„THE ROLE AND FUNCTION OF ADIPOSE TISSUE-DERIVED DPP4 IN INTRA- AND INTERORGAN CROSSTALK“

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Diana Röhrborn

aus Gera

Düsseldorf, Dezember 2016

Dieser Arbeit wurde angefertigt am

Deutschen Diabetes-Zentrum

Paul-Langerhans-Gruppe für Integrative Physiologie

Leibniz-Zentrum für Diabetes-Forschung

an der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der

Heinrich-Heine-Universität Düsseldorf

Referent: Prof Jürgen Eckel

Korreferent: Prof. Ulrich Rüther

Tag der mündlichen Prüfung: 09.02.17

„Der Zweifel ist der Beginn der Wissenschaft. Wer nichts anzweifelt, prüft nichts. Wer nichts prüft, entdeckt nichts. Wer nichts entdeckt, ist blind und bleibt blind."

- Teilhard de Chardin (1881-1955), frz. Theologe, Paläontologe u. Philosoph -

ZUSAMMENFASSUNG

Weißes Fettgewebe galt lange Zeit als reines Energiespeicher-Depot. Heutzutage ist weithin anerkannt, dass weißes Fettgewebe ein endokrines Organ darstellt, das eine Vielzahl an Proteinen freisetzt, die sogenannten Adipokine. Das Fettgewebe ist ein heterogenes Organ, das sich aus veschiedenen Zelltypen zusammensetzt, zu denen sowohl Präadipozyten, Fibroblasten, Immunzellen als auch reife Adipozyten zählen. Bei positiver Energiebilanz kommt es zu einer dysfunktionellen Ausdehnung des Fettgewebes, welches durch chronische niedriggradige Inflammation, Insulinresistenz und Veränderung des sekretorischen Profils charakterisiert ist. Durch die Kommunikation mit anderen insulin-sensitiven Geweben, wie Leber und Skelettmuskel sind übergewichtige Individuen besonders prädestiniert, chronische metabolische Erkrankungen zu entwickeln. Zu diesen gehören Typ 2 Diabetes mellitus (T2DM), Herz-Kreislauf Erkrankungen und nicht-alkoholische Fettleber. In unserer westlichen Gesellschaft hat sich Fettleibigkeit zu einer der größten Gesundheitsbelastungen entwickelt, die mittlerweile epidemische Ausmaße annimmt. Um dieses komplexe Krankheitsbild zu bekämpfen, ist es nötig, die zugrunde liegenden molekularen Mechanismen, die Fettleibigkeit mit metabolischen Erkrankungen verbinden, zu verstehen. In diesem Zusammenhang entwickelten sich Adipokine, die vom Fettgewebe freigesetzt werden, zu einem Forschungsschwerpunkt in den letzten Jahrzehnten.

Durch Proteomanalyse des humanen Adipozytensekretoms wurde Dipeptidylpeptidase 4 (DPP4) als neues Adipopkin identifiziert. DPP4 ist eine ubiquitär exprimierte Zelloberflächen-Protease. Aufgrund ihrer katabolen Eigenschaft gegenüber den Inkretin-Hormonen, welche maßgeblich für die postprandiale Insulinfreisetzung verantwortlich sind, wurde DPP4 zu einem wichtigen therapeutischen Ziel für die Behandlung von T2DM. DPP4 wird nicht nur auf der Zelloberfläche exprimiert, sondern auch in die Zirkulation freigesetzt, wo es sowohl para- als auch endokrine Funktionen in Zielorganen erfüllt. Frühere Studien weisen außerdem darauf hin, dass DPP4 ein neuer Faktor sein könnte, der Fettleibigkeit mit Parametern von metabolischen Erkrankungen, wie BMI, Taillenumfang und Insulinsensitivität verbindet. Dennoch sind der Mechanismus und die Regulation der Freisetzung von DPP4 weitestgehend unbekannt. Aus diesem Grund war der Ausgangspunkt dieser Arbeit, den Freisetzungsmechanismus und dessen Regulation *in vitro* aufzuklären. Die Ergebnisse legen nahe, dass konstitutive

Freisetzung von löslichem DPP4 (sDPP4) über hydrolytische Spaltung von der Zelloberfläche erfolgt, welche auch „shedding“ genannt wird. Ich konnte zeigen, dass dieser Prozess verschiedene Proteasen involviert, wie beispielsweise Matrixmetalloproteasen oder Cathepsine, und Zelltyp-spezifisch ist. Außerdem deuten meine Ergebnisse darauf hin, dass die beteiligten Enzyme über eine katalytische Kette aktiviert werden. Ein identifizierter regulatorischer Faktor der DPP4 Freisetzung in vaskulären Zellen ist Hypoxie. Diese führt zu einer gesteigerten Expression von Metalloproteasen, was zu einer erhöhten DPP4 Freisetzung beiträgt.

Obwohl frühere Studien bereits belegen konnten, dass das Fettgewebe eines der wichtigen Quellen für sDPP4 darstellt, blieb die Rolle von DPP4 innerhalb des Fettgewebes weitestgehend unbekannt. Ein weiteres Untersuchungsziel dieser Thesis war die Charakterisierung der Rolle von DPP4 im Fett *in vitro* durch siRNA-vermittelte Reduktion von DPP4 in Präadipozyten. Um klarzustellen, ob die beobachteten Effekte durch die enzymatische Aktivität von DPP4 vermittelt werden, wurden Schlüsselxperimente unter Verwendung von etablierten DPP4 Inhibitoren wiederholt. Die erhobenen Daten zeigen, dass DPP4 keine zentrale Rolle in der Adipozyten-Differenzierung zu spielen scheint. Weiterhin konnten keine Effekte einer DPP4 Reduktion auf Ebene der TNFα-induzierten Inflammation hinsichtlich NFκB Aktivierung oder Expression von NFκB Zielgenen beobachtet werden. Effekte einer siRNA-induziertem Reduktion von DPP4 hinsichtlich der Änderung des Adipozyten Sekretoms können auf Grundlage meiner Ergebnisse derzeit nicht ausgeschlossen werden. Interessanterweise führt die Verminderung von DPP4 zu einer verbesserten Insulin Signalweiterleitung, welche zumindest teilweise über die enzymatische Aktivität vermittelt wird. Zusammen mit unseren bereits publizierten Ergebnissen zu direkten Effekten von sDPP4 auf den Insulinsignalweg postuliere ich, dass die vorhandene Menge an DPP4 speziell in fettleibigen insulin-resistenten Patienten einen Mediator der Insulinresistenz darstellt.

Mäuse mit einem kompletten DPP4 Knockout (KO) sind vor diät-induzierter Fettleibigkeit geschützt, indem metabolische Parameter wie Glukosetoleranz und hepatische Fettakkumulation verbessert werden. Die Interpretation der Ergebnisse mit diesem Mausmodel gestaltet sich allerdings schwierig, da auch Nahrungsaufnahme, Körpergröße und Immunzellen vom KO beeinflusst werden. Zusätzlich kann keine Aussage zu organspezifischen Effekten eines DPP4 KO getroffen werden. Aufgrunddessen war ein weiteres Ziel die lokale und systemische Rolle von fettgewebs-

spezifischem DPP4 unter Generierung eines gewebe-spezifischen KO zu untersuchen. Um weiterhin die Rolle von fettgewebs-spezifischem DPP4 in der Fettleibigkeit zu untersuchen, wurden die Mäuse für 24 Wochen einer Hoch-Fett-Diät (HFD) ausgesetzt. In diesem Zusammenhang konnte trotz erhöhtem Körpergewicht eine verbesserte orale Glukosetoleranz in den KO Tieren unter HFD beobachtet werden. Interessanterweise scheint das gewebs-spezifisches Modell speziell die hepatische Insulinsensitivität in Hinsicht auf endogene Glukoseproduktion zu verbessern. Ich postuliere, dass dies über eine verminderte Freisetzung von Insulin-like Growth Factor Binding Protein 3 (IGFBP-3) aus dem Fettgewebe mediiert wird, welche zu erhöhtem Level von freiem Insulin-like Growth Factor 1 (IGF1) führt. Daraus resultiert eine Verbesserung der Glucose Toleranz und der hepatischen Insulinresistenz. Die Beobachtungen deuten außerdem darauf hin, dass es zu einer vorteilhaften Fettgewebsmodellierung unter HFD kommt. Diese zeichnet sich durch ein optimiertes Verhältnis von M1 zu M2 Makrophagen, verminderter Fibrose und einer reduzierten Adipozytengröße aus. Besonders hervor zu heben ist diesbezüglich, dass die beobachteten Effekte unabhängig von der Inkretin-Achse zu sein scheinen und eher direkt über die Wirkung von DPP4 oder über alternative Substrate erfolgen.

Zusammengefasst verdeutlichen die erzielten Erkenntnisse die negative Rolle von DPP4 in der Entwicklung der Fettleibigkeit. Diese Arbeit zeigt, dass DPP4 vor allem in der Fettleibigkeit lokal den Metabolismus und die Struktur des Fettgewebes beeinflusst. Außerdem konnten meine Ergebnisse maßgeblich zu einem besseren Verständnis der DPP4 Freisetzung beitragen, was besonders im Hinblick auf die Kommunikation mit anderen Geweben von großem Interesse ist. Beispielhaft wurde dies in dieser Arbeit hinsichtlich der Wirkung von DPP4 auf die hepatische Insulinsensitivität im fettgewebs-spezifischen KO Mausmodell gezeigt. Diese Thesis bekräftigt weiterhin die Rolle von DPP4 als therapeutisches Mittel für die Behandlung von metabolischen Erkrankungen wie T2DM, welche auch über die Inkretin-vermittelte Wirkung hinausgeht.

SUMMARY

White adipose tissue (AT) has long been considered as a mere energy storage organ. Nowadays, it is widely accepted that AT is a real endocrine organ releasing various metabolically active proteins, the so-called adipokines. AT is a heterogeneous organ comprising different cell types such as preadipocytes, fibroblasts, immune cells and mature adipocytes. Due to positive energy balance AT mass is expanding during obesity, leading to a dysfunctional tissue, which is characterized by a chronic low-grade inflammation, insulin resistance and an altered secretory profile. Via crosstalk to other insulin sensitive tissues like liver and skeletal muscle, obese individuals are predisposed to the development of chronic metabolic diseases like type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) or non-alcoholic fatty liver disease (NAFLD). In our western society obesity became one of the greatest health burdens reaching epidemic dimensions now. To combat this complex disease scenario it is necessary to understand the underlying molecular mechanisms linking obesity to metabolic diseases. In this context, adipokines released by AT became a major research topic in the last decades.

By proteomic profiling of the human adipocyte secretome, dipeptidylpeptidase 4 (DPP4) has been identified as a novel adipokine. DPP4 is a ubiquitously expressed cell-surface protease. Due to its catabolism of the incretin hormones, which are important for post-prandial insulin secretion, it became a therapeutic target for the treatment of T2DM. DPP4 is not only expressed as a cell-surface molecule, but is also released into the circulation, thus exerting para- and endocrine functions on target tissues. Previous studies also revealed that DPP4 might be a novel factor linking obesity to parameters of metabolic diseases like BMI, waist circumference and insulin sensitivity. However, the mechanism and regulation of DPP4 release were largely unknown. Therefore the starting point of this thesis was to elucidate the mechanism and regulation of DPP4 release *in vitro*. Our findings suggest that constitutive sDPP4 release occurs via hydrolytic cleavage from the cell surface, in a process which is termed shedding. I postulate that this process involves different types of proteases like matrix metalloproteases and cathepsins in a cell-type specific manner. Furthermore, I propose a catalytic cascade being involved in the activation of the responsible sheddases. One regulating factor of sDPP4 release in vascular cells seems to be hypoxia, which leads to an upregulation of MMP gene expression, thereby increasing DPP4 shedding.

Although previous studies support the notion that AT is an important source of circulating DPP4, its functional role within AT remained elusive. A second topic of this thesis was to study the effects of DPP4 ablation on AT *in vitro* by siRNA-mediated silencing of DPP4 in preadipocytes. To verify if the observed effects are mediated through DPP4 enzymatic activity selected experiments were repeated with established DPP4 inhibitors. The present thesis reveals that DPP4 seems to play no role in adipocyte differentiation *in vitro*. There have also been observed no effects of DPP4 silencing on TNFα-induced inflammation in respect to NFκB activation or gene expression of NFκB target genes. Effects of DPP4 on the adipocyte secretome can not be excluded with the obtained data. Interestingly, DPP4 knock-down improves insulin signaling in mature adipocytes which seems to be at least partly mediated through its enzymatic activity. Together with previously published results on direct effects of sDPP4 on insulin signaling, I postulate that the amount of DPP4 is potentially a regulating factor in insulin sensitivity especially in obese insulin-resistant patients.

Whole body knock-out (KO) animals revealed that DPP4 ablation protects from diet-induced obesity and improves metabolic parameters like glucose tolerance and hepatic lipid accumulation. However, the interpretation of these findings is difficult because of an effect of the KO on food intake, body size and immune cells. Furthermore, these studies fail to ascribe the observed effects to a particular DPP4 releasing tissue. Therefore, another aim of the thesis was to elucidate local and systemic effects of AT-derived DPP4 with a tissue-specific KO approach. To further reveal the importance of AT-derived DPP4 in the development of obesity, adipose-specific DPP4 KO mice were challenged with 24 weeks of high-fat diet (HFD) feeding. In this context improved oral glucose tolerance was observed despite increased body weight in the KO animals under HFD. Most interestingly, the AT-specific KO model seems to selectively improve hepatic insulin sensitivity via improving endogenous glucose production. I propose that this might be mediated by a downregulated insulin-like growth factor binding protein 3 (IGFBP-3) release from AT into the circulation. In this respect the findings further indicate that AT-specific DPP4 KO leads to a beneficial AT remodeling under HFD with improved M1 vs M2 macrophage ratio, less fibrosis and a significant reduction of adipocyte diameter. Of note is, that the observed effects seem to be independent of incretin hormones and might rather be mediated via direct effects of DPP4 or via alternative DPP4 substrates.

Taken together, the findings highlight the importance of DPP4 as a negative modulator during the development of obesity. This thesis indicates that DPP4 acts locally on the metabolism and structure of AT especially in obesity. Furthermore, my results contributed significantly to a better understanding of DPP4 release which is important in understanding the underlying effects of DPP4 in the cross-talk to other tissues. As an example of this cross-talk DPP4 was identified as a mediator of hepatic insulin resistance via cross-talk mechanisms in the AT specific KO mouse model. This thesis further strengthens the role of DPP4 as a therapeutic target for the treatment of metabolic disease like T2DM also beyond its incretin-mediating action.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADA	adenosine deaminase
AS160	Akt substrate of 160kDa
AT	adipose tissue
ATGL	adipocyte triglyceride lipase
cAMP	cyclic adenosine-mono-phosphat
CM	conditioned medium
CVD	cardiovascular disease
ECM	extracellular matrix
ER	endoplasmatic reticulum
GIP	glucose-dependent insulinotropic peptide
GLP-1	glucagon-like peptide 1
Glut	glucose transporter
HFD	high-fat diet
Hif1α	hypoxia inducible factor 1α
HOMA-IR	homeostatic model assessment-insulin resistance
HSL	hormone sensitive lipase
IL	interleukin
InsR	insulin receptor
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
Loxl1	lysyl oxidase homolog 1
MHO	metabolically healthy obese
mTOR	mammalian target of rapamycin
MUNW	metabolically unhealthy normal weight
NEFA	non-esterified fatty acids
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
oGTT	oral glucose tolerance test
PDE3b	phosphodiesterase 3B
PI3K	phosphoinositide 3-kinase
PKA	proteinkinase A
PKC	proteinkinase C
PLIN1	perilipin

Rab	ras-related in brain
SMC	smooth muscle cell
T2DM	type 2 diabetes mellitus
TNFα	tumor necrosis factor α

LIST OF FIGURES

1. GENERAL INTRODUCTION

1.1. ADIPOSE TISSUE AND ITS DYSFUNCTION IN OBESITY

Adipose tissue (AT) is a heterogeneous cell population consisting of energy storing, lipid-loaden mature adipocytes, its prescursors the preadipocytes, collagenous and reticular fibre, neurons, blood vessels, lymph nodes, immune cells and fibroblasts (1;2). There are two distinct types of classical AT in mammals, classical brown, which is mainly responsible for thermoregulation (3) and classical white. The latter will be the focus of this thesis and has the main energy regulating function via storage and mobilization of triglycerides (TG). Another important feature of white AT is only known since several years and shifted the relevance of AT from a mere energy storing depot to an active endocrine organ. This feature is the secretion of various factors, the so called adipokines (2).

During obesity optimal body composition shifts to increasing adipose tissue (AT) mass. The classification of patients is defined according to their percentage of body fat, which is defined by certain cut-offs depending on gender and age (4). Obesity in males is defined by the cut-off of 20.6-24.2% fat per bodyweight depending on age whereas in females it ranges from 36-37%. Obesity is one of the greatest health burdens in our western society since it is linked to the development of chronic diseases like type 2 diabetes mellitus (T2DM)(5), cardiovascular disease (CVD)(6), non-alcoholic fatty liver disease (NAFLD) (7;8) and certain types of cancer (9). The following sections deal with the complex alterations of AT occurring during the progression of obesity and leading to insulin resistance within AT. Insulin resistance is influenced by various mechanisms. On the one hand insulin action itself on adipocytes in respect of lipolysis and glucose uptake is impaired, but also adipocyte hypertrophy, hypoxia, fibrosis as well as macrophage infiltration and the development of a chronic low-grade inflammation are contributing to insulin resistance. All of these aspects will shortly be discussed in the following sections.

1.1.1. INSULIN ACTION ON GLUCOSE UPTAKE AND LIPOLYSIS

Insulin is produced by the beta cells of the pancreas and reaches its target tissues via the blood stream. Post-prandially insulin levels are rising which leads to a subsequent

activation of Akt within the target cells (10). Interestingly, insulin is increasing the blood-flow after meal ingestion or physical activity and thereby enhances its own delivery and that of other signaling substrates to the target tissues (11;12). The increase in post-prandial AT blood-flow is also associated with insulin sensitivity independent of body-weight as shown by Karpe and colleagues (13). Furthermore, it is well known that ectopic fat accumulation leads to impaired insulin sensitivity in tissues like liver and muscle (14;15). There is a tight cross-talk between anabolic processes mediated by insulin and catabolic processes mediated by catecholamines. On the one hand insulin is able to counteract lipolysis, and on the other hand meditators of lipolysis are able to interfere with glucose uptake. This complex association was already described by Randle in 1963 and is known as the "randle cycle" and was redefined throughout the years by more in depth knowledge (16). The first part of this section will deal with lipolysis and how insulin affects this process.

