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HOW TO GET FAT

ADIPOSE TISSUE EXPANSION AND METABOLIC HEALTH

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presented by

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Abstract

Obesity, the excessive accumulation of body fat is the key predictor for the metabolic syndrome (MS), and a major global healthcare challenge. The MS is the combination of central obesity with impaired glucose homeostasis as well as the cardiovascular risk factors elevated plasma lipids and elevated blood pressure. Exactly why and how these symptoms cluster together is still incompletely understood. The expansion of adipose tissue (AT) can happen via hyperplasia, which is an increase of the number of adipocytes, or via hypertrophy, which is an increase of their size. Small and plentiful adipocytes are the result of hyperplasia and are associated with good metabolic health, whereas hypertrophic, large adipocytes are associated with poor metabolic health. In my thesis I explored these two different modes of AT expansion and their link to metabolic health.

In the first study I investigated the effect of AT stromal vascular fraction derived extracellular matrix (ECM) on the differentiation of adipocyte precursors residing in AT. We found that the ECM modulates the differentiation of precursors in a tissue specific manner. Visceral (Vis) AT derived ECM reduced the differentiation of precursors when compared to subcutaneous (Sc) AT or brown AT. We were also able to show that this effect is mediated by collagenous ECM and increased upon the addition of vitamin C. Lastly, we identified Flotillin 2 (FLOT2) a membrane-microdomain associated protein, as part of the cellular signalling machinery that reacts to different ECM cues.

The second study focuses on adipose tissue hypertrophy, and how it could be causally involved in metabolic impairments caused by obesity. Systemic glucose tolerance is already impaired after 3 days of high fat diet (HFD) feeding in mice. We analysed insulin dependent AKT phosphorylation in liver, muscle, and Sc and Vis AT at this time point, finding noticeable deficits only in the liver. However, when we measured insulin dependent eNOS-phosphorylation as a surrogate for endothelial insulin sensitivity, we found that Vis endothelium was virtually unresponsive, while in Sc phosphorylation of eNOS was still insulin-responsive, albeit reduced. This correlated with adipocyte hypertrophy which in Vis adipose tissue occurs after a single high fat diet (HFD) meal, and is much more pronounced than Sc after 3 days. We hypothesised that adipocyte hypertrophy is linked to AT endothelial stress and insulin resistance which was supported by detection of increased ROS production in AT, in conjunction with platelet activation. In contrast, treatment with the antiplatelet and vasodilator drug dipyridamole ameliorated glucose tolerance, indicating a functional role for endothelial stress in mediating HFD induced insulin resistance.

In the last project, we tested the effect of different diets on glucose tolerance after 3 days of feeding. There is an on-going controversy between proponents of ketogenic, low carb, and low fat, high carbohydrate diets and people alternately blame sugar or fat on metabolic

impairments. To address this question, we fed animals with a ketogenic diet (KD), a HFD, regular chow or added 5% glucose to the drinking water of chow fed mice, and measured plasma parameters and glucose tolerance after 3 days. To our surprise, we found that both HFD and KD showed impaired glucose tolerance, while animals with added glucose appeared normal.

In summary, I present data showing that the ECM influences tissue specific capacity for precursor differentiation, identified endothelial insulin resistance as a very early event concurring with adipocyte hypertrophy and found that KD and HFD show a similar response on glucose tolerance after very short feeding.

Zusammenfassung

Adipositas, die übermäßige Ansammlung von Körperfett ist der wichtigste Risikofaktor für das metabolische Syndrom (MS), und ein globales Gesundheitsproblem. Das MS ist die Kombination von zentraler Adipositas zusammen mit gestörtem Glukosehaushalt sowie den kardiovaskulären Risikofaktoren erhöhte Blutfette und erhöhter Blutdruck. Wieso genau diese Symptome zusammen auftreten wird noch nicht vollständig verstanden. Der Zuwachs des Fettgewebes (FG) kann über Hyperplasie passieren, was eine Erhöhung der Anzahl von Adipozyten ist, oder über Hypertrophie, einer Erhöhung ihrer Größe. Kleine und viele Adipozyten sind das Ergebnis von Hyperplasie und mit gesundem Metabolismus assoziiert, während hypertrophe, große Adipozyten mit schlechter metabolischen Gesundheit verbunden sind. In meiner Arbeit erkunde ich diese zwei verschiedenen Arten der Fettgewebeexpansion und ihren Zusammenhang mit metabolischer Gesundheit.

In der ersten Studie untersuchte ich die Wirkung von aus der stromal-vaskulären Fraktion des FG gewonnener Extrazellulärmatrix (EZM) auf die Differenzierung von Adipozyten-Vorläufern aus dem Fett. Wir fanden heraus, dass die ECM die Differenzierung von Vorläufern auf gewebespezifische Art und Weise beeinflußt. EZM aus viszeralem (Vis) FG, reduzierte die Differenzierung von Vorläufern im Vergleich zu EZM aus subkutanem (Sk) oder braunem FG. Wir konnten weiters zeigen, dass dieser Effekt durch kollagenhaltige ECM vermittelt, und durch die Zugabe von Vitamin C erhöht wird. Außerdem identifizierten wir Flotillin 2 (FLOT2) ein Membran-Mikrodomänen assoziiertes Protein, als Teil der zellulären Maschinerie, welche die Signale von verschiedenen ECM-Effekten verarbeitet.

Die zweite Studie konzentriert sich auf die Hypertrophie von FG, und wie diese kausal an metabolischen Beeinträchtigungen durch Fettleibigkeit beteiligt sein könnte. Die systemische Glukosetoleranz ist in Mäusen bereits nach 3 Tagen der Hochfett-Diät (HFD)-Fütterung beeinträchtigt. Wir analysierten Insulin-abhängige AKT-Phosphorylierung in Leber, Muskel und Sc und Vis FG zu diesem Zeitpunkt, und fanden nur in der Leber Defekte. Als wir jedoch Insulin-abhängige eNOS-Phosphorylierung als Ersatz für endotheliale Insulinempfindlichkeit bestimmten, fanden wir, dass Vis-Endothel praktisch nicht mehr reagierte, während Sk Phosphorylierung von eNOS noch durch Insulin erhöht wurde, wenngleich die Phosphorylierung insgesamt reduziert war. Dies korrelierte mit der Hypertrophie der Adipozyten, die in Vis Fettgewebe bereits nach einer einzigen Mahlzeit einer Hochfettdiät (HFD) auftrat, und auch nach 3 Tagen HFD in Vis viel stärker ausgeprägt ist als in Sc. Daher stellten wir die Hypothese auf, dass Fettzell-Hypertrophie mit endothelialem Stress und Insulinresistenz zusammenhängt. Erhöhte ROS Produktion im FG nach Hochfettdiät, sowie Aktivierung von Blutplättchen unterstützen diese Hypothese. Weiters führte die Behandlung mit Dipyridamol, einem gerinnungshemmenden und gefäßerweiternden Wirkstoff, zu

verbesserter Glukosetoleranz, was für eine funktionelle Rolle von endothelialem Stress in der HFD-vermittelten Insulinresistenz spricht.

Im letzten Projekt testeten wir die Wirkung von verschiedenen Diäten auf die Glukosetoleranz nach 3 Tagen der Fütterung. Zwischen den Befürwortern von ketogener, Low-Carb Diät, und Low-Fat Kohledydratreichen Diäten herrscht Uneinigkeit, und abwechselnd werden Zucker oder Fett für Übergewicht und Stoffwechselstörungen verantwortlich gemacht. Um dieser Frage zu nachzugehen, fütterten wir Tiere mit einer ketogenen Diät (KD), HFD, regulärem Futter, und gaben 5% Glucose zum Trinkwasser von Mäusen mit regulärem Futter. Nach drei Tagen testeten wir die Glukosetoleranz der Tiere. Zu unserer Überraschung fanden wir, dass sowohl mit HFD als auch mit KD gefütterte Tiere gestörte Glukosetoleranz zeigten, während die Tiere mit zusätzlicher Glukose normal schienen.

Zusammenfassend präsentiere ich Daten die zeigen, dass die EZM einen Einfluss auf die gewebespezifischen Unterschiede in der Vorläuferdifferenzierung hat, dass endotheliale Insulinresistenz im Fett ein sehr frühes Ereignis im MS ist, das zeitgleich mit der Fettzell-Hypertrophie auftritt, und dass HFD- und KD-gefütterte Tiere nach drei Tagen ähnliche Reduktion ihrer Glukosetoleranz zeigen.

Abbreviations

ABHD5	1-acylglycerol-3-phosphate O-acyltransferase
AC	adipocyte
AdC	adenylate cyclase
ADP	adenosine dophosphate
AKT	PKB, protein kinase B
AT	adipose tissue
ATGL	Adipose trigycerida lipase
ATP	adenosine triphosphate
BAT	brown adipose tissue
BH4	tetrahydrobiopterin
BHB	β-hydroxybutyrate
cAMP	cyclic AMP
cGMP	cyclic GuandineMonoPhosphate
CNS	central nervous system
COL1	collagen 1
COL4	collagen 4
c-Raf	rapid accelerated fibrosarcoma kinase
db	diabetic gene
DIO	diet induced obesity
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
Erk	extracellular signal regulated kinase
F3	coagulation factor 3
FABP4	fatty acid binding protein 4
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FFA	free fatty acids
FLOT2	flotillin 2
FN	fibronectin
FoxO	forkhead transcription factor
GCGR	glucagon receptor
GLUT	glucose transporter
GPCR	G protein coupled receptor
Grb10	Growth factor receptor-bound protein 10
Grb2	Growth factor receptor-bound protein 2
GSV	glucose storage vesicle
HCAR	hydroxycarboxylic acid receptor
HDL	high density lipoprotein
HFD	high fat diet
HOMA-IR	homeostatic model of assessment for insulin resistance
HSL	hormone sensitive lipase
HSP90	heat shock protein 90
IL1β	interleukin 1 beta
IL6	interleukin 6
ILK	integrin linked kinase
IPGTT	intraperetoneal glucose tolerance test
IR	insulin receptor
IRS1	insulin receptor substrate 1
KD	ketogenic diet
LDL	low density lipoprotein
LDLR	LDL-receptor
lepR	leptin receptor
LPL	lipoprotein lipase
Mek	MAPK/ERK mitogen activated protein kinase kinase
MS	metabolic syndrome
NO	nitric oxide
ob	obese gene
PDE	phosphodiesterase
PDK1	phosphoinositide-dependent protein kinase
PI3K	phosphatidylinositol 3 kinase
PKA	protein kinase A
PSCK9	Proprotein convertase subtilisin/kexin type 9
Ras	rat sarcoma kinase
RMR	resting metabolic rate
ROS	reactive oxygen species
Sc	subcutaneous adipose tissue
Sos	son of sevenless guanodine exchange factor
SREBP	sterol regulatory element-binding protein
SVF	stromal vascular fraction
T2D	type 2 diabetes
TCA	tricarboxylic acid cycle
TG	triglycerides
TNFα	tumour necrosis factor alpha
UCP1	uncoupling protein 1
Vis	visceral adipose tissue
vitC	vitamin C
vitE	vitamin E
VMH	ventromedial hypothalamus
WAT	white adipose tissue
β-MHC	myosin heavy chain beta

1. Introduction

1.1. Global Obesity, the Metabolic Syndrome, and Scope of the Thesis

Between 1980 and 2013 worldwide rates of overweight or obesity have increased by more than 8% across both sexes, to the point where obesity is now considered a major global healthcare challenge.[1] In addition, a recent study suggests that this trend of increasing rates of obesity is not likely to stop or reverse in the near future (Figure 1). [2]

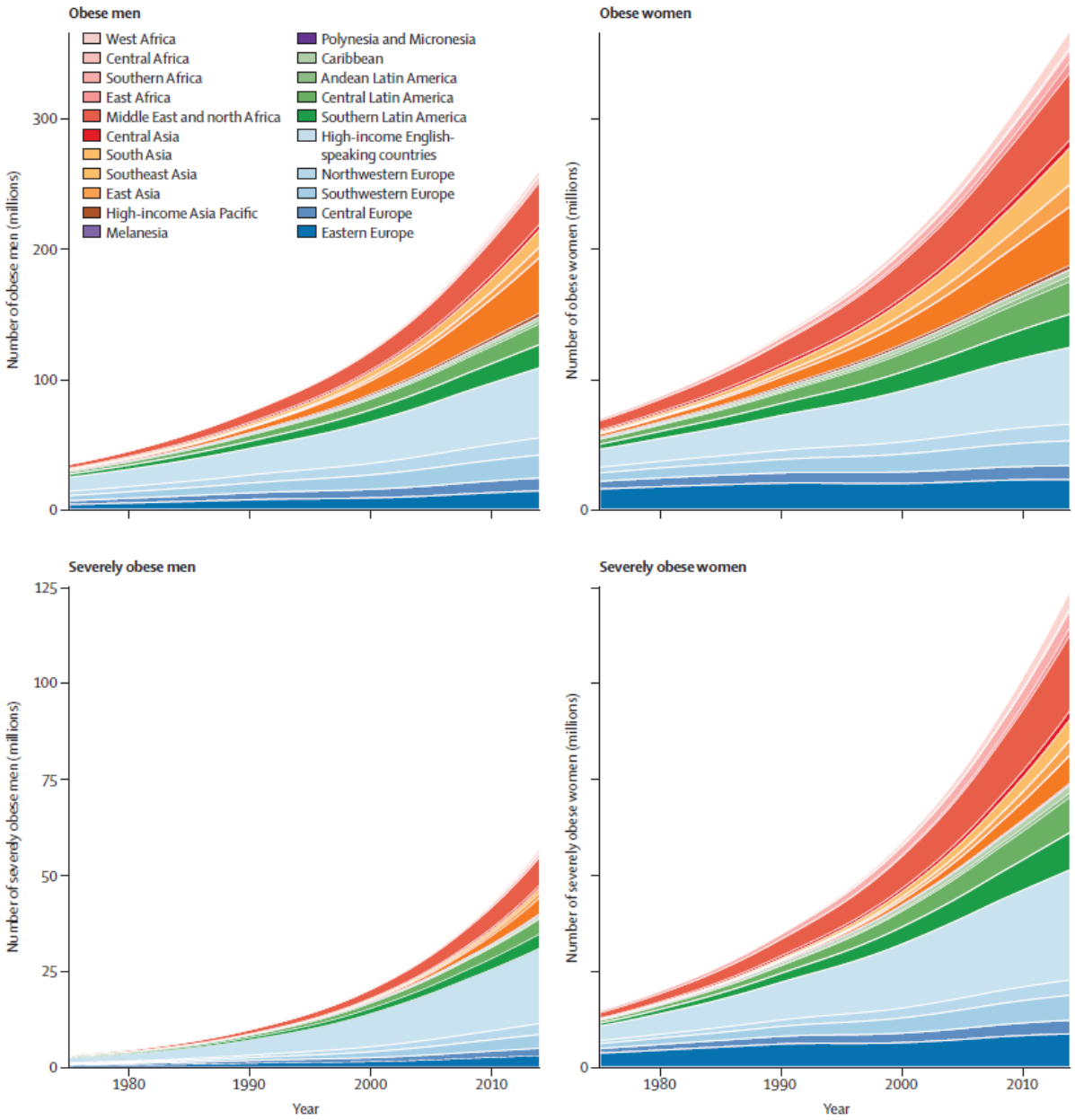

Figure 1: Trends in global obesity during the last 30 years. Taken from [2]

Obesity results when calorie intake chronically exceeds calorie expenditure. Simple as this sounds, the underlying cause of the global obesity epidemic is far from being understood.[3] In an attempt to understand the reasons for the rise of obesity, people have looked at both sides of the equation, calorie intake and calorie expenditure. One popular assumption is that the sedentary lifestyle prevalent in western culture with little physical activity is the key culprit. However, a recent study measuring daily energy expenditure in Hadza people, living in a pre-agricultural hunter-gatherer lifestyle, and comparing these data with energy expenditure of preindustrial agriculture utilizing people and people living in western industrialized society, found that the hunter-gatherer lifestyle people had similar daily energy expenditures to people in western societies, with both lying slightly below that of preindustrial farmers.[4] This suggests that at least on the side of simply counting calories, reduced physical activity does not explain the rise in obesity, leaving increased caloric intake as the likely cause. The observation that developing countries also developed increased rates of obesity when they adopted a more "western" diet in recent history also gives strength to the argument that, rather than physical activity and lifestyle factors, there are certain kinds of obesogenic foods that, when consumed regularly, lead to a loss of energy homeostasis and the development of obesity. This is certainly the case in mice, where switching animals from their regular chow diet to a diet containing 60% of calories as fat (60% HFD) is enough to cause obesity and insulin resistance in most strains.[5]

That being said, it is very likely that physical activity, or lack thereof, contributes to obesity in a way that goes beyond just the calories expended; by modulating systemic levels of hormones, activating repair mechanisms and muscle growth and modulating substrate usage for energy generation systemically physical activity can counter weight gain by restoring or improving general physiological mechanisms of energy homeostasis.

Overall, whatever the underlying reason, obesity is essentially a breakdown of homeostatic mechanisms in the body leading to an overall increase in body fat mass. An increase in calories burned need not reduce body fat if it is accompanied with an increase in calories consumed, just as a – perhaps forced – decrease in calorie intake will often lead to a decrease in total energy expenditure, and is difficult to maintain voluntarily.[6,7]

Perhaps the global research effort focusing on obesity would not be quite as intense as it is presently, even given the staggering increase over the last decades, if obesity were merely an aesthetic problem. The main problem that arises with obesity is that it is a key predictor for a condition termed the metabolic syndrome (MS). The MS is characterised by excess visceral adiposity, increased blood-pressure, insulin resistance, elevated fasting plasma glucose and insulin levels, and elevated plasma lipid levels.[8] It is the key risk factor for developing type II diabetes (T2D), and together with T2D, it is the leading cause for cardiovascular disease and related mortality. It is this strong correlation between obesity, and in particular visceral obesity,

metabolic complications, and cardiovascular disease that causes the strong interest of the biomedical sciences into researching this condition.[9]

The hallmark of obesity is increased body fat mass. While late stages of obesity and obesity associated with diabetes correlate with a state of increased ectopic lipid deposition – which is lipid deposition in organs other than adipose tissue (AT), such as muscle and liver, obesity typically begins with an expansion of existing AT depots. AT expansion consists of two modes: hyperplasia – an increase of the number of adipocytes, and hypertrophy – an increase of adipocyte size.

My thesis focuses on these two aspects of adipose tissue expansion. In my first project, I focus on adipocyte hyperplasia by looking at a particular aspect of preadipocyte differentiation, namely the influence of the extracellular matrix microenvironment generated by different adipose tissue depots on the differentiation of tissue-resident preadipocytes into mature adipocytes. In my second project, I focus on hypertrophy, investigating whether hypertrophy of adipocytes acutely causes endothelial stress or coagulatory events, and whether these stressors contribute to the pathology of obesity and the metabolic syndrome.

1.2. Adipose Tissue Function

Adipose tissue is categorized into white AT (WAT) and brown AT (BAT), and further into subcutaneous (Sc, situated under the epidermis) and visceral (Vis, residing inside the peritoneum) AT. The most commonly studied depots for each of these categories in the mouse are the inguinal fat pads, located between the thighs and body on the posterior side for Sc, the perigonadal fat pads, surrounding the gonads inside the peritoneum, for Vis, and the interscapular BAT, located between the shoulder blades.

Originally thought of only as an energy storage organ, the recent discovery of various hormones secreted from adipose tissue, termed adipokines, has led to the understanding that AT also plays an important role as an endocrine organ. The first adipokine described was leptin, which was discovered through positional cloning as the gene product of the *obese* (*ob)* gene.[10] Leptin is expressed by mature adipocytes and the temporal average of its levels correlates with total body adipose tissue mass. It acts as sensor of systemic energy storage, by acting on the leptin receptor (lepR) – the gene product of the *diabetic (db)* gene – expressed strongly in the brain. There, it regulates food intake, energy expenditure, and also reproduction. The primary central effect of leptin is to reduce food intake and increase energy expenditure.[11]

Perhaps the second most metabolically important adipokine is adiponectin. Like leptin, it is expressed in mature adipocytes. It acts via the adiponectin receptor, a 7-transmembrane receptor that is however not a G-protein coupled receptor (GPCR), and is expressed in a large

number of tissues. Adiponectin modulates glucose homeostasis, systemic insulin signalling, as well as lipid metabolism.[12]

There is a large number of other adipokines secreted from adipose tissue, among them inflammatory cytokines such as tumour necrosis factor α (TNFα) or interleukin-6 (IL6), which have a variety of systemic effects, but these are not secreted by adipocytes exclusively and may also originate from other cells resident in adipose tissue.

In terms of tissue architecture, AT consists of terminally differentiated mature adipocytes and the stroma, comprising a dense network of adipocyte-surrounding vasculature (endothelial, smooth muscle cells and pericytes), AT resident fibroblasts, as well as a pool of AT resident macrophages and immune cells. Morphologically, there is a key difference between WAT and BAT mature adipocytes; white adipocytes are large (40-150 μm diameter) and contain one big lipid droplet surrounded by a thin layer of cytosol, whereas brown adipocytes are smaller (20-40 μm diameter), and contain many small lipid droplets surrounded by cytosol and many mitochondria, which give BAT its brown colour.

In addition to the aforementioned functions of AT as an endocrine regulator of energy homeostasis, the primary function of WAT is the storage of metabolically available energy in the form of triglycerides (TG). WAT endothelium expresses lipoprotein lipase (LPL) which tethers lipid-filled chylomicrons circulating in the blood after a high fat meal. LPL hydrolyses triglycerides inside the lipoproteins to free fatty acids (FFA) and glycerol, which are taken up by adipocytes, re-esterified into triglycerides, and stored inside lipid droplets. WAT endothelium also interacts with other lipoproteins, such as high density Lipoprotein (HDL) and low density lipoprotein (LDL) exchanging lipids and cholesterol, depending on the physiological context. Moreover, WAT can perform lipogenesis, creating triglycerides from glucose or other sugars. During fasting the main function of AT is to degrade triglycerides from the lipid droplet and release FFAs and glycerol, a process termed lipolysis.[13]

In contrast, BAT is not an energy storage organ, but rather has its main function as thermogenesis. In response to pro-thermogenic stimuli such as signals from direct innervation, but also systemic factors such as β-adrenalin, BAT increases metabolic flux towards the mitochondrial proton gradient which it converts to heat by uncoupling the mitochondrial membrane potential from adenosine triphosphate (ATP)-production through the use of uncoupling protein 1 (UCP1).[14]

1.3. Extracellular Matrix Signalling

Expansion of AT, both trough hypertrophy and hyperplasia, requires physical changes to the tissue to allow for the increased space taken up by larger or more cells. Tissue maintenance and homeostasis is a complex process entailing the adaptation of tissues and their resident cells to the specific stresses and workloads to which they are exposed. This requires a delicate balance between removal of old cells and their replacement by proliferation and differentiation of precursor- or tissue stem cells. Several types of interactions are known to influence the state of a cell within a tissue: systemic cues, local interactions of cells with their neighbouring cells, and interactions between cells and the extracellular matrix (ECM). It is known that most cells need both common growth factors present in the serum, which are systemic cues, as well as direct signals from the surface to which they are attached, in order to stay alive and not undergo apoptosis – this circumstance is termed anchorage dependence.

Extracellular matrix is present in every tissue, but is most enriched in connective tissue and the basement membrane of epithelia where it is known to play an important role in organizing cell proliferation and differentiation. There is a vast number of different proteins and polysaccharides composing the ECM. They are typically categorized into collagens, proteoglycans, non-collagenous glycoproteins and elastic fibres. ECM components are secreted by tissue resident cells and organize into three-dimensional scaffolds. Most of the proteins contributing to the ECM are known to interact with cells directly via specific binding motifs involved in signalling between the ECM and the cell. The ECM is also known to bind and sequester growth factors, influencing their availability and epitopes.[15]

For a long time, it was thought that the ECM has two main functions: physical and mechanical support of the tissue, and anchorage for cells to grow. However, it has become apparent that the ECM also plays a fundamental role in influencing cell differentiation, thus contributing to tissue identity even at the cellular level.

In recent years, a wealth of data has accumulated showing that cells not only need to bind extracellular matrix in order to grow, but that they actually sense and respond to the structural, biochemical and mechanical properties of these surfaces in three dimensions. They do this by exerting force and pulling on their growth substrate through mechanisms involving actin- and myosin dynamics.[16–18] For example, various differentiation experiments using different stem cell types have shown that very rigid surfaces trigger responses indicative of a bone cell fate, while softer surfaces tip the balance towards neuronal or fat cell development.[19]

Since a fundamental role of the ECM is to serve as a scaffold and growth surface for cells, it is clear that any change in the amount, size or shape of a tissue and its cells necessitates a corresponding change in ECM structure or composition. Indeed, in tissue homeostasis, there is a balance between the degradation of the ECM – orchestrated by secreted matrix

metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) – and the synthesis and secretion of new ECM.[20]

Given the fact that the ECM is under constant construction and remodelling by the cells constituting the tissue, it begs the question of how it can influence or dictate cell state and behaviour simultaneously. An interesting concept was put forth to solve this apparent dilemma, termed dynamic reciprocity. According to this concept, the tissue identity of cells is partly mediated by bidirectional crosstalk between the ECM and membrane bound ECM-receptors on the cells, signalling to the nucleus and influencing the expression profile of the cell, which in turn influences and perpetuates the state of the surrounding ECM by synthesizing and secreting its components.[21] Since it was postulated, this model has been extended to include systemic cues from growth factors, morphogens or other endocrine signals as well as paracrine signals and signalling mediated by cell-cell interactions.

