Th1) cells, and $Foxp3^+$ regulatory T cells (Tregs). The infiltration of $CD8^+$ T cells in AT was shown to precede macrophage influx and M1 polarization in obesity, reminiscent of the B cell influx (44,45). *In vitro*, AT-derived $CD8^+$ T cells stimulated macrophage differentiation and M1 polarization of monocytes, suggesting that $CD8^+$ T cells are involved in macrophage recruitment and polarization in obesity (45). *In vivo*, CD8-deficient mice indeed showed impaired M1 polarization of ATMs. Furthermore, depletion of $CD8^+$ T cells in obese mice improved insulin sensitivity, whereas adoptive transfer of $CD8^+$ T cells induced insulin resistance in CD8-deficient mice (45). Thus, $CD8^+$ T cells seem to contribute to the initiation and propagation of AT inflammation and insulin resistance, in part via ATM recruitment and M1 polarization. Of note, CD8-deficient mice on a HFD still develop moderate insulin resistance, and adoptive transfer of $CD8^+$ T cells in $Rag^{-/-}$ mice that lack all B and T lymphocytes does not aggravate the pre-existing insulin resistance phenotype in $Rag^{-/-}$ mice (45,46). Thus, $CD8^+$ T cells may require collaboration with other AT-resident immune cells to exert their inflammatory role in AT.

IFN-γ-producing $CD4^+$ T (Th1) cells represent the second T-lymphocyte subset identified in AT. Similarly to $CD8^+$ T cells, IFN-γ^+ $CD4^+$ T cell numbers in AT increase with obesity. Notably, AT-resident $CD4^+$ T cells show an increasing bias towards antigen-specific (clonotypic) T cell receptor (TCR) repertoires in obesity. Thus, it has been suggested that AT dysfunction and inflammation in obesity drive antigen-

specific selection and expansion of AT-resident $CD4^+$ T cells, including Th1 cells (46). Furthermore, Th1 cells have been implicated in AT inflammation and insulin resistance, similarly to $CD8^+$ T cells. First, IFN-γ-deficient mice show reduced AT inflammation and ameliorated insulin sensitivity (47). Second, antibody-mediated skewing of AT-resident T cells from IFN-γ^+ $CD4^+$ T cells to regulatory T cells ameliorates insulin sensitivity (46,48). Taken together, decreased IFN-γ and Th1 cell expression in AT is associated with decreased AT inflammation and improved insulin sensitivity. As discussed above for the IFN-γ producing mast cells in AT, the specific role of IFN-γ in AT needs elucidation, but may involve modulation of oxidative metabolism and microvessel growth (32,34).

The third T cell subset involved in AT immune homeostasis are the $Foxp3^+$ regulatory T cells (Tregs). In contrast to the AT-resident $CD8^+$ and IFN-γ^+ $CD4^+$ T cells described above, Treg numbers decrease with obesity, and Tregs prevent AT inflammation and insulin resistance (48). Whereas antibody-mediated T cell polarization towards a regulatory phenotype ameliorates insulin sensitivity, Treg depletion was shown to aggravate AT inflammation and insulin resistance in mice (48). Three different mechanisms have been proposed. First, AT-resident Tregs improved glucose uptake of adipocytes *in vitro* (48). Second, AT-resident Tregs showed an inverse correlation with M1 polarized ATM numbers in humans, and, similarly to many other AT-resident immune cells, may act on AT inflammation through interaction with ATMs (49). Third, the inverse correlation between IFN-γ^+ and $Foxp3^+$ T cell numbers in AT suggests that Tregs could prevent Th1 skewing of the AT-resident T cells. In obesity, AT dysfunction and inflammation may drive antigen-specific selection and expansion of Th1 cells at the expense of Treg populations, explaining the decreased numbers of AT Tregs observed in obese mice and men (48,49). Taken together, Tregs can be added to the list of AT-resident immune cells that prevent AT inflammation and insulin resistance and disappear in obesity, such as M2 ATMs, eosinophils, and possibly iNKT cells.

In conclusion, the discovery of adaptive immune cells in AT, with a bias towards AT-specific antigen receptors, sheds new light on AT immune homeostasis. Apart from the metabolic danger signals that can drive activation of AT-resident innate and bridging immune cells, AT-specific antigens seem to control the selection and expansion of adaptive immune cells. Taken together, AT inflammation in obesity shows many similarities to other inflammatory responses, however, the regulation by metabolic danger signals and AT-specific antigens makes an important difference.

BRIDGING IMMUNITY

Bridging immune cells are also named innate-type B and T cells because they exhibit characteristics that are ascribed to both innate and adaptive immunity. Similarly to adaptive immune cells, they express B or T cell receptors generated by V(D)J recombination. Unlike adaptive immune cells, however, bridging immune cells are unable to develop immunological memory. Furthermore, their B and T cell receptors have a restricted repertoire of antigen specificities and resemble innate pattern

recognition receptors in their responsiveness to conserved danger signals (50). In AT, three bridging immune-cell types have been described, each with distinct roles and relatively sparsely represented compared to ATM populations: γδ T cells, invariant natural killer T (iNKT) cells, and B-1 cells.

Recently, γδ T cells were identified as the main IL-17-producing cells in AT (51,52). The IL-17 release of γδ T cells can be induced by IL-1β, without further TCR engagement (53). Because IL-1β in AT is released upon lipid-mediated inflammasome activation (Box 1), it is tempting to assume that the IL-17 release of γδ T cells in AT is driven by lipid spillover. At the same time, the different immunological effects of IL-17-producing γδ T cells, including stimulation of IL-1, IL-6, IL-23 and TGF-β release by resident macrophages (53), make it difficult to predict the consequences of γδ T cell activation in AT. Although the inflammatory effects of IL-17 are widely studied, particularly in cardiovascular disorders (54,55), and one study suggests that IL-17 reduces AT expansion and ameliorates insulin sensitivity (51), the local effects of IL-17 on AT immune homeostasis need further study.

In contrast to γδ T cells, iNKT cells appear to be enriched in mouse and human AT, compared to the circulation (52,56). The enrichment of iNKT cells in AT may come as no surprise because the abundance of lipid antigens pre-eminently suits lipid-sensitive iNKT cells, which respond to lipid/CD1d complex binding by the release of immune-polarizing cytokines (57). Nevertheless, the role of AT-resident iNKT cells in AT inflammation and insulin resistance is debated: some studies showed no differences in insulin sensitivity between wild type mice and iNKT-deficient mice on a HFD (58–60), whereas other studies showed improved insulin sensitivity in iNKT-deficient obese mice (61–63) or improved insulin sensitivity in obese mice upon administration of iNKT cell ligands (60,64). These discrepancies may be explained by differences in experimental conditions (diet composition, duration of diet), indigenous microbiota, and the low and possibly variable numbers of AT-resident iNKT cells in obesity (56,60) that can only affect AT inflammation and insulin resistance upon ligand-mediated activation. The decreased AT-resident iNKT cell numbers in obesity suggest that AT-resident iNKT cells may play a more prominent role in AT immune homeostasis in lean mice and men, but this hypothesis requires further investigation.

The final bridging cell type in AT are B-1 cells, which are also enriched in AT compared to the circulation (40). AT-resident B-1 cells may well assist ATMs in their housekeeping functions, as do mast cells. First, B-1 cells express a limited B cell receptor (BCR) repertoire that is enriched for polyspecific BCR to self antigens such as oxidized lipids and apoptotic cells. B-1 cells secrete large amounts of polyreactive IgM antibodies that promote phagocytosis of apoptotic cells (65). Second, IgM antibodies secreted from B-1 cells are preferentially localized in regions of CLS, indeed suggesting involvement of B-1 cells in ATM-mediated clearance of adipocyte remains (40). Third, B-1 cells are TLR-4 proficient and fulfill housekeeping functions together with macrophages in many other tissues (65). However, AT IgM levels are not affected by obesity, and administration of IgM antibodies to mature B cell-deficient mice does not influence insulin sensitivity, in contrast to the B-2 cell-derived IgG

antibodies discussed above (40). Thus, B-1 cells may fulfill housekeeping functions in AT in concert with ATMs, but appear not to be directly involved in obesity-induced AT inflammation and insulin resistance.

In conclusion, both iNKT cells and B-1 cells are enriched in AT. The potential of these bridging immune cells to elicit immune responses rapidly, together with their ability to respond to conserved danger signals, presumed to include metabolic danger signals, make them interesting players in AT immune homeostasis. Nevertheless, their specific roles in AT remain to be established.

CONCLUDING REMARKS

AT-resident immune cells such as ATMs, mast cells, and B-2 cells fulfill important housekeeping functions in AT. In addition to clearance of apoptotic adipocytes, these immune- cells have been implicated in ECM modeling, angiogenesis, adipogenesis, and the preservation of insulin sensitivity in lean subjects. In obesity, however, AT-resident immune cell populations shift in phenotype and numbers (Figure 1). Obesity provides bacterial and metabolic danger signals that activate a plethora of inflammatory cascades and drive M1 polarization of ATMs (Box 1, Box 3) which function as the sentinels of AT inflammation. Furthermore, AT-resident regulatory T cells and the IL-4 producing eosinophils are downregulated in obesity, whereas inflammatory cell types such as IFN-γ^+ Th1 cells and $CD8^+$ T cells prevail. Thereby, the obesity-induced shift in AT-resident immune cell phenotypes and numbers presents as a prototypic Th1 response (Figure 2). In contrast to Th1 responses against bacteria, however, obesity results in prolonged inflammation and insulin resistance. Thereby, obesity may turn an adaptive strategy into a metabolic disaster (Box 2). After all, prolonged AT inflammation and insulin resistance underlie most comorbidities in obese patients.

Box 4 Outstanding questions

- In the studies on AT-resident immune cells performed so far, whole-body depletion or knockout models were used. Do other immune-cell populations, such as liver-resident immune cells, also contribute to the observed effects on insulin resistance?
- Immune-cells may regulate metabolic parameters at different levels, ranging from appetite, adipocyte growth, and lipid metabolism, to AT inflammation. Until now, most studies focused on the effects of AT-resident immune cells on ATMs and AT inflammation. Do AT-resident immune cells also modulate metabolism at other levels?
- Obesity-induced insulin resistance is particularly associated with visceral AT (VAT) expansion and inflammation, and most studies thus far have focused on the role of VAT-resident immune cells. In contrast to VAT, expansion of subcutaneous AT (SCAT) has been associated with improved insulin sensitivity, and transplantation of SCAT was shown to confer these metabolic benefits (89). Are the different metabolic actions of SCAT and VAT related to depot-specific immune-cell populations and immune-cell responses?
- Tissue-specific danger signals often drive the effector class switch (Th1/Th2) of local immune responses (80). Several metabolic danger signals that drive innate immune-cell polarization in obesity have been identified (Box 3). But what are the AT-specific antigens that drive T cell selection and expansion in AT?
- Most inflammatory responses serve to restore proper tissue function. Is obesity-induced AT inflammation merely destructive, or does the inflammatory Th1 response in AT also serve to restore AT function?

Although oustanding questions remain (Box 4), the inflammatory nature of obesity offers new opportunities for breaking the links between obesity and its metabolic sequelae. In addition to anti-inflammatory drugs such as NF-κB inhibitors and IL-1 receptor antagonists that have already been shown to improve inflammatory and glycemic parameters (66,67), targeted immunomodulatory interventions that block the prolonged exposure to inflammatory danger signals may further enhance the metabolic and cardiovascular outcome of obese patients.

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CHAPTER 3

Natural killer T cells in adipose tissue prevent insulin resistance

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ABSTRACT

Lipid overload and adipocyte dysfunction are key to the development of insulin resistance and can be induced by a high-fat diet. CD1d-restricted invariant natural killer T (iNKT) cells have been proposed as mediators between lipid overload and insulin resistance, but recent studies found decreased iNKT cell numbers and marginal effects of iNKT cell depletion on insulin resistance under high-fat diet conditions. Here, we focused on the role of iNKT cells under normal conditions. We showed that iNKT cell-deficient mice on a low-fat diet, considered a normal diet for mice, displayed a distinctive insulin resistance phenotype without overt adipose tissue inflammation. Insulin resistance was characterized by adipocyte dysfunction, including adipocyte hypertrophy, increased leptin, and decreased adiponectin levels. The lack of liver abnormalities in CD1d-null mice together with the enrichment of CD1d-restricted iNKT cells in both mouse and human adipose tissue indicated a specific role for adipose tissue-resident iNKT cells in the development of insulin resistance. Strikingly, iNKT cell function was directly modulated by adipocytes, which acted as lipid antigen-presenting cells in a CD1d-mediated fashion. Based on these findings, we propose that, especially under low-fat diet conditions, adipose tissue-resident iNKT cells maintain healthy adipose tissue through direct interplay with adipocytes and prevent insulin resistance.

INTRODUCTION

More than one-third of the U.S. population has insulin resistance, a condition that is predominantly caused by obesity and is associated with adipocyte dysfunction together with chronic low-grade adipose tissue (AT) inflammation (1-3). Lipid-induced adipocyte dysfunction appears instrumental to the inflammatory response in AT (4), which is characterized by inflammasome activation (5) and the release of fatty acids and cytokines (adipokines) that impair insulin receptor signaling, ultimately resulting in the development of metabolic syndrome (6-8).

Distinct mechanisms impart control of immune homeostasis within AT, some of which were recently uncovered. AT-resident Tregs together with eosinophils control the development of local inflammation by counteracting the influx of $CD11c^+$ (M1) inflammatory macrophages, $CD8^+$ T cells, $CD4^+$ T cells, and B cells, thereby preventing AT inflammation and insulin resistance (9-16). How adipocyte dysfunction relates to immune homeostasis, however, remains incompletely understood, and a self-reactive cell type involved in orchestrating immune homeostasis in AT has not yet been reported.

Various findings prompted us to study the role of lipid antigen-reactive invariant natural killer T cells (iNKT) cells in controlling AT inflammation and insulin resistance. First, the abundance of lipid antigens in AT pre-eminently suits lipid-sensitive invariant T cells such as iNKT cells, as they are triggered to release immune-polarizing cytokines by lipid/CD1d complex binding (17-19). Second, CD1d-restricted iNKT cells have roles in multiple metabolic disease models, including type 1 diabetes mellitus (20-23). Third, many tissues harbor resident T cells that can respond to stress-induced self molecules rather than foreign antigens and ensure a tissue-specific effector class (Th1, Th2, or tolerogenic) response (24). iNKT cells are known to fulfill this role in the liver, representing up to 40% of liver-resident T cells in mice (19). Fourth, we were intrigued by the apparent enrichment of iNKT cells in mouse and human AT compared with peripheral blood (our unpublished observations and refs. 25, 26), especially in lean mice and humans. Fifth, recent studies showed that under high-fat diet (HFD) conditions, CD1d-restricted iNKT cell function only marginally affects the development of insulin resistance (26-28). Accordingly, we hypothesized that AT-resident CD1d-restricted iNKT cell function may be particularly relevant under normal diet conditions.

We employed CD1d-null and Jα18-null mice, antibody depletion of iNKT cells in WT mice, and human AT to address the role of AT-resident CD1d-restricted iNKT cells. Our mouse-based data show a unique role for CD1d-restricted iNKT cells in the maintenance of healthy adipocytes and prevention of insulin resistance, especially under low-fat diet (LFD) conditions, considered a normal diet for mice (29). Furthermore, coculture of human CD1d-restricted iNKT cells with adipocytes revealed a potential mechanism linking adipocyte dysfunction to immune cell homeostasis, showing that CD1d-proficient adipocytes can function as lipid APCs for iNKT cells.

RESULTS

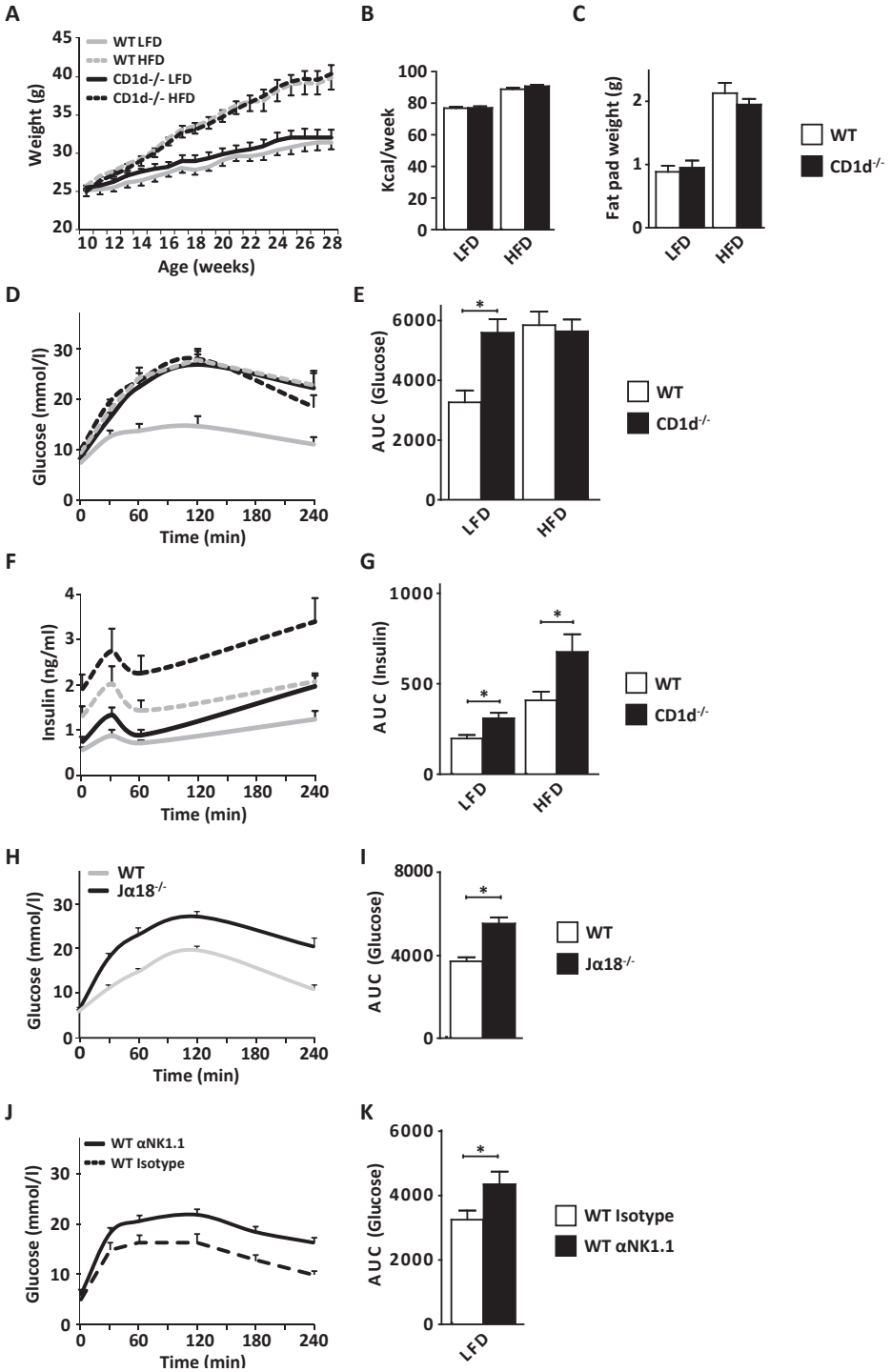
iNKT cell knockout and antibody-mediated depletion result in insulin resistance in lean mice

We addressed the impact of CD1d-restricted iNKT cells on AT homeostasis and insulin resistance using CD1d-null (30) and WT C57BL/6 mice. The mice were fed normal chow until 11 weeks of age, followed by 19 weeks of LFD or HFD. Weight gain, caloric intake, and epididymal fat pad weight were similar among the genotypes, for both LFD and HFD mouse groups (Figure 1, A-C). Strikingly, glucose tolerance measured via an intraperitoneal glucose tolerance test (IP-GTT) was clearly impaired in the CD1d-null mice compared with their WT counterparts, especially under LFD conditions (Figure 1, D-G). Under HFD conditions, CD1d-null mice showed higher insulin levels during the IP-GTT than their WT counterparts, but maintained comparable glucose levels (Figure 1, D-G), in accordance with previous studies (26-28). To corroborate these findings, we used of Jα18-null mice, which are selectively deficient in type 1 iNKT cells (31). These animals also showed impaired glucose tolerance after 18 weeks of LFD compared with their WT counterparts (Figure 1, H and I) while maintaining comparable body weight (data not shown).

The insulin resistance in lean CD1d-null and Jα18-null mice was confirmed using an established iNKT depletion model, comparing antibody-treated (anti-NK1.1) with isotype control-treated LFD-fed WT mice (32, 33). Partial depletion of AT-resident iNKT cells resulted in impaired glucose tolerance compared with isotype control treatment (Figure 1, J and K, and Supplemental Figure 1A). When a gain-of-function approach was pursued by *in vivo* activation of iNKT cells in WT mice on LFD through injection of the CD1d-restricted iNKT cell ligand α-galactosyl ceramide (αGalCer), glucose tolerance did not improve (Supplemental Figure 2C and ref. 26), probably due to the fact that WT animals are highly insulin sensitive under LFD conditions. Taken together, these findings indicate that CD1d-restricted iNKT cells protect against insulin resistance, especially in LFD-fed mice.

As iNKT cells reside not only in AT (Figure 2) but also in the liver, and both organs are critically involved in the regulation of whole-body lipid and glucose homeostasis (34), we investigated circulating lipids and liver function in CD1d-null mice, focusing on LFD conditions. While circulating triglycerides were slightly elevated in CD1d-null mice on

Figure 1 iNKT cell knockout and antibody-mediated depletion result in insulin resistance in lean mice ▶ For A-G, *n* = 10 mice per group; total 40 mice. (A) Weight gain of WT and CD1d-null mice on the LFD and HFD regimens. Mice were weighed weekly. (B) Weekly caloric intake of WT and CD1d-null mice on the LFD and HFD regimens. (C) Epididymal fat pad weights of the WT and CD1d-null mice on a LFD and HFD regimen, measured after termination. (D and E) IP-GTTs were performed after 17 weeks of LFD or HFD. Plasma glucose concentrations and the AUC for the various groups are shown. (F and G) Plasma insulin levels during the IP-GTT are shown, together with the AUC. (H and I) IP-GTT of WT and Jα18-null mice after 18 weeks of LFD. Shown are plasma glucose concentrations and the AUC for the 2 groups. *n* = 15 mice per group; total 30 mice. (J and K) IP-GTT of WT (Isotype) and iNKT cell-depleted (αNK1.1) WT mice. Note that this antibody also depletes NK cells. Shown are plasma glucose concentrations and the AUC for the 2 groups. *n* = 10 mice per group; total 20 mice. **P* < 0.05.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WT HFD
CD1d-/- LFD
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Weight (g)
Age (weeks)
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Kcal/week
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C
Fat pad weight (g)
WT
CD1d-/-
D
Glucose (mmol/l)
Time (min)
E
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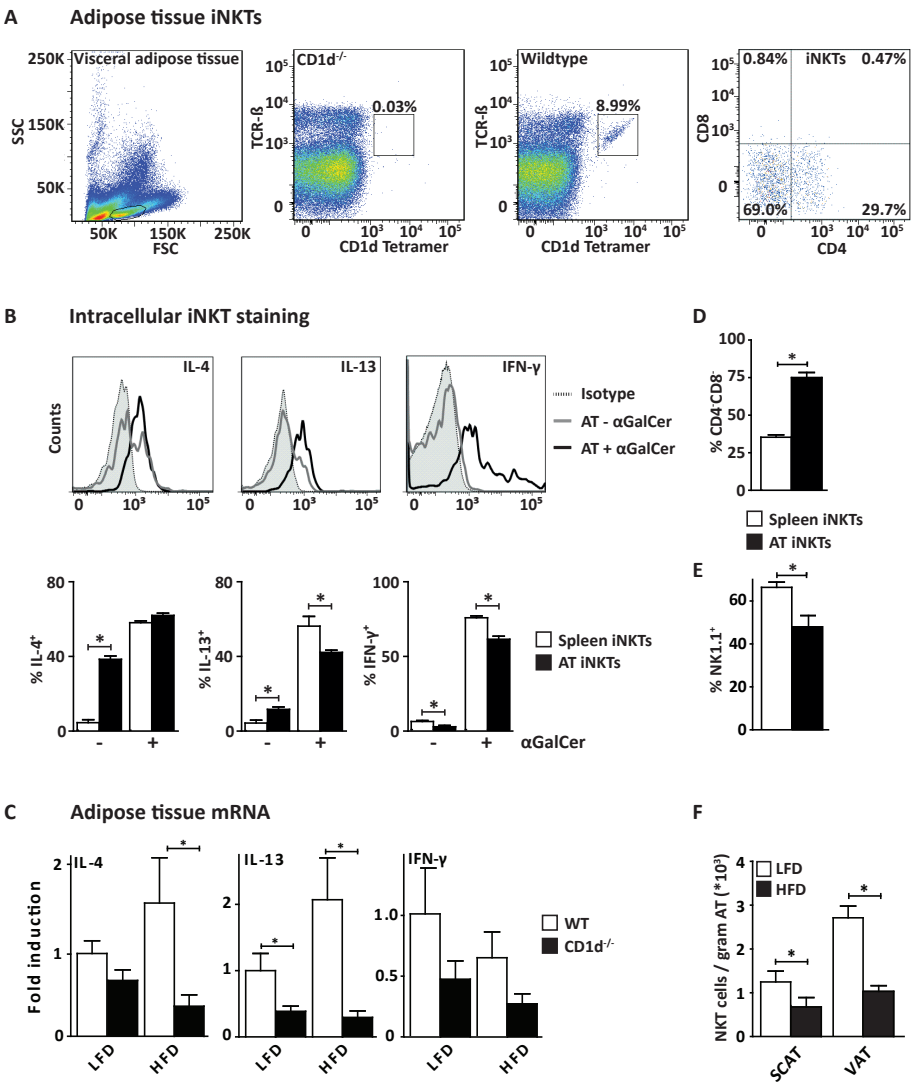


Figure 2 AT-resident iNKT cells show an activated phenotype and are downregulated on a long-term HFD

(A) Stromal vascular fraction of visceral adipose tissue (VAT) from WT and CD1d-null mice was stained for TCRβ, CD1d tetramer, CD4, and CD8. FSC, forward scatter; SSC, side scatter. Numbers in graphs indicate the percentage of cells in that gate. Second and third panel, percentage of TCRβ+ cells; fourth panel, CD4 and CD8 staining of iNKT cells. (B) Intracellular staining of spleen and visceral AT-extracted iNKT cells from 4 WT mice, injected intraperitoneally with αGalCer (5 μg) or vehicle. Shown are representative histograms and averages in bar graphs. (C) Quantitative RT-PCR on VAT of WT and CD1d-null mice on the LFD and HFD regimens. *n* = 9 mice per group; total 36 mice. (D and E) Percentage of $CD4^{-}CD8^{-}$ and $NK1.1^{+}$ iNKT cells (gated on TCRβ and CD1d/αGC-loaded tetramer) extracted from spleen and VAT of WT mice on a LFD. *n* = 10 mice per group; total 20 mice. (F) Number of iNKT cells per gram of SCAT and VAT of WT mice on LFD and HFD regimens. *n* = 10 mice per group; total 20 mice. **P* < 0.05.

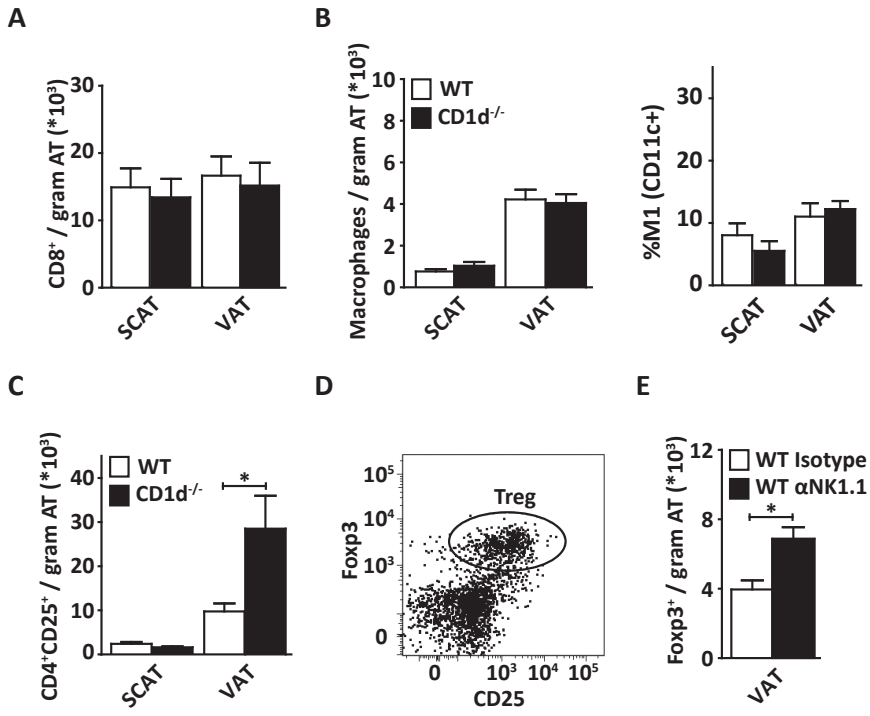
a LFD, circulating FFA and cholesterol levels were not (Supplemental Figure 1, B-D). More importantly, CD1d-null mice on a LFD showed none of the pathological alterations that are associated with liver-mediated insulin resistance (35): neither liver histology, weight, and lipid content nor the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and the liver inflammatory markers lipocalin-2 (*Lcn2*) and serum amyloid A (*Saa2*) were altered in CD1d-null mice on a LFD, compared with their WT counterparts (Supplemental Figure 1, E-K). In WT and CD1d-null mice under HFD conditions, though, liver pathology was observed; for some parameters, this was most pronounced in CD1d-null mice (Supplemental Figure 1, E-K). These findings argue against a principal role for the liver in the insulin-resistant phenotype observed in LFD-fed CD1d-null and iNKT cell-depleted mice. We therefore focused on the role of AT-resident iNKT cells.

AT-resident iNKT cells show an antiinflammatory phenotype and are downregulated on a long-term HFD

AT-resident iNKT cells constitute 5%-10% of the visceral AT-resident (VAT-resident) T lymphocyte pool, as indicated by αGC-loaded CD1d tetramer and TCRβ staining (Figure 2A). Compared with spleen-derived iNKT cells, which are predominantly $CD4^+$ and clearly express NK1.1, AT-resident iNKT cells exhibit a phenotype biased toward CD4/CD8 double-negative (~70%) with reduced NK1.1 expression (~50%) (Figure 2, A, D, and E). Next, AT-resident iNKT cell cytokine production was analyzed. AT-resident iNKT cells showed an antiinflammatory phenotype with high levels of intracellular IL-4 and IL-13 and lower levels of IFN-γ compared with splenic iNKT cells (Figure 2B). Production of all 3 cytokines was increased upon *in vivo* treatment with αGalCer, both in AT and splenic iNKT cells (Figure 2B). Functionality of AT-resident iNKT cells (i.e. cytokine production) was confirmed by *ex vivo* stimulation experiments (Supplemental Figure 2B). To address the contribution of iNKT cells to cytokine production in AT, we performed quantitative RT-PCR analysis on AT from WT and CD1d-null mice. A decrease in IL-4 and IL-13 mRNA levels in particular was observed in CD1d-null mice, indicating that iNKT cells contribute to the presence of these cytokines in AT (Figure 2C). We noticed a decrease in iNKT numbers in VAT and in subcutaneous AT (SCAT) in WT mice fed a HFD compared with a LFD (Figure 2F), which was not due to TCRβ downregulation (data not shown). In addition, reduced expression of signaling lymphocytic activation molecule f1 (SLAMf1) (36, 37) on the remaining iNKT cells was observed (Supplemental Figure 2A). Thus, low iNKT cell numbers and activity under HFD conditions may partly explain the relatively small differences in glucose tolerance observed between WT and CD1d-null mice on a long-term HFD.

CD1d-null mice exhibit enhanced AT Treg numbers, preventing worsening of insulin resistance

We next asked whether iNKT cells prevent the insulin resistance in CD1d-null mice on a LFD through an immune-modulatory mechanism, as shown in other tissues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E
CD4+CD25+ / gram AT (*10³)
Foxp3
Treg
CD25
Foxp3+ / gram AT (*10³)
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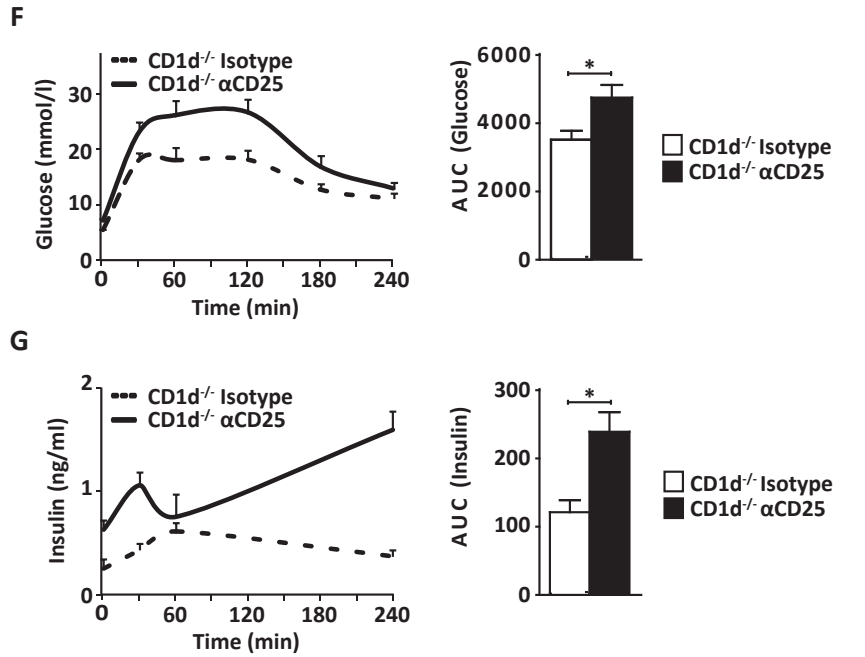
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(17-19). Under HFD conditions, AT infiltration of $CD8^+$ T cells, followed by infiltration of macrophages that exhibit an M1-polarized phenotype, plays a pivotal role in the development of insulin resistance (13, 38). However, CD1d-null mice on a LFD exhibited neither AT $CD8^+$ T cell infiltration nor increased macrophage numbers and M1 polarization (Figure 3, A and B, and Supplemental Figure 3A). Only under HFD conditions did we observe increased macrophage numbers and M1-polarization in CD1d-null mice compared with their WT counterparts (Supplemental Figure 3, A and B). Instead, higher $CD4^+CD25^+$ T cell numbers were detected in AT of CD1d-null mice, on both LFD and HFD (Figure 3C and Supplemental Figure 3B). $CD4^+CD25^+$ T cells extracted from AT expressed high levels of Foxp3 (Figure 3D), and the number of $CD4^+Foxp3^+$ T cells (but not their MFI) was increased in the iNKT cell-depleted mice (Figure 3E and data not shown). Thus, complete absence of iNKT cells in CD1d-null mice and antibody-mediated iNKT cell depletion both result in an enrichment of Tregs in AT. Considering the protective role of Tregs in AT (9), we next addressed whether the increased Treg numbers prevent worsening of the insulin resistance phenotype. Upon anti-CD25 antibody-mediated depletion of Tregs in the CD1d-null mice on a LFD, an aggravation of the insulin-resistant phenotype observed in CD1d-null mice was seen (Figure 3, F and G, and Supplemental Figure 3C). Pointing to the importance of iNKT cells for this effect, depletion of Tregs in the WT mice on a LFD had no effect (Supplemental Figure 3D). Taken together, the results indicated that CD1d-null mice on a LFD have a unique AT phenotype that, unlike the well-studied HFD phenotype, is characterized not by increased influx of proinflammatory $CD8^+$ T cell or macrophage populations, but rather by increased Treg numbers. The increase in AT Tregs observed upon iNKT cell depletion may serve to prevent further aggravation of insulin resistance.

Absence of CD1d-restricted iNKT cells is associated with adipocyte dysfunction in lean mice

Using an Affymetrix microarray platform, we explored further the AT phenotype in CD1d-null mice on a LFD. Microarray analysis underscored that the AT phenotype in lean insulin-resistant CD1d-null mice is different from the HFD-associated disease pattern. Classical inflammatory markers upregulated under HFD conditions, including *Tnfa*, Emr1 hormone receptor (*F4/80*), integrin alpha X (*Cd11c*), *Ccl2*, serum amyloid a 3 (*Saa3*), and a disintegrin and metallopeptidase domain 8 (*Adam8*) (39-41) were

◀ **Figure 3 CD1d-null mice exhibit enhanced AT Treg numbers, preventing worsening of insulin resistance** (A-C) n = 10 mice per group; total 20 mice. (A) Number of $CD8^+$ T cells ($TCR\beta^+$) per gram of SCAT and VAT, for WT and CD1d-null mice on a LFD. (B) Number of macrophages ($F4/80^+$) per gram of SCAT and VAT, and percentage of M1-polarized ($CD11c^+$) macrophages for WT and CD1d-null mice on a LFD. (C) Number of $CD4^+CD25^+$ T cells ($TCR\beta^+$) per gram of SCAT and VAT for WT and CD1d-null mice on a LFD. (D) Representative results of staining of Foxp3 and CD25 expression on AT-derived $CD4^+$ T cells ($TCR\beta^+$) in CD1d-null mice. (E) Number of Tregs ($TCR\beta^+CD4^+Foxp3^+$) per gram of VAT for WT (Isotype) and iNKT cell-depleted (αNK1.1) WT mice. n = 10 mice per group; total 20 mice. (F and G) IP-GTT of CD1d-null (Isotype) and Treg-depleted (αCD25) CD1d-null mice on a LFD. Shown are plasma glucose and insulin concentrations, together with the AUC for the 2 groups. n = 10 mice per group; total 20 mice. $^*P < 0.05$.

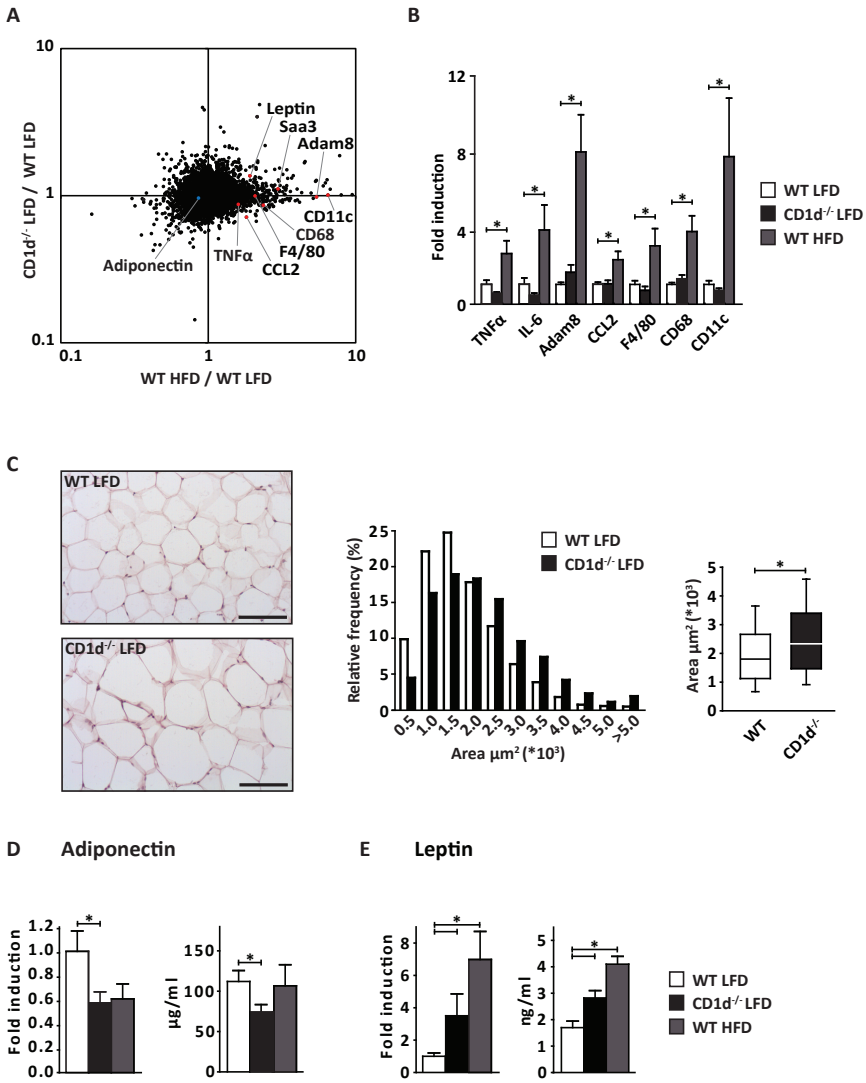


Figure 4 Absence of CD1d-restricted iNKT cells is associated with adipocyte dysfunction in lean mice
(A) Microarray-based fold change versus fold change scatter plot comparing gene expression profiles in WT HFD group (*x* axis) and CD1d-null LFD group (*y* axis). Genes of interest encoding classical inflammatory markers or adipokines are highlighted in red (upregulated) or blue (downregulated). Fold changes represent the mean of 4-6 mice per experimental group. (B) Quantitative RT-PCR of selected classical inflammatory markers in AT. Mean expression in WT LFD mice was set at 1. Fold changes were normalized for housekeeping gene expression (*36B4*). *n* = 9 mice per group; total 27 mice. (C) H&E staining of VAT from WT and CD1d-null mice after 19 weeks of LFD feeding. Scale bars: 100 µm. VAT adipocyte sizes (area per adipocyte, µm2) in LFD-fed WT and CD1d-null mice are presented. Box plots show the median area per adipocyte for both groups, and 10th to 90th percentiles. *n* = 10 mice per group; total 20 mice. (D and E) Leptin and adiponectin mRNA expression in VAT were determined by quantitative RT-PCR (*n* = 9 mice per group; total 27 mice). Leptin and adiponectin protein levels were analyzed in plasma from LFD-fed CD1d-null mice and WT mice on a LFD and HFD. *n* = 10 mice per group; total 30 mice.

not different in LFD-fed CD1d-null mice and their WT counterparts (Figure 4, A and B). The effect of longterm HFD on the transcriptional profile was remarkably similar in both genotypes (Supplemental Figure 4), in accordance with the reduced AT-resident iNKT numbers detected under longterm HFD conditions (Figure 2F). Next, we focused on adipocyte function in LFD-fed CD1d-null mice. Along with inflammatory changes, adipocyte dysfunction, characterized by adipocyte hypertrophy and altered adipokine secretion (1), is key to the development of insulin resistance. Indeed, enlarged adipocytes (larger area per adipocyte and lower number of adipocytes per field) were found in the CD1d-null mice on a LFD compared with their WT counterparts (Figure 4C and Supplemental Figure 4C), but there was no difference in epididymal fat pad weight (Figure 1C) or total fat mass as determined by dual energy X-ray absorptiometry (DEXA) scanning (Supplemental Figure 4D). No changes in several genes involved in lipogenesis (stearoyl-coenzyme A desaturase 1, *Scd1*; fatty acid synthase, *Fas*), lipid droplet formation (perilipin 1, *Lipin1*, *Pparg*), and thermogenesis (uncoupling protein 1, *Ucp1*; *Ppara*) were detected in LFD-fed CD1d-null mice, except for a significant increase in *Lipin1* (Supplemental Figure 4E), an adiposity gene involved in triglyceride synthesis (42). Lipolysis, as determined by plasma glycerol levels, was also not significantly changed (Supplemental Figure 4F). Remarkably, the adipocyte dysfunction in LFD-fed CD1d-null mice was reflected by decreased levels of the insulin-sensitizing adipokine adiponectin and increased levels of the insulin-desensitizing leptin (43), at both the mRNA level in the AT and the protein level in the plasma (Figure 4, D and E). Thus, development of insulin resistance in the absence of iNKT cells may originate from adipocyte dysfunction, in particular altered adipokine secretion.