AT lipolysis is the catabolic pathway which leads to a sequential breakdown of triglycerides into glycerol and fatty acids (17). It is the key regulator of energy mobilization and storage and therefore lipolysis is under tight control according to nutritional status of an individual. In T2DM patients there is an insufficient suppression of lipolysis which results in an increased fatty acid spillover to the circulation (18;19). Akt despite being a key component in the phosphoinositide 3-kinas (PI3K) pathway which leads to glucose uptake also phosphorylates phosphodiesterase 3 b (PDE3b). PDE3b catalyzes cAMP hydrolysis, which is usually built during fasting upon activation of adenylyl cyclase via β-adrenergic receptor activation (20). Thereby insulin acts antilipolytic in AT. However there are also Akt-independent pathways described by which insulin acts on lipolysis. Especially at low submaximal levels of catecholamines insulin might rather act through proteinkinase C-dependent pathways or through the regulation of lipid droplet trafficking by Rab proteins (21). Another inhibitory pathway of lipolysis might be mediated through lactate (22). Preferentially under conditions where glucose uptake is enhanced lactate production is upregulated. Lactate is able to activate the G-protein-coupled receptor GPR81 and thereby counteracts the effect of catecholamines on β-adrenoceptors (22). Not only the suppression of lipolysis by insulin is disturbed in T2DM patients, but also basal fat cell lipolysis is elevated in obesity and is closely associated with insulin resistance (14;23). Since it is well-known that fat cell size positively correlates with basal lipolysis rate this could be one possible reason for elevated basal lipolysis in obese subjects (24). However this is not applicable for the

sensitivity to insulin. It was shown on the contrary that larger adipocytes are more sensitive to the inhibitory action of insulin on lipolysis (25;26). Disturbancies in antilipolytic action of insulin as seen in obese individuals seem to be rather mediated through impaired insulin binding (27). Furthermore, it is reported that in metabolically healthy humans insulin acts very quickly as an antilipolytic agent after glucose ingestion via alterations at the post-receptor level, which is not the case in obesity (28).

Although AT accounts for only approximately 10% of whole body glucose disposal capacity, glucose uptake is still crucial for normal AT function (29). Impaired glucose uptake is altering esterification of fatty acids, lipid biosynthesis (30) and endocrine function of AT (31). The following section will deal with glucose uptake and how lipid products generated during lipolysis may counteract this process in AT.

Insulin-stimulated glucose uptake in AT is dependent on the presence of the glucose transporter isoform 4 (Glut4). This protein is absent from fibroblastic, pre-adipocyte states and is progressively upregulated upon adipocyte differentiation (32). Another glucose transporter isoform is also expressed in AT and accounts for basal glucose uptake. Glut1 is already detectable in adipocyte precursor cells and is gradually decreased during adipocyte differentiation. At least human adipocyte precursor cells are not sensitive to insulin and basal glucose uptake is decreased throughout differentiation. During obesity Glut4 protein levels are reduced and thereby glucose disposal rate is diminished in AT. This was shown in diabetic rats where Glut4 levels in the membrane fraction of adipose cells are markedly reduced even in the insulin-stimulated state (33). In contrast, it was reported that Glut1 levels are unaffected. It seems that the intracellular Glut4 pool in diabetic rats is decreased and therefore less Glut4 is available for translocation (33). In humans it was shown that already in patients with impaired glucose tolerance Glut4 mRNA levels are diminished, thus the regulation of glucose transport is already affected at pretranslational level (34). Mullins and colleagues found out that lipid products generated during lipolysis lead to a dissociation of mammalian target of rapamycin (mTOR) complexes. Especially via decrease of mTORC2 a subsequent decrease of glucose uptake occurs (35). MTORC2, which consists of mTOR and rictor together with other proteins, is responsible for phosphorylation of Akt at Ser473 upon insulin stimulation (36). This in turn is required for activation of Akt substrate of 160kDa (AS160) and translocation of Glut4 to the plasma membrane which results in glucose uptake.

1.1.2. ADIPOSE TISSUE HYPERTROPHY VERSUS HYPERPLASIA

Obesity is a heterogeneous condition which is influenced by genetics, lifestyle habits and behavior and is characterized as an excessive accumulation of body fat (37). Although AT is a heterogenous tissue, adverse effects which are observed due to obesity are mainly linked to adipocytes (38). Interestingly not all obese individuals develop T2DM and there is also a subgroup of individuals who are lean, but not cardiometabolically healthy. These two subgroups are classified as metabolically healthy obese (MHO) or metabolically unhealthy normal weight (MUNW) individuals (37). MHO individuals are characterized by a BMI above 30 but normal insulin sensitivity, fasting glucose and blood lipids (39) whereas MUNW individuals have a BMI below 25, which is still accompanied by elevated plasma triglycerides, and LDL-Cholesterol with lowered HDL-Cholesterol (40).

There are two possible ways how AT can react on positive energy balance. First, mature adipocytes could increase in diameter by storing more triacylglycerol in lipid droplets, which would make them hypertrophic (41). Indeed, large lipid-laden adipocytes can be predominant in white AT and may exceed a volume of 800pL (42). And second, new mature adipocytes can be generated from pre-adipocytes by differentiation which forms a hyperplastic AT (41). More smaller adipocytes are protective against metabolic abnormalities in respect of lipid profile, glucose and insulin levels as normally seen in hypertrophic obesity (43). Functional adipogenesis is therefore crucial to prevent development of insulin resistance. It has been demonstrated that polymorphisms in PPARγ, which renders it unfunctional, are associated with higher risk to develop T2DM (44). In contrast to that, polymorphisms without loss-of-function have no effect on insulin sensitivity. Additionally, the level of circulating adiponectin, which is a key mediator of adipogenesis, is a strong predictor of insulin sensitivity (45). Larger adipocytes are characterized by an increased basal and catecholamine-stimulated lipolysis (46). Especially in the postprandial period lipid, storage capacity in obese individuals is reduced. This might be due to the fact that adipocytes have a maximum size threshold which could already be exceeded during obesity (47). On top of that, the lipolysis rate is increased during fasting in obese AT so that there is a higher efflux of non-esterified fatty acids (NEFA) into other tissues and this could impair whole body

insulin sensitivity (48). There is evidence, that hypertrophy is more likely to lead to insulin resistance and T2DM than hyperplasia (49;50). Interestingly MHO individuals tend to have lower ectopic fat accumulation in liver and skeletal muscle than their metabolically unhealthy obese counterparts (39;51). Studies suggest that MHO individuals preserve their white AT function via increasing hyperplasia instead of hypertrophy (49;52). This is achieved e.g. via increasing the expression of genes related to adipocyte differentiation (50). The mean and maximum volume of adipocytes is associated with whole body insulin sensitivity, increased parameters of inflammation and oxidative stress in the circulation, and increased macrophage number within AT (42). One possible explanation might be the activation of stress kinases like JNK or p38MARK during hypertrophy and adipocyte differentiation which leads to an alteration of the secretome, to insulin resistance and stimulates macrophage infiltration (53-55).

Another important explanation for the existence of MHO and MUNW individuals might be the location of AT. It has been demonstrated that MUNW have increased VAT (56;57). It was also shown that in MHO and MUO individuals matched for percentage of body fat MHO have reduced amounts of VAT (58). Although it is hypothesized that MHO preferentially store excess fat in SAT, the literature is controversial and some studies did not find differences in SAT between MHO and MUO (51;59-61).

A novel study by Lackey and colleagues suggests that another difference between MHO and MUO might be ECM characteristics like tensile strength, collagen and proteolytic enzymes (62).

Blood vessels within white AT might also function as an adipocyte progenitor niche providing expanding AT with new pre-adipocytes (38;63). Another important function of blood vessels is the supply with oxygen and there might be a difference between MHO and MUO in angiogenic potential as proposed by Sun and colleagues (64).

1.1.3. FIBROSIS AND HYPOXIA

Hypoxia and fibrosis are tightly linked to each other in the progression of obesity. Fibrosis is defined as the excessive deposition of fibrous connective tissue within a certain organ or tissue. In AT, fibrosis results from a higher synthesis of fibrillary components like collagen I, II and VI (65). To accommodate hypertrophic adipocytes properly during nutrient overload, the extracellular matrix (ECM) is underlying constant remodeling. During obesity adipocyte size increases to 140-180µm in diameter and at

some point the size of these adipocytes exceeds the possibility to efficiently vascularize and innervate these cells (66). Efficient oxygen diffusion is possible until approximately 100µm apart from blood vessels (67). Therefore at some point during obesity AT becomes hypoxic (64). This leads to an increase in hypoxia-inducible factor 1 α (Hif1α) expression (68). Hypoxia also leads to inflammation via upregulation of IL-6 and macrophage inflammation factor 1 (MIF1) by Hif1α (69). Inflammation further triggers expression and secretion of collagens by macrophages and preadipocytes, thus highlighting the correlation of AT fibrosis and inflammation (70). Furthermore hypoxia stimulates fibrosis of AT since lysyl oxidase homolog 1 (Loxl1), which crosslinks collagens and elastin (68), is a Hif1α target gene. Obesity in humans is associated with increased fibrosis via excessive collagen deposition (71). Fibrosis limits AT expandability and makes it more "stiff", which leads to adipocyte cell death and triggers inflammation (65). On the other hand hypoxia induces angiogenesis to counteract AT growth and oxygen demand (72). Therefore obese subjects are characterized by increased vessel size in AT in comparison to lean individuals (73). However, this counteraction is not enough to sufficiently react on the oxygen need of the expanding tissue. VAT seems to be more prone to fibrotic strokes than SAT and limited hypertrophy also increases the risk for ectopic lipid accumulation (74). However the exact molecular link between obesity and fibrosis is mainly unknown.

1.1.4. CHRONIC LOW-GRADE INFLAMMATION

During the progression of obesity, AT undergoes massive metabolic and morphologic changes due to hypertrophy, apoptosis and stresses such as inflammation and hypoxia. These changes are summarized in Fig 1. Taken together, these changes in AT lead to an alteration of the secretory profile towards a more pro-inflammatory status (75). Pro-inflammatory cytokines like interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) are known to inhibit adipocyte differentiation and to induce apoptosis in pre- and mature adipocytes (76-78). These modifications lead to a decrease in hyperplastic potential of AT and thereby reduces its storage capacity resulting in higher NEFA production (79). NEFA on the one hand can than systemically impair insulin sensitivity and further elevate the risk to develop T2DM, but on the other hand NEFA also have local effects like elevating MCP-1 and IL-6 levels in pre-adipocytes and further support development of a pro-inflammatory secretome (80).

Furthermore, the population of immune cells in white AT tissue changes during the progression of obesity and metabolic dysfunction. Whereas in healthy white AT, resident macrophages are mainly of the anti-inflammatory M2 phenotype, during obesity a switch of these macrophages towards a more pro-inflammatory M1 subset occurs (81). In addition to a phenotypic switch also the number of macrophages is elevated during low-grade chronic inflammation. This occurs both due to local proliferation as well as recruitment of circulating macrophages (82). Pro-inflammatory cytokines like MCP-1 attract more macrophages to the inflamed AT and macrophages themselves also produce MCP-1, and thereby close the vicious cycle (83;84). Macrophages are typically infiltrating AT especially around necrotic adipocytes which are called crown-like structures (85).

In addition to macrophages, another important cell type associated with insulin resistance and T2DM are CD4+ T-cells (86;87). Also other cells of the innate and adaptive immune system are involved in the pathology of T2DM and obesity. These cells are e.g. B-cells, neutrophils and dendritic cells, as recently reviewed by Apostopoulos (88). The amount of these cells positively correlates with the severity of the metabolic disorder (37).

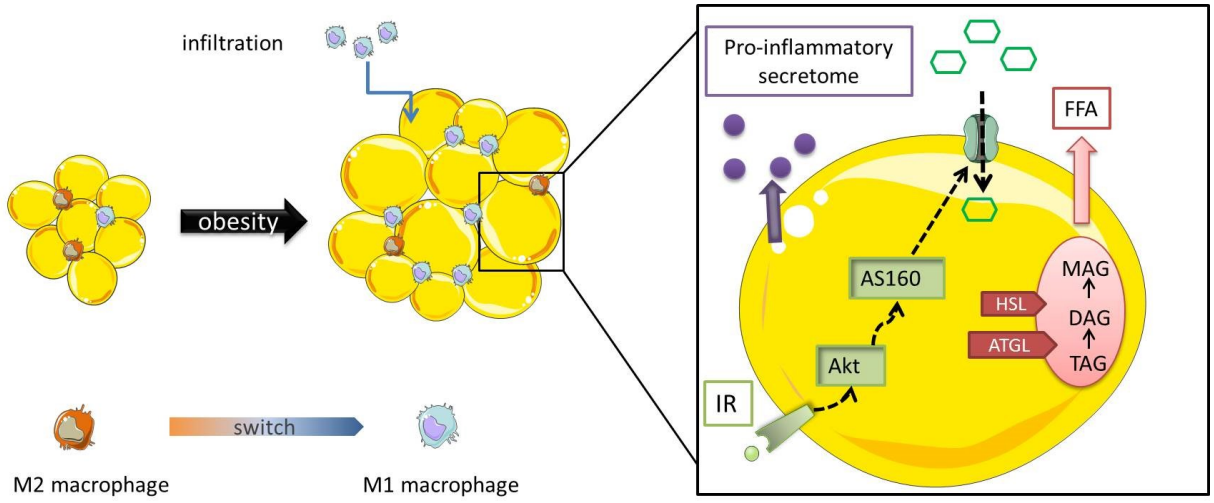


FIGURE 1: ADIPOSE TISSUE AND ITS DYSFUNCTION IN OBESITY
During obesity adipocytes are increasing in size and number. Furthermore, a phenotypic switch of macrophages from anti-inflammatory M2 to more pro-inflammatory M1 occurs, plus new macrophages are recruited into AT. Within adipocytes more pro-inflammatory adipokines are released. Due to impaired insulin sensitivity more free fatty acids are released and glucose uptake is diminished. FFA free fatty acids; MAG monoacylglycerol; DAG diacylglycerol; TAG triacylglycerol; HSL hormone sensitive lipase; ATGL adipose triglyceride lipase; PLIN perilipin; IR insulin resistance; Akt protein kinase B; AS160 Akt-substrate of 160kDa

1.2. ADIPOSE TISSUE IN THE CROSS-TALK TO CELLS AND ORGANS

Through the release of circulating factors, AT is not only able to regulate its own function but also to interact with surrounding or distant organs and tissues. Although the list of newly identified secreted factors is expanding rapidly most of them are incompletely characterized. It is well-accepted that many of these factors released by AT have an impact on the function of other organs. However, it is often unknown how these factors emerge AT or how and if these factors are involved in adipogenesis, AT growth or function. The next section deals with the role of AT as an endocrine organ, the general export mechanisms for the secreted factors, and gives examples of some factors and their role in intra- and inter-organ crosstalk. This complex interplay is summarized in Fig 2.

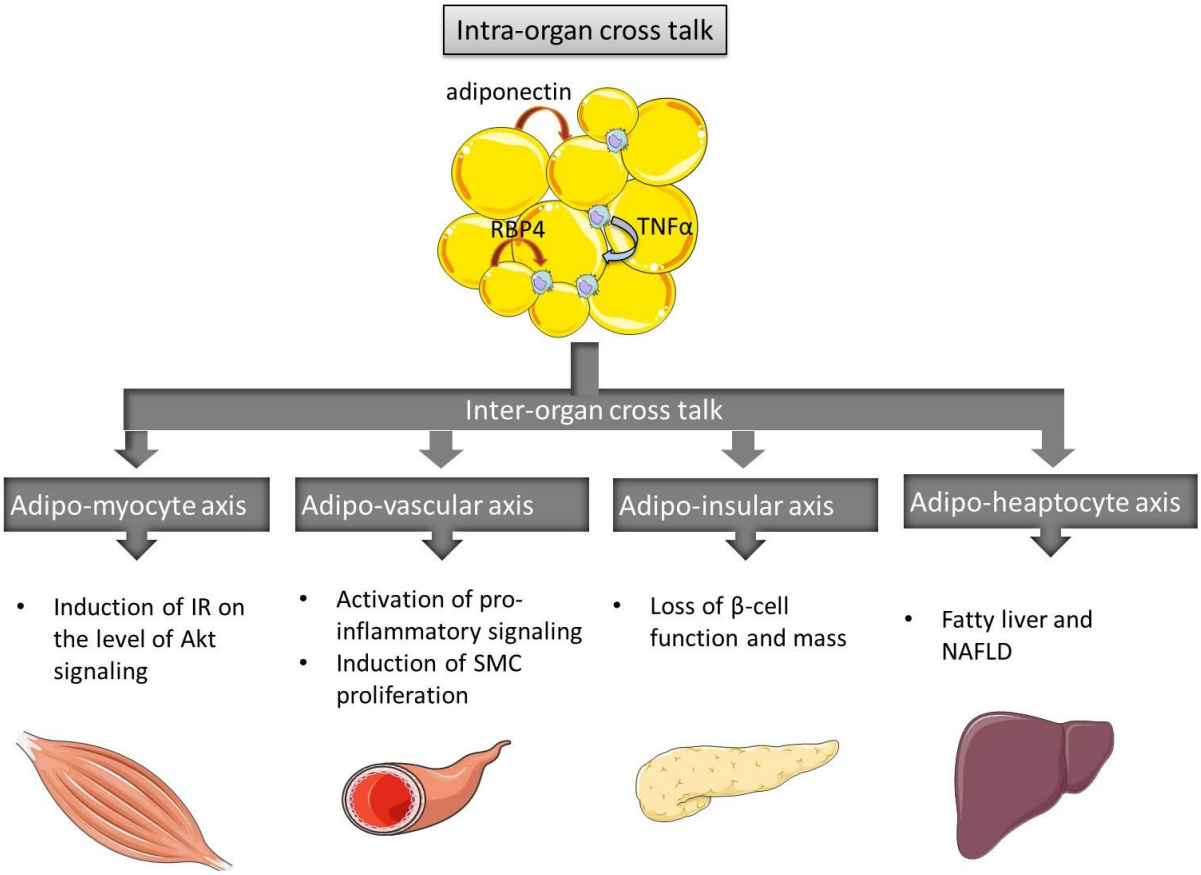


FIGURE 2: ADIPOSE TISSUE IN THE CROSS-TALK TO OTHER CELLS
AT is an active endocrine organ releasing a variety of factors. Thereby it is able to communicate locally with adipocytes or immune cells within AT or systemically with target tissues like skeletal muscle, vascular cells, pancreas or liver. RBP4 retinol binding protein 4; TNFα tumor necrosis factor α; NAFLD non-alcoholic fatty liver disease

1.2.1. ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

For a long time it was believed that AT is an inactive tissue mainly responsible for the storage and mobilization of lipids according to need. Already in 1987, when AT was identified as the major site of sex steroid metabolism and as producer of adipsin, this view changed (89). The discovery and characterization of leptin in 1994 opened up the road to a new era of research on AT (90). Nowadays, it is widely accepted that AT is an endocrine organ and the number of identified secreted factors, the so called adipokines, is increasing at a tremendous rate over the last decades (91-93). The released adipokines are able to act locally within AT or systemically on other peripheral target tissues thus influencing developmental, metabolical or inflammatory processes. Furthermore, adipocytes are equipped with receptors necessary to receive signals from other cells and distant organs thus enabling it to not only actively interfere in processes, but also to react on signals (94). Although many of the secreted factors are derived from the non-adipocyte fraction of AT, still all components are one functional unit and thus AT is a true endocrine organ. Attempts to identify novel adipokines and to characterize the whole secretome revealed hundreds of secreted factors (91). It could be shown that the majority of the identified factors have an altered expression in obesity. Most significantly, upregulated gene sets at mRNA level are associated with ECM organization, cell-cell adhesion, and acute inflammatory response (95). If one keeps in mind that the body is composed of >25% AT in obese individuals and obesity is associated with numerous diseases, it is necessary to understand the role and function of these adipokines.