1.4. Insulin Signalling

Insulin, arguably the most important anabolic hormone, is a peptide hormone consisting of a longer α and a shorter β chain, covalently connected by disulphide bonds. It is released by beta cells in the Langerhans islets of the pancreas in response to elevated plasma glucose levels. Inside the beta cell, the ATP/ADP ratio is linked to extracellular glucose concentration via the low affinity constitutive glucose transporter GLUT2, glycolysis, the tricarboxylic acid (TCA) cycle and mitochondrial respiration. Increased levels of ATP cause a depolarization of the plasma membrane via ATP-dependent K^+ channels. This leads to the opening of voltage-gated Ca^{2+} channels and to an increase of Ca^{2+} inside the cytosol, causing insulin secretory vesicles to fuse with the plasma membrane and release insulin. Insulin can then enter the bloodstream quickly through the highly fenestrated microvasculature of the endocrine pancreatic islets.[22,23]

Insulin fulfils a variety of roles in different tissues; it can act as a growth factor, as well as a metabolically active hormone, but its most well characterized function is its effect on plasma glucose concentration. Insulin was first isolated by Banting and Best in 1921, as the active component of an extract of pancreatic langerhans islets in dogs that has the ability to lower blood glucose.[24] Since then, the mechanism of how insulin performs this lowering of plasma glucose has been largely elucidated. Insulin acts via binding its receptor (IR), a receptor tyrosine kinase, expressed in virtually all tissues but at concentrations spanning two orders of magnitude, with the lowest expression on erythrocytes and the highest on adipocytes and hepatocytes. The IR is a heterotetrameric glycoprotein connected by disulfide bonds consisting of two extracellular α subunits, which contain the ligand binding site, and two β subunits, that cross the plasma membrane and exhibit kinase activity leading to auto-phosphorylation of

several of its tyrosine residues. Upon binding of insulin, the receptor phosphorylates its cytosolic β-subunits, which subsequently phosphorylate other intracellular subtstrates.[25] According to the different intracellular downstream effects, the actions of IR activation can be broadly classified into a growth factor arm and a metabolic arm.

On the metabolic arm, the first downstream effector of IR is a protein called Insulin receptor substrate 1 (IRS1), which is phosphorylated. Phosphorylated IRS1 is bound by and activates phosphatidylinositol-3 kinase (PI3K), which in turn phosphorylates and activates phosphoinositide-dependent protein kinase (PDK1) and protein kinase B (PKB, commonly called AKT). This results in the translocation of glucose transporter 4 (GLUT4) storage vesicles to the plasma membrane and increased glucose uptake (Figure 2). This translocation of GLUT4 is most important in skeletal muscle, which accounts for over 90% of insulin-stimulated glucose uptake[26], cardiac muscle, and to a lesser extent, adipose tissue, which corresponds also to the tissue distribution of GLUT4 expression.[27]

Within the adipocyte, the most important effect of insulin signalling is inhibitory control of lipolysis. Lipolysis is generally triggered by increased plasma glucagon, catecholamines and β- adrenalin binding to glucagon- and the α2, β2 and β3 adrenalin-receptors, which are G-protein coupled receptors linked to downstream adenylate cyclase (AdC). Their activation causes an increase of cytosolic cyclic AMP (cAMP) concentrations and activation of protein kinase A (PKA), which in turn phosphorylates and activates hormone sensitive lipase (HSL) and perilipin (PLN), the functionally important surface protein coating the lipid droplet (LD). Phosphorylation of PLN causes conformational changes of the LD, allowing for HSL and other lipases to engage in lipolysis. In addition, phosphorylation of PLN also causes it to release bound 1-acylglycerol-3-phosphate O-acyltransferase (ABHD5), a coactivator of adipose triglyceride lipase (ATGL), which recruits ATGL to the lipid droplet, to participate in lipoysis.[28,29] Insulin blocks this pathway through phosphorylation of phosphodiesterase 3 (PDE3) by AKT, which hydrolyses cAMP, leading to reduced cAMP levels and PKA-dependent phosphorylation (Figure 2).[29] In addition, Insulin has also been shown to cause a decrease in transcription of ATGL.[30]

In the liver, insulin also acts to suppress lipolysis, but its key role is to suppress hepatic gluconeogenesis as well as glycogenolysis in order to reduce hepatic glucose output. The regulation of this process is complex. While a direct effect of insulin on hepatic glucose output using selective knockout of IR in hepatocytes has been demonstrated[31], there are also important indirect effects known to contribute to insulin's ability to suppress hepatic gluconeogenesis. On the one hand, increased insulin release corresponds to a pronounced decrease of glucagon in the plasma, reducing the pro-gluconeogenic signalling in the hepatocyte. In addition, insulin signalling leads to reduced release of FFA and glycerol from AT, and reduced release of gluconeogenic precursors from muscle, causing a decrease in

hepatic glucose production due to decreased availability of substrates and energy.[32] On the other hand, and perhaps even more importantly, insulin action in the brain strongly influences hepatic glucose production via direct innervation. In the mediobasal hypothalamus it acts on insulin-sensitive neurons by opening ATP-activated potassium channels, which causes suppression of hepatic glucose production that is dependent on intact vagal efferent neurons.[33,34,35]

However, besides these convergent actions of insulin in the periphery and the central nervous system (CNS), in causing reduced hepatic glucose production, the effects of insulin are somewhat distinct in the periphery and the brain. Centrally, insulin has a net catabolic effect as it acts to reduce food intake and increase energy expenditure[36], whereas peripherally, insulin is a strongly anabolic hormone, acting on cells and organs to increase nutrient uptake and inhibit lipolysis.

Insulin also acts as an important growth factor, both in the brain and peripherally. The growth factor action of insulin signalling is mediated by GRB2 and IRS1 binding, which are upstream of the classical extracellular signalling regulated kinase (ERK) mitogen activated kinase (MAPK) signalling cascade, connecting SOS to RAS, RAF, MEK1/2 and finally phosphorylating ERK1/2. This results in the dimerization if ERK1/2 which translocates to the nucleus and transcriptionally mediates the growth factor signalling effects of insulin (Figure 2).

In addition to all of these growth and metabolic effects of insulin, insulin also acts specifically on the endothelium as a vasodilator. It does so by causing the phosphorylation of endothelial nitric oxide synthase (eNOS) at serine 1177 (S1178 in humans). Phosphorylation of S1177 leads to increased production of the vasodilator nitric oxide (NO·) that causes vascular smooth muscle relaxation and consequent vasodilation (Figure 2). This insulin dependent vasodilation plays an important role in mediating insulin's function of stimulating nutrient uptake by tissues, by increasing blood flow and nutrient transport to the tissue. In fact, vasodilation and skeletal blood flow can independently modulate skeletal glucose uptake, and impaired endothelial insulin signalling reduces glucose uptake in skeletal muscle.[37–40]

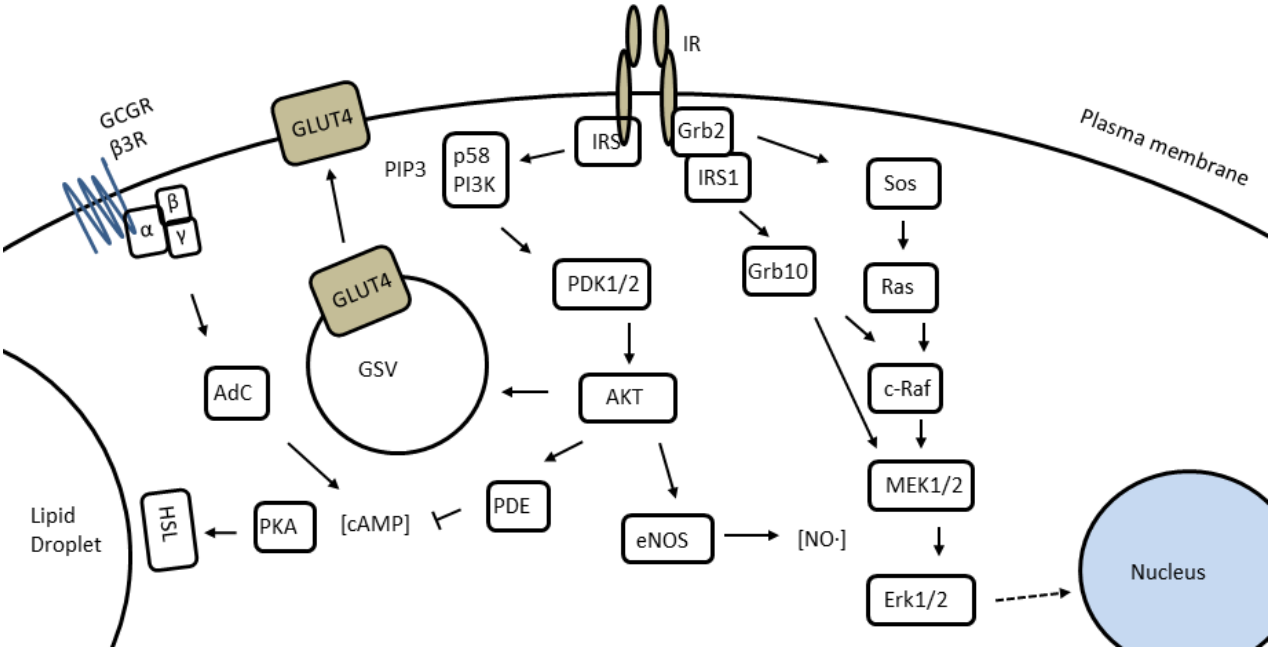

Figure 2: Intracellular insulin signaling pathway.: Effects of insulin signaling via the IR on glucose uptake (GLUT4 translocation), lipolysis (HSL), growth (Erk1/2), and eNOS are depicted.

1.5. Inflammation in Obesity

In recent years, it has become clear that chronic inflammation of AT is an important aspect of the development of the metabolic syndrome, insulin resistance and T2D.[41] The first seminal work to clearly demonstrate a link between adipose tissue inflammation and the metabolic syndrome was the finding that obese adipose tissue produces the pro-inflammatory cytokine tumour necrosis factor α (TNFα) in 1995.[42] Since then a lot of findings have been added to this observation, to the extent that a causal link between systemic inflammation and the metabolic syndrome is now generally accepted.[43]

A verified contributor to obesity-caused inflammation are AT-resident macrophages, in particular in the visceral AT.[44] Healthy adipose tissue in a state of balanced nutrition contains macrophages that are in a resting state, termed M2, expressing a range of anti-inflammatory cytokines and repressing inflammation. In a state of increased adipose tissue mass and obesity, these macrophages become activated M1 macrophages, releasing pro-inflammatory cytokines – most prominently TNFα, IL6, and interleukin 1 beta (IL1β) – and leading to inflammation and recruiting additional immune cells such as activated macrophages or T-lymphocytes into the adipose tissue.[45] One specific population of macrophages that has been widely reported to contribute to adipose tissue inflammation are F4/80+, CD11c+ activated macrophages that are actively recruited to obese adipose tissue[46], but further studies revealed

that there is a continuum of functional states in AT macrophages that seems more dependent on the general nutritional state than on the specific expressed surface markers.[47]

Several studies have shown that the inflammatory state of adipose tissue has a strong impact on the overall metabolic profile in general, and the development of decreased glucose tolerance, insulin resistance and T2D. It has been demonstrated that immunotherapy acting against the inflammation state in Vis AT can ameliorate and even reverse the insulin resistant state caused by diet induced obesity (DIO).[48,49]

However, which specific factors or elements initially cause adipose tissue inflammation is a question that is still incompletely understood. One of the early hypothesis was that hypertrophy causes adipocyte apoptosis or necrosis and that AT inflammation is caused by macrophages acting to clear debris at so-called crown like structures,[50] but it has since been demonstrated that adipocyte apoptosis can be separated from AT inflammation. One study showed that cyclophylin-D -/- animals, which have greatly reduced adipocyte death still show high levels of AT inflammation, and another study used inducible caspase activation in adipocytes to show that adipocyte death causes macrophage invasion, but polarizes macrophages towards an anti-inflammatory state.[51,52] There is a lot of data implicating free fatty acid binding by pathogen-associated-molecular-pattern receptors of the innate immune system, like the Toll-like-receptor family, as a cause for AT inflammation.[44] Further popular ideas as to what causes AT inflammation are increased endoplasmic reticulum (ER)-stress,[53] and hypoxia,[54] but a clear primary cause has not been established. It is likely that there are a variety of signals converging on AT leading to increased pro-inflammatory signalling.

While it is generally accepted that the systemic inflammation present in the obese state originates in the adipose tissue and that circulating pro-inflammatory cytokines mediate its effect throughout the body, another organ whose state of inflammation during the metabolic syndrome has come into focus is the brain, and in particular the hypothalamus. It was demonstrated that blocking hypothalamic inflammatory signalling along the IKKβ/NF-κB axis protects from high fat diet (HFD) induced obesity and insulin resistance[55] and reduces diet induced elevation of high blood pressure.[56] Recently it has been shown that the hypothalamus might actually show signs of inflammation at the same time or even earlier than inflammation of the AT is apparent,[57] raising the interesting question whether hypothalamic inflammation might be a different way diet links to obesity and the metabolic syndrome.

1.6. Insulin Resistance in Obesity

Impaired glucose tolerance and insulin resistance is the hallmark of the metabolic syndrome. Obesity associated insulin resistance affects a large variety of tissues in several ways, and manifests along all the different metabolic axis of signalling insulin affects, namely glucose clearance, glucose production, lipolysis, and vasodilation. Its progression in the onset of diet induced obesity is typically slow, and physiologic effects are blunted over the course of the development of the metabolic syndrome due to increasing compensatory insulin output by the pancreatic beta cell. T2D typically develops at a late stage of the metabolic syndrome, as a consequence of increased beta cell damage and failure of the pancreas to increase insulin levels sufficiently to maintain normoglycemia.[58]

The exact origins of diet induced insulin resistance are incompletely understood, but a large body of work exists describing various contributing aspects. The current most prominent view of the aetiology is that physiological limits to lipid storage inside adipose tissue lead to an overflow of lipids or FFA into other tissues – so called ectopic lipid deposition – most prominently liver and muscle, and that ectopic lipid deposition causes insulin resistance in these respective tissues.[59]

However, complicating matters is the circumstance that many groups report systemic and local insulin resistance at time points arguably preceding maximal lipid storage capacity of AT by a long margin. Using direct insulin infusion into the mediobasal hypothalamus Ono et al. showed that insulin resistance is already detectable in the mouse brain after one day of HFD feeding.[60] Clegg et al. showed that three days of high fat diet already blunted effects of insulin infused into the third ventricle of the hypothalamus on food intake in rats.[61] Lee at al. showed impaired systemic glucose tolerance after 3 days of HFD feeding and used three different immunocompromised mouse models, targeting immune cells of hematopoietic origin, to demonstrate that inflammation was necessary for insulin resistance caused by long term high fat diet feeding, but blocking inflammation did not alleviate short term insulin resistance in mice.[62] On the other hand, Wiedemann et al. targeted inflammation by deleting CD95 specifically on adipocytes, using the fatty acid binding protein4 (FABP4) promoter, and demonstrated amelioration of insulin resistance after 3 days of HFD in the mouse.[63]

Taken together, these data indicate that there are two separate, if overlapping processes contributing to insulin resistance in HFD induced obesity, one of them slow and requiring inflammatory contribution of bone marrow derived immune cells, and the other fast, probably involving the hypothalamus and possibly requiring inflammatory processes in the adipocyte.

While the immediate effects of the fast process remain poorly understood, the long term temporal development of insulin resistance in developing the metabolic syndrome is fairly well documented and reviewed elsewhere.[64] Summing up briefly, in the brain, insulin's effects in

reducing food intake and suppress hepatic gluconeogenesis as well as increase energy expenditure become blunted. In the endothelium, a variety of processes, such as insulin mediated NO· dependent vasodilation become impaired.[64] In skeletal muscle, insulin dependent glucose uptake and glycogen synthesis are reduced, and in the cardiac muscle, insulin dependent increase of glucose consumption and decrease in FFA oxidation are blunted. Macrophages show increased LDL uptake and ER-stress. In the adipocyte, insulin's ability to suppress lipolysis is severely blunted, and in the liver, insulin's ability to suppress gluconeogenesis is impaired – in concert with reduced hypothalamic insulin signalling, as well as insulin-dependent reduction of LDL-receptor (LDLR) expression (Figure 3).

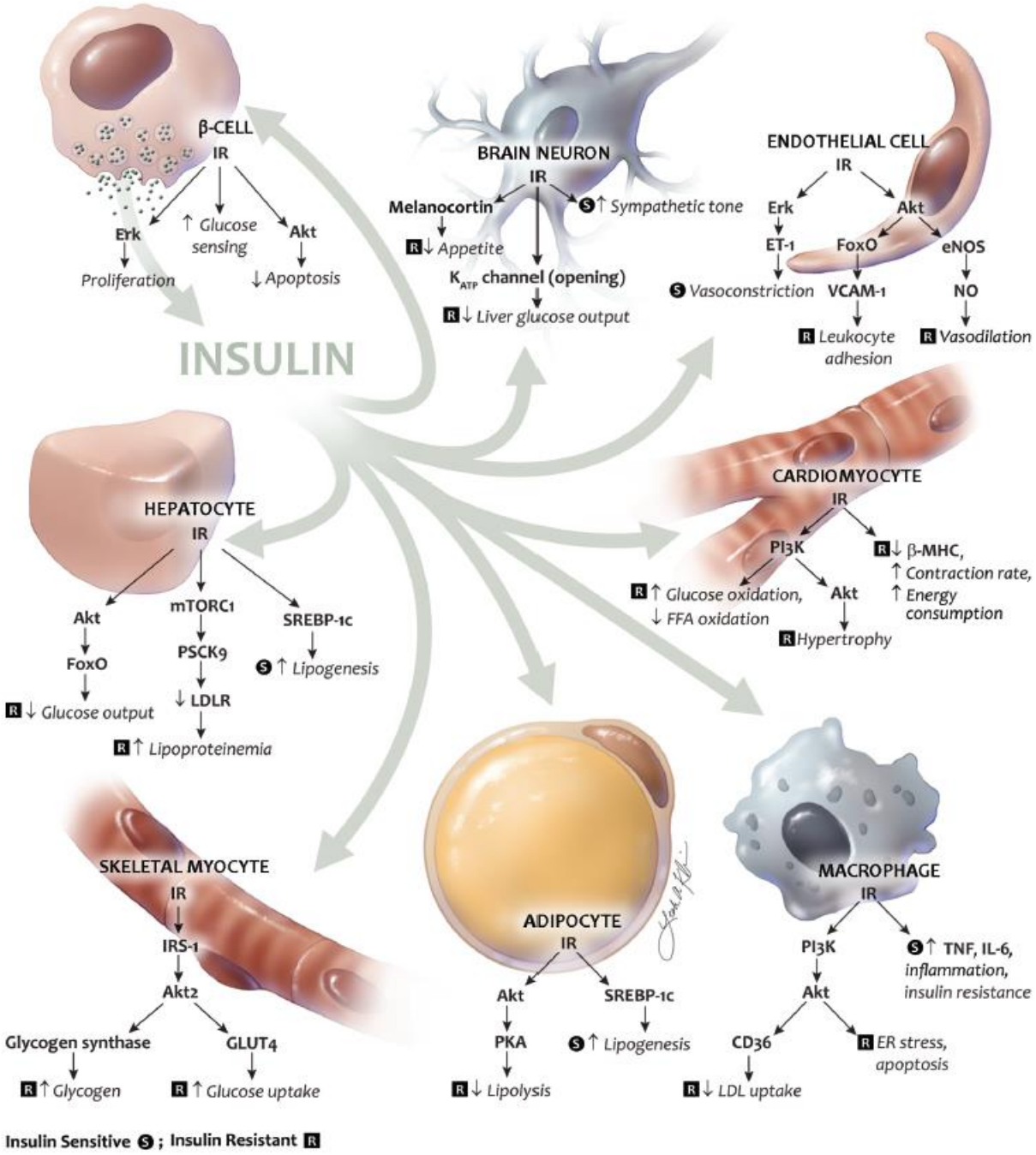

Figure 3: Insulin signalling impairments in various tissues in obesity. R indicates that these action of insulin are impaired in insulin resistance; S indicates that they remain sensitive. Figure taken from [64]

1.7. Coagulation

The second major aspect of the metabolic syndrome, besides the metabolic aspect, is the cardiovascular aspect. Indeed, it is cardiovascular complications that are often the clinical endpoint in studying the metabolic syndrome, and they are the leading cause of mortality related to the metabolic syndrome and T2D.[65,66,67] Cardiovascular mortality is typically caused by aberrant clot formation – thrombosis – blocking essential blood vessels supplying either the brain of the heart, so it is effectively a result of disorders of the coagulation system.

Coagulation refers to the process of blood clotting, whereby blood changes from being liquid to becoming a gel and forming a clot. Together with the regulation of vascular tone and blood pressure, and platelet activation, it is a key component of haemostasis – the process of preventing blood loss from damaged blood vessels and their repair. Coagulation is a complex process involving a cascade of enzymes called coagulation factors that act as proteases and typically become active through cleavage, acting upon their downstream factors and ultimately resulting in the cleavage of fibrinogen by active thrombin, leading to formation of cross-linked fibrin which forms a large protein mesh and the physical basis for a blood clot – also termed thrombus. Activated platelets contribute to clot formation by expressing on their surface, as well as secreting, a variety of clotting factors, and also releasing small molecule coagulation and vasoactive compounds such as ADP, polyphosphate, serotonin or thromboxane. They also directly bind fibrinogen, fibrin, and sub-endothelial ECM proteins or activated endothelium, thereby tethering the blood clot to the site of blood vessel damage. The coagulation cascade has two converging pathways, the tissue factor pathway – historically termed extrinsic – and the intrinsic pathway – historically called the contact activation pathway, since it was originally discovered through plasma interacting with inert surfaces such as glass. The tissue factor pathway acts through the exposure of tissue factor, which is expressed on sub-endothelial cells, and activated platelets and endothelial cells, and converts factor VII, present in the plasma into its active form, which activates factor X (FX), which in turn activates thrombin, the endpoint of the cascade, cleaving fibrinogen and triggering fibrin formation. The intrinsic pathways can be thought of as a positive feedback loop. Activated surfaces – ostensibly sub-endothelial ECM, incipient fibrin-clots, neutrophil extracellular nets, or polyphosphate (although this finding has been questioned recently[68]) – are bound by circulating high-molecular-weight kininogen (HMWK), prekallikrein, and factor XII (FXII), leading to FXII, FXI and FIX activation and converging with the tissue factor pathway at FX activation, again resulting in the formation of thrombin (Figure 4).[69,70]

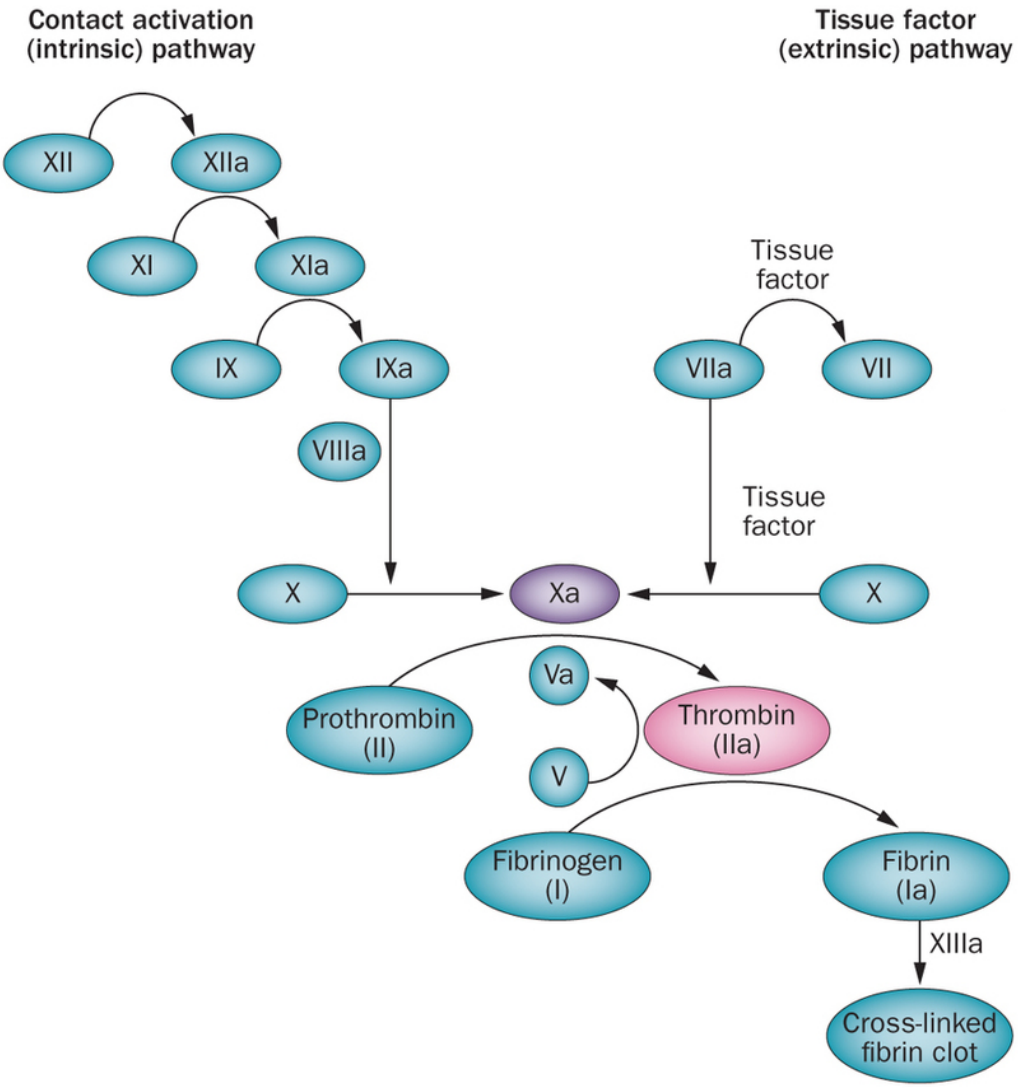

Figure 4: The coagulation cascade. Adapted from [71].

1.8. Endothelial Stress and NO· signalling

Another relevant topic in understanding the physiology of the metabolic syndrome is endothelial stress signalling. Besides the obvious contribution of endothelial disorders to cardiovascular disease, such as vascular inflammation or the formation of atherosclerotic plaques,[72] the endothelium actually plays a fundamental role in insulin signalling even in the healthy individual. Since the origin of insulin is the pancreatic beta cell, in order to act on any tissue, insulin must first access the relevant cells, passing though the endothelium. This trans-endothelial transport of insulin has been studied, primarily in the context of muscle[73,74,75] and brain,[76,77] and has been shown to be saturable, dependent on the insulin receptor, and involve AKT signalling and insulin receptor endocytosis.