Enrichment of CCR2$^+$ iNKT cells in human AT

Having investigated mouse AT-resident iNKT cells, we set out to study the role of iNKT cells in human AT. We first assessed the relative number of iNKT cells as a fraction of lymphocytes in paired blood and abdominal SCAT samples obtained from healthy donors (n = 6). iNKT cell numbers were enriched approximately 10-fold in AT compared with blood (flow cytometry analyses, using TCR Vα24/Vβ11 and CD3/αGC-loaded CD1d tetramer staining) (Figure 5, A and B). In AT and blood, 30% of the iNKT population consisted of CD4$^+$ iNKT cells, with the remaining fraction mostly representing CD4$^-$CD8$^-$ iNKT cells (Figure 5, A and C). We considered the possibility that iNKT cells are recruited to AT. To this end, we determined the expression of a range of chemokine receptors on iNKT cells purified from blood and AT, including CCR2, CCR4, CCR5, CCR7, CXCR2, CXCR3, CXCR6, and CX3CR1, as well as CD62L and CD11b (44). Significantly increased expression levels on iNKT cells from AT compared with blood were observed for CCR2, the chemokine receptor for the AT-secreted MCP-1 (45), and for the chemokine receptors CXCR2 and CXCR6, while expression levels of the other chemokine receptors and CD62L and CD11b were similar in iNKT cells from AT and blood (Figure 5D and Supplemental Figure 5A). Thus, MCP-1/CCR2-mediated and CXCR2 and CXCR6-mediated chemotaxis may provide a mechanism for the recruitment of iNKT cells to AT.

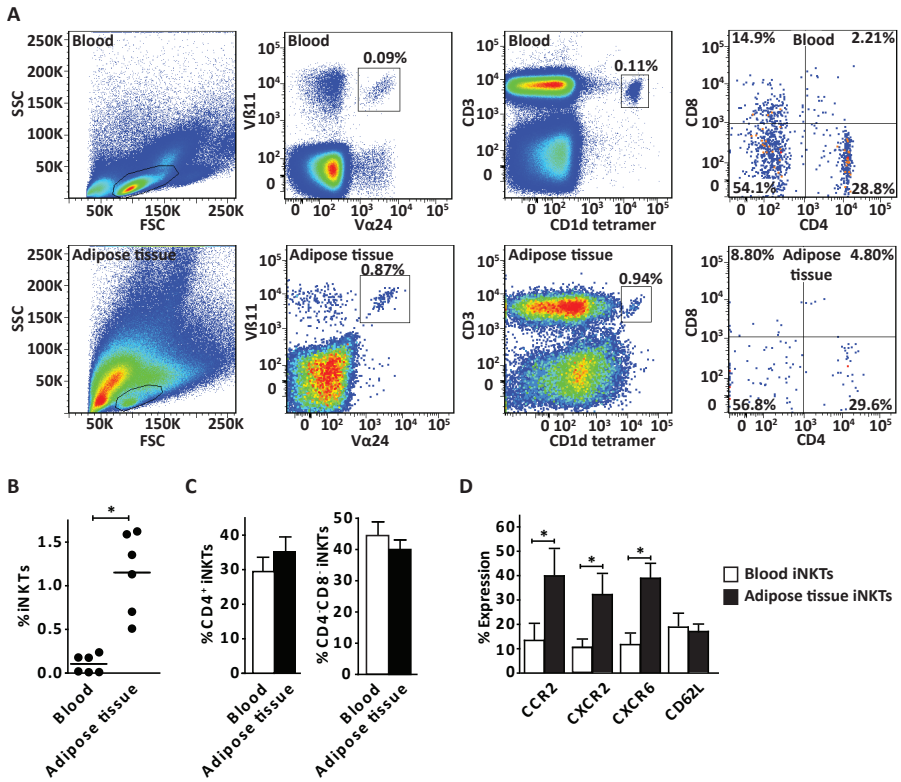
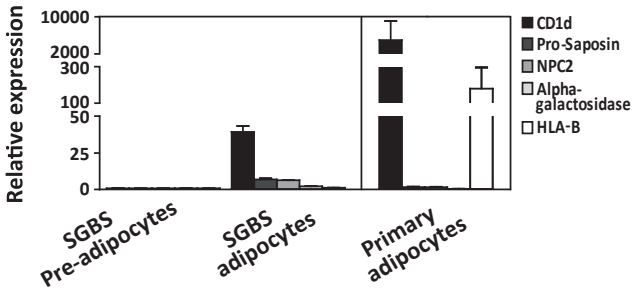


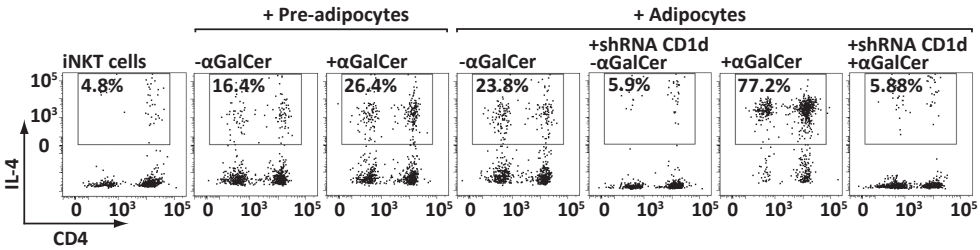
Figure 5 Enrichment of CCR2⁺ iNKT cells in human AT
(A) Blood and AT-derived iNKT cells were stained for Vα24, Vβ11, CD1d tetramer, and CD4 and CD8 expression. iNKT cell percentages of the total $CD3^+$ population are shown. For B-D, $n = 6$ healthy donors. (B and C) iNKT cell contribution to the total $CD3^+$ T cell population in blood and AT, and the percentage of $CD4^+$ and CD4/CD8 double negative iNKT cells. (D) Chemokine receptor and CD62L expression on blood and AT-derived iNKT cells (gated on CD3 and CD1d/αGC-loaded tetramer). $^*P < 0.05$.

Figure 6 Adipocytes can modulate iNKT cell function in a CD1d-dependent manner ▶
(A) Quantitative RT-PCRs of *CD1d* and its lipid-loading machinery genes pro-saposin, *NPC2*, and α-galactosidase in human SGBS preadipocytes, mature adipocytes, and primary subcutaneous adipocytes isolated from 3 human subjects. *HLA-B* mRNA levels were included as a negative control. Fold changes were normalized for housekeeping genes (*36B4* and β2 actin). (B) Intracellular IL-4 staining of iNKT cells cocultured with undifferentiated SGBS preadipocytes and mature adipocytes, with and without prior loading of the (pre)adipocytes with the CD1d-restricted iNKT cell ligand αGalCer. CD1d knockdown in the adipocytes depleted intracellular IL-4 staining in the cocultured iNKT cells. (C) IL-4, IL-13, and IFN-γ levels in the supernatants of iNKT cells cocultured with undifferentiated SGBS preadipocytes and mature adipocytes. Antibody blocking and CD1d knockdown of CD1d in mature adipocytes result in a significant decrease in IL-4, IL-13, and IFN-γ levels in the supernatants, while CD1d overexpression results in an increase. Data represent the mean results of 5 different iNKT cell lines cocultured with the (pre)adipocytes. $^*P < 0.05$.

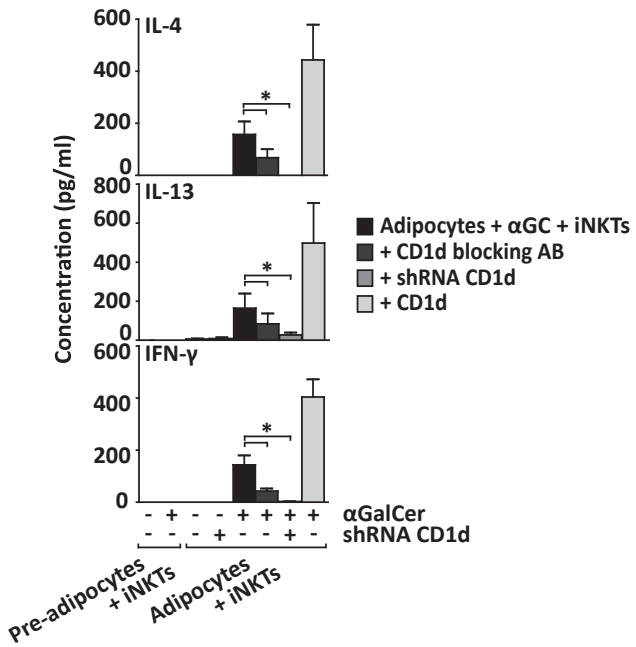
A Quantitative RT-PCR CD1d machinery



B Intracellular IL-4 staining iNKT cells



C Cytokines in supernatant



Human adipocytes express CD1d and can regulate iNKT cell function

Adipocytes are a major constituent of AT and contain lipids that may serve as CD1d antigenic ligands. Therefore, we tested the possibility that adipocytes directly modulate iNKT cell function by presenting lipid antigens. First, the expression of CD1d and its lipid-loading machinery was analyzed in human SGBS (pre)adipocytes, a well-established adipocyte cell culture model (46), and in human primary adipocytes by quantitative RT-PCR. Several known components required for antigen loading (pro-saposin, *NPC2*, α-galactisodase) as well as *CD1D* itself were expressed at background levels in undifferentiated preadipocytes, but were readily detectable in mature human SGBS adipocytes and primary adipocytes (Figure 6A). Of note, CD1d depletion by lentivirally transduced CD1d shRNA in human preadipocytes did not influence their differentiation potential, adipocyte marker expression, or adiponectin secretion, suggesting that CD1d expression is not essential for differentiation and function of cultured (pre)adipocytes (Supplemental Figure 5, B and C). The upregulation of CD1d and its loading machinery as measured by increased mRNA gene transcription in mature human adipocytes suggests that adipocytes can function as lipid APCs for iNKT cells. To test this possibility, we generated short-term iNKT cell lines (>70% CD1d-restricted iNKT cells) from 5 healthy blood donors (47) and cocultured them with human adipocyte cell lines, where indicated displaying reduced CD1d expression upon shRNA-mediated knockdown or overexpressing CD1d (Supplemental Figure 5B and Supplemental Figure 6A). After loading of mature adipocytes with αGalCer (48) and 18 hours of coculture, the production of IL-4, IL-13, and IFN-γ by the iNKT cells was assessed. Strikingly, iNKT cells cocultured with mature adipocytes showed the highest intracellular IL-4, IL-13, and IFN-γ levels. The intracellular cytokine levels were found to be reduced upon CD1d blocking and depleted to background levels by CD1d knockdown (Figure 6B and Supplemental Figure 6B). Interestingly, basal cytokine levels were also decreased by CD1d knockdown, suggesting that adipocytes can present lipid autoantigens (Figure 6B and Supplemental Figure 6B). Cytokine measurements in the supernatant were affirmative, again showing only IL-4, IL-13, and IFN-γ release for iNKT cells cocultured with mature adipocytes. Also here cytokine release was reduced upon CD1d blocking and fully depleted by CD1d knockdown (Figure 6C and Supplemental Figure 6C). Taken together, our results show that mature human adipocytes express functional CD1d and can act as lipid APCs, modulating iNKT cell function.

DISCUSSION

In recent years, various AT-resident immune cells have been implicated in the regulation of lipid and glucose homeostasis (9, 13, 14). Here we show that AT-resident CD1d-restricted iNKT cells protect against the development of insulin resistance by preventing adipocyte dysfunction, especially under LFD conditions. The high numbers of CD1d-restricted iNKT cells in both mouse and human AT raise three interesting questions.

First, why do iNKT cells accumulate in AT? The abundance of lipid antigens, together with the lipid APC function of CD1d-proficient adipocytes, may contribute importantly to this phenomenon. In mouse and human liver, lipid processing and high CD1d expression by various cell types also create an iNKT niche (48, 49). Moreover, the enrichment of $CCR2^+$ iNKT cells in human AT suggests chemotaxis by AT-secreted MCP-1, reminiscent of AT-resident macrophages (50). The plethora of AT-secreted factors (43) may well include additional molecules affecting chemotaxis, proliferation, and function of AT-resident iNKT cells.

Second, are liver or muscle, as important regulators of glucose tolerance along with AT, involved in the insulin resistance observed in CD1d-null mice? LFD-fed CD1d-null mice showed none of the pathological alterations associated with liver-mediated insulin resistance. A primary role for muscle tissue, another key regulator of whole-body insulin sensitivity, also seems unlikely, as iNKT cell numbers in muscle appear to be low (51). Nevertheless, we cannot exclude secondary roles for the liver and muscle in the insulin resistance following CD1d deficiency. As in obesity, the adipocyte dysfunction in the LFD-fed CD1d-null mice, characterized by adipocyte hypertrophy and altered adipokine secretion, may well affect the insulin sensitivity of secondary tissues such as liver and muscle (1, 43). We have thereby come to the third question.

What function do iNKT cells exert in AT? Our results indicate that iNKT cells should be included in the list of AT-resident immune cells mediating glucose tolerance: AT-resident macrophages (15, 16), T cells (9, 13, 14), B cells (52), eosinophils (10), and mast cells (53). AT-resident iNKT cells appear to be unique, though, in their communication with adipocytes. The expression of functional CD1d in adipocytes and modulation of iNKT cell function fuel the hypothesis that adipocytes, via lipid presentation to iNKT cells, exert control over the local immune response in AT (24). Similar to other tissues (54), iNKT cells may exert immunoregulatory roles in concert with AT-resident Tregs. Indeed, the increased Treg numbers in AT upon depletion or knockdown of iNKT cells suggest partial compensation of the iNKT loss by Tregs. Accordingly, depletion of AT-resident Tregs in CD1d-null mice on a LFD aggravated the already existing insulin resistance. The adipocyte dysfunction in CD1d-null mice observed here, however, also fuels an alternative hypothesis. Along with indirectly affecting adipocyte function via immune modulation, iNKT cells may also directly control adipocyte function: the iNKT cytokine IL-4, secreted at high levels by AT-resident compared with spleen-derived iNKT cells, is known to improve insulin sensitivity via STAT6 activation (55). IL-13, which is also produced by AT-resident iNKT cells under basal conditions, may also affect adipocyte function, but this area has not been explored so far. Interestingly, we found that AT-resident iNKT cells are also capable of producing IFN-γ when stimulated with αGalCer. As IFN-γ has been associated with insulin resistance in cultured adipocytes (56, 57) and *in vivo* (14, 58), these findings suggest that, in contrast to the protective role we observed under LFD conditions, iNKT cells could contribute to the development of insulin resistance under other conditions. Indeed, some recent reports indicate that depletion of iNKT cells can improve insulin sensitivity under HFD

conditions (59-61). The exact role of iNKT cells under HFD conditions is, however, unclear, as other studies, and the present study, failed to detect a prominent effect of iNKT cell depletion on glucose homeostasis (26-28). Of note, the decreased number of AT-resident iNKT cells in obese human individuals and in mice under long-term HFD conditions may explain the marginal effects of iNKT cell depletion under HFD conditions (refs. 25, 26, and the present study). Future studies are required to establish the exact roles of iNKT-produced cytokines in mediating adipocyte (dys) function and insulin resistance under HFD conditions.

Finally, iNKT cells are known to bridge innate and adaptive immunity, as they can rapidly release high doses of immune polarizing cytokines upon lipid/CD1d complex binding (17-19). Upon prolonged stimulation, iNKT cell numbers are known to decrease and iNKT cells become anergic (62). This physiological role of iNKT cells, together with the insulin-resistant phenotype of CD1d-null iNKT cell-deficient mice under LFD conditions, supports a key role for AT-resident iNKT cells as a first line of defense against adipocyte dysfunction, AT inflammation, and insulin resistance. Importantly, AT-resident iNKT cell function seems to depend on diet composition, duration of the diet, and possibly also indigenous gut microbiota (63). The protective role of iNKT cells appears most explicit under long-term LFD conditions, as Kotas *et al* recently failed to observe a protective role of iNKT cells under normal chow conditions in 7 to 10 week old mice (28). The antiinflammatory phenotype of AT-resident iNKT cells we observed under unstimulated conditions, with high IL-4 and IL-13 production, together with the strong upregulation of IFN-γ production upon stimulation with the exogenous lipid antigen αGalCer, fuels the hypothesis that AT-resident iNKT cell function is determined by dietary factors, possibly in combination with AT lipid autoantigens. The capacity of adipocytes to modulate iNKT cell function in a CD1d-mediated fashion offers a tempting adipocyte-centered perspective on the mechanisms behind iNKT cell activation. CD1d-dependent adipocyte-iNKT cell interactions may play a key role in the maintenance of healthy AT under LFD conditions.

METHODS

Animal studies

WT C57BL/6J mice (8 weeks; Charles River), CD1d-null mice (30), and Jα18-null mice (31) that had been backcrossed to C57BL/6J for 10-12 generations were age matched and fed standard chow until age 10-11 weeks and subsequently fed a LFD (10 kcal% fat; Research Diets, D12450B) or HFD (45 kcal% fat; Research Diets, D12451) for 18-19 weeks. For the IP-GTT, mice (age 28 weeks) were fasted overnight, glucose was injected intraperitoneally (0.5 g/kg body weight), and blood glucose levels were measured before and at multiple time points after glucose injection (Accu-chek, Roche). Plasma was frozen at multiple time points for insulin measurements. For the iNKT cell and Treg depletion studies (Figure 1, J and K, Figure 3, F and G, Supplemental Figure 1A, and Supplemental Figure 3C), WT C57BL/6J mice (8 weeks; Charles River) were fed standard chow until age 10 weeks and subsequently fed LFD for 6 weeks. In the fifth

week of LFD feeding, weight-matched groups received 3 intraperitoneal injections of 300 µg αNK1.1 antibody (clone PK136), 3 injections of 250 µg αCD25 antibody (clone PC61), or isotype antibody injections, in agreement with established iNKT and Treg depletion models (32, 64). (Note that the αNK1.1 antibody also depletes NK cells.) In the sixth week of LFD feeding, all mice underwent an IP-GTT (1 g/kg body weight glucose) before sacrifice. For the *in vivo* challenge with αGalCer, WT C57BL/6J mice (10 weeks) fed LFD for 18 weeks were injected intraperitoneally with either vehicle (PBS; n = 10) or αGalCer (n = 10) and underwent an IP-GTT (1 g/kg body weight glucose) 3 days afterward. For DEXA, fat mass was measured by DEXA scan under general anesthesia (isoflurane/N2O/O2) using a PIXImus imager (GE Lunar).

Isolation of mouse leukocytes and flow cytometry

Murine visceral (epididymal) and subcutaneous AT was collected, washed in PBS, and digested for 45 minutes with collagenase type II (Sigma-Aldrich) and DNAse I (Roche). Stromal vascular cells (SVCs) were pelleted by centrifugation, incubated for 20 minutes with NH_4Cl erythrocyte lysis buffer, and passed through a 100-µm cup filter (BD). Simultaneously, spleens were minced through a 70-µm mesh filter (BD) and collected in NH_4Cl lysis buffer. Subsequently, AT SVCs and spleen cells were washed in FACS buffer (2% fetal calf serum and 0.1% NaN_3 in PBS); preincubated with 10% rat serum in FACS buffer; and stained with mAbs specific for TCRβ, NK1.1, CD3, CD8, CD4, CD25, and a CD1d tetramer (NIH) for lymphocyte phenotyping (for some samples, this was followed by intranuclear staining of Foxp3) or stained with mAbs for CD206, F4/80, TCRβ, a CD1d tetramer (NIH), CD150, and CD11c for macrophage phenotyping. Cells were analyzed by flow cytometry with a FacsCanto II (BD) flow cytometer and FACSDiva (BD) and FlowJo (Tree Star Inc.) software.

αGalCer stimulation of iNKT cells for intracellular staining

WT C57BL/6J mice (10 weeks) received an intraperitoneal injection of either 5 µg αGalCer (n = 4) or vehicle (n = 4). The next day, the mice were sacrificed, and AT SVCs and spleen cells were extracted and dissolved in RPMI medium containing 10% fetal calf serum, 1% penicillin/streptomycin, and 0.1% GolgiPlug (BD) for 2 hours. Subsequently, after preincubation with 10% rat serum in FACS buffer, cells were stained with mAbs for TCRβ, NK1.1, and a CD1d tetramer (NIH) to identify the iNKT cells, followed by intracellular staining for IL-4 (BD), IFN-γ (BD), IL-13 (BioLegend), and the corresponding isotype antibodies to determine intracellular iNKT cytokine levels.

Ex vivo stimulation and intracellular cytokine staining of AT-resident iNKT cells

Extracted AT SVCs and spleen cells of WT C57Bl/6J mice (9 weeks, n = 4) were dissolved in RPMI medium including 10% fetal calf serum and 1% penicillin/streptomycin, and incubated with 5 ng/ml PMA, 1 µg/ml ionomycin, and 0.1% GolgiStop (BD) for 4 hours.

Subsequently, intracellular cytokine levels were measured as described above for the αGalCer stimulation.

RNA extraction, quantitative PCR, and microarray analysis

Snap-frozen epididymal AT was homogenized and RNA was extracted using Trizol (Invitrogen). RNA was purified on an RNeasy Micro column (QIAGEN), RNA integrity was checked with a Bioanalyzer (Agilent), and cDNA synthesis was performed with iScript (Bio-Rad). Quantitative PCR with SYBR Green (Bio-Rad) was run on a MyiQ machine (Bio-Rad). Primers for quantitative RT-PCR were designed with the universal probe library (Roche) and are described in Supplemental Table 1. Microarray experiments were performed as described before (65). RNA samples from 4-6 mice per experimental group were used for microarray analysis. One hundred nanograms of RNA per sample was hybridized to an Affymetrix GeneChip Mouse Gene 1.1 ST 24 array plate according to the manufacturer's instructions. Arrays were normalized with the robust multiarray average (RMA) method (66,67). Probe sets were defined according to Dai *et al* (68) with CDF version 13.0.2 based on Entrez identifiers. The probes present on these arrays target 21,212 unique genes. Genes were only taken into account if the intensity value was greater than 20 on at least 3 arrays and the interquartile range of the intensity values was greater than 0.1 (log2) across the experiment. These criteria were met by 14,444 genes. Microarray data have been submitted to the Gene Expression Omnibus database (GEO number GSE39534).

Mouse plasma measurements

Mouse EDTA plasma was harvested after centrifugation and stored at -80°C until analysis. Mouse plasma adipokines were measured with Milliplex mouse adipokine kits (Millipore), according to the manufacturer's instructions. Measurements and data analysis were performed on a Bio-Plex system in combination with Bio-Plex manager software version 4.1.1 (Bio-Rad). AST and ALT levels in mouse EDTA plasma were measured at the diagnostic laboratory of the University Medical Center Utrecht with a Beckman Coulter DxC chemistry analyzer. Plasma lipoproteins were separated using fast protein liquid chromatography (FPLC). Pooled plasma (0.2 ml) was injected into a Superose 6B 10/300 column (GE Healthcare Life Sciences) and eluted at a constant flow of 0.5 ml/min with PBS (pH 7.4). The effluent was collected in 0.5 ml fractions and FFA, triglyceride, and cholesterol levels were determined (Instruchemie). Plasma glycerol was measured with a commercially available kit from Instruchemie.

Adipocyte morphometry, AT and liver immunohistochemistry, and liver triglycerides

Morphometry of individual adipocytes was performed as described (69). H&E staining of AT and liver sections was performed using standard protocols. Oil-red-O (ORO) stock solution was prepared by dissolving 0.5 g ORO (Sigma-Aldrich, O-0625) in 100 ml isopropanol. ORO working solution was prepared by mixing 30 ml ORO

stock with 20 ml dH_2O, followed by filtration. Sections (5 µm) were cut from frozen liver sections embedded in O.C.T. Sections were air dried for 30 minutes, followed by fixation in 4% formaldehyde for 10 minutes (4% formaldehyde). Sections were immersed in ORO working solution for 15 minutes, followed by 2 rinses with dH2O. Hematoxylin staining of nuclei was subsequently carried out for 5 minutes, followed by several rinses with dH_2O. Sections were mounted in aqueous mountant (Imsol). Liver triglycerides were determined in liver homogenates prepared in buffer containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl at pH 7.5 using a commercially available kit (Instruchemie) according to the manufacturer's instructions.

Human subjects

Human abdominal subcutaneous AT samples and blood (sodium heparin) were obtained from 6 healthy female donors during elective abdominoplastic surgery in Bergman Beauty Clinics, Bilthoven, the Netherlands. Immune cell isolations were performed immediately after surgery.

Isolation of human lymphocytes and flow cytometry

SCAT was collected and finely minced, washed in DPBS (Invitrogen), and digested with collagenase type II (Sigma-Aldrich) and DNAse I (Roche). SVCs were filtered over a 100 µm cup filter (BD) and pelleted by centrifugation. After NH_4Cl erythrocyte lysis, SVCs were filtered over a 50 µm cup filter (BD) and pelleted by centrifugation. Simultaneously, PBMCs were isolated from the patients' blood as described previously (70). SVCs and PBMCs were washed in FACS buffer, preincubated with 10% mouse serum in FACS buffer, and stained with mAbs specific for Vα24, Vβ11, CD3, CD4, CD8, CD56, CD95, and a CD1d tetramer (NIH). Furthermore, adhesion factor and chemokine receptor expression was studied with mAbs specific for CD62L, CD11b, CCR2, CCR4, CCR5, CCR7, CXCR2, CXCR3, CXCR6, and CX3CR1. Cells were analyzed by flow cytometry as described above.

Human adipocytes, lentiviral overexpression, and knockdown of human CD1d

The human preadipocyte SGBS cell line was cultured and differentiated into adipocytes as described previously (46, 71). Full-length cDNA encoding human CD1d was cloned into a pLenti CMV vector (Addgene). The shRNA construct for human CD1d was provided in a pLKO.1 vector (Sigma-Aldrich, clone NM_001766.2-814s1c1). Lentiviral particles were produced in 293T cells. After lentiviral infection, SGBS preadipocytes were kept on 2 µg/ml puromycin. Stably transduced cells were used for the adipocyte-iNKT cell interaction studies. The adiponectin secretion of SGBS adipocytes transduced with empty vector and CD1d shRNA constructs was measured making use of a recently developed and validated multiplex immunoassay (72).

(Pre)adipocyte-iNKT cell interaction study

Untransduced, CD1d shRNA-transduced, and CD1d-overexpressing human SGBS (pre) adipocytes were incubated with αGalCer for 24 hours and with and without CD1d blocking antibody (1 μg/ml CD1d mAb clone 51.1, BioLegend) for 1 hour. Subsequently, iNKT cell lines (>70% CD1d-restricted iNKT cells) from 5 different blood donors, generated as described previously (47), were incubated with the preadipocytes and adipocytes for 18 hours in RPMI medium (Invitrogen) supplemented with 10% human AB+ serum, 100 μg penicillin/ml, and 100 μg streptomycin/ml (Invitrogen) and IL-2 (10 U/ml). For the last 6 hours, media were supplemented with 0.1% GolgiStop (BD). Finally, the suspended iNKT cell fraction was pelleted for intracellular cytokine staining, and the supernatant was stored at -80°C until analysis of cytokine levels. Cytokine levels were measured with a cytokine multiplex immunoassay, as described recently (72).

Statistics

Data are presented as mean ± SEM, unless otherwise indicated. Statistical significance between 2 groups was determined using 2-tailed Student's *t* tests for normally distributed data and Mann-Whitney *U* tests for nonparametric analyses. *P* values less than 0.05 were considered significant.

Study approval

All mouse study protocols were approved by the Utrecht University Ethical Committee for Animal Experimentation (protocol 2010.III.07.083 and 2011.III.06.061) and were in accordance with Dutch laws on animal experimentation. The study protocol for collection of human samples was approved by the local medical Ethical Committee of the University Medical Center Utrecht (protocol 10-159/C), and oral and written consent was obtained.

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SUPPLEMENTAL INFORMATION

Natural killer T cells in adipose tissue prevent insulin resistance

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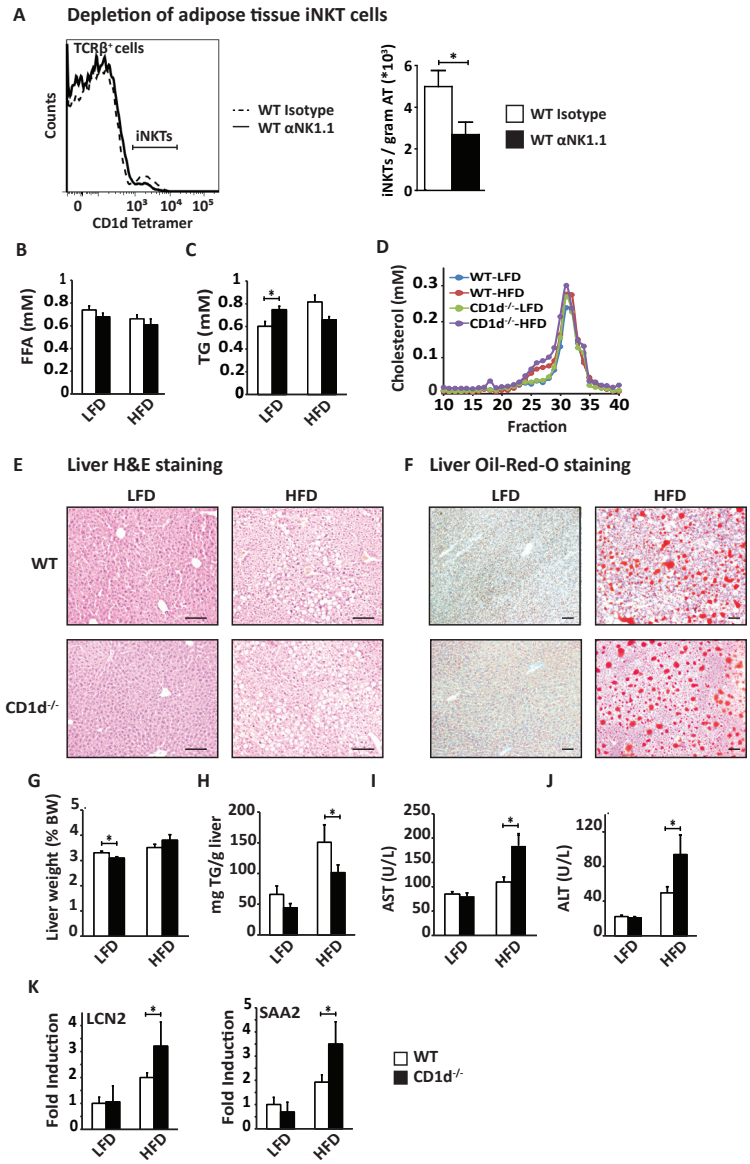
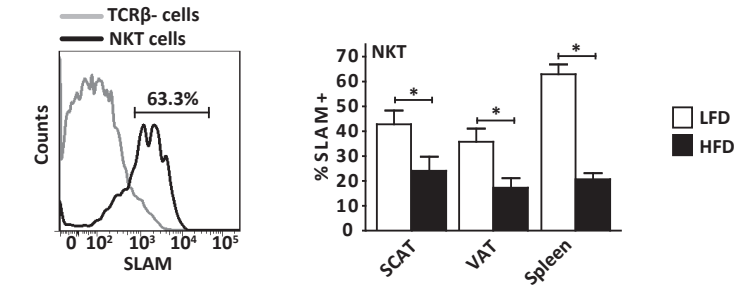


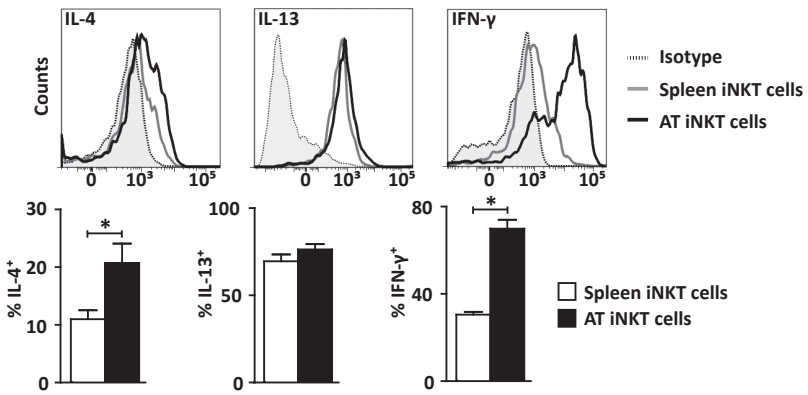
Supplemental figure 1

(A) Histogram represents the percentage AT-derived iNKT cells (CD1d/αGC-loaded tetramer) of the AT-resident T cells (TCRβ+) in WT mice injected with an isotype or αNK1.1 antibody. Bar graph represents absolute numbers of AT-resident iNKT cells in the WT mice injected with an isotype or αNK1.1 antibody. N=10 mice per group, total 20 mice. (B) Plasma free fatty acid (FFA) levels. (C) Plasma TG levels. (D) Plasma FPLC lipoprotein profiling. For each group of mice, 10µl of pooled plasma was used. (E) H&E and (F) Oil-Red-O staining of representative liver sections of the WT and CD1d-null mice fed LFD or HFD for 19 weeks (scale bars indicate 100 µm). (G-K) N=10 mice per group, total 40 mice. (G) Liver weight as percentage of total body weight. (H) Liver triglyceride (TG) content. (I) Plasma aspartate aminotransferase (AST) levels. (J) Plasma alanine aminotransferase (ALT) levels. (K) Changes in gene expression of selected inflammatory genes as determined by quantitative RT-PCR. Mean expression in WT LFD mice was set at 1.

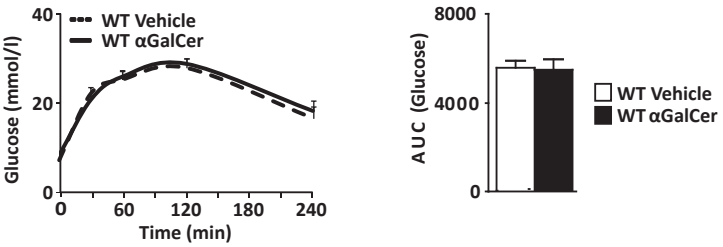
A **SLAM expression on adipose tissue and spleen iNKT cells**



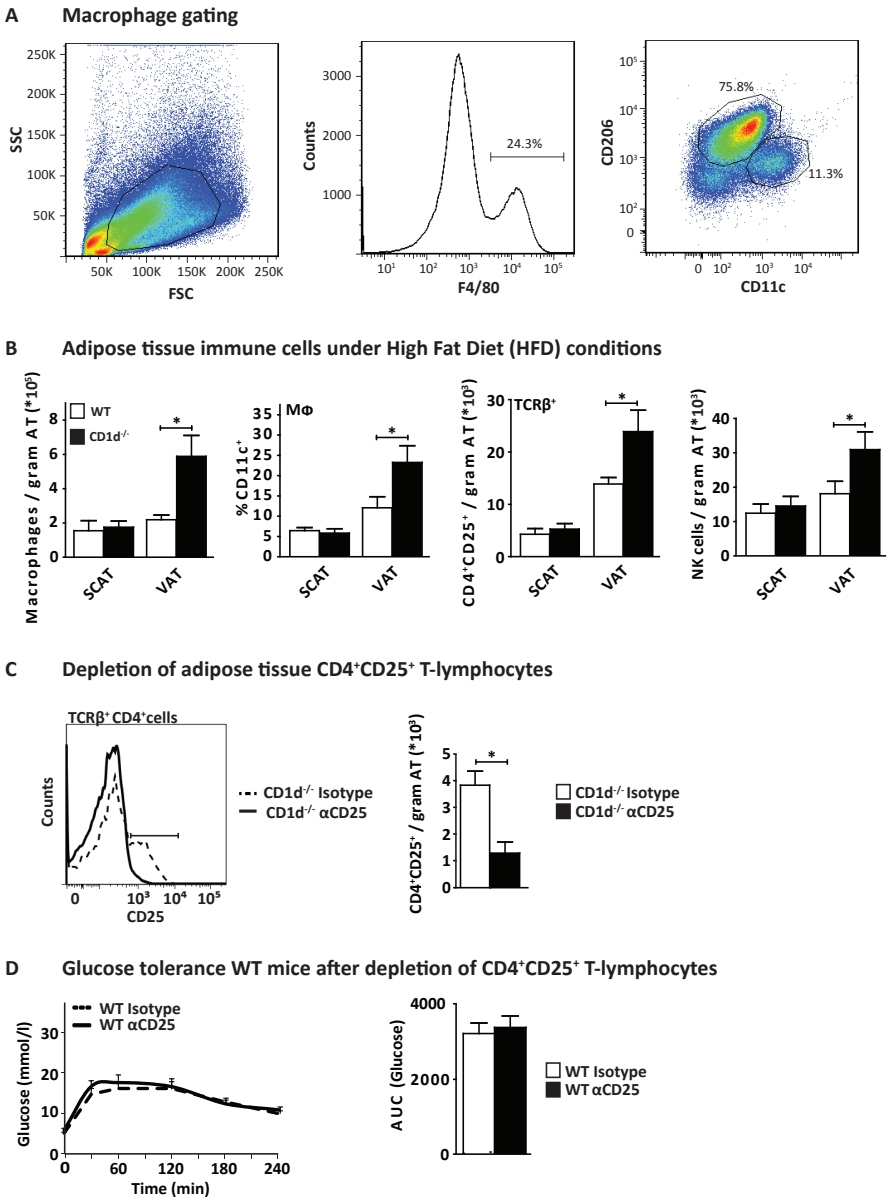
B ***Ex-vivo*** **PMA/Ionomycin stimulation of iNKT cells**



C **Glucose tolerance WT mice after αGalCer injection**

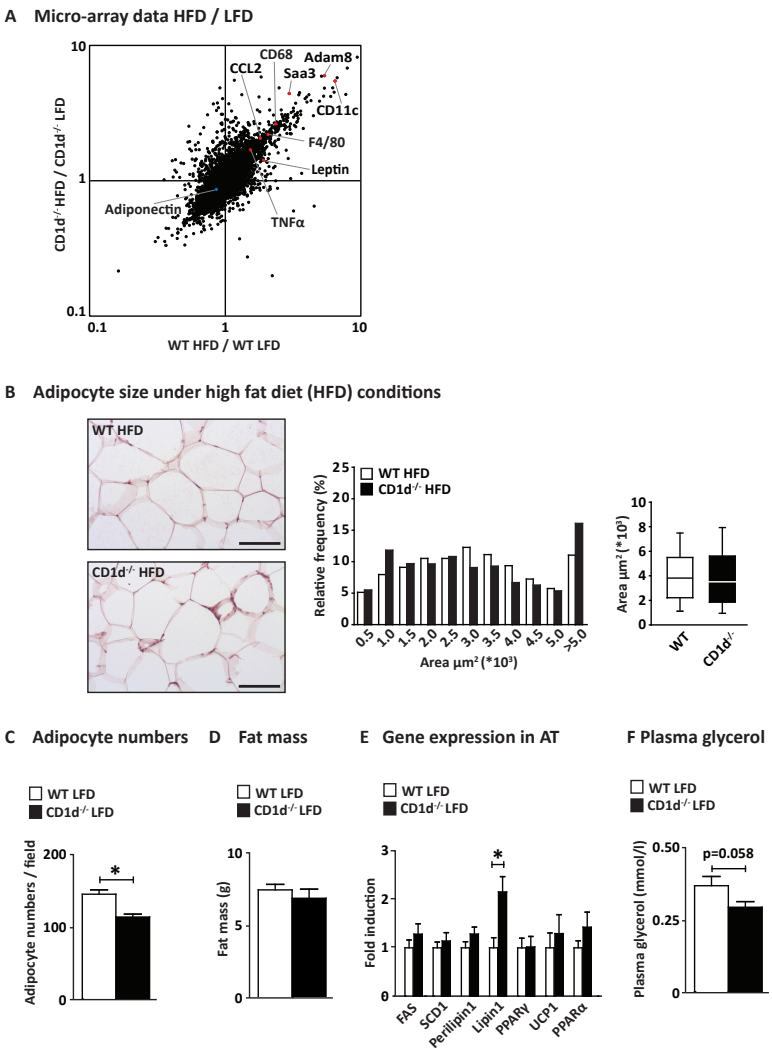


Supplemental figure 2
(A) SLAMf1 (CD150) expression on AT-derived iNKT cells. The histogram displays the gating of SLAMf1, i.e. the SLAMf1 expression on TCRβ$^-$ cells versus iNKT cells. Bar graphs represent SLAMf1 expression on iNKTs derived from SCAT, VAT and spleen. N=10 WT mice per group, total 20 mice. (B) Intracellular cytokine staining of spleen and visceral AT-extracted iNKTs from 4 WT mice, after 4 hours *ex vivo* stimulation with PMA and ionomycin. Shown are both representative histograms and averages in bar graphs. (C) Intra-peritoneal glucose tolerance test of WT mice fed LFD for 18 weeks injected with vehicle (PBS) or αGalCer (50μg). Shown are plasma glucose concentrations and the AUC for the two groups. N=10 mice per group, total 20 mice.