1.2.2. Different secretory pathways for adipokines

As already mentioned above, research on the human adipokinome revealed numerous novel adipokines. However, the precise mechanism of secretion of many adipokines is still unknown. There are several mechanisms by which adipokines can exit the cell into the extracellular space. These mechanisms can be separated into the classical, or endoplasmatic reticulum (ER)-Golgi-mediated pathway, and non-classical secretory pathways.

To exit adipocytes via the classical pathway, adipokines need to be equipped with an N-terminal signal sequence (96). Thereby these factors are translocated to the ER, correctly folded and assembled and then exit the compartment via COPII-coated vesicles to enter the Golgi complex (97). Inside this compartment adipokines are sorted

according to their destination and packed into vesicles. These vesicles fuse with the plasma membrane afterwards to release their content into the extracellular space (98). One representative adipokine of this export mechanism is adiponectin (99).

For the non-classical secretion mechanism, there are several possibilities with at least three distinct ways described until know, namely transporter-mediated export, microvesicle/exosome release and selective post-translational hydrolysis from the cell surface (100). All of these mechanisms have in common, that the exported factors lack the typical signal sequence which targets them to the ER and are therefore also called "leaderless". Furthermore, this makes them also insensitive to brefeldin A or monensin treatment (101). One example for transporter-mediated export is fibroblast growth factor 2 (FGF2), which is exported via the plasma membrane Na^+/K^+-ATPase which was shown by treatment with the inhibitor ouabain (102;103). Il-1β is a classical representative of vesicle mediated transport. It is able to exit the cell via at least three possible vesicle routes like lysosomes, shed plasma membrane vesicles, or exosomes from multi-vesicular bodies (104). One non-classical secretory pathway is especially interesting in the context of the presented PhD thesis. The selective post-translational hydrolysis from the cell surface, called shedding, is a proposed export route for dipeptidylpeptidase 4 (DPP4) (105). As a member of the type II transmembrane protease family of proteins, DPP4 contains a predicted signal sequence which is located at the transmembrane domain and serves as the anchor sequence for DPP4 inside the plasma membrane (106). It was already speculated that DPP4 is released via shedding, but the exact underlying mechanism is unknown (107). Furthermore, it was already shown that it is insensitive to brefeldin A treatment in skeletal muscle cells (108). Another member of this secretory pathway is TNFα which is released into the extracellular space by proteolytic cleavage at Ala76-Val77 by TNFα converting enzyme (TACE) (109).

The release of adipokines can be constitutively active or it can be triggered by a certain stimulus. These stimuli can originate from the cross-talk with other cells within adipose tissue and this will be discussed in the following section.

1.2.3. INTRA-ORGAN CROSS-TALK

AT it is a heterogeneous tissue which does not only consist of adipocytes and precursor cells, but is also highly infiltrated by immune cells. Via the secretion of cytokines and adipokines these different cell-types are in close communication. This cross-talk is not unidirectional but a rather complex interplay between the different partners (110).

The cross-talk can influence AT on different levels like mediation of adipogenesis (111) or inflammation (112). Furthermore, some mediators are also known to have an impact on adipocyte function like lipolysis and glucose uptake (113;114).

Although also adipocytes are able to release TNFα and IL-6, the main sources of these cytokines within AT are macrophages (115;116). TNFα is known to inhibit adipogenesis by preventing peroxisome proliferator activated receptor (PPAR) γ and CCAAT/enhancer binding protein (C/EBP) α expression (117). Both TNFα and IL-6 are associated with insulin resistance in AT (118;119). The release and expression of cytokines and adipokines is tightly regulated and can be influenced bidirectional via cytokines or adipokines. Especially TNFα is able to increase the release of some adipokines like chemerin (120), or decrease the release of adipokines like retinol binding protein 4 (RBP4) (121). Adipokines can also impair macrophage secretory function. As such RBP4 is able to induce MCP-1, TNFα and IL-6 release in macrophages (122). IL-6 is able to regulate its own expression and release, as it was shown by several groups (118;123). The impact of cytokines on adipocyte function can be versatile. In this respect TNFα is able to impair lipolysis by interfering with the expression and activity of LPL and fatty acid binding transport proteins (113). But it is also involved in regulation of glucose uptake. Several mechanisms are known by which TNFα can interfere with glucose uptake. First, TNFα is able to lower PPARγ expression and activity, and thereby reduces Glut4, insulin receptor (InsR) and IRS1 levels (124). Second, it actively inhibits tyrosine phosphorylation of the InsR and IRS-1, and thereby impairs insulin signaling (125;126). Furthermore, TNFα elevates intracellular ceramides and diacylglycerol levels which are impairing insulin signaling (127;128). In obesity, lipolysis is dysregulated and basal as well as isoprenaline-stimulated lipolysis are upregulated, which might be mediated by IL-6 (129).

Well-characterized adipokines which are positively mediating adipogenesis are adiponectin and chemerin. A known negative mediator of adipogenesis is RBP4. Low levels of circulating adiponectin are correlated with obesity, insulin resistance, T2DM and CVD (130;131). Since mature adipocytes are the main source of this adipokine, the

role and function of adiponectin within AT have been studied intensively. Fu and coworkers found out that adiponectin promotes adipocyte proliferation and differentiation and is able to prolong gene expression of C/EBP2, PPARγ and sterol regulatory element-binding protein (SREBP1c) (132). Furthermore, adiponectin is able to suppress PPARγ coactivator (PGC-1α) expression, all of which is leading to an augmented programmed gene expression of adipogenesis markers (132). Additionally, mature adipocytes are characterized by a higher number and increased size of lipid droplets. Adiponectin also mediates insulin-stimulated glucose uptake by elevating Glut4 expression and recruitment to the plasma membrane (132).

Chemerin is mainly derived from mature adipocytes (120). It is influencing adipogenesis by impairing the mitotic expansion phase during the initial days of differentiation, thus potentially providing new adipocytes during phases of hypertrophy (133).

RBP4 is a negative regulator of adipogenesis, which is upregulated during differentiation of adipocytes (134). High levels of RBP4 are associated with obesity, T2DM and metabolic syndrome (135;136). RBP4 affects adipogenesis by downregulating the expression of differentiation markers like aP2 and PPARγ and by reducing the lipid content of adipocytes (137). Furthermore it impairs insulin signaling locally within AT by blocking phosphorylation of IRS-1.

1.2.4. INTER-ORGAN CROSS-TALK TO OTHER TISSUES

Since the discovery of the first adipokines and the acceptance of AT as a real endocrine organ, researchers all over the world are interested in the cross-talk of AT with other tissues and organs. Especially visceral AT is known to play a central role in the progression and pathogenesis of metabolic diseases (138;139). However, the cross-talk scenario is an extremely complex multidirectional network of factors and the field of interacting factors is mere endless. Every day the list of factors secreted by other tissues and influencing each other is broadening. Despite the classical cytokines and the well-known adipokines and myokines (140), nowadays there are also hepatokines (141), cardiokines (142), or batokines (143) described. To add more complexity to this whole scenario one has to keep in mind that most of the factors are not exclusively produced by one tissue but by several, and especially adipokines and myokines are overlapping, contributing both to the circulating level (108). By proteomic profiling of the secretome of different tissues novel factors are easily identified, but the role within the cross-talk remain often poorly understood. To study inter-organ cross-talk some basic tools have

been established and most of them are either *in vitro* or *ex vivo*. The co-culture of adipocytes with cells of the tissue of interest is the first important tool for investigation of cross-talk mechanisms. The advantage of this method is the bidirectional way of action of the released factors. Another option is to use conditioned medium (CM) either generated *in vitro* during the culture of adipocytes or *ex vivo* from AT explants. Whereupon, the latter has the advantage of getting all factors released from all cells within AT. However, both of these CM techniques lack the bidirectionality. Though, the complexity of the inter-organ cross-talk makes it very difficult to study isolated factors *in vivo*. The following section deals with different axis of interaction between AT and other tissues.

1.2.4.1. ADIPO-MYCOCYTE AXIS

Skeletal muscle besides liver is the main target organ for glucose disposal and thus important for the regulation of insulin sensitivity in the body (144). Co-culture experiments of myocytes and adipocytes have revealed an interaction on the insulin signaling axis, as adipokines seem to induce insulin resistance by affecting Akt phosphorylation and Glut4 translocation thus lowering glucose uptake (145). This notion was further supported by experiments with CM generated from adipocytes that is also able to induce insulin resistance on the level of Akt phosphorylation and downstream (146). Furthermore, CM generated from adipocytes increases oxidative stress and ceramide level paralleled with reduced mitochondrial capacity and elevated lipotoxic potential of palmitate (147). A well-known adipokine which promotes insulin sensitivity in skeletal muscle cells is adiponectin (148).

Resistin is at least in rodents an AT-derived factor which is clearly linked to insulin resistance (149). It impairs insulin-stimulated glucose transport in muscle of rodents via regulating IRS-1 and Akt activity and via impairing Glut4 trafficking (150).

1.2.4.2. ADIPO-VASCULAR AXIS

During obesity, a switch towards a more pro-inflammatory secretion profile of AT occurs which is associated with endothelial dysfunction and inflammation (112). Some adipokines such as fatty acid binding protein 4 (FABP4) and lipocalin-2 are well-known for their activation of pro-inflammatory signaling pathways in vascular cells and are predictors of cardio-vascular disease (151-154). Through the release of classical vasoactive factors or adipokines, or the release of fibrinolysis regulating factors,

adipocytes affect vascular homeostasis thus actively promote the progression of atherothrombotic disease (155;156). One example for the action of adipokines on vascular cells is their impact on smooth muscle cells (SMC). Our group was able to show that CM generated from adipocytes affects SMC in various ways. CM is able to induce insulin resistance on the level of Akt and PRAS40 phosphorylation via the dysregulation of certain miRNAs (157). Furthermore, CM-induced proliferation and elevated migration seems to be mediated by dipeptidylpeptidase 4, vascular endothelial growth factor and CD36 (158). Lamers and co-workers could show that CM not only increases VEGF release but also the expression level of VEGF receptor, thus potentiating the proliferative effect of VEGF on SMC. Furthermore, activation of NFκB and p38MAPK signaling was elevated by CM.

1.2.4.3. ADIPO-INSULAR AXIS

During the progression of T2DM, loss of β-cell function and mass occurs due to glucotoxicity, lipotoxicity and islet cell amyloid (159). Adiponectin is one example of a positive cross-talk from adipocytes to the pancreas. On the one hand adiponectin is able to protect β-cells by inhibiting apoptotic effects of cytokines, palmitate or high glucose (160;161). On the other hand adiponectin actively promotes insulin secretion via elevated insulin gene expression and exocytosis of insulin granules (162). This might also lead to the rescued impairment of insulin secretion by FFA and cytokines (160). In a study by Schinner and colleagues the cross-talk of Ins-1 cells and adipocytes was investigated via the generation of CM. They revealed that adipocytes release Wnt-signaling molecules which induce β-cell proliferation and insulin secretion which might link obesity to hyperinsulinemia (163).

1.2.4.4. ADIPO-HEPATOCYTE AXIS

Cross-talk of AT and liver is essential for the regulation of glucose and lipid metabolism. Thus, elevated pro-inflammatory markers in the circulation are associated with fatty liver and non-alcoholic fatty liver disease (NAFLD) (164). In a study by Zhou and colleagues it could be shown that HepG2 cells have impaired insulin sensitivity upon treatment with adipocyte-derived CM (165). Another group could induce steatosis in hepatocytes with CM which was generated from white AT explants (166). They postulate that the observed effect is mediated by MCP-1, which lowered Apolipoprotein

B secretion and increases phosphoenolpyruvate carboxykinase mRNA expression thus leading to triglyceride accumulation in hepatocytes.

It is well-known that adiponectin sensitizes hepatocytes for insulin signaling and acts as an anti-inflammatory agent via lowering the levels of TNFα and IL-6 (110;167). Adiponectin is involved in the regulation of genes involved in glucose and lipid metabolism, which was demonstrated by RNA sequencing analysis in adiponectin KO mice (168). Recently Nishijima-Matsunobu and coworkers established a three-dimensional culture model to investigate the cross-talk of white AT and hepatocytes (169). Thereby they confirmed an induction of lipotoxicity especially by VAT and a downregulation of FA production in VAT by hepatocytes to counteract the lipotoxic effects. These model could be very helpful for future research on cross-talk scenarios of AT with other tissues.

1.3. DIPEPTIDYLPEPTIDASE 4

Dipeptidylpeptidase 4 (DPP4) is a well-known target for the treatment of T2DM since several years. It is well known for its deactivation of the incretin hormones glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are to a large extent responsible for the postprandial insulin release (170). Thereby DPP4 inhibitors like sitagliptin, which was the first approved gliptin, prolong the half-life of incretins and are used as monotherapy (171) or in combination with e.g. metformin to treat T2DM (172). Interestingly, DPP4 was one of the highest scored adipokines identified by comprehensive proteomic profiling of the adipocyte secretome (91). The following section deals with a short characterization of DPP4 in respect of structure and function. Furthermore, the multi-faceted role of DPP4 in the context of T2DM will be highlighted.

1.3.1. GENERAL ASPECTS OF DPP4

Dipeptidylpeptidase 4 (DPP4) was first described by Hopsu-Havu and Glenner in 1966 (173). This type II transmembrane protease belongs to the serine peptidase subfamily S9B, where it groups together with fibroblast activation protein α (FAP), DPP8, DPP9, DPP6 and DPP10. All of them share a typical α/β hydrolase fold.

The 110kDa protein consists of four domains, namely the short cytoplasmic domain [aa 1-6], the transmembrane domain [aa 7-28], a flexible stalk region [aa 29-39] and the

extracellular domain [aa 40-766] (107). The latter can be further subdivided into a highly glycosylated region, a cysteine-rich region, and the catalytic region. The domain architecture of DPP4 is displayed in Fig 3.

Although DPP4 contains a signal peptide which targets the protein to the ER and is responsible for the translocation across the cell membrane, this signal is not cleaved off like in classically secreted proteins, but serves as an anchor inside the cell membrane (107). The hydrolytic cleavage of DPP4 occurs within the flexible stalk region and releases a soluble, fully functional form of DPP4 (sDPP4) into the circulation (107).

DPP4 is ubiquitously expressed on numerous cell types. It is believed that substitution with different carbohydrates, which account for approximately 20% of the total molecular mass, are responsible for the heterogeneity depending on the location of DPP4 on different cell types (174). The DPP4 gene is located on chromosome 2 with a size of 70kb, which consists of 26 exons (107). Consensus sites for different transcription factors are located within the promoter region of DPP4. Amongst them e.g. NFκB and epidermal growth factor receptor (EGFR) are known consensus sites (175).

Dimers are the predominant form of DPP4 and dimerization is necessary for enzymatic activity of the protease (176). Dimerization occurs through the cysteine-rich region of DPP4 either with another DPP4 molecule, or as a heterodimer with other partners like FAP or adenosine deaminase (ADA) (177;178).

DPP4 is a multifunctional molecule which, on one hand, cleaves as an exopeptidase dipeptides from the N-terminal penultimate position of its substrates. Thereby it can generate novel bioactive compounds or inactivate its substrates (105). On the other hand, DPP4 exerts enzyme-independent functions either through binding of partner molecules as membrane-bound form or through binding of receptors as soluble form. This complex function of DPP4 is also displayed in Fig.3. By binding to ADA, DPP4 is involved in tissue remodeling through MMP activation (179) or mediates inflammatory processes like T-cell proliferation (180). Furthermore, DPP4 plays a role in glucose homeostasis via adenosine through ADA binding (181;182). Via the binding to receptors on its target cells, soluble DPP4 is involved in different intracellular signaling processes, and thereby exerts paracrine or endocrine effects. However, the knowledge on potential receptors for DPP4 is scarce and so far only mannose-6 phosphat/insulin-like growth factor 2 receptor and protease activated receptor 2 are described as potential acceptors for DPP4 (183;184).

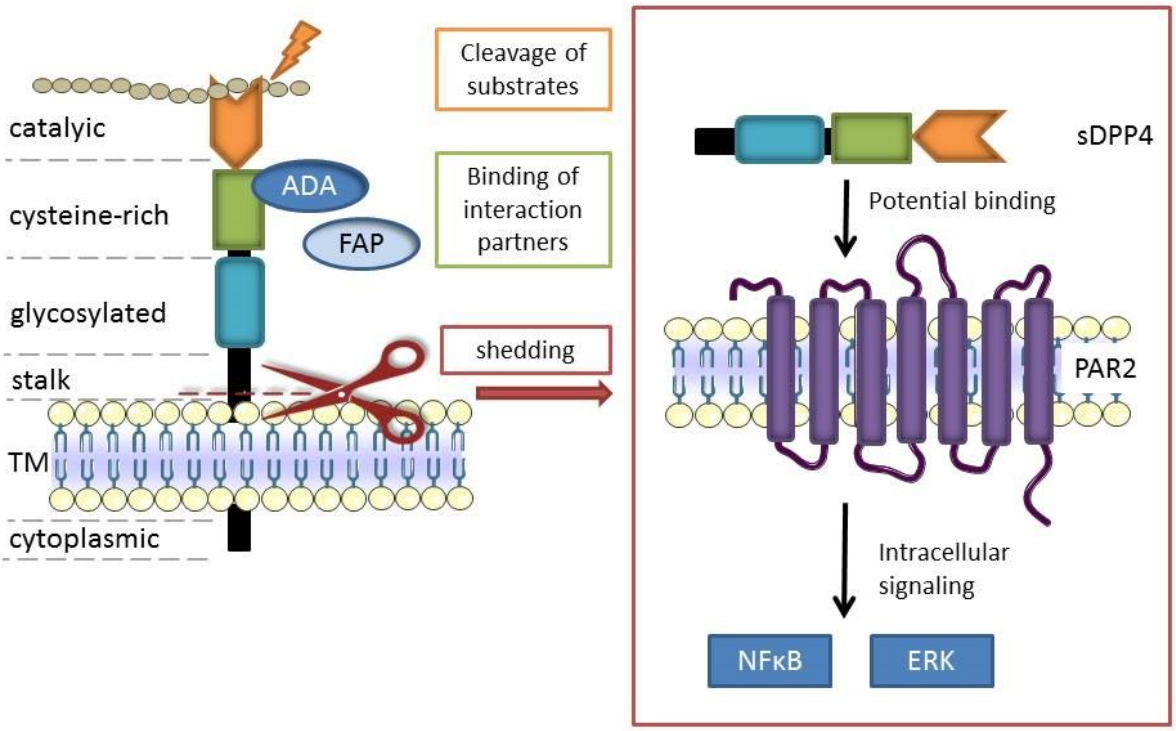


FIGURE 3: DPP4 AS A MULTIFUNCTIONAL ENZYME.
DPP4 domain architecture displays its multifunctional properties as binding partner for various proteins like FAP and ADA and its proteolytic action on numerous substrates. Upon shedding sDPP4 is released, which than is able to bind to potential receptors like PAR2. Thereby DPP4 is able to initiate intracellular signaling processes. TM transmembrane domain; ADA Adenosine Deaminase; FAP fibroblast activation protein: sDPP4 soluble DPP4; PAR2 protease activated receptor 2; NFκB nuclear factor of activated B-cells; ERK Extracellular-signal Regulated Kinase

1.3.2. DPP4 AS A NOVEL ADIPOKINE

As already mentioned above, DPP4 is ubiquitously expressed on numerous cell types. In 2011 our group was the first to describe DPP4 as a novel adipokine potentially linking obesity to the metabolic syndrome (185). In this study it was shown, that DPP4 expression and release is upregulated in a differentiation-dependent manner. Although macrophages also release DPP4, it was postulated that the major contribution of sDPP4 from adipose tissue arises from mature adipocytes, since they account for 2/3 of the cellular content within AT (185). Furthermore, it was shown that DPP4 release can be triggered by inflammatory stimuli like TNFα but also by metabolic stimuli like insulin. Both of these stimuli are especially interesting in the context of AT dysregulation during

the progression of obesity. Recombinant sDPP4 exerted direct effects on adipocytes themselves, on skeletal muscle cells (SkMc) and SMC, where it induced insulin resistance in a dose-dependent manner. This could be blocked by a specific DPP4 inhibitor. Additionally sDPP4 induced proliferation in SMC (185). Taken together, these observations illustrate the potential of sDPP4 to be involved in obesity-associated diseases like T2DM and atherosclerosis, which will be discussed in the following section in more detail.