Endothelium can respond to changes in flow by vasodilation or vasoconstriction. A key component in regulating the vascular response to blood flow is flow-mediated shear stress. Various mechanosensing receptors expressed on the surface of endothelial cells can signal

into the cell, causing changes in concentration of two key mediators of vascular shear stress, NO·, and various reactive oxygen species (ROS). The main source of NO· in the endothelium is eNOS. NOS are enzymes that catalyse the reaction of L-Arginine with O_2 to L-Citrulline and NO·, accepting electrons from NADPH, when homodimerized (coupled) and in the presence of the cofactor tetrahydrobiopterin (BH_4). NOS monomers or complexes lacking BH_4 instead produce the ROS superoxide (O_2^-). Various studies have shown that fluid shear stress can directly cause the phosphorylation of eNOS at S1177, leading to increased NO· production[78,79], but the process is also dependent on calcium dependent calmodulin binding and thus sensitive to intracellular Ca^{2+} concentrations that are also downstream of mechanosensing.[80] ROS sources upon variable or steady increased flow in endothelium are the mitochondrial proton gradient, NADPH-oxidases and uncoupled eNOS.[81] While NO· is itself a free radical that can freely diffuse across the plasma membrane, its primary mechanism of action in endothelial and adjacent smooth muscle cells is to act as a vasodilator via the activation of soluble guanylate cyclase (sGC) which produces the second messenger cyclic guanidine monophosphate (cGMP) upon NO· binding (Figure 5). The complex interplay of vascular ROS and NO· signalling is still incompletely understood, with chemical modifications of proteins such as S-nitrosations likely to play a role, but both of these signals have been shown to become deregulated in the context of diet induced obesity and there is increasing awareness of the important role of endothelial stress on the onset of insulin resistance and the metabolic syndrome.[82]

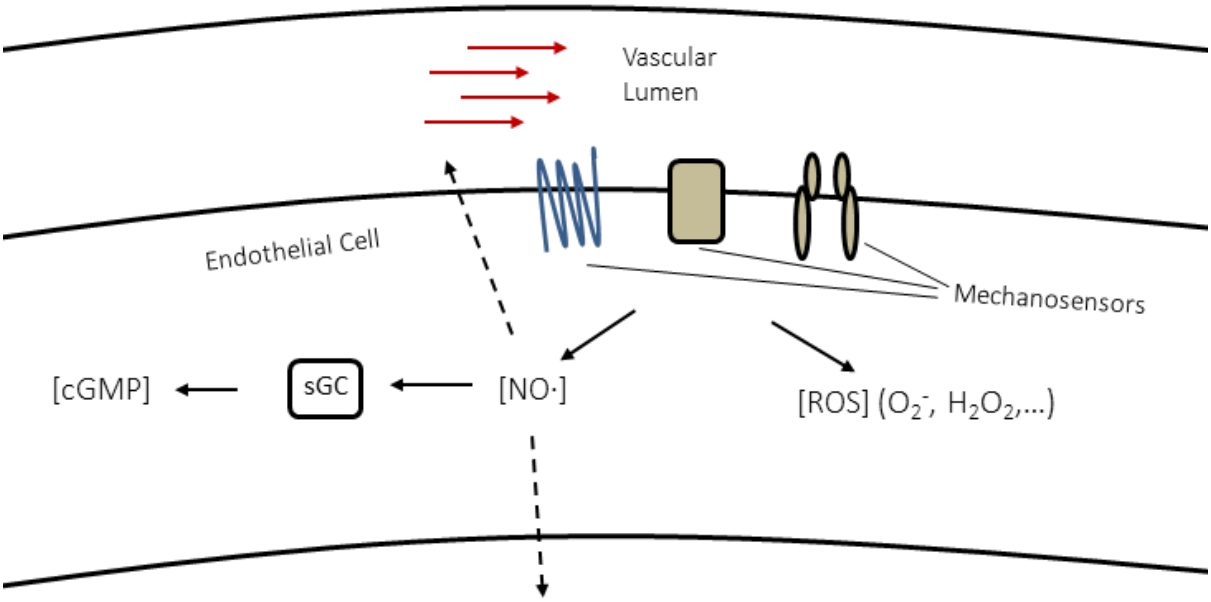

Figure 5: Signalling events downstream of increased endothelial shear stress. Changed flow leads to increased eNOS-dependent NO· production and ROS production. NO·binds to and activates sGC, leading to increased cGMP levels.

2. Depot Specific Differences in the Adipogenic Potential of Precursors are mediated by Collagenous Extracellular Matrix and Flotillin-2 dependent Signaling

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Contribution

Gerald Grandl developed the hypothesis, performed most experimental work, analysed the data and wrote the manuscript.

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Depot Specific Differences in the Adipogenic Potential of Precursors are mediated by Collagenous Extracellular Matrix and Flotillin-2 dependent Signaling

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Abstract

Objective – Adipose tissue shows a high degree of plasticity and adipocyte hyperplasia is an important mechanism for adipose tissue expansion. Different adipose depots respond differently to an increased demand for lipid storage. Orchestrating cellular expansion in vivo requires extracellular matrix (ECM) remodeling and a high degree of interaction between cells and ECM.

Methods – We studied decellularized primary adipose stromal cell derived ECM of different adipose depots and reseeded them with primary adipose precursors. We tested ECM effect on adipocyte differentiation, and analyzed ECM composition using proteomic and immunohistochemical approaches to identify factors in the ECM influencing adipogenesis.

Results – We show that the ECM of an adipose depot is the major determinant for the differentiation capacity of primary preadipocytes. Visceral adipose tissue stromal cells differentiate less than subcutaneous cells, which in turn are less adipogenic than BAT-derived cells. This effect is based on the ECM composition of the respective depot and not dependent on the precursor origin. Addition of vitamin C pronounces the pro-adipogenic effects of the ECM, indicating the importance of collagenous ECM in mediating the effect. Using a proteomic global and a targeted downstream analysis we identify Flotillin 2 as a protein enriched in pro-adipogenic ECM which is involved in orchestrating ECM to preadipocyte signaling.

Conclusions – We show that adipose tissue SVF secretes collagenous ECM which directly modulates terminal differentiation of adipocyte precursors in a depot specific manner. This data demonstrates the importance of the tissue microenvironment in preadipocyte differentiation.

Keywords

Adipocyte Precursors
Extracellular matrix
Collagen
Stem Cell Niche
Flotillin 2

1. Introduction

The global prevalence of obesity, which is an excessive expansion of an individual's adipose tissue stores, has been rising at alarming rates over the last decades and is predicted to reach even higher proportions in the future [1]. Adipose tissue (AT) can be broadly categorized into white AT (WAT) and brown AT (BAT), with white AT further separated into subcutaneous (Sc) and visceral (Vis) AT (localized inside the peritoneal cavity). The main cell type of AT are terminally differentiated adipocytes, which are composed of a small cytosol and one or more large lipid droplets for triglyceride storage. The AT stroma contains tissue resident fibroblasts, endothelial and smooth muscle cells as well as macrophages [2].

AT shows a high degree of plasticity and is able to respond to an increased demand to store calories with rapid expansion. The two modes of AT expansion are hypertrophy, which is an increase in cell size, and hyperplasia, which is an increase in cell numbers. Increasing the number of adipocytes requires the activation of tissue resident adipose precursor cells, which are believed to be of a mesenchymal origin and give rise to committed preadipocytes. Tracing studies using a PPARγ-GFP mouse line initially identified adipocyte precursor cells to be of perivascular origin [3]. The first defined population of AT resident fibroblasts thought to be committed preadipocytes was identified in 2008 [4], and has since been specified further [5]. Further studies have revealed a Lin- PDGFRα+ fibroblast residing in AT perivascular space that is able to give rise to both brown and white adipocytes [6].

Terminal differentiation of preadipocytes is a complex process critically involving the nuclear receptor PPARγ [7], and entails the morphological changes from a fibroblast-type precursor to a round, large cells filled with lipid droplets for triglyceride storage. Besides the complex intracellular changes it has been shown that interaction of the differentiating cell with its surrounding extracellular matrix (ECM), as well as extensive ECM remodeling play important roles in this process [8], [9].

Involvement of ECM in orchestrating the correct response of tissue resident cells to various stimuli has become a focus of intense study, and various ECM properties have been shown to directly influence precursor or stem cell behavior [10]. In addition, prolonged states of inflammation, tissue damage or remodeling often lead to a state termed fibrosis, which is excessive deposition of ECM, and is frequently observed concomitantly with high levels of obesity and dysfunctional adipose tissue [11].

In obesity, a physiological environment favoring AT expansion, different adipose depots have been shown to expand differently. Sc AT grows slower and is described to show more of a hyperplastic response while Vis AT grows more rapidly and shows more extensive hypertrophy. These two modes of expansion also correlate to some extent with clinical outcomes, where hypertrophic Vis AT is associated with poor metabolic health while Sc AT is sometimes even ascribed a beneficial role [12].

Given the important role ECM is known to play in terminal differentiation of preadipocytes, as well as the known differences in the response of different adipose depots we employed a strategy of deriving extracellular matrices from primary cells of different adipose depots to investigate its role in adipose precursor differentiation.

2. Material and Methods

Materials

All chemicals were purchased from Sigma-Aldrich unless specified otherwise.

Animals

C57Bl/6 mice were housed in a pathogen-free animal facility on a 12-h/12-h light/dark cycle with free access to food and water, at an ambient temperature of 23°C.

Western Blot Analysis

Whole cell lysates were prepared as previously described[13]. For western blot analysis the following antibodies were used: FLOT2 (Santa Cruz Biotechnology, SC-25507, 1:1000) and Hsp90 (Cell Signalling Technology, #4877 S).

Cell Culture

All cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all Invitrogen). 3T3-L1 cells or primary SVF were seeded plates coated with cross-linked gelatin and grown to confluence prior to differentiation. Two days post-confluence cells were treated with induction cocktail (1 µg/ml insulin, 1 µM dexamethasone and 115 µg/ml isobutyl-methylxanthine) for 2 days followed by insulin media (1 µg/ml insulin) for 2 days and DMEM for 3 days. For ECM reseeding FACS-sorted cells were plated into decellularized ECM at 50 000 or 100 000 cells per well. For FLOT2 knockdown the siRNA TGGGCAAGAACGTACAGGACATTAA and a scrambled control were used at 100nM.

Primary Cell Isolation

For SVF preparation adipose tissue subcutaneous (inguineal), visceral (perigonadal) and brown (interscapular) fat pads were dissected, finely minced and incubated with collagenase (1mg/ml) in collagenase buffer (25 mM NaHCO3, 12mM KH2PO4, 1.2mM gSO4 1,2 mM, 4.8mM KCl 120 mM NaCl, 1.4mM CaCl2 5mM Glucose, BSA 2.5 %, pH = 7.4) for 1h at 37°C. Cells were washed in DMEM, 10% FBS, filtered through 40µM cell strainers, erythrocytes were lysed by washing in erythrocyte lysis buffer (154 mM NH4Cl, 10 mM KHCO3, 0.1mM EDTA), and plated in DMEM, 10% FBS, 1% penicillin/streptomycin.

ECM extraction

SVF-derived ECM was prepared on a protocol based on [14]. Briefly, primary SVF of WT B57Bl/6 mice, aged 7 to 12 weeks, was plated at 100 000 cells per well (corresponding to a confluence of 80-90 % after attachment of cells) on 96-well plates coated with cross-linked

gelatin. Regular DMEM with FBS, or DMEM supplemented with FBS and vitamin C (50 µg/µl) was changed every two days. At day 7 ECMs were decellularized by lysing the cells using an alkaline buffer (PBS with 0.5% Triton, 20mM NH_4OH), followed by at least 8 washes with PBS. To improve ECM purity, nucleotide digestion using benzonase (Merck-Millipore) was performed after the second wash, using 750U/ml in benzonase buffer, (PBS with 1mM MgSO4, 1mM CaCl2), 200ul per well for 30 minutes at 37°C.

FACS

Primary adipose SVF was prepared as described. Cells were stained in FACS buffer (PBS, 3% FBS, 1% penicillin/streptomycin, 1mM EDTA) for 15 minutes at 4°C, washed, strained through 35 µm cell strainers and sorted on a FACS Diva system (BD). Single stains were done on BD-FACS beads. Fluorescently labeled antibodies (CD45-FITC, CD31-FITC, Ter119-FITC, CD140a-PE, CD34-APC, Sca1-ACP/Cy7) were bought at BioLegend.

Real-time PCR

mRNA was isolated and transcribed into cDNA using the Multi-MACS cDNA kit (Miltenyi). Expression levels of mRNA were assessed by real-time PCR using SybrGreen (Invitrogen) according to the manufacturer's protocol. Expression was normalized to 36B4.

Adipocyte Differentiation Analysis

Differentiated cells were fixed with 4% formaldehyde prior to staining with Hoechst (nuclei), BODIPY (lipid droplets) and Syto60 (cytosol) (Invitrogen). Images were taken per well with an automatic imaging system (Operetta, Perkin Elmer) and analyzed for lipid droplet content using the Operetta software.

Immunofluorescence

Decellularized ECMs were stained with primary antibodies against fibronectin (Santa Cruz Biotechnoloy, SC-9086, 1:500), Collagen I, IV and VI (Abcam, ab21286, ab 6586 and ab6588, 1:500) at 4°C overnight, washed with PBS and stained with A488-labeled secondary antibody (Invitrogen, A-21206). Images were taken per well with an automatic imaging system (Operetta, Perkin Elmer).

Immunoprecipitations

All immunoprecipitations were performed from postconfluent 3T3-L1 cells. Lysis was done in RIPA buffer (50mMTris [pH 7.4], 150mMNaCl, 1% Triton X-100, with 60 mM octyl glucopyranoside), based on [15]. Briefly, cell lysate was incubated overnight at 4°C on an overhead rotator with 5µg of antibody (Santa Cruz Biotechnology FLOT2 SC-25507 or rabbit

IgG control SC-2027). Next, 60 µl of Protein G-PLUS Agarose (Santa Cruz) (pre-washed twice with RIPA buffer) was incubated with lysate for 2 h at 4°C on overhead rotator. Agarose beads were washed 5 times (RIPA, 1000 g spin for 2 min between washes) and proteins were eluted in supernatant for western blots in 100 µl of 2x Laemmli buffer (boiled for 10 min before a brief full speed spin to pellet the remaining agarose). For mass spectrometry, agarose beads were transferred to Bio-Spin columns (Bio Rad, #732-6204) for 5 washes with 50 mM ammonium bicarbonate buffer. Proteins were digested overnight at 37°C with 0.5 µg trypsin in 50 mM ammonium bicarbonate buffer and peptides in supernatant were e collected and frozen.

Mass Spectrometry

For proteomic experiments ECMs were grown in 5ml cell culture dishes. ECM samples were digested in the cell culture dish with 0.5 µg/ul trypsin in ammonium bicarbonate buffer at 37°C overnight. ECMs were scraped off the plates, sonicated in Eppendorf tubes, digested for another 3h at 37°C. Peptides were clarified by centrifugation at 13 000 g for 10 minutes.

Trypsin digested samples (ECM or pulldown) were reduced with 5mM TCEP (tris(2-carboxyethyl)phosphine) and alkylated with 10mM Iodoacetamide. Peptides were purified with 3–30 µg UltraMicroSpin columns (The Nest Group), dried in a speed-vac and resolubilized in 0.1% formic acid.

For reversed-phase chromatography a high-performance liquid chromatography (HPLC) column (75-µm inner diameter, New Objective), which was packed in-house with a 15-cm stationary phase (ReproSil-Pur C18-AQ, 1.9 µm , Dr. Maisch), was used,connected to aneasy-nLC 1000 system (Thermo Scientifc). The HPLC was coupled via a nanoelectrospray ion source (Thermo Scientific) to either a Q-exactive plus mass spectrometer (Thermo Scientific; ECM sample data acquisiton) or an Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific; FLOT2-IP sample data acquisition. After loading the peptides on the column with 95% buffer A (98% H_2O, 2% acetonitrile, 0.1% formic acid), they were eluted at flow rate of 300 nl/min over a 80 min linear gradient from 5–35% buffer B (2% H_2O, 98% acetonitrile, 0.1% formic acid). Mass spectra were acquired in a data-dependent acquisition mode with one cycle consisting of one MS1 scan followed by 10 MS/MS scans.

MSconvert, as part of the Proteowizard (64bit, Version 3.0.6002) package [16] was used to convert raw data into the open mzXL format, which were searched with Comet (2015.01 rev. 2)[17] against the mouse UniProtKB/Swiss-Prot protein databases (release 2014_04). Search parameters for peptide-spectrum matching included cysteine carbamidomethylation (+57.021 Da) as a static amino acid modification as well as methionine, lysine and proline oxidation (+15.995 Da) as variable modification. For probability scoring of peptides and proteins the Trans-Proteomic Pipeline (Version 4.7)[18] was used including PeptideProphet and ProteinProphet, and protein identifications were filtered to a false-discovery rate of ≤1%. For

MS1 based label-free quantification of the identified peptides, MS1 ions signals corresponding to peptide features were extracted with theProgenesis QI for proteomics software (Nonlinear dynamics). After intensity normalization, peptide signal intensities belonging to the same protein were summarized followed by statistical analysis of the data.

Gene Onthology Analysis

GO-annotation was performed using the Mus musculus pantherdb database [19].

Bioethics

All animal studies were approved by the Canton of Zurich Veterinary Office.

Statistical Analysis

Results are given as mean ± standard error. Statistical analyses were performed using a two-tailed Student's *t*-test.

3. Results

3.1 Depot specific differentiation potential is inherent to depot-resident Lin- fibroblasts and Lin- PDGFRα+ populations

Depot specific differences of differentiation potential of preadipocytes from different adipose tissues have been reported in tissues of both human and mouse origin [20], [21],[22], with preadipocytes of a Sc origin typically showing higher degrees of differentiation than Vis derived preadipocytes. In contrast, the ex-vivo differentiation capacity of BAT derived preadipocytes has not been compared to that of Sc and Vis derived preadipocytes. Several studies indicate that differences in preadipocyte abundance in Sc and Vis depots are not the cause for altered differentiation capacities, however this is complicated by the use of surface markers [20], [23]. For Lin- PDGFRα+ fibroblasts, which have been shown to be upstream of all mature adipocytes by linage tracing in PDGFRα–Cre:mT/mG mice [5], these comparisons are lacking. Since the adipocyte niche has also been described to be perivascular, and since immune cells have been reported to be important for regulating adipogenesis we quantified the abundance of endothelial (CD45-, CD31+), immune (CD45+) and Lin- PDGFRα+ preadipocytes in Sc, Vis and BAT AT depots (**Figure 1A**). We found that the Vis AT has a higher content of immune cells than the Sc, and both WAT depots have a higher content than BAT (**Figure 1B**). CD31+ CD45- endothelial cells, are highest in BAT, lower in Sc and lower still in Vis AT. Inversely, Lin- fibroblasts, known to contain the Lin- PDGFRα+ precursors, are highest in Vis, lower in Sc and much lower in BAT. In addition, Vis SVF also has a higher percentage of PDGFRα+ within its Lin- population than Sc, and both white depots have a much higher percentage than BAT (**Figure 1A)**.

The marked difference of endothelial and immune cells between Sc and Vis lead us to suspect that the differences in adipogenic potential of the ECMs is modulated by the presence of endothelial or immune cells. Therefore, we isolated the Lin- PDGFRα+ population from WAT and compared its differentiation capacity to complete SVF. As shown in **Figure 1C, D** we observed that the differences in differentiation are maintained in these isolated cells, suggesting that the differences in the ex vivo adipogenic potential of SVF from different AT depots reside within the Lin- PDGFRα+ population and does not stem from the contribution of immune cells.

3.2 Addition of vitamin C enhances adipocyte differentiation and orderly deposition of collagens in adipose SVF ex vivo

Preadipocyte differentiation has been shown to be dependent on the production of collagen, as well as interaction with ECM [24]. Since vitamin C (vitC) is an essential cofactor in the posttranslational modification of collagens, as a first approach to probe for the involvement of ECM in mediating depot specific adipogenesis differences, we differentiated primary SVF of

Vis, Sc and BAT in the presence or absence of vitC (**Figure 2A**, **B**). We could show that addition of vitC enhances differentiation in SVF of all three depots and that, as previously reported, Sc SVF differentiates better than Vis, while BAT SVF differentiated much more than both the Sc and Vis derived SVF with vitC. To test whether the antioxidant properties of vitC are responsible for the observed effect, we differentiated 3T3-L1 preadipocytes in the presence of vitamin E (vitE), a strong antioxidant, or vitC. Since only vitC led to an induction of adipogenesis (**Figure S1A**) we decided to investigate contributions of the ECM on adipogenesis.

In order to test whether the ECM deposited by different SVFs influences differentiation of adipose precursors we implemented a protocol to decellularize primary SVF to obtain SVF-derived ECM. As an initial approach we performed immunohistochemistry on decellularized primary ECM focusing on fibronectin (FN), as well as two collagens (COL1 and COL4). Fibronectin is one of the most abundant ECM proteins and has been reported to be deposited by fibroblasts very early upon culturing. Collagen I is a collagen belonging to the fibrillar type, being deposited as a broad mesh, and Collagen IV is one of the main components of the basement membrane deposited by endothelial cells. Immunohistochemistry analysis of the ECMs, showed that the addition of vitC did not affect the amount or morphology of Fibronectin deposition, but strongly affected both the amount and the morphology of collagen deposition. VitC increased both the amount of collagen deposition but also the shape of the deposition, with bundles of collagens being more focused and pronounced **(Figure 2C)**. A blatant morphological difference of the vitC ECM is the appearance of a network of elongate bundles of basement membrane collagens (COL1 and COL4) **(Figure 2C)**. We hypothesized these cells to be endothelial cells, and confirmed this by staining with CD31, a well described endothelial cell marker **(Figure S2A)**. Given the strong visual phenotype of endothelial cell basement membrane collagens on the decellularized ECMs, as well as the fact that in plated SVF endothelial cells clearly attach and form distinct ECM, we next tested to see how removing endothelial cells affect differentiation of plated SVF. We could show that removing endothelial cells from SVF by FACS-sorting CD31- cells enhanced differentiation in both tissues (**Figure 2D, E**) to an extent that it was similar to plated Lin- PDGFRα+ SVF (**Figure 1C, D**). Nevertheless, the depot specific differences were retained (**Figure 2D, E**), corroborating our finding that Lin+ cells are not responsible for the observed depot specific differences, ex vivo.

3.3 SVF-derived ECM modulates differentiation capacity of adipocyte precursor cells

In order to unequivocally test whether the ECM deposited by different SVFs influences differentiation of adipose precursors we implemented a protocol to reseed primary, FACS-sorted adipose Lin- PDGFRα+ precursors into decellularized primary SVF-derived ECM (**Figure 3A**) and induce differentiation. Since Vis AT has the highest amount of these cells and since Vis SVF shows the lowest amount of differentiation (**Figure 2B**), we analyzed the capacity of Vis Lin- PDGFRα+ adipocyte precursors to differentiate in ECM environments from all three depots. We found that the origin of the ECM largely recapitulated the differences in differentiation observed in different tissue SVF, as Vis precursors differentiated better in Sc and BAT than Vis ECM **(Figure 3B)**. To quantify the effects, we extracted RNA from reseeded cells after 2 days of differentiation and measured levels of PPARγ and FABP4, two proteins which are induced early in adipocyte differentiation. Reseeding adipocyte precursors into their respective ECMs shows that ECMs grown with added vitC enhances differentiation (**Figure 3C, D**). Measuring FABP4 and PPARγ levels from Vis precursors in all three ECMs, grown with added vitC, confirmed our observation that BAT ECM is more adipogenic than ECMs from both WAT depots **(Figure 3E).** Taken together, our data indicate that the ECM environment provided by the SVF influences the differentiation behavior of preadipocytes, ex vivo and recapitulates the capacity of SVF from different AT depots to differentiate, ex vivo.

3.4 Proteomic analysis of SVF-derived ECM

In order to elucidate the different effect of SVF-ECM on preadipocyte differentiation we performed label-free shotgun proteomics of Sc, Vis and BAT grown with and without added vitC. We detected peptides mapping to more than 1600 different proteins over all three tissues. The two most abundant proteins in all samples were fibronectin (FN) and collagen 1 (COL1) in samples generated in the presence and absence of vitC, respectively **(Figure 4A, Supplementary Table 1)**. Highly abundant proteins not regulated by vitC were ECM proteins such as fibrillin and vimentin and components of the cytoskeleton like actin or myosin, which are still adhering to decellularized membrane. Addition of vitC caused a marked increase of the amount of detected collagens (but no shift in types), concomitant with collagen-associated extracellular proteins, most prominently periostin, biglycan and decorin. Gene-Onthology analysis of all detected proteins mapped a number of detected proteins to compartments other than the ECM **(Figure 4B)**. This is partly due to contamination of cytosolic proteins attached to the decellularized ECMs non-specifically, most prominently histones, ribosomal proteins and heat-shock proteins, which are known contaminants in protein interaction studies [25], but partly reflects the fact that the functional interactions between the ECM and cells seem to partially stay attached during decellularization, accounting for the presence of cytolinker and cytoskeletal proteins. When analyzing the differences in ECM composition between the

different depots our datasets revealed a large overlap in the proteome between the different depots **(Figure 4C)**, with that derived from BAT having a more unique protein signature than the two WAT depots. Collectively, these data show that addition of vitC causes a strong increase in collagen deposition on SVF-derived ECMs, and shows a conserved core of proteins abundant in all adipose SVF-derived ECMs.