Supplemental figure 3

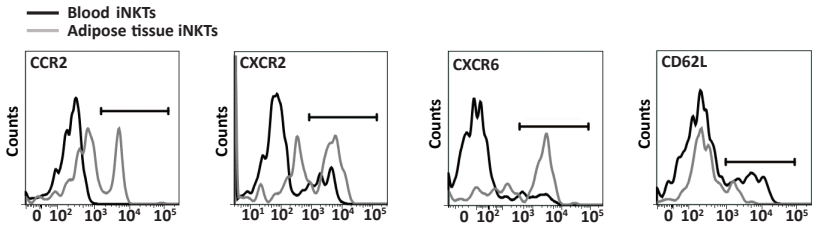
(A) Macrophage gating (F4/80$^+$) and macrophage CD11c expression on AT-derived stromal vascular cells (SVC). (B) Macrophage numbers, macrophage polarization, CD4$^+$CD25$^+$ numbers and NK cell numbers in SCAT and VAT derived from WT and CD1d-null mice on a HFD. (C) Histogram represents the percentage AT-derived CD25$^+$ cells (of TCRβ$^+$CD4$^+$ cells) of the CD1d-null mice injected with an isotype or αCD25 antibody. Bar graph represents absolute numbers of AT-resident CD4$^+$CD25$^+$ cells in the WT mice injected with an isotype or αCD25 antibody. N=10 mice per group, total 20 mice. (D) Intra-peritoneal glucose tolerance test of WT (Isotype) and Treg-depleted (αCD25) WT mice on a LFD. Shown are plasma glucose concentrations, together with the AUC for the two groups. N=10 mice per group, total 20 mice.



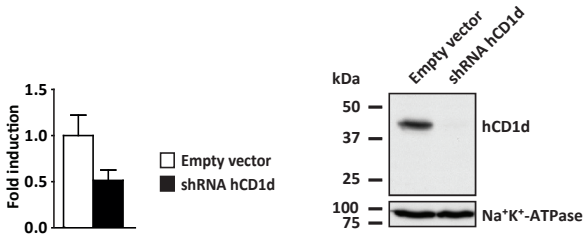
Supplemental figure 4

(A) Microarray-based fold change versus fold change scatter plot comparing gene expression profiles between WT HFD group (*x* axis) and CD1d-null HFD group (*y* axis). Genes of interest encoding classical inflammatory markers or adipokines are highlighted in red (upregulated) or blue (downregulated). Fold changes represent the mean of 4-6 mice per experimental group. (B) H&E staining of VAT from WT and CD1d-null mice after 19 weeks of HFD feeding. Scale bars indicate 100µm. VAT adipocyte sizes (area per adipocyte, µm²) in HFD-fed WT and CD1d-null mice are presented in a histogram and boxplot. Boxplots show the median area per adipocyte for both groups, and 10th to 90th percentiles. N=4 mice per group (random), total 8 mice. (C) Adipocyte numbers per field. The number of adipocytes per field was calculated using the total number of cells counted per microscopic field. The average adipocyte number per field of 33 fields was used for WT animals whereas 40 fields were included for the CD1d-null animals. (D) Total fat mass of the WT and CD1d-null animals after 18 weeks of LFD, as measured by dual energy X-ray absorptiometry (DEXA) scanning. (E) Quantitative RT-PCR of selected lipogenic, lipid droplet and thermogenic genes in adipose tissue. Mean expression in WT LFD mice was set at 1. Fold inductions were normalized for housekeeping gene expression (36B4). N=9 mice per group, total 18 mice. (F) Plasma glycerol levels of the LFD-fed WT and CD1d-null mice.

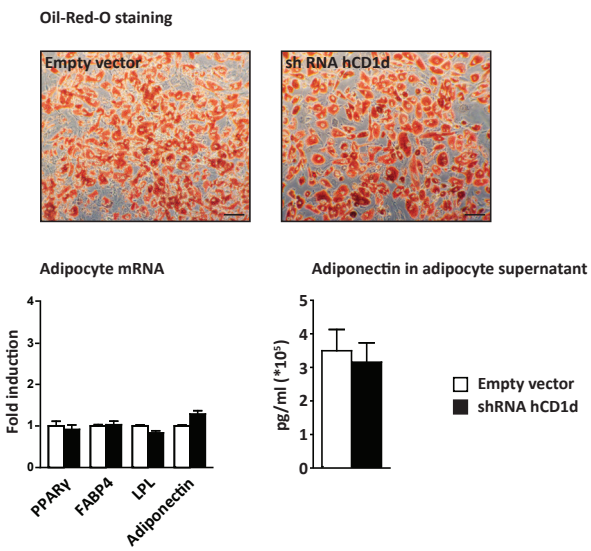
A **Gating chemokine receptors and CD62L on human iNKT cells**



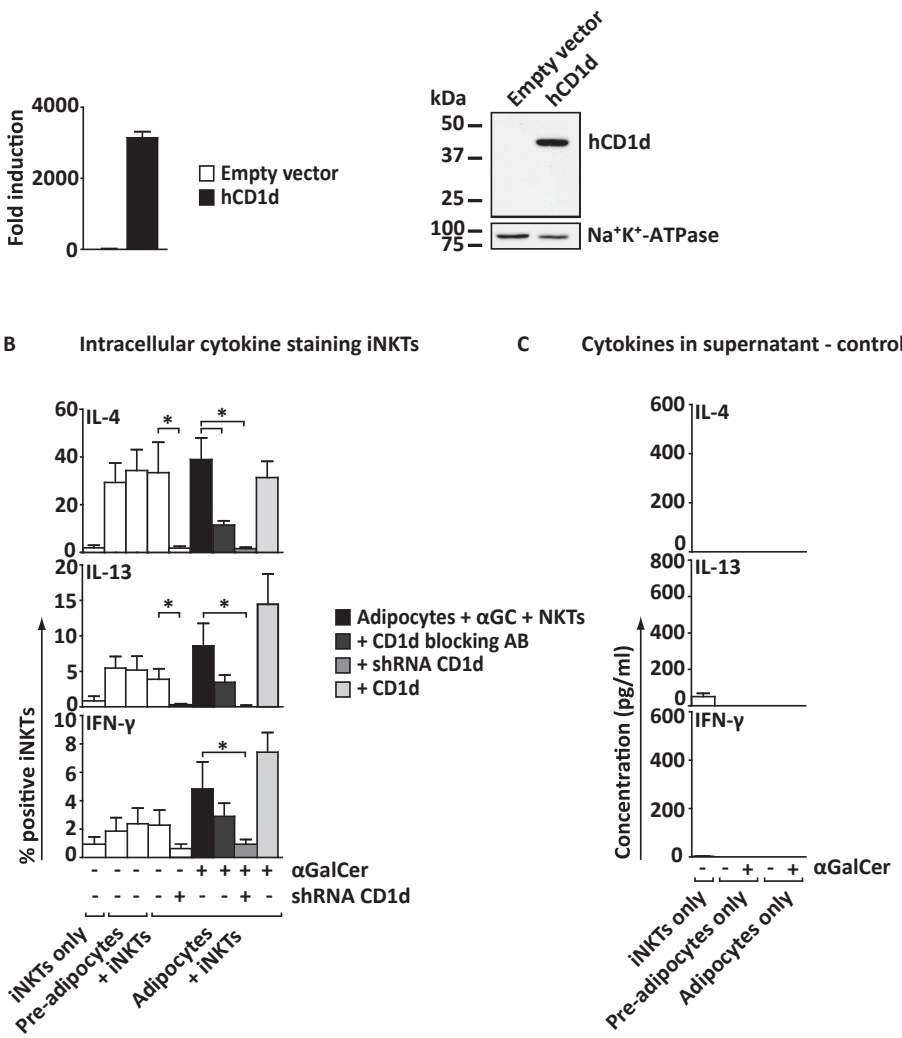
B **CD1d knockdown in human SGBS adipocytes**



C **No effect of CD1d knockdown on adipocytes**



Supplemental figure 5
(A) Chemokine receptor and CD62L gating on human AT-derived and blood iNKT cells. (B) shRNA knockdown of human CD1d. The quantitative RT-PCR (left panel) shows the knockdown of human CD1d in mature SGBS adipocytes. The Western blot (right panel) shows the effectiveness of the shRNA on protein level, by knockdown of human CD1d in HeLa cells stably expressing human CD1d. Na^+K^+-ATPase is presented as a loading control. (C) Upper panel, Oil-Red-O staining of differentiated mature SGBS adipocytes transduced with scrambled sh RNA or sh RNA for human CD1d. Lower left panel, quantitative RT-PCRs for a few adipoycte differentiation genes are shown. Mean expression in the scrambled sh RNA transduced cells was set at 1. Fold inductions were normalized for housekeeping gene expression (36B4). Lower right panel, adiponectin levels in the supernatant of the adipocytes are shown, after 24hr of incubation.



Supplemental figure 6

(A) Quantitative RT-PCR (left panel) showing the overexpression of human CD1d in undifferentiated SGBS pre-adipocytes. Western blot (right panel) shows the overexpression on protein level, again in undifferentiated SGBS pre-adipoyctes. Na^+K^+-ATPase is presented as a loading control. (B) Intracellular IL-4, IL-13 and IFN-γ staining of iNKT cells alone, and cocultured for 18hr with undifferentiated SGBS pre-adipocytes and mature adipocytes, with and without prior loading of the (pre)adipocytes with the CD1d-restricted iNKT cell ligand α-Galactosyl Ceramide (αGalCer, αGC). CD1d blocking and knockdown in the adipocytes depleted intracellular cytokine staining in the co-cultured iNKT cells. Bars represent the mean results of 5 different iNKT cell lines cocultured with the (pre)adipocytes. (C) IL-4, IL-13 and IFN-γ levels in the supernatants of iNKT cells alone, and undifferentiated SGBS pre-adipocytes and mature adipocytes alone, with and without loading of the (pre)adipocytes with αGalCer. No cytokines were detected, except for low levels of IL-13 and IFN-γ in the iNKT cell alone supernatant.

Supplemental Table 1 Primer sequences for quantitative RT-PCRs

Gene	Forward primer	Reverse primer
mLeptin	AGAAGATCCCAGGGAGGAAA	TGATGAGGGTTTTGGTGTCA
mAdiponectin	GCAGAGATGGCACTCCTGGA	CCCTTCAGCTCCTGTCATTCC
mIL-4	CCCCAGCTAGTTGTCATCCTG	CGCATCCGTGGATATGGCTC
mIL-13	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
mIFNG	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
mLCN2	GGGAAATATGCACAGGTATCCTC	GCCACTTGCACATTGTAGCTC
mSAA2	TGGCTGGAAAGATGGAGACAA	AAAGCTCTCTCTTGCATCACTG
mTNFα	CAACCTCCTCTCTGCCGTCAA	TGACTCCAAAGTAGACCTGCCC
mIL6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
mADAM8	AGTTCCTGTTTATGCCCCAAAG	AAAGGTTGGCTTGACCTGCT
mCCL2	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCCTTCTTG
mF4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
mCD68	CATCCCCACCTGTCTCTCTC	CCATGAATGTCCACTGTGCT
mCD11C	TCAACCAGCACCAGACAGAG	AAACATCCTGTAATGGCTTGTG
mFAS	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
mSCD	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
mPerilipin	CAAGCACCTCTGACAAGGTTC	GTTGGCGGCATATTCTGCTG
mLipin	CGCCAAAGAATAACCTGGAA	TGAAGACTCGCTGTGAATGG
mPPARγ	CGCTGATGCACTGCCTATGA	AGAGGTCCACAGAGCTGATTCC
mUCP1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
mPPARα	CACGCATGTGAAGGCTGTAA	CAGCTCCGATCACACTTGTC
m36B4	AGCGCGTCCTGGCATTGTGTGG	GGGCAGCAGTGGTGGCAGCAGC
hNPC2	CAGTGAAAAGCGAATATCCCTCTA	TTTGGTTTTTGTCATCCTGAAGT
hCD1d	GTGGCCTCCTTGAGTCA	ACAGGCTTTGGGTAGAATC
hProsaposin	GCCAGAACACAGAGACAGCA	GCTGTGGTTTCTGCCAAGAT
hGLA	TGGAAAATTTGGCAGATGGT	AAAGAGGCCACTCACAGGAG
hHLA-B	CTACCCTGCGGAGATCAC	TAGGACAGCCAGGCCAGCAACA
hPPARG2	CCTATTGACCCAGAAAGCGATT	CATTACGGAGAGATCCACGGA
hFABP4	CCTTTAAAAATACTGAGATTTCCTTCA	GGACACCCCCATCTAAGGTT
hLPL	ATGTGGCCCGGTTTATCA	CTGTATCCCAAGAGATGGACATT
hAdiponectin	CCTGGTGAGAAGGGTGAGAA	CACCGATGTCTCCCTTAGGA
hB2M	TTCTGGCCTGGAGGCTATC	TCAGGAAATTTGACTTTCCATTC
hBeta-Actin	GATCGGCGGCTCCATCCTG	GACTCGTCATACTCCTGCTTGC

PART II

SYSTEMIC EFFECTS OF ADIPOSE TISSUE INFLAMMATION

CHAPTER 4

A multiplex immunoassay for human adipokine profiling

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ABSTRACT

Background

Adipose tissue secretory proteins, called adipokines, play pivotal roles in the pathophysiology of obesity and its associated disorders such as metabolic syndrome, type 2 diabetes, and cardiovascular disease. Because methods for comprehensive adipokine profiling in patient plasma and other biological samples are currently limited, we developed a multiplex immunoassay for rapid and high-throughput measurement of 25 adipokines in only 50 µL of sample.

Methods

(Pre)adipocyte and *ex vivo* cultured adipose tissue supernatants were generated and together with plasma from 5 morbidly obese patients and 5 healthy and normal weight controls used to develop the adipokine multiplex immunoassay and test its usefulness in biological samples. We assessed adipokine dynamic ranges, lower limits of detection and quantification, cross-reactivity, intra- and interassay variation, and correlation with adipokine ELISAs.

Results

The limits of quantification and broad dynamic ranges enabled measurement of all 25 adipokines in supernatants and patient plasmas, with the exception of TNF-α in plasma samples. Intraassay variation was <10% for all adipokines; interassay variation was <15%. The multiplex immunoassay results correlated significantly with ELISA measurements. Plasma adipokine profiling showed significantly higher concentrations of the novel adipokines cathepsin S (5.1×10^4 vs 4.3×10^4 ng/L, $P = 0.003$) and chemerin (4.1×10^5 vs 2.7×10^5 ng/L, $P = 0.0008$) in morbidly obese patients than normal weight controls, besides the established differences in adiponectin and leptin concentrations.

Conclusions

Our findings underscore the relevance of the novel adipokines cathepsin S and chemerin, but foremost the potential of this novel method for both comprehensive adipokine profiling in large patient cohorts and for biological discovery.

INTRODUCTION

Adipose tissue, with its secretory proteins called "adipocytokines" or "adipokines," has over the last decade emerged as an important endocrine organ (1, 2). Expansion of adipose tissue, as seen in obese individuals, results in qualitative and quantitative changes in a number of adipokines (2, 3). The implications vary because of differences in mode of action; adiponectin regulates hepatic glucose metabolism and has antiinflammatory effects on the vascular wall (4), whereas leptin exhibits systemic effects on appetite and metabolism (3), and the novel adipokines chemerin and cathepsin S affect adipogenesis and atherosclerotic plaque growth and destabilization, respectively (5-7). The adipokine profile changes induce local and systemic low-grade inflammation, which contributes to obesity-associated pathologies such as metabolic syndrome, type 2 diabetes, and cardiovascular disease (3, 8). In light of the search for cardiovascular biomarkers that augment information obtained from clinical parameters and illuminate disease mechanisms, adipokines are considered promising candidates (9, 10).

Although robust and high-throughput methods for adipokine profiling could strongly fuel research into obesity and its associated disorders, comprehensive adipokine profiling remains difficult. ELISAs are widely used for their user-friendliness, low detection limits, and robustness compared with other antibody-based and mass spectrometry techniques (11); however, they have difficulty with adipokine diversity because they measure 1 adipokine at a time. Bead-based multiplex immunoassays (MIAs) share the sandwich ELISA principle and are characterized by similar user-friendliness and robustness (12-14). In addition, MIAs render superior binding kinetics and limits of quantification compared with solid-carrier immunoassays like ELISA because the antibody-coated microspheres are suspended in the analyte sample during the assay. More importantly, the use of carboxylated microspheres with distinct fluorescent labels enables simultaneous measurement of many proteins. For these reasons, MIA has established itself as an excellent profiling tool (12-14).

Here we report the development of the first comprehensive human adipokine MIA, enabling measurement of 25 adipokines in only 50 µL of sample. To show its usefulness in biological samples, we furthermore present adipokine profiling of (pre) adipocyte supernatants, *ex vivo* cultured adipose tissue, and human plasma samples.

MATERIALS AND METHODS

Patient samples

We obtained fasting patient heparinized blood samples during the daytime, with informed consent and permission of the local medical ethics committee (protocol number 09-210). We recruited healthy age- and sex-matched normal weight controls from laboratory personnel. All heparinized blood samples were centrifuged 400*g* for 10 min, after which the plasma was aliquoted and stored at -80 °C until analysis. Before analysis, all plasma samples were centrifuged on a 0.22-µm nylon membrane (Spin-X column, Corning) to remove cellular debris. To prevent interference from heterophilic antibodies, we removed immunoglobulins by 1-h incubation on protein L-coated plates (Pierce) (15).

Subcutaneous abdominal adipose tissue was residual material from a 38-year-old woman undergoing abdominoplastic surgery. Fifty grams of the adipose tissue was washed twice with PBS, cut in pieces of approximately 5 by 5 by 5mm, and incubated for 24 h on a shaker in 25 mL DMEM/F12 (Dulbecco) containing 1% penicillin/streptomycin (Invitrogen) and 20 nM insulin (Sigma). The supernatant was aliquoted and stored at -80 °C.

Cell and tissue culture

Culturing and differentiation of the human Simpson-Golabi-Behmel Syndrome (SGBS) preadipocyte cell line was performed as described (16, 17). On day 8 of differentiation, medium was conditioned for 24 h, aliquoted, and stored at -80 °C. We assessed differentiation by Oil-Red-O staining, as described (18).

Conjugation of antibodies to microspheres

Carboxylated polystyrene microspheres (xMAP, Luminex) were covalently conjugated to commercially available capture antibodies, as described (19). We performed all conjugations with 25 µg capture antibody dissolved in 250 µL PBS and 2.5 x 10^6 beads, except for retinol binding protein 4 (RBP-4), which required 100 µg antibody for optimal assay performance.

Multiplex immunoassay

Supernatants were analyzed undiluted. Plasma samples were analyzed both undiluted and 100x diluted in serum diluent (R&D Systems) and PBS (1:1), the latter for measurement of plasma adipokines present in very high concentrations, i.e., adipsin, cathepsin S, leptin, adiponectin, chemerin, plasminogen activator inhibitor 1 (PAI-1), RBP-4, serum amyloid A1 (SAA-1), tissue inhibitor of metalloproteinase 1 (TIMP-1), and thrombopoietin.

All assays were carried out at room temperature and protected from light. We prepared calibration curves as 2-fold dilution series in serum diluent with PBS (1:1). Plasma samples (45 µL) were preincubated with 5 µL rat/mouse (1:1) serum for 5 min at room temperature for further reduction of heterophilic antibody binding. We transferred 50 µL of calibrator, blank, or sample to 96-well 1.2-µm filter plates (Millipore) prewetted with PBS and added a mix containing 1000 microspheres per adipokine (total volume 10 µL/well) to all wells. The plate was incubated for 1 h with continuous shaking. We then added 10 µL of a cocktail of biotinylated antibodies (16.7 mg/L each) to the wells, followed by 1-h incubation. After incubation, the plate was washed and incubated for 10 min with streptavidin-PE (50 µg/L, Becton Dickinson) 100x diluted in high-performance ELISA (HPE) buffer (Sanquin). After washing, we measured the fluorescence intensity of the beads in a final volume of 100 µL HPE buffer. All washing steps were performed with 1% BSA/0.05% Tween/0.001% sodium azide in PBS in an automated plate washer (Bio-Plex Pro II wash station, Bio-Rad).

Measurements and data analysis of all assays were performed on the Bioplex system in combination with Bioplex manager software version 4.1.1 by use of a 5-parametric curve fitting (Bio-Rad).

Assay characteristics

Dynamic range. The MIA adipokine dynamic ranges were defined by the concentration ranges the calibration curves covered. To optimize dynamic ranges, we titrated all MIA adipokine calibrator series (13 points) to a maximum fluorescence intensity of at least 3000.

Cross-reactivity. To determine assay cross-reactivity, we tested the response of adipokine microspheres to single recombinant adipokines. Single recombinant adipokines were dissolved in HPE buffer and tested at concentrations of 500 000 ng/L for adipokines with highest calibration point ≥500 000 ng/L, at 50 000 ng/L for adipokines with highest calibration point between 5000 and 500 000 ng/L, and at 5000 ng/L for adipokines with highest calibration point ≤5000 ng/L. We calculated percentage of cross-reactivity as the ratio of fluorescence intensity in response to a single recombinant adipokine compared with the maximum fluorescence intensity.

Assay reproducibility. To assess reproducibility of the MIA adipokine panel, we measured triplicates of 11 samples on 3 consecutive days. We assessed intraassay variation as the mean CV for the triplicates and interassay variation as the mean CV for the 3 consecutive runs.

Lower limit of detection and quantification. We assessed the lower limit of detection (LLOD) and quantification (LLOQ) for all adipokines. For all adipokines, we calculated the blank average MFI (n = 26 over 3 consecutive runs), MFI standard deviation (SD), and 2xSD. Adipokine LLODs were determined by interpolation of the mean blank MFI + 2xSD in the 5-parameter logistic adipokine standard curves. The LLOQ was calculated as 3xLLOD (20).

ELISAs

A comparison of MIA and ELISA for most of the cytokines and chemokines in the MIA adipokine panel has been published by our group (15, 19). ELISAs available for the other adipokines, i.e., adiponectin, adipsin, granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF), leptin, PAI-1, resistin, TIMP-1, and thrombopoietin (all R&D Systems), were performed according to the manufacturer's protocols. Absorbance of the ELISAs was read at 450 nm on an iMark microplate reader (Bio-Rad). We determined ELISA and MIA correlations by measuring several dilutions of all plasma (n = 10) and supernatant (n = 3) samples. On average, 26 measurements were used to calculate the correlations.

Statistics

We studied differences in adipokine concentrations between obese individuals and sex- and age-matched nonobese controls using paired *t*-tests. *P* values were adjusted

for multiple testing using Benjamini and Hochbergs false discovery rate correction (21). Pearson correlation coefficients were determined to assess correlation between the MIA and ELISA measurements. SPSS 15.0.1 (SPSS Software) and GraphPad Prism 4.02 (GraphPad Software) were used for statistical analysis.

4

RESULTS

MIA development and validation

To develop a MIA for adipokines, 25 recombinant adipokine proteins plus specific capture and detection antibody pairs (Table 1) were tested for lower limits of detection and quantification, cross-reactivity, and intra- and interassay variation. All recombinant protein-antibody combinations were optimized in single bead assays before being included in the multiplex panel.

The MIA calibration curves showed high maximum fluorescence intensities and broad dynamic ranges (Fig. 1). The LLODs and LLOQs (Table 2) showed that the majority of adipokines could be measured in low concentration and fluorescence ranges. The supernatant and plasma concentrations of the 5 adipokines with a higher LLOQ (adiponectin, chemerin, PAI-1, SAA-1, and RBP-4, LLOQ >100 ng/L) all exceeded their LLOQ, so these adipokines could be measured reliably as well.

We assessed cross-reactivity by testing high concentrations of a given recombinant adipokine against the full panel of adipokine capture and detection antibodies. Most of the recombinant adipokine proteins did not display cross-reactivity with antibodies directed against other adipokines. Only 2 nonspecific (PAI-1 recombinant protein, RBP-4 antibody pair) and 3 specific cross-reactions (>1%) were observed (Table 3). Resistin antibodies displayed 10.8% cross-reactivity to PAI-1 recombinant protein, indicating that resistin measurements might be affected in the case of high PAI-1 concentrations. It should be noted, however, that in all other cases cross-reactions were observed at recombinant adipokine concentrations that exceed physiological concentrations, thereby reducing the chance of cross-reactivity in physiological samples.

Intraassay variation (CV%) was <10% for all adipokines, with a mean intraassay variation of 4.6%. Interassay variation was <15%, with a mean interassay variation of 7.7% (Table 2). The measured intra- and interassay variations are comparable to or lower than the variations usually reported for adipokine ELISAs (22).

MIA results correlated significantly with ELISA adipokine measurements of the supernatants and plasma samples (Table 2), but 4 categories of adipokines could be distinguished. For most of the cytokines and chemokines, ELISAs with the same antibody pairs used for the MIA were available, resulting in optimal correlations (Pearson $R > 0.9$, $P < 0.0001$), as published before (15). For adiponectin, adipsin, HGF, leptin, PAI-1, resistin, TIMP-1, and thrombopoietin, the antibody pairs used for ELISA and MIA may be identical, but the antibody clones were not disclosed by the ELISA manufacturer. These adipokines showed significant but variable correlation (Pearson R between 0.59 and 0.93) between MIA and ELISA, probably due to MIA-ELISA antibody (dis)similarities. Next, for cathepsin S, chemerin, interleukin (IL)-1RA,

Table 1 Adipokine MIA reagents

Adipokine	Recombinant protein	Capture antibody			Detection antibody		
	Source[a]	Host	Clone	Source[a]	Host	Clone	Source[a]
Adiponectin	RnD	Mouse	166126	RnD	Mouse	166128	RnD
Adipsin	RnD	Mouse	255706	RnD	Goat	Polyclonal	RnD
Cathepsin S	RnD	Goat	Polyclonal	RnD	Goat	Polyclonal	RnD
Chemerin	RnD	Mouse	365317	RnD	Goat	Polyclonal	RnD
GM-CSF	BD	Mouse	6804.11	RnD	Mouse	3209.1	RnD
HGF	E-bio	Mouse	24612	RnD	Goat	Polyclonal	RnD
IFN-γ	BD	Mouse	NIB42	BD	Mouse	4S.B3	BD
IL-1RA	RnD	Mouse	CRM17	eBio	Rat	Polyclonal	eBio
IL-1β	Miltenyi	Mouse	CLB/IL1B-8	SQ	Mouse	CLB/IL1B-4	SQ
IL-6	BD	Rat	MQ2-13A5	BD	Rat	MQ2-39C3	BD
IL-8	NIBSC	Mouse	CLB/IL8-1	SQ	Sheep	Polyclonal	SQ
IP-10	BD	Mouse	4D5/A7/C5	BD	Mouse	6D4/D6/G2	BD
Leptin	RnD	Mouse	44802	RnD	Mouse	44804	RnD
MCP-1	RnD	Mouse	23007	RnD	Goat	Polyclonal	RnD
M-CSF	RnD	Mouse	21113	RnD	Goat	Polyclonal	RnD
MIF	RnD	Mouse	12302.2	RnD	Goat	Polyclonal	RnD
MIP-1α	RnD	Mouse	14215	RnD	Goat	Polyclonal	RnD
NGF	RnD	Mouse	25623	RnD	Goat	Polyclonal	RnD
PAI-1	RnD	Mouse	242816	RnD	Goat	Polyclonal	RnD
RBP-4	RnD	Mouse	393005	RnD	Goat	Polyclonal	RnD
Resistin	Adipogen	Mouse	184335	RnD	Goat	Polyclonal	RnD
SAA-1	Peprotech	Mouse	3H64	Lifespan	Mouse	6i7	Lifespan
TIMP-1	RnD	Mouse	63515	RnD	Goat	Polyclonal	RnD
TNF-α	Miltenyi	Mouse	Mab1	BD	Mouse	Mab11	BD
Thrombopoietin	RnD	Mouse	34817	RnD	Mouse	34840	RnD

[a] RnD= R&D Systems, BD = BD Biosciences, eBio = eBioscience, Miltenyi = Miltenyi Biotec, Lifespan = Lifespan Technologies, SQ = Sanquin Reagents.

monocyte chemoattractant protein 1 (MCP-1), M-CSF, macrophage migration inhibitory factor (MIF), nerve growth factor (NGF), RBP-4, and SAA-1, ELISAs with the same antibody pairs as the MIA were not available and were not performed. Finally, GM-CSF concentrations in the supernatants and plasma samples were below ELISA limit of detection.

Table 2 Adipokine MIA characteristics

Adipokine	Dynamic range (ng/L) High - low[a]	Intra-assay variation CV% (n=33)[b]	Inter-assay variation CV% (n=11)[b]	LLOD (ng/L)[c]	LLOQ (ng/L)[c]	MIA vs ELISA[d] Pearson R	MIA vs ELISA[d] *P* value
Adiponectin	200 000-49	3.4	3.4	109.2	327.7	0.59	0.0332
Adipsin	20 000-5	5.7	10.2	19.5	58.5	0.69	0.0195
Cathepsin S	10 000-2	4.1	11.5	<2	<6	ND	ND
Chemerin	200 000-49	2.8	4.6	95.1	285.4	ND	ND
GM-CSF	5 000-1	4.4	11.4	12.0	35.9	ND	ND
HGF	10 000-2	4.3	6.9	<2	<6	0.69	0.0045
IFN-γ	10 000-2	4.5	10.4	<2	<6	1.00[e]	<0.0001[e]
IL-1RA	10 000-2	6.3	9.4	<2	<6	ND	ND
IL-1β	5 000-1	4.6	3.8	0.1	0.4	1.00[e]	<0.0001[e]
IL-6	10 000-2	4.2	8.7	<2	<6	0.98[e]	<0.0001[e]
IL-8	5 000-1	6.7	4.6	0.1	0.4	0.99[e]	<0.0001[e]
IP-10	5 000-1	4.8	6.8	0.1	0.3	1.00[e]	<0.0001[e]
Leptin	100 000-24	5.9	8.9	<1	<3	0.84	<0.0001
MCP-1	5 000-1	4.1	12.0	1.0	3.1	ND	ND
M-CSF	10 000-2	4.6	5.6	3.3	10.0	ND	ND
MIF	20 000-5	3.5	4.0	14.3	42.8	ND	ND
MIP-1α	20 000-5	6.7	8.3	20.2	60.6	0.97[e]	<0.0001[e]
NGF	5 000-1	4.7	12.0	<1	<3	ND	ND
PAI-1	2 000 000-488	7.7	4.2	91.7	275.2	0.70	0.024
RBP4	2 000 000-488	3.4	6.4	13074.7	39223.9	ND	ND
Resistin	20 000-5	3.9	6.6	0.4	1.1	0.89	0.0002
SAA1	500 000-122	6.2	11.8	2496.2	7488.5	ND	ND
TIMP-1	20 000-5	2.4	7.5	25.3	75.8	0.93	<0.0001
TNF-α	5 000-1	4.5	4.4	<1	<3	0.96[e]	<0.0001[e]
Thrombopoietin	5 000-1	2.3	8.8	<1	<3	0.60	0.041

[a] Dynamic range is expressed as the highest to lowest concentration covered by the calibration curve.
[b] Intra- and interassay variation were calculated after measuring 11 different samples in triplicate for three consecutive days, and are expressed as the coefficient of variation (%).
[c] Lower limit of detection (LLOD) and quantitation (LLOQ) were calculated after measuring a total of 26 blanks over three consecutive days and are expressed as a concentration (ng/L). Arrows (<) indicate the LLOD and/or LLOQ could not be calculated by interpolation in the 5-parameter logistic adipokine standard curves, as they were too low to fit in the logistic curves.
[d] ND = Not determined. ELISA kits with the same antibody-pair clones as used for the MIA were either not available or were not able to detect adipokine levels in the supernatants and plasma samples (GM-CSF).
[e] MIA and ELISA results were compared in an earlier publication of our group (15).

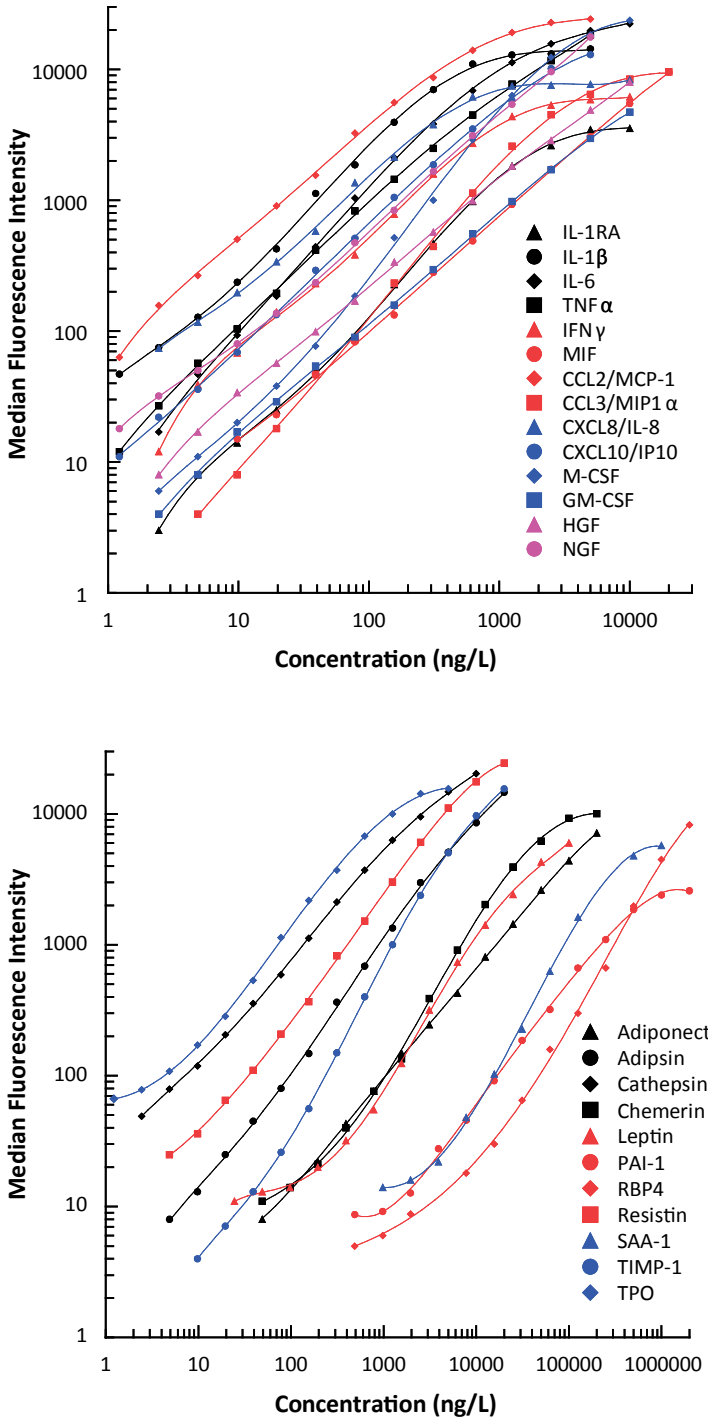

Figure 1 Adipokine MIA calibration curves

Table 3 Adipokine MIA cross-reactivity

		Fluorescent signal on adipokine microsphere (%)[a]										
		Adipo[b]	Adips	CatS	Chem	GMCSF	HGF	IFNγ	IL1RA	IL1β	IL6	IL8
Adipokine recombinant protein	**Adiponectin**		0.2	0.1	0.1	0.0	0.1	0.2	0.0	0.1	0.0	0.1
	Adipsin	0.2		0.0	0.2	0.0	0.1	0.2	0.0	0.0	0.0	0.1
	Cathepsin S	0.1	0.1		0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.2
	Chemerin	0.2	0.1	0.1		0.0	0.7	0.3	0.1	0.1	0.0	0.2
	GM-CSF	0.1	0.1	0.0	0.1		0.1	0.2	0.0	0.0	0.0	0.1
	HGF	0.1	0.1	0.0	0.1	0.0		0.2	0.0	0.1	0.0	0.1
	IFN-γ	0.3	0.2	0.1	0.2	0.0	0.2		0.3	0.3	0.1	0.5
	IL-1RA	0.2	0.1	0.1	0.2	0.0	0.1	0.2		0.1	0.0	0.2
	IL-1β	0.2	0.1	0.1	0.2	0.0	0.2	0.2	0.0		0.0	0.2
	IL-6	0.2	0.1	0.1	0.2	0.0	0.1	0.2	0.0	0.1		0.2
	IL-8	0.1	0.1	0.0	0.2	0.0	0.1	0.2	0.0	0.1	0.0	
	IP-10	0.2	0.1	0.1	0.2	0.0	0.1	0.2	0.0	0.1	0.0	0.2
	Leptin	0.1	0.1	0.0	0.1	0.0	0.0	0.2	0.0	0.1	0.0	0.1
	MCP-1	0.2	0.1	0.1	0.2	0.0	0.1	0.2	0.0	0.1	0.0	0.2
	M-CSF	0.1	0.1	0.1	0.2	0.0	0.1	0.2	0.0	0.1	0.0	0.2
	MIF	0.2	0.1	0.1	0.2	0.0	0.1	0.2	0.0	0.1	0.0	0.2
	MIP-1α	0.2	0.2	0.1	0.3	0.0	0.1	0.3	0.1	0.1	0.0	0.3
	NGF	0.2	0.1	0.0	0.1	0.0	0.1	0.2	0.0	0.1	0.0	0.1
	PAI-1	*2.5*	1.0	0.9	1.0	*1.3*	*1.6*	0.8	*4.6*	*2.2*	*1.4*	*4.2*
	RBP-4	0.1	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.1
	Resistin	0.2	0.1	0.0	0.1	0.0	0.1	0.2	0.0	0.1	0.0	0.3
	SAA-1	0.5	0.2	0.1	0.3	0.0	0.2	0.3	0.0	0.2	0.0	0.3
	TIMP-1	0.1	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.1
	TNF-α	0.2	0.2	0.1	0.2	0.0	0.1	0.2	0.0	0.1	0.0	0.2
	Thrombopoietin	0.2	0.1	0.0	0.2	0.0	0.1	0.2	0.0	0.1	0.0	0.2

[a] Columns represent percentages of cross-reactivity of adipokine microspheres in response to high concentrations of single recombinant adipokines (rows), expressed as the ratio of fluorescence intensity compared to the microspheres' maximum fluorescence intensity. Cross-reactions (>1%) are displayed in *italics*.