Interestingly, serum levels of DPP4 are elevated in obese patients and positively correlate with clinical and biochemical markers like BMI, adipocyte surface area and insulin levels (185). DPP4 expression in AT biopsies of obese patients is significantly higher in in visceral than in subcutaneous fat, and the release of DPP4 from these biopsies correlates with parameters of the metabolic syndrome (MetS) (185;186). Together with the fact that insulin-sensitive obese patients release significantly less DPP4 than obesity-matched insulin-resistant patients, DPP4 may be a marker for visceral obesity, insulin resistance and MetS (186).

1.3.3. DPP4 AND ITS ROLE IN DIABETES

1.3.3.1. DPP4 AS A DRUG-TARGET FOR THE TREATMENT OF T2DM

DPP4 is a serine-type protease which cleaves dozens of substrates equipped with appropriate residues at the penultimate position. Although there is a clear preference for proline at this position also other residues, which possess a similar stereochemistry are accepted (187). The scientific interest in DPP4 dramatically rose with the discovery that it cleaves the incretin hormones, GLP-1 and glucose-dependent insulinotropic peptide (GIP) in 1993 by Mentlein and coworkers (188). Incretin hormones are responsible for approximately 50% of the postprandial insulin secretion and are very rapidly inactivated upon DPP4 cleavage. This discovery promoted the development of DPP4 inhibitors as a treatment option for T2DM. After sitagliptin was approved in 2006, more and more of these inhibitors with improved pharmacologic functions came to the market (189;190). They can be classified into two subgroups, peptidomimetics (sitagliptin, teneligliptin, vildaglitpin, saxagliptin and anaglitpin) and non-peptidomimetics (alogliptin and linagliptin) (reviewed by (191)). The disadvantage of the first developed inhibitors was their unstability and unselectivity for DPP4. Because they possessed an electrophilic trap such as a nitrile group, they build a covalent bond

with Ser630 of the catalytic triad, which is very conserved and also present in family members like DPP8 and DPP9 (192). This was improved during the further development of the inhibitors. Gliptins are orally administered drugs that prevent the rapid degradation of incretins, thereby raising the postprandial levels of active GLP-1 and GIP which leads to an improved glycemic control. Upon cleavage by DPP4 both GLP-1 and GIP lose their receptor activating properties (193;194). It is even reported that cleaved GIP is a weak antagonist of its receptor *in vivo* (195). The majority of effects seen upon DPP4 inhibitor treatment are addressed to the rise in GLP-1 levels. DPP4i are able to lower DPP4 activity by 70-90% and a clear benefit of a gliptin therapy is the indifference on bodyweight and a low risk of hypoglycemia in patients (196). As already mentioned before, DPP4 serum levels are elevated in obese patients and circulating levels and DPP4 release from AT is linked to parameters of the MetS (185). So it can be speculated that enlarged visceral adipocytes might substantially contribute to augmented sDPP4 levels during obesity (197). Interestingly, there are more and more beneficial effects of DPP4i reported that go beyond the incretin axis. *Ex vivo* and *in vitro* studies on effects of acute DPP4 inhibition by vildagliptin revealed Src-Akt-eNOS mediated nitric oxide release, which leads to vasodilation (198). Another example is a study by Shirakawa and colleagues, who could show that AT hypertrophy and linoleic-acid induced inflammation is prevented upon des-fluoro sitagliptin treatment in a specific mouse model (199). Because of a lack of GLP-1 receptors in AT, these effects have to be mediated incretin-independently, most likely through alternative DPP4 substrates.

In addition to the incretin hormones, there are other substrates of DPP4 that might be linked to its important role in diabetes. One of these substrates is stromal cell derived factor 1 α (SDF-1α), which is reported to protect stem-cell derived insulin-producing cells from glucotoxicity (200). Furthermore, SDF-1α enhances β-cell survival via Akt activation (201). Also the polypeptide family with its members peptide YY (PYY) and neuropeptide Y (NPY) are involved in β-cell survival and improved glucose homeostasis and are known physiological targets of DPP4. Upon DPP4 truncation, the affinity of these peptides towards their receptors is shifted thereby altering their biological function (202;203). Substance P is another physiological substrate of DPP4, which is reported to promote insulin resistance in preadipocytes *in vitr*o (204).

All of these data indicate that obesity-associated processes like inflammation, AT hypertrophy and insulin resistance are closely linked to DPP4 expression and release by AT.

1.3.3.2. DPP4 DEFICIENCY IN ANIMAL MODELS

DPP4 deficiency is preferentially studied in rodents. In rats with a loss of function mutation in the DPP4 gene, which leads to a global KO phenotype, insulin resistance was induced by high fat diet (HFD)-challenge (205). These DPP4-deficient rats showed an improved homeostatic model assessment-insulin resistance (HOMA-IR) value and improved oral glucose tolerance test (oGTT) which was attributed to higher levels of active GLP-1. This in turn also leads to improved insulin levels. Furthermore, it could be shown that these rats are characterized by an improved lipid profile and improved insulin sensitivity. Additionally, improved adipocyte maturation could be observed in parallel to less AT inflammation in DPP4-deficient rats (206). The same group also observed an improved liver function under HFD (207). Basically the same improvements were also shown in global DPP4 KO mice. In addition to displaying an improved glucose tolerance (208), also improved insulin sensitivity with sustained islet morphology was described in global DPP4 KO mice (209). KO mice also showed an improved liver biology in respect of lipid accumulation and gene expression levels, like already described for DPP4-deficient rats (209). A drawback of these animal models is, however, that central regulation of food intake is impaired by DPP4 KO. This impedes to dissect the role of DPP4 in metabolic complications under diet-induced obesity, as animals consume less food and gain less weight. Another disadvantage of these models is the fact that the global KO also affects immune cells. Infiltration of these cells plays a central role in obesity-releated AT inflammation and might be modulated by DPP4 deficiency. Furthermore, the role of selected organs in the contribution to circulating levels of DPP4 remains unclear.

1.4. OBJECTIVES

Obesity is a hallmark of the metabolic syndrome and increasing at an alarming rate worldwide. The fact that it increases the risk for cardiometabolic diseases or the development of T2DM highlights how important it is to understand the progression and causes of this disease. During obesity, AT needs to store excess lipids in lipid droplets and this leads on one hand to AT hypertrophy, but also to a recruitment and differentiation of novel adipocytes. However, at some point AT is no longer able to sustain its normal function, and not only more free fatty acids (FFA) and triglycerides are released, but also a shift towards a more proinflammatory secretion of adipokines occurs. Both facts are associated with increasing insulin resistance in peripheral organs such as skeletal muscle or liver. Currently we believe that adipokines display the missing link between obesity, insulin resistance and β-cell dysfunction during AT expansion (210). To combat the metabolic complications it is necessary to understand the role and function of adipokines. One of these adipokines which was identified by comprehensive proteomic profiling of the adipocyte secretome was DPP4 (91). Because of its incretin inactivating property, DPP4 is already a key therapeutic target for the treatment of T2DM. Furthermore, it was already known that it is dysregulated in obese subjects especially in visceral AT and that mature adipocytes might be the most important source of sDPP4 within AT (185;186).

- As a member of the type II transmembrane protease family DPP4 is not classically secreted, but cleaved off the cell surface in a process called shedding. However, which enzymes are contributing to the shedding of DPP4, and how this process is regulated remains elusive. The first objective of this thesis was to identify the enzymes involved in DPP4 shedding from human adipocytes and human SMC, and to elucidate the regulation of this process *in vitro*. Therefore, human adipocytes and SMC were treated with different protease inhibitors and the impact on DPP4 release was assessed. Furthermore, the influence of low oxygen levels on the release of DPP4 and the expression of involved proteases was analyzed.

- Although DPP4 is a well characterized adipokine, which potentially links obesity to the metabolic syndrome, and might be a marker for visceral obesity and insulin resistance, the role of DPP4 within adipose tissue is unknown so far. The second objective of this thesis was to elucidate the role of DPP4 within human adipocytes *in vitro*. This was addressed by siRNA-mediated silencing of DPP4 throughout adipocyte differentiation and the impact on differentiation,

inflammation and secretory output of adipocytes was analyzed. Furthermore, the influence of DPP4 silencing on adipocyte function in respect of insulin signaling was assessed. Additionally, to investigate whether the seen effects can be allocated to the enzymatic function of the protein, selected experiments were repeated after administration of the well-known DPP4 inhibitors sitagliptin and saxagliptin.

Although generation of global DPP4 knock-out (KO) mice has already shown an improvement in glucose tolerance, and a resistance to diet-induced obesity and insulin resistance (209), it remains difficult to dissect the role of DPP4 on peripheral metabolism since the food intake is affected by the KO. Furthermore, a global KO also leads to an ablation of DPP4 in immune cells, which are critical components in the development of AT inflammation during the progression of obesity. It is also unclear how much AT contributes to the level of circulating DPP4, and how this affects general insulin sensitivity in peripheral organs like liver or skeletal muscle.

- Based on our previously published data it might be speculated that AT is a key source for circulating DPP4 during obesity. To address which role AT-derived DPP4 plays in the development of obesity-associated disorders like T2DM, we developed a unique AT-specific DPP4 KO mouse model. These mice were challenged with a high fat diet (HFD) to characterize the impact of AT-derived DPP4 on the metabolic phenotype. Furthermore, the role of DPP4 within AT was assessed *in vivo* and *ex vivo* by analyzing the AT phenotype and metabolic parameters like marker gene expression.

2. PUBLISHED STUDIES

2.1. STUDY 1: DPP4 IN DIABETES

DPP4 IN DIABETES

Diana Röhrborn [1], Nina Wronkowitz[1], Juergen Eckel[1*]

[1] Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Düsseldorf,Germany

*correspondence: Prof . Dr. Juergen Eckel, Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Auf'm Hennekamp 65, D-40225 Düsseldorf, Germany, e-mail: eckel@uni-duesseldorf.de

Published in: Front Immunol**. 2015 Jul 27;6:386. doi: 10.3389/fimmu.2015.00386. eCollection 2015. Review**.

Keywords: CD26/DPP4, soluble DPP4, type 2 diabetes mellitus, incretins, DPP4 inhibitors/Gliptins, multifunctional enzyme

Dipeptidyl peptidase 4 (DPP4) is a glycoprotein of 110 kDa, which is ubiquitously expressed on the surface of a variety of cells. This exopeptidase selectively cleaves N-terminal dipeptides from a variety of substrates, including cytokines, growth factors neuropeptides, and the incretin hormones. Expression of DPP4 is substantially dysregulated in a variety of disease states including inflammation, cancer, obesity and diabetes. Since the incretin hormones GLP-1 and GIP are major regulators of post-prandial insulin secretion, inhibition of DPP4 by the gliptin family of drugs has gained considerable interest for the therapy of type 2 diabetic patients. In this review, we summarise the current knowledge on the DPP4 - incretin axis, and evaluate most recent findings on DPP4 inhibitors.
Furthermore, DPP4 as a type II transmembrane protein is also known to be cleaved from the cell membrane involving different metalloproteases in a cell-type specific manner. Circulating, soluble DPP4 has been identified as a new adipokine which exerts both para- and endocrine effects. Recently, a novel receptor for soluble DPP4 has been identified and data are accumulating that the adipokine-related effects of DPP4 may play an important role in the pathogenesis of cardiovascular disease. Importantly, circulating DPP4 is augmented in obese and type 2 diabetic subjects and it may represent a molecular link between obesity and vascular dysfunction. A critical evaluation of the impact of circulating DPP4 is presented and the potential role of DPP4 inhibition at this level is also discussed.

INTRODUCTION

Dipeptidylpeptidase (DPP) 4, which is also known as CD26, is a ubiquitously expressed glycoprotein of 110 kDa, which was first characterized by Hopsu-Havu and Glenner 1966 (1). DPP4 is a type II transmembrane protein, which is also cleaved off the membrane and released into the circulation by a process called shedding (2;3). The importance of DPP4 for the scientific and medical community raised substantially since the approval of DPP4 inhibitors for the treatment of type 2 diabetes mellitus (T2DM). These so called gliptins increase the incretin levels and therefore prolong the postprandial insulin action. Since soluble DPP4 is characterized as an adipokine (4) and also correlates with parameters of the metabolic syndrome (5), it might also be an important molecular biomarker. DPP4 is a multifunctional enzyme which serves as a binding partner for numerous peptides amongst which are adenosine deaminase (ADA) and extracellular matrix proteins (2;6;7). Moreover, as a serine protease DPP4 cleaves numerous substrates, which further amplifies its complexity of action. Thus, DPP4 is involved in signaling processes, immune cell activation, and its dysregulated expression and release is associated with numerous diseases.

In the present review we wanted to emphasize the complex function of DPP4 with special focus on its association to T2DM. Furthermore, we wanted to offer a different perspective of the current view of DPP4 beyond the inhibition of its protease activity (8-10). The first part of the present review is dealing with general informations about DPP4 and its numerous biological functions in regard to T2DM and its treatment. The last section collects the current knowledge about how DPP4 with its pleiotropic functions, as described before, affects several organs, thereby playing a pivotal role in the development of T2DM and its comorbidities.

GENERAL INFORMATION ON DPP4

BIOLOGY OF DPP4

The following part will deal with the domain architecture and respective relevance of these domains for the functionality of DPP4.

DPP4 (EC3.4.14.5) is a type II transmembrane protein which groups together with fibroblast activation protein α (FAP), the resident cytoplasmic proteins DPP8 and DPP9 and the non-enzymatic members DPP6 and DPP10 to the serine peptidase subfamily S9B. All of these proteins share a typical α/β hydrolase fold (2;6). The DPP4 protein consists mainly of 4 domains: a short cytoplasmic domain [1-6], a transmembrane domain [7-28], a flexible stalk segment [29-39], and the extracellular domain [40-766], which can be further separated by a highly glycosylated region, the cysteine-rich region and the catalytic region (Fig. 1).

As a member of the type II transmembrane proteins DPP4 contains a typical signal peptide which is necessary for the targeting to the endoplasmatic reticulum and the initiation of the translocation across the cell membrane. In contrast to the classically secreted proteins the signal peptide is not cleaved off, but serves as a membrane anchor. We were able to show that the circulating

form of DPP4 (sDPP4), which lacks the cytoplasmic domain and the transmembrane region, is cleaved off the membrane of human adipocytes and smooth muscle cells in a process called shedding by involvement of matrix metalloproteases (3).

Within the transmembrane domain (TMD) it could be shown that proline residues play an important role for the translocation of membrane-anchored proteins such as DPP4. Chung and colleagues (11) studied single proline substitution throughout the TMD of DPP4. They were able to show that translocation and integration into the membrane are determined by the hydrophobicity, conformation and also the location of proline within the TMD. Furthermore, the position of proline relative to other prolines and the location of highly hydrophobic residues within the TMD are important for correct translocation and membrane-integration of DPP4.

In addition to the TMD also the glycosylation of DPP4 is also important for the correct trafficking of DPP4. Carbohydrates account for approximately 20% of the total molecular mass of DPP4 and cause heterogeneity of this protein depending on the location on different cell types. Two highly conserved glutamate residues (205 and 206) within the glycosylated region are essential for the activity of DPP4 (12). Interestingly, six of the nine N-glycosylation sites are located within the glycosylated region. These glycosylation sites are mostly conserved among species. They are necessary for folding, stability and intracellular trafficking (13). Other modifications like sialylation and/or O-glycosylation have an impact on targeting DPP4 to the cell membrane. Sialyation of DPP4 increases

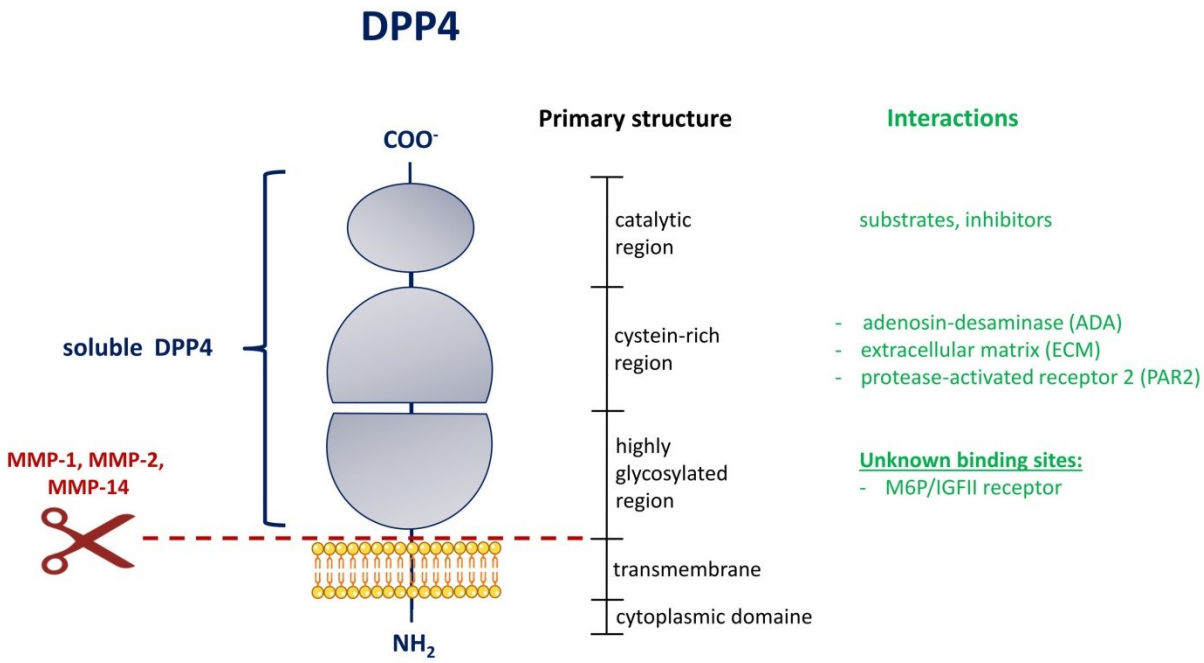


FIGURE 1 | Domain structure of DPP4 [adapted from Ref. (2)]. Schematic representation of the membrane-bound DPP4 monomer. The extent of the circulating and soluble form of DPP4 is illustrated on the left in blue. The shedding of DPP4 from the membrane by indicated matrix metalloproteinases is shown by a scissor symbol in red. The vertical black bar on the right represents the primary structure with the delineation of the different regions. In green are interactions collected, which occur in the indicated region of the DPP4 structure. MMP, matrix metalloproteinase; M6P/IGFII, mannose-6 phosphate/insulin-like growth factor 2.

significantly with age and hypersialyation occurs in patients with HIV-infection (14).
Not only glycosylation and residues within the TMD are important for the cellular function of DPP4 but also dimerization. DPP4 can be found as monomer, as homodimer or even as homotetramer on the cell surface of cells. DPP4 needs dimerization for enzymatic activity and this form is the predominant form of DPP4 (15). Dimerization occurs upon interaction with DPP4 itself or with other binding partners e.g. FAP (16;17), and occurs via interaction with the cysteine rich region. Through its interaction with several proteins DPP4 can act also in an enzymatic activity-independent way. Through this interaction DPP4 is linked to various mechanisms like immune response and tumor invasion. The heterodimerization and interaction with different binding partners will be discussed in a later section.
The serine in the active site of DPP4 is located in the sequence Gly-Trp-Ser-Tyr-Gly and is part of the catalytic triad (Ser 630, Asp 708, His 740) within the catalytic region of DPP4. DPP4 is an exopeptidase which cleaves dipeptides from the penultimate N-terminal position of its substrates and thereby either inactivates these peptides and/or generates new bioactive compounds (7). There are numerous different DPP4 substrates known to date and they will be addressed in a separate section within this review.