3.5 Flotillin 2 is enriched in pro-adipogenic ECM and mediates vitC dependent effects on differentiation

One protein that was consistently up-regulated in +vitC ECMs, to a level similar to collagens was Flotillin 2 (FLOT2). Therefore, we analyzed whether FLOT2 was also differentially regulated between the three depots. Indeed, we found significantly higher levels of FLOT2 in Sc and BAT ECM compared to Vis (**Figure 5A**). We also checked FLOT2 expression in vivo, by Western Blot analysis of whole tissue extract (**Figure S3A**). Here we detect slightly higher levels in Vis versus Sc, which is probably due to the fact that FLOT2 is ubiquitously expressed, and reflects the different cellular composition of these tissues. FLOT2 is a transmembrane protein localized to lipid rafts in the plasma membrane. This cellular localization, combined with the proteome-data, prompted us to speculate that FLOT2 plays a role in organizing the signaling machinery necessary to interpret specific ECM to cell signaling cues. To further understand the role of FLOT2 we performed co-Immunoprecipitation (co-IP) assays against FLOT2 in 3T3-L1 cells, and analyzed the precipitated proteins by mass spectroscopy, followed by running an algorithm designed to remove false interactions commonly detected in co-IP assays [26] (**Figure S3B,C**). GO-analysis of detected proteins revealed them to map to signaling pathways such as integrin signaling, axon guidance pathways, Ras, and cytoskeletal Rho signaling, which is consistent with our hypothesis (**Figure 5B**). Therefore, in order to test the functional involvement of FLOT2 in the differentiation process we used siRNA mediated knock down of FLOT2 in 3T3-L1 preadipocytes (**Figure S3D**) and differentiated them in the presence and absence of vitC. Interestingly, ablation of FLOT2 completely abolished the effect of vitC on adipocyte differentiation **(Figure 5C, D)** suggesting that this protein might indeed be one responsible factor mediating the effects of ECM on adipocyte differentiation potential. In summary, we found that FLOT2 is differently regulated in decellularized ECMs of different depots, with more pro-adipogenic ECMs showing much higher levels of FLOT2, and necessary for vitC enhanced differentiation in vivo, suggesting that it plays an important role in mediating the signaling of different ECM properties.

4. Discussion

In this study we show that the ECM of primary AT SVF directly influences differentiation of adipogenic precursors, which provides an explanation for existing findings that cells of distinct adipose depots show varying capacities for differentiating into mature adipocytes.

Interestingly, Lin- PDGFRα+ cells, which have been shown to contain adipocyte precursors [5],[6] are significantly more abundant in Vis than Sc adipose tissue, even though Sc Lin- PDGFRα+ cells have repeatedly been demonstrated to have a higher rate of differentiation than Vis derived cells [20],[23]. Two recent studies focusing on adipose tissue expansion during the very early time-points of obesity development (three days and two weeks of high fat diet (HFD) feeding), [27],[28] demonstrate on the other hand, that cell division and BrdU incorporation almost exclusively occurs in Vis, but not Sc adipose tissue. Based on these data it has been speculated that HFD feeding triggers local and systemic changes that cause an activation of the stem cell pool in adipose tissue and triggers proliferation and differentiation of preadipocytes. This is supported by a recent study which demonstrated that blocking inflammation, specifically in the adipose tissue, leads to reduced adipogenesis upon high fat diet feeding and a more unfavorable systemic metabolic outcome, suggesting that inflammatory processes are involved in such a regulation [29]. Related to this, an adipogenic niche consisting of PDGFRα+ fibroblasts expressing the osteopontin receptor CD44, as well as M2 polarized macrophages secreting osteopontin, was recently identified, and it was demonstrated that proliferation of these cells is enhanced in Vis over Sc adipose tissue upon triggering adipocyte hyperplasia [30]. Another study, comparing hyperplasia between Sc and Vis AT demonstrated more increased hyperplasia and turnover in the Vis than the Sc AT [31], in addition to showing that Sc AT is more easily exhaustible than Vis AT. These data taken together with our findings suggest that not the precursors themselves present in AT, but rather the microenvironment which is shaped by the ECM can confer the signal which is required to induce adipogenesis.

Furthermore, based on this compilation of data, it is tempting to speculate that the Lin- PDGFRα+ cell population contains a niche cell population which signals to maintain stemness and proliferative potential of precursor cells and blocks differentiation. Such a population would have to be more abundant in Vis AT than in Sc AT or BAT and would respond to dietary cues such as lipids derived from the diet. A better characterization of the Lin- PDGFRα+ subpopulations will be needed to identify such cells.

Furthermore, our data of EMCs grown with and without vitamin C suggest that collagens play an essential role in conferring these properties which is in line with recent findings, in vivo [32]. One caveat when interpreting our findings is the fact that cells seeded into the ECM themselves will modulate the matrix and thus contribute to the observed phenotype.

Most cells require stable cell-matrix adhesion contacts for survival and proper function. The canonical pathways for ECM to cell signaling at the plasma membrane are focal adhesion kinase (FAK) and integrin linked kinase (ILK). In addition, the transcription factors YAP and TAZ have recently been shown to play an important role in regulation the signaling machinery for sensing and reacting to different matrix stiffness cues [33]. However, knock-downs of these factors, while affecting differentiation of 3T3-L1 cells compared to unspecific siRNAs, do not modulate the collagen dependent vitC response, suggesting that the collagen dependent effect of ECMs we observe in preadipocytes from different depots is probably not mediated by these proteins.

In addition to the matrix functionalities such as receptor binding motifs, growth factor sequestering, or protease dependent release of signaling peptides, matrix stiffness of the ECM is an important property [34],[35]. Recent findings suggest that an important factor contributing to stiffness sensing may however just lead to changes in the availability of binding sites for mechanosensing complexes on matrices with larger pore sizes [36]. While we did not investigate mechanical properties, we found the plasma transmembrane and lipid raft associated protein FLOT2 to be regulated in accordance with the adipogenicity of adipose depot-derived ECMs. FLOT2 is associated to the plasma membrane by two n-terminal transmembrane hairpin loops as well as n-terminal myristoylation and palmitoylation, while the c-terminus is cytosolic. FLOT2 and its homologue FLOT1 were first described in regenerating ganglion cells, and independently identified as proteins in detergent resistant membrane fractions that float in density gradients [37],[38]. Because of their prominent role in lipid raft formation Flotillins have been studied in the context of cell proliferation, migration and cell-cell interaction and they have been shown to interact or associate with both cell surface signaling molecules GPCRs [39], receptor tyrosine kinases, and intracellular actin binding proteins such as myosin [40] or γ-catenin [41].

Interestingly, while we found a roughly 2-fold increase of FLOT2 in pro-adipogenic ECMs, FLOT1 abundance was not changed, suggesting a differential regulation of certain membrane microdomains in relation to matrix composition and assembly. We hypothesize that FLOT2 might organize a matrix signaling hub integrating a variety of ECM signals. Our co-IP data support this interpretation even in light of the fact that we performed co-IP with an antibody against an endogenous transmembrane protein, which is a detergent-resistant microdomain-protein and thus might have a limited coverage. A recently published paper outlining a human interactome by performing co-IPs for a wide range of cellular targets, using GFP-tagged overexpressed proteins and computationally matching mutual hits, reports that FLOT2 interacts with a variety of small GTPases and G-protein subunits, as well as actin binding proteins implicated in mechanosensing, cytoskeletal dynamics, and cell motility, lending further

support to our hypothesis that FLOT2-dependent signaling contributes to sensing and interpreting ECM properties [42].

In summary, we demonstrate that the depot specific differences of the potential of adipocyte precursors to form mature adipocytes is mainly dependent on the ECM, and that integration of the necessary ECM to cell signaling cues might in part be mediated through transmembrane protein Flot2.

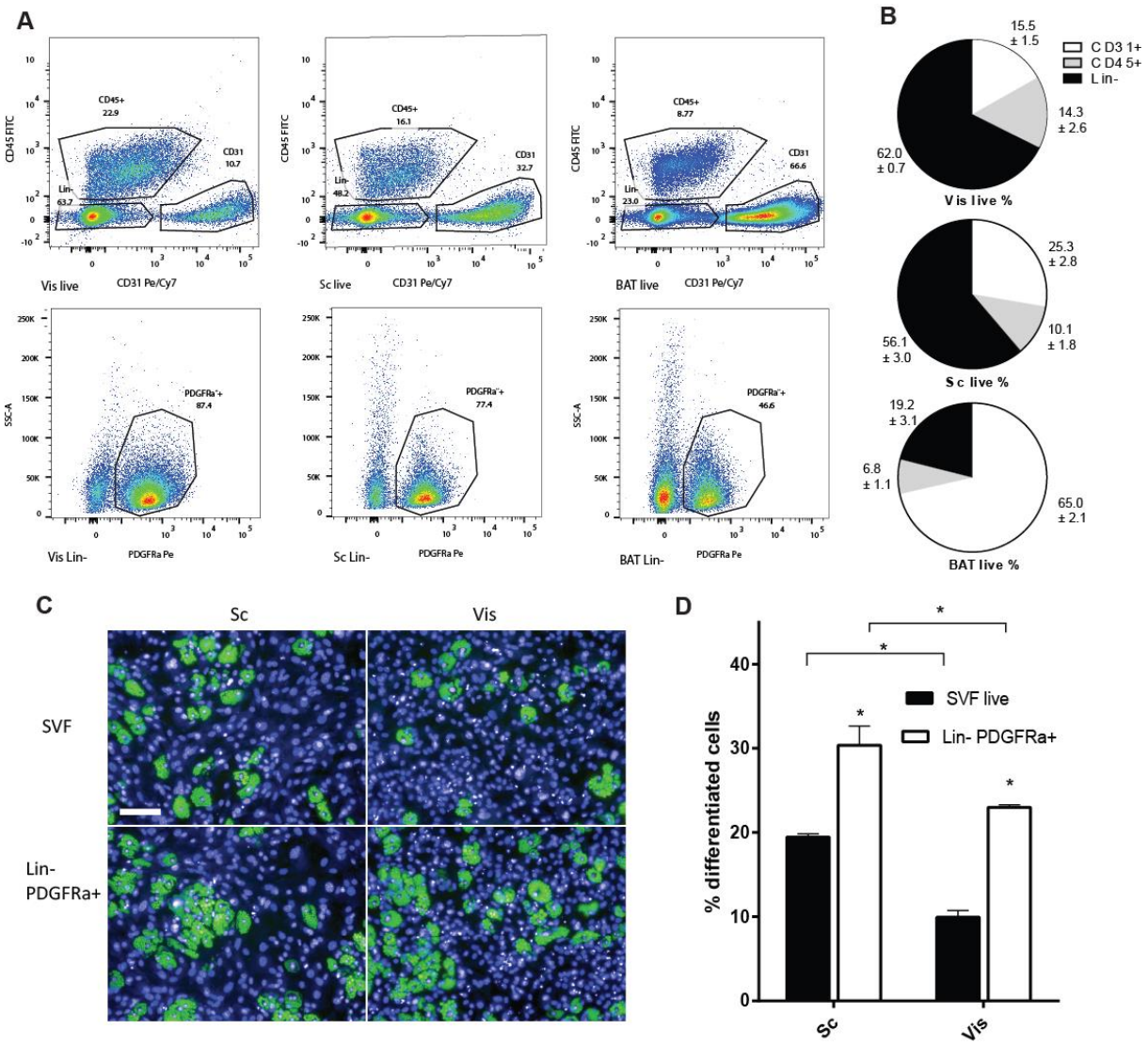


Figure 1: Depot specific differentiation potential is inherent to depot-resident Lin- fibroblasts and Lin- PDGFRα+ populations. (**A**) FACS-analysis of Sc, Vis and BAT SVF, stained for CD31 (A488), CD45 (PE), PDGFRα (APC) and Sytox Blue (**B**) Quantification of SVFs for cellular composition (n = 4). (**C**) Differentiation of Sc and Vis Lin- PDGFRα+ cells with vitC; lipid (green) and nuclei (blue) staining. (**D**) Quantification of Lin- PDGFRα+ cell differentiation (n = 4). * $P < 0.05$ by Student's *t*-test. Bar graphs shown are mean ± s.e.m. Scale bar = 100um.

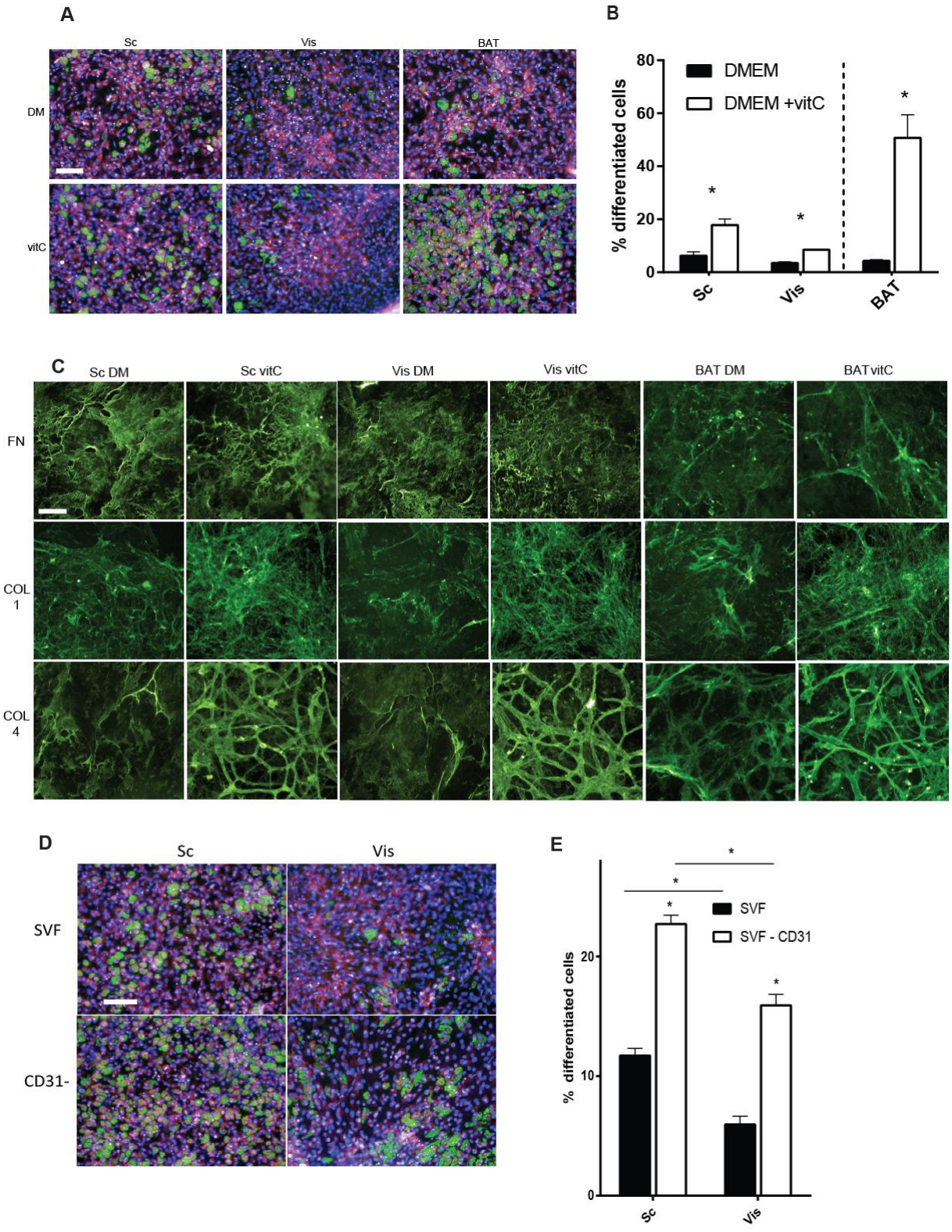


Figure 2: Addition of vitamin C enhances adipocyte differentiation and orderly deposition of collagens in adipose SVF ex vivo. SVF of Sc, Vis and BAT differentiated in the presence and absence of vitC. (**A**) Representative images: lipid (green), nuclei (blue) and cytosol (pink) staining. (**B**) Quantifiaction. (**C**) Images of decellularized ECM of Sc, Vis and BAT SVF, stained for Collagen I (COL1), Fibronectin (FN) and Collagen VI (COL4), with Alexa 488 (green). (**D**) SVF of Sc and Vis differentiated in the presence of vitC with and without depletion of CD31+ cells. Lipid (green), nuclei (blue) and cytosol (pink) staining. (**E**) Quantification of SVF differentiated with and without CD31+ cell depletion (n=4). * $P < 0.05$, ** $P < 0.01$ by Student's *t*-test. Bar graphs shown are mean ± s.e.m. Scale bar = 100um.

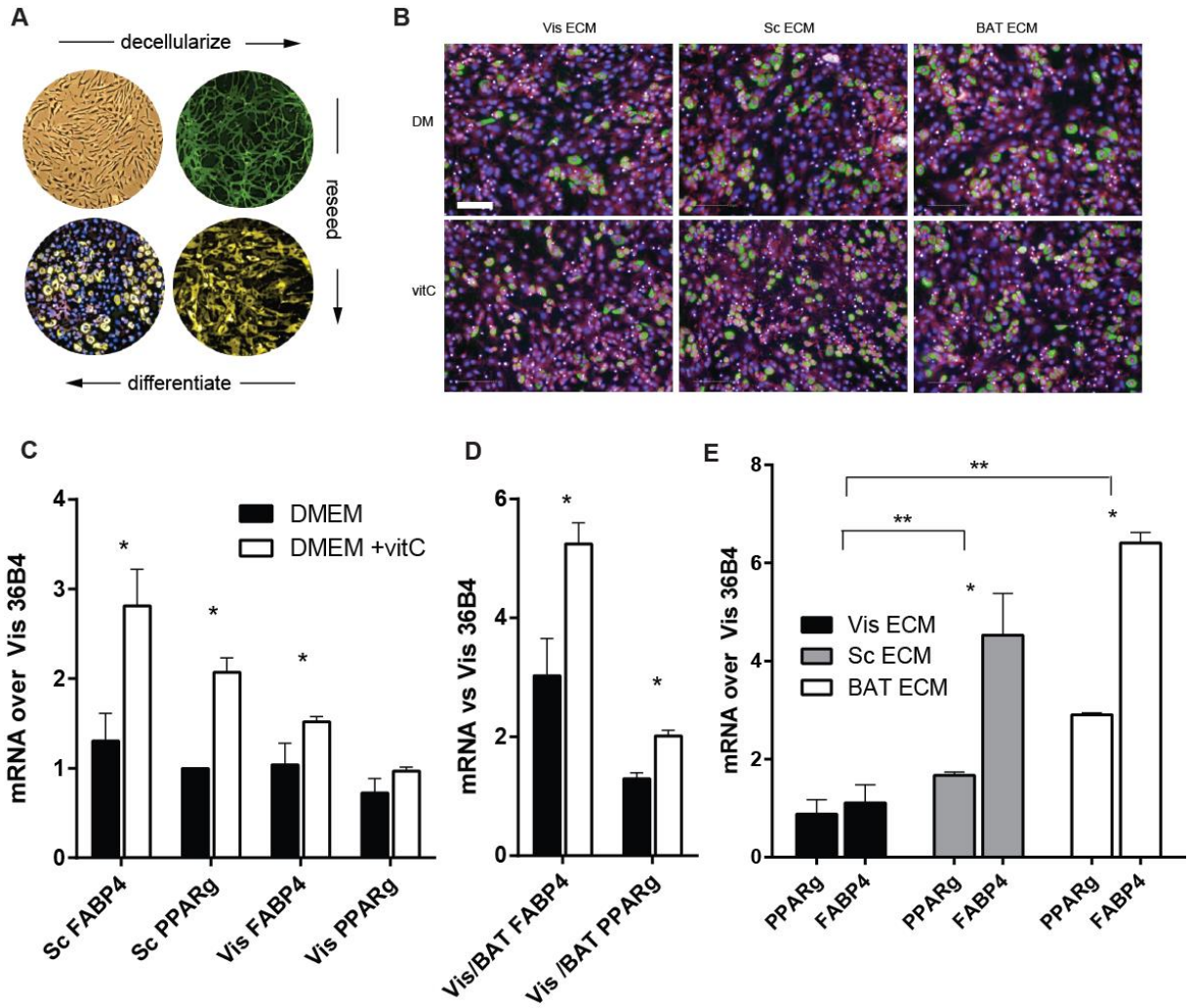
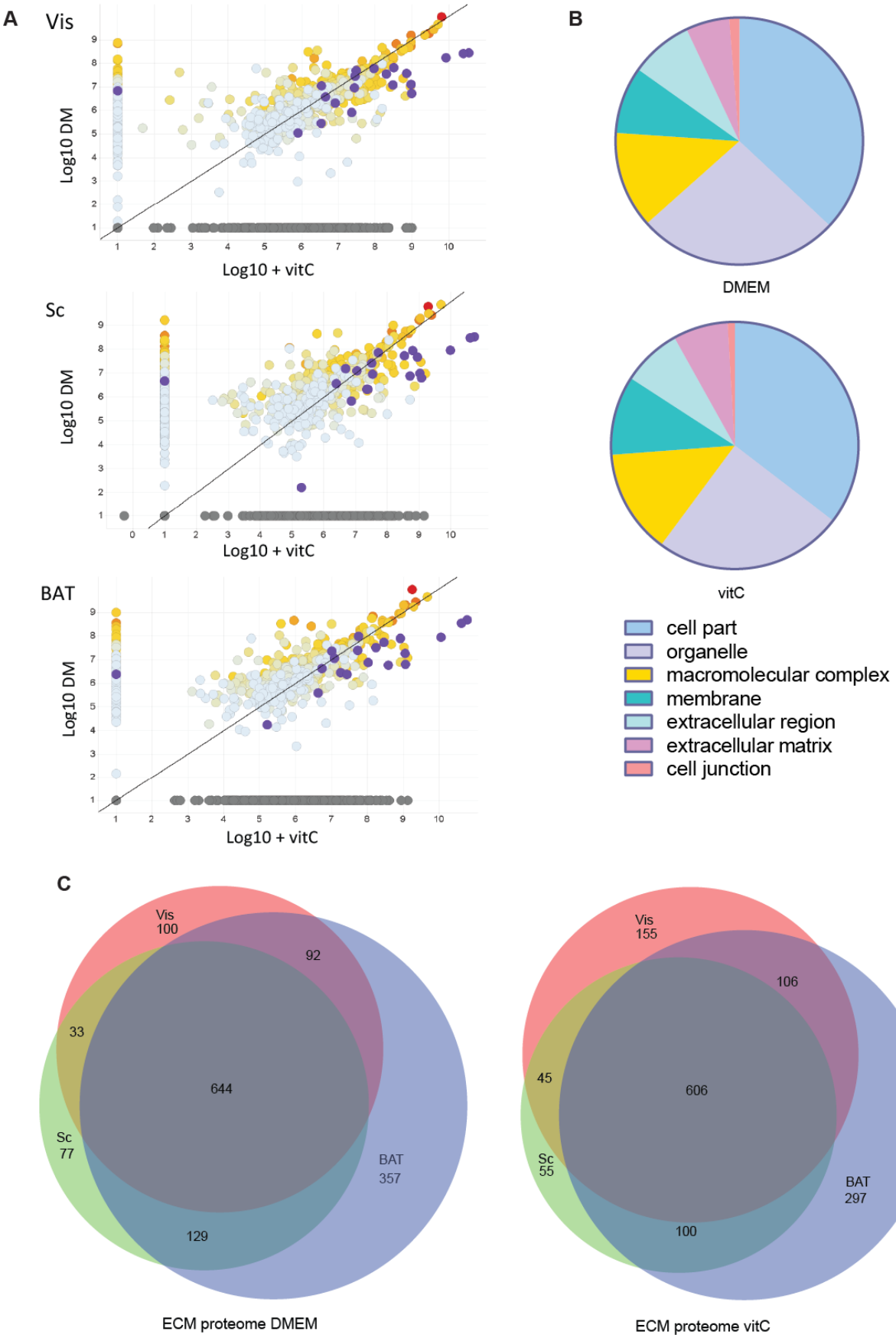


Figure 3: SVF-derived ECM modulates differentiation capacity of adipose stem cells. (**A**) Scheme of SVF-decellularization and reseeding with FACS-sorted adipocyte precursors. (**B**) Lin- PDGFRα+ Vis adipocyte precursor cells differentiated in ECMs ± vitC, representative images: lipid (green), nuclei (blue) and cytosol (pink) staining. (**C-E**) qPCR for FABP4 and PPARγ of RNA extracted from differentiating precursors (**C**) Lin- PDGFRα+ seeded into ECM ± vitC (n = 3). (**D**) Vis Lin- PDGFRα+ cells seeded into BAT ECMs ± vitC. (n=3) (**E**) Vis Lin- PDGFRα+ cells seeded into ECMs +vitC of Sc, Vis and BAT. (n=3) * $P < 0.05$ by Student's *t*-test, Bar graphs shown are mean ± s.e.m. Scale bar = 100um.


A
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Log10 DM
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Sc
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Log10 + vitC
BAT
Log10 DM
Log10 + vitC
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organelle
macromolecular complex
membrane
extracellular region
extracellular matrix
cell junction
C
Vis
100
92
33
644
Sc
77
BAT
357
129
ECM proteome DMEM
Vis
155
106
45
606
Sc
55
BAT
297
100
ECM proteome vitC

Figure 4: Proteomic analysis of SVF-derived ECM does not reveal a strong depot specific signature. (**A**) Scatter plots of detected proteins in SVF-derived ECM of Sc, Vis and BAT, grown in DMEM and DMEM +vitC, showing fibronectin (red), collagens (purple) and other proteins (hues of yellow, grey and blue). Log10 scale, non-detected samples (0) are set to 1. (**B**) Panther classification – Annotations of all proteins detected in the three tissues, grown with and without added vitC. (**C**) Venn-Diagrams of SVF-ECM from Sc, Vis and BAT grown in DMEM and DMEM + vitC.