[b] The header usually has shortened names for the protein column on the left; the order for the header and the protein column is exactly the same.

	Fluorescent signal on adipokine microsphere (%)[a]													
	IP10	**Lept**	**MCP1**	**MCSF**	**MIF**	**MIP1α**	**NGF**	**PAI**	**RBP4**	**Res**	**SAA1**	**TIMP1**	**TNFα**	**Tpo**
	0.0	0.1	0.0	0.0	0.3	0.3	0.1	0.2	*1.8*	0.1	0.2	0.1	0.0	0.0
	0.0	0.1	0.0	0.0	0.3	0.3	0.1	0.3	*1.8*	0.1	0.2	0.1	0.0	0.1
	0.0	0.1	0.0	0.0	0.2	0.3	0.1	0.2	*1.4*	0.1	0.2	0.1	0.0	0.0
	0.1	0.2	0.0	0.0	0.1	0.4	0.0	0.6	*1.1*	0.3	0.1	0.2	0.0	0.1
	0.0	0.1	0.0	0.0	0.3	0.3	0.1	0.2	*1.5*	0.1	0.1	0.1	0.0	0.0
	0.0	0.1	0.0	0.0	0.2	0.3	0.1	0.1	*1.4*	0.0	0.1	0.1	0.0	0.0
	0.1	0.1	0.0	*3.4*	0.7	0.4	0.1	0.6	*2.1*	0.7	0.2	0.1	0.0	0.1
	0.0	0.1	0.0	0.0	0.3	0.3	0.1	0.3	*2.1*	0.3	0.2	0.1	0.0	0.0
	0.1	0.1	0.0	0.0	0.4	0.3	0.1	0.3	*2.1*	0.2	0.3	0.1	0.0	0.0
	0.1	0.1	0.0	0.0	0.3	0.3	0.1	0.3	*2.1*	0.2	0.2	0.1	0.0	0.0
	0.0	0.1	0.0	0.0	0.3	0.3	0.1	0.2	*2.0*	0.2	0.2	0.1	0.0	0.0
		0.1	0.0	0.0	0.3	0.4	0.1	0.3	*2.0*	0.3	0.3	0.1	0.0	0.0
	0.0		0.0	0.0	0.2	0.3	0.1	0.2	*1.8*	0.1	0.1	0.1	0.0	0.0
	0.0	0.1		0.0	0.4	0.3	0.1	0.3	*2.4*	0.3	*3.7*	0.1	0.0	0.0
	0.1	0.1	0.0		0.3	0.3	0.1	0.2	*1.8*	0.2	0.2	0.1	0.0	0.0
	0.1	0.1	0.0	0.0		0.3	0.1	0.3	*2.0*	0.2	0.2	0.1	0.0	0.0
	0.1	0.1	0.0	0.0	0.5		0.1	0.4	*2.5*	0.3	0.3	0.1	0.0	0.1
	0.0	0.1	0.0	0.0	0.3	0.3		0.3	*1.9*	0.2	0.2	0.1	0.0	0.0
	0.5	*2.1*	0.0	0.3	*2.2*	*1.4*	0.4		*2.4*	*10.8*	0.9	0.7	0.1	0.4
	0.0	0.1	0.0	0.0	0.2	0.2	0.1	0.1		0.1	0.1	0.1	0.0	0.0
	0.0	0.1	0.0	0.0	0.5	*1.4*	0.1	0.2	*1.8*		0.6	0.1	0.0	0.1
	0.1	0.5	0.0	0.0	0.4	0.4	0.2	0.6	*2.1*	0.5		0.2	0.0	0.1
	0.0	0.0	0.0	0.0	0.1	0.2	0.1	0.2	*1.3*	0.0	0.1		0.0	0.0
	0.1	0.1	0.0	0.0	0.4	0.3	0.1	0.4	*2.0*	0.2	0.3	0.1		0.1
	0.1	0.1	0.0	0.0	0.3	0.3	0.1	0.3	*1.8*	0.2	0.2	0.1	0.0	

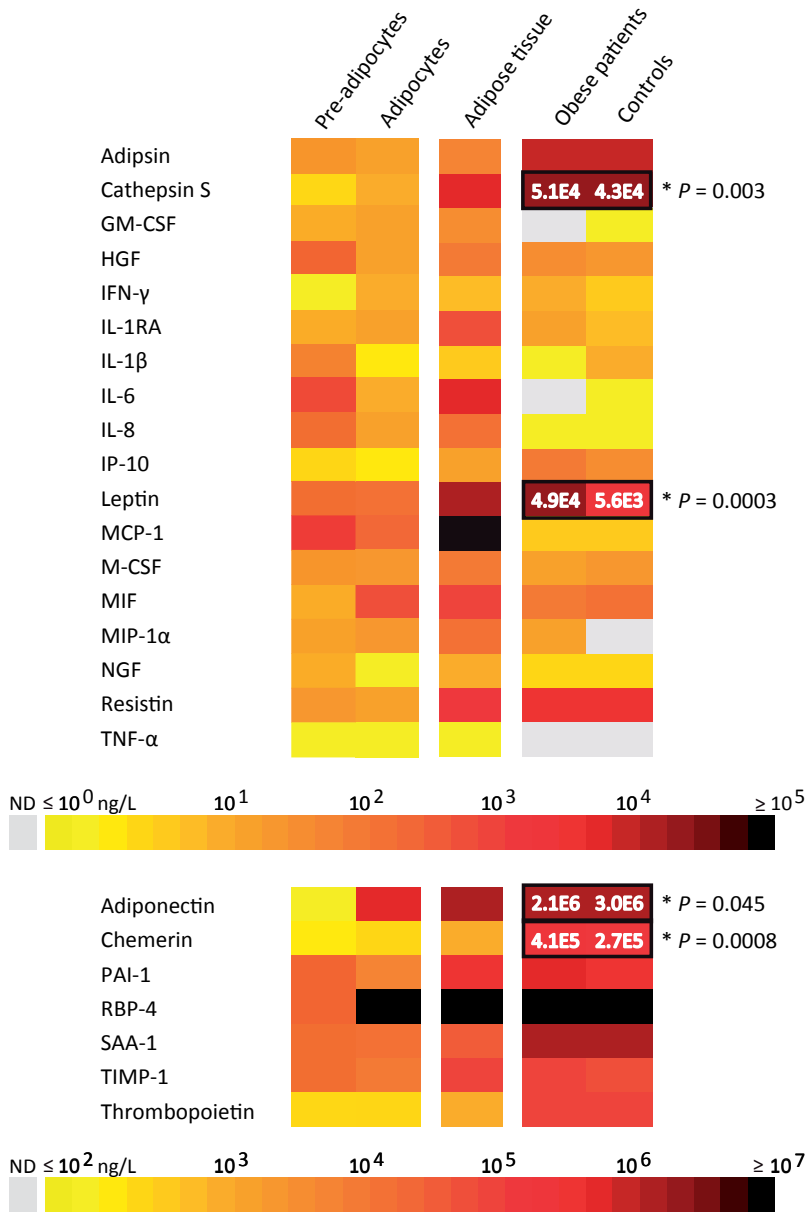

Figure 2 Adipokine profiles of various biological samples
Adipokine concentrations are displayed in 2 groups. The upper panel displays adipokines present in concentrations between 1 and 100 000 ng/L, and the lower panel displays adipokines present in concentrations between 100 and 10 000 000 ng/L. For all significant differences between obese patients and controls, mean adipokine concentrations and corresponding *P* values are shown (paired *t*-tests). ND, not determinable (levels below detection limit).

Human adipokine profiling in biological samples

To investigate whether the human adipokine MIA could be applied to various biological samples, we generated adipokine profiles of preadipocyte, adipocyte, and adipose tissue supernatants together with plasma adipokine profiles of morbidly obese patients and healthy, normal-weight sex- and age-matched controls. First, supernatants were collected of human SGBS cells, either before or after differentiation into mature adipocytes (Supplemental Fig. 1). All 25 adipokines were detected in the supernatants, either before or after differentiation (Fig. 2, depicted as heat map). SGBS preadipocytes secreted more IL-1β, IL-6, IL-8, MCP-1, and PAI-1 than differentiated adipocytes, whereas adipocytes secreted more adiponectin and RBP-4. When adipokine profiling was performed on supernatant of *ex vivo* cultured adipose tissue, several adipokines were detected that were also found to be secreted by SGBS adipocytes (Fig. 2). In addition, high concentrations of cathepsin S, IL-6, MCP-1, and PAI-1 were found (Fig. 2), probably reflecting the presence of preadipocytes and stromal vascular cells in the tissue sample. Finally, we performed adipokine profiling of 5 morbidly obese women and 5 sex- and age-matched normal-weight controls (Table 4). Except for GM-CSF, IL-6, macrophage inflammatory protein 1α (MIP-1α), and tumor necrosis factor α (TNF-α), all adipokines could be detected in both obese patient and control plasma samples (Fig. 2). Furthermore, morbidly obese patients showed significantly lower concentrations of adiponectin and higher concentrations of leptin and the novel adipokines cathepsin S and chemerin. When corrected for multiple testing, differences in leptin ($P = 0.006$), cathepsin S ($P = 0.026$), and chemerin ($P = 0.010$) concentrations remained significant, whereas differences in adiponectin concentrations could not be established anymore ($P = 0.227$), probably because of the small sample size.

Table 4 Participant characteristics

	Number	Mean age, years (range)	Mean BMI, kg/m^2 (range)	Sex
Obese patients	5	34 (23-47)	48.0 (39.2-66.9)	All female
Normal-weight healthy controls	5	34 (24-47)	21.8 (20.1-24.8)	All female
Adipose tissue donor	1	38	28.4	Female

DISCUSSION

In this study, we report the development and validation of an MIA for comprehensive profiling of human adipokines. The assay shows advantages over adipokine ELISAs and other techniques by enabling measurement of 25 adipokines in only 50 µL of sample. This large adipokine MIA adds to the smaller commercial adipokine MIA

panels (up to 8 adipokines) that have been described so far (22). Furthermore, the assay is characterized by low limits of quantification, cross-reactivity, and intra- and interassay variation together with potential flexibility - i.e., newly discovered adipokines (e.g., visfatin) could be added to the MIA panel as soon as appropriate capture and detection antibodies become available.

There are a few general considerations for cytokine and adipokine measurements with ELISA and MIA techniques. First, long-term storage (>2 years at -80 °C) and repeated freeze-thawing can influence the measurements (23). Second, matrix characteristics such as protein content and pH affect protein concentrations detected (12). Sample dilution thus requires standardization. We chose to measure all adipokines undiluted except for adipsin, cathepsin S, leptin, adiponectin, chemerin, PAI-1, RBP-4, SAA-1, TIMP-1, and thrombopoietin, which occur in high concentrations in human plasma. Third, to avoid interference of heterophilic antibodies, preincubation of the plasma samples on protein L-coated plates followed by a blocking step with rodent serum is needed, as described (15). Finally, antibody-based assays like ELISA and MIA render semiquantitative results. Although relative sample differences can be compared, absolute concentrations vary even between ELISA kits (24, 25). One of the reasons for the latter is illustrated by the MIA-ELISA correlation studies we performed. As described earlier, comparing MIA and/or ELISA assays using different antibody clones renders lower correlations (12, 13, 22, 24, 25). Meaningful MIA and/or ELISA comparisons for validation purposes thus require analogous antibody clones. In our study, the MIA adipokines that, for this reason, could not be compared with ELISAs still showed good reproducibility, specificity, and limits of quantification, as required generally for new antibody-based assays like ELISA and MIA (26).

The human adipokine MIA showed its value in several ways. When profiling the supernatants of human SGBS preadipocytes and adipocytes, we found that high concentrations of adiponectin were secreted by adipocytes, whereas preadipocytes secreted more IL-6, IL-8, PAI-1, and MCP-1, in agreement with previous studies (27). Taking note of the fact that adipokine profiles of SGBS adipocytes and primary adipocytes might show differences, our findings do support a role for the adipokine MIA in studying the effects of bioactive compounds (e.g., lipids) on adipokine profiles in simplified model systems, such as cultured (pre)adipocytes. Furthermore, we observed interesting differences between the plasma adipokine profiles of morbidly obese patients and sex- and age-matched controls. While as a proof of concept our data confirmed the well established link between obesity and plasma leptin concentrations (3, 4), our data also underlined the potential relevance of cathepsin S and chemerin in obesity and its associated disorders (7, 28-30). We therefore conclude that this novel method can be of value for both comprehensive adipokine profiling in large patient cohorts and biological discovery in cellular model systems.

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SUPPLEMENTAL INFORMATION

A multiplex immunoassay for human adipokine profiling

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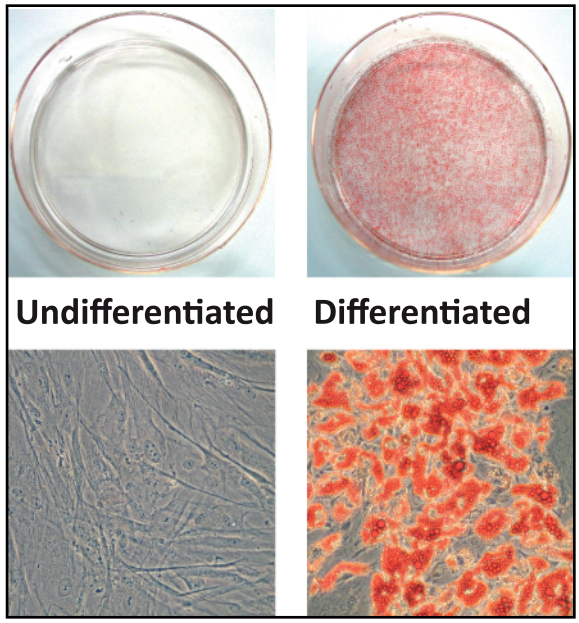

Supplemental figure 1 Adipocyte differentiation
Upper panels display Oil-Red-O staining of 10 cm dishes with human SGBS cells, before (left panel) and after differentiation into adipocytes (right panel). Lower panels display higher magnifications (200x).

CHAPTER 5

Altered plasma adipokine levels and *in vitro* adipocyte differentiation in pediatric type 1 diabetes

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ABSTRACT

Context

Type 1 diabetes (T1D) is considered a proinflammatory condition. Adipose tissue involvement seems evident because adiponectin levels correlate with disease remission and administration of leptin suppresses the low-grade systemic inflammation in mice with T1D. Whether adipose tissue involvement in T1D already occurs at a young age is yet unknown.

Objective

The aim was to explore the extent of adipokine alterations in pediatric T1D and gain more insight into the mechanisms underlying the involvement of adipose tissue.

Design and participants

First, plasma adipokine profiling (24 adipokines) of 20 children with onset T1D, 20 children with long-standing T1D, and 17 healthy controls was performed using a recently developed and validated multiplex immunoassay. Second, the effects of diabetic plasma factors on preadipocyte proliferation and differentiation were studied *in vitro*.

Results

In children with onset and long-standing T1D, plasma adipokine profiling showed increased levels of various adipokines acting at the crossroads of adipose tissue function and inflammation, including CCL2/monocyte chemoattractant protein-1 and the novel adipokines cathepsin S, chemerin, and tissue inhibitor of metalloproteinase-1 ($P < 0.05$). Furthermore, onset and long-standing diabetic plasma significantly induced preadipocyte proliferation and adipocyte differentiation *in vitro* ($P < 0.05$). Two candidate plasma factors, glucose and the saturated fatty acid palmitic acid, did not affect proliferation or adipocyte differentiation *in vitro* but were found to increase CCL2 (monocyte chemoattractant protein-1) secretion by adipocytes.

Conclusions

The adipogenic effects of diabetic plasma *in vitro* and the altered adipokine levels *in vivo* suggest adipose tissue involvement in the low-grade inflammation associated with T1D, already in pediatric patients.

INTRODUCTION

Type 1 diabetes is increasingly considered to be a proinflammatory disease (1). Compelling evidence for this view comes from the observation that circulating levels of proinflammatory proteins such as TNF-α, C-reactive protein, IL-1β, and IL-6 are enhanced and that cytokine secretion by peripheral blood mononuclear cells is increased in type 1 diabetes (2-6).

In type 2 diabetes, low-grade systemic inflammation reflects adipose tissue inflammatory processes. Adipocyte hypertrophy and insulin resistance are known to boost the production of inflammatory proteins by adipose tissue, thereby establishing adipose tissue as a key mediator between type 2 diabetes, cardiovascular disease, and inflammation (7, 8).

In contrast to type 2 diabetes, adipose tissue involvement in the low-grade systemic inflammation observed in type 1 diabetes has been investigated far less. Yet there are strong indications for adipose tissue involvement. From a theoretical point of view, type 1 diabetes accompanying features like glucose dysregulation and dyslipidemia (9) may well impact adipose tissue function and inflammatory processes (8, 10). Indeed, alterations in circulating levels of the adipose tissue-secreted cytokines (adipokines) leptin and adiponectin in adult and pediatric type 1 diabetes indicate adipose tissue involvement in the low-grade systemic Inflammation observed (11). Interestingly, adiponectin levels were found to specifically correlate with disease remission (12, 13). Furthermore, recent studies in mice suggest that administration of adipose tissue-secreted factors in type 1 diabetes can even improve inflammatory and metabolic parameters; leptin therapy was found to reduce low-grade systemic inflammation and improve the metabolic balance (14). Although findings in mice may not easily translate to humans (15), these studies emphasize the emergence of adipose tissue function and adipokines in type 1 diabetes as an important new field of research.

To explore the extent of adipokine alterations in pediatric type 1 diabetes and to gain more mechanistic insight in the involvement of adipose tissue, we employed two novel approaches. First, we compared adipokine profiles in plasma of children at onset of type 1 diabetes, long-standing pediatric type 1 diabetes patients, and healthy controls (HC). For this, a recently developed and validated multiplex adipokine immunoassay (16) was used to analyze 24 different adipokines. Second, to investigate whether changes in adipocyte differentiation may account for the altered adipokine levels, we conducted *in vitro* adipocyte differentiation assays with patient and control plasma pools. In addition, we studied whether the effects of diabetic plasma on adipocyte differentiation and adipokine production could be reproduced by varying two plasma factors associated with type 1 diabetes - glucose and free fatty acids.

SUBJECTS

Heparinized daytime blood samples were obtained from 20 children with onset type 1 diabetes mellitus, 20 children with long-standing type 1 diabetes (> 1 yr after onset), and 17 HC (Table 1). Using the International Society for Pediatric and Adolescent

Table 1 Patient characteristics

	Onset T1D		Long-standing T1D		Healthy controls	
	Female	Male	Female	Male	Female	Male
n	10	10	10	10	10	7
Age (yr)	13.0 (7.8-16.4)	13.4 (10.0-15.4)	15.2 (12.0-18.8)	14.1 (9.4-17.9)	12.9 (7.9-16.5)	9.5 (6.5-12.9)
Age at onset T1D (yr)	13.0 (7.8-16.4)	13.4 (10.0-15.4)	9.7 (3,0-13)	8.3 (1.7-14.8)	NA	NA
Duration T1D (yr)	0	0	5.4 (3.1-11)	5.9 (1.7-12.8)	NA	NA
HbA1c (%)	11.8 (8.2-17.3)	10.7 (8.5-13.8)	9.0 (7.4-14)	8.7 (6.9-11.2)	NA	NA
BMI (SD-score)	-0.7 (-2.9 to +0.5)	-0.6 (-2.2 to +1.2)	0.8 (-0.8 to +2,6)	0.6 (-1.2 to +2.2)	-0.2 (-1.3 to +1.8)	0.4 (-0.4 to +1.7)
Total cholesterol (mmol/L)	4.2 (3.3-5.4)	4.3 (3.4-5.0)	4.3 (3.3-6.0)	4.3 (3.2-5.4)	4.3 (3.6-5.2)	3.9 (3.5-4.3)
Triglycerides (mmol/L)	1.1 (0.4-2.6)	0.8 (0.4-1.2)	0.8 (0.5-1.3)	0.9 (0.5-1.6)	0.8 (0.4-1.3)	0.7 (0.4-1.2)
HDL (mmol/L)	1.3 (0.9-1.8)	1.3 (0.9-1.9)	1.4 (1.2-1.8)	1.5 (1.1-2.3)	1.5 (0.9-1.8)	1.3 (1.0-1.4)
LDL (mmol/L)	2.4 (1.8-3.5)	2.7 (2.0-3.5)	2.5 (1.6-4.3)	2.4 (1.7-3.4)	2.5 (2.1-3.1)	2.3 (1.9-2.6)
Free fatty acids (mmol/L)	0.9 (0.3-2.1)	0.6 (0.2-1.1)	0.5 (0.3-0.7)	0.4 (0.3-0.5)	0.4 (0.2-0.9)	0.5 (0.2-1.1)

Table 1. Data are expressed as mean (range). Because BMI and lipid profiles are age-related and partly gender-related, all patient groups are divided for gender. NA, not available; T1D, type 1 diabetes; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Diabetes (ISPAD) criteria for diagnosis of type 1 diabetes mellitus, the day of diagnosis ("onset") was defined as the day on which hyperglycemia was detected (17). For onset type 1 diabetes patients, a blood sample was obtained shortly after diagnosis (median, 1 d; range, 0-12 d).

Nonfasting blood samples were drawn at the participating hospitals at random times of the day together with samples for routine diagnostics, including glycosylated hemoglobin (HbA1c) as a glycemic state indicator. Body mass index (BMI) was calculated as weight in kilograms/height in meters^2, and SD values were calculated based on Dutch reference values (18). Written informed consent was obtained from all children and/or their parents. The study was approved by all local medical ethics review boards (METC 01/020-K). The study design was retrospective and cross-

sectional. Researchers were blinded for the patient groups when performing the laboratory measurements described below.

METHODS

Patient plasma samples

Heparinized blood samples were centrifuged at 150 *g* for 10 min, after which plasma was aliquoted and stored at -80°C until analysis. Before analysis, plasma samples were centrifuged on a 0.22-µm nylon membrane (Spin-X column; Corning Life Sciences, Lowell, MA) to remove cellular debris. To prevent interference from heterophilic antibodies, Ig were removed by 1-h incubation on protein L-coated plates (Pierce, Rockford, IL), as described previously (19). Lipid levels were measured using routine diagnostics.

Adipocyte differentiation and plasma stimulation

The human preadipocyte SGBS cell line was cultured and differentiated as described previously (20). In short, preadipocytes were grown to confluence. Differentiation was initiated with a 5-d pulse of dexamethasone, rosiglitazone, 3-isobutyl-1-methylxanthine, T_3, biotin, and D-panthotenic acid in DMEM/F12 medium (Invitrogen, Carlsbad, CA) and continued with 4 d of T_3, biotin, and D-panthotenic acid stimulation only.

At d 7, pooled patient or control plasma (5 random patients per plasma pool) was added to the cell supernatants to a final concentration of 20% for 24 h. At d 8, cells were washed with PBS once and incubated with fresh differentiation medium. At d 9, supernatants were harvested and stored at -80°C until the adipokine multiplex immunoassay analysis. Cells were fixed with 4% paraformaldehyde for 30 min and stored in PBS.

Free fatty acids

Before incubation with the adipocytes, oleic acid and palmitic acid were dissolved in potassium hydroxide in 100% ethanol, and complexed with BSA at 37°C for 1 h in a fatty acid:BSA 3:1 molar ratio, as described (21).

Effects of hyperglycemia and free fatty acid on adipocyte differentiation

Human preadipocytes were cultured and differentiated as described above. At d 7, cells were exposed to different concentrations of glucose (low glucose, 1 g/liter; normal glucose, 4.5 g/liter; or high glucose dose, 20 g/liter) or free fatty acids (vehicle, KOH in 100% ethanol; oleic acid, 250 µM; or palmitic acid, 250 µM) for 24 h. Please note that 1 g/liter glucose represents a low concentration of glucose in *in vitro* adipocyte cultures (16, 20), whereas being a relatively normal dose *in vivo* (5.7 mmol/liter). At d 8, cells were washed once with PBS and incubated with fresh differentiation media. At d 9, supernatants were harvested and stored at -80°C until the adipokine multiplex immunoassay analysis. Cells were fixed with 4% paraformaldehyde for 30 min and stored in PBS.

Quantification of adipocyte differentiation

Fixed cells were either stained with Oil-Red-O as described previously (20) or stained with Nile Red and 4',6-diamidino-2-phenylindole for automated fluorescence microscopy (Cellomic ArrayScan, VTI HCS Reader; Thermo Scientific, Rockford, IL) to quantify adipocyte differentiation. This was carried out using the Cellomics Target Activation Bioapplication package. Essentially, this application identifies the 4',6-diamidino-2-phenylindole-stained nuclei. An *x* pixel mask was created around the nucleus, and the intensity of Nile Red staining within this mask was reported. The percentage of responders was based on a cutoff of *y* for the average pixel intensity/ total intensity of Nile Red within the mask. Details on the algorithm settings are given in the Supplemental information.

Adipokine multiplex immunoassay

Adipokine levels were measured with the Bio-Plex system in combination with Bio-Plex Manager software version 5.0 (Bio-Rad Laboratories, Hercules CA), as described recently (16, 19). Adipocyte supernatants were measured undiluted. Plasmas were measured undiluted for most of the adipokines. For measurement of plasma adipokines naturally occurring in very high concentrations [*i.e.* tissue inhibitor of metalloproteinase-1 (TIMP-1), chemerin, plasminogen activator inhibitor-1 (PAI-1), adiponectin, adipsin (complement factor D), serum amyloid A1 (SAA-1), retinol binding protein 4 (RBP-4), cathepsin S, thrombopoietin, and leptin], patient and control plasmas were diluted 100 times. For measurement of osteopontin (OPN), all plasma samples were diluted five times.

Statistical analyses

Statistical evaluation was performed using GraphPad Prism software, version 4.02 (GraphPad Software, La Jolla, CA) and SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL). Basic descriptive statistics were used to describe the patient populations. For the adipokine levels and lipids, paired data were analyzed with the Wilcoxon signed-ranks test; unpaired data were analyzed with the Mann-Whitney *U* test. For the adipocyte differentiation, differences in the number of differentiated adipocytes per microscopic field between groups were analyzed with a one-way ANOVA and Tukey's multiple comparison *post hoc* test. A P value < 0.05 was considered statistically significant.

RESULTS

Plasma adipokine levels

We compared plasma adipokine profiles of HC with newly diagnosed and long-standing type 1 diabetes patients, as shown in Fig. 1 and Table 2. Plasma concentrations of several adipokines were significantly altered between type 1 diabetes patients and HC.

In both onset and long-standing type 1 diabetes patients, adiponectin, leptin, RBP-4, cathepsin S, TIMP-1, chemerin, SAA-1, and PAI-1 levels were significantly increased compared with HC. Interestingly, leptin, SAA-1, and PAI-1 levels were

higher in long-standing compared with newly diagnosed patients. CCL2 (monocyte chemoattractant protein-1) levels were higher in patients with long-standing disease compared with newly diagnosed patients and HC, and macrophage inhibitory factor (MIF) levels were significantly enhanced in patients with longstanding disease compared with HC only. Finally, OPN showed increased levels in patients at onset of disease compared with long-standing diabetes patients.

Table 2 Adipokine and cytokine levels in plasma

	Units	Healthy controls	Onset	Long-standing disease
IL-1RA [aa]	pg/ml	45 (6.0- 421)	46 (2.3- 417)	42 (6.0-114)
IL-1β	pg/ml	6.3 (0.4- 73)	2.7 (0.4- 22)	7.2 (0.4-87)
IL-6 [aa]	pg/ml	17 (6.0- 161)	6.7 (0.4- 26)	16 (2.0-192)
IL-10	pg/ml	39 (0.1- 407)	13 (0.1- 95)	28 (0.9-312)
TNF-α [aa]	pg/ml	4.7 (0.5- 33)	2.9 (0.1- 3.0)	3.9 (1.0-23)
IFN-γ [aa]	pg/ml	7.4 (6.0- 25)	6.0 (6.0)	9.7 (4.5-81)
MIF	ng/ml	1.3 (0.0- 3.1) [a]	1.5 (0.1- 3.4)	2.5 (0.6-11) [a]
CCL2	pg/ml	18 (1.0- 45) [a]	27 (4.4- 65) [b]	45 (13-100) [a,b]
CCL3 [aa]	pg/ml	91 (61- 280) [a,c]	61 (61) [c]	60 (54-97) [a]
CXCL8 (IL-8)	pg/ml	28 (0.4- 160)	14 (0.3-103)	21(0.4-99)
CXCL10	pg/ml	371 (100- 2333)	359 (108- 2210)	281 (125-712)
GM-CSF [aa]	pg/ml	36 (34- 36)	35 (5.5- 63)	35 (0.7-125)
OPN	ng/ml	83 (16- 253)	71 (18-288) [b]	44 (15-77) [b]
Resistin	ng/ml	190 (58- 452)	213 (111-624)	178 (75-359)
Thrombopoietin	µg/ml	4.0 (1.0- 7.6)	5.4 (1.1-6.4)	6.0 (5.2-7.1)
Adipsin	ng/ml	79 (3- 183)	111 (12-154)	126 (107-152)
Chemerin	ng/ml	98 (13- 256) [a,c]	220 (118-326) [c]	255 (126-452) [a]
SAA-1	µg/ml	10 (3.3- 18) [a,c]	16 (7.0-18) [b,c]	18 (18) [a,b]
RBP-4	µg/ml	81 (31- 161) [a,c]	180 (133-245) [c]	184 (154-213) [a]
Leptin	ng/ml	52 (22- 96) [a,c]	90 (22-118) [b,c]	112 (80-220) [a,b]
TIMP-1	ng/ml	202(51- 356) [a,c]	389 (261-482) [c]	389 (265-495) [a]
Cathepsin S	ng/ml	86 (52- 120) [a,c]	114 (25-160) [c]	127 (114-152) [a]
Adiponectin	µg/ml	2.7 (0.7- 8.3) [a,c]	6.4 (2.5-19) [c]	5.7 (2.5-9.6) [a]
PAI-1	µg/ml	1.8 (266- 3.6) [a,c]	3.0 (31-6.4) [b,c]	4.0 (2.7-5.8) [a,b]

Table 2. Plasma adipokine and cytokine levels are shown in HC, patients with new-onset type 1 diabetes, and patients with long-standing type 1 diabetes. Data are displayed as mean (range). IFN-γ, interferon-γ; GM-CSF, granulocyte-macrophage colony stimulating factor; CXCL10, γ interferon inducible protein 10 (IP10); CCL3, macrophage inflammatory protein 1α. [aa] ≥75% of all values below detection limit. [a] $P < 0.05$ between long-standing type 1 diabetes and HC. [b] $P < 0.05$ between onset type 1 diabetes and long-standing disease. [c] $P < 0.05$ between onset type 1 diabetes and HC.

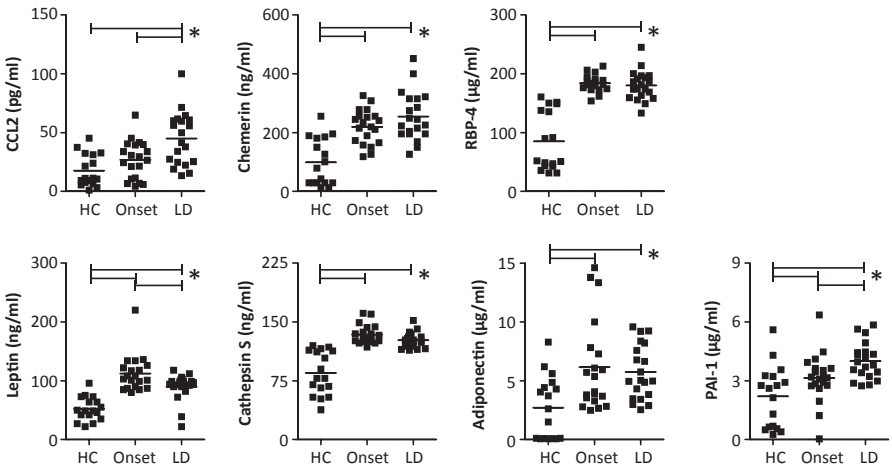

Figure 1 Adipokine and cytokine levels in plasma
Plasma levels of CCL2, chemerin, RBP-4, leptin, cathepsin S, adiponectin, and PAI-1 in HC, patients with new onset type 1 diabetes (onset), and patients with long-standing type 1 diabetes (LD) are shown. *Lines* depict mean value per group. *, $P < 0.05$.

Differences in adipokine profiles between the three groups could not be attributed to differences in age or BMI-SD (linear regression analysis, data for BMI-SD shown in Supplemental Table 1). No significant differences between the study populations were found for any of the other adipokines studied (IL-1RA, IL-1β, IL-6, IL-10, TNF-α, interferon-γ, MIF, CXCL8 (IL-8), CXCL10, gamma interferon inducible protein 10 (IP10), granulocyte-macrophage colony stimulating factor, resistin, thrombopoietin, and adipsin).

In conclusion, both onset and long-standing pediatric type 1 diabetes patients showed extensive alterations in plasma adipokine levels compared with HC. Apart from increased free fatty acid levels in type 1 diabetes patients at onset compared with long-standing disease ($P = 0.04$), these alterations were not accompanied by plasma lipid alterations (Table 1).

Diabetic plasma induces adipocyte differentiation *in vitro*

The differences in plasma adipokine levels observed between type 1 diabetes patients and HC (Fig. 1 and Table 2) might be related to plasma effects on the adipocytes in type 1 diabetes patients. To investigate this possibility, we examined the effects of diabetic plasma versus control plasma on adipocyte differentiation. Pooled diabetic plasma induced adipocyte differentiation, as assessed by Oil-Red-O staining, whereas pooled HC plasma had no effect (Fig. 2, A-C). To quantify these differences, pooled plasmas were added in 12-fold, and differentiation was assessed by Nile Red staining and quantified by automated microscopy. Onset as well as long-standing diabetic pooled plasma significantly induced the number of differentiated adipocytes per microscopic

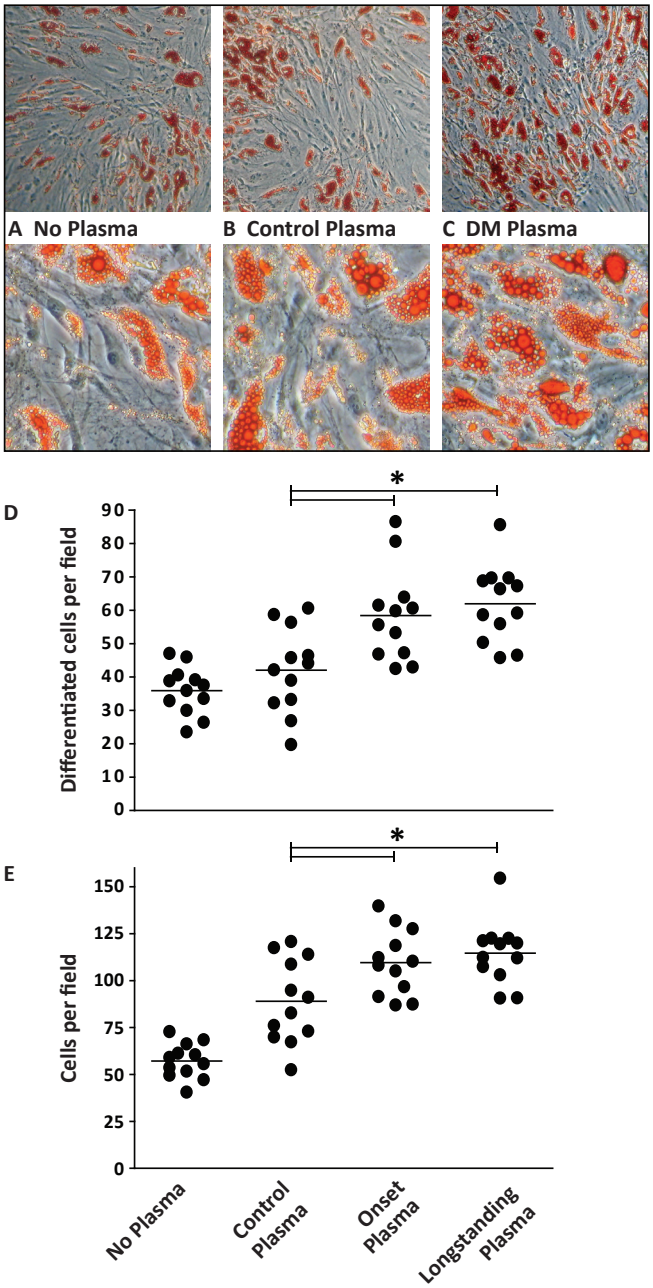

Figure 2 Diabetic plasma induces adipocyte differentiation *in vitro*
(A-C) Oil-Red-O staining of adipocyte differentiation without additional plasma (A) and with addition of pooled plasma of HC (B) or pediatric type 1 diabetes patients (C). Images were made with the 50x and 200x zoom objective, respectively. (D-E) Differentiated number of adipocytes per microscopic field for various pooled plasmas (D), together with the total number of cells per microscopic field for these plasmas (E). *, $P < 0.05$.

field within 24 h, compared with control plasma and the nonplasma control (Fig. 2D). In addition, onset and long-standing diabetic plasma significantly induced preadipocyte proliferation compared with HC plasma and the nonplasma control (Fig. 2E). Thus, plasma of type 1 diabetes patients induced adipocyte differentiation and preadipocyte proliferation, and this effect was already detectable at the onset of type 1 diabetes.

Plasma-induced adipokine secretion by adipocytes

To test whether the differences in plasma adipokine levels between diabetic patients and controls could be attributed to plasma effects on adipocytes, we performed multiplex adipokine analysis on plasma-conditioned adipocytes. The levels of adiponectin, CCL2, and TNF-α secreted by adipocytes upon incubation with diabetic plasma were significantly higher compared with control plasma treatment, reflecting the differences in adiponectin and CCL2 levels observed in plasma (Fig. 3 and Supplemental Table 2). The trend for higher RBP-4 release of long-standing diabetic plasma-conditioned adipocytes and higher cathepsin S release of onset diabetic plasma-conditioned adipocytes also reflected the differences in plasma RBP-4 and cathepsin S levels. Yet MIF levels were higher in control plasma-conditioned adipocytes compared with

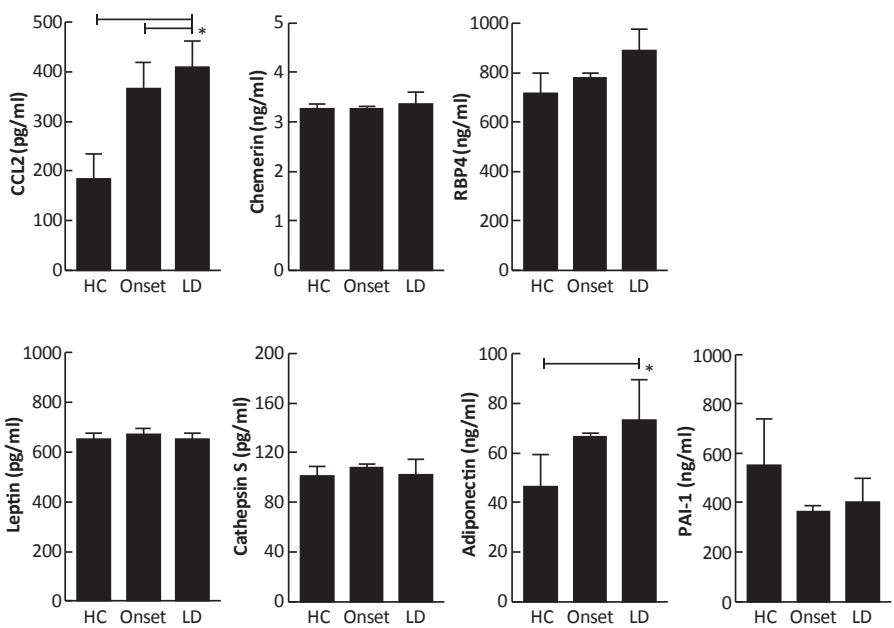

Figure 3 Adipokine and cytokine levels in adipocyte culture supernatants
Levels of CCL2, chemerin, RBP-4, leptin, cathepsin S, adiponectin, and PAI-1 in supernatants of adipocyte cultures after incubation with pooled plasma of HC, patients with new onset type 1 diabetes (onset), and patients with long-standing type 1 diabetes (LD). Data represent mean values ± SD. *, $P < 0.05$.

onset diabetic plasma, and no differences in the adipocyte release of leptin, chemerin, SAA-1, and PAI-1 were observed, whereas these adipokines did show higher levels in diabetic compared with control plasma (Fig. 3 and Supplemental Table 2).

Taken together, our findings indicate that diabetic plasma can induce alterations in adipocyte function (proliferation, differentiation, adipokine profile), which may contribute to the proinflammatory condition in type 1 diabetes.