DPP4 EXPRESSION AND ITS REGULATION

DPP4 is ubiquitously expressed on numerous different cell types among which are epithelial cells, fibroblasts and leukocyte subsets. Mechanisms that regulate DPP4 gene transcription and enzymatic activity are not fully understood so far and may be dependent on the studied cell type.
The human DPP4 gene is located on chromosome 2, spans 70 kb and consists of 26 exons (2). The DPP4 promoter region contains consensus sites for different transcription factors like NFκB, SP-1, EGFR and AP-1 factor NF-1(18). At least in chronic b lymphocytic leukemia cells it could be shown that there is a consensus interferon γ-activated sequence (GAS) which is a binding motif for STAT1. The interferons α, β and γ stimulate STAT1α binding to this region and thus lead to an increased DPP4 expression and activity (19). Interleukin (IL) 12, which is a key factor in differentiation of naïve T-cells into the Th1 subtype, is also able to upregulate DPP4 expression. Therefore DPP4 is important in immune cell activation (20;21). Our group was able to show that release of soluble DPP4 is increased upon TNFα stimulation and insulin *in vitro* (4). However IL-12 and TNFα also seem to play a regulatory role in translation and translocation of DPP4. In activated lymphocytes IL-12 upregulates DPP4 translation whereas TNFα decreases cell surface expression, which might be due to elevated sDPP4 release (22). Also transcription factors, such as HIF-1α and HNFs target DPP4 expression (23), which fits to the observation of our group that hypoxia induces DPP4 release in human smooth muscle cells, which might be mediated by MMPs (3).

NON-ENZYMATIC INTERACTIONS OF DPP4

Through its cysteine-rich region which is separated from the catalytic region, DPP4 is able to interact with different proteins, and further broadens its spectrum of

activity and highlights its multifunctional role in different processes.

BINDING PARTNERS OF MEMBRANE-BOUND DPP4

The best studied interaction in this regard is certainly the binding of DPP4 and adenosine deaminase (ADA). It was already identified in 1993 by Morrison and colleagues (24). Importantly, the interaction of DPP4 and ADA preserves the enzymatic function of both binding partners. It has been shown that residues 340-343 of DPP4 are essential for the interaction with ADA. Regulation of the DPP4/ADA interaction occurs e.g. via tetramerisation of DPP4 or glycosylation at Asn281 which interferes with ADA binding (25). Also the HIV envelope glycoprotein gp120, which interacts with DPP4 on lymphocytes via its C3 region, is able to inhibit the association with ADA (2). Upon ADA binding, activation of plasminogen-2 occurs, which raises plasmin levels. This leads to a degradation of matrix proteins and an activation of matrix metalloproteases (MMP), thereby indicating that the interaction of DPP4 and ADA might be involved in tissue remodeling (26).

Furthermore, ADA catalyzes the irreversible deamination of adenosine and 2'-deoxyadenosine and is therefore a crucial player in the cellular and humoral immunity. Via interaction with CD45 the complex of ADA and DPP4 enhances T-cell activation. Interestingly, DPP4 is also able to promote T-cell proliferation independent from ADA binding or even its enzymatic activity (27). Zhong *et al.* were able to show that the interaction of DPP4 and ADA on dendritic cells might potentiate inflammation in obesity upon activation and proliferation of T-cells, which could be competitively inhibited by exogenous sDPP4, but not by inhibiting DPP4 enzymatic function (28). Furthermore, ADA activity is elevated in T2DM patients and may serve as a marker of inflammation and obesity (29).

Beside its role in inflammation adenosine is also an important player in glucose homeostasis. Already in 1988 it was shown that by lowering endogenous adenosine levels, ADA contributes to a reduced insulin sensitivity of glucose transport stimulation (30). Additionally, adenosine seems to facilitate insulin action in adipocytes (31). Another study could show a correlation of increased ADA activity in T2DM with fasting plasma glucose, HbA1c, aspartate and alanine aminotransferase. DPP4-inhibitors exert no additional effects on ADA activity despite glycemic control or HbA1c-dependent effects (32). All these studies emphasize that the effects of ADA/DPP4-interaction are independent of DPP4 enzymatic activity.

Another known interaction partner of DPP4 is Caveolin-1 which is present on antigen presenting cells (APCs) and binds to residues 630 and 201-211 of DPP4 expressed on T-cells. Thereby an upregulation of CD68 occurs and initiates a signaling cascade which might be implicated in the pathogenesis of arthritis, and may be relevant for other inflammatory diseases as well (33). Intracellular signaling is also initiated by DPP4 via interaction with Caspase recruitment domain containing protein 11 (CARMA-1) (6).

Another well-known interaction of DPP4 is with extracellular matrix proteins like collagen and fibronectin (34;35). The interaction of DPP4 with fibronectin was

revealed via nitrocellulose binding assays in rat hepatocytes and seems to play a role in the interaction of these cells with the ECM and with matrix assembly (36). Interaction of DPP4 with FAPα leads to a local degradation of ECM and thus migration and invasion of endothelial cells (37).

POTENTIAL RECEPTORS FOR SDPP4

Since DPP4 is shedded from the membrane of cells with intact enzymatic and cysteine-rich region, it can also exert biological functions in a paracrine or endocrine manner. These functions might also involve intracellular signaling events in the targeted cells. Therefore it would be of great importance to know receptors of sDPP4 to better understand the multiple role of sDPP4 on different cells and in different disease conditions where serum levels are elevated. However, there is not much known about DPP4 receptors so far.

Ikushima *et al.* were able to show that DPP4 needs to associate with mannose-6 phosphat/IGFIIR to exhibit its function as T-cell activator. This is due to the fact that for this activation, internalization of DPP4 is necessary, but DPP4 lacks a signal for exocytosis. The binding with M6P/IGFIIR occurs via M6P residues in the carbohydrate moiety of DPP4 and the complex is than internalized and able to exert its biological function (38).

Our group showed that at least in human vascular smooth muscle cells protease activated receptor 2 (PAR2) might be activated by sDPP4. We were able to show that sDPP4-mediated ERK activation and proliferation, as well as upregulation of inflammatory cytokines could be prevented by silencing of PAR2. The same was shown by use of a specific PAR2 antagonist. We propose that sDPP4 acts as an activator of PAR2, since a sequence within the cysteine-rich region of DPP4 is highly homologous to the auto-activating tethered ligand of PAR2 (39).

GENETIC ALTERATIONS OF DPP4 AND PREDISPOSITION TO T2DM ASSOCIATED DISEASES

There are only few studies aiming to identify modifications in the DPP4 gene and their association with T2DM. Some of these are reviewed in the following section.

In 2009 Bouchard *et al.* analyzed single nucleotide polymorphisms (SNPs) in the DPP4 gene and searched for association with blood pressure, lipids and diabetes-related phenotypes in obese individuals, to verify whether DPP4 gene polymorphisms could explain the individual risks of obese patients to develop metabolic complications. Three of the analyzed SNPs showed significant association with plasma total-cholesterol levels or plasma triglyceride level or total cholesterol level. But none of the polymorphisms or cardiovascular disease risk factors showed a significant correlation with DPP4 mRNA levels in omental adipose tissue. Therefore, the authors concluded that at least in their studied group DPP4 gene polymorphisms seem to be unrelated to the inter-individual risk of developing obesity-related metabolic complications (40).

In another study visceral adipose tissue DNA of 92 severely obese, non-diabetic female patients was analyzed for methylation rate in the DPP4 promoter CpG island, and compared between different DPP4 polymorphisms. These cysteine and guanine rich regions are prone to epigenetic modification like methylation, and thus inactivate or

activate transcription of certain genes. Different methylation levels of the DPP4 gene were identified in three DPP4 SNPs. Interestingly, the methylation level was negatively associated with DPP4 mRNA abundance and positively with plasma total/HDL-cholesterol ratio. These observations suggest that plasma lipid profile is improved by a higher methylation status of the investigated CpGs (41). Two years later the same group analyzed DPP4 gene methylation levels between obese subjects with and without the metabolic syndrome in visceral adipose tissue. They observed no significant difference in the percentage of methylation levels of the CpGs within or near the second exon of the DPP4 gene between non-diabetic severely obese subjects with or without metabolic syndrome. However, they were able to show a correlation between plasma cholesterol levels and the percentage of methylation when the subjects were classified into quartiles (42). This further underpins a link between epigenetic modification of the DPP4 gene and plasma lipid metabolism.

Aghili N. *et al.* analyzed 875 patients with angiographically documented coronary artery disease (CAD), and divided them in two subgroups dependent of their myocardial infarction (MI) status. By a genome-wide association study, loci which predispose to MI were assessed and associated with SNPs in the DPP4 gene. They found that polymorphisms in the DPP4 gene increase the risk of MI and progression of atherosclerosis in terms of plaque stability in patients with already existing CAD. Especially one SNP was identified in both dominant and additive inheritance modes, which associates with low plasma DPP4 levels and which may increase the risk of MI in CAD patients (43).

Dyslipidemia which is characterized by excessive lipids in the blood is a common feature of T2DM. The status of this risk factor is quantifiable by the measurement of apolipoprotein B (ApoB) in the blood. In a very recent study by Baileys and colleagues they aimed to identify novel SNPs associated with ApoB level. Especially in South Asians which tend to develop risk factors for T2DM and MI at younger ages and lower BMI they found an association of a DPP4 SNP with ApoB level (44).

THE DPP4 DEFICIENCY IN ANIMAL MODELS

To date there are several studies dealing with the question which role DPP4 plays *in vivo.* Animal models are useful tools to study the involvement of DPP4 in different organs. Upon triggering different diseases like insulin resistance (IR) or myocardial infarction (MI) it is possible to understand the role of DPP4 in these comorbidities of T2DM.

DPP4 DEFICIENCY IN RATS

A major part of the literature is dealing rather with DPP4-KO in rats than in mice. Most research groups work with the F344/DuCrj (DPP4 deficient)-strain. Rats developing insulin resistance due to high-fat diet (HFD) feeding showed improved HOMA-IR values and blood glucose levels in oral glucose tolerance test (oGTT) and more active GLP-1 and insulin in plasma (45). The same improved glucose tolerance with increased GLP-1 and leptin levels was found in DPP4 depleted Dark Agouti rats with diet-induced obesity (46).

Another research group also found improvement in serum lipid profile despite increased visceral fat. They also performed insulin tolerance tests (ITT) in addition to GTT and saw an increased phosphorylation of Akt and reduced expression of gluconeogenic genes, concluding that DPP4-KO improved insulin sensitivity. Furthermore, the KO rats showed increased adipocyte maturation by increased expression of genes involved in triglyceride uptake and in PPARγ expression and increased adiponectin and leptin levels. In addition, adipose tissue is less inflamed illustrated by lower TNFα, IL-6, PAI1 and CCL7 levels (47). The observed effects were attributed to elevated GIP levels in the KO rats. Furthermore, the same group could also show attenuated liver damage under HFD challenge in the KO rats due to improved bile secretory function. They postulate that the enhanced export of bile acids out of hepatocytes and a reduction of bile acid synthesis via inhibition of CYP7A1, which converts cholesterol to bile acids, were mediated by increased GLP-1 in DPP4 KO rats (48). Interestingly, at least Yasuda and colleagues also saw a significant reduced food intake in the KO rats irrespective of the diet (45) which might be due to changed receptor-specificity of NPY, which was shown to be more potent in KO rats to influence food intake and feeding motivation (49). Although several independent working groups saw increased NPY levels in KO rats (49-51), the effect on food intake is controversial (45;50). When diabetes is induced via streptozotocin (STZ) treatment in F344/DuCrj-DPP4-deficient rats, onset of hyperglycaemia was delayed, but KO rats showed impaired creatinine clearance and more severe dyslipidemia, which might be caused by a dysregulated expression of factors involved in steroid and lipid metabolism (52;53). The authors concluded that DPP4 might be responsible for preservation of renal function. Another effect of the whole-body KO of DPP4 in rats is induction of behavioural changes like a blunted stress phenotype (46;51) and also effects on the immune-regulatory system like blunted NK cell and T-cell function and differential leukocyte subset composition or altered cytokine levels (46;47).

DPP4 DEFICIENCY IN MICE

Most of the observations already described in deficient(54;55) rats are also true in whole-body DPP4 KO mice. Marguet *et al.* showed enhanced glucose tolerance, lower plasma glucose and higher plasma insulin and GLP-1 after a 15 min oral glucose load without further characterizing the diet, age or sex of the used C57BL/6 DPP4 KO mice (56). Conarello and colleagues found less weight-gain independent of the diet, and marked hypertrophy in the HFD-fed KO mice in epididymal white (eWAT) and brown adipose tissue (BAT). Importantly, they admitted that the reduction in caloric intake accounted for ~70% of the observed changes in bodyweight. Although they still observed differences in the bodyweight between KO and WT when they used pair-feeding, they carried out their further analysis in ad libitum fed mice and it is therefore difficult to judge the influence of DPP4 irrespective of bodyweight. However, they found improved insulin sensitivity and islet morphology, and improved liver biology in respect to lipid content and marker gene expression (57). The observation that DPP4 might be involved in the immune-

regulatory system was also investigated in DPP4-KO mice, which were treated with pokeweed mitogen which stimulates growth and proliferation of B-cells. DPP4 seems to be involved in maturation and migration of immune cells, cytokine secretion and percentages of spleen lymphocytes (58).

All these studies have in common that they use whole-body KO animals. The disadvantage here is that one cannot distinguish between direct effects of the KO and side effects caused for example by different immune cell status or decreased caloric intake. To really decipher the role of DPP4 in different tissues and their crosstalk with other target tissues it is of great importance to study tissue-specific KO models.

Because of this and because we were the first to describe DPP4 as a novel adipokine linked to parameters of the metabolic syndrome (4), we decided to develop an adipose tissue-specific KO mouse model. The AT-specific DPP4 KO mouse was generated using a Cre-lox strategy under control of the aP2 promoter on the C57BL/6J background. Interestingly, we found out that KO mice gained significantly more weight, fat and lean mass under HFD with no effect on energy expenditure or food intake. However, KO mice showed improved HOMA-IR and lower fasting insulin. The observations that within AT KO mice display a shift towards significantly more smaller adipocytes, and an increased expression of M2 macrophage marker genes, points towards a beneficial role of DPP4 deletion in adipose tissue remodeling during HFD (54;55).

ENZYMATIC FUNCTION OF DPP4

DPP4 exerts its enzymatic action by clipping dipeptides from the penultimate position of its substrates. The active center, which is housed in an internal cavity, is surrounded by the β-propeller domain and the catalytic domain. Inhibitors and substrates enter/leave the active center by a so-called "side opening" (59;60). The following section deals with known substrates of DPP4 in respect of T2DM, and with DPP4 as a drug target for T2DM treatment, which will include current knowledge on DPP4-inhibitors and the impact of DPP4 on organs involved in complications of T2DM.

DPP4 SUBSTRATES

In theory, numerous peptides are potential DPP4 substrates since they contain the cleavable amino acid sequence at their penultimate position, but not for all of them it could be shown that DPP4 is able to cleave them *in vivo*. There seems to be a size limitation at least for cytokines, where DPP4 is more prone to cleave substrates of around 24 amino acids (aa) length. Furthermore the substrate recognition is also dependent on the aa sequence around the penultimate position (61;62). It turned out to be difficult to find physiological targets of DPP4 in the literature, reasons of that are excellently summarized in a recent review by Mulvihill and Drucker (6). We decided to focus here on the (potential) substrates of DPP4, which might play a role in T2DM or its complications. The list of DPP4 substrates mentioned here is not fully complete and aims to highlight the importance of DPP4 in T2DM also beyond its well-known incretin effect.

INCRETIN HORMONES

The incretin hormones account for approximately 50 % of the insulin secretion after meal intake and are secreted from the gut within minutes after the meal intake. Through binding to distinct receptors on beta-cells in the pancreas they stimulate insulin secretion and suppress glucagon release depending on the blood glucose level. Most potent in their glucose-lowering action are glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Both peptides belong to the same glucagon peptide superfamily and share significant aa character.

GLP-1 is secreted from L-cells of the gut into the bloodstream. Upon binding to G-protein coupled receptors on the beta cells, intracellular cAMP level is elevated and the protein kinases Epac1 and 2 are activated, which leads to an increase of insulin secretion. Furthermore, GLP-1 enhances beta-cell mass by mediating proliferation and differentiation and inhibiting apoptosis (8). By inhibiting gastric emptying GLP-1 also improves blood sugar excursion, delays food absorption, and is therefore a regulator of satiety and appetite also through the hypothalamus (63).

GIP Is a 42 aa peptide which mainly originates from enteroendocrine K-cells (64). Subjects with diabetes or impaired glucose tolerance show significantly reduced levels of meal-stimulated circulating GIP and the levels are negatively correlated with the severity of insulin resistance in the patients (65;66). GIP has in contrast to GLP-1 no effect on glucagon secretion, but also regulates fat metabolism in adipocytes (67).

Since inhibition of DPP4 due to genetic deletion or use of DPP4-inhibitors was shown to elevate GLP-1 / GIP levels in numerous studies, this effect is the main focus of developing therapeutic targets for treatment of T2DM. There are numerous reviews which focus on DPP4 and GLP-1 mediated effects and this will topic not be further discussed here.

STROMAL CELL-DERIVED FACTOR 1A (SDF-1A)/ CXCL12

This factor is important in the homing of stem cells and is thus discussed in the literature as one of the mediators of cardioprotective effects addressed to the use of DPP4-inhibitors. It is a well-known physiological target of DPP4 (68;69). SDF-1α also plays a role in diabetes itself by protecting stem-cell derived insulin-producing cells from glucotoxicity under high glucose conditions (70) or promoting pancreatic beta cell survival in mice via Akt activation (71). Furthermore, it was shown that some genetic variants of SDF-1α are associated with late stage complications in T2DM patients (72;73).