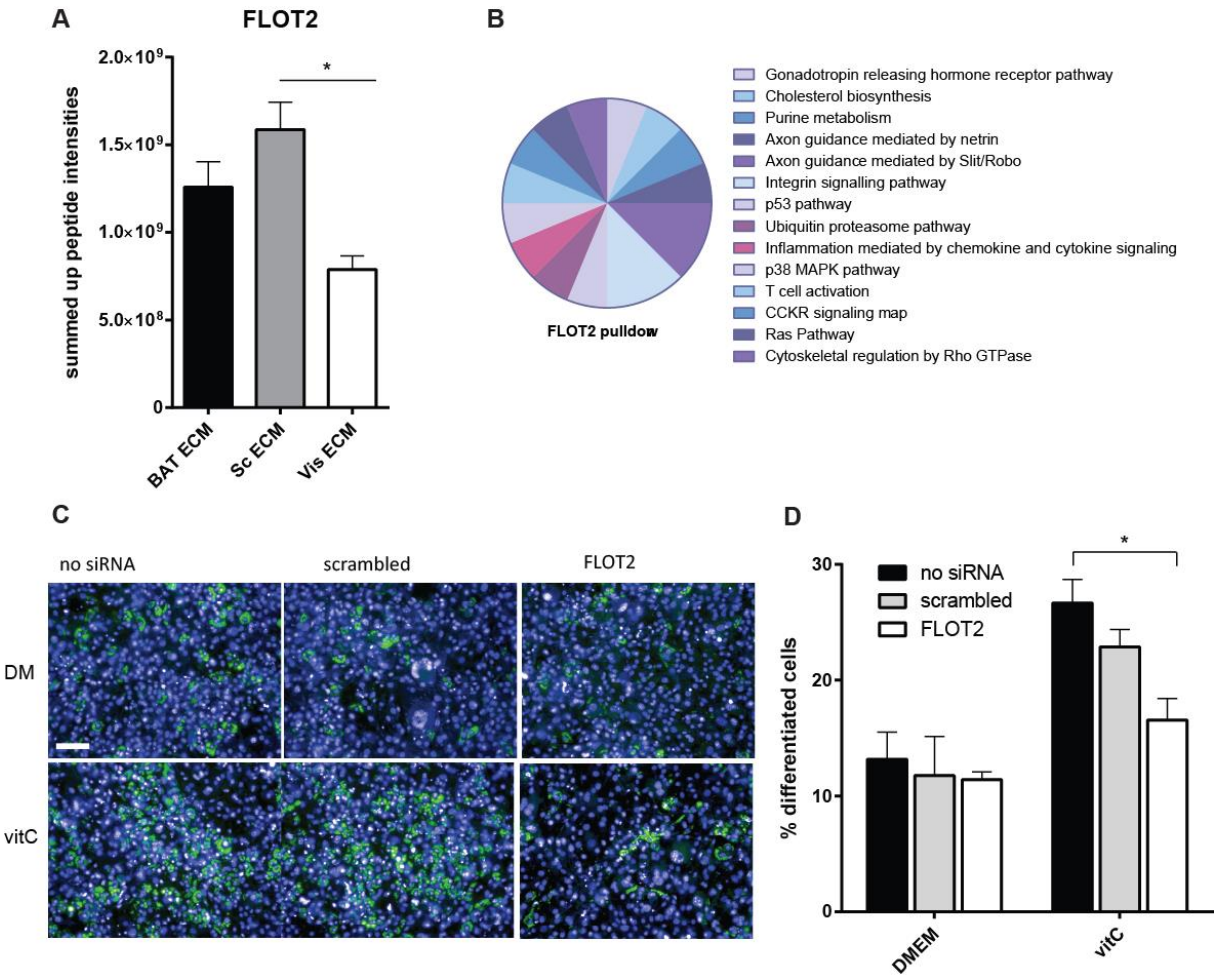
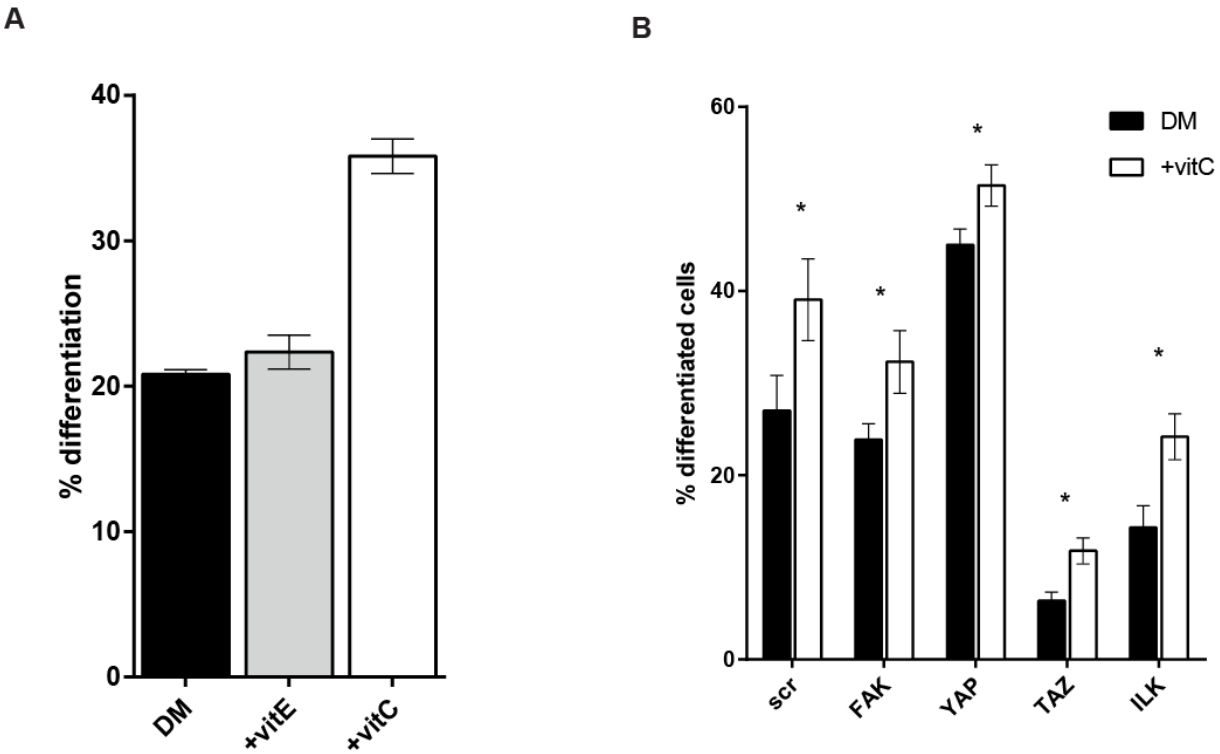
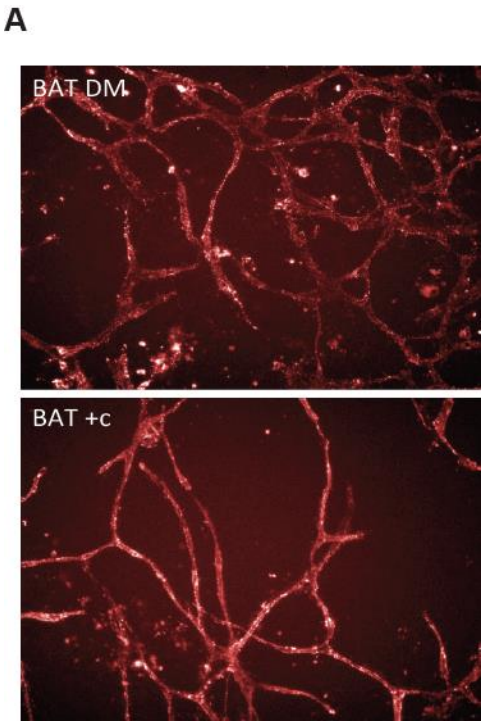


Figure 5: Flotillin 2 is enriched in pro-adipogenic ECM and mediates vitC dependent effects on differentiation

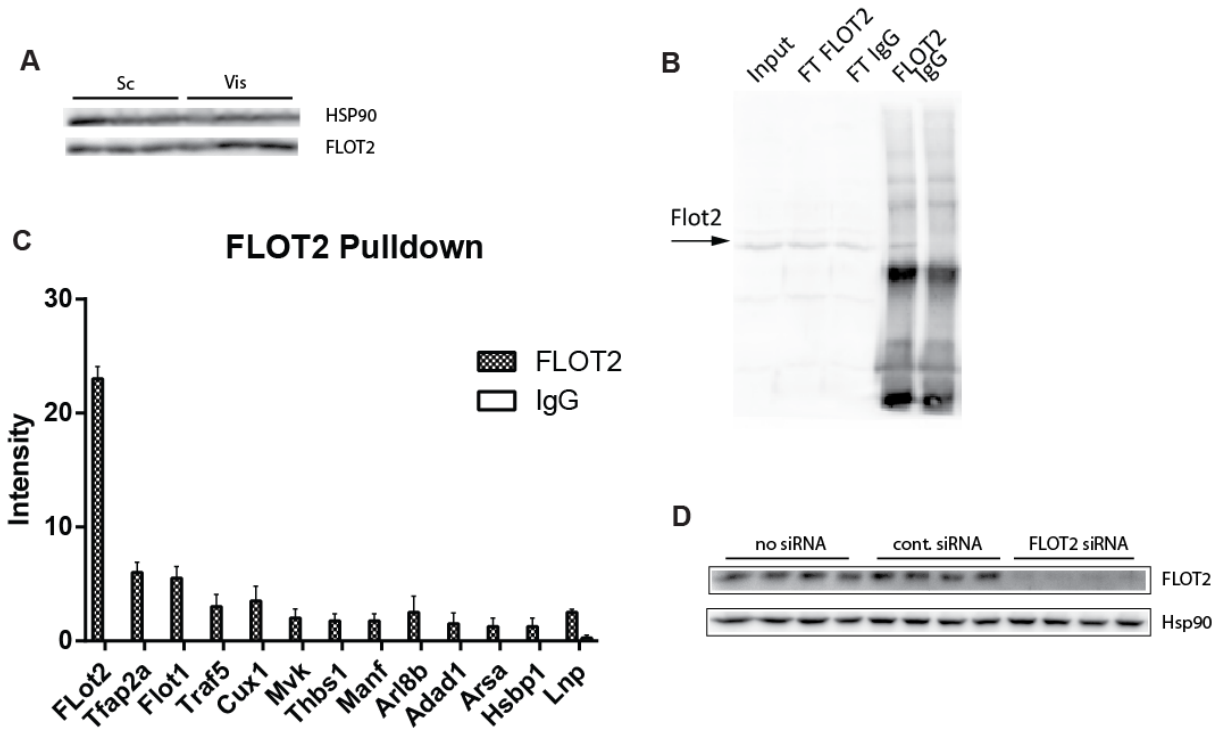
(**A**) Summed up petide intensities of FLOT2 in decellularized ECMs of Sc, Vis and BAT grown in DMEM +vitC. (**B**) Panther-classifications of the signaling pathways of detected FLOT2 interacting proteins (n=4). (**C**) 3T3 cells differentiated in the presence and absence of vitC with siRNA against FLOT2 (n=3). (**D**) Representative images: lipid (green) and nucleus (blue) staining of differentiated 3T3-cells. Bar graphs shown are mean ± s.e.m. * $P < 0.05$ by Student's *t*-test. Bar graphs shown are mean ± s.e.m. Scale bar = 100um.



Supplementary Figure 1: (**A**) 3T3-L1 cells differentiated in regular DMEM, DMEM + vitamin E and DMEM + vitamin C. (**B**) Effect of siRNA mediated knockdown of FAK, YAP, TAZ, ILK and on differentiation in 3T3-L1 cells.



Supplementary Figure 2: (**A**) Plated SVF-cells stained for A647 labeled CD31 (for endothelial cells), demonstrating attachment of endothelial cells.



Supplementary Figure 3: (**A**) FLOT2 expression in vivo: protein extracts of Sc and Vis AT of 8 week old mice, stained with anti-FLOT2 and anti-Hsp90. (**B**) Western Blot of Flotillin 2 Immunoprecipitation from 3T3-L1 cell extracts with vitamin C: Input (InP), flow-through FLOT2 antibody (FT), flow-through IgG antibody (FT IgG), FLOT2-beads (FLOT2), IgG-beads (IgG). (**C**) Proteins most enriched in FLOT2 vs IgG pulldown (n=4). (**D)** Western Blot of siRNA mediated knockdown of FLOT2 in 3T3-L1 cells.

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Author Contributions

G.G. developed the hypothesis, performed experimental work, analyzed the data and wrote the manuscript, B.W. supervised the proteomic experiments, S.M. and H.M. performed mass spectrometric data acquisition and analysis, C.M. performed experimental work and analyzed data and C.W. initiated and supervised the project, analyzed the data and wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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Adipose Tissue Endothelial Stress as the Driver of Insulin Resistance in the Metabolic Syndrome

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Contribution

Gerald Grandl developed the hypothesis and project, performed experiments, analysed data and wrote the manuscript.

This article is in preparation.

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Adipose Tissue Endothelial Stress as the Driver of Insulin Resistance in the Metabolic Syndrome

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Summary

The association of the metabolic syndrome (MS), a combination of impaired glucose homeostasis with cardiovascular risk factors such as elevated plasma lipids, elevated blood pressure, and central obesity, in particular visceral (Vis) adipocyte hypertrophy, is well established, but their causal contribution to the pathology is still incompletely understood. We found that after 3 days of HFD feeding systemic glucose tolerance is impaired, together with insulin dependent AKT phosphorylation in the liver, while subcutaneous (Sc), Vis adipose tissue (AT), and muscle show intact AKT phosphorylation. Analysing insulin dependent eNOS phosphorylation, we observed that endothelial insulin sensitivity in the Vis adipose tissue is already severely impaired after 3 days of HFD, while in Sc adipose tissue it is still responsive. This was correlated with adipocyte hypertrophy which in Vis adipose tissue occurs after a single high fat diet (HFD) meal, and is much more pronounced than Sc after 3 days. We hypothesised that hypertrophy is linked to AT endothelial stress and insulin resistance, which was supported by increased ROS production in AT in conjunction with platelet activation. In contrast, treatment with the antiplatelet and vasodilator drug dipyridamole ameliorated glucose tolerance, indicating a functional role for endothelial stress in mediating HFD induced insulin resistance.

Keywords

Adipose Tissue Hypertrophy

Metabolic Syndrome

Insulin Resistance

eNOS

Endothelial Stress

Diet Induced Obesity

ROS

1. Introduction

In the last decades, global rates of obesity have been rising at an alarming speed, with no indication of a reversal of this trend in the near future (NCD Risk Factor Collaboration, 2016). Central obesity is the key risk factor for developing the metabolic syndrome (MS), a clustering of the cardiovascular risk factors of central obesity together with elevated plasma triglycerides, reduced plasma high density lipoprotein (HDL), increased blood pressure or raised fasting blood glucose (IDF, 2006). The metabolic syndrome predisposes towards the development of type two diabetes (T2D) and represents a considerable disease burden, particularly increasing the risk of cardiovascular mortality (Gerstein et al., 2012; Holman RR, Paul SK, 2008).

The aetiology of the metabolic syndrome is still incompletely understood, but the association of adipocyte hypertrophy in visceral adipose tissue and resulting inflammation and elevation of systemic pro-inflammatory markers have been implicated. The functional link of adipose tissue (AT) inflammation to obesity-induced insulin resistance was first established by (Hotamisligil et al., 2012), and has since been confirmed in multiple studies (Olefsky and Glass, 2010). Although the causes of AT inflammation remain to be completely elucidated, AT hypertrophy, in particular of visceral (Vis) AT typically correlates with inflammation and insulin resistance. The timeline and causal interaction of adverse effects in diet induced obesity (DIO) is far from clear. A commonly accepted model of the development of insulin resistance states that overloading of adipose tissue by lipids causes eventual lipid spill-over into other tissues, leading to so called ectopic lipid deposition and causing metabolic impairments in these tissues (Longo and Shulman, 2014).

However, (Ono et al., 2008) reported impaired hypothalamic insulin signalling already after 1 day of high fat diet (HFD) feeding, a time-point at which adipose tissue inflammation has not yet been demonstrated, and recently (Thaler et al., 2011) described signs of hypothalamic injury a few days after beginning HFD feeding. (Lee et al., 2011) reported impaired systemic glucose clearance already after 3 days of HFD and demonstrated that this early insulin resistance is not ameliorated by various ways of blocking immune-cell dependent inflammatory responses, while insulin resistance caused by long term HFD feeding is. On the other hand, (Wiedemann et al., 2013) report adipose tissue inflammation after 4 days of HFD and use Fabp4-Cre mediated Fas deletion to prevent this inflammation and protect from the effects of HFD feeding. Together, these data indicate that there are two separate, if overlapping processes contributing to insulin resistance in HFD induced obesity, one of them slow and requiring inflammatory contribution of bone marrow-derived immune cells, and the other fast, probably involving the hypothalamus and possibly AT inflammation.

Besides the interplay of inflammatory processes and insulin resistance, the MS is also known to cause severe changes in the coagulation system, contributing to the key causes of mortality associated with the MS, which are cardiovascular (Carr, 2001). In addition T2D has also been shown to be closely linked to vascular disease, which is thought to be a direct consequence of elevated plasma glucose and lipid levels (Creager et al., 2003). Moreover, there is a large body of work looking at endothelial function in response to meals, with various studies showing that both elevated blood glucose and free fatty acids (FFA) contribute to impaired endothelial and increased platelet reactivity in the postprandial state (Gill et al., 2004; Jackson et al., 2007; Mah and Bruno, 2012; Nappo et al., 2002; Spectre et al., 2012).

Considering these various contributing factors, specifically Vis adipocyte hypertrophy together with inflammation and adverse endothelial effects, we hypothesised that the cellular and metabolic dynamics caused by increased lipid transport to AT cause direct endothelial stress and perhaps damage, leading to the aforementioned complications.

2. Results

2.1 Short term high fat diet feeding impairs systemic glucose tolerance, hepatic insulin dependent AKT phosphorylation and insulin dependent eNOS phosphorylation in AT

Changes in glucose tolerance have been reported after short periods of HFD exposure. To study this phenomenon, we fed C57/Bl6 mice with HFD for 3 days and performed an intraperitoneal glucose tolerance test (IPGTT). We found that systemic glucose tolerance is significantly impaired after only 3 days on a HFD (**Figure 1 A, B**), suggesting the development of a systemic insulin resistance. To further study the involvement of insulin, we quantified plasma insulin levels after a 6 hour fast of mice after 3 days of HFD feeding. As shown in **Figure 1C**, we observed a significant increase in insulin levels concomitant with an increase in fasting plasma FFA (**Figure 1D**). To elucidate whether insulin resistance occurs even earlier, we measured fasting glucose levels after only a very brief fast (3 to 4 hours) in mice, starting from day 1 of HFD feeding (**Figure 1E**). Interestingly, already after 1 day of HFD feeding we observed a significant increase of fasting plasma glucose levels after a brief fast, and analysis of the animals for three days more revealed that this was not a transient phenomenon, but persisted. Since this only established a systemic impairment of insulin and glucose signalling in very early HFD feeding and various suggestions have been made as to the first affected organs, we next decided to test levels of phosphorylated AKT-S473, a phosphorylation-site that is confirmed to be downstream of insulin signalling (Alessi et al., 1996), in liver, muscle, Sc and Vis after IP injection of insulin or saline in fasted animals by Western Blot (**Figure 1F**). Interestingly, we could not detect an obvious impairment in insulin-dependent AKT phosphorylation after 3 days of HFD feeding in either Vis, Sc or muscle. However, in liver we observed a clear reduction in insulin-dependent AKT phosphorylation, consistent with other findings reporting early selective hepatic insulin resistance in DIO. Since AT is a complex mixture of cells and since it has been reported that endothelial insulin resistance precedes the onset of peripheral insulin resistance (Kim et al., 2008), we next analysed insulin-mediated phosphorylation of eNOS at S1177 in Sc and Vis AT (**Figure 1G**) as a surrogate marker for endothelial insulin resistance in AT. The first striking observation was that phosphorylation of eNOS-1177S is much lower overall, both in basal and insulin-stimulated conditions, without a significant reduction in total eNOS levels (**Figure S1**). Quantification of the p1177S-eNOS signal normalized to heat shock protein 90 (HSP90) (**Figure 1H**), reveals that in spite of lower total levels of p1177S-eNOS, Sc AT is still able to respond to insulin with eNOS-S1177 phosphorylation, while this effect is already absent in Vis AT. These data show that insulin-mediated AKT phosphorylation is impaired in liver, but not other insulin responsive tissues after 3 days of HFD feeding. However, despite normal insulin signalling to AKT, which most likely reflects mature adipocyte function, the insulin dependent effect on eNOS phosphorylation is

severely impaired in Vis AT, while Sc AT eNOS is still phosphorylated in response to insulin. Taken together, this suggests that AT endothelium becomes insulin resistant at a time when insulin mediated phosphorylation of AKT is still functional.

2.2 Adipocyte hypertrophy in Vis AT already occurs after one HFD meal and precedes hypertrophy in Sc AT

HFD feeding is known to cause both hypertrophy and hyperplasia in AT, with hypertrophy, in particular of Vis AT, being linked to HFD induced insulin resistance. A lot of DIO studies in mice focus on long term feeding, often 12 weeks or more, but little is known about AT dynamics in an early response to HFD. Therefore, we first compared effects of hypertrophy in chow fed mice with mice on a HFD for 6 weeks and mice on a HFD for 16 weeks. We quantified adipocyte (AC) hypertrophy in Sc and Vis AT (**Figure S1**) and found that visceral AT has a more marked increase of hypertrophy than Sc AT, and that hypertrophy begins earlier in Vis AT. Based on these findings, we next decided to carefully analyse Vis and Sc AC sizes during early time points of HFD feeding. Comparing AC sizes of Vis and Sc of overnight fasted mice to mice fed either chow, HFD or HFD with 10% added Glucose 3 hours after feeding (to measure acute post-prandial effect of AC size) (**Figure 2A, 2B, 2C**), revealed that in Vis AT a shift in AC sizes away from smaller size and towards larger size is already apparent after a single meal, albeit to a much smaller extent than long-term HFD feeding, while we found no effect on Sc AC sizes after a single meal (**Figure 2D, 2E, 2F**). After 3 days of HFD feeding we also detected a noticeable increase in Vis AC size, and to a much lesser extent in Sc AC size (**Figure 2G, 2H**). Taken together, we show a much more pronounced hypertrophy in Vis AT compared to Sc AT, which correlates with the changes in endothelial insulin resistance.

2.3 Short-time HFD feeding causes an increase in AT ROS production and changes in systemic hemostasis while platelets are present in both basal and HFD fed AT interstitium

Given our findings of the dynamics of AC hypertrophy in Vis and Sc AT, we hypothesized that the rapid size-increase of cells in AT causes changes in flow through the blood vessels in these tissues, resulting in increased stress or injuries. As endothelial shear stress has been reported to cause increased ROS production (Hsieh et al., 2014) we investigated whether H_2O_2 production was changed in Vis and Sc AT 3 hours after feeding with either a HFD or a chow meal (**Figure 3A**). We found that 3 hours after a HFD meal H_2O_2 production is significantly increased compared to a chow meal in Vis, but not Sc AT. When testing postprandial ROS production in Vis and Sc AT of animals after 3 days of HFD versus chow feeding we found three days of HFD feeding increases ROS production in both the Vis and Sc AT (**Figure 3B**),

although similar to our other findings the effect is more pronounced in Vis AT. Since blood vessel injuries trigger coagulation and lead to platelets attaching to exposed sub-endothelial space, we asked if we could observe interstitial platelet binding in AT after HFD feeding. To that end, we performed immunohistochemical analysis on Vis AT staining for endothelial cells and platelets (**Figure 3C**). To our surprise, we found large numbers of attached platelets in the AT interstitium in both chow and HFD fed conditions. The high heterogeneity of platelet attachment, dependent on the proximity to and density of endothelial cells, precluded attempts to quantify levels of attached platelets in the different conditions. To exclude the possibility that the platelet binding was an artefact of the whole-mount staining procedure, we repeated stainings on normal tissue and tissue from animals perfused with phosphate buffer to remove the blood (**Figure S3**), and confirmed our finding of interstitially attached platelets in perfused tissues. To further corroborate our data and also look at endothelial cells, we next performed transmission electron microscopy (TEM) on Vis AT of fasted versus HFD-fed postprandial mice (**Figure 3D**). We were able find clear evidence of extravascular attached and activated platelets, confirming our immunohistochemical findings, but our coverage of endothelial cells was not sufficient to draw conclusions concerning vascular injury based on our TEM data. Finding this evidence of coagulatory events even at baseline, compared with reported findings of increased coagulation in mice and humans on a HFD (Cleuren et al., 2015; Miller, 1998) prompted us to assess systemic changes in coagulation. Therefore, we analysed mean bleeding times in mice after 3 days of HFD versus chow mice (**Figure 3E**), and found that coagulation is already slightly increased in these mice. Overall, these data show HFD feeding induced AT ROS production in a way that correlates with AT hypertrophy, while platelet attachment, and ostensibly vascular micro-injuries, occur frequently in Vis AT. Moreover, 3 days of HFD feeding are already sufficient to have an effect on systemic haemostasis, highlighting the importance of the interaction between effects in Vis AT and the systemic coagulation.

2.4 Oral treatment with dipyridamole protects from HFD induced insulin resistance without affecting Vis AT hypertrophy

In order to test whether endothelial stress in concert with a more pro-coagulatory systemic state might be causally involved with impaired insulin glucose tolerance caused by short term HFD feeding, we treated mice with dipyridamole (DP) by gavage during HFD feeding (HFD + DP). DP is a drug commonly used as a coronary vasodilator and antithrombotic. DP is effective through various mechanisms, such as inhibition of PDE5 and PDE9 or inhibition of adenosine reuptake. We performed IPGTT on mice fed with HFD, HFD + DP or chow for 3 days (**Figure 6A, 6B**) and found that treatment with DP alleviated the impaired glucose tolerance after 3 days of HFD feeding. We also quantified hypertrophy of AT in these mice, to test whether DP

treatment is effective in spite of existing AT hypertrophy and found comparable levels of Vis AT hypertrophy in DP and HFD + DP mice. These findings suggest that vascular stress is indeed functionally linked to metabolic impairments caused by short term HD feeding in mice, and that treating the vascular stress can improve metabolic function in C57/Bl6 mice.

Discussion

In this study we present evidence for a role of AT endothelial stress in the aetiology of obesity induced insulin resistance and the MS. We corroborate existing findings (Lee et al., 2011; Wiedemann et al., 2013), showing that HFD leads to systemic metabolic complications in relation to glucose clearance very rapidly and point to an involvement of the liver. This is consistent with findings of Wiedemann et al. but also with work implicating the hypothalamus as a very early target of HFD (Ono et al., 2008), since suppression of liver gluconeogenesis has been shown to be strongly dependent on signals from the brain (Pocai et al., 2005). Selective insulin resistance of the liver, in particular concerning failure to suppress gluconeogenesis is also consistent with the aetiology of insulin resistance described more broadly in the literature (Ferris and Kahn, 2016).