Glucose and free fatty acids alter adipokine secretion *in vitro*

Both the glucose dysregulation and dyslipidemia associated with type 1 diabetes may affect adipose tissue function. Therefore, we studied whether the effects of diabetic plasma on adipocyte differentiation and adipokine production could be reproduced by incubation with glucose or lipids. For lipids, we focused on free fatty acids because this was the only lipid class that was significantly induced in the (onset) patient group (Table 1). Similar to the plasma incubations described above, adipocytes were incubated for 24 h with various glucose doses (1, 4.5, or 20 g/liter), the unsaturated fatty acid oleic acid (250 μM), the saturated fatty acid palmitic acid (250 μM), or the fatty acid vehicle. Next, cell proliferation, adipocyte differentiation, and adipokine secretion were assessed (Fig. 4). We selected seven adipokines that were found altered in diabetic plasma compared with controls (adiponectin, CCL2, RBP-4, cathepsin S, chemerin, leptin, and PAI-1). In contrast to type 1 diabetic plasma (Fig. 2), glucose or free fatty acid treatment did not affect cell proliferation or adipocyte differentiation (Fig. 4). High glucose levels and palmitic acid did, however, increase CCL2 secretion by adipocytes (Fig. 4). In addition, high glucose levels specifically reduced RBP-4 secretion. These results indicate that type 1 diabetic plasma can alter adipokine secretion by adipocytes, either directly (*e.g.* glucose, palmitic acid) or indirectly through its effects on adipogenesis (plasma).

DISCUSSION

Adipose tissue-secreted factors in type 1 diabetes have emerged as a promising new field of research and may comprise potential targets for intervention (14). Here, we report altered plasma adipokine profiles in children with onset and long-standing type 1 diabetes versus HC, making use of a recently developed and validated multiplex immunoassay for 24 adipokines (16). To the best of our knowledge, this is the first study exploring such a wide range of adipokines in pediatric type 1 diabetes. Our *in vitro* studies indicate that plasma factors in pediatric type 1 diabetes can affect adipokine profiles, either directly (*e.g.* glucose, palmitic acid) or indirectly through their effects on differentiation (plasma), providing a possible explanation for the altered adipokine profiles observed *in vivo*.

We identified three novel adipokines that are altered in children with type 1 diabetes. First, whereas cathepsin S has recently been implicated in immune regulation in mouse models for type 1 diabetes (22) and plays a key role in various adipose tissue-mediated inflammatory and atherogenic processes (23, 24), this is the

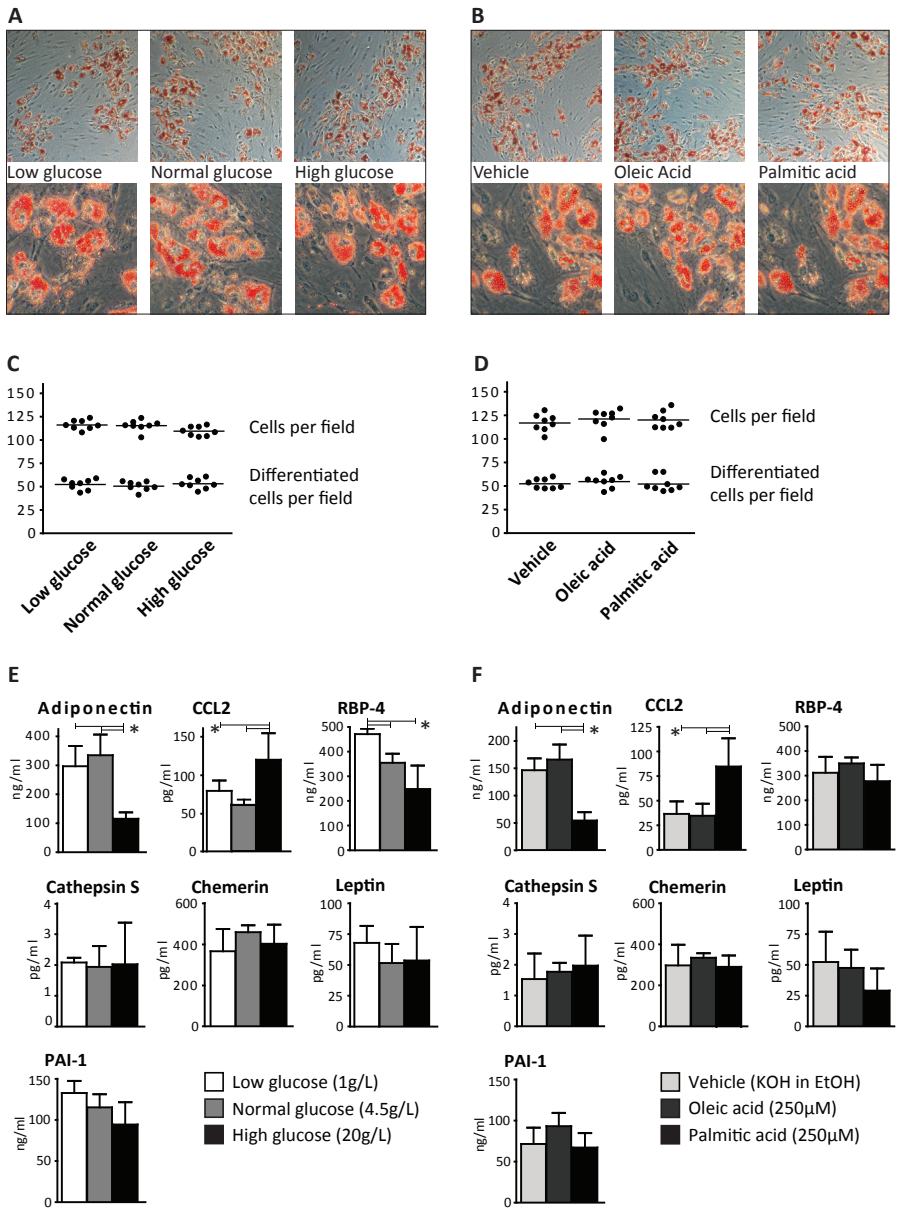

Figure 4 Glucose and the saturated fatty acid palmitic acid alter adipokine secretion *in vitro*
(A) Oil-Red-O staining of adipocytes incubated for 24 h with low glucose (1 g/liter), normal glucose (4.5 g/liter), or high glucose doses (20 g/liter). Images were made with a 50x and 200x zoom objective, respectively. (B) Oil-Red-O staining of adipocytes incubated with vehicle (KOH in 100% ethanol), unsaturated oleic acid (250 µM), or saturated palmitic acid (250 µM). (C-D) Quantification of the cell proliferation and adipocyte differentiation upon 24-h incubation with the various glucose doses and fatty acids. (E-F) Measurement of adipokines in the supernatants of the adipocytes after 24-h incubation with the various glucose concentrations and fatty acids. Data represent mean values ± SD. *, $P < 0.05$.

first time that cathepsin S levels in type 1 diabetes patients have been determined and found to be increased. Chemerin, a second adipokine at the crossroads of adipose tissue and inflammation (25) that has not been analyzed in type 1 diabetes patients so far, was significantly increased in both onset and long-standing type 1 diabetes children compared with HC. Third, elevated levels of TIMP-1 have been reported in adults with type 1 diabetes before (26, 27) but are now also observed in children with type 1 diabetes. TIMP-1 is produced by adipocytes (16), its levels are elevated in obesity (27), and it has been considered to be part of an antiinflammatory pathway counteracting β-cell death (28). As for the inflammatory proteins CCL2, MIF, and PAI-1, which are known to be elevated in adult type 1 diabetes patients (11, 13, 29, 30), this study now also found elevated levels in pediatric type 1 diabetes patients. Furthermore, we confirmed elevated adiponectin levels in pediatric type 1 diabetes patients. In contrast, RBP-4 and SAA-1 levels were found to be higher in children with type 1 diabetes than in HC, whereas in adult type 1 diabetes patients unaltered or even lowered levels have been reported (31, 32).

Although the metabolic disturbances in patients with onset type 1 diabetes may be expected to result in an extra derailment of adipokine levels, we found children with onset and long-standing type 1 diabetes to display comparable adipokine profiles. We considered various explanations. First, the adipokine profiles may be comparable because they are more severely affected in long-standing type 1 diabetes patients than expected. This may be explained by the accelerator hypothesis, which assumes a continuum between β-cell function and insulin resistance (33). Accordingly, the manifest β-cell dysfunction in onset patients, followed by insulin treatment, may lead to weight gain (34), insulin resistance, and further derailment of the adipokine levels in long-standing patients. We performed correlation studies of BMI and HbA1c with adipokines (Supplemental Fig. 1, A and B), and also of BMI with HbA1c (Supplemental Fig. 1C). The positive correlation between BMI-SD and HbA1c in the long-standing patient group ($P = 0.03$) suggests that weight gain may indeed worsen the phenotype in long-standing patients. However, due to the relatively small group size, no definite conclusions can be drawn on this point. A second explanation may be the variation between onset patients. Although we strictly followed the ISPAD criteria for onset patients (17), the variation in days after diagnosis (0-12 d) may have dampened the effects of the metabolic disturbances seen directly after diagnosis. Furthermore, whereas blood withdrawal was nonfasting and at random times during the day in all patient and control groups, standardized morning and fasting blood withdrawal might have reduced the variation and would have helped to study the effects of individual variables on the adipokine levels. Finally, the lack of data on residual β-cell function together with the retrospective and cross-sectional study design prevents drawing conclusions on the role of weight gain and insulin resistance in accelerating the onset of type 1 diabetes.

Nevertheless, the multitude of altered adipokines in children with type 1 diabetes strongly suggests adipose tissue involvement in the low-grade systemic inflammation observed in type 1 diabetes, and our *in vitro* experiments with diabetic plasma provide further support for the involvement of adipose tissue. Glucose and free fatty acids are

candidate factors to explain the marked adipogenic effects of type 1 diabetic plasma. Gogitidze *et al* (30) recently reported glucose dysregulation effects on adipokine secretion, and lipids have long been known for their adipogenic properties (10, 35). In our model system, neither glucose nor the saturated fatty acid palmitic acid affected proliferation or differentiation, indicating that (combinations of) other plasma factors are required for these effects.

Although the effects of glucose and palmitic acid on the CCL2 secretion by adipocytes suggest that these diabetic plasma factors can directly contribute to the adipokine secretion by adipocytes, the decreased adiponectin release upon glucose and palmitic acid incubation once more indicates that (combinations of) other plasma factors are required to explain the *in vivo* adipokine levels and the *in vitro* adipogenic effects of diabetic plasma. Unfortunately, limited amounts of plasma available (*i.e.* ethical restrictions with respect to blood withdrawal in children) precluded fractionation experiments to identify the adipogenic plasma factors. Therefore, further experiments in adult type 1 diabetes patients are needed to identify the diabetic plasma factors specifically responsible for the observed effects on adipogenesis and adipokine profiles.

The adipogenic effects of diabetic plasma raise three interesting hypotheses. First, enhanced preadipocyte proliferation may partly account for the increased levels of circulating inflammatory cytokines in type 1 diabetes. Preadipocytes are known as potent producers of CCL2, and to a lesser extent TNF-α (16, 36). In accordance with the proliferative effect of diabetic plasma on preadipocytes, we observed enhanced TNF-α and CCL2 release by the (pre)adipocytes (Fig. 2 and Supplemental Table 2) and increased plasma CCL2 levels in the diabetic children (Fig. 1 and Table 2). Second, enhanced adipocyte differentiation may also contribute to the altered adipokine profiles in diabetic patients. Both adiponectin and RBP-4 are, for example, produced by differentiated adipocytes (16, 36). In accordance with the diabetic plasma-induced adipocyte differentiation, we observed enhanced adiponectin and RBP-4 release by the (pre)adipocytes (Fig. 3 and Supplemental Table 2), and increased plasma RBP-4 and adiponectin levels in the diabetic children (Table 2). Concurrently, it is important to note that adipocyte differentiation models do not suffice to study the diabetic plasma-induced secretion of, for example, chemerin, SAA-1, and PAI-1. *In vivo*, these adipokines are mainly secreted by adipose tissue stromal vascular cells (36-38). The heterogeneity of the stromal vascular fraction, *i.e.* varying compilations of stem cells, preadipocytes, leukocytes, and endothelial cells, prevented us from studying the effects of diabetic plasma on this fraction. Third, it is interesting to consider a general implication of increased adipogenesis for diabetic children. Childhood type 1 diabetes is associated with a significant increase in BMI within 3-6 months (34). Adipocyte number is known to be a major determinant for fat cell mass, BMI, and supposedly insulin resistance at a later age (39, 40). Increased adipogenesis in diabetic children may thus contribute to a higher fat cell mass and insulin resistance later in life.

In conclusion, our data fuel the hypothesis that the assumed continuum between type 1 and type 2 diabetes is partly explained by the adipose tissue involvement in both diseases. As recently emphasized (40), studying adipose tissue involvement may be key to developing novel targets for prevention and treatment.

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SUPPLEMENTAL INFORMATION

Altered plasma adipokine levels and *in vitro* adipocyte differentiation in pediatric type 1 diabetes

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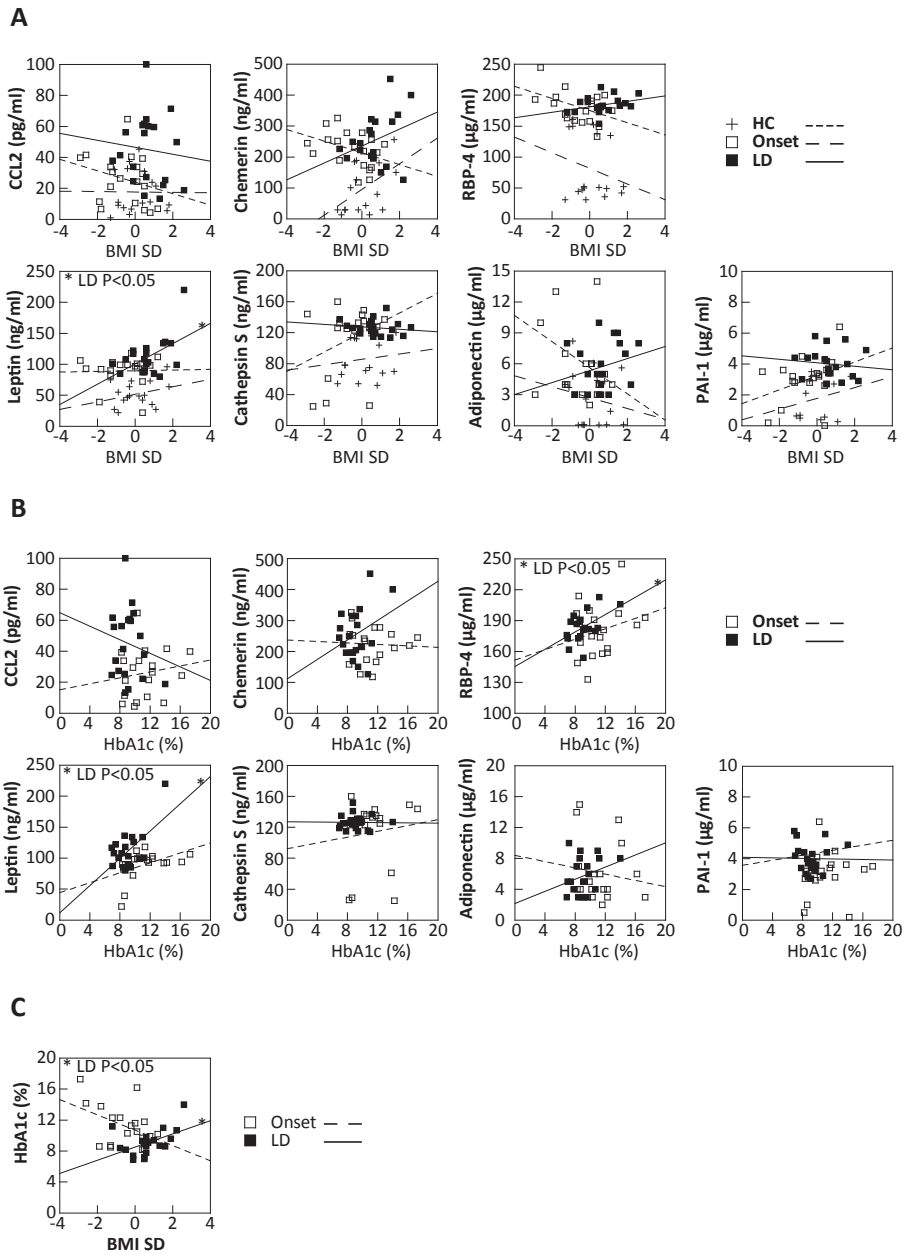

Supplemental Figure 1 Correlation of BMI and HbA1c with plasma adipokine levels
(A-B) Plasma levels of CCL2, chemerin, RBP-4, leptin, cathepsin S, adiponectin and PAI-1 in healthy controls (HC), patients with new onset type 1 diabetes (onset) and patients with longstanding type 1 diabetes (LD) are shown in relation to BMI-SD and HbA1c. (C) Correlation between BMI-SD and HbA1c for all type 1 diabetes patients (both onset and long standing type 1 diabetes). *, $P < 0.05$.

Supplemental Table 1 Univariate logistic regression analysis for Hba1c and BMI

	Hba1c			BMI		
	Onset	LD	HC	Onset	LD	HC
IL-1RA	-0.19 / 0.43	-0.11 / 0.69	NA	0.30 / 0.21	0.16 / 0.51	-0.20 / 0.10
IL-1β	0.12 / 0.63	-0.04 / 0.72	NA	-0.03 / 0.91	0.18 / 0.46	0.21 / 0.42
IL-6	-0.21 / 0.39	-0.05 / 0.76	NA	0.30 / 0.21	0.13 / 0.57	0.21 / 0.42
IL-10	0.00 / 0.99	-0.06 / 0.68	NA	0.11 / 0.65	0.15 / 0.51	0.24 / 0.36
TNF-α	0.27 / 0.26	-0.02 / 0.83	NA	-0.22 / 0.35	0.15 / 0.53	0.24 / 0.35
IFN-γ	-0.14 / 0.56	-0.03 / 0.82	NA	0.33 / 0.15	0.15 / 0.52	0.07 / 0.79
MIF	-0.49 / 0.03	0.34 / 0.60	NA	0.15 / 0.76	-0.33 / 0.09	0.09 / 0.46
CCL2	0.16 / 0.52	0.03 / 0.49	NA	-0.33 / 0.15	-0.10 / 0.68	-0.01 / 0.97
CCL3	NA	0.01 / 0.95	NA	NA	0.08 / 0.74	0.16 / 0.55
CXCL8 (IL-8)	-0.28 / 0.24	0.02 / 0.85	NA	0.27 / 0.26	0.12 / 0.61	-0.05 / 0.86
CXCL10	-0.14 / 0.58	0.14 / 0.42	NA	0.12 / 0.62	0.30 / 0.20	0.37 / 0.14
GM-CSF	-0.48 / 0.04	-0.07 / 0.79	NA	0.07 / 0.76	-0.38 / 0.09	-0.19 / 0.47
OPN	-0.27 / 0.27	0.18 / 0.74	NA	0.35 / 0.54	0.02 / 0.15	0.08 / 0.72
Resistin	-0.41 / 0.08	-0.23 / 0.63	NA	0.27 / 0.26	-0.08 / 0.73	0.03 / 0.92
Thrombopoietin	0.32 / 0.18	0.03 / 0.10	NA	0.05 / 0.84	0.20 / 0.40	0.09 / 0.73
Adipsin	0.32 / 0.18	-0.04 / 0.30	NA	-0.01 / 0.98	0.10 / 0.68	0.13 / 0.62
Chemerin	-0.05 / 0.83	0.06 / 0.17	NA	-0.27 / 0.25	0.32 / 0.17	0.49 / 0.05
SAA-1	0.41 / 0.08	NA	NA	-0.19 / 0.42	NA	0.09 / 0.74
RBP-4	0.26 / 0.28	0.38 / 0.02	NA	-0.41 / 0.07	0.08 / 0.73	-0.23 / 0.38
Leptin	0.45 / 0.05	0.11 / 0.01	NA	0.00 / 0.99	0.51 / 0.02	0.29 / 0.26
TIMP-1	-0.18 / 0.46	0.28 / 0.04	NA	-0.22 / 0.35	0.41 / 0.07	-0.17 / 0.53
Cathepsin	0.11 / 0.64	-0.03 / 0.95	NA	0.32 / 0.17	-0.16 / 0.51	1.00 / 0.62
Adiponectin	-0.15 / 0.55	0.25 / 0.12	NA	-0.34 / 0.14	0.29 / 0.22	-0.19 / 0.48
PAI-1	0.15 / 0.84	-0.23 / 0.98	NA	0.30 / 0.57	-0.11 / 0.63	0.24 / 0.44

Supplemental Table 1. Depicted are Pearson correlation coefficients / *P* values per adipokine. Onset, onset type 1 diabetes; LD, long-standing type 1 diabetes; HC, healthy control; NA, not available or cannot be determined.

Supplemental Table 2 Adipokine and cytokine levels in adipocyte culture supernatants.

	Units	Healthy controls	Onset	Long-standing disease
IL-1RA	pg/ml	23 (21-25)	23 (21-25)	22 (21-25)
IL-1β	pg/ml	4.6 (3.6-6.6)	4.0 (3.9-4.1)	3.8 (3.3-4.1)
IL-6	pg/ml	55 (35-77)	67 (64-73)	70 (41-92)
IL-10	pg/ml	20 (19-20)	21 (19-22)	19 (16-21)
TNF-α	pg/ml	5.3 (4.5-5.7) [a]	6.2 (6.0-6.5) [a]	5.6 (4.5-6.0)
MIF	pg/ml	1192 (1052-1497) [a]	876 (734-1029) [a]	920 (826-1125)
CCL2	pg/ml	185 (137-251) [a,b]	367 (304-426) [a]	410 (385-412) [b]
CCL3	pg/ml	620 (348-793)	608 (542-668)	557 (493-655)
CXCL8 (IL-8)	pg/ml	1034 (737-1289)	866 (799-928)	785 (689-958)
CXCL10	pg/ml	18 (16-20)	16 (14-17)	15 (12-17)
GM-CSF	pg/ml	33 (30-35)	32 (31-32)	31 (29-34)
OPN	pg/ml	545 (507-570)	567 (556-579)	540 (485-592)
Resistin	pg/ml	1192 (1052-1497)	876 (734-1029)	920 (826-1125)
Thrombopoietin	ng/ml	7.3 (6.9-7.4)	7.3 (7.2-7.3)	7.1 (6.6-7.6)
Adipsin	ng/ml	331 (306-352)	342 (331-352)	338 (304-360)
Chemerin	ng/ml	3.2 (3.1-3.4)	3.3 (3.2-3.3)	3.4 (3.2-3.7)
SAA-1	ng/ml	31 (28-34)	32 (31-33)	31 (27-35)
RBP-4	ng/ml	718 (634-823)	778 (745-796)	881 (788-963)
Leptin	ng/ml	0.7 (0.6-0.7)	0.7 (0.7)	0.7 (0.6-0.7)
Cathepsin S	pg/ml	102 (92-107)	108 (106-111)	102 (89-114)
Adiponectin	ng/ml	46 (28-59) [a]	66 (65-68) [a]	73 (58-88)
PAI-1	ng/ml	549 (380-714)	365 (292-380)	403 (338-546)

Supplemental Table 2. Cytokine and adipokine levels in adipocyte cultures after incubation with pooled plasma of healthy controls, patients with new onset type 1 diabetes and patients with longstanding type 1 diabetes. Data are mean values (range). Not included in table: IFN-γ, all values below detection limit; TIMP-1, all values above detection limit. [a] $P < 0.05$ between onset type 1 diabetes and healthy controls. [b] $P < 0.05$ between longstanding type 1 diabetes and healthy controls.

Supplemental information

Settings for automated fluorescence microscopy (Thermo Scientific Cellomic ArrayScan; VTI HCS Reader) to quantify adipocyte differentiation using Cellomics Target Activation Bioapplication software.

Image Acquisition	
Objective	10x
Camera Name	ORCA-ER;1.00
Acquisition Camera Mode	Standard (1024x1024;2x2)
AutoFocus Camera Mode	AutoFocus (1024x1024;4x4)
AutoFocus Field Interval	0

AutoFocus Parameters	
Fine Focus Step Size	17.6
Fine Focus Plane Count	9
Coarse Focus Step Size	70.4
Coarse Focus Plane Count	9
Smart Focus Plane Count	21
Use Extended Range Focusing	False
Apply Backlash Correction	False
AutoFocus Method	STANDARD
Use Relaxed Pass/Fail Criteria	False
Focus Edge Threshhold	0
Focus Adjustment	0
Focus Score Min Ratio	0.2
Focus Score Mid Ratio	0.4
Focus Score Max Ratio	0.5
Focus Exposure Time for AutoExpose (seconds)	0.1

Scan Limits	
Max Fields for Well	20
Min Objects for Well	500
Max Sparse Fields for Well	No Limit
Min Objects for Field	N/A
Max Sparse Wells for Plate	N/A

Channel 1: Nuclei	
Dye	XF93 - Hoechst
Apply Illumination Correction	False
Apply Background Correction	True
Gain	25
Use Apotome	False
Z Offset	0.00

Exposure Parameters	
Method	Fixed
Exposure Time (seconds)	0.02074

Object Identification	
Method	FixedThreshold
Value	70

Object Selection Parameter	Min	Max
ObjectAreaCh1	100	550
ObjectShapeP2ACh1	1	10
ObjectShapeLWRCh1	1	10
ObjectAvgIntenCh1	0	4095
ObjectVarIntenCh1	0	32767
ObjectTotalIntenCh1	0	10000000000

Display Options		
Composite Color (Hex)	#0000FF	Blue
RejectedObject	#FF7F00	Orange
MaskCh2	#00FF00	Green

Channel 2: nile red	
Dye	XF93 - TRITC
Apply Illumination Correction	False
Apply Background Correction	True
Gain	25
Use Apotome	False
Z Offset	0.00

Exposure Parameters	
Method	Fixed
Exposure Time (seconds)	0.069027

Object Identification		
Method	None	
Value	0	
Object Selection Parameter	Min	Max
AvgIntenCh2	50	4095
TotalIntenCh2	9500	10000000000

Display Options		
Composite Color (Hex)	#80FF80	Light green
SelectedObject	#0000FF	Blue
RejectedObject	#FF7F00	Orange
MaskCh2	#00FF00	Green

Assay	
Assay Algorithm	TargetActivation.V3
Assay Version	6.1 (Locally Installed Version: 6.1.0.3018)
Focus Channel	1
#Channels	2

Assay Parameters	
UseReferenceWells	0
MinRefAvgObjectCountPerField	2
UseMicrometers	0
PixelSize	1.29
Type_1_EventDefinition	0.926
Type_2_EventDefinition	0
Type_3_EventDefinition	0
ObjectTypeCh1	0
BackgroundCorrectionCh1	10

Assay Parameters	
ObjectSmoothFactorCh1	5
ObjectSegmentationCh1	5.1
RejectBorderObjectsCh1	1
ObjectAreaCh1LevelHigh	150
ObjectAreaCh1LevelLow	70
ObjectAreaCh1LevelHigh_CC	1
ObjectAreaCh1LevelLow_CC	1
ObjectShapeP2ACh1LevelHigh	1.26
ObjectShapeP2ACh1LevelLow	1.1
ObjectShapeP2ACh1LevelHigh_CC	1
ObjectShapeP2ACh1LevelLow_CC	1
ObjectShapeLWRCh1LevelHigh	1.67
ObjectShapeLWRCh1LevelLow	1.19
ObjectShapeLWRCh1LevelHigh_CC	1
ObjectShapeLWRCh1LevelLow_CC	1
ObjectTotalIntenCh1LevelHigh	75000
ObjectTotalIntenCh1LevelLow	27300
ObjectTotalIntenCh1LevelHigh_CC	1
ObjectTotalIntenCh1LevelLow_CC	1
ObjectAvgIntenCh1LevelHigh	630
ObjectAvgIntenCh1LevelLow	305
ObjectAvgIntenCh1LevelHigh_CC	1
ObjectAvgIntenCh1LevelLow_CC	1
ObjectVarIntenCh1LevelHigh	330
ObjectVarIntenCh1LevelLow	55
ObjectVarIntenCh1LevelHigh_CC	1
ObjectVarIntenCh1LevelLow_CC	1
BackgroundCorrectionCh2	25
MaskModifierCh2	7
TotalIntenCh2LevelHigh	12100
TotalIntenCh2LevelLow	0
TotalIntenCh2LevelHigh_CC	1
TotalIntenCh2LevelLow_CC	1
AvgIntenCh2LevelHigh	125
AvgIntenCh2LevelLow	0
AvgIntenCh2LevelHigh_CC	1
AvgIntenCh2LevelLow_CC	1
VarIntenCh2LevelHigh	60
VarIntenCh2LevelLow	0
VarIntenCh2LevelHigh_CC	1
VarIntenCh2LevelLow_CC	1

CHAPTER 6

Systemic inflammation in childhood obesity: circulating inflammatory mediators and activated CD14^{++} monocytes

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ABSTRACT

Aims/hypothesis

In adults, circulating inflammatory mediators and activated CD14^{++} monocytes link obesity to its metabolic and cardiovascular complications. However, it is largely unknown whether these inflammatory changes already occur in childhood obesity. To survey inflammatory changes during the early stages of obesity, we performed a comprehensive analysis of circulating inflammatory mediators, monocyte populations and their function in childhood obesity.

Methods

In lean and obese children aged 6 to 16 years (n = 96), 35 circulating inflammatory mediators including adipokines were measured. Hierarchical cluster analysis of the inflammatory mediator profiles was performed to investigate associations between inflammatory mediator clusters and clinical variables. Whole-blood monocyte phenotyping and functional testing with the Toll-like receptor 4 ligand lipopolysaccharide were also executed.

Results

First, next to leptin, the circulating mediators chemerin, tissue inhibitor of metalloproteinase 1, EGF and TNF receptor 2 were identified as novel inflammatory mediators that are increased in childhood obesity. Second, cluster analysis of the circulating mediators distinguished two obesity clusters, two leanness clusters, and one mixed cluster. All clusters showed distinct inflammatory mediator profiles, together with differences in insulin sensitivity and other clinical variables. Third, childhood obesity was associated with increased CD14^{++} monocyte numbers and an activated phenotype of the CD14^{++} monocyte subsets.

Conclusions/interpretation

Inflammatory mediator clusters were associated with insulin resistance in obese and lean children. The activation of CD14^{++} monocyte subsets, which is associated with increased development of atherosclerosis in obese adults, was also readily detected in obese children. Our results indicate that inflammatory mechanisms linking obesity to its metabolic and cardiovascular complications are already activated in childhood obesity.

INTRODUCTION

Obesity induces local inflammation in adipose tissue (1, 2). As adipokines, i.e. inflammatory mediators produced by adipose tissue, are secreted into the circulation, this local obesity-induced inflammation in adipose tissue is conveyed to other sites in the body, ranging from cardiovascular tissue to circulating immune cells (3). The resulting low-grade systemic inflammation is a pivotal link between obesity, type 2 diabetes and cardiovascular disease (4, 5). In obese adults, low-grade inflammation is characterised by higher levels of inflammatory adipokines such as leptin, which is an important risk marker for the development of type 2 diabetes (6). Furthermore, monocytes in obese adults are in a pro-inflammatory state (7, 8). Monocytes can be divided into three populations, based on their levels of CD14 and CD16: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) (9-11). Interestingly, greater numbers and activation of the $CD14^{++}$ populations were associated with hyperglycaemia and increased atherosclerosis in obese adults (12-14). Taken together, circulating inflammatory mediators, such as adipokines, and activated $CD14^{++}$ monocytes seem to act as inflammatory agents linking obesity to its metabolic and cardiovascular complications.

Whereas most of the knowledge on obesity-induced inflammation comes from studies in adults, childhood obesity provides an excellent opportunity to study inflammatory changes during the early stages of obesity, without confounding lifestyle habits such as smoking and co-existing inflammatory conditions like arthritis (15). Earlier studies on small sets of circulating mediators showed greater levels of inflammatory mediators, including high-sensitivity C-reactive protein (hsCRP), leptin, IL-6 and IL-8, together with decreased levels of the insulin-sensitising adipokine adiponectin, and higher leucocyte numbers in obese children than in lean controls (15-18). These studies indicated that low-grade systemic inflammation is already present during childhood obesity. Considering the link between low-grade inflammation and both diabetes and cardiovascular disease, inflammatory changes at a young age may provide potential avenues for early detection and prevention of these complications (19, 20).

Here, we used two novel approaches to perform an in-depth analysis of inflammatory mediator profiles in childhood obesity and to study the involvement of monocyte subpopulations in low-grade systemic inflammation. First, we compared plasma inflammatory mediator profiles of obese children with those of lean controls. For this, 35 inflammatory mediators, including adipokines, were measured using a recently developed multiplex immunoassay (MIA) (21). We also conducted cluster analysis of the inflammatory mediators to see whether clinically different obese and lean control groups can be distinguished on the basis of their mediator profiles. Second, circulating monocytes in obese children and lean controls were phenotyped in detail, using a recently published flow cytometry procedure (22), and submitted to functional testing with the Toll-like receptor 4 (TLR-4) ligand lipopolysaccharide (LPS). Taken together, this is the first comprehensive study of systemic inflammation in childhood obesity to address circulating inflammatory mediator profiles and monocyte activation status.

METHODS

Participants

This cross-sectional study included 96 children aged 6 to 16 years, all patients of the Paediatric Outpatient Department of the Meander Medical Center (Amersfoort, the Netherlands). The BMI and BMI SD for age and sex (BMI-SD) were calculated for all children, based on results of the Fifth Dutch Growth Study (2008-2010) (23). Childhood obesity was defined as BMI-SD > 2.5, which roughly corresponds to the international definition of obesity as BMI > 30 kg/m^2 projected to 18 years of age (23, 24). The population of 96 children consisted of 30 healthy lean children (BMI-SD -2.0 to 2.0), 60 age- and sex-matched obese children (BMI-SD > 2.5) (Table 1), and six other children who either had a BMI-SD between 2 and 2.5, or could not be age- and sex-matched (Supplemental Table 1). Most of the analyses were performed with data from all 96 children. To compare lean and obese children, only the age and sex-matched groups (30 lean children versus 60 obese children) were used.

Table 1 Clinical characteristics

	Lean controls (n=30)		Obese children (n=60)	
	Males	**Females**	**Males**	**Females**
Number	14	16	27	33
Age (years)	11.3 ± 2.8	11.5 ± 3.5	12.1 ± 2.6	12.4 ± 2.7
BMI-SD [a]	0.0 ± 1.1	0.6 ± 0.9	3.7 ± 0.6 $^{+}$	3.3 ± 0.4 $^{++}$
Waist circumference (cm)	62 ± 8	64 ± 9	90 ± 12 $^{+}$	86 ± 11 $^{++}$
Total body fat (%)	25.3 ± 7.1	30.0 ± 4.5	39.2 ± 5.1 $^{+}$	40.7 ± 3.0 $^{++}$
QUICKI	0.44 (0.41-0.44)	0.37 (0.34-0.45)	0.33 (0.30-0.34) $^{+}$	0.32 (0.31-0.33) $^{++}$
Systolic blood pressure (%) [b]	48.5 ± 29.3	61.3 ± 31.4	79.7 ± 19.7 $^{+}$	77.6 ± 24.6
Diastolic blood pressure (%) [b]	38.8 ± 21.4	46.4 ± 27.0	48.4 ± 28.7	52.6 ± 27.7
Total cholesterol (mmol/l)	3.9 ± 0.9	4.3 ± 0.7	4.2 ± 0.9	4.2 ± 0.7
HDL (mmol/l)	1.4 ± 0.3	1.7 ± 0.9	1.2 ± 0.3 *	1.2 ± 0.2 **
LDL (mmol/l)	2.2 ± 0.7	2.4 ± 0.6	2.5 ± 0.8	2.5 ± 0.7
Triacylglycerol (mmol/l)	0.6 (0.4-0.7)	0.8 (0.5-1.1)	1.0 (0.6-1.4) *	0.9 (0.7-1.3)
AST (U/l)	28 (25-37)	25 (20-31)	28 (23-32)	23 (19-28)
ALT (U/l)	19 (17-22)	14 (12-17)	24 (19-38) *	18 (14-30)
hsCRP (mg/l)	0.15 (0.15-0.48)	0.33 (0.16-0.73)	1.70 (0.78-3.20) $^{+}$	2.05 (0.88-3.8) $^{++}$

Table 1. Normally distributed data are shown as mean ± SD. Non-parametric data are shown as median (interquartile range). For clinical characteristics of the six children who either had a BMI-SD between 2 and 2.5, or could not be age- and sex-matched, see Supplemental Table 1. [a] Standard deviation scores, normalized for age and sex. [b] Percentiles, normalized for age, height and sex. * $P < 0.05$ (male lean versus male obese). ** $P < 0.05$ (female lean versus female obese). $^{+}P < 0.001$ (male lean versus male obese). $^{++}$ $P < 0.001$ (female lean versus female obese). AST, aspartate-aminotransferase.

Exclusion criteria for all participants were: acute or chronic inflammatory and/or infectious conditions (e.g. asthma, Morbus Pfeiffer), endocrine disorders (e.g. hypothyroidism), growth disorders (e.g. growth hormone deficiency) and intoxication (smoking, drug use). Written informed consent was obtained from all children and/or their parents. The study was approved by the Institutional Medical Ethical Review Board (METC 09/217 K).

Clinical variables

For bioelectrical impedance measurements (total body fat %), a foot-hand bio-impedance analyser was used in accordance with the manufacturer's instructions (Model BIA 101; Akern, Florence, Italy) (25). Blood pressure was measured in participants while seated and after a 5 min rest, using an automated oscillometric method (Dinamap; GE Healthcare, Amersham, UK) with an appropriately sized cuff. The lowest reading of three measurements was used. Blood pressure was normalised for age, height and sex, and expressed as a percentile. Waist circumference was measured to the nearest 1 mm in light expiration at the midpoint between the lowest rib and the iliac crest, in accordance with previous studies (26).

Blood samples

Routine laboratory testing included: fasting glucose, fasting insulin, HbA1c, hsCRP, lipids and liver enzymes. For the other analyses, blood samples were taken in sodium-heparin tubes between 08:00 and 10:00 hours following an overnight fast. For inflammatory mediator profiling (n = 96), heparinised blood samples were centrifuged at 150 g for 10 min, after which plasma was aliquotted and stored at -80°C until analysis. For monocyte phenotyping (n = 83, 30 lean, 49 age- and sex-matched obese children, four extra children) and functional studies (n = 66, 25 lean, 41 age- and sex-matched obese children), whole-blood staining for flow cytometry and whole-blood stimulation with the TLR-4 ligand LPS were performed immediately.

Inflammatory mediator profiling

Plasma sample preparation was performed exactly as described previously (21, 27). Inflammatory mediator levels were measured with an MIA as described recently (21). The MIA shows a high sensitivity compared with regular ELISAs, although we cannot rule out the possibility that ELISAs may incidentally show a better sensitivity (21). Plasma samples were measured undiluted for most of the mediators. For the measurement of mediators naturally occurring in very high concentrations, 100 or 1000 times dilutions were used. Adipsin, cathepsin S, chemerin, leptin, plasminogen activator inhibitor-1 (PAI-1), retinol binding protein 4 (RBP-4), resistin, serum amyloid A1 (SAA-1), tissue inhibitor of metalloproteinase 1 (TIMP-1), thrombopoietin, soluble intercellular adhesion molecule (sICAM) and soluble vascular cell adhesion molecule (sVCAM) were measured in a 100 times dilution, and adiponectin and soluble CD14 (sCD14) in a 1000 times dilution.

Monocyte phenotyping

The monocyte phenotyping protocol was adapted from Heimbeck *et al* (22) and performed in the dark at 4°C. In short, 100 µl whole blood was incubated for 20 min with titrated antibody mixes of: CD14 pacific blue (Biolegend, San Diego, CA, USA), HLA-DR PE-Cy7 (Biolegend), CD16 APC (Caltag, Buckingham, UK) and CD3 Percp-Cy5.5 (Biolegend), together with either CD62L FITC (Biolegend) and CD11b PE (BD Biosciences, Heidelberg, Germany), or chemokine (C-C motif) receptor 5 (CCR5) FITC (BD Biosciences) and CCR2 PE (RnD, Minneapolis, MN, USA), or chemokine (C-X3-C motif) receptor 1 (CX3CR1) FITC and CXCR2 PE (both from Biolegend); alternatively, isotype control antibodies were added. After the staining, samples were analysed with a flow cytometer (FACS Canto II; BD Biosciences). Monocytes were gated on the basis of their forward or sideward scatter properties and CD14/CD16 staining, after exclusion of CD3-positive and HLA-DR-negative cells. Monocyte surface marker levels were measured and quantified as the median fluorescence intensity for all surface markers.

Monocyte functional studies

For monocyte functional studies, we tested the TLR-4 response of obese children and lean control monocytes, using a protocol adapted from Scholtes *et al* (28). In short, 0.5 ml whole blood was stimulated for 4 h with 100 ng/ml of the Escherichia coli-derived TLR-4 ligand LPS (Sigma-Aldrich, St Louis, MO, USA). Subsequently, blood samples were centrifuged at 150 *g* for 10 min, after which plasma was aliquotted and stored at -80°C until analysis. IL-6 and TNF-α levels in the plasma were measured with the MIA described above.