NPY AND PYY

Neuropeptide Y and Peptide YY are members of the polypeptide family. They are highly expressed in the hypothalamus but are also present in peripheral tissues like islets. NPY regulates energy balance, memory and learning, while PYY reduces appetite, inhibits gastric motility, and increases water and electrolyte absorption in the colon (74). Both NPY together with PYY play a role in beta cell survival and in glucose homeostasis (74). NPY is able to suppress insulin secretion acutely (75). Both polypeptides have in common that DPP4 truncation shifts their receptor specificity and thus alters their biological role in different cellular processes. *In vitro* experiments in adipocytes could show that DPP4 inhibition has an impact on lipid metabolism mediated by NPY (76;77).

SUBSTANCE P

Substance P is a physiological target of DPP4 which is sequentially converted to SP [3-11] and SP[5-11] *in vivo* in F344-DPP4 positive rats (78). SP is a neurotransmitter and –modulator which is involved in neurogenic inflammation. Serum levels in diabetes are controversially discussed with one study showing a decrease in diabetic patients (79), and another one showing an increase in fasting blood samples with correlation to diabetic risk factors like BMI and blood pressure (80). This discrepancy in serum levels could be addressed to the fact that it is not always stated which form of SP (full length vs. truncated) is measured. However, SP was shown to promote insulin resistance *in vitro* in human preadipocytes by interacting with proteins that are involved in the inhibitory phosphorylation of IRS-1. Furthermore, SP can directly inhibit insulin-dependent glucose metabolism in rat adipocytes (81). SP also promotes diabetic corneal wound healing, as shown by Yang and colleagues (82).

BRAIN NATRIURETIC PEPTIDE (BNP)

BNP is responsible for vasodilation, natriuresis and suppresses renin secretion. It is so far only a predicted DPP4 substrate, which was cleaved *in vitro* by DPP4 to BNP[3-32]. This truncation was inhibited by a DPP4-inhibitor in a dose-dependent manner (83). Truncated forms of BNP with lower enzymatic activity are discussed as an indicator of heart failure severity. In 2013, dos Santos *et al*. could show an improved cardiac performance in sitagliptin-treated rats, which they attributed to increased levels of active BNP (84).

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP)

PACAP is very rapidly degraded by DPP4 to the fragments [3-27], [5-27] and [6-27]. These fragments lack PACAPs insulinotropic ability, but are no feasible treatment options for T2DM because of their actions on glucose homeostasis and glucagon secretion (85). Several studies have shown that PACAP is a powerful stimulator of insulin secretion, which enhances glucose uptake in adipocytes and augments antilipolytic action of insulin (86;87). After DPP4-inhibitor treatment in mice, PACAP-induced insulin secretion was enhanced (88). However, a proof that PACAP also plays a role in humans is lacking so far.

REGULATED ON ACTIVATION, NORMAL T-CELL EXPRESSED AND SECRETED (RANTES)/CCL5

RANTES recruits leukocytes into inflammatory sites and is cleaved by DPP4 to RANTES[3-68]. Due to this truncation, RANTES [3-68] is not able anymore to increase cytosolic calcium concentrations and to induce chemotaxis of human monocytes *in vitro*. This is explained by a shift in receptor subtype-specificity towards enhanced activation of CC-Motiv-Chemokin-Receptor 5 (CCR5) (89). Elevated serum levels of RANTES in T2DM are associated with postprandial hyperglycaemia (90). Interestingly, RANTES and its receptor CCR5 are important mediators of obesity-induced inflammation which was shown in CCR5 KO mice (91). Levels of RANTES and CCR5 were reduced in adipose tissue of obese patients upon exercise (92). RANTES reduces glucose-stimulated GLP-1 secretion *in vitro* and *in vivo* in mice, by acting most probably through the intestinal glucose transporter SGLT1 (93).

EOTAXIN/CCL11

Eotaxin mediates mobilization of eosinophils into the bloodstream, which was shown to be increased in DPP4-deficient F344 rats (94). DPP4 cleaves eotaxin to eotaxin[3-74]. However there was no significant correlation of eotaxin seen in patients with T2DM or impaired glucose tolerance in the KORA cohort (95).

DPP4 AS A DRUG TARGET FOR THE TREATMENT OF T2DM

DEACTIVATION OF DPP4 ENZYMATIC ACTIVITY

DPP4-inhibitors

Major DPP4 substrates are the so called incretin hormones which are key regulators of postprandial insulin release. DPP4 inhibition leads to greater bioavailability of these proteins and therefore prolongs the half-life of insulin action. The majority of effects seen upon DPP4-inhibitor treatment are ascribed to an increase in GLP-1 levels. Because of this, DPP4 became a major target for the treatment of T2DM. This section deals with the most recent knowledge around DPP4-inhibitors, their mode of action - if known - and the newest developments in the inhibition of DPP4 enzymatic activity. There are numerous modifications and potential optimizations of the five so far approved gliptins reported. However, most of them are not in clinical trials yet and not much is known about their advantage in a head to head comparison to established gliptins. Therefore we decided to focus on the most recent data on approved gliptins in this review. The data are also summarized in Tab. 1.

DPP4-inhibitors lower DPP4 activity by 70-90%. They do not pass the blood-brain barrier and have no direct effect on satiety or on altering gastric emptying (8). The benefit for diabetes therapy clearly is their indifference on bodyweight gain and the low risk of hypoglycemia. There are five gliptins approved so far for clinical use, namely sitagliptin, vildagliptin, saxagliptin, linagliptin and alogliptin. Another gliptin, teneligliptin is only approved in the Japanese and Korean market. Despite the same mode of action, the different gliptins diverge in their pharmacodynamic and pharmacokinetic properties, which might be clinically relevant for some patients (9;96). The peptide mimetic compounds vilda-, saxa and teneligliptin were identified by replacement experiments of peptide-based substrates, whereas the non-peptide mimetic compounds sita, alo- and linagliptin where derived from initially found inhibitors of random screenings. The diverse chemical structures also explain the unique binding modes of the inhibitors to DPP4 (10).

The six inhibitors have been classified into 3 classes depending on their different binding modes in the DPP4 active center (10). Class one contains vilda- and saxagliptin which only bind to the S1 and S2 subsites, and form a covalent bond with the nitrile group of their cyanopyrrolidine moiety and Ser630 of DPP4. Saxagliptin has a 5-fold higher activity in blocking DPP4 than vildagliptin. Group 2 contains alo- and linagliptin, which also interact with the S1' subsite or even in case of linagliptin with the S2' subsite. The uracil rings of both gliptins induce a conformational change in the Tyr547 of the S1' subsite. Because of the additional interaction of linagliptin with S2' subsite it has a 8-fold higher activity than alogliptin. The third classe has the highest inhibitory function towards DPP4 because both sita-

as well as teneligliptin interact with the S2 extensive subsite of the DPP4 active center, and an increasing number of interactions seems to increase the potency of the gliptin (10). Teneligliptin which is only approved for T2DM treatment in the Japanese and Korean market so far, also has a unique structure characterized by a J-shape and an anchor lock domain, which explains the strong inhibitory function and the low IC_{50} value of this drug (for review see (97)). The binding of the DPP4 S2 extensive subsite of some inhibitors also guarantees a high specificity towards DPP4 since other close-related peptidases like DPP8, DPP9 and FAP lack this subsite. All DPP4-inhibitors have in common that they build salt bridges with Glu-residues in the S2 subsite (10). At least for sitagliptin it is also known, that it lowers the level of free fatty acids (FFA) and thereby also comprises insulin sensitizing properties (98). Furthermore, sitagliptin was shown to have potent anti-inflammatory properties by suppressing expression of pro-inflammatory genes in mouse and humans (98;99). In patients with renal impairment, which is a very common complication of T2DM, sitagliptin is more suitable than sulphonylureas (100).

Although some authors claim that DPP4-inhibitors are only beneficial in early stages of diabetes, this could be rebutted by the work of Kumar and Gupta (101). They could show beneficial effects of three gliptins (sita-, saxa- and vildagliptin) in lowering HbA1c also in patients with longstanding T2DM for more than 10 years. Thus, DPP4 inhibition also plays an important role irrespective of the duration of diabetes.

What has to be mentioned in respect of the beneficial roles of DPP4-inhibitors is that more and more studies about their beneficial pleiotropic effects are upcoming, which is also discussed in the following section of this review dealing

Table 1 Summarized properties of gliptins

Inhibitor	Approved since	Binding mode	Kind of inhibition	Route of excretion	IC50 value	Reference
Sitagliptin	2006 FDA	S1, S2, and S2 extensive subsites	Competitive inhibition	Mostly renal route	19 nM	(10, 98–100)
Vildagliptin	2007 European medicines agency	Only S1 and S2 subsite	Substrate–enzyme blocker	Mostly renal route	62 nM	(10, 209)
Saxagliptin	2009 FDA	Only S1 and S2 subsite	Substrate–enzyme blocker	Mostly renal route	50 nM	(10, 209)
Linagliptin	2011 FDA	S1, S2, and S1′ subsites	–	Through biliary route	1 nM	(10)
Alogliptin	2013 FDA	S1, S2, and S1′ subsites	Competitive inhibition	Mostly renal route	24 nM	(8, 10)
Teneligliptin	2012 Japan 2014 Korea	S1, S2, and S2 extensive subsites	Very potent because of unique anchor- lock domain and J-shape of molecule	Mostly renal route	0.37 nM	(97)

with different organs. There are reports that gliptins themselves have effects on lipid profile and blood pressure as well as on inflammatory processes (102). In addition to the incretins there are some DPP4 substrates, like SDF1α, which might explain potential cardioprotective effects which are discussed for gliptins. However, cardiovascular outcomes are still widely debated and controversially evidenced. Ongoing long-term studies will further shed light on the respective role of DPP4i beyond glucose homeostasis. Furthermore one has to keep in mind that also DPP4 has direct effects independent of its enzymatic activity, like activation of downstream signaling events upon receptor binding, which are not well understood so far. Which role DPP4 inhibition plays on T2D relevant organs/comorbidities will be the topic of the following sections.

Alternative modes of DPP4 inhibition

Very recently Pang and colleagues published a different strategy to inhibit DPP4 activity. They used DPP4-targeted immune therapy by vaccines in a C57BL/6J mouse model and were able to show comparable effects like in treatments with gliptins regarding GLP-1 plasma levels and postprandial glucose excursion and insulin sensitivity in HFD fed mice. Furthermore they observed no side effects on immune cell activation by the DPP4 vaccine. An advantage of this

method is the long lasting effect of the vaccine in the mouse model, which could, if transferable to human patients, be a convenient alternative to the daily intake of gliptins (103). Further research in developing alternatives towards Gliptins especially for long-acting medications would be an interesting new approach to improve life-style of patients.

Incretin-Based Therapies: Comparing DPP4i and GLP-1 Analogs

It is well accepted that incretin-based therapies are able to lower blood glucose levels and are therefore a treatment option for T2DM. There are mainly two approaches to target the incretin system (1) via inhibiting the enzymatic action of DPP4 and thereby upregulating GLP-1 levels physiologically and (2) via increasing GLP-1 levels pharmacologically. While GLP-1 receptor agonists (GLP-1-RA) directly target GLP-1, GLP-1-independent effects are also possible with the use of DPP4 inhibitors (DPP4i). These drugs might also affect the level of other DPP4 substrates and might therefore have a more complex mode of action. However, there have been a lot of attempts to compare the effects of GLP-1-RA versus DPP4i in clinical studies. The results of these head-to-head comparisons are summarized in many current reviews (110–114). Most of these comparative studies agree that GLP-1 analogs are more effective in respect of glycemic con- trol. Both incretin-based therapies are equally potent in lowering blood pressure and total cholesterol (110). Furthermore, both have the advantage of low incidence of hypoglycemia (110, 111). The results for body-weight lowering effects of DPP4i are heteroge- neous throughout the studies (113), whereas beneficial effects on body-weight are well accepted for GLP-1-RA (110–112, 114). Therefore, some authors tend to prefer GLP-1-RA over the use of DPP4i (112). However, one should be aware of the fact, that GLP- 1-RA have a higher incidence of gastrointestinal adverse events like nausea (110, 112, 114), which might be disadvantageous for elderly people who may be more prone to these side effects (114). Furthermore,

there are reports that DPP4i might also have car- dioprotective effects (113), which will also shortly be discussed in section "Effect of DPP4 Inhibition on the Cardiovascular System" of this review. Despite the clear beneficial effects of incretin-based therapies, there are also concerns reported in respect of the risk for long-term complications like pancreatitis (115). These potential risks might, however, outweigh the benefits. These controversial discussions are well summarized by the reviews of Nauck and Butler (115, 116). To really assess which medication is of more importance always depends on the special patient characteristic.

Impact of DPP4 on T2DM relevant organs and associated comorbidities

DPP4-inhibitors exert glucose regulatory actions by prolonging the effects of GLP-1 and GIP, ultimately increasing glucose-mediated insulin secretion and suppressing glucagon secretion (104). Beside the glucose-lowering properties of DPP4-inhibitors, emerging evidence suggests that incretin-based therapies may also have a positive impact on inflammation, cardiovascular and hepatic health, sleep, and the central nervous system (105). However, the underlying mechanisms of these effects cannot be fully explained by lower blood glucose levels or increased GLP-1 bioavailability or signaling, and has to be further elucidated. Thus, the next section is focused on the role of DPP4 action in T2DM relevant organs and associated comorbidities.

Adipose Tissue

Adipose tissue is the primary storage organ for excess energy. While the role of adipose tissue as a central source of energy has been recognized for centuries, in the past decade it has become increasingly clear that adipose tissue also displays characteristics of an endocrine organ releasing a number of adipose tissue-specific factors, known as adipokines. During the progression of obesity, the ability of adipocytes to function as endocrine cells and to secrete multiple biologically active proteins is affected (106). Thus, adipose tissue has been shown to be a central driver of T2DM progression, establishing and maintaining a chronic state of low-level inflammation (107).

DPP4 expression and release within adipose tissue

Recently, we showed that DPP4 is highly expressed in human primary adipocytes (4). Furthermore, DPP4 expression in adipose tissue is increased in obese compared to lean individuals in both subcutaneous and visceral adipose tissue (4;5). Interestingly, visceral fat of obese patients exhibits the highest DPP4 level. According to the increased expression, we could identify sDPP4 as a novel adipokine released from primary human adipocytes. *In vitro*, the DPP4 release increased substantially during fat cell differentiation, and comparison with preadipocytes and adipose tissue macrophages showed that adipocytes most likely represent the major source of DPP4 released from the intact organ to the circulation. Furthermore, the release of sDPP4 was elevated in adipose tissue explants of obese patients compared to lean controls and correlates with various classical markers of the metabolic syndrome, namely BMI, waist

circumference, plasma triglycerides, and HOMA as an index of insulin resistance, as well as with fat cell volume and the adipokine leptin (4;5).

How DPP4 expression affects adipocyte homeostasis can only be speculated. DPP4 might be involved in adipose tissue lipolysis. DPP4 recruits ADA, a monomeric enzyme catalyzing deamination of adenosine to inosine and ammonia (108;109). It has been shown that DPP4-bound ADA has a 1000-fold greater activity than free ADA (110), which in turn may modulate the well established antilipolytic effects of adenosine. Moreover, DPP4 is a strong inhibitor of the antilipolytic activity of neuropeptide Y (NPY) (76), which is one of the best peptide substrates of the enzyme (89). In this regard, Rosmaninho-Salgadoa and colleagues demonstrated that DPP4 stimulates lipid accumulation and PPAR-γ expression through cleavage of NPY suggesting that sDPP4 might stimulate adipocyte differentiation (77). However it is noteworthy that the authors of this study were using tremendously high and non-physiological concentrations of sDPP4. On the contrary, a recent published study showed that DPP4 expression was strongly up-regulated during adipocyte dedifferentiation *in vitro*. Hence, the authors concluded that DPP4 might be a major component in adipose tissue remodeling and cell plasticity (111). Nevertheless, enhanced abundance of DPP4 within adipose tissue of obese subjects may be involved in adipose tissue remodeling and substantially augments the lipolytic activity of enlarged adipocytes (54in press;55).

Moreover, dendritic cells and macrophages resident in visceral adipose depots exhibit an increased DPP4 expression in response to inflammation or in the obese state (28). Since it is known that DPP4 exerts immunomodulating properties, Zhong *et al.* showed that membrane bound DPP4 is co-localized with membrane bound ADA on human dendritic cells resulting in an increased T-cell proliferation (28). Thus, it can be speculated that DPP4 might also play an important role in the chronic low-grade inflammation taking place in obesity and T2DM.

Adipose tissue as a relevant source of circulating DPP4

Serum levels of sDPP4 are altered in many pathophysiologic conditions, including different types of cancer, allergic asthma, or hepatitis C (7). Our group was the first analyzing circulating sDPP4 in the context of obesity and the metabolic syndrome. DPP4 serum levels of morbidly obese men are elevated compared with lean controls and significantly correlated with BMI, the size of adipocytes in subcutaneous and visceral fat, and the adipocyte hormones adiponectin (negatively) and leptin. These data suggest that sDPP4 is related not only to increased body weight but also to other important parameters of adipose tissue physiology. In addition, sDPP4 release and serum concentration can be reversed to normal levels by surgery-induced weight loss (4). Thus, in obesity, both circulating levels of sDPP4 and sDPP4 release by adipose tissue are increased and correlate strongly with the metabolic syndrome but can be reduced to control levels by substantial weight loss. Thus, indicating that enlargement of visceral adipocytes in obesity may substantially contribute to the augmented level of circulating sDPP4 in obese patients.

Endocrine effects of soluble DPP4

Although there is clear evidence that increased circulating levels of sDPP4 are associated with hallmarks of obesity and type diabetes, such as whole-body insulin resistance, elevated BMI and adipocyte hypertrophy, there are only few studies investigating the endocrine effects of sDPP4. We were the first showing that DPP4 consistently impairs insulin signaling at the level of Akt in primary human adipocytes (4). Enzymatic activity of sDPP4 appears to be involved in this process; however, since this work was done *in vitro* it is most unlikely that the sDPP4-induced impairment of insulin action is due to an increased bioavailability of any DPP4 substrate. It might rather be that DPP4-inhibitors may also affect the binding properties of sDPP4 to its receptors, namely M6P/IGFII receptor (38) or PAR2 (39). For the latter, it is not only known that PAR2 signaling induces insulin resistance in adipocytes (112), but PAR2 might also be a substantial contributor to inflammatory and metabolic dysfunction (113). Although there is a hint that circulating sDPP4 itself might affect adipose tissue function, the exact mechanism has to be further investigated.

Impact of DPP4 inhibition on adipose tissue

To further investigate the role of DPP4 in adipose tissue, several studies with DPP4-inhibitors were conducted. Interestingly, the administration of the DPP4-inhibitior des-fluoro-sitagliptin ameliorates linoleic acid-induced adipose tissue hypertrophy in β-cell–specific glucokinase haploinsufficient mice, a model of non-obese T2DM (114). Moreover, des-fluoro-sitagliptin protects against linoleic acid-induced adipose tissue inflammation illustrated by $CD8^+$ T-cell infiltration. Due to the loss of GLP-1 receptors in adipose tissue the authors exclude the involvement of GLP-1 and claim that the observed effects are due to the huge variety of DPP4 substrates. Thus, DPP4 inhibition might have pleiotropic effects in adipose tissue. A similar outcome has been observed in C57BL/6 mice fed a high-fat diet. After linagliptin treatment a significantly lower expression of the macrophage marker F4/80 was found compared with vehicle treatment. In line with these data, the authors demonstrated an increased insulin sensitivity after linagliptin treatment suggesting that DPP4 and adipose tissue inflammation play a pivotal role in the induction of insulin resistance. In 3T3-L1 cells, a murine peadipocyte cell line, Rosmaninho-Salgado *et al.* demonstrated that the DPP4-inhibitor vildagliptin reduces lipid accumulation by inhibiting adipogenesis, without affecting lipolysis through NPY cleavage and subsequent NPY Y2 receptor activation (77).