We further extend these findings by pointing to a role of endothelium and endothelial stress in mediating these adverse reactions to HFD feeding. In particular Lee et al. have shown that impaired glucose tolerance occurs as soon as 3 days on a HFD, yet they also showed that this early effect is independent of bone marrow derived lymphocyte involvement. While 3 days is much sooner than most people's reports of diet induced systemic inflammation and also than broadly detected inflammation in adipose tissue (Gregor and Hotamisligil, 2011), Wiedemann et al. report that adipose tissue inflammation is responsible for the early insulin resistance in HFD feeding, also describing increased hepatic gluconeogenesis as the proximate reason for the metabolic abnormalities. They use [B6.Cg-Tg(Fabp4- cre)1Rev/J mice to drive Cre expression in cells expressing Fabp4 crossed with mice carrying a floxed Fas allele to knock out Fas specifically in adipocytes. However, it has recently been shown by FACS analysis, looking at different defined cell populations, that the Fabp4 Cre is expressed very highly in CD31+ endothelial cells of adipose tissue (Jeffery et al., 2014). This suggests that perhaps the phenotype reported by Wiedemann et al. is at least in part due to the effect of the Cre expression in endothelial cells.

We are not the first to report an early involvement of endothelial stress in the development of the metabolic syndrome. (Kim et al., 2008) report that endothelial inflammation and insulin resistance precedes large scale inflammation and insulin resistance in other tissues, and invoke direct effects of free fatty acids on endothelial cells as the driver (Kim et al., 2005). Moreover, there are a number of papers implicating impaired NO production or impaired NO signalling in the pathologies associated with obesity. (Sansbury et al., 2012) show that global overexpression of eNOS protects against DIO in mice. (Ayala et al., 2007) showed that chronic treatment with sildenafil alleviated metabolic impairments caused by a HFD in mice, and sildenafil has also been shown to have effects on insulin resistance in humans (Ramirez et al., 2015). Two studies investigated the effect of supplementation of arginine, which is the

substrate of eNOS and necessary for NO production on obesity, in a genetic model, the Zucker diabetic fatty rat (Ramirez et al., 2015), and in DIO in rats (Jobgen et al., 2009), finding improved metabolism in both cases. Investigating a different way to increase systemic NO signalling (Geldenhuys et al., 2014) showed that UV radiation, which has been shown to strongly increase systemic bioavailability of NO (Liu et al., 2014), protects against DIO in a dose dependent manner and independently of vitamin D.

The general involvement of endothelial impairment and remodelling in the context of obesity is still incompletely understood (Cao, 2013). To give a brief excerpt, increasing adipose tissue vascular density by inducing angiogenesis has been shown to have a beneficial impact in various models, while reduced vascular density correlates with a poorer metabolic outcome (Sung et al., 2013; Robciuc et al., 2016), and seems to be caused by obesity.

While we present strong evidence for an early involvement of endothelial impairments in AT in the MS, the exact cause of these impairments remains to be elucidated. We hypothesized increased endothelial stress or vascular injury due to mechanical forces acting on adipose tissue, and (Hara et al., 2011) published data showing increased activity of the mechanosensor-linked GTPase Rho that would support such a hypothesis. Our data on adipose hypertrophy in concert with the closely correlated phenotypes of increased AT ROS production certainly points in this direction but the fact that we find decreased, rather than increased levels of eNOS-p1177 is contrary to what we would expect from a pure mechanical shear stress response. It is also possible that the lipids interacting with and crossing through the vasculature affect the endothelial cells directly, causing eNOS uncoupling and leading to both increased ROS production as well as decreased eNOS phosphorylation. Taking into account the correlation between adipocyte hypertrophy we observe, and the impaired eNOS signalling and increased ROS, would have an interesting implication concerning the often evoked mechanism of ectopic lipid deposition. Perhaps overloading of AT already occurs to a small extent at very short timeframes, leading to an increased lipid load on other tissues, such as the liver or hypothalamus, where it causes increased endothelial stress and local pro-inflammatory signals. Persistent lipid overload would lead to an increase in AT lipid storage, albeit under conditions of continued endothelial stress, and in the long term cause bona fide inflammation with its described metabolic consequences. Postprandial FFA are known to be largely derived from lipoprotein lipase (LPL) derived lipolysis in the AT endothelium, where a fraction of FFA escapes uptake by adipocytes and enters the blood stream (Karpe et al., 2011). Perhaps this process is already affected in the very short term and hypertrophy together with changed endothelial reactivity affects FFA uptake in AT already after a few HFD meals, leading to changed systemic FFA load. Tissue specific uptake of dietary lipids in humans is consistent with this hypothesis; in relation to tissue mass intra-abdominal AT took up much more lipids

than both abdominal Sc, and Sc in the extremities after a lipid meal (Jensen et al., 2003). This pattern of lipid uptake correlates exactly with the temporal dynamics in hypertrophy we observed and might explain why Vis adiposity in more associated to poor metabolic health than Sc. In summary, we show increased ROS production and impaired eNOS signalling in AT very early in DIO, and propose a causal role of AT endothelial stress in the development of insulin resistance and the metabolic syndrome.

Experimental Procedures

Materials

All chemicals were purchased from Sigma-Aldrich unless specified otherwise.

Animals

C57BL/6 mice were housed in a pathogen-free animal facility on a 12-h/12-h light/dark cycle with free access to food and water, at an ambient temperature of 23°C. Mice were fed standard chow (Kliba-Nafag purified diet #2222; 18% protein, 7% fat, 58% NFE), a 60% calories high fat diet (Kliba-Nafag purified diet #2127; 23.9% protein, 35% fat, 23.2% NFE). Dipyridamole was gavaged 200mg/kg in acid water (pH 2.5) with 5% polyetheylenegylcol b.i.d., based on (Venkatesh et al., 2010).

Western Blot Analysis

Whole cell lysates were prepared as previously described (Meissburger et al., 2011). For western blot analysis the following antibodies were used: AKT1 (Cell Signaling, 1:1000), AKT1 p-S473 (Cell Signaling, 1:1000), Hsp90 (Cell Signalling Technology, #4877 S), eNOS p-S1177 (Cell Signaling, 1:1000), eNOS (Cell Signaling).

Tissue and Plasma Collection

Blood was collected from the tail vein in tubes containing 0.5M EDTA and centrifuged to derive plasma. For post mortem plasma collection animals were sacrificed by CO_2 asphyxiation and blood was taken from the heart. For tissue collection animals were sacrificed by CO_2 asphyxiation and tissues were dissected.

Plasma Analysis

Blood Glucose was measured using an Aviva AccuCheck glucose strip system. Insulin was measured using a MSD kit (K152BZC). Free Fatty acids were measured using the Wako Nefa kit #9196, Trigycerides were measured with the Cobas Roche/Hitachi Kit #11489232 and Cholesterol was measured using the Cobas Roche/Hitachi Kit #11877771.

H_2O_2 measurement

We used a protocol based on (Furukawa et al., 2004) Animals were sacrificed by cervical dislocation, pieces of roughly 5x5mm of Sc and Vis AT were dissected, placed in Krebs-Ringer HEPES buffer (pH 7.4) with 100µM Amplex Red (Sigma) and 0.2U/ml Horse Radish Peroxidase (Sigma), cut into small pieces and left for 30-60 at 37°C in the dark. The solution was analysed on a Synergy Mx plate reader at 544nm excitation and 590nm emission with a standard of H_2O_2 in the same buffer.

Adipocyte Size Frequency Distribution Analysis

Paraffin sections of BAT were stained with Hematoxylin and Eosin and images were taken on a Zeiss Axio M1 inverted microscope. Pictures were analysed in FIJI using a macro to segment images and determine adipocyte area. Frequency distribution analysis was performed and data was plotted in Graphpad Prism.

Glucose Tolerance Test

Animals were fasted for 6h and injected with 1g/kg body weight of glucose in 0.9% saline. Blood samples were taken from the tail vein for blood glucose measurements at regular intervals.

Immunofluorescence

Adipose Tissue was stained using a whole mount technique. Small tissue chunks (2x2mm) were placed in PBS with 2% FBS and primary antibody to CD41 (Abcam) and CD31 (BD) at 4°C overnight. Samples were washed and stained with fluorescently labelled secondary antibody (A488 and A594) for 2h at 4°C. Samples were mounted on microscopy slides in Vectashield and imaged on a Zeiss Spinning Disk Axio Observer Z1 (inverse) microscope.

Electron Microscopy

Animals were perfused with Na-Cacodilate –Sucrose buffer (0,1M pH 7.4, 300mosm, 4% formaldehyde, 1% glutaraldehyde), Vis AT was dissected and fixed for an additional 2h in perfusion buffer. Samples were treated with 1% OsO4 for 1h on ice, washed in H_2O and treated for 1h with 1% uranylacetate, washed in H_2O and dehydrated by successive washes to 100% ethanol (70%, 80%, 100% 30min each). Samples were incubated in propylene and a 1:1 ethanol : Epon mix for 30 min, dragged through Epon with DMP30 and baked at 60°C for 28h. Ultrathin sections (70nm) were cut, sections stained with 3% uranylacetate and Reynold's lead, and mounted on iron grids. Images were acquired by TEM using a XX system.

Mean Bleeding Time Assay

C57/Bl6 mice were anaesthetized by intraperitoneal injection of Xylozine/Ketamine and placed on a box with the tail tip protruding. The distal 5mm of the tail tips were amputated and the tails placed into falcon-tubes containing PBS. Bleeding was observed and time of cessations of bleeding recorded. Experiment was performed with experimentor blinded to the identity (diet) of the mice.

Author Contributions

G.G. developed the hypothesis and project, performed experiments, analysed data and wrote the manuscript, C.W supervised the project, analysed data and wrote the manuscript.

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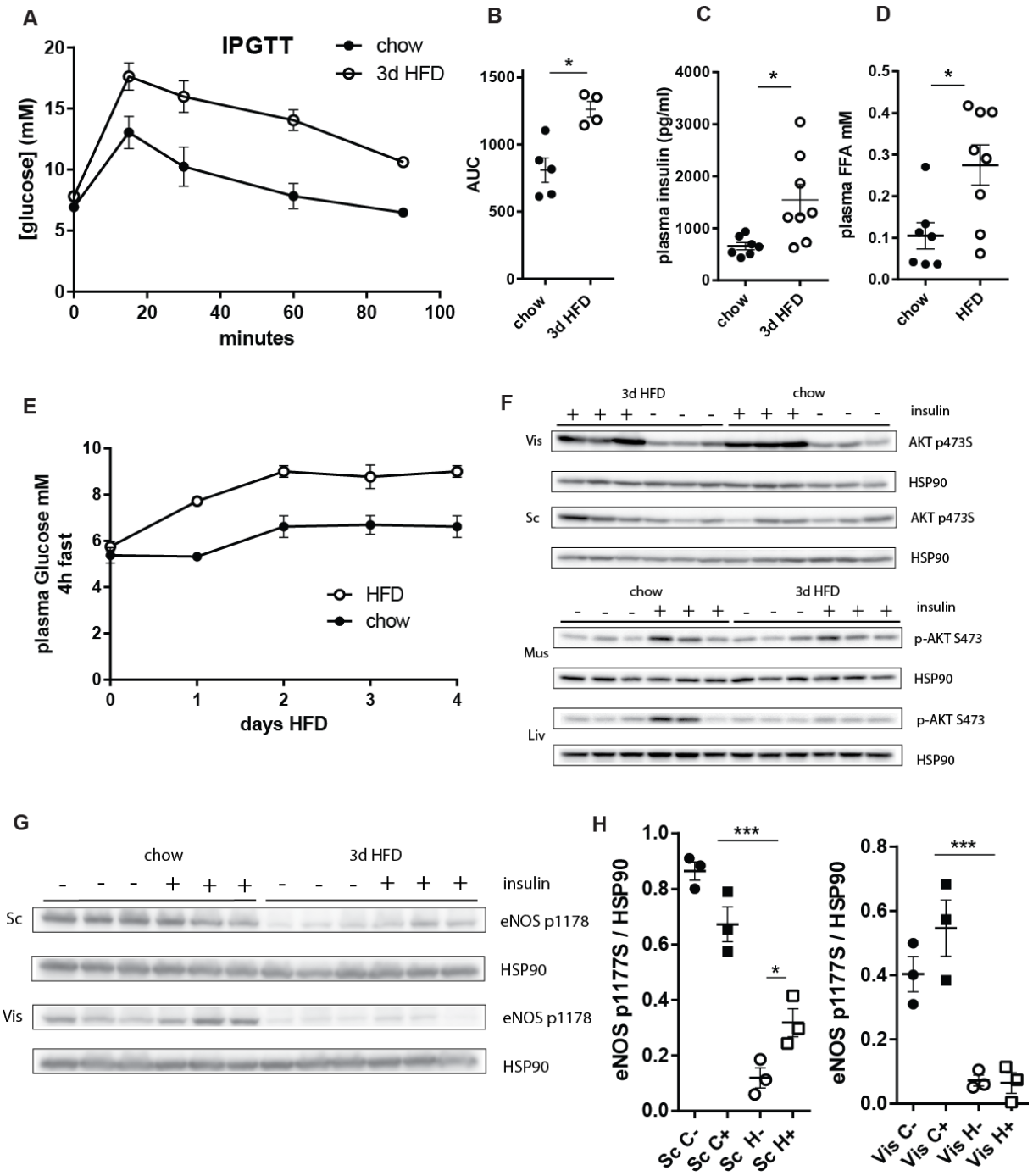


Figure 1. Short term high fat diet feeding impairs systemic glucose tolerance, hepatic insulin dependent AKT phosphorylation and insulin dependent eNOS phosphorylation in AT

(**A**) IPGTT of C57/BL6 mice after 3 days of HFD feeding and control, n = 4 – 5. (**B)** Area under the curve of IPGTT. (**C**) and (**D**): Plasma insulin (**C**) and FFA (**D**) levels after a 6 hour fast of C57/BL6 mice after 3 days of HFD feeding and control, n = 7 – 8. (**E**) Timeline of plasma glucose levels of C57/BL6 mice on a HFD and control after a 4 hour fast, n = 5 – 6. (**F**) Western blot of p-S473 of AKT of Sc, Vis, liver and muscle of C57/BL6 mice on a HFD for 3 days and control, after a 6h fast injected with either saline or insulin. (**G**) Western Blot for p-eNOS 1177 and Hsp90 of Sc and Vis AT of mice on a HFD or chow for 3d, injected with either insulin or saline after a 6h fast. (**H**) Densitometric quantification of p-eNOS Western Blot levels normalised to Hsp90. Data plotted as mean ± SEM, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ by Student's t-test.

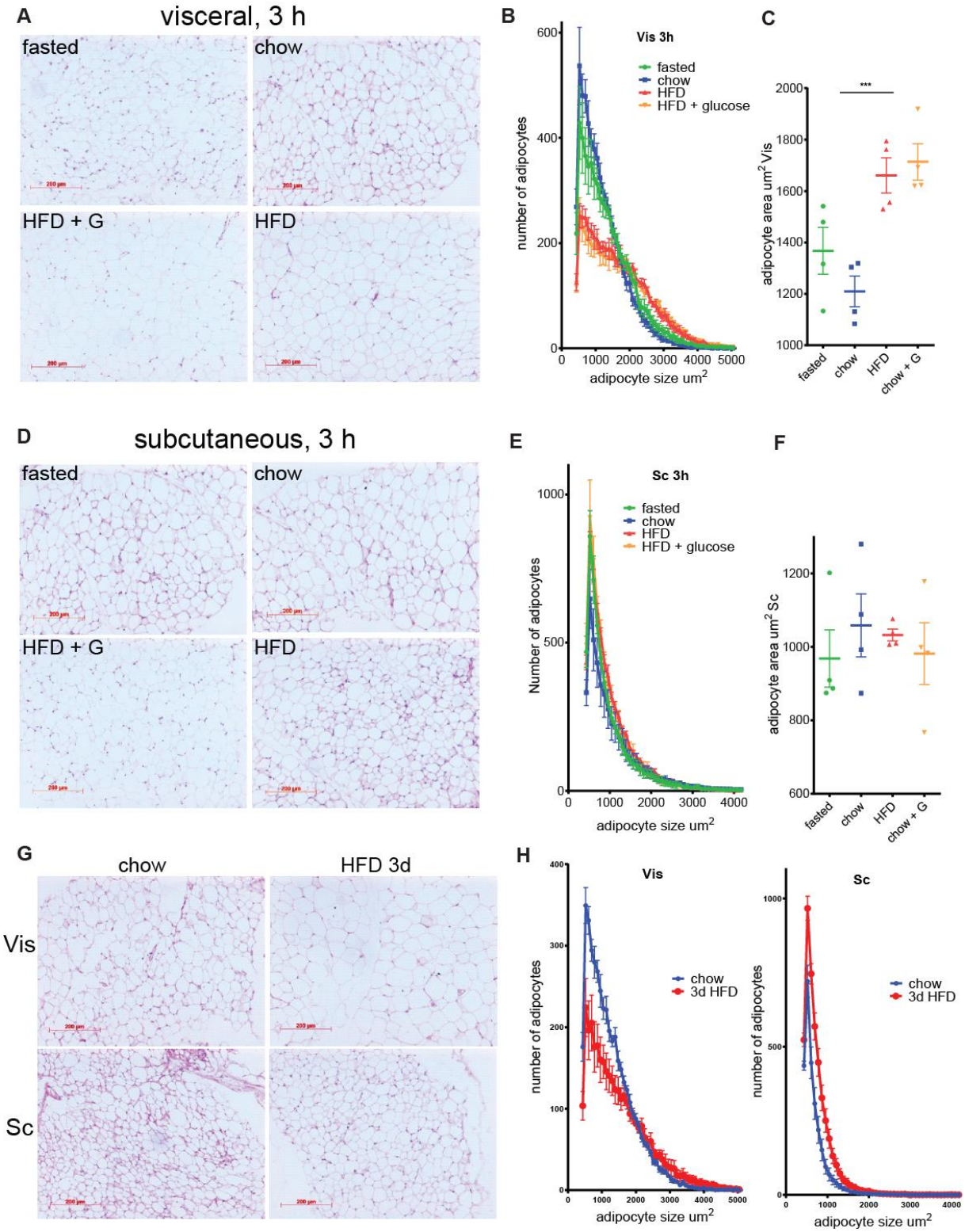


Figure 2: Adipocyte hypertrophy in Vis AT already occurs after one HFD meal and precedes hypertrophy in Sc AT

(**A**) and (**D**) H&E stainings of Vis AT (**A**) and of Sc AT (**D**) paraffin sections of C57/BL6 mice either fasted overnight, or fed after an overnight fast with either chow, HFD or HFD with added glucose, representative images. (**C**) and (**F**) Scatter plot showing mean adipocyte size of Vis and Sc. (**B**) and (**E**) quantitative analysis showing frequency plots of adipocyte areas of Vis (**B**) and Sc AT (**E**), 24 images per mouse, n = 4. (**G**) H&E stainings of Vis and Sc AT paraffin sections of C57/BL6 mice on chow of after 3d of HFD. (**H**) Quantitative analysis showing histograms adipocyte area of mice on chow or 3d HFD, n = 5. Scale bar = 200µm. Data plotted as mean ± SEM, *** $p < 0.001$ by Student's t-test.

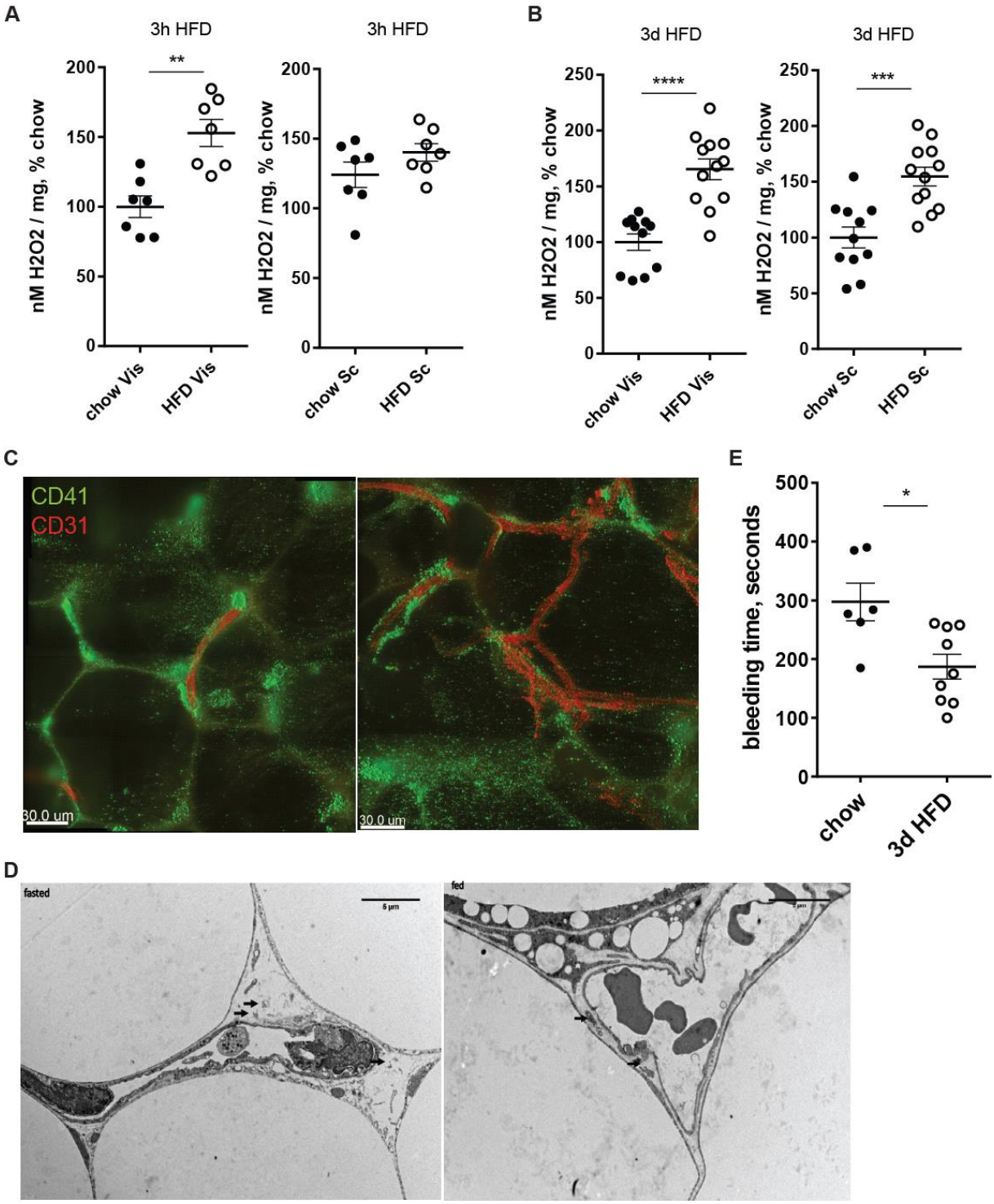


Figure 3: Short-time HFD feeding causes an increase in AT ROS production and changes in systemic hemostasis while platelets are present in both basal and HFD fed AT interstitium.

(**A**) H_2O_2 production by Amplex Red measurement of Vis and Sc adipose tissue explants 3 hours after HFD feeding, normalized to chow levels, n = 7. (**B**) H_2O_2 production by Amplex Red measurement of Vis and Sc adipose tissue explants after 3 days of HFD feeding, normalized to chow levels, n = 11 – 12. (**C**) Representative images of Vis AT whole mounts stained for platelets (CD41- A488) and endothelial cells (CD31-A568) of mice on chow or 3 weeks of HFD, scale bar = 30µm. (**D**) TEM images of Vis AT of fasted mice and mice fed a HFD for 3 hours, scale bar = 5µm. (**E**) Mean bleeding time of mice on a HFD for 3 days and chow-fed mice, n = 6 – 9. Data plotted as mean ± SEM, * $p < 0.01$ by Student's t-test.