Statistical analyses

Differences between obese children and controls were studied in the matched obese and control groups, and calculated with an independent-sample Student's *t* test for normally distributed data or with a Mann-Whitney *U* test for non-parametric comparisons. Correlations between two variables were assessed as a Pearson correlation coefficient for normally distributed data and as a Spearman correlation coefficient for non-parametric comparisons.

For regression analysis of the inflammatory mediators with BMI-SD, the age- and sex-dependence of the inflammatory mediators was determined by comparing the levels of inflammatory mediators between boys and girls, and performing correlation analysis of the inflammatory mediators with age. Next, simple correlation analysis with BMI-SD (Pearson, Spearman) was performed for inflammatory variables not depending on age or sex. For inflammatory variables depending on age and/or sex, partial correlations with BMI-SD were calculated, while controlling for age and sex.

For inflammatory mediator profiling (Table 2) and monocyte phenotyping (Supplemental Table 2), the *P* values were corrected for multiple testing using Benjamini and Hochberg's false discovery rate correction for multiple testing (29).

Non-supervised hierarchical cluster analysis was performed for the whole study population and for all inflammatory mediators, using a method adapted from Van den Ham *et al* (30). As quality control testing revealed that participants and mediators with > 10% missing values clustered together and interfered with the analysis, these participants (n = 8, seven obese children, one other child) and mediators (IFN-γ, IL-6, TNF-α, RBP-4, SAA-1, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 1α (MIP-1α), extracellular newly identified RAGE binding protein (EN-RAGE), chemokine (C-X-C motif) ligand 8 (CXCL8)) were excluded from the cluster analysis. Where values were missing in < 10% of participants, measurement values below the detection limit were set to the lowest detection limit. The data were log-transformed and normalised per mediator to have a mean of 0 (i.e., $y_1 = x_1 - \frac{1}{n}\sum_{i=0}^{n} x_i$ and a range of -1 to 1 (i.e., $z_1 = \frac{y_i}{\max_{1 \le i \le n \mid y_i \mid}}$).

Subsequently, the normalised data were hierarchically clustered using Ward's (minimum variance) linkage and 1-ρ as distance measure, where ρ stands for pairwise complete Pearson correlation. To assess the robustness of mediator and patient clusters, bootstrapping (n = 10 000) was applied and the consensus clusters used for further analyses. Heatmaps were created by combining the mediator and study population clustering. To compare the distribution of clinical variables for obesity (BMI-SD, waist circumference, body fat percentage) and insulin sensitivity (QUICKI) between two different clusters, a Mann-Whitney *U* test was used.

Statistical analyses were performed with SPSS 15.0 for Windows (SPSS, Chicago, IL, USA) and R, a free software environment for statistical computing and graphics (31). Bootstrapping was performed using the Pvclust package (32).

RESULTS

Circulating inflammatory mediators

Obese children and lean controls had characteristic differences in clinical variables (Table 1). First, together with age- and sex-corrected BMI-SD, waist circumference and total body fat percentage were increased in the obese children. Second, obese boys and girls had lower insulin sensitivity than lean controls (P < 0.001), as determined by QUICKI, a well established surrogate measure for insulin sensitivity in adults and children (33, 34). Third, obese boys had higher systolic blood pressure corrected for age and height (P < 0.001), higher triacylglycerol levels (P = 0.018) and higher alanine-aminotransferase (ALT) levels (P = 0.020). Finally, obese boys and girls had lower HDL-cholesterol levels (P = 0.030 and P = 0.004, respectively), and higher hsCRP levels (P < 0.001) than lean controls (Table 1).

Profiling of circulating inflammatory mediators, including adipokines, also showed characteristic differences between obese children and lean controls. As expected (35), leptin levels were more than twofold higher in obese children than in lean controls (Table 2). Furthermore, after correction for multiple testing, several novel childhood obesity-associated inflammatory mediators were identified. Plasma levels of IL-18,

Table 2 Circulating inflammatory mediators

		Mean / median		Regression (BMI-SD)	
Mediator	**Units**	**Lean**	**Obese**	**Age / sex correction**	**Correlation coefficient**
Cytokines					
IFN-γ [d]	pg/ml	0.95 (0.95-1.96)	0.95 (0.95-0.95)	None	-0.112
IL-6 [d]	pg/ml	0.13 (0.13-62.1)	0.13 (0.13-52.2)	None	-0.032
IL-10	pg/ml	11.9 (9.6-19.7)	11.4 (9.3-15.8)	Age	0.012
IL-18	pg/ml	313 (235-375)	373 (313-476) *	None	0.217
TNF-α [c]	pg/ml	2.3 (1.1-4.2)	1.3 (0.01-2.6)	None	-0.305 *
Adipokines					
Adiponectin [b]	µg/ml	33.5 ± 10.8	29.5 ± 10.0	Age	-0.134
Adipsin [a]	ng/ml	615 (595-643)	605 (575-624)	None	-0.179
Cathepsin S	ng/ml	56.4 ± 11.7	60.0 ± 11.2	None	0.240 *
Chemerin	µg/ml	2.8 ± 0.4	3.0 ± 0.5 *	None	0.273 *
FABP-4 [a]	ng/ml	23.6 (20.5-27.9)	24.0 (21.5-27.0)	None	-0.060
HGF	pg/ml	282 (223-339)	365 (260-523) *	None	0.154
Leptin	ng/ml	123 (111-159)	298 (210-452) [+]	Sex	0.627 [+]
Omentin	pg/ml	3.8 (3.3-4.4)	4.0 (3.5-4.5)	None	0.032
PAI-1 [a]	µg/ml	168 ± 45.3	151 ± 41.0	None	-0.181
RBP-4 [c]	µg/ml	153 (139-180)	156 (143-168)	Age	-0.048
Resistin [a]	ng/ml	919 (901-963)	925 (890-985)	None	0.037
SAA-1 [d]	µg/ml	68.4 (35.4-100)	40.0 (24.7-100)	None	-0.212
TIMP-1	ng/ml	23.0 (7.6-26.2)	25.6 (21.7-30.3) *	None	0.238 *
Trombopoietin	µg/ml	12.3 (12.0-12.9)	12.3 (11.7-12.8)	None	-0.178
Monocytes					
GM-CSF [d]	pg/ml	0.12 (0.12-2.7)	0.12 (0.12-0.12)	None	-0.143
M-CSF [a]	pg/ml	56.0 (44.5-72.2)	50.1 (35.9-69.2)	None	-0.212
MIF [b]	pg/ml	877 (311-1262)	1083 (591-1696)	None	0.119
MCP-1/CCL2 [a]	pg/ml	408 ± 144	380 ± 185	None	-0.150
MIP-1α/CCL3 [c]	pg/ml	49.4 (20.0-76.3)	40.5 (12.9-84.2)	None	-0.025
MIP-1β	pg/ml	77.9 (63.3-95.8)	95.5 (67.2-144)	None	0.128
sCD14 [b]	µg/ml	7.4 (6.0-9.6)	7.7 (6.5-9.9)	None	0.209
Chemotaxis/other					
EGF [a]	pg/ml	57.2 ± 38.6	90.5 ± 61.1 *	None	0.228 *
EN-RAGE [c]	pg/ml	35.5 (2.28-64.8)	6.6 (0.12-48.5)	None	-0.160
CXCL8/IL-8 [c]	pg/ml	44.5 (23.5-69.7)	64.9 (34.4-211) *	Age	0.122
IP-10	pg/ml	291 (199-388)	395 (291-471) *	None	0.206

		Mean / median		Regression (BMI-SD)	
Mediator	Units	Lean	Obese	Age / sex correction	Correlation coefficient
TNF-R1 [a]	ng/ml	2.6 ± 0.7	2.7 ± 0.8	None	0.042
TNF-R2	ng/ml	2.6 ± 0.6	3.0 ± 0.6 *	None	0.273 *
sICAM	µg/ml	3.0 (2.8-3.2)	3.1 (2.9-3.3)	None	0.010
sVCAM	µg/ml	5.1 ± 0.5	5.2 ± 0.5	Age	0.072
VEGF [b]	pg/ml	113 (57.6-159)	156 (87.0-238)	None	0.104

Table 2. Circulating inflammatory mediator levels for age- and sex-matched lean (*n*=30) and obese (*n*=60) children. For clarity, mediators are classified according to their origin and function: classical cytokines, adipokines, mediators affecting monocyte function, and mediators involved in chemotaxis / other. Normally distributed data are shown as mean ± SD. Non-parametric data are shown as median (interquartile range). For regression analysis with BMI-SD, inflammatory mediators of the 6 children who either had a BMI-SD between 2 and 2.5, or could not be age- and sex-matched were also included (total of 96 children). Mediators for which values were missing, mostly due to undetectable levels, are indicated as follows: [a] 1-3% missing values, [b] 3-10% missing values, [c] 10-30% missing values, [d] >30% missing values. * $P < 0.05$. + $P < 0.001$. CCL, chemokine (C-C motif) ligand; FABP-4, fatty acid binding protein 4; MCP-1, monocyte chemoattractant protein-1.

chemerin, hepatic growth factor (HGF), TIMP-1, EGF, CXCL8 (IL-8), IFN-γ-induced protein 10 (IP-10) and TNF receptor 2 (TNF-R2) were higher in obese children than in lean controls. For five of these mediators (leptin, chemerin, TIMP-1, EGF and TNF-R2, fold inductions 2.4, 1.1, 1.1, 1.6 and 1.2, respectively), the association with childhood obesity seemed particularly relevant, as linear regression analysis also showed a positive correlation with BMI-SD (Table 2, Supplemental Fig. 1).

In conclusion, next to leptin, the inflammatory mediators chemerin, TIMP-1, EGF and TNF-R2 were identified as circulating mediators that are increased in childhood obesity and correlate with BMI-SD. Differences in these mediators could not be attributed to differences in age or sex between obese children and lean controls, as the groups were age- and sex-matched and none of the five increased inflammatory mediators was correlated with age. Only leptin was correlated with sex, i.e. had higher levels in girls than in boys, as reported before (35), but this sex-effect did not influence the positive correlation between leptin and BMI-SD (Table 2).

Cluster analysis distinguishes clinically different obese and lean control groups

To identify clusters of inflammatory mediators and investigate whether obese children and controls can be distinguished via such clusters, non-hierarchical cluster analysis of the inflammatory mediator profiles was performed.

First, we identified three robust clusters of inflammatory mediators that met the requirements of bootstrapping probability (BsP) > 50% (Supplemental Fig. 2a). Correlation analysis on the three robust clusters was performed to verify the results (Supplemental Fig. 2b). As expected, all three robust clusters showed a

high correlation between the inflammatory mediators: adipsin and thrombopoietin (BsP 100, $R^2 = 0.91$); cathepsin S and chemerin (BsP 57, $R^2 = 0.53$); and the third cluster consisting of IL-18, TNF-R2 and IP-10 (BsP 80; correlation between IL-18 and TNF-R2, $R^2 = 0.46$ and correlation between IL-18 and IP-10, $R^2 = 0.36$; Supplemental Fig. 2b). Taken together, cluster analysis showed three robust clusters of inflammatory mediators. Two of these clusters seemed particularly interesting (adipsin and thrombopoietin, cathepsin S and chemerin), as they were part of a larger cluster of inflammatory mediators, including leptin, which roughly distinguished lean and obese children, as shown in a heatmap (Fig. 1a).

Second, cluster analysis of the inflammatory mediator profiles distinguished lean and obese children, and showed five distinct participant clusters (Fig. 1a). Obese children were predominantly concentrated in clusters I and II, while clusters III and IV mainly comprised lean controls, and cluster V was mixed (Fig. 1a, g). As chemerin, leptin, TIMP-1, EGF and TNF-R2 concentrations were increased in childhood obesity and correlated with BMI-SD (Table 2), the levels of these mediators for all five clusters were studied in detail (Fig. 1b-f, Supplemental Fig. 3b). Furthermore, the relationships between inflammatory mediators and clinical variables were investigated (Fig. 1g-k). Comparison of the obesity clusters I and II showed that several inflammatory mediators, e.g. leptin, TIMP-1, EGF, chemerin, macrophage migration inhibitory factor (MIF) and vascular endothelial growth factor (VEGF), occurred at high levels in cluster I, but not in cluster II (Fig. 1d, e, Supplemental Fig. 3b). The high inflammatory mediator levels in obesity cluster I coincided with a trend towards lower insulin sensitivity (QUICKI) than that in cluster II ($P = 0.07$) (Fig. 1h). Similarly, the inflammatory mediator profiles of leanness clusters III and IV distinguished clinically different lean control groups. Thus the lean controls in cluster III had higher levels of inflammatory mediators such as TIMP-1 and TNF-R2 (Fig. 1d and f), but mainly were conspicuous for their high levels of the insulin-sensitising adipokine adiponectin, compared with leanness cluster IV (Fig. 1b). The high levels of adiponectin in cluster III coincided with a trend towards lower waist circumference ($P = 0.09$) and significantly higher insulin sensitivity (QUICKI, $P = 0.01$) (Fig. 1h and i).

In summary, high levels of inflammatory mediators (leptin, TIMP-1, EGF, VEGF and MIF) coincided with a trend towards lower insulin sensitivity in obesity cluster I versus II, while high levels of insulin-sensitising adiponectin coincided with higher insulin sensitivity in leanness cluster III versus IV. In other words, obesity cluster II and leanness cluster III seem to represent healthy groups, compared with their counterparts.

Monocyte phenotyping and functional analysis

To study the involvement of monocyte subsets in the low-grade inflammation occurring in childhood obesity, monocyte subtype numbers were determined and extensive monocyte phenotyping (i.e. surface marker expression) was performed on whole blood samples. First, monocytes were gated as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocyte subsets, in accordance with

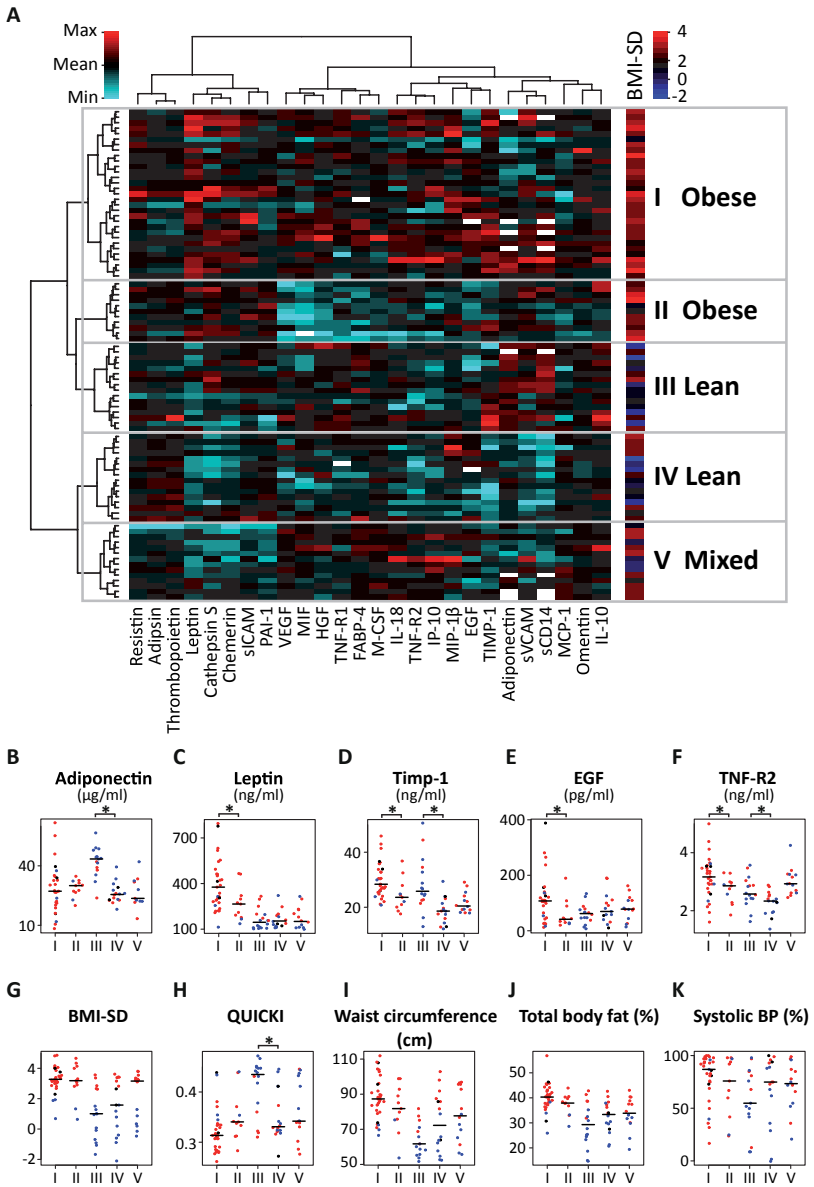

Figure 1 Cluster analysis of the inflammatory mediators

(A) Hierarchical cluster analysis of the inflammatory mediator profiles was performed in 88 of the 96 children, resulting in two obesity clusters, two leanness clusters and one mixed cluster. The heatmap shows log-transformed inflammatory mediator levels for all individual children. White blocks represent missing values. **(B)** Distribution of adiponectin, **(C)** leptin, **(D)** TIMP-1, **(E)** EGF and **(F)** TNF-R2 over the five clusters, and **(G-K)** distribution of clinical characteristics over the five clusters as labeled. Medians are represented by a black bar. Blue and red dots represent age- and sex-matched lean (*n*=30) and obese (*n*=53) children, respectively. Black dots represent children who either had a BMI-SD between 2 and 2.5, or could not be age- and sex-matched (*n*=5). **P* < 0.05.

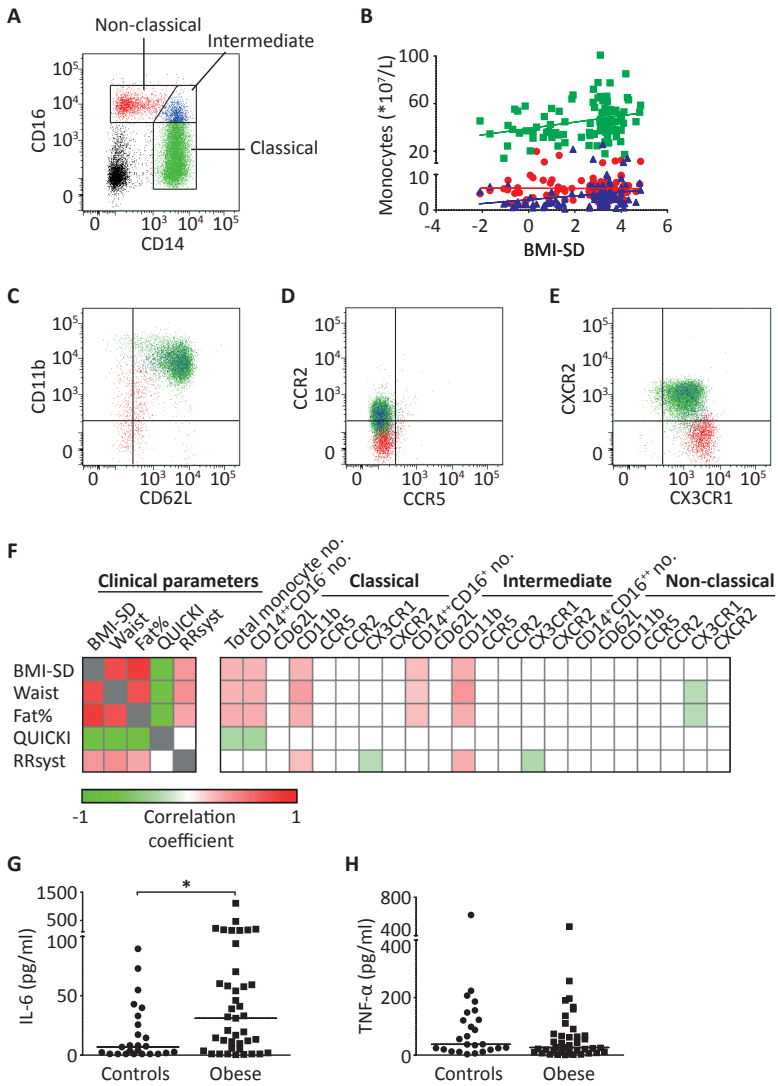

Figure 2 Monocyte populations in childhood obesity

(A) Gating of classical ($CD14^{++}CD16^{-}$; green), intermediate ($CD14^{++}CD16^{+}$; blue) and non-classical ($CD14^{+}CD16^{++}$; red) monocyte subsets. (B) Correlation of the monocyte population numbers with BMI-SD, with significant correlations between both classical (correlation coefficient 0.270) and intermediate (correlation coefficient 0.220) monocyte numbers and BMI-SD. (C-E) Representative phenotype (i.e. surface marker expression of CD11b, CD62L, CCR2, CCR5, CXCR2 and CX3CR1) of the classical (green), intermediate (blue) and non-classical (red) monocytes. Quadrants express isotype control staining. (F) Correlation coefficients between monocyte numbers (no.), monocyte phenotype (surface marker expression) and clinical variables. Only significant correlations are shown ($P < 0.05$). Monocyte phenotyping data of 30 lean and 49 age- and sex-matched obese children, and four children who either had a BMI-SD between 2 and 2.5, or could not be age- and sex-matched (total 83 children), were available and used for the analyses. RRsyst, systolic blood pressure. (G) IL-6 and (H) TNF-α levels upon 4 h of TLR-4 stimulation with LPS, performed in 25 lean and 41 obese children (total 66 children). * $P < 0.05$.

previous studies (Fig. 2a) (9, 10, 13). Quantification of monocyte subset numbers revealed that the numbers of $CD14^{++}$ classical and intermediate monocytes, but not those of non-classical monocytes, correlated positively with BMI-SD (Fig. 2b). Second, the surface expression of the following adhesion factors and chemokine receptors that are involved in atherosclerosis was studied: CD62L, CD11b, CCR2, CCR5, CX3CR1 and chemokine (C-X-C motif) receptor 2 (CXCR2) (Fig. 2c-e) (36, 37). Subsequently, correlations were calculated between, on the one hand, monocyte subset numbers and adhesion factor and chemokine expression, and, on the other hand, the clinical variables BMI-SD, waist circumference, total body fat percentage, QUICKI and systolic blood pressure. Correlation coefficients for all significant correlations ($P < 0.05$, without multiple testing correction) are shown in a heatmap (Fig. 2f). After correction for multiple testing, classical monocyte numbers and CD11b abundance on the classical and intermediate monocyte subsets remained as the monocytes markers most significantly correlated with BMI-SD (Supplemental Table 2).

To verify the increased numbers and activated phenotype of classical monocytes in obese children functionally, whole-blood samples were stimulated with the TLR-4 ligand LPS. Although other TLR-4-proficient cells can also secrete IL-6 and TNF-α, monocytes account for most of the cytokine production in response to whole-blood stimulation with LPS (28), with classical monocytes secreting high levels of IL-6, while non-classical monocytes produce more TNF-α (10). The IL-6 and TNF-α levels after LPS stimulation therefore give an indication of the balance between classical and non-classical monocytes. Interestingly, intermediate monocytes do not have striking levels of IL-6 or TNF-α production upon LPS stimulation (10), but their involvement was not tested with this assay. In line with the high numbers and activated phenotype of classical monocytes observed in obese children, we observed higher IL-6 levels upon LPS stimulation in them than in lean controls, but no differences in TNF-α levels (Fig. 2g and h).

Taken together, especially classical ($CD14^{++}CD16^{-}$) monocytes seem to be involved in the low-grade systemic inflammation seen in childhood obesity, as this subset showed increased numbers, increased CD11b levels and high IL-6 production in response to LPS in obese children compared with lean controls. While intermediate ($CD14^{++}CD16^{+}$) monocytes also exhibited greater expression of the activation marker CD11b, the non-classical ($CD14^{+}CD16^{++}$) monocytes showed no alterations in number or phenotype in obese children.

DISCUSSION

Across diverse adult patient groups, circulating inflammatory mediators and activated $CD14^{++}$ monocytes have been identified as inflammatory agents linking obesity to its metabolic and cardiovascular complications (3, 12, 13). To the best of our knowledge, this is the first comprehensive study of inflammatory mediator profiles and monocyte populations in childhood obesity, thereby providing novel insights into the inflammatory changes that occur during the early stages of obesity.

First, next to leptin, the mediators chemerin, TIMP-1, EGF and TNF-R2 were identified as novel inflammatory mediators that are increased in childhood obesity. All of these mediators act at the crossroads of metabolism and inflammation, and have been associated with decreased insulin sensitivity in mouse models, obese adults and obese children (38-41). Interestingly, high chemerin levels were recently associated with endothelial activation in obese children (42). The relatively subtle differences observed by us in the levels of these circulating proteins between lean and obese individuals are in agreement with other studies (39, 42). Local concentrations (i.e. in the target tissue) may display larger differences, but this remains to be established. Our second insight was that, in cluster analysis of the inflammatory mediators, clinically different obese and lean control groups emerged. Comparing the two obesity clusters, high levels of inflammatory mediators such as leptin, TIMP-1 and EGF coincided with a trend towards lower insulin sensitivity. In the two leanness clusters, high levels of the insulin-sensitising adipokine adiponectin coincided with higher insulin sensitivity. Our third insight was that childhood obesity is associated with increased classical monocyte numbers and an activated phenotype of the classical and intermediate monocyte subsets, including increased IL-6 production upon TLR-4 stimulation. Taken together, our findings reveal an association between inflammatory mediator clusters and insulin resistance, both in obese and lean control groups. Furthermore, activation of $CD14^{++}$ monocyte subsets, which is associated with increased atherosclerosis in obese adults (12, 13), was readily detected in obese children.

Our comprehensive approach enabled the identification of several novel inflammatory mediators in childhood obesity. Moreover, cluster analysis showed the relevance of inflammatory mediators for important clinical variables such as insulin sensitivity (QUICKI). Nevertheless, this study has two weaknesses. First, obese children and lean controls were age- and sex-matched, but age- and sex-mediated effects on inflammatory mediators could not always be excluded. The small differences in age and sex distribution between the obese and lean control clusters, though statistically non-significant, may have influenced the mediator clustering (Supplemental Fig. 3a). Second, the relatively small obese and lean control groups, and the wide age-range of our participants could have masked differences between lean and obese children. Thus while we report a significant correlation between chemerin and BMI-SD, an even stronger correlation was recently observed in a larger cohort with older children (42). At the same time, subtle differences in inflammatory mediators may also have been missed due to the relatively small groups with a wide age-range. For example, we were unable to confirm the increased IL-10 levels previously observed in obese adolescent girls (18). Moreover, based on previous studies (43), we would also have expected lower adiponectin levels in obese children than in controls, but merely observed a trend (P = 0.09). Interestingly, however, cluster analysis revealed that, compared with the other clusters, leanness cluster III was striking for its high adiponectin levels, coinciding with high insulin sensitivity.

Increased numbers of circulating monocytes have been reported in obese children and adults (8, 44). While $CD14^{++}$ monocytes have been implicated in the development of atherosclerosis in obese adults (12-14), this is, to our knowledge, the first study showing that $CD14^{++}$ monocytes numbers are already increased in childhood obesity. We also showed that $CD14^{++}$ monocytes in obese children are striking for their high levels of CD11b, compared with lean controls. CD11b is an α-integrin that plays a key role in cell-adhesive interactions and the migration of cells to inflammatory sites (45). Recently, however, CD11b was also identified as a negative regulator of TLR-4-mediated inflammatory responses. In fact, CD11b is upregulated upon TLR-4 stimulation (28) and inhibits inflammatory pathways downstream of TLR-4 (46). Taken together, the high CD11b expression on $CD14^{++}$ monocytes in obese children may propagate monocyte migration to inflammatory sites, such as vascular lesions or adipose tissue. Alternatively, the increased monocyte CD11b expression in childhood obesity may reflect TLR-4 stimulation and serve to inhibit TLR-4-mediated inflammatory pathways. In obesity, increased translocation of bacterial LPS from the gut and spillover of saturated fatty acids can induce TLR-4 signaling (47-49) and may contribute to the observed upregulation of CD11b on $CD14^{++}$ monocytes. Yet it is important to note that IL-6 secretion in response to TLR-4 stimulation is still increased in obese children (Fig. 2d). Thus, increased monocyte CD11b expression does not prevent a higher TLR-4-mediated inflammatory response in obese children. Furthermore, three intriguing questions with respect to monocyte activation in childhood obesity remain. First, while previously investigated in adults (12-14), the effects of $CD14^{++}$ monocytes on vascular function in obese children require further study. Second, definitions of the recently identified intermediate monocyte subset vary and its role in vascular disease is as yet unclear (10, 11). Our results suggest that intermediate monocytes are upregulated in childhood obesity and activated like classical monocytes, but the question of whether these cells fulfill a specific role in childhood obesity requires further investigation. Third, as adipokines are known to modulate leucocyte function (3), monocyte function may be affected by inflammatory mediator profiles. Indeed, we observed the highest number of intermediate monocytes in obesity cluster I, coinciding with its inflammatory mediator profile (Supplemental Fig. 4a). Moreover, leanness cluster III showed the lowest number of classical monocytes, coinciding with its high adiponectin levels (Supplemental Fig. 4a). Nevertheless, no differences in monocyte phenotype (e.g. CD11b expression) were observed between the clusters (Supplemental Fig. 4b), and further investigation is needed to address the interaction between adipokines and circulating monocytes in childhood obesity.

In conclusion, this study addressed two inflammatory modes of action in childhood obesity. On the one hand, inflammatory mediator clusters were found to correlate with insulin resistance in obese and lean control groups. On the other hand, activation of $CD14^{++}$ monocytes, which is associated with increased atherosclerosis development in obese adults (12, 13), was readily detectable in childhood obesity. Thus inflammatory mediators and activated $CD14^{++}$ monocytes seem to be part

of the inflammatory link between obesity and its metabolic and cardiovascular complications, and may provide potential avenues for early detection and prevention of these complications.

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SUPPLEMENTAL INFORMATION

Systemic inflammation in childhood obesity: circulating inflammatory mediators and activated CD14^{++} monocytes

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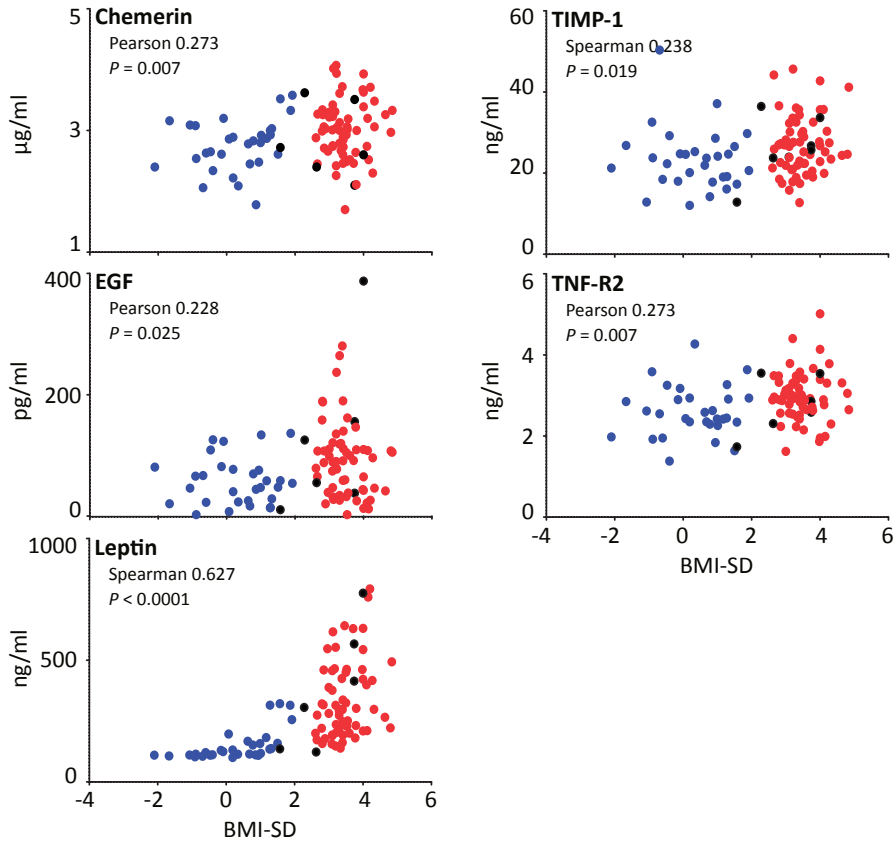
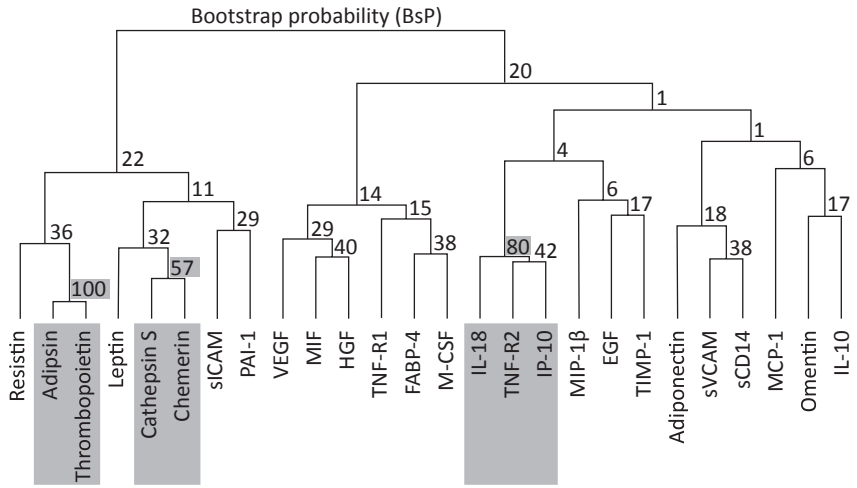
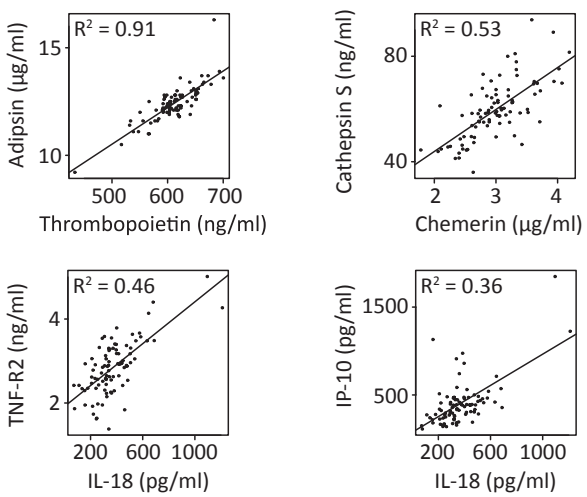


Supplemental Figure 1
Correlation of chemerin, EGF, leptin, TIMP-1 and TNF-R2 with BMI-SD. All subjects (*n*=96) are included in the analysis. Blue and red dots represent the age- and sex-matched lean (*n*=30) and obese (*n*=60) children, respectively. Black dots represent the additional children (*n*=6).

A **Bootstrap analysis (*n* = 10 000)**

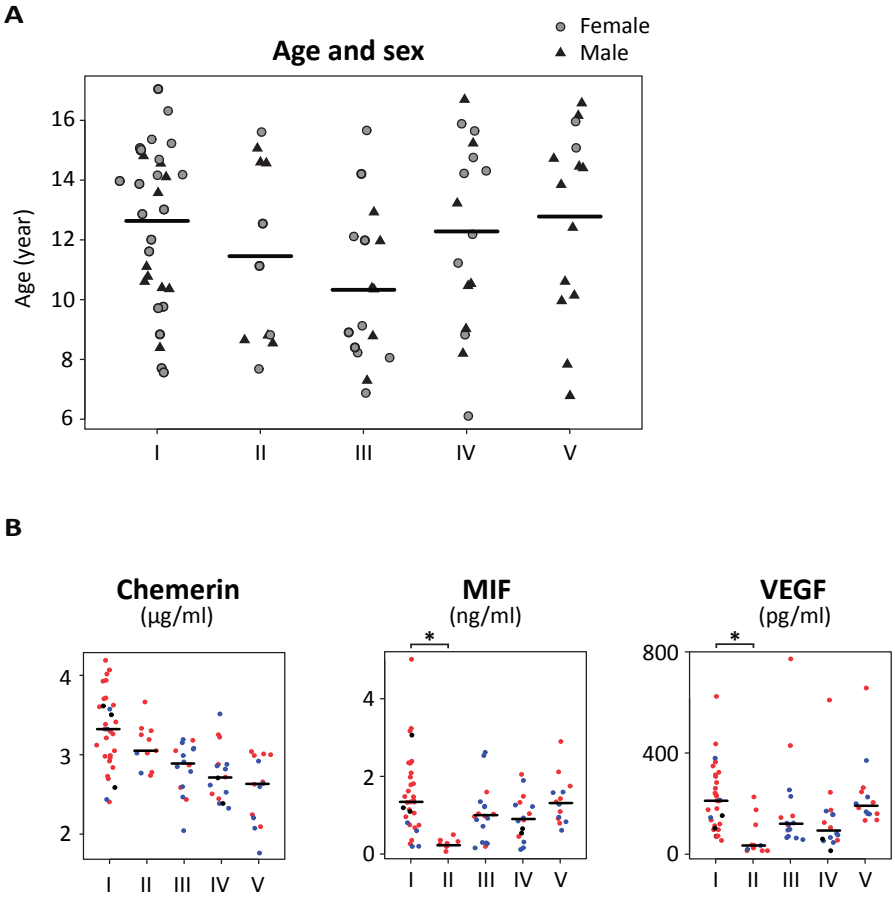


B **Correlation analysis of adipokines with a BsP > 50**



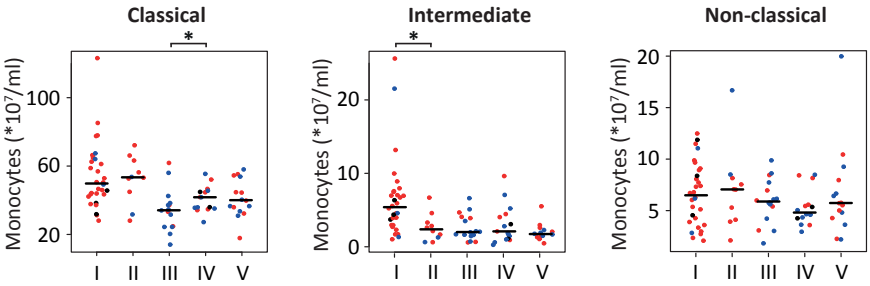
Supplemental Figure 2

Cluster analysis of the inflammatory mediators, which was performed in 88 of the 96 children (30 lean and 53 age- and sex-matched obese children, and 5 additional children). The data was resampled 10 000 times to obtain consensus clustering. Bootstrap probabilities (BsP) represent clustering frequencies (%) of specific mediators. A BsP > 50 indicates that these mediators clustered together in > 50% of the 10 000 clustering samples, and is considered robust clustering. (A) Bootstrap probabilities for all inflammatory mediator clusters. (B) Correlation analysis of inflammatory mediator clusters with a BsP > 50. All correlations are significant ($P < 0.05$).

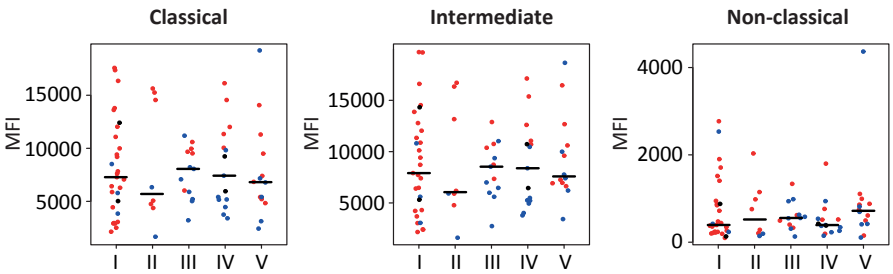


Supplemental Figure 3
(A) Age and sex distribution over the 2 obesity clusters (I+II), 2 leanness clusters (III+IV), and the mixed cluster (V). Please see Figure 2 for the clustering analysis. (B) Chemerin, MIF and VEGF distribution over the five clusters. Cluster analysis was performed in 88 children. Blue and red dots represent the age- and sex-matched lean (*n*=30) and obese (*n*=53) children, respectively. Black dots represent the additional children (*n*=5). * $P < 0.05$.

A Monocyte numbers in the patient clusters



B Monocyte CD11b expression in the patient clusters



Supplemental Figure 4
(A) Monocyte numbers for the 2 obesity clusters (I+II), 2 leanness clusters (III+IV), and mixed cluster (V). (B) CD11b expression on monocyte subpopulations for the five clusters. For 82 of the 88 subjects included in the cluster analysis, monocyte numbers and phenotyping data (surface marker expression) were available. Blue and red dots represent the age- and sex-matched lean (*n*=30) and obese (*n*=47) children, respectively. Black dots represent the additional children (*n*=5). * $P < 0.05$.