With the recognition that adult humans also have brown adipose tissue, an organ with substantial capacity to dissipate energy, brown adipose tissue gained considerable interest as a novel target to treat or prevent obesity and its associated diseases. In 2013, the group around Shimasaki was the first reporting that des-fluoro-sitagliptin attenuated body adiposity, without affecting food intake, in C57BL/6 mice with diet-induced obesity (115). The increase in energy expenditure could be explained by enhanced levels of PPAR-α, PGC-1, and uncoupling protein-1 (UCP-1) in brown adipose tissue as well as elevated levels of proopiomelanocortin in the hypothalamus. The beneficial effects of

des-fluoro-sitagliptin on energy expenditure could only partly be ascribed to increased GLP-1 levels and have to be further validated. Shortly afterwards, Fukuda-Tsuru *et al.* could confirm these date in the same animal model by administration of teneligliptin (116). Moreover, in this study teneligliptin also reduces fat mass and suppresses high-fat diet-induced adipocyte hypertrophy.
Collectively, there is clear evidence that DPP4 expression and release by adipose tissue plays a key role in obesity and T2DM associated processes, such as inflammation, adipocyte hypertrophy and insulin resistance. However, the underlying mechanism of these beneficial effects is not fully understood and remains unclear in most of the publications.

Pancreatic islets

β-cells play a central role in the etiology of T2DM. Due to failure of β-cell sensitivity to glucose and loss of β-cell mass, insulin secretion of these cells is not sufficient to counter balance insulin resistance, finally leading to T2DM. Although DPP4-inhibitors are now widely used for glycemic control, many debates are ongoing about their exact mode of action and their beneficial effects on pancreatic β-cells.

Regulation of DPP4 expression within pancreatic islets

Interestingly, within the pancreatic islets DPP4 localization differs between species. Islets of rodents showed a near-exclusive expression of DPP4 in β-cells, with little expression in α-cells. In contrast, human and pig islets express DPP4 almost exclusively in α-cells (117;118). The species difference in the localization of DPP4 expression, and the possible physiological consequence of that difference, is unclear. Moreover, in a recent published study it has been demonstrated that DPP4 activity was detectable in the conditioned medium of human islets suggesting that DPP4 is released from human islets as well (119). Under pathological conditions, islets of obese mice chronically fed a high-fat diet exhibit an increased DPP4 activity. The contrary was found in human islets from type 2 diabetic donors, showing a decreased DPP4 activity (118).

Impact of DPP4 inhibition on pancreatic islets

Accumulating *in vitro* and pre-clinical data show that DPP4 inhibition has beneficial effects on T2DM induced β-cell dysfunction and apoptosis. Omar and colleagues demonstrated that DPP4 is not only present and active in mouse and human islets, but inhibition of islet DPP4 activity also has a direct stimulatory effect on insulin secretion, which is GLP-1 dependent (118). The same effect could be observed with a two week des-fluoro-sitagliptin treatment leading to increased insulin exocytosis by β-cells from *db*/*db* diabetic mice (120). Furthermore, it could be shown that DPP4 inhibition is clearly associated with significantly increased β-cell mass and function in several models of T2DM (121-123). These beneficial effects were associated with the transcriptional activation of anti-apoptotic and pro-survival genes, as well as the suppression of pro-apoptotic genes in β-cells (124). Additionally, Shah and collaborators showed that the DPP4-inhibitor linagliptin protects isolated human islets from gluco-, lipo-, and cytokine-toxicity (119). Accordingly, Akarte *et al.* reported anti-oxidative properties of vildagliptin shown

by a dose-dependent decrease in nitric oxide concentrations in both serum and pancreatic homogenates of vildagliptin-treated diabetic rats (125).

Beside these pre-clinical and *in vitro* studies, only few is known about the beneficial effect of DPP4-inhibitors on β-cells in human. In the short-term, 12-weeks vildagliptin treatment leads to a small increase in the capacity for insulin secretion (126). Treatment with vildagliptin over a longer period of time could also confirm an increased β-cell function in humans as a result of improved sensitivity of β-cells to glucose (127;128). However, this effect was not maintained after washout period, indicating that this increased capacity was not a disease modifying effect on beta cell mass and/or function. In the SAVOR-TIMI 53 trial, which was originally performed to assess the cardiovascular safety of saxagliptin, Leibowitz and colleagues recently reported that DPP4 inhibition may attenuate the progression of diabetes (129). This was evidenced by a decreased requirement for intensification of treatment associated with better preservation of glycemic control, as well as better sustained *β*-cell function as reflected in the fasting HOMA-2*β* during the 2-year follow up period.

The exact mechanism how DPP4-inhibitors augment insulin secretion and increase *β*-cell mass *in vitro* and *in vivo* is still not fully understood, since not all these effects could be explained by elevated GLP-1 level or improved glycemic control associated with less glucotoxicity.

Liver

Non-alcoholic fatty liver disease (NAFLD) describes a disorder with excessive deposition of fat within the liver with increasing prevalence in parallel to obesity and diabetes, which are major risk factors for NAFLD (130). Indeed, NAFLD is now the most common cause of chronic liver disease (131) and is present in one quarter to one half of diabetes patients (132). In the obese state, elevated triglyceride degradation in adipose tissue causes an increased hepatic uptake of fatty acids leading to fat accumulation within the tissue. Furthermore, reactive oxygen species (ROS), produced during lipid oxidation, are assumed to induce hepatocyte death and inflammatory reactions. Liver cirrhosis can be defined as the end stage of chronic liver diseases and is caused by progressive fibrosis. This process is characterized by excessive accumulation of ECM and activated hepatic stellate cells (133;134) that ultimately results in nodular regeneration with loss of function (135).

Regulation of DPP4 expression in the liver

Although DPP4 exhibits a widespread organ distribution, the liver is one of the organs that highly expresses DPP4 (136). In the healthy human liver, intense staining for DPP4 was found in hepatic acinar zones 2 and 3, but not in zone 1. This heterogeneous lobular distribution suggests that DPP4 might be involved in the regulation of hepatic metabolism (137). Furthermore, mRNA expression levels of DPP4 were significantly increased in NAFLD livers compared to that in control livers (138). In accordance to that, DPP4 expression levels of NAFLD patients were negatively correlated with HOMA-IR and BMI, and positively correlated with total cholesterol levels, but not with alanine aminotransferase (ALT), lactate

dehydrogenase (LDH) or triglyceride levels. Moreover, under conditions of high glucose, DPP4 expression was increased in HepG2 cells. However, other nutritional conditions, such as high insulin or the presence of fatty acids and cholesterol did not affect DPP4 expression in these cells. Thus, the authors claim that enhanced DPP4 expression in NAFLD liver may rather be associated with insulin resistance than triglyceride accumulation, and may promote the progression of liver disease via subsequent deteriorations in glucose metabolism. How increased DPP4 expression might affect liver function is still unknown. There are only a few hints that DPP4 might play a role in fibronectin-mediated interaction of hepatocytes with extracellular matrix (2;36;139). Beside DPP4 expression, there is only indirect evidence that hepatocytes also release DPP4 to the circulation, which will be further discussed in the next section.

Serum level of DPP4 in liver disease

As previously discussed, hepatic DPP4 mRNA expression level in the livers is significantly higher in patients with NAFLD compared to healthy subjects (138). This up-regulation of hepatic DPP4 expression is thought to be responsible for elevated DPP4 serum level in patients with liver disease (140-142). In line with this observation, serum DPP4 activity can be correlated with hepatic steatosis and NAFLD grading (143). Similarly, in patients with NAFLD, DPP4 activity in serum correlates with markers of liver damage such as serum gamma-glutamyltranspeptidase and alanine aminotransferase levels, but do not correlate with fasting blood glucose levels and HbA1c values (143;144). Thus, hepatic DPP4 expression in NAFLD may be directly associated with increased DPP4 serum level and may be involved in hepatic lipogenesis and liver injury.

Impact of DPP4 inhibition on liver function

Since DPP4-Inhibitors are widely used in clinical practice, this drug was also investigated as a potential new therapeutic strategy against the development of liver fibrosis and steatosis. Kaji and collaborators demonstrated that sitagliptin markedly inhibits liver fibrosis development in rats via suppression of hepatic stellate cell proliferation and collagen synthesis (145). These suppressive effects were associated with dephosphorylation of ERK1/2, p38 and Smad2/3 in the hepatic stellate cells. Additionally, hepatic steatosis could be prevented in several different animal models by DPP4-inhibition (114;146;147). Shirakawa and colleagues studied the effects of sitagliptin in glucokinase$^{+/-}$ diabetic mice with diet-induced hepatic steatosis (114). Here, sitagliptin prevented fatty liver in both wild-type and glucokinase$^{+/-}$ mice paralleled by decreased expression of sterol regulatory element-binding protein-1c, stearoyl-CoA desaturase-1, and fatty acid synthase, and increased expression of peroxisome proliferator-activated receptor-α in the liver. Furthermore, in a mouse model of non-alcoholic steatohepatitis further studies indicated that linagliptin improves insulin sensitivity and hepatic steatosis in mice with diet-induced obesity (148) and ameliorates liver inflammation (149). The underlying mechanism of these beneficial effects has been further investigated by Ohyama *et al.* in *ob/ob* mice (150). The novel DPP4-inhibitor MK-0626 attenuates hepatic steatosis by enhancing AMPK

activity, inhibiting hepatic lipogenic gene expression, increasing triglyceride secretion from liver, and elevating serum adiponectin levels.

Clinical data are very limited; however, several non-randomized trials conducted in small groups of diabetic patients demonstrated that DPP4-inhibitors improved the levels of liver transaminases and liver fat (151-153). Accordingly, Iwasaki *et al.* found a decrease in ballooning and nonalcoholic steatohepatitis scores in post-treatment liver biopsies (152;153). Recently, in a comprehensive retrospective review of 459 type 2 diabetic patients, treated with DPP4-inhibtors, it was shown that DPP4-inhibitors improved the abnormality of the liver transaminases AST and ALT independent of HbA1c and body weight (154). Again in the majority of publications the authors postulate that these beneficial actions were mediated through potentiation of direct GLP-1 actions on hepatocytes; however, it seems unlikely that hepatocytes express the canonical GLP-1 receptor (155).

In conclusion, accumulating studies indicate that DPP4-inhibitors are clinically useful for patients with T2DM accompanied by liver dysfunction based on fatty liver, and that DPP4 inhibition affects liver function regardless of diabetic status and obesity.

Cardiovascular system

Cardiovascular complications (CVD) are common in patients with T2DM and a major cause of mortality (156). Atherosclerosis is the dominant cause of CVD and usually develops many years before any clinical symptoms are manifest. The underlying pathogenesis of atherosclerosis involves an imbalanced lipid metabolism and a maladaptive immune response entailing a chronic low-grade inflammation of the arterial wall. Endothelial cells and intimal smooth muscle cells represent the major cell types of the artery wall preserving vessel wall homeostasis. Together with leukocytes they are the major players in the development of this disease. Beside atherosclerosis, T2DM also exacerbates heart failure associated with diastolic heart failure and coronary microangiopathy (157-159).

Regulation of DPP4 expression and release in vascular cells

DPP4 is expressed in both microvascular endothelial cells of different human tissues, such as liver, spleen, lung, brain, heart (157;159), and in human vascular smooth muscle cells (3). Under conditions of high glucose, DPP4 expression and activity were increased in human glomerular endothelial cells (160). Additionally, in STZ-induced diabetic rats activity of membrane-bound DPP4 was increased, thereby reducing cardiac SDF-1 concentrations and causing impaired angiogenesis (161). Also hypoxia has been shown to regulate DPP4 expression in vascular cells. Regarding endothelial cells, there are conflicting data on the influence of hypoxia on DPP4 expression. In human microvascular endothelial cells as well as human umbilical vein endothelial cells, Eltzschig and colleagues showed that hypoxia increased DPP4 mRNA and protein level (162), whereas another study by Shigeta *et al.* observed a decreased protein level of DPP4 under hypoxic conditions in the same cells (161). However, in human vascular smooth muscle cells we observed an increased

DPP4 expression in response to hypoxia (3). In this particular study, we could also show that DPP4 is released from human vascular smooth muscle cells. However, only very little is known about the physiological role of the membrane bound DPP4 within the vasculature. There is only one study showing that DPP4 forms a complex with adenosine deaminase capable of degrading extracellular adenosine to inosine in endothelial cells. Increased inosine levels in turn are known to induce vasoconstriction due to mast cell degranulation (163).

Effect of DPP4 inhibition on the cardiovascular system

In several *in vitro* and pre-clinical studies DPP4-inhibitors have been shown to exert important protective effects on the cardiovascular system. In this regard, it has been shown that DPP4-inhibitors decrease myocardial infarct size, stabilize the cardiac electrophysiological state during myocardial ischemia, reduce ischemia/reperfusion injury, and prevent left ventricular remodeling following myocardial infarction (164;165). Additionally, DPP4-inhibitors also exert vascular protective properties, including anti- inflammatory and anti-atherosclerotic effects and the ability to induce vascular relaxation (166;167). To confirm cardiovascular safety or even protection of DPP4-inhibitors in humans, several cardiovascular outcome studies were conducted. However, several clinical trials, namely SAVOR-TIMI53, EXAMINE or VIVIDD in patients with established cardiovascular disease failed to confirm a cardio-protective effect (168-170). As sDPP4 is an adipokine upregulated in obesity and T2DM that triggers insulin resistance and metabolic complications (4;5), it might be speculated that the beneficial effects of DPP4-inhibitors would be higher in those early phases of the metabolic disorders previous to the development of established cardiovascular disease.

However, whether these beneficial effects observed in pre-clinical settings are due to increased levels of different DPP4 substrates or inhibition of direct effects of DPP4 remains unclear and will be assessed in more detail in the following section.

DPP4 substrates: GLP-1 dependent effects of DPP4-inhibitors

Since several studies have identified a role for GLP-1 receptor (GLP1R) signaling in DPP4-dependent cardioprotection, it is suggested that GLP-1 itself has favorable cardiovascular effects. Indeed, mRNA transcripts of the GLP-1R have been detected in the heart of rodents (171;172) and humans (173). Furthermore, GLP-1R has also been localized to mouse aortic smooth muscle and endothelial cells, as well as monocytes and macrophages (174).

Regarding myocardial infarction and heart failure, pre-clinical studies have demonstrated that DPP4-deficient rats subjected to 45 minutes of ischemia with 2 hours or reperfusion exhibited cardio-protection illustrated by reduced infarct size, improved cardiac performance, and reduced levels of BNP compared to control rats (175). These beneficial effects could be partially reversed by co-administration of the GLP-1R antagonist exendin [9-39]. Accordingly, administration of exendin [9-39] reversed the sitagliptin-induced improvement in ventricular function in Sprague Dawley rats with transient cardiac ischemia (176). Additionally, in a

rat model of chronic heart failure, GLP-1 analogues were able to improve cardiac function and morphology, with a concomitant amelioration of hyperglycemia and hyperinsulinemia (177).

Regarding the vascular system, continuous infusion of the GLP-1 analogue exendin-4 reduced monocyte adhesion to aortic endothelial cells, associated with a reduction in atherosclerotic lesion size in non-diabetic C57BL/6 and *ApoE*$^{-}$/$^{-}$ mice. Furthermore, treatment for 1 h with exendin-4 reduced the expression of the pro-inflammatory cytokines TNFα and MCP- 1 in response to LPS (174). In addition, exendin-4 stimulates proliferation of human coronary artery endothelial cells through endothelial nitric oxide synthase (eNOS)-, protein kinase A (PKA)- and PI3K/Akt-dependent pathways (178;179). Accordingly, in humans, preliminary data confirm the ability of GLP-1 to protect from high glucose-induced endothelial dysfunction in the post-meal phase (180). In a model of vascular injury, it has been shown that continuous infusion of exendin-4 reduces neointimal formation at 4 weeks after injury without altering body weight or various metabolic parameters (181). From *in vitro* studies, Goto *et al.* suggest that this effect was mediated by the ability of GLP-1 to suppress platelet derived growth factor (PDGF)-induced proliferation of vascular smooth muscle cells. In contrast, in a pre-clinical study combining HFD and STZ treatment in ApoE$^{-/-}$ failed to detect evidence for GLP-1R-dependent reduction of lesion size in the thoracic or abdominal aorta (155). The authors discuss that the duration of treatment, the dose of the GLP-1 agonist or the age of mice might be responsible for the lack of anti-atherogenic activity in this study.

However, in patients with heart failure, pilot studies also suggest cardio-protection by GLP-1 infusion (182;183). Accordingly, a large retrospective analysis indicates that patients treated with the GLP-1 analogue exenatide had a significant 20% reduction of CVD events compared with patients on other glucose-lowering agents (184). Nevertheless, studies showing cardiovascular protective effects of GLP-1 were carried out using either native GLP-1 or recombinant GLP-1 analogues at high concentrations or in a way that induced supraphysiological GLP-1 signaling. Considering that DPP4 inhibition restores GLP-1 signaling within the physiological range, beneficial effects of DPP4-inhibitors might be different to those of GLP-1 analogues.

DPP4 substrates: SDF-1- and BNP-dependent effects of DPP4-inhibitors

But beside GLP-1, there are further substrates of DPP4 which might play a role in the favorable cardiovascular effects of DPP4-inhibitors. Two of the most promising candidates are stromal derived factor (SDF)-1α and brain natriuretic peptide (BNP). SDF-1 is a chemokine that promotes angiogenesis and attracts endothelial progenitor cells (EPC) by binding to its receptor C-X-C motife chemokine receptor type 4 (CXCR4). EPC are derived from the bone marrow and are known to promote vascular repair and neoangiogenesis. When vascular damage occurs, local growth factors and cytokines signal the bone marrow to release EPC targeted to the injured sites. EPC then differentiate into mature endothelial cells and assist in the reconstruction of the

vasculature (185). In mice, genetic deletion or pharmacologic inhibition of DPP4 is able to increase the homing of CXCR4+ EPC at sites of myocardial damage, resulting in a reduced cardiac remodeling and improved heart function and survival (186). In a human study, Fadini *et al.* demonstrated that type 2 diabetic patients receiving a 4 week course of therapy with the DPP4-inhibitor sitagliptin show increased SDF-1α plasma concentrations and circulating EPC levels (185). Additionally, SDF-1 engineered to be resistant to DPP4 cleavage, and delivered by nanofibers, improves blood flow in a model of peripheral artery disease (187). Collectively, these studies implicate a rationale to use DPP4-inhibitors for vascular repair through stimulation of EPC and neovascularization.
BNP, another substrate of DPP4, plays an important role in regulating body fluid homeostasis and vascular tone through binding and subsequent activation of the cGMP- coupled natriuretic peptide receptor type A (NPR-A) (188). BNP is secreted predominantly by ventricular cardiomyocytes in response to increased wall stress. Thus, elevated BNP is a sensitive marker of heart failure and appears to play a role in cardiac remodeling and healing after acute myocardial infarction (189-191). DPP4 cleavage of the physiologically active BNP(1–32) to BNP(3–32) effectively lowers plasma cGMP levels, reduces diuresis and natriuresis, and inhibits vasodilatation (83;188).