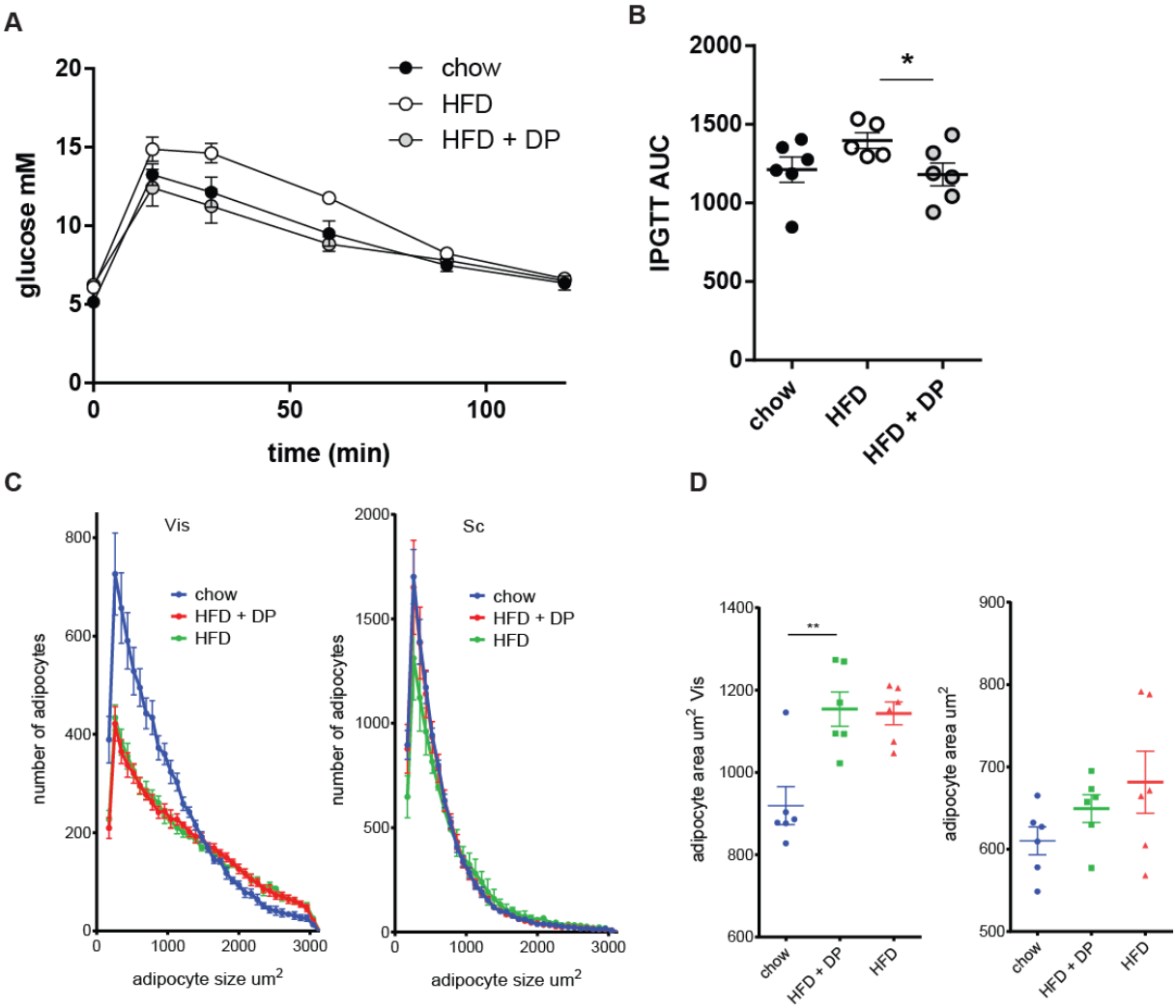
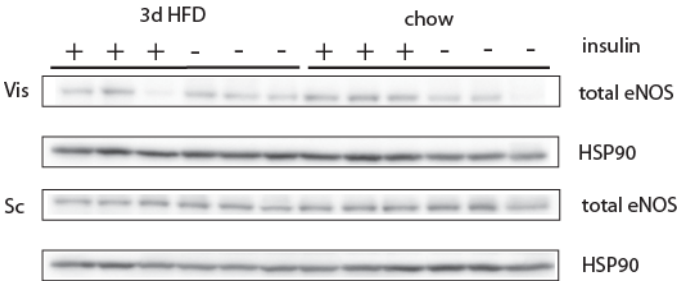
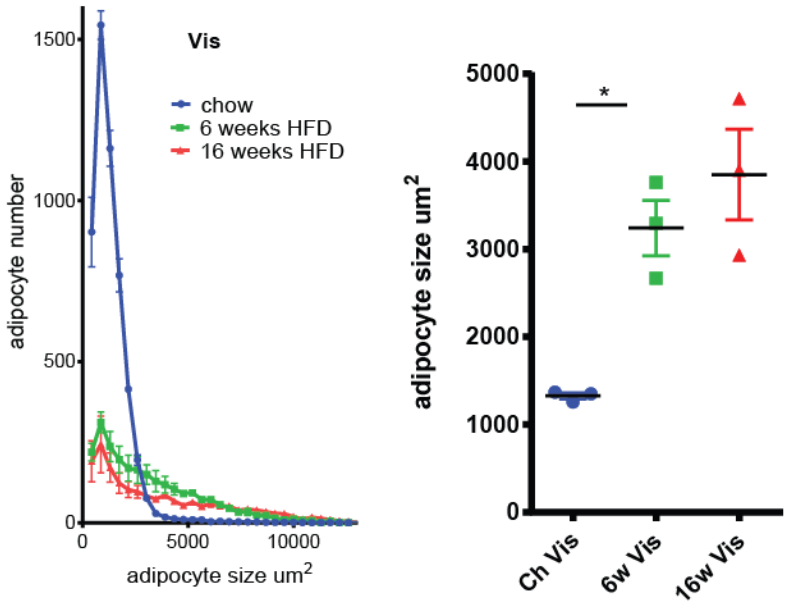


Figure 4: Oral treatment with dipyridamole protects from HFD induced insulin resistance without affecting Vis AT hypertrophy
(**A**) IPGTT of C57/BL6 mice after 3 days of HFD feeding, 3 days of HFD with bidaily gavage of dipyridamole (HFD + DP) and control, n = 5 – 6. (**B**) Area under the curve of IPGTT. Data plotted as mean ± SEM, * $p < 0.05$, ** $p < 0.01$ by Student's t-test

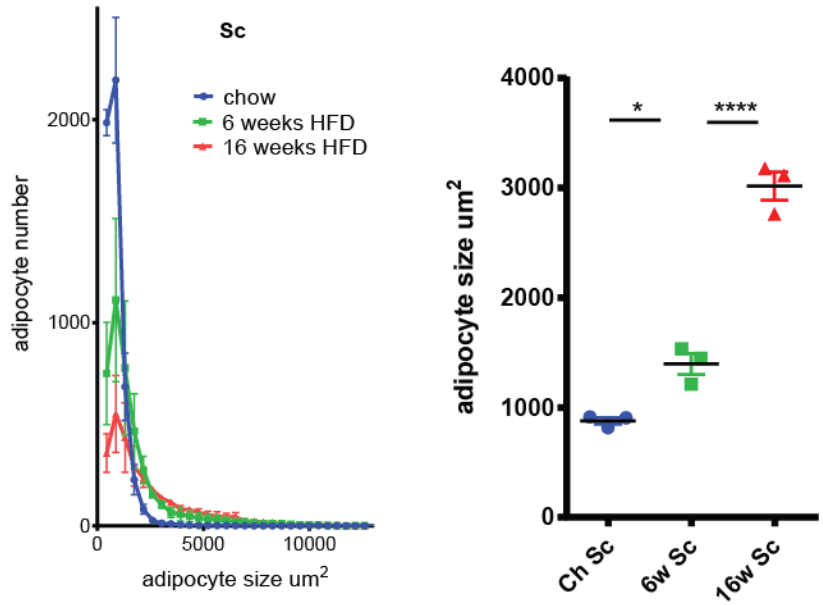


Supplementary Figure 1: (**A**) Western Blot for total eNOS and Hsp90 of Sc and Vis AT of mice on a HFD or chow for 3d, injected with either insulin or saline after a 6h fast.

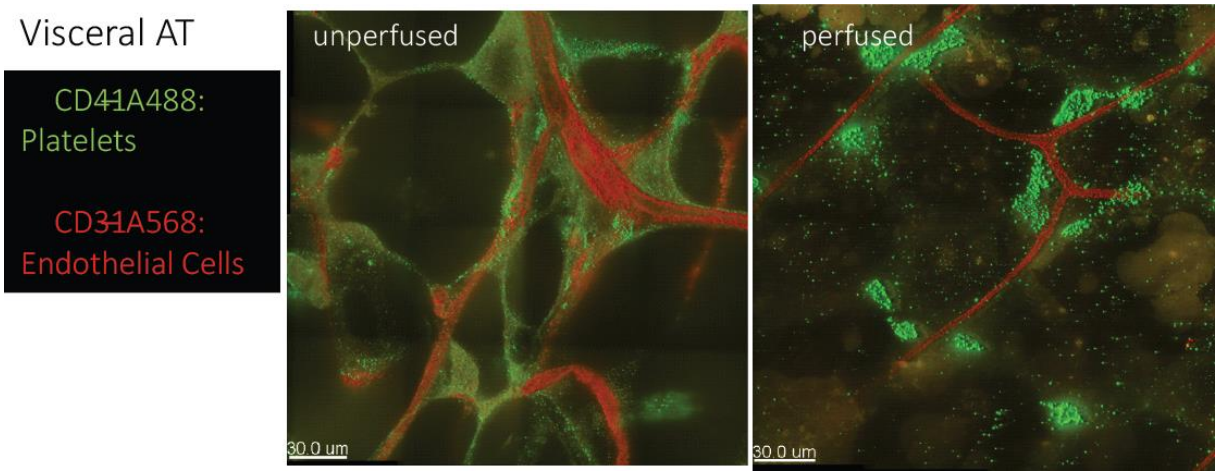
A



B



Supplementary Figure 2: Frequency plot and scatter plot of adipocyte sizes of Vis AT (**A**) and Sc AT (**B**) of chow mice and mice on a HFD for 6 weeks and 16 weeks.



Supplementary Figure 3: Vis AT whole mounts stained for platelets (CD41 A488) and endothelial cells (CD31 A568) of tissue from an unperfused and a perfused animal.

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3. Short time Ketogenic and High Fat but not Glucose feeding cause impaired Glucose Tolerance in Mice

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Contribution

Gerald Grandl developed the hypothesis and project, performed experiments, analysed data and wrote the manuscript.

This article is in preparation.

This chapter is a reprint of the manuscript.

Short time Ketogenic and High Fat but not added Glucose feeding cause impaired Glucose Tolerance in Mice

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Summary

A ketogenic diet (KD), containing very low amounts of carbohydrates, medium amounts of protein and very high amounts fat, induces ketogenesis and gluconeogenesis. Because it generally causes weight and body fat loss in the obese it is enjoying increasing popularity as a dietary intervention in humans. However, conflicting data concerning its effect on glucose metabolism and insulin sensitivity have been published, with some studies showing increased insulin sensitivity, while other studies show hepatic insulin resistance and increased glucose production after several weeks of KD consumption. Here we tested the effect of short term KD, high fat diet (HFD) feeding, and glucose added to the drinking water, on glucose tolerance in mice. We find that KD feeding similar to HFD feeding and contrary to glucose feeding causes impaired systemic glucose tolerance even after just 3 days. These data suggest that either the damage caused by KD and HFD happens very fast, or that impaired glucose tolerance is part of the metabolic adaptation to the consumption of a KD.

Keywords

Ketogenic Diet

Insulin Resistance

Hepatic Gluconeogenesis

Free Fatty Acids

3. Introduction

Recent years have seen an animated controversy between proponents of low carb, high fat ketogenic diets and low fat, high carbohydrate diets, with both sides claiming health benefits and good weight loss results when compared to a standard western diet, which is high in both fat and carbohydrates. [1–3]

The ketogenic diet (KD), a diet containing very low amounts of carbohydrates in order to induce liver ketogenesis and gluconeogenesis, was developed in the 1920s as a treatment for epilepsy in children and young adults [4], but in recent years it has seen a surge in use as a weight loss diet in humans. While the efficacy of KD in causing weigh loss is accepted [1,3,5–7], there is much controversy about the overall effects on health of a ketogenic diet in particular if consumed over long times, because of the large amounts of lipids contained in such a diet, which are historically linked to increased vascular disease and cardiovascular mortality risk [7,8]. Moreover, while KD feeding has been associated with improved insulin sensitivity in addition to its positive effects on body weight, there is some controversy surrounding the matter. Kennedy AR et al. [10] showed that KD improved glucose tolerance and insulin signalling in mice when compared to mice maintained on a high fat diet (HFD), and Badman et al. [11], showed that KD increases glucose sensitivity in *ob/ob* mice independently of weight loss. However, Jornayvaz et al. [12] showed that in C57/Bl6 mice 5 weeks on a KD induced hepatic insulin resistance, decreased glucose tolerance and increased gluconeogenesis in spite of preventing weight gain and increasing energy expenditure. Garbow et al. [13] found increased hepatic steatosis and inflammation and decreased glucose tolerance in spite of systemic maintained insulin sensitivity in mice on a KD for 12 weeks. Recently, another study showed that in New Zealand Obese mice, a polygenic model of obesity, a KD increases weight gain and does not improve glucose-related metabolic parameters [14].

Given this controversy, and the obvious interest in low carb KDs because of their widespread and increasing use as human dietary intervention, we decided to compare metabolic parameters in C57/Bl6 mice maintained on a HFD, a KD, or normal chow with 5% glucose in the drinking water to simulate a low fat, high carbohydrate, high sugar diet for just 3 days, to test whether metabolic effects are already noticeable in this short timeframe.

4. Results

2.1 Short time Ketogenic Diet or High Fat Diet feeding causes increased fasting plasma free fatty acids in C57/Bl6 mice

First we assessed metabolic parameters in unchallenged mice after a 6 hour fast, comparing mice fed a standard chow diet to mice fed a 60% high fat diet (HFD), a low carbohydrate ketogenic diet (KD), or mice on standard chow with 5% glucose (chow + G) in the drinking water for 3 days. Adding glucose to the drinking water caused a roughly 4-fold increase in water consumption in this group, when compared to mice on chow with regular water (**Fig S1B**). We found that after 3 days of KD feeding, plasma glucose was significantly reduced compared to chow, HFD, or chow + G (**Figure 1A**), whereas plasma insulin levels (**Figure 1B**) were not significantly different after a 6h fast, but were elevated in HFD fed mice. Using these parameters to calculate the HOMA-IR metric to quantify insulin resistance suggests that KD-fed mice are slightly more insulin sensitive than chow fed mice after 3 days, mice with glucose in the drinking water appear normal, and HFD fed mice show slightly impaired insulin signalling according to HOMA-IR (**Figure 1C**). However, HOMA-IR is based only on a correlation between fasted glucose and insulin levels, and thus not necessarily reflective of actual glucose tolerance. When analysing plasma lipids, we found no difference in triglycerides (TG) (**Figure 1D**) or cholesterol (**Figure 1E**) after a 6 hour fast, but we found a highly significant almost 10-fold increase in plasma free fatty acids (FFA) in the fasted state for KD, and a significant increase in HFD (**Figure 1F**). These data indicate that in the basal state, 3 days of a KD seem to have a beneficial effect on glucose levels, suggesting a healthy, glucose tolerant state, in spite of markedly increased plasma FFAs, while HFD fed mice have a much smaller increase in plasma FFA but appear insulin resistant because of an increase in fasting insulin levels. On the other hand, adding glucose to the drinking water did not show an adverse effect.

2.2 Short time High Fat Diet or Ketogenic Diet feeding causes impaired glucose tolerance in an IP-GTT in C57/Bl6 mice, while adding Glucose to the drinking water does not

Next we challenged animals with an intraperitoneal glucose tolerance test (IPGTT) to measure glucose tolerance (**Figure 2A, 2B**). We found a marked and significant decrease in glucose clearance of HFD and KD fed animals when compared to chow fed animals and chow fed animals drinking 5% glucose. This means that in spite of seemingly improved insulin sensitivity when looking in the basal state, already three days of KD feeding is sufficient to cause impaired glucose tolerance in C57/Bl6 mice, while HFD fed mice also show impaired glucose clearance, consistent with their HOMA-IR. Adding glucose to the drinking water of chow fed mice does not appear to cause any problems in glucose tolerance in this short feeding regimen.

5. Discussion

In this study we looked at effects of 3 days of HFD and KD feeding and glucose added to the drinking water of normal chow mice on systemic glucose signalling and insulin sensitivity parameters. We confirmed existing findings regarding short term HFD feeding and extended findings regarding KD feeding by showing impaired glucose tolerance as a consequence of KD feeding in mice is already manifest after 3 days. Impaired glucose tolerance is consistent with hepatic insulin resistance and increased gluconeogenesis as reported by [12], although the methods we used do not allow us to conclusively show this to be the case.

Our findings concerning KD feeding, with a phenotype that appears to be more insulin sensitive in the basal state when measuring HOMA-IR are consistent with what [12] showed after 5 weeks of KD. By assessment of indirect metrics of insulin resistance such as HOMA-IR in the basal state, KD fed animals appeared to be healthier and more insulin sensitive than those on chow whereas a glucose tolerance test revealed impairments. While we and others found KD to cause these effects on liver insulin signalling and gluconeogenesis [12,13], beneficial effects of KD were only found when comparing KD to models of diet induced obesity (DIO), such as HFD or the leptin deficient ob/ob mouse [10,11] after several weeks of developing obesity. Peripheral insulin resistance in the insulin responsive tissues muscle, AT and liver is a consequence of DIO, and has been shown to be causally linked to AT and systemic inflammatory processes [15]. On the other hand, impaired glucose tolerance after just 3 days of HFD feeding has recently been shown to not be dependent on inflammation [16]. Importantly, in the 3 day regimen we find similar effects of glucose tolerance between HFD and KD feeding.

In order to clearly pinpoint where the defect originates, we need to perform hyperinsulinemic euglycemic clamp studies on animals after 3 days of the different regimens, to see if the effect is linked to hepatic gluconeogenesis like reported after 5 weeks [12].

It is relevant to consider whether the impaired glucose tolerance caused by short term KD feeding compared to healthy animals observed here is physiological and beneficial, or a sign of metabolic impairment caused by KD. Physiologically, increasing gluconeogenesis during a state of low carbohydrate feeding and ketosis makes absolute sense. A KD mimics fasting metabolism [17], and maintaining normoglycemia is a physiological imperative during starvation and fasting. During acute starvation (24 hours), the liver provides glucose from glycogenolysis, as well as gluconeogenesis, a process that also occurs in the kidneys. Gluconeogenesis uses as substrates glycerol, released from adipocytes, amino acids from muscle, as well as lactate in the Cori cycle, which is returned to the liver from mainly erythrocytes and leukocytes or anaerobic oxidation in muscle. As a result of the Cori cycle, during fasting glucose is completely oxidised almost exclusively in the nerves, whereas the

rest of the body is fuelled by oxidation of FFAs. During prolonged fasting the use of muscle derived amino acids for gluconeogenesis, in concert with total hepatic and kidney glucose output, drops drastically and large amounts of ketones are made to be oxidised in nerve cells [18]. This metabolic adaptation to fasting takes on the order of magnitude of around 3 days and this is the state mimicked by a KD [19]. The key hormone in regulating this process is most likely insulin [18].

In the last decade, the molecular mechanism of how insulin controls hepatic gluconeogenesis has been largely elucidated, with both direct effects through insulin receptors on the liver, as well as indirect effects through substrate release, affecting both the lipolysis in the fat [20], and proteolysis in the muscle [21], with muscle inhibition of proteolysis apparently being sensitive to smaller increments of insulin than muscle glucose uptake [22]. Moreover, indirect effects of insulin through insulin action in the hypothalamus and vagal efferents play an important role in the insulin dependent suppression of hepatic glucose output [23]. While plasma insulin levels on KD are probably slightly higher than those during true starvation since protein alone also causes a small insulin spike, and protein potentiates the insulin spike caused by carbohydrate ingestion [24], the temporal average of plasma insulin levels in KD fed animals will be much closer to those of fasted animals than those on a high carbohydrate diet. This could arguably account for the metabolic adaptations occurring during KD feeding. In accordance with this, a few very interesting studies from the beginning of the beginning of the 20th century showed that after long fasts of 24 hours or more or very low carbohydrate feeding in humans, glucose tolerance is impaired [25,26], suggesting that KD feeding and fasting share a similar physiological response in respect to glucose tolerance.

Beside impaired glucose tolerance, the second parameter were we noticed similar changes of KD or HFD compared to chow fed animals were plasma FFAs after a 6 hour fast. We saw a roughly 10-fold increase in KD and a 2-fold increase in HFD. The primary source of FFAs is the adipose tissue (AT), and FFAs constitute the form of transport of calories from AT to the rest of the body [27]. However, in the postprandial period there is an additional source of plasma FFAs, termed spillover FFAs. Spillover FFAs are FFAs that escape the adipocyte lipid uptake during release of lipoprotein lipase (LPL) catalysed lipolysis of triglycerides, typically in chylomicrons, in the adipose capillary. They join the plasma pool and it is estimated that spillover FFAs could constitute up to 50% of total plasma FFA in the postprandial period [28,29]. Interestingly, increased plasma lipids and also plasma infusion of lipids, has long been known to cause insulin resistance, and increased hepatic glucose production [30,31]. Most of the work done with lipid infusion focuses on insulin sensitivity in the muscle and it seems that inflammatory processes play a crucial role [32,33]. In particular TLR4, a receptor of the innate immune system, seems to link inflammatory processes to lipid-induced insulin resistance since it was shown that lipids can activate TLR4 signalling andTLR4-/- mice are protected from

insulin resistance following lipid-infusion [34]. Very recently a paper also looked at hepatic insulin resistance being caused by lipid infusion, and found that iNOS, a key inflammatory enzyme, is involved [35]. It is important to note however, that all these experiments were performed during euglycemic, hyperinsulinimc clamps and therefore might not accurately reflect the KD or HFD fed state. Moreover, both studies reporting insulin resistance following KD feeding report normal glucose clearance in muscle, and increased gluconeogenesis from liver [10,11].

Considering the metabolic adaptations to KD and the data discussed so far, as well as the fact that this metabolic adaptation apparently happens within just days after starting a KD, it is tantalizing to speculate that high plasma FFAs themselves might cause selective insulin resistance in respect to hepatic glucose output as a metabolic adaptation to a low carb diet. If plasma FFAs serve as an indicator of low relative glucose availability it would make sense to maintain gluconeogenesis in spite of a brief influx of external glucose in order to replenish glycogen stores and perhaps this is a mechanism applicable to both HFD and KD feeding.

Taken together, here we present data showing that short term high glucose added to a low fat diet do not seem to cause metabolic impairments, whereas low carb KD causes impaired glucose tolerance similar to a HFD, and raise the possibility that this could be not due to acute damage but could also be a physiological metabolic consequence of the diet.

6. Experimental Procedures

Materials

All chemicals were purchased from Sigma-Aldrich unless specified otherwise.

Animals

C57BL/6 mice were housed in a pathogen-free animal facility on a 12-h/12-h light/dark cycle with free access to food and water, at an ambient temperature of 23°C. Mice were fed standard chow (Kliba-Nafag purified diet #2222; 18% protein, 7% fat, 58% carbohydrate), a ketogenic diet with 70% of calories as fat (Teklan, TD.96355; 15.3% protein, 67,4% fat, 0.6% carbohydrate), a 60% calories high fat diet (Kliba-Nafag purified diet #2127; 23.9% protein, 35% fat, 23.2% carbohydrate), or standard chow with 5% D-glucose added in the drinking water.

Plasma Collection

Blood was collected from the tail vein in tubes containing 0.5M EDTA and centrifuged to derive plasma. For post mortem plasma collection animals were sacrificed by CO_2 asphyxiation and blood was taken from the heart.

Plasma Analysis

Blood Glucose was measured using an Aviva AccuCheck glucose strip system. Insulin was measured using a MSD kit (K152BZC). Free Fatty acids were measured using the Wako Nefa kit #9196, Trigycerides were measured with the Cobas Roche/Hitachi Kit #11489232 and Cholesterol was measured using the Cobas Roche/Hitachi Kit #11877771.

Glucose Tolerance Test

Animals were fasted for 6h and injected with 1g/kg body weight of glucose in 0.9% saline. Blood samples were taken from the tail vein for blood glucose measurements at regular intervals.

Author Contributions

G.G. developed the hypothesis and project, performed experiments, analysed data and wrote the manuscript, C.W supervised the project, analysed data and wrote the manuscript.

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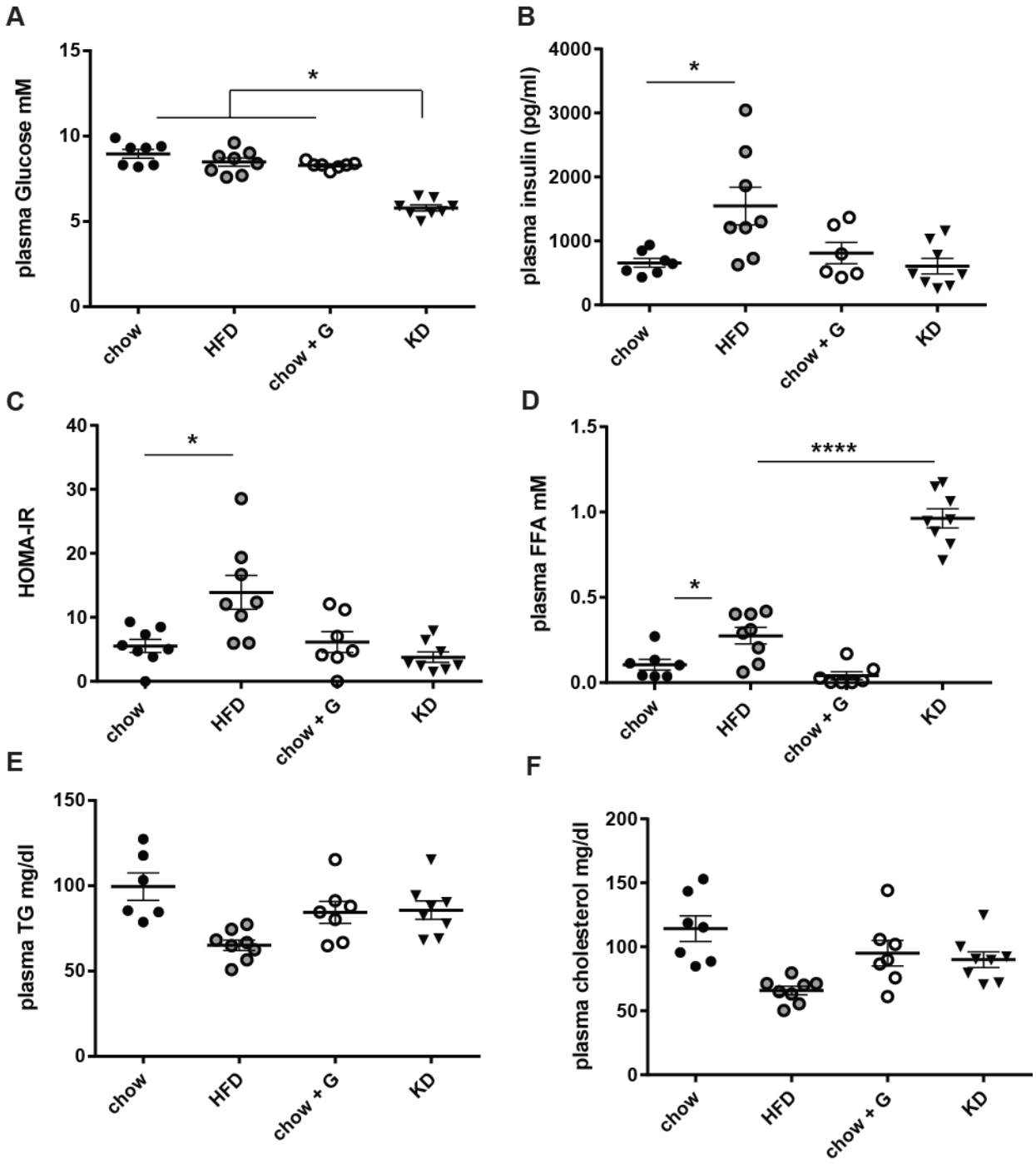

Figure 1: Short time Ketogenic Diet or High Fat Diet feeding causes increased fasting plasma FFA in C57/Bl6 mice

Plasma metabolic parameters in chow versus 3 days KD, HFD-fed mice and chow-fed mice with 5% glucose in the drinking water (chow + G) after a 6h fast. (**A**) glucose levels (**B)** plasma insulin, (**C**) calculated HOMA-IR, (**D**), plasma triglycerides (TG), (**E**) plasma cholesterol (CHOL), (**F**) plasma FFA, n = 7 – 8. Data plotted as mean ± SEM, * $p < 0.05$, **** $p < 0.0001$ by Student's t-test.