Supplemental Table 1 Clinical characteristics

	Additional children
Number	6
Age (years)	13.9 ± 3.3
BMI-SD [a]	3.0 ± 1.0
Waist Circumference (cm)	89 ± 18
Total body fat (%)	36.7 ± 7.0
QUICKI	0.32 (0.27-0.42)
Systolic blood pressure (%) [b]	86.0 ± 9.7
Diastolic blood pressure (%) [b]	54.2 ± 28.3
Total cholesterol (mmol/L)	5.4 ± 1.0
HDL (mmol/L)	1.5 ± 0.2
LDL (mmol/L)	3.3 ± 0.8
Triacylglycerol (mmol/L)	0.7 (0.6-2.8)
AST (U/L)	31 (19-42)
ALT (U/L)	28 (13-49)
hsCRP (mg/L)	2.78 (1.06-3.68)

Supplemental Table 1. In addition to Table 1 which shows the characteristics of the 30 lean and 60 age- and sex-matched obese children, Supplemental Table 1 shows the characteristics of the 6 additional children. Normally distributed data is shown as mean ± SD. Non-parametric data is shown as median (interquartile range). [a] Standard deviation scores, normalized for age and sex. [b] Percentiles, normalized for age, height and sex. AST, aspartate-aminotransferase.

Supplemental Table 2 Monocytes

		Mean / median		Regression (BMI-SD)	
	Units	Lean	Obese	Age / sex correction	Correlation coefficient
All monocytes	*10^9/L	0.46 ± 0.13	0.54 ± 0.2 *	None	0.262 +
Classical monocytes	*10^7/L	39.6 ± 12.8	48.6 ± 17.9 *	None	0.270 +
CD62L	MFI	1176 (827-2227)	1572 (699-2177)	None	0.003
CD11b	MFI	6185 ± 3422	8966 ± 4389 *	Age	0.244 *
CCR5	MFI	137 ± 20	141 ± 18	None	0.153
CCR2	MFI	218 ± 102	251 ± 83	None	0.153
CX3CR1	MFI	1025 (856-1178)	857 (684-1131)	Sex	-0.074
CXCR2	MFI	1028 ± 307	1076 ± 297	None	-0.013
Intermediate monocytes	*10^7/L	3.0 ± 4.0	4.8 ± 4.2	None	0.220 *
CD62L	MFI	793 ± 779	871 ± 797	None	0.098
CD11b	MFI	6889 ± 3470	9948 ± 4626 *	Age	0.279 *
CCR5	MFI	168 ± 36	168 ± 35	None	0.012
CCR2	MFI	190 ± 103	230 ± 82	None	0.206
CX3CR1	MFI	1366 ± 511	1157 ± 499	None	-0.151
CXCR2	MFI	766 ± 339	920 ± 321	None	0.125
Non-classical monocytes	*10^7/L	6.5 ± 4.0	5.9 ± 2.4	None	-0.008
CD62L	MFI	233 ± 54	242 ± 65	None	0.135
CD11b	MFI	686 ± 875	747 ± 581	Age	0.022
CCR5	MFI	172 ± 32	167 ± 27	None	0.014
CCR2	MFI	49 ± 25	52 ± 33	None	0.096
CX3CR1	MFI	3266 ± 1131	2844 ± 1354	None	-0.121
CXCR2	MFI	107 ± 35	119 ± 53	None	0.133

Supplemental Table 2. Monocyte numbers and phenotype (surface marker expression) of 30 lean and 49 age- and sex-matched obese children. Shown are means ± SD for normally distributed data, and median (interquartile range) for non-parametric data. For regression analysis with BMI-SD, available monocyte data of 4 additional children who either had a BMI-SD between 2 and 2.5, or could not be age- and sex-matched, were included as well (total of 83 children). All *P* values were corrected for multiple testing. MFI = median fluorescence intensity. * $P < 0.05$. + $P < 0.01$.

PART III

IMPLICATIONS FOR CHILDHOOD OBESITY

CHAPTER 7

Vitamin D deficiency in childhood obesity is associated with high levels of circulating inflammatory mediators, and low insulin sensitivity

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* Both authors contributed equally

Submitted

ABSTRACT

Hypothesis

Childhood obesity is accompanied by low-grade systemic inflammation, which contributes to the development of insulin resistance and cardiovascular complications later in life. As vitamin D exhibits profound immunomodulatory functions and vitamin D deficiency is highly prevalent in childhood obesity, we hypothesized that vitamin D deficiency in childhood obesity coincides with enhanced systemic inflammation and reduced insulin sensitivity.

Methods

In a cross-sectional study of 64 obese and 32 healthy children aged 6-16 years, comprehensive profiling of 32 circulating inflammatory mediators was performed, together with assessment of 25(OH)D levels and measures for insulin sensitivity.

Results

Vitamin D deficiency was highly prevalent in obese (56%) versus healthy control children (16%). First, vitamin D deficient obese children showed a lower insulin sensitivity than non-deficient obese children, as measured by a lower quantitative insulin sensitivity check index (QUICKI). Second, the association between vitamin D deficiency and insulin resistance in childhood obesity was confirmed with multiple regression analysis. Third, vitamin D deficient obese children showed higher levels of the inflammatory mediators cathepsin S, chemerin and soluble vascular adhesion molecule (sVCAM), compared with non-deficient obese children. Finally, hierarchical cluster analysis revealed an overrepresentation of vitamin D deficiency in obese children expressing inflammatory mediator clusters with high levels of cathepsin S, sVCAM and chemerin.

Conclusion

Vitamin D deficiency in childhood obesity was associated with enhanced systemic inflammation and reduced insulin sensitivity. The high cathepsin S and sVCAM levels may reflect activation of a pro-inflammatory, pro-diabetic and atherogenic pathway that could be inhibited by vitamin D supplementation.

INTRODUCTION

Vitamin D deficiency is endemic in childhood obesity, with 51% of obese US children showing a vitamin D deficiency, compared to 9% of all US children (1). Recently, an association between vitamin D deficiency and decreased insulin sensitivity in childhood obesity was reported (2). The association between vitamin D deficiency and the development of type 2 diabetes (T2D) was underscored in a large and prospective population-based cohort study in adults (3).

Vitamin D deficiency may affect glucose homeostasis in several ways. First, as vitamin D plays a key role in calcium metabolism and insulin secretion is a calcium-dependent process, it was hypothesized that vitamin D deficiency hampers insulin secretion by β-cells in a calcium-dependent manner (4). Second, vitamin D deficiency was associated with decreased peripheral insulin action, either via reduced insulin receptor expression or via impaired signaling downstream of the insulin receptor (4). Third, vitamin D is well known for its immune modulatory functions (5). Considering the deleterious effects of systemic inflammation on insulin resistance in obesity (6), it was proposed that vitamin D deficiency aggravates insulin resistance in obesity through enhanced systemic inflammation (4, 7).

Here, we focused on the latter hypothesis. In a cross-sectional study of 64 obese and 32 age- and sex-matched healthy control children, we analyzed circulating levels of vitamin D and 32 systemic inflammatory mediators, together with measures for insulin sensitivity. To our knowledge, this is the first study performing comprehensive profiling of inflammatory mediators in relation to vitamin D status, showing that vitamin D deficiency in childhood obesity is accompanied by both reduced insulin sensitivity and enhanced systemic inflammation.

METHODS

Subjects

Childhood obesity was defined as a body-mass index (BMI) > 2.5 standard deviations of the mean BMI (BMI-SD) for age and sex, in accordance with the international definition of childhood obesity as a BMI of > 30 kg/m^2 projected to 18 years of age (6, 8, 9). In a cross-sectional study, 64 obese children and 32 age- and sex-matched healthy controls with a BMI-SD < 2.5, aged 6-16 years, were included at the pediatric outpatient department of the Meander Medical Center, Amersfoort, the Netherlands. Exclusion criteria were inflammatory or infectious conditions, endocrine disorders, growth abnormalities, and intoxications. Vitamin D deficiency was defined as 25(OH) D levels ≤ 37.5 nmol/L (≤ 15 ng/ml), in accordance with international guidelines (1, 10). One vitamin D deficient and one non-deficient obese patient reported the use of oral insulin sensitizing drugs (metformin). None of the children reported the use of vitamin D supplementation. Written informed consent was obtained from all children and their parents. The study was approved by the institutional medical ethical review board (METC 09/217K).

Clinical parameters

For bioelectrical impedance measurements (total body fat percentage), a foot-hand bio-impedance analyzer was used, in accordance with the manufacturer's instructions (Analyzer Model BIA 101; Akern Srl, Florence, Italy). Skin tone was categorized in light, mid color and dark (11). Waist circumference standard deviations (SD) for age and sex were calculated with Cole's LMS method and data of the Dutch national growth study (9, 12).

Blood samples

Blood samples were taken in sodium-heparin tubes upon overnight fasting. 25(OH) D levels were measured with an Elecsys vitamin D assay (Roche Diagnostics, Mannheim, Germany) (13). Undercarboxylated and carboxylated osteocalcin levels were determined using ELISA kits (Takara Bio USA, Madison, WI). Total osteocalcin levels represent the sum of carboxylated and undercarboxylated osteocalcin. Routine laboratory testing included fasting glucose, fasting insulin, triglycerides, high and low-density lipoprotein cholesterol (HDL, LDL) and hsCRP levels. The quantitative insulin sensitivity check index (QUICKI) and homeostasis model assessment of insulin resistance (HOMA-IR) were calculated as described earlier (14). Circulating inflammatory mediators were measured in plasma using a recently developed and validated multiplex immunoassay (15).

Statistical analyses

Throughout the manuscript, deficient obese children (n = 36) were compared with non-deficient obese children (n = 28) to study the effect of vitamin D in obese children. Next, non-deficient obese children were compared with non-deficient healthy controls (n = 27), to study the effect of obesity. Please note that the 5 deficient healthy control children were excluded from further analyses, as this group was considered too small to compare with the deficient obese children (Figure 1).

As most clinical parameters and inflammatory mediators showed a non-parametric distribution, Mann-Whitney U tests, or the Fisher's exact test if applicable, were used to assess differences between groups. Benjamini and Hochberg's False Discovery Rate correction was used to correct P values for multiple testing (6). Multiple linear regression analysis was performed to examine the relation between QUICKI and vitamin D status, correcting for BMI-SD, age, sex, skin tone and undercarboxylated osteocalcin. Similarly, the relation between inflammatory mediators and vitamin D status was assessed, correcting for BMI-SD, age and sex.

Statistical analyses were performed with SPSS 15.0 for Windows (SPSS, Chicago, IL, USA) and R, a free software environment for statistical computing and graphics (16). Non-supervised hierarchical cluster analysis of the inflammatory mediator profiles was executed as recently described (6), using the Pvclust package (17).

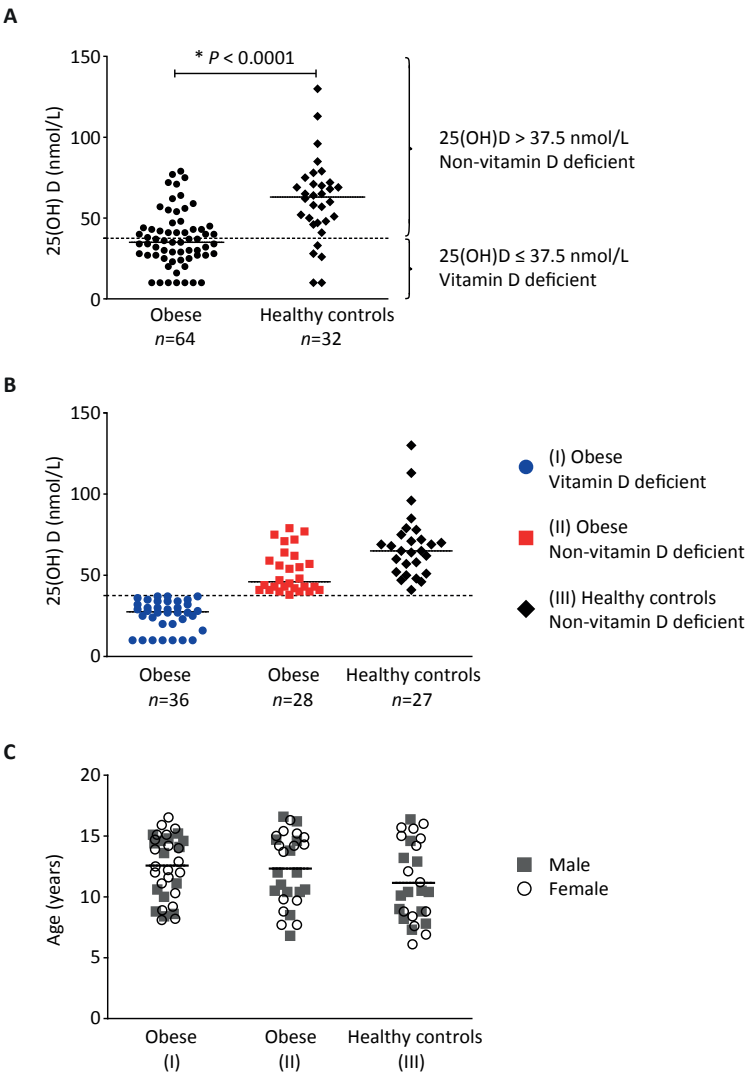

Figure 1 Vitamin D deficiency in obese children
(A) 25(OH)D levels of obese children (*n*=64) and healthy controls (*n*=32), showing significantly lower 25(OH)D levels in obese children. In accordance with international guidelines, vitamin D deficiency was defined as a 25(OH)D level ≤ 37.5 nmol/L. Obese and healthy children with 25(OH)D levels > 37.5nmol/L are referred to as non-deficient. (B) 25(OH)D levels of the deficient obese children (group I, *n*=36), non-deficient obese children (group II, *n*=28), and non-deficient healthy controls (group III, *n*=27). Throughout the manuscript, groups were compared as follows: 1) deficient obese children versus non-deficient obese children, to study the effect of vitamin D in obese children, and 2) non-deficient obese children versus non-deficient healthy controls, to study the effect of obesity. Please note that the 5 deficient healthy control children were excluded from further analyses, as this group was considered too small to compare with the deficient obese children. (C) Age and sex distribution of the three groups. (A-C) Lines represent medians.

RESULTS

Vitamin D deficiency is associated with lower insulin sensitivity

Vitamin D deficiency showed 56% prevalence in obese children, compared with 16% in healthy children (Table 1, Figure 1a). The vitamin D status of obese children seemed relevant, as deficient obese children (i.e. vitamin D deficient, *n* = 36) showed a lower

Table 1 Clinical characteristics

	Obese		Healthy controls
	Vitamin D deficient	Non-vitamin D deficient	Non-vitamin D deficient
Number of children (%)	36 (56%)	28 (44%)	27 (84%)
Age (year, mean ± SD)	12.6 (± 2.6)	12.3 (± 3.0)	11.1 (± 3.2)
Sex (% female)	61.1	50.0	51.9
BMI-SD	3.40 (3.10-3.94)	3.39 (3.09-3.76)†	0.68 (-0.60-1.27)†
Waist circumference (SD)	2.58 (2.38-2.91)	2.46 (2.14-2.89)†	0.02 (-0.76-0.81)†
Fat percentage (%)	40.4 (38.0-42.8)	39.1 (36.3-41.5)†	27.8 (23.4-32.6)†
25(OH)D (nmol/L)	27.5 (20.0-33.5)$^{+}$	46.0 (41.3-61.3)$^{+,\dagger}$	65.0 (52.0-75.0)†
Total osteocalcin (ng/ml)	48.5 (39.7-60.2)*	40.9 (31.9-52.5)*	47.9 (36.6-56.4)
Undercarboxylated osteocalcin (ng/ml)	19.5 (11.8-28.4)*	15.4 (9.39-19.5)*	16.5 (9.95-23.5)
Fasting glucose (mmol/L)	5.30 (5.10-5.60)	5.20 (5.00-5.40)†	4.90 (4.60-5.10)†
Fasting insulin (mU/L)	15.0 (11.0-26.0)*	11.0 (5.00-18.0)*,†	2.00 (2.00-9.00)†
QUICKI	0.32 (0.29-0.33)*	0.33 (0.31-0.37)*,†	0.43 (0.34-0.44)†
HOMA-IR	3.56 (2.44-6.44)*	2.64 (1.20-4.32)*,†	0.51 (0.44-2.00)†
Triglycerides (mmol/L)	1.05 (0.70-1.45)	0.80 (0.60-1.40)	0.70 (0.50-0.90)
HDL cholesterol (mmol/L)	1.20 (1.10-1.38)	1.20 (1.00-1.40)†	1.50(1.30-1.70)†
LDL cholesterol (mmol/L)	2.50 (2.05-3.18)	2.50 (2.00-3.30)	2.40 (2.20-2.60)
hsCRP (mg/L)	2.32 (1.14-3.52)	1.59 (0.70-2.73)†	0.19 (0.15-0.68)†
Skin tone (%)			
Light	34.3	57.1†	92.6†
Mid color	48.6*	17.9*	7.4
Dark	17.1	25.0$^{\#}$	0.0$^{\#}$
Season of inclusion (%)			
Spring	22.2	46.4†	11.1†
Summer	19.4	21.4	48.1
Fall	13.9	7.1	22.2
Winter	44.4	25.0	18.5

Table 1. Clinical characteristics of the obese children, divided in a vitamin D deficient (25(OH)D ≤ 37.5 nmol/L) and a non-deficient group (25(OH)D > 37.5nmol/L), and of the non-deficient healthy controls. Data for the groups are presented as median (interquartile ranges), unless indicated otherwise. * P<0.05, $^{+}$ P<0.01 (between both obese groups). $^{\#}$ P<0.05, † P<0.01 (between non-deficient obese and healthy control groups).

insulin sensitivity than non-deficient obese children (i.e. non-vitamin D deficient, *n* = 28), as measured by a lower quantitative insulin sensitivity check index (QUICKI) and a higher HOMA-IR (Table 1), which are surrogate markers for insulin sensitivity (14). Differences in insulin sensitivity could not be explained by clinical parameters such as age, sex, BMI-SD, waist circumference SD (a measure for visceral adiposity (18)), fat percentage, lipid profile, and season of inclusion, which were all comparable between deficient and non-deficient obese children (Table 1, Figure 1c).

Interestingly, deficient obese children showed significantly higher levels of undercarboxylated osteocalcin and total osteocalcin levels than non-deficient obese children (Table 1). As undercarboxylated osteocalcin appears to be part of an adaptive process to counter glucose intolerance (19), differences in undercarboxylated osteocalcin levels may mask differences in insulin sensitivity. Furthermore, deficient obese children showed more mid-colored skin tones than non-deficient obese children (Table 1), which may also influence vitamin D levels and/or insulin sensitivity (2). Therefore, multiple regression analysis in all children was performed, to study the association between vitamin D deficiency and insulin sensitivity, independent of possible confounders such as undercarboxylated osteocalcin and skin tone, next to BMI-SD, age, and sex (Supplemental Table 1). Importantly, multiple linear regression analysis underscored the association between vitamin D deficiency and insulin resistance: QUICKI depended on vitamin D status even when adjusting for BMI-SD, age, sex, undercarboxylated osteocalcin, and skin tone ($P = 0.045$, $\beta = 0.188$, $R^2 = 0.555$, Supplemental Table 1). Next to vitamin D status, only BMI-SD and age were significantly associated with QUICKI ($P < 0.001$ and $P = 0.030$ respectively, Supplemental Table 1).

In conclusion, vitamin D deficiency was highly prevalent in obese Dutch children, and associated with reduced insulin sensitivity. While non-deficient obese children (*n* = 28) showed lower insulin sensitivity, lower HDL levels, and higher hsCRP levels than non-deficient healthy children (*n* = 27) (Table 1), deficient obese children (*n* = 36) showed even lower insulin sensitivity (Table 1), independent of other relevant measures such as BMI-SD, age, sex, undercarboxylated osteocalcin and skin tone (Supplemental Table 1).

Vitamin D deficiency is associated with enhanced levels of circulating inflammatory mediators

Immunomodulatory effects of vitamin D may partly explain the association between vitamin D deficiency and insulin resistance in childhood obesity (4, 5, 7). To assess the effects of vitamin D deficiency on systemic inflammation in childhood obesity, 32 circulating inflammatory mediators were measured. The set of inflammatory mediators was established in earlier studies, and comprises multiple adipokines and cytokines acting at the crossroads of metabolism and inflammation (6, 15). Deficient obese children exhibited higher plasma levels of cathepsin S, chemerin, RBP-4 and sVCAM than non-deficient obese children (Table 2, Supplemental Figure 1). After correcting for multiple testing, differences in cathepsin S, RBP-4 and sVCAM remained significant, while chemerin showed a trend ($P = 0.09$).

Table 2 Circulating inflammatory mediators

		Obese		Healthy controls	All groups	
		Vitamin D deficient	Non-vitamin D deficient	Non-vitamin D deficient	Multiple linear regression	
	Units				β	R^2
Cytokines						
IL-6 [d]	pg/ml	0.13 (0.13-60.0)	0.13 (0.13-41.9)	0.13 (0.13-74.8)	-0.075	0.030
IL-10	pg/ml	12.1 (9.69-15.4)	10.6 (8.67-16.8)	12.6 (9.39-20.4)	-0.018	0.218
IL-18	pg/ml	385 (316-479)	339 (295-482)[#]	262 (215-355)[#]	-0.057	0.074
TNF-α [c]	pg/ml	1.10 (0.15-3.62)	1.49 (0.15-4.01)	2.31 (1.45-4.17)	-0.098	0.108
Adipokines						
Adiponectin [b]	µg/ml	28.7 (22.6-34.7)	26.8 (21.3-33.3)	32.2 (25.7-43.3)	-0.172	0.245
Adipsin	ng/ml	604 (578-624)	606 (536-633)	616 (592-644)	-0.143	0.203
Cathepsin S	ng/ml	62.5 (56.2-72.2)[+]	56.2 (48.7-61.3)[+]	57.7 (46.3-60.6)	-0.341	0.141[++]
Chemerin	µg/ml	3.13 (2.74-3.47)[*]	2.87 (2.50-3.11)[*]	2.80(2.48-3.00)	-0.229	0.079
FABP-4 [a]	ng/ml	23.0 (20.9-26.4)	25.7 (22.6-27.3)	22.8 (20.4-27.6)	0.081	0.058
HGF	pg/ml	375 (242-530)	354 (262-534)[#]	282 (221-340)[#]	-0.064	0.033
Leptin	ng/ml	309 (239-486)	252 (184-424)[†]	130 (111-159)[†]	-0.112	0.381
MIF [b]	pg/ml	1084 (473-1781)	1044 (636-1492)	841 (298-1240)	-0.088	0.039
MCP-1	pg/ml	381 (196-477)	352 (215-545)	406 (307-476)	-0.008	0.135
Omentin	pg/ml	4.06 (3.43-4.55)	3.81 (3.32-4.53)	3.79 (3.33-4.40)	0.042	0.015
PAI-1	µg/ml	160 (136-183)	141 (117-175)[#]	177 (136-194)[#]	-0.173	0.035
RBP-4 [c]	µg/ml	166 (149-175)[*]	148 (137-158)[*]	153 (140-184)	-0.175	0.128
Resistin	ng/ml	939 (892-1030)	905 (876-961)	917 (898-963)	-0.087	0.041
TIMP-1	ng/ml	27.6 (22.7-32.6)	24.6 (21.0-27.2)	21.9 (17.8-26.6)	-0.248	0.095
Thrombopoietin	µg/ml	12.3 (11.8-12.8)	12.2 (11.5-12.7)	12.4 (12.0-13.0)	-0.125	0.071
Other						
CXCL8 (IL-8) [b]	pg/ml	80.5 (35.1-243)	55.6 (28.5-142)	43.5 (20.0-63.3)	0.107	0.080
EGF [a]	pg/ml	89.5 (39.7-118)	85.0 (39.0-117)[†]	46.1 (20.3-66.9)[†]	-0.112	0.052
EN-RAGE [d]	pg/ml	6.57 (0.12-41.8)	6.07 (0.12-50.8)	34.2 (0.36-68.1)	-0.027	0.077
IP-10	pg/ml	394 (307-478)	389 (271-458)	278 (190-386)	-0.025	0.057
M-CSF [a]	pg/ml	48.9 (34.6-70.9)	48.7 (36.9-68.4)	57.4 (48.9-74.1)	-0.124	0.076
MIP-1α [d]	pg/ml	44.0 (9.77-133)	36.3 (26.2-61.8)	47.9 (19.2-76.3)	0.152	0.049
MIP-1β	pg/ml	111 (67.0-177)	81.1 (60.8-118)	71.5 (60.5-93.4)	-0.062	0.149
sCD14 [b]	µg/ml	7.68 (6.46-9.94)	8.37 (6.37-10.3)	7.38 (5.30-9.48)	0.074	0.154
sICAM	µg/ml	3.06 (2.94-3.27)	2.98 (2.83-3.16)	3.06 (2.86-3.21)	0.011	0.011
sVCAM	µg/ml	5.29 (5.02-5.55)[+]	4.98 (4.64-5.14)[+]	4.97 (4.67-5.42)	-0.342	0.273[++]
TNF-R1	ng/ml	2.76 (2.13-3.40)	2.69 (2.16-2.98)	2.46 (2.09-2.98)	-0.119	0.049
TNF-R2	ng/ml	2.97 (2.63-3.40)	2.88 (2.49-3.18)[†]	2.42 (1.98-2.85)[†]	-0.094	0.045
VEGF [b]	pg/ml	165 (93.2-284)	125 (61.7-200)	108 (54.5-157)	-0.130	0.029

To study the effect of obesity independent of vitamin D deficiency, differences in circulating inflammatory mediators between the non-deficient obese and healthy children were assessed as well. In non-deficient obese children, higher levels of IL-18, HGF, leptin, EGF and TNF-R2 were observed, and lower levels of PAI-1 (Table 2). After correction for multiple testing, differences in leptin, EGF, TNF-R2 and PAI-1 remained significant, while IL-18 and HGF showed a trend (P = 0.07 and 0.05 respectively). Taken together, the effect of vitamin D deficiency on circulating inflammatory mediators in childhood obesity seemed different from the effect of obesity itself. While obesity was associated with altered levels of leptin, EGF, TNF-R2, PAI-1, IL-18 and HGF, in accordance with a recent study (6), vitamin D deficiency in childhood obesity coincided with higher levels of cathepsin S, chemerin, RBP-4 and sVCAM.

Multiple linear regression analysis in all children supported the vitamin D dependency of cathepsin S, chemerin and sVCAM: cathepsin S and sVCAM depended on vitamin D status, even when adjusted for BMI-SD, age and sex, while chemerin levels showed a trend towards vitamin D dependency (P = 0.08) (Table 2). RBP-4 levels did not depend on vitamin D status after adjustment for the aforementioned potential confounders (P = 0.160). In conclusion, vitamin D deficiency in childhood obesity was associated with enhanced levels of circulating inflammatory mediators, specifically cathepsin S, chemerin and sVCAM.

Clustering of inflammatory mediators distinguishes vitamin D deficient obese children

As an alternative approach to study the association between vitamin D deficiency and inflammation, the inflammatory mediator profiles of obese children were submitted to hierarchical cluster analysis. Interestingly, cathepsin S and sVCAM, the inflammatory mediators most strongly associated with vitamin D deficiency (see above), showed a bootstrapping probability of 65%, which is indicative for robust clustering (Figure 2a) (6). They comprised an inflammatory mediator cluster together with TIMP-1, leptin, chemerin, resistin, sICAM and PAI-1, which distinguished two clusters of obese children (Figure 2b). Cluster I represented a mixed group, including both deficient and non-deficient children. Cluster II contained predominantly deficient obese children (P = 0.008). Taken together, cluster analysis of the inflammatory mediators underscored the link between vitamin D deficiency and inflammation. In obese children expressing inflammatory mediator clusters with high levels of cathepsin S, sVCAM, and chemerin, vitamin D deficiency was overrepresented.

◀ **Table 2.** Levels of circulating inflammatory mediators for all three groups are displayed as median (interquartile ranges). For clarity, mediators are classified according to their origin and function: classical cytokines, adipokines, and other mediators. Multiple linear regression analysis of the inflammatory mediators with vitamin D status was performed to control for BMI-SD, age and sex, as possible confounders. Shown are the standardized β and R^2 for the inflammatory mediators. * P<0.05, $^{+}$ P<0.01 (between both obese groups). $^{\#}$ P<0.05 and † P<0.01 (between non-deficient obese and healthy control groups). ** P<0.05, $^{++}$ P<0.01 (all groups, multiple linear regression analysis). Mediators for which values were missing, mostly due to undetectable levels, are indicated as follows: [a] 1-3% missing values, [b] 3-10% missing values, [c] 10-20% missing values, [d] >20.0% missing values.

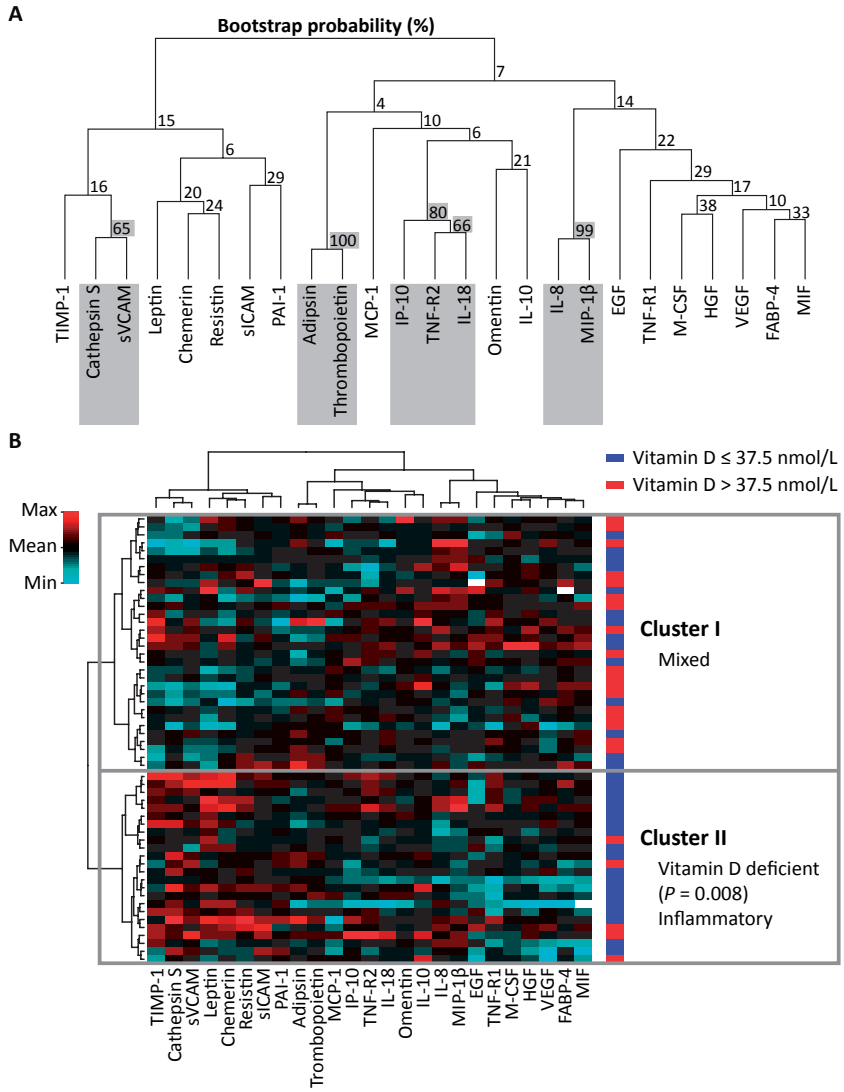

Figure 2 Cluster analysis of inflammatory mediators

(A) Bootstrap analysis of circulating inflammatory mediators, to identify clusters of related inflammatory mediators. Shown are bootstrap probabilities (%) for clusters of inflammatory mediators. Of note, inflammatory mediators with > 10% missing values (e.g. RBP-4, 10 values above upper detection limit) interfered with the cluster analysis and were excluded from the analysis. Grey boxes mark inflammatory mediators that cluster together with a bootstrap probability > 50%, indicative for robust clustering. (B) Hierarchical cluster analysis of the inflammatory mediator profiles of all obese patients, to identify clusters of related patients. Displayed are log-transformed inflammatory mediator levels in a heat-map. White boxes represent missing values. Hierarchical cluster analysis showed 2 clusters of patients: a mixed cluster (I) and an inflammatory cluster with patients exhibiting high levels of inflammatory mediators among which cathepsin S, sVCAM and chemerin (II). Cluster II showed an overrepresentation of vitamin D deficient obese children (*P*=0.008). (A-B) Please note that the bootstrap analysis of inflammatory mediators displayed in panel A is also part of panel B. In fact, the order of the inflammatory mediators in panel B is determined by the bootstrap analysis displayed in panel A.

DISCUSSION

The high prevalence of vitamin D deficiency (56%) observed in this Dutch cohort of obese children equals the reported 51% prevalence in obese US children (1). Moreover, this study confirmed the recently reported association between vitamin D deficiency and insulin resistance in childhood obesity (2). The novelty of this study is its focus on the immune modulatory role of vitamin D, which may partly explain the relation between vitamin D deficiency and insulin resistance in childhood obesity (4, 5, 7). Vitamin D deficiency in obese children coincided with enhanced systemic inflammation, independent of BMI-SD, age, and sex, factors which are known to affect inflammation and metabolic outcome (6, 18). The systemic inflammation was specifically reflected by increased levels of circulating cathepsin S, chemerin and sVCAM, which all have been associated with insulin resistance in several patient groups (20-22). Clustering of the inflammatory mediator profiles confirmed the link between vitamin D deficiency and systemic inflammation, as vitamin D deficiency was overrepresented in obese children with explicitly inflammatory profiles. In conclusion, our results fuel the hypothesis that vitamin D deficiency lowers insulin sensitivity in obese children through enhanced systemic inflammation.

Interestingly, the association between vitamin D deficiency and high cathepsin S and sVCAM levels is supported by recent fundamental studies. Vitamin D was shown to induce the expression of cystatin D, which is a high-affinity inhibitor of cathepsin S (23, 24). Accordingly, vitamin D deficiency may enhance cathepsin S activity in obese children through reduced expression of cystatin D. In animal models and human studies, enhanced cathepsin S activity was implicated in the development of diabetes and atherosclerosis, and high levels of cathepsin S were associated with an increased mortality risk (25, 26). Thus, high cathepsin S levels in vitamin D deficient obese children may reflect activation of a pro-inflammatory, pro-diabetic and atherogenic pathway that could be inhibited by vitamin D supplementation. Similarly, vitamin D was shown to attenuate the expression of sVCAM by human endothelial cells (27). sVCAM is highly expressed in atherosclerotic plaques, plays a key role in mononuclear cell adhesion, and contributes to the progress of atherosclerotic lesions (28, 29). Thus, the high sVCAM levels may reflect a second pro-inflammatory and atherogenic pathway that could be inhibited by vitamin D supplementation. Of note, mechanisms underlying the association between vitamin D deficiency and high chemerin levels are currently unknown, and require further investigation.

Notably, this study does not exclude a calcium-dependent effect of vitamin D deficiency on insulin receptor expression or signaling, as these parameters were not investigated. Furthermore, because the number of vitamin D deficient healthy controls was too small to include in subsequent analyses (n = 5, Figure 1a), this study specifically focused on the impact of vitamin D deficiency for obese children. Whether vitamin D deficiency is also associated with enhanced systemic inflammation in non-obese children requires further investigation. Next, food

intake as well as outdoor activity and exercise levels, which all can influence 25(OH)D levels (1), were not registered during this study. Lastly, due to its cross-sectional design, a causal relationship between enhanced systemic inflammation and insulin resistance could not yet be established. Prospective studies are needed to that end. As a proof-of-concept study though, our data provide novel insights in the pathophysiological mechanisms that may link vitamin D deficiency to insulin resistance. Moreover, this study underscores the consequences of vitamin D deficiency in childhood obesity, which seem to exceed mere effects on calcium metabolism and bone growth (10, 30).

Finally, this study is of value for the assessment of vitamin D supplementation studies in obese children. In analogy with a recent study in adults with T2D, which showed reduced expression of inflammatory markers such as RBP-4 and enhanced insulin sensitivity upon vitamin D supplementation (7), supplementation studies in obese children are currently ongoing (e.g. NCT01386736, NCT00994396, NCT00858247, NCT01217840, www.clinicaltrials.gov). These prospective studies could further establish the relationship between vitamin D deficiency, systemic inflammation and insulin resistance. More importantly, vitamin D supplementation may provide a novel avenue for the prevention of T2D and atherosclerosis in obese children in the near-future (31).

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SUPPLEMENTAL INFORMATION

Vitamin D deficiency in childhood obesity is associated with high levels of circulating inflammatory mediators, and low insulin sensitivity

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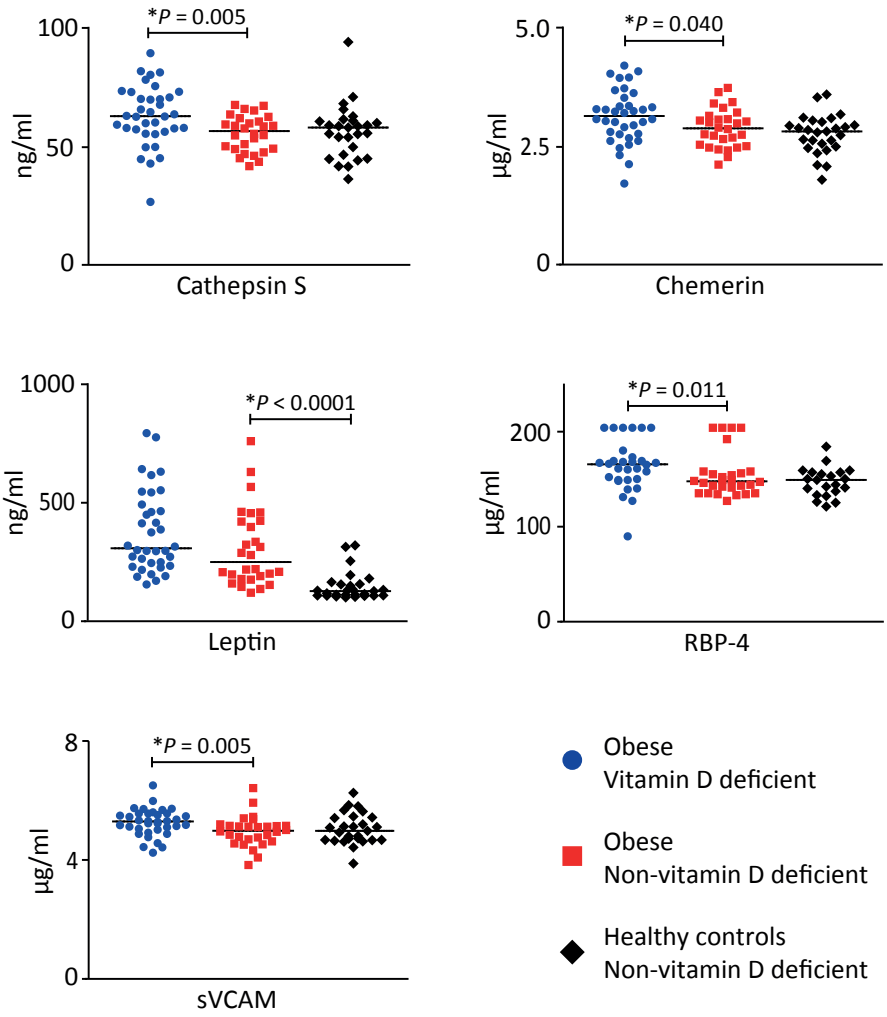

Supplemental Figure 1 Circulating inflammatory mediators
Levels of 5 inflammatory mediators in the three groups. Four of these inflammatory mediators (cathepsin S, chemerin, RBP-4 and sVCAM) showed significantly higher levels in the vitamin D deficient obese group, as compared to the non-vitamin D deficient obese children, but no differences between non-vitamin D deficient obese and healthy control children. Leptin levels, on the contrary, were significantly lower in the non-vitamin D deficient healthy control children, but showed no significant differences between the two obese groups. Lines represent medians. For RBP-4, 10 values were above detection limit, and have been set to the upper detection limit.

Supplemental table 1 Multiple linear regression analysis

Model: Insulin sensitivity (QUICKI) =

$\beta_0 + \beta_1$* Vitamin D status + β_2 * BMI-SD + β_3 * Age + β_4 * Sex + β_5 * Undercarboxylated osteocalcin + β_6 * Skin tone

Independent variable	Standardized β	Significance (*P* value)
Vitamin D status	0.188	0.045
BMI-SD	-0.591	<0.001
Age	-0.171	0.030
Sex	-0.129	0.101
Undercarboxylated osteocalcin	0.051	0.530
Skin tone	0.072	0.397

Supplemental Table 1. Multiple linear regression analysis of the insulin sensitivity check index (QUICKI) with the independent variables vitamin D status, BMI-SD, age, and sex, and the potential confounders undercarboxylated osteocalcin and skin tone. As shown, QUICKI depends significantly on vitamin D status, BMI-SD and age, but not on sex nor the potential confounders undercarboxylated osteocalcin and skin tone. R^2 of the model: 0.555.