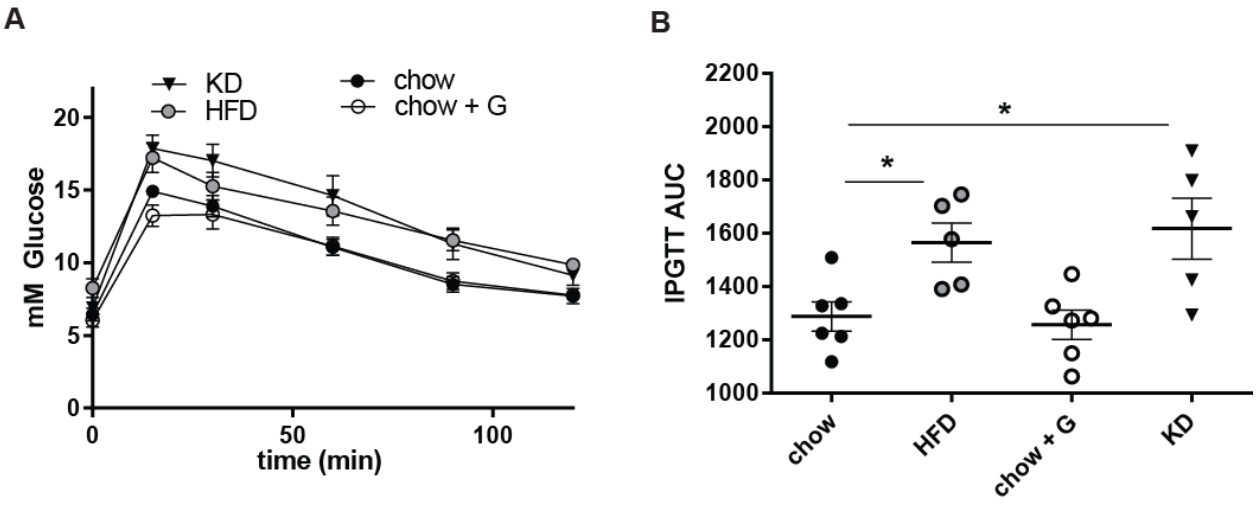

Figure 2: Short time Ketogenic Diet or High Fat Diet feeding causes impaired glucose clearance in IP-GTT in C57/Bl6 mice

(**A**) IPGTT of mice fed chow, KD, HFD or chow + 5% glucose (chow +G) for 3 days, after a 6 hour fast. (**B**) Area under the curve (AUC), n = 5 – 6. Data plotted as mean ± SEM, * $p < 0.05$ by Student's t-test.

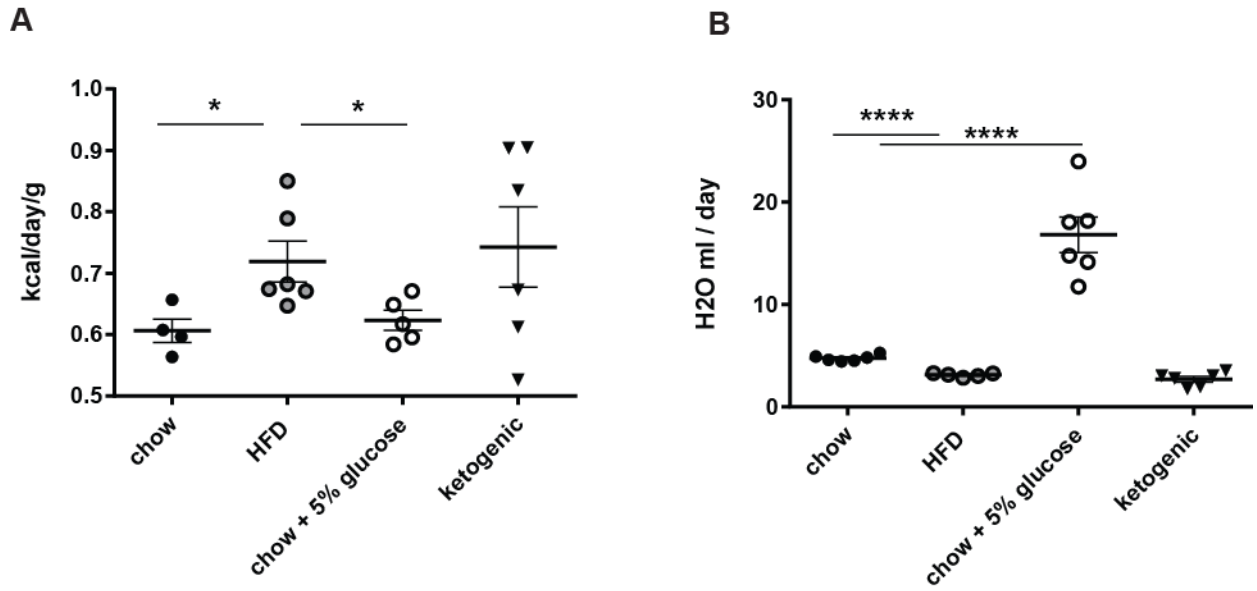

Figure S1: Food intake and water intake

(**A**) Per day average calorie intake per mouse for mice fed chow, a HFD, KD, or chow with 5% glucose in the drinking water, normalized to mouse body weight. (**B**) Water intake per day.

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5. Thesis Discussion

In this thesis I explore the question of how obesity develops and why it is often unhealthy, focusing on the two modes of AT expansion, which are hyperplasia and hypertrophy. In the first paper (chapter 2) the focus is on AT ECM and how ECM-precursor interactions influence the terminal differentiation of precursors. In this paper we found that the ECM has a profound effect in modulating differentiation, and we could identify FLOT2 as a membrane protein involved in ECM to precursor signalling.

The concept of how stem and precursor cells are organized in a tissue, maintain themselves and respond to growth and differentiation signals is that of the stem cell niche, a cellular microenvironment that maintains the stemness of stem cells, while allowing their daughter cells to progress along the way towards post-mitotic cells.[83] One might reasonably assume that a stem cell niche should allow growth of cells, while blocking their differentiation. These are the conditions we found for Vis AT derived ECM. Vis ECM allowed for less differentiation than Sc and even less than BAT. Recent data from other labs, however, showed that Vis AT has much more proliferation than Sc after animals are put on a HFD,[84,85] and that the stem cell pool of Sc is more easily exhausted.[86] While the second finding fits very well to what we described, implying a better maintenance and replenishment of precursors in Vis AT, the fact that Vis AT responds faster with proliferation of precursors in vivo, while ex vivo Vis ECM blocks differentiation at first seems contradictory. These phenotypes can be reconciled by assuming that HFD rapidly triggers a signal to activate the stem cell niche and precursor pool, acting on the Vis AT before, or stronger than the Sc AT, and that the tissue specific ECM signals we studied in chapter 2 likely represent the basal, unchallenged state of the adipose stem cell niche. Unfortunately, we were not able to identify the exact properties of the ECM that contribute to the differentiation-inhibiting quality of Vis ECM. We did not find large differences in the macromolecular composition of the different ECMs, therefore it is likely that either small, ECM binding proteins or growth factors were responsible, or that certain cells in the SVF modulate the ECM microenvironment through cleavage of ECM components or bound factors. Likely, a combination of both factors contribute. Highlighting the importance of modulating ECM, matrix metalloproteases (MMPs), enzymes that degrade or cleave ECM, as well as their inhibitors, are a growing research subject.[87]

Besides considering the effectors of the niche in the basal state, it is tempting to speculate what may constitute the signals caused by HFD feeding that trigger the response of the stem cell niche. In the second paper (chapter 3) we found that HFD feeding causes a noticeable shift in cells size distribution towards less smaller and more larger cell sizes as soon as 3 hours after initiating HFD feeding, whereas a size shift, to a much smaller extent, was only noticeable in Sc AT after three days. One possibility is that the physical expansion of adipocytes itself is

detected by the microenvironment, perhaps by mechanosensing in endothelial or smooth muscle cells, or through interaction of the ECM and attached cells, possibly involving the tissue resident macrophages. Another possibility would be that perhaps the different ATs have a different temporal progression of dietary lipid uptake. If the size dynamics are reflective of lipid uptake and transport, which happens in the form of LPL generated FFA, this would mean that there would be a larger amount of FFAs present on Vis AT at earlier times during HFD feeding. This would constitute an important signal, since FFAs are known PPAR activators,[88] and PPARγ in particular has been shown to be a key transcription factor in controlling preadipocyte differentiation.[89] Available data from human studies support this view, showing that specific activity of fat tissue after a meal containing [3H]-labeled triolein was 200% higher in intra-abdominal AT than abdominal Sc AT, which in turn was 150% higher than Sc on the leg, and intra-abdominal AT took up 17 % of fat from a lipid meal, even though accounting for only 11% of body AT mass.[90]

The reason it is relevant to understand AT hyperplasia is because hyperplastic AT, that is to say AT consisting of many, small adipocytes, is the main predictor for what is termed healthy obesity. This correlation is evidenced both by animal studies such as adiponectin[91] and Mito-NEET[92] overexpression or RORγ knockout,[93] as well as clinical data from humans.[94,95] The mechanism of how exactly hyperplasia, or small adipocyte size contributes to increased health in obesity is far from elucidated, but two factors are plausible. Firstly, smaller adipocyte size correlates with lower AT and systemic inflammation, which is linked to better peripheral insulin sensitivity. Secondly, smaller adipocyte size is linked to increased FFA clearance and lower plasma FFA levels, which also correlates with improved insulin sensitivity.[96] There may also be changes in additional AT derived hormones that are differentially regulated in small versus large adipocytes.

Moving away from healthy obesity, in the second paper (chapter 3) we asked the question how obesity, and more specifically obesity manifesting as increased Vis obesity with a high degree of hypertrophy, is linked to the unhealthy aspects of the MS. As mentioned in the introduction, the MS means an increased cardiovascular risk profile with abdominal obesity together with impaired glucose tolerance and insulin sensitivity. We hypothesized that AC hypertrophy directly causes AT endothelial stress and that this stress is functionally linked to systemic insulin resistance and glucose tolerance. We measured the time-course of hypertrophy in Vis versus Sc and showed that both ROS production as well as impaired eNOS signalling correlate temporarily with the hypertrophy. We also show that modulating endothelial stress and NO signalling pharmacologically can protect from impaired glucose tolerance after short term HFD feeding.

Following up this work and looking in a model that raises plasma FFA without raising obesity, in the next paper we tested the effects of short term feeding with a ketogenic diet (KD) on

glucose tolerance, and to our surprise we found that KD affected systemic glucose tolerance to an extent comparable to HFD feeding. Given this result of impaired insulin signalling by a diet not known to cause obesity, we should consider the following questions carefully: What kind of a diet causes obesity, healthy or otherwise; how are vascular impairments linked to FFAs and impaired glucose metabolism; and is the KD actually healthy?

We can say with a relatively high degree of certainty that the proximate hormonal cause of DIO is insulin. This is supported by a large amount of data, coming from different directions. It was recently shown that mice lacking one copy of the insulin gene don't produce the large increase in fasting hyperglycaemia typically observed during HFD feeding, and are protected from DIO.[97] In humans, patients suffering from insulinoma, which is a tumour of the pancreatic beta cell resulting in abnormally high plasma insulin levels, typically present with abdominal obesity, although this may also be a consequence of increased meal frequency, which relieves symptoms of hyperinsulinism.[98] In Pima Indians, known for having the highest incidence of obesity worldwide, increased fasting insulin levels predict onset of obesity,[99] and increased post-challenge insulin release predicts development of obesity in glucose tolerant offspring of parents with T2D.[100] In our own study (chapter 3), we see that fasting insulin levels are already increased after 3 days of HFD feeding, at which point there is not yet a significant change in body weight. The connection is also apparent in clinical trials; two separate, large clinical trials performed in obese humans, comparing intensive glucose management in T2D by standard care such metformin treatment with insulin treatment showed a significant, albeit modest weight gain for the groups receiving insulin.[66,101]

If we follow the line of reasoning that insulin is the proximate cause of obesity, we must next rephrase the question for the ultimate cause, i.e. how a diet causes obesity, to read: How does an obesogenic diet lead to increased insulin levels? In order to address this question, we should first establish a consensus as to what kind of diet is obesogenic. Very broadly speaking, the answer is that a diet that is high in both fats and carbohydrates at the same time is obesogenic, whereas diets that are sufficiently low in either of the two constituents are not. While this seems straightforward, there is a longstanding controversy on the respective benefits of low carb or low fat diets, both for weight loss and general health. At the heart of the problem is the fact that major medical institutions already issued a recommendation for a low fat, high carb diet in order to combat obesity several decades ago, yet in these decades obesity levels did not got down, but rather went up.[102] In addition, the interpretation of these guidelines was often that dietary fats of any kind in large amounts are bad for one's health, a statement that is no longer supported by the available evidence.[103] In recent years, proponents of the opposite side gained momentum, both because the original guidelines did not seem to solve the problem, and because of the highly publicised successes of low carb or ketogenic diets such as the Atkins Diet, or the Paleo Diet.[104] This momentum is now in full swing with ideas

such as “all carbs are bad” having recently entered the mainstream. The reality however, is more complicated than “carbs are bad” or “fats are bad”. Most studies comparing low carb and low fat diets head to head find very little advantage for one diet over the other, with both kinds of diets typically achieving weight loss, improved cardiovascular biomarkers and improved insulin sensitivity.[105–107] Meta-studies comparing various diets can be found to support an added benefit for either diet.[108–112] Looking at a case where the two diets were very strictly controlled for a short duration, a recent paper comparing a diet producing a calorie deficit and keeping consumed calories equal for both low fat or low carb, found that, calorie for calorie, reduction of dietary fat leads to more weight loss than reduction of carbohydrates.[113]

The weight loss effect of a low carb diet is readily explained. Consuming low amounts of carbohydrates leads to an acute and long-term reduction of plasma insulin, which is the most important anabolic and adipogenic hormone. This of course leaves out the question of cardiovascular health, since lipids, and in particular the build-up of atherosclerotic plaques, are widely believed to be drivers of cardiovascular mortality. The main argument to explain cardiovascular benefits of low carb diets states that any therapy achieving weight loss will improve cardiovascular health, but conclusive data of whether a low carb diet is healthy in the long run is still not available.[114] Importantly, in the last years several papers were published studying specific parameters of vascular health such as plaque build- or vascular reactivity, rather than plasma lipid profiles which have only correlative value, and found that low carb or KD actually might adversely affect important parameters of vascular health.[115–117]

Another very important aspect of a diet that should be discussed here is not whether the diet works in a controlled setting of high compliance, but how easy it is for people to maintain. A key overlooked nutrient in this context, and in all of the above discussion, is protein. The fact of the matter is that most low carb, KDs are also fairly high in protein content. Protein is an important contributor to satiety,[118] and indeed there are some studies suggesting that actually the protein content is the parameter responsible for the efficacy of low carb diets, since it leads to increased satiety and reduced overall caloric intake.[119] They also argue that a dilution of protein in processed foods by carbohydrates or fats is mainly responsible for the obesity epidemic.[120]

Our data studying short term KD (chapter 4) at first sight casts some doubt on the benefits of the ketogenic or low carb diets. The apparent glucose intolerance after just 3 days of a KD suggests that there are some physiological parameters that are adversely affected. While one possible culprit is endothelial stress caused by excess lipids, treated in the discussion parts of both chapter 3 and 4, there is also the possibility that selective hepatic insulin resistance resulting in increased glucose output is a normal physiological response to KD.

In considering this scenario, it is instructive to look back to the early days of the study of diabetes and glucose metabolism. In the 1920s Staub found that while 20g of glucose given orally after an overnight fast would not cause a noticeable increase in plasma glucose, successively longer periods of fasting would cause increased plasma glucose levels after this challenge.[121] Several other studies added to this finding, showing that starvation or severe carbohydrate restriction for several days cause marked hyperglycaemia after a glucose challenge – a condition termed starvation diabetes (reviewed in Peters JP, 1945; Lundbeck K, 1948).[121,122] The key differences in plasma metabolites underlying these conditions of prolonged fasting or KD are increased FFA and ketones, as well as decreased glucose and insulin, any of which could cause the observed effect. Moreover, it was also shown that exhaustive exercise or a long walk before an oral glucose challenge would increase the hyperglycaemic reaction.[37] This effect of a single bout of exercise on oral glucose tolerance was also recently reported to be abolished in insulin resistant or diabetic subjects.[123] Metabolite signalling mediators of this effect could be ketone bodies or FFA, both of which are reported to go up after fasting or prolonged exercise.[124,125] Also contributing could be a shift of muscle – and to a lesser extent the whole body – metabolism from carbohydrate toward FFA oxidation. A shift like that is likely the result of transient, or persistent, low plasma glucose concurrent with low insulin levels, as it occurs during exercise or fasting. Given that we saw a drastic increase in FFA after 3 days of KD (chapter 4), and both fasting and exercise produce a significant increase in plasma FFA[126–129], one hypothesis is that perhaps elevated FFAs themselves are interpreted by some part of the physiologic homeostat – likely neurons of the ventromedial hypothalamus (VMH) – as a carbohydrate deficiency signal and trigger hepatic glucose output. This would explain the results obtained after short-time KD feeding, also in the absence of any obvious damage. Moreover, it is possible that the early selective hepatic insulin resistance with increased glucose output observed during HFD feeding may be caused by the same mechanism. In support of this idea, GPR40-/- mice, GPR40 having been identified as a free fatty sensor,[130] are protected from impaired glucose homeostasis caused by a HFD.[131] On the other hand, in humans, two days of fasting as well as 90 minutes of intense exercise produce similar plasma levels of around 1-2mM of β-hydroxybutyrate (BHB),[132] the most abundant ketone body. This suggests that perhaps BHB itself acts as a signalling metabolite to increase hepatic glucose production during times of increased glucose need. The receptor for BHB has been identified as GPCR109, also known as hydroxycarboxylic acid receptor 2 (HCAR2),[133] which had previously been shown to be a receptor binding the widely used lipid lowering drug niacin and being responsible for its effect.[134] It is very interesting to note here that one of the most frequent side effects reported for niacin in multiple clinical trials is an increase in plasma glucose levels and incidence of diabetes.[135] This is consistent with a role for an endogenous activator of HCAR2 signalling such as BHB in mediating an increase in

hepatic glucose output. Addressing the question of how exactly ketone bodies influence glucose homeostasis is beyond the scope of this thesis, but dissecting the molecular pathways involving HCAR2 in both the VMH and the liver seem promising strategies. Another interesting question is whether such a role for BHB is also involved in the impairment of glucose tolerance caused by HFD feeding. Plasma BHB levels have been reported to increase in mice, in a study after 16 weeks of HFD feeding[136] and cats, in a study after 3 weeks of HFD feeding.[137] Moreover, the group of Barry Levin recently published very interesting findings arguing that ketone bodies in the VMH already increase after very short periods of HFD feeding due to increased production by astrocytes from FFA.[138] Thus, it is plausible that astrocyte-derived BHB could trigger signals in the VMH that are similar to signals caused by several days of fasting or intense exercise, causing an increase in hepatic glucose output and impaired glucose tolerance, and contributing to the aetiology of T2D.

Considering that the first paper (chapter 2) is already published while the other two are still pending publication I want to give a brief outlook regarding further experiments ostensibly required. In chapter 3 I will repeat the short term HFD feeding experiments with gavage of dipyridamole with a cohort size of at least 6 individuals and repeat the glucose tolerance measurements. In addition, I plan to perform measurements of insulin-dependent phosphorylation of eNOS to investigate whether dipyridamole can also rescue this HFD-feeding caused impairment. For the story concerning short term KD feeding the key experiment is to perform a hyperinsulinemic-euglycemic clamp. This experiment will allow us to address the question whether the impaired glucose tolerance observed after 3 days of KD feeding is indeed due to impaired suppression of hepatic glucose output, and whether insulin-dependent glucose uptake into tissues still functions normally. As an additional test of tissue specific insulin resistance I also want to measure insulin-dependent phosphorylation in the target tissues fat, liver and muscle after three days of KD feeding.

In ending the discussion, I want to briefly outline the concept of the so called metabolic set point. The concept states that there is a set weight that the body will defend.[139] It arose out of the observation that animals, when calorically restricted, will rapidly gain weight if allowed to eat ad libitum again, but only until they match the weights if their non-restricted controls.[140] A similar effect can be observed in transiently overfed animals.[141] Interestingly, the mechanism of weight defence was also present if animals were calorically matched to normally fed animals.[142] In this study, different diets had comparable outcomes in refeeding efficiency, except for low protein, were it was reduced, and >30% fat, where it was increased. These results are important for understanding how obesity can develop, since they both demonstrate that the homeostat has considerable control over not only food intake but also energy expenditure, and that dietary composition can at least modulate some aspect of this. When rodents are put on a high fat diet, they only consume increased calories (starting at roughly

30% more than on chow, corresponding to the increase in food calorie density) only during the first 7 to 14 days of HFD feeding and subsequently adjust their calorie intake to levels of animals kept on a regular chow and therefore consuming less absolute mass of food than animals on chow.[57] In spite of this, the bulk of weight gain happens in the period where calorie intake of HFD animals is matched to chow animals. The reason for this is that animals kept on HFD have reduced energy expenditure when compared to chow fed animals, an effect that can be rescued when animals are only given time restricted access to HFD food.[143] On the human side, a similar problem can be observed in what is termed the yoyo-effect, where people often gain back the weight they lost quite rapidly and seemingly defend their high weight.[144] Parts of the reason for the yoyo-effect are probably psychological, and I venture that a big contribution to it is that people often return to the exact dietary habits that caused the overweight in the first place, rather than sticking with the weight-loss promoting habits. Nonetheless, within that framework, strong negative changes in energy expenditure following severe and rapid weight loss have also been described in humans, most recently in a follow up study of participants of a weight loss TV-show, where negative energy expenditure adaptations of almost -500 kcal per day on average were reported 6 years after the weight loss occurred, in spite of the fact that participants on average had already gained two thirds of the weight back.[145] This change in energy expenditure was a result of changed resting metabolic rate (RMR), rather than reduced activity, suggesting that the study participant's homeostat was still defending a higher weight. In terms of the physiological mediator of this reduction in (RMR) the authors make no statement, but likely processes are reduced BAT or muscle thermogenesis.

Taken together, in my thesis I outlined how different diets and the physiological response to them can cause or modulate obesity, and how obesity might cause both metabolic and cardiovascular problems. It is clear that much remains to be understood. Perhaps learning if there is a shared mechanism of increased hepatic gluconeogenesis activated by both KD and HFD, and elucidating this mechanism, will help combat obesity not just by making it healthier, but ostensibly by also finding what causes the disturbances in the weight set point.

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1999 – 2003	Sir-Karl-Popper-School, Vienna

Experience

04/2009 – 07/2010	Master's Thesis Research, Harvard Medical School, Boston, Department of Systems Biology, Professor Pamela A. Silver *"A Directed Evolution Approach to Engineering Oxygen Resistant Fe-Fe Hydrogenases"*
3/2008 – 5/2008	Visiting Student and Research Assistant, Centro Nacional de Biotecnología, Madrid, Dr. Mariano Esteban *"Cloning and Purification of Vaccinia-C10L using a pET27-vector His-Tag system"*
7/2007 – 8/2007	Visiting Student and Research Assistant, Insitute of Molecular Pathology, Vienna, Dr. Barry Dickson *"Promoter characterization and data analysis for an RNAi based screen for behavioral phenotypes in courtship-song in Drosophila"*
6/2006	Visiting Student and Research Assistant, Max F. Perutz Laboratories, Vienna, Prof. Gerhard Wiche *"The Role of Plectin in the assembly of Sarcomeric and Costameric Structures"*

Awards and Honors

Novartis Prize for Best Talk – Molecular Translational Biomedicine Graduate School Zürich Retreat

PhD Fellowship recipient of the Ernst Schering Foundation

Austrian Marshall Plan Scholarship awarded for my diploma thesis project

Master's Degree University of Vienna – Magna Cum Laude

High School Examination Certificate – Magna Cum Laude

Publications

Grandl, G. *et al.* Depot Specific Differences in the Adipogenic Potential of Precursors Are Mediated by Collagenous Extracellular Matrix and Flotillin-2 Dependent Signaling. *Mol. Metab.* 1–11 (2016). doi:10.1016/j.molmet.2016.07.008

Kumpf S, Mihlan M, Goginashvili A, **Grandl G**, Gehart H, Godel A, Schmidt J, Müller J, Bezzi M, Ittner A, Guccione E, Wolfrum C, Ricci R. "Hairless promotes PPARγ expression and is required for white adipogenesis." EMBO Rep. 2012

Barstow B, **Grandl G**[1], Agapakis CM[1], Boyle PM[1], Silver PA, and Wintermute EH. "A synthetic system links FeFe-hydrogenases to essential *E. coli* sulfur metabolism." Journal of Biological Engineering [1] *contributed equally*, 2011

Conference Presentations

Selected Talk - Retreat of the Molecular Translational Biomedicine Graduate School Zürich

Poster presentation, SynBERC Meeting 2009, Massachusetts Institute of Technology, Boston, MA *"Engineering Oxygen Resistance in Hydrogenases through Directed Evolution"*

Skills

Molecular biology and biochemistry lab techniques

Matlab and Mathematica modeling and data processing

Intermediate Python programming skills

German (native), English, Spanish (fluent), French (advanced)