CHAPTER 8

General discussion: Childhood obesity as an inflammatory disorder

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Submitted

ABSTRACT

Childhood obesity has a deleterious impact on population health. As obesity is increasingly considered as an inflammatory disorder, we provide an immunological perspective on childhood obesity, and discuss the potential of immune modulation in childhood obesity. First, adipose tissue (AT) is presented as the sentinel of obesity-induced inflammation. In obesity, lipid spillover and adipocyte stress induce adipose tissue (AT) inflammation. Through the release of inflammatory lipids and adipokines, AT inflammation spreads to other tissues such as the liver and cardiovascular system. The resulting low-grade systemic inflammation comprises a pivotal link between obesity and its metabolic and cardiovascular complications. Second, potential immune modulatory interventions in childhood obesity, ranging from recombinant IL-1 receptor antagonists to salsalate treatment, vitamin D supplementation and dietary manipulation of gut microbiota, are reviewed. Finally, advantages and disadvantages of immune modulation will be weighed. While immune modulatory interventions provide promising avenues for the prevention of metabolic and cardiovascular complications in the near-future, their safety, cost-effectiveness and implementation in the integrative treatment of childhood obesity require careful consideration.

INTRODUCTION

The world is caught in an obesity epidemic and unfortunately, children hardly stay behind. Over the last three decades, the prevalence of childhood obesity increased in most developed and developing countries worldwide (1, 2). In 2009-2010, the prevalence of obesity in US children and adolescents reached a peak of 16.9% (3). Though the prevalence may be leveling off in some countries (3, 4), childhood obesity has a vast impact on population health. It is associated with psychosocial complications, orthopaedic complaints, and an increased risk of type 2 diabetes, cardiovascular disease and premature death later in life, among others (5-7).

Treatment options for childhood obesity include behavioral programs, lifestyle interventions, pharmacological treatment with lipase or serotonin inhibitors, and surgical treatment (6). A recent Cochrane review concludes that combined behavioral lifestyle programs produce a variable but clinically meaningful reduction in weight loss compared to standard care or self-help (8). The addition of lipase or serotonin inhibitors slightly improves the outcome, but is associated with adverse effects such as oily stool (42%), abdominal pain (11%) and faecal incontinence (9%) for lipase inhibitors, and tachycardia (6%), dry mouth (5%) and constipation (5%) for serotonin inhibitors (6, 8). For surgical treatment, long-term prospective studies are required to establish whether the resulting weight loss outweighs risks of surgical complications and life-long nutritional deficiencies (6). Considering the impact of childhood obesity and the limitations of current interventions, novel treatment options are urgently needed. In order to develop these, research into mechanisms linking childhood obesity to its metabolic and cardiovascular complications, as important contributors to morbidity and mortality, is imperative (5, 7, 9).

In the last decade, promising advancements were made in the field of immunometabolism, which investigates the interface between metabolism and immunology (10). In fact, obesity is increasingly considered as an inflammatory disorder (11). Obesity induces inflammation of adipose tissue (AT) (12-14). Through the release of inflammatory lipids and immune modulatory proteins called adipokines (15), AT inflammation spreads to other tissues ranging from liver and muscle to the cardiovascular system and circulating immune cells, and thus drives low-grade systemic inflammation (10-13). The low-grade systemic inflammation provides a pivotal link between obesity and its metabolic and cardiovascular complications (9, 10). In mice and adults, anti-inflammatory treatment options for obesity were already explored, and indicated that suppression of AT inflammation or systemic inflammation in obesity prevents the development of its metabolic sequelae (16-18). Thus, immune modulatory interventions are promising novel treatment options for obese patients. Here, we will discuss the relevance and potential of immune modulation in childhood obesity.

ADIPOSE TISSUE INFLAMMATION IN OBESITY

AT has long been considered a neutral lipid storage organ, evolved to store energy as triglycerides in times of excess and release energy as fatty acids when needed. Over

the last decades however, AT emerged as a pivotal player in whole-body glucose and lipid homeostasis. Four interconnected mechanisms appear to play an important role.

First, AT has an active role in food intake and energy expenditure. In fact, food craving, energy expenditure and adiposity are all related to adipocyte numbers, which are significantly enhanced in obesity (19). Adipocyte numbers are set during childhood and hardly change in lean and obese adults, even after marked weight loss (19). Thus, childhood is a window of opportunity for the prevention of adipocyte hyperplasia, i.e. high adipocyte numbers, and the prevention of adult obesity. In line with this observation, longitudinal studies show that childhood obesity is one of the best predictors for adult obesity (20). The metabolic effects of adipocyte hyperplasia have been attributed to the endocrine role of adipocytes. Adipocytes are potent producers of the endocrine mediator leptin, which decreases appetite and increases energy expenditure (15). Adipocyte hyperplasia is associated with prolonged hyperleptinemia, which induces leptin resistance and opposes leptin action (21). Taken together, the association between adipocyte hyperplasia and obesity is partly explained by leptin resistance, which increases food craving and decreases energy expenditure.

Second, obesity is associated with increased triglyceride storage in adipocytes, leading to adipocyte hypertrophy (22). Adipocytes have a remarkable capacity to synthesize and store triglycerides upon feeding. During short-term high caloric feeding, adipocytes quickly increase triglyceride synthesis and reduce circulating lipid levels (12). Upon prolonged high caloric feeding though, adipocyte triglyceride synthesis decreases and lipolysis increases, leading to hyperlipidemia. Adipocyte stress, driven by lipid spillover and adipocyte hypertrophy, seems to underlie this phenomenon (12, 23). Adipocyte stress not only aggravates lipid spillover through increased lipolysis, but also enhances the secretion of inflammatory proteins such as IL-1β, TNF-α and monocyte chemoattractant protein 1 (MCP-1) (12, 24). Both the increased lipid spillover and inflammatory adipokine release are key mediators in the development of insulin resistance (15, 25). In summary, AT inflammation and insulin resistance are induced by hypertrophic, stressed adipocytes through the release of inflammatory lipids and adipokines.

Third, adipocyte stress propagates AT inflammation via the recruitment and activation of AT-resident immune cells. The role of AT-resident immune cells was first recognized a decade ago, with the discovery of accumulated inflammatory macrophages in obese AT (26). Since then, multiple AT-resident immune cell populations have been identified (27). The interactions between adipocytes and AT-resident immune cells culminate in so-called crown-like structures (CLS), consisting of apoptotic hypertrophic adipocytes encircled by AT macrophages (ATMs), mast cells and probably also other immune cells (26). In obese AT, the number of CLS increases together with the number of hypertrophic adipocytes, and correlates with the development of AT inflammation, systemic inflammation, and insulin resistance (26, 28). Thus, the recruitment and activation of inflammatory immune cells in obese AT aggravates obesity-associated AT and systemic inflammation.

Fourth, the released inflammatory lipids and adipokines reach the circulation and this way spread AT inflammation to other tissues, including the liver, immune cells and cardiovascular system (12, 15). Thereby, the inflammatory cascades initiated by hypertrophic

and stressed adipocytes in obese AT finally result in low-grade systemic inflammation, which links obesity to its metabolic and cardiovascular complications (Figure 1).

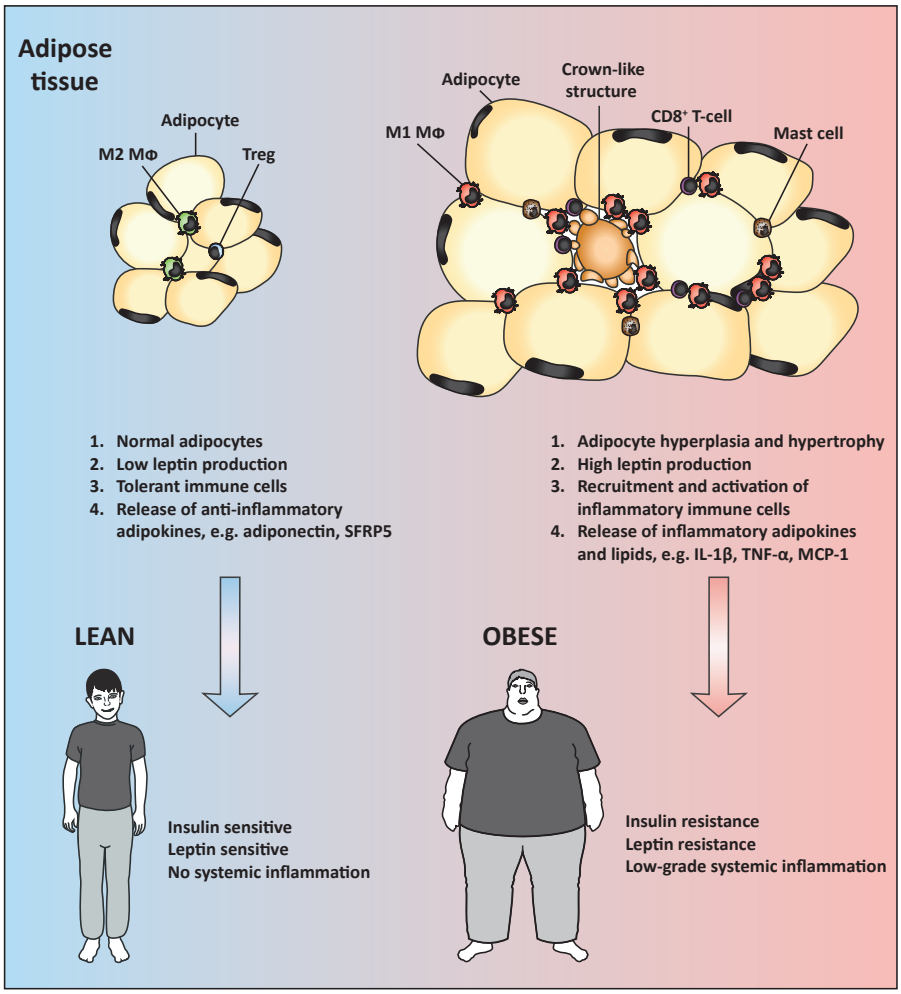

Figure 1 Adipose tissue inflammation in obesity
Schematic representation of adipose tissue (AT) in lean and obese subjects, depicting the four interconnected mechanisms that underlie AT-inflammation in obese subjects. First, AT in obese subjects contains higher adipocyte numbers (hyperplasia) and enlarged adipocytes (hypertrophy) than in lean subjects. Second, adipocyte hyperplasia and hypertrophy in obesity lead to a high leptin release in AT, and eventually to systemic leptin resistance. Third, tolerant immune cells in lean AT (M2 Mϕ, anti-inflammatory M2 macrophages; Treg, regulatory T-cells) are replaced by inflammatory immune cells in obese AT (M1 Mϕ, inflammatory M1 macrophages; mast cells, $CD8^+$ T-cells), part of which encircles apoptotic adipocytes in so-called crown-like structures (CLS). Fourth, inflammatory lipids and adipokines released by obese AT spread inflammation to other tissues such as the liver and cardiovascular system, and propagate low-grade systemic inflammation. Together, these four mechanisms importantly contribute to the insulin resistance, leptin resistance and low-grade systemic inflammation observed in obese subjects.

The discovery that inflammatory mechanisms affect AT control of whole-body glucose and lipid homeostasis has far-reaching consequences. In fact, obesity is increasingly considered as an inflammatory disorder, next to a metabolic disease (13). The link between obesity, AT inflammation and low-grade systemic inflammation is confirmed in numerous clinical studies, mostly in adults (29), but also in obese children. Already in pre-pubertal children, obesity is associated with an influx of inflammatory macrophages and lymphocytes (9, 30). Moreover, obese children from 3 years and older have elevated levels of circulating inflammatory mediators such as CRP, leptin, TNF-α, IL-6, MCP-1 and the endothelial adhesion molecule VCAM (9, 31). Taken together, clinical studies in children and adults underscore the classification of obesity as an inflammatory disorder, with AT as the sentinel of obesity-induced inflammation driving systemic inflammatory cascades. More importantly, these clinical studies suggest that suppressing inflammation may reduce the development of metabolic and cardiovascular complications in obesity. Here, we will focus on the potential of immune modulatory interventions in childhood obesity.

IMMUNE MODULATION IN OBESITY

Immune modulatory interventions for the prevention of metabolic and cardiovascular complications can be divided in three groups: biological agents, other immune modulating agents, and dietary manipulation of gut microbiota.

Biological agents

As TNF-α, IL-1β and IL-6 play an important role in the development of both AT and systemic inflammation (15), they are candidate targets for intervention. Moreover, registered biological agents that block the activity of these cytokines are available. While metabolic and cardiovascular effects of biological agents in pediatric patient groups are still under investigation, studies in adults showed promising results.

TNF-α inhibitors (infliximab, etanercept) are for instance used for rheumatoid arthritis (RA) treatment, and known to improve metabolic and cardiovascular outcome as well as clinical parameters of RA in adults (32-34). Recent studies suggest that TNF-α inhibition also ameliorates glycemic parameters in obese adults, and patients with metabolic syndrome (35, 36). In obese adults with type 2 diabetes though, TNF-α blocking did not improve insulin sensitivity (29, 37). Furthermore, some studies suggested that high doses of TNF-α inhibitors can aggravate pre-existing congestive heart failure (38, 39). Clearly, more data are necessary, and wide-spread use of TNF-α inhibitors in RA may enable evaluation of their long-term effects in the near future.

For IL-1 inhibition, a recombinant human IL-1 receptor antagonist (IL-1RA, anakinra) is available, with other biological agents in late stages of development. While cardiovascular outcome data from RA registries are still scarce (39), a randomized controlled trial showed reduced systemic inflammation and improved glycemia and beta-cell secretory function in obese adults with type 2 diabetes (18). Reduced systemic inflammation and improved beta-cell secretory function sustained upon

treatment withdrawal, as shown in a 39-week follow-up study (40). Again though, long-term efficacy and side-effects require further study.

For IL-6 inhibition, clinical data are available since the introduction of a monoclonal antibody against the IL-6 receptor (tocilizumab) in 2009. A few reports suggested improved glycemic parameters in adults with RA after treatment with tocilizumab (39, 41). Yet the effects of IL-6 blocking are controversial, as the IL-6 release by muscle during exercise enhances insulin sensitivity, which suggests that IL-6 can also exhibit beneficial effects on glucose metabolism (42). Furthermore, IL-6-deficient mice develop obesity and insulin resistance, while weight gain and dyslipidemia were observed in clinical studies with tocilizumab (43). Thus, IL-6 blocking may have serious side-effects.

Taken together, IL-1 receptor antagonists so far seem the most promising biologicals to improve parameters of glucose control in obesity. However, long-term efficacy and side-effects require further study, especially in pediatric patient groups. Both the side-effects and high costs may be an irreconcilable disadvantage for the use of biological agents in childhood obesity.

Other immune modulating agents: salsalate and vitamin D

A second group of immune modulating agents consists of salicylate derivates and vitamin D. High doses of salicylates can inhibit the inflammatory IKKβ/NF-κB axis, and are since long known to ameliorate glycemic parameters in patients with type 2 diabetes (44-46). As acetylated salicylates (aspirin) also inhibit cyclooxygenase enzymes, resulting in side-effects such as prolonged bleeding time, non-acetylated salicylates appear advantageous (47). Of the non-acetylated salicylates, salsalate is preferred as it does not compromise the gastric mucosal layer (47). Recently, salsalate treatment was shown to reduce systemic inflammation and improve glycemic parameters, both in obese adults with and without type 2 diabetes (17, 47-49). Considering that salsalate is generic and inexpensive, and has an established safety profile, it seems a promising treatment option for childhood obesity.

Next, vitamin D supplementation is proposed as an immune modulating intervention in obesity, with beneficial effects on glucose homeostasis. Obesity is associated with a high prevalence of vitamin D deficiency, both in children and adults (50, 51). The high prevalence seems related to increased sequestration of vitamin D in obese AT, which decreases its bioavailability, but other mechanisms may also be involved (50). Recently, it was shown that vitamin D deficiency in obese children is associated with the development of insulin resistance, independent of measures for adiposity (52). Similarly, vitamin D deficiency in adults was associated with the development of metabolic syndrome (53). Supplementation of vitamin D was shown to suppress circulating inflammatory mediators in adults with type 2 diabetes, in accordance with the established immune modulatory effects of vitamin D (54-56). In line with this finding, combined vitamin D and calcium supplementation in pre-diabetic adults improved glycemic parameters (57, 58). Taken together, combined vitamin D and calcium supplementation in obese children may help to suppress inflammation and thereby improve glycemic parameters, in addition to its beneficial effects on bone health (59, 60).

Dietary manipulation of gut microbiota

Finally, gut microbiota emerged as a potential therapeutic target for weight loss and immune modulation in obesity. The relevance of gut microbiota is illustrated by germ-free mice, which are protected against diet-induced obesity (61). The protection against obesity is partly explained by digestive functions of microbiota, including the production of short-chain fatty acids that comprise substrates for gluconeogenesis and lipogenesis upon uptake (62). Several studies reported reduced amounts of intestinal Bacteroidetes species and *Faecalibacterium prausnitzii* in obese patients (62). Skewing of the microbiota may underlie the enhanced expression of microbiota-derived genes involved in nutrient harvesting, which is observed in obese patients (63). Next to their role in energy harvesting, microbiota also promote low-grade systemic inflammation in obesity. Obesity is associated with increased translocation of endotoxins from the gut, either via uptake with chylomicrons or via increased gut permeability, which confers a state of metabolic endotoxemia (64). The endotoxemia contributes to the low-grade systemic inflammation and insulin resistance in obesity (65), and can be alleviated by antibiotic treatment (66). Recently, non-digestible food ingredients (prebiotics) and bacteriocin-producing microorganisms (probiotics) were shown to improve the composition of gut microbiota in obese mice (67, 68). In addition, prebiotics reduced weight gain and systemic inflammation in obese mice, while glycemic parameters improved (67). In obese patients, changes in gut microbiota composition upon treatment with prebiotics or probiotics have not yet been reported, but reduced weight gain and systemic inflammation together with improved glycemic parameters are frequently observed (69). In conclusion, the effects of prebiotics and probiotics on human gut microbiota need to be assessed. Yet dietary manipulation of gut microbiota may provide novel avenues for the treatment of obesity.

FUTURE PERSPECTIVE ON CHILDHOOD OBESITY

The emerging concept of obesity as an inflammatory disorder has evoked a search for immune modulatory interventions that could break the link between obesity and its metabolic and cardiovascular complications. Here, we reviewed the potential of biologicals, salsalate and vitamin D, and immune modulating diets to that end. With respect to the biologicals, recombinant IL-1RA showed the most promising results in obese adults. Several randomized controlled trials (RCTs) addressing the safety and efficacy of IL-1RA and other IL-1β inhibitors in adults are currently ongoing (Table 1). A large disadvantage though, lies in the costs of biologicals such as IL-1RA. In contrast, salsalate treatment, vitamin D supplementation and dietary interventions have an established safety profile, and come at much lower costs. Salsalate treatment does not enhance bleeding risk nor compromise the gastric mucosal layer, unlike other salicylates (47). While earlier studies addressed the efficacy of 14-weeks salsalate treatment in adults with type 2 diabetes (17), RCTs into the long-term effects of salsalate on glycemic and cardiovascular parameters in adults with type 2 diabetes are

currently performed (Table 1). Vitamin D supplementation and dietary interventions may even be closer to application in childhood obesity, as intervention trials for these immune modulatory treatments are already ongoing in obese children and adolescents (Table 1). Interestingly, the listed prebiotic and probiotic intervention trials combine mapping the gut microbiota and studying metabolic and inflammatory parameters upon intervention (Table 1). Taken together, while the efficacy of biologicals and salsalate in childhood obesity requires further study, vitamin D supplementation and probiotics in childhood obesity are already under investigation.

In conclusion, fundamental research and clinical studies revealed that low-grade systemic inflammation is prevalent in childhood obesity. Accordingly, immune modulatory interventions provide promising avenues for the prevention of metabolic and cardiovascular complications of childhood obesity in the near-future. From a

Table 1 Immune modulatory intervention trials for the prevention of metabolic and cardiovascular complications

Treatment	Trial phase	Subjects	Number of subjects	Treatment duration (weeks)	Reference
Biological agents					
IL-1RA	II	Adults with T2DM	12	4	NCT00928876
IL-1β antibody	II	Adults with T2DM	140	48	NCT00995930
IL-1β antibody	II	Adults with T2DM	80	48	NCT01144975
IL-1β antibody	II	Adults with T2DM	325	26	NCT01066715
Other agents					
Salsalate	II	Adults with T2DM	80	12	NCT00330733
Salsalate	III	Adults with T2DM	284	48	NCT00799643
Salsalate	III	Adults with T2DM	278	130	NCT00624923
Vitamin D	IV	Obese children (7+)	110	18	NCT01386736
Vitamin D	III	Obese adolescents	40	26	NCT00994396
Vitamin D	III	Obese adolescents	130	12	NCT00858247
Vitamin D	III	Obese adolescents	40	24	NCT01217840
Vitamin D + calcium	III	Adolescents	30	8	NCT01107808
Dietary interventions					
Prebiotic/probiotic	III	Obese adults	60	6	NCT01433120
Probiotic	III	Obese adolescents	51	52	NCT01020617

Table 1. To survey ongoing immune modulatory interventions, www.clinicaltrials.gov was consulted. For biological agents and salsalate, no trials in children or adolescents are currently performed. With respect to vitamin D supplementation, only trials in children and adolescents are listed. For prebiotic and probiotic interventions, only studies investigating the effects on gut microbiota together with metabolic or cardiovascular outcome are listed. T2DM, type 2 diabetes mellitus. 7+, aged 7 years and older.

clinician's perspective though, these novel treatment options evoke challenging questions. At this moment, obese children have a clear incentive to lose weight. What will happen to this incentive when immune modulatory interventions could prevent metabolic and cardiovascular complications without losing weight? Finally, next to metabolic and cardiovascular complications, childhood obesity has adverse effects on almost every organ (6). What is the best way to treat complications that are not targeted by immune modulation, such as psychosocial problems and orthopaedic complaints? Summarizing, immune modulatory interventions show promise for the near-future, but their safety, cost-effectiveness and implementation in the integrative treatment of childhood obesity require careful consideration. In analogy to surgical interventions in childhood obesity which are commonly reserved for treatment-resistant children (6), immune modulatory interventions may be most effective as a second or third line of therapy. At last, it is encouraging to know that novel treatment options seem to be on their way.

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SUMMARY & SAMENVATTING

SUMMARY

The worldwide prevalence of childhood obesity increased dramatically over the last three decades, in the wake of a global obesity epidemic. The US bears the palm in the developed world, with 16.9% obese children in 2009-2010, and worrying projections for the coming decades. Childhood obesity has a deleterious impact on population health, as it is associated with psychosocial problems, orthopaedic complaints, and an increased risk of type 2 diabetes, cardiovascular disease and premature death later in life. Considering this impact and the limitations of current interventions, novel treatment options are urgently needed. In order to develop these, research into mechanisms linking obesity to its metabolic and cardiovascular complications is imperative. This thesis aims at exploring a promising novel avenue for intervention: the immunological role of adipose tissue.

In **chapter 1**, current knowledge on the immunological role of adipose tissue (AT) is summarized. AT is far more than a lipid storage organ. In lower organisms such as *Drosophila melanogaster*, the so-called 'fat body' comprises lipid storage and immunological moieties in one functional unit. AT in higher organisms such as mice and humans is reminiscent of the integrated functionality seen in lower organisms. Adipocytes secrete various immune modulating proteins (adipokines) that play a key role in whole-body energy homeostasis as well as immunological processes. Furthermore, the enlargement of adipocytes seen in obesity (adipocyte hypertrophy) serves to enhance lipid storage, and activates a plethora of inflammatory cascades in parallel. Finally, AT hosts multiple immune cell types, and actively recruits inflammatory immune cells in obesity. Taken together, in obesity, AT propagates local and systemic inflammation via distinct inflammatory mechanisms.

In the first part of this thesis, we focus on specific inflammatory mechanisms in AT. In **chapter 2**, the role of AT-resident immune cells is reviewed. In lean individuals, AT-resident immune cells have housekeeping functions ranging from apoptotic cell clearance to extra-cellular matrix remodeling and angiogenesis. Obesity however provides metabolic and bacterial danger signals that mimic bacterial infection, and drive a shift towards pro-inflammatory immune cells. This inflammatory shift may partly be explained by the 'energy-on-demand' model: in case of life-threatening bacterial infections, temporary AT inflammation and the resulting insulin resistance can serve to mobilize nutrients, and fuel the activated immune system. Hence *Homo sapiens* seems not up to the prolonged presentation of metabolic and bacterial danger signals in obesity, which drives prolonged local and systemic inflammation. The chronic low-grade inflammation in obese patients propagates insulin resistance, and underlies many metabolic and cardiovascular complications seen in obese patients. **Chapter 3** highlights the pivotal role of invariant natural killer T (iNKT) cells in AT homeostasis, and the prevention of AT inflammation and insulin resistance. We show that iNKT cell-deficient mice on a low-fat diet, considered a normal diet for mice, display a distinctive phenotype characterized by adipocyte dysfunction and insulin resistance. Moreover, AT-resident iNKT cells have an anti-inflammatory phenotype, and can interact with adipocytes in a CD1d-mediated fashion. These findings fuel the hypothesis that adipocytes, via lipid presentation to

iNKT cells, exert control over the local immune response in AT. The adipocyte-iNKT cell interactions seem to play a key role in the maintenance of healthy AT under normal conditions. In obesity however, AT-resident iNKT cell numbers decrease, dysfunctional adipocytes take center stage, and AT inflammation and insulin resistance arise.

In the second part of this thesis, we focus on the systemic effects of AT inflammation. In order to study AT-derived circulating inflammatory mediators, we developed and validated a multiplex immunoassay (MIA) for adipokines, which is reported in **chapter 4**. The adipokine MIA enables rapid and high-throughput measurement of more than 25 adipokines in only 50 µL of blood, cell supernatant, or other biological samples. In **chapter 5**, we test the adipokine MIA in children with onset and long-standing type 1 diabetes (T1D), together with healthy controls. The onset and long-standing T1D patients show increased levels of circulating adipokines acting at the crossroads of metabolism and inflammation, including monocyte chemoattractant protein-1 (MCP-1) and the novel adipokines cathepsin S, chemerin, and tissue inhibitor of metalloproteinase-1 (TIMP-1). Moreover, onset and long-standing diabetic plasma induce preadipocyte proliferation and adipocyte differentiation *in vitro*. Taken together, analogous to the role of AT in obesity, AT seems also involved in the low-grade systemic inflammation observed in children with T1D. In **chapter 6**, the low-grade systemic inflammation in childhood obesity is studied in detail. Circulating inflammatory mediator and immune cell profiles of obese children are compared with age- and sex-matched healthy controls. First, next to leptin, the circulating mediators chemerin, TIMP-1, EGF and TNF receptor 2 are identified as novel inflammatory mediators that are increased in childhood obesity. Second, cluster analysis of the circulating mediators shows a correlation of systemic inflammation with clinical variables such as insulin resistance. Third, activation of $CD14^{++}$ monocytes, which is associated with increased atherosclerosis in obese adults, is readily detectable in obese children. Thus, the circulating inflammatory mediators and activated $CD14^{++}$ monocytes seem part of the inflammatory link between obesity and its metabolic and cardiovascular complications.

In the third part of this thesis, the implications of the immunological role of AT for childhood obesity are discussed in detail. As vitamin D exhibits profound immunomodulatory functions, the impact of vitamin D deficiency in childhood obesity is studied in **chapter 7**. Vitamin D deficiency is highly prevalent in obese children (56%) compared with healthy controls (16%), and is associated with enhanced systemic inflammation together with insulin resistance. The high cathepsin S and sVCAM levels observed in vitamin D deficient obese children may reflect activation of a pro-inflammatory, pro-diabetic and atherogenic pathway that could be inhibited by vitamin D supplementation. Finally, **chapter 8** discusses potential immune modulatory interventions in childhood obesity. Intervention trials studying vitamin D supplementation and dietary manipulation of gut microbiota in childhood obesity are currently ongoing, and may be close to application in obese children. Furthermore, recombinant IL-1RA and salsalate are known to suppress systemic inflammation in obese adults, and to ameliorate glycemic parameters. Though their safety, cost-effectiveness and implementation in the integrative treatment of childhood obesity require careful consideration, immune modulatory interventions provide a promising novel avenue for the prevention of metabolic and cardiovascular complications in childhood obesity in the near-future.

SAMENVATTING

Wereldwijd is het aantal kinderen met obesitas de afgelopen 3 decennia sterk toegenomen, in het kielzog van de obesitas epidemie bij volwassenen. De VS spant van alle westerse landen de kroon, met 16.9% obese kinderen in 2009-2010, en een verwachte verdere stijging de aankomende jaren. De toename van obesitas bij kinderen heeft ernstige gezondheidseffecten, aangezien obesitas is geassocieerd met psychosociale problemen, orthopedische klachten, en een toegenomen risico op type 2 diabetes (T2D), hart- en vaatziekten en vroegtijdig overlijden op latere leeftijd. Vanwege de gezondheidsproblemen en tekortschietende behandelmogelijkheden op dit moment is wetenschappelijk onderzoek naar nieuwe behandelmogelijkheden hard nodig. Hiervoor is onderzoek naar de mechanismes die leiden tot T2D en hart- en vaatziekten essentieel. Dit proefschrift presenteert een nieuwe invalshoek: de immunologische rol van vetweefsel.

In **hoofdstuk 1** wordt de immunologische rol van vetweefsel geïntroduceerd. Vetweefsel is veel meer dan alleen een orgaan voor vetopslag. De 'fat body' in lagere organismen zoals *Drosophila melanogaster* (fruitvlieg) dient voor vetopslag, en speelt tegelijkertijd een centrale rol in het afweersysteem. Vetweefsel in hogere organismen zoals muis en mens is verder gedifferentieerd, maar heeft nog steeds immunologische functies. Adipocyten (vetcellen) secreteren verschillende immuno-actieve stoffen (adipokines), die een belangrijke rol spelen in glucose metabolisme én immunologische processen. Daarnaast leidt vetcelhypertrofie (grote vetcellen) bij obesitas tot de activatie van verschillende ontstekingsprocessen. Ten slotte rekruteert vetweefsel bij obesitas verschillende inflammatoire immuuncellen, die de vetweefselontsteking bij obesitas versterken. Vetweefsel speelt zo een centrale rol bij de locale en systemische inflammatie die gezien wordt bij obesitas.

Het eerste deel van dit proefschrift richt zich op specifieke inflammatoire mechanismes in vetweefsel. In **hoofdstuk 2** wordt de rol van vetweefsel-specifieke immuuncellen besproken. In normale individuen vervullen deze immuuncellen een soort 'onderhoudsdienst': ze zorgen voor angiogenese (de aanmaak van nieuwe bloedvaten), spelen een rol in de modellering van extracellulaire matrix (de ruimte tussen cellen), en ruimen dode cellen op. Bij obesitas komen er echter metabole en bacteriële componenten vrij die lijken op een bacteriële infectie, en die de immuuncellen prikkelen om in plaats van onderhoudsfuncties een ontstekingsproces op gang te brengen. Deze inflammatoire verandering kan worden verklaard met behulp van het zogenaamde 'energy-on-demand' model: ontsteking van vetweefsel bij een bacteriële infectie lijkt bedoeld om voedingsstoffen te mobiliseren die het energie-behoeftige immuunsysteem kunnen voeden. Zo kan een bacteriële invasie snel het hoofd worden geboden. De mens lijkt echter niet opgewassen tegen de langdurige blootstelling aan metabole en bacteriële prikkels bij obesitas, die leiden tot langdurige ontsteking van het vetweefsel. De chronische ontsteking in obese patiënten leidt onder andere tot de ontwikkeling van T2D en hart- en vaatziekten. **Hoofdstuk 3** richt zich op de beschermende rol van 'invariant natural killer T' (iNKT) cellen in vetweefsel. Zij communiceren direct met vetcellen door vetten te

presenteren op het CD1d-eiwit, en onderdrukken ontsteking in vetweefsel. Muizen zonder iNKT cellen hebben slecht functionerende vetcellen, en ontwikkelen T2D. Deze bevindingen ondersteunen de hypothese dat vetcellen, door communicatie met de iNKT cellen, de immuuncellen in het vetweefsel aansturen. De interactie tussen vetcellen en iNKT cellen lijkt een belangrijke rol te spelen bij het gezond houden van vetweefsel onder normale omstandigheden. Bij obesitas neemt het aantal iNKT cellen in vetweefsel snel af, ontstaan grote en disfunctionele vetcellen, en raakt het vetweefsel ontstoken.

In het tweede deel van dit proefschrift richten we ons op de systemische effecten van vetweefselontsteking. Om de circulerende ontstekingsstoffen uit vetweefsel te kunnen bestuderen hebben we allereerst een multiplex immunoassay (MIA) ontwikkeld, die in staat stelt om snel meer dan 25 adipokines te meten in slechts 50 µL bloed, cel supernatant, of andere biologische monsters. De ontwikkeling en validatie van deze techniek wordt beschreven in **hoofdstuk 4**. Vervolgens hebben we deze techniek getest in kinderen met type 1 diabetes, zoals beschreven wordt in **hoofstuk 5**. Zowel kinderen die kort als lang type 1 diabetes hebben, laten in vergelijking met gezonde kinderen verhoogde spiegels adipokines zien in hun bloed, waaronder monocyte chemoattractant protein-1 (MCP-1), en de nieuwe adipokines cathepsine S, chemerine, en tissue inhibitor of metalloproteinase-1 (TIMP-1). Daarnaast stimuleert bloedplasma van kinderen met type 1 diabetes de groei en ontwikkeling van vetcellen *in vitro*. Vetweefsel lijkt zo een belangrijke rol te spelen in de ontwikkeling van ontsteking bij kinderen met type 1 diabetes, net als bij obesitas. In **hoofdstuk 6** wordt de systemische ontsteking bij kinderen met obesitas uitgebreid in kaart gebracht. Circulerende adipokines en immuuncellen van obese kinderen worden vergeleken met gezonde kinderen. In de eerste plaats worden naast de bekende hoge leptine spiegels ook verhoogde spiegels van de minder bekende adipokines TIMP-1, EGF en TNF receptor 2 gevonden bij obese kinderen. Ten tweede laat cluster analyse van alle circulerende adipokines een correlatie zien tussen ontsteking en insuline ongevoeligheid, naast andere klinische variabelen. Ten slotte worden bij de obese kinderen geactiveerde $CD14^{++}$ monocyten gevonden, die geassocieerd zijn met de ontwikkeling van hart- en vaatziekten bij volwassenen. Concluderend, de systemische ontsteking bij kinderen met obesitas lijkt een belangrijke schakel tussen obesitas en de ontwikkeling van T2D en hart- en vaatziekten op latere leeftijd.

In het derde deel van dit proefschrift worden de implicaties van de immunologische rol van vetweefsel voor kinderen met obesitas besproken. In **hoofdstuk 7** richten wij ons specifiek op obese kinderen met een vitamine D deficiëntie (te lage vitamine D spiegel). Vitamine D onderdrukt ontsteking. Helaas heeft ongeveer de helft van de obese kinderen vitamine D deficiëntie, wat is geassocieerd met toegenomen ontsteking en insuline ongevoeligheid. Vitamine D toediening zouden kunnen helpen om de ontsteking in obese kinderen te onderdrukken, en de insulinegevoeligheid te verhogen. Met name spiegels van de ontstekingsstoffen cathepsine S en sVCAM kunnen mogelijk worden verlaagd door toediening van vitamine D tabletten. Ten slotte worden in **hoofdstuk 8** potentiële immuun-modulatoire interventies besproken

voor kinderen met obesitas. Interventiestudies naar vitamine D toediening bij kinderen met obesitas, en studies om door middel van probiotica en prebiotica (speciale diëten) de darmbacteriën van obese kinderen te beïnvloeden, worden op dit moment uitgevoerd en kunnen mogelijk over enkele jaren al worden toegepast. Daarnaast is bekend dat recombinant IL-1RA en salsalaat systemische ontsteking in obese volwassenen onderdrukken, en de insulinegevoeligheid verhogen. Hoewel de veiligheid, kosteneffectiviteit en inpassing in de integrale behandeling van kinderen met obesitas nader onderzocht dient te worden, zijn immuun-modulatoire interventies veelbelovend voor de preventie van T2D en hart- en vaatziekten in de nabije toekomst.

ADDENDUM

LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
APC	Antigen presenting cell
AST	Aspartate aminotransferase
AT	Adipose tissue
ATM	Adipose tissue-resident macrophage
αGC	α-Galactosyl ceramide
BCR	B cell receptor
BMI	Body-mass index
BMI-SD	Body-mass index standard deviation for age and sex
BP	Blood pressure
BsP	Bootstrapping probability (%)
CCL	Chemokine (C-C motif) ligand
CCL2	Monocyte chemoattractant protein 1; MCP-1
CCL3	Macrophage inflammatory protein 1α; Mip-1α
CCR	Chemokine (C-C motif) receptor
CLS	Crown-like structures
CV%	Coefficient of variation (%)
CXCL	Chemokine (C-X-C motif) ligand
CXCL10	Interferon gamma-induced protein 10; IP-10
CXCL8	Interleukin 8
CXCR	Chemokine (C-X-C motif) receptor
DC	Dendritic cell
DEXA	Dual energy X-ray absorptiometry
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EN-RAGE	Extracellular newly identified RAGE binding protein
ER	Endoplasmic reticulum
FA	Fatty acid
FABP-4	Fatty acid binding protein 4
FDR	False discovery rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HbA1c	Glycosylated hemoglobin
HC	Healthy control
HFD	High-fat diet
HGF	Hepatocyte growth factor
HOMA-IR	Homeostasis model assessment of insulin resistance
HPE buffer	High performance ELISA buffer
hsCRP	High-sensitivity C-reactive protein
IFN-γ	Interferon gamma
IL	Interleukin

IL-1RA	Interleukin 1 receptor antagonist
iNKT cell	Invariant natural killer T cell
IP-GTT	Intraperitoneal glucose tolerance test
IP-10	Interferon gamma-induced protein 10; CXCL10
LFD	Low-fat diet
LLOD	Lower limit of detection
LLOQ	Lower limit of quantitation
LPS	Lipopolysaccharide
MALT	Mucosa associated lymphoid tissue
MCP-1	Monocyte chemoattractant protein 1; CCL2
M-CSF	Macrophage colony-stimulating factor
MFI	Median fluorescence intensity
MIA	Multiplex immunoassay
MIF	Macrophage migration inhibitory factor
MIP-1α/β	Macrophage inflammatory protein 1α/β
OPN	Osteopontin
PAI-1	Plasminogen activator inhibitor 1
PAT	Perinodal adipose tissue, surrounding lymph nodes
QUICKI	Quantitative insulin sensitivity check index
RBP-4	Retinol binding protein 4
RCT	Randomized controlled trial
SAA-1	Serum amyloid A1
SCAT	Subcutaneous adipose tissue
sCD14	Soluble CD14
SGBS	Simpson-Golabi-Behmel Syndrome
sICAM	Soluble intercellular adhesion molecule
sVCAM	Soluble vascular cell adhesion molecule
TCR	T cell receptor
TG	Triglycerides
TIMP-1	Tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptor
TNF/TNF-α	Tumor necrosis factor α
TNF-R	Tumor necrosis factor receptor
Treg	Regulatory T cell
T1D/T1DM	Type 1 diabetes mellitus
T2D/T2DM	Type 2 diabetes mellitus
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor

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Prijs de HEER, mijn ziel,
prijs, mijn hart, zijn heilige naam.
Prijs de HEER, mijn ziel,
vergeet niet één van zijn weldaden.
Psalm 103:1,2

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CURRICULUM VITAE

Henk Schipper was born the 7th of June in 1981 as the eldest of 11 children in Emmen, in the north of the Netherlands. In 1999 he completed grammar school (*cum laude*) at the Greijdanus College in Zwolle, the Netherlands. Subsequently, he studied medicine and philosophy at the Rijksuniversiteit Groningen. During his medical training he was awarded a personal research grant (De Cock Stichting) to study immune dysregulation in children with *Mycobacterium ulcerans* infections in Ghana, West-Africa, under supervision of prof. dr. T.S. van der Werf. After graduation from medical school in 2006 (*cum laude*), he continued and finished this research project, which strongly encouraged his interest in fundamental immunological research. Furthermore, he was awarded a personal study grant (Stichting Pro Rege) to complete his master philosophy at the Rijksuniversiteit Groningen in 2007, with a specialization in philosophy of medicine & medical ethics, and a thesis on patient autonomy in genetic screening situations, under supervision of prof. dr. M.V.B.P.M van Hees and prof. dr. M.A. Verkerk. In 2008, he started his PhD studies at the departments of paediatric immunology and metabolic diseases, under supervision of prof. dr. A.B.J. Prakken and dr. E. Kalkhoven. Results are presented in this thesis. Henk is married to Emma van Houwelingen, and together they have two sons: Ties (2009) and Jelle (2012). In fact, Henk, Ties and Jelle hope to skate the Elfstedentocht together around 2028.

LIST OF PUBLICATIONS

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