Endocrine effects of soluble DPP4 on cardiovascular homeostasis

Although it is well established that serum levels of sDPP4 are altered in several pathological conditions and that sDPP4 is released from vascular cells, only a minor part of research has focused on potential endocrine effects of this proteolytic enzyme.
Considering that DPP4 is discussed in immunomodulation, it might be speculated that the inhibition of DPP4 modulates responses occurring within early or late atherosclerotic lesions. In low-density lipoprotein receptor-deficient (LDLR-/-) mice, Shah *et al.* could demonstrate that exogenously injected DPP4 increases monocyte migration *in vivo* (167). Although these pro-migratory properties of DPP4 could be completely inhibited by sitagliptin, the underlying mechanism of these effects remains unclear. Moreover, the combined treatment of sDPP4 and lipopolysaccharide (LPS) leads to increased expression and secretion of the pro-inflammatory cytokines TNFα and IL-6. This up-regulation was achieved by elevated levels of ERK, c-Fos, NF-κB p65, NF-κB p50, and CUX1, all factors known to bind to the promotor of TNFα and IL-6 (167). In accordance to that, Ikushima and collaborators observed that sDPP4 binds to the M6P/IGF-IIR resulting in enhanced transendothelial T cell migration (192). In a further study, sDPP4 binding to M6P/IGF-IIR leads to elevated ROS levels in HUVECs. In both studies, binding of DPP4 to this particular receptor was completely prevented by a DPP4-inhibitor (193).

In human vascular smooth muscle cells, we could show that sDPP4 activates the MAPK and NF-κB signaling cascade resulting in pro-atherogenic changes in human vascular smooth muscle cells illustrated by an increased proliferation, the induction of iNOS and elevated expression and secretion of pro-inflammatory cytokines (39). Additionally, we observed that all these detrimental effects of sDPP4 were PAR2 mediated, since both a PAR2 antagonist and PAR2 silencing completely prevented the sDPP4-induced effects. In collaboration with the group of Sánchez-Ferrer we further showed that sDPP4 exhibits direct effects on vascular function illustrated by vascular reactivity of murine mesenteric arteries (194). sDPP4 impaired the endothelium-dependent relaxation to acetylcholine in a concentration-dependent manner by up to 75%, without modifying endothelium-independent relaxation to sodium nitroprusside. Again enzymatic activity of DPP4 appears to be involved in this process. Similarly, the cyclooxygenase inhibitor indomethacin and the thromboxane A2 receptor antagonist SQ29548 abrogated the impairing action of DPP4. These data suggest that DPP-4 directly impairs endothelium-dependent relaxation

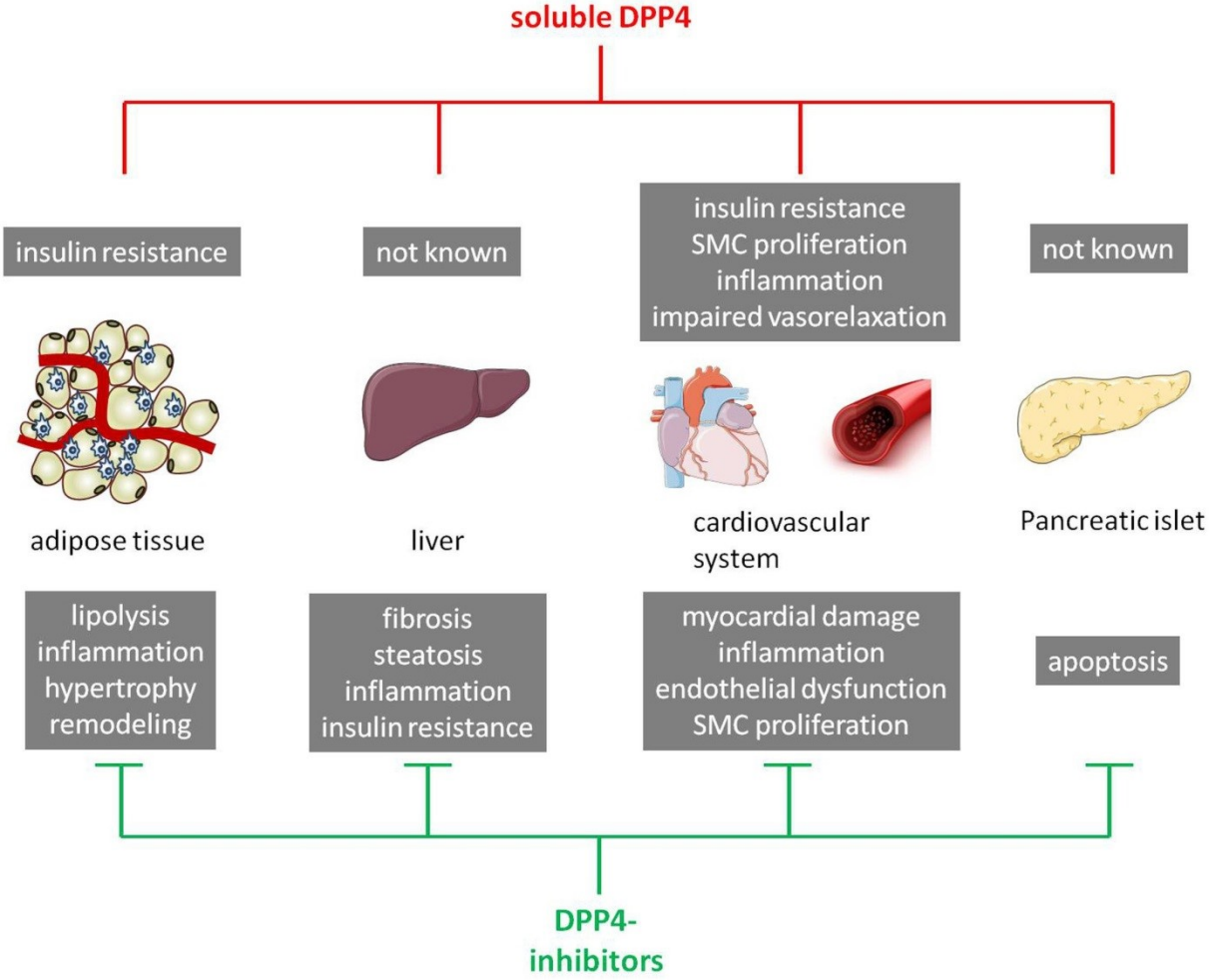


FIGURE 2 | Schematic overview of the impact of soluble DPP4 and DPP4 inhibitors on T2DM-relevant organs/tissues. In the upper panel, direct effects of soluble DPP4 (in red) on different organs/tissues are presented (gray boxes). The lower panel shows known effects of DPP4 inhibitors (in green) in these particular organs/tissues (gray boxes). SMC, smooth muscle cells.

through a mechanism that involves cyclooxygenase activation, and likely the release of a vasoconstrictor prostanoid. Since sDPP4 has been reported not only to contribute to monocyte migration, and macrophage-mediated inflammatory reactions, but also stimulates proliferation of human coronary artery smooth muscle cells as well as impairs endothelium-dependent vasorelaxation, it might be speculated that sDPP4 itself acts as a risk factor for atherosclerosis.

Collectively, this section emphasizes that both membrane-bound and sDPP4 and its inhibition are not only playing an important role in glucose homeostasis, but also in several other processes and organs involved in the pathogenesis of T2DM (Fig. 2). This supports the notion that DPP4 exhibits pleiotropic properties that are not fully understood so far and have to be further elucidated in the future.

Conclusion

DPP4, originally identified as an enzyme nearly 50 years ago, has now been recognised to exert pleiotropic functions with substantial impact for a variety of diseases. The complexity of DPP4 action stems from i) a long list of substrates cleaved by the enzyme including hormones, growth factors, and cytokines, ii) an additional function of this protein being a binding partner at the surface of different cells, specifically immune cells, and iii) the recent discovery that DPP4 is an adipokine with different endocrine functions. Thus, an integrated view on this molecule is required to more precisely understand its impact for metabolic diseases like type 2 diabetes. For this disease, DPP4 inhibition has gained substantial interest, mostly related to the DPP4 substrate GLP-1. As shown here, other substrates like SDF-1 and BNP should also be taken into account and may help to better understand the therapeutic potential of DPP4 inhibitors. In this context, the direct effects of DPP4 inhibitors require to be assessed in more detail, and several aspects like the cardio-protective function of DPP4 inhibition remains controversial. Finally, soluble DPP4 is emerging as a new research line, putting this molecule to the list of adipo-cytokines with pro-inflammatory and proliferative function. Combining the accumulated knowledge on DPP4 will lead to an improved understanding of its impact for health and disease.

Acknowledgements

This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen (Ministry of Science and Research of the State of North Rhine-Westphalia), and the Bundesministerium für Gesundheit (Federal Ministry of Health).

References

(1) Hopsu-Havu VK, Glenner GG. A new dipeptide naphthylamidase hydrolyzing glycyl-prolyl-beta-naphthylamide. Histochemie 1966;7(3):197-201.

(2) Lambeir AM, Durinx C, Scharpe S, De M, I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci 2003 Jun;40(3):209-94.

(3) Rohrborn D, Eckel J, Sell H. Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells. FEBS Lett 2014 Nov 3;588(21):3870-7.

(4) Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, et al. Dipeptidyl peptidase 4 is a novel adipokine potentially linking

obesity to the metabolic syndrome. Diabetes 2011 Jul;60(7:1917-25.
(5) Sell H, Bluher M, Kloting N, Schlich R, Willems M, Ruppe F, et al. Adipose dipeptidyl peptidase-4 and obesity: correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro. Diabetes Care 2013 Dec;36(12):4083-90.
(6) Mulvihill EE, Drucker DJ. Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. Endocr Rev 2014 Dec;35(6):992-1019.
(7) Cordero OJ, Salgado FJ, Nogueira M. On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 2009 Nov;58(11):1723-47.
(8) Capuano A, Sportiello L, Maiorino MI, Rossi F, Giugliano D, Esposito K. Dipeptidyl peptidase-4 inhibitors in type 2 diabetes therapy--focus on alogliptin. Drug Des Devel Ther 2013 Sep 17;7:989-1001.
(9) Scheen AJ. A review of gliptins in 2011. Expert Opin Pharmacother 2012 Jan;13(1):81-99.
(10) Nabeno M, Akahoshi F, Kishida H, Miyaguchi I, Tanaka Y, Ishii S, et al. A comparative study of the binding modes of recently launched dipeptidyl peptidase IV inhibitors in the active site. Biochem Biophys Res Commun 2013 May 3;434(2):191-6.
(11) Chung KM, Huang CH, Cheng JH, Tsai CH, Suen CS, Hwang MJ, et al. Proline in transmembrane domain of type II protein DPP-IV governs its translocation behavior through endoplasmic reticulum. Biochemistry 2011 Sep;%20;50(37):7909-18.
(12) Abbott CA, McCaughan GW, Gorrell MD. Two highly conserved glutamic acid residues in the predicted beta propeller domain of dipeptidyl peptidase IV are required for its enzyme activity. FEBS Lett 1999 Sep 24;458(3):278-84.
(13) Fan H, Meng W, Kilian C, Grams S, Reutter W. Domain-specific N-glycosylation of the membrane glycoprotein dipeptidylpeptidase IV (CD26) influences its subcellular trafficking, biological stability, enzyme activity and protein folding. Eur J Biochem 1997 May 15;246(1):243-51.
(14) Smith RE, Talhouk JW, Brown EE, Edgar SE. The significance of hypersialylation of dipeptidyl peptidase IV (CD26) in the inhibition of its activity by Tat and other cationic peptides. CD26: a subverted adhesion molecule for HIV peptide binding. AIDS Res Hum Retroviruses 1998 Jul 1;14(10):851-68.
(15) Chien CH, Huang LH, Chou CY, Chen YS, Han YS, Chang GG, et al. One site mutation disrupts dimer formation in human DPP-IV proteins. J Biol Chem 2004 Dec 10;279(50):52338-45.
(16) Scanlan MJ, Raj BK, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, Healey JH, et al. Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. Proc Natl Acad Sci U S A 1994 Jun 7;91(12):5657-61.
(17) Ghersi G, Dong H, Goldstein LA, Yeh Y, Hakkinen L, Larjava HS, et al. Seprase-dPPIV association and prolyl peptidase and gelatinase activities of the protease complex. Adv Exp Med Biol 2003;524:87-94.
(18) Bohm SK, Gum JR, Jr., Erickson RH, Hicks JW, Kim YS. Human dipeptidyl peptidase IV gene promoter: tissue-specific regulation from a TATA-less GC-rich sequence characteristic of a housekeeping gene promoter. Biochem J 1995 Nov 1;311 (Pt 3):835-43.
(19) Bauvois B, Djavaheri-Mergny M, Rouillard D, Dumont J, Wietzerbin J. Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. Oncogene 2000 Jan 13;19(2):265-72.
(20) Mattern T, Reich C, Duchrow M, Ansorge S, Ulmer AJ, Flad HD. Antibody-induced modulation of CD26 surface expression. Immunology 1995 Apr;84(4):595-600.
(21) Cordero OJ, Salgado FJ, Vinuela JE, Nogueira M. Interleukin-12 enhances CD26 expression and dipeptidyl peptidase IV function on human activated lymphocytes. Immunobiology 1997 Nov;197(5):522-33.
(22) Salgado FJ, Vela E, Martin M, Franco R, Nogueira M, Cordero OJ. Mechanisms of CD26/dipeptidyl peptidase IV cytokine-dependent regulation on human activated lymphocytes. Cytokine 2000 Jul;12(7):1136-41.
(23) Gu N, Tsuda M, Matsunaga T, Adachi T, Yasuda K, Ishihara A, et al. Glucose regulation of dipeptidyl peptidase IV gene expression is mediated by hepatocyte nuclear factor-1alpha in epithelial intestinal cells. Clin Exp Pharmacol Physiol 2008 Dec;35(12):1433-9.
(24) Morrison ME, Vijayasaradhi S, Engelstein D, Albino AP, Houghton AN. A marker for neoplastic progression of human melanocytes is a cell surface ectopeptidase. J Exp Med 1993 Apr 1;177(4):1135-43.
(25) Engel M, Hoffmann T, Wagner L, Wermann M, Heiser U, Kiefersauer R, et al. The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism. Proc Natl Acad Sci U S A 2003 Apr 29;100(9):5063-8.
(26) Gorrell MD. Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. Clin Sci (Lond) 2005 Apr;108(4):277-92.
(27) Yu DM, Slaitini L, Gysbers V, Riekhoff AG, Kahne T, Knott HM, et al. Soluble CD26 / dipeptidyl peptidase IV enhances human lymphocyte proliferation in vitro independent of dipeptidyl peptidase enzyme activity and adenosine

deaminase binding. Scand J Immunol 2011 Feb;73(2):102-11.
(28) Zhong J, Rao X, Deiuliis J, Braunstein Z, Narula V, Hazey J, et al. A potential role for dendritic cell/macrophage-expressing DPP4 in obesity-induced visceral inflammation. Diabetes 2013 Jan;62(1):149-57.
(29) Belle LP, Bitencourt PE, De Bona KS, Moresco RN, Moretto MB. Association between HbA1c and dipeptidyl peptidase IV activity in type 2 diabetes mellitus. Clin Chim Acta 2012 Jun 14;413(11-12):1020-1.
(30) Ciaraldi TP. The role of adenosine in insulin action coupling in rat adipocytes. Mol Cell Endocrinol 1988 Nov;60(1):31-41.
(31) Heseltine L, Webster JM, Taylor R. Adenosine effects upon insulin action on lipolysis and glucose transport in human adipocytes. Mol Cell Biochem 1995 Mar 23;144(2):147-51.
(32) Lee JG, Kang DG, Yu JR, Kim Y, Kim J, Koh G, et al. Changes in Adenosine Deaminase Activity in Patients with Type 2 Diabetes Mellitus and Effect of DPP-4 Inhibitor Treatment on ADA Activity. Diabetes Metab J 2011 Apr;35(2):149-58.
(33) Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Yoshikawa N, Shimizu N, et al. CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1. Proc Natl Acad Sci U S A 2004 Sep 28;101(39):14186-91.
(34) Loster K, Zeilinger K, Schuppan D, Reutter W. The cysteine-rich region of dipeptidyl peptidase IV (CD 26) is the collagen-binding site. Biochem Biophys Res Commun 1995 Dec 5;217(1):341-8.
(35) Cheng HC, Abdel-Ghany M, Pauli BU. A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis. J Biol Chem 2003 Jul 4;278(27):24600-7.
(36) Piazza GA, Callanan HM, Mowery J, Hixson DC. Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. Biochem J 1989 Aug 15;262(1):327-34.
(37) Ghersi G, Zhao Q, Salamone M, Yeh Y, Zucker S, Chen WT. The protease complex consisting of dipeptidyl peptidase IV and seprase plays a role in the migration and invasion of human endothelial cells in collagenous matrices. Cancer Res 2006 May 1;66(9):4652-61.
(38) Ikushima H, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, et al. Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation. Proc Natl Acad Sci U S A 2000 Jul 18;97(15):8439-44.
(39) Wronkowitz N, Gorgens SW, Romacho T, Villalobos LA, Sanchez-Ferrer CF, Peiro C, et al. Soluble DPP4 induces inflammation and proliferation of human smooth muscle cells via protease-activated receptor 2. Biochim Biophys Acta 2014 Sep;1842(9):1613-21.
(40) Bouchard L, Faucher G, Tchernof A, Deshaies Y, Lebel S, Hould FS, et al. Comprehensive genetic analysis of the dipeptidyl peptidase-4 gene and cardiovascular disease risk factors in obese individuals. Acta Diabetol 2009 Mar;46(1):13-21.
(41) Turcot V, Bouchard L, Faucher G, Tchernof A, Deshaies Y, Perusse L, et al. DPP4 gene DNA methylation in the omentum is associated with its gene expression and plasma lipid profile in severe obesity. Obesity (Silver Spring) 2011 Feb;19(2):388-95.
(42) Turcot V, Tchernof A, Deshaies Y, Perusse L, Belisle A, Marceau P, et al. Comparison of the dipeptidyl peptidase-4 gene methylation levels between severely obese subjects with and without the metabolic syndrome. Diabetol Metab Syndr 2013 Feb 4;5(1):4-5.
(43) Aghili N, Devaney JM, Alderman LO, Zukowska Z, Epstein SE, Burnett MS. Polymorphisms in dipeptidyl peptidase IV gene are associated with the risk of myocardial infarction in patients with atherosclerosis. Neuropeptides 2012 Dec;46(6):367-71.
(44) Bailey SD, Xie C, Pare G, Montpetit A, Mohan V, Yusuf S, et al. Variation at the DPP4 locus influences apolipoprotein B levels in South Asians and exhibits heterogeneity in Europeans related to BMI. Diabetologia 2014 Apr;57(4):738-45.
(45) Yasuda N, Nagakura T, Yamazaki K, Inoue T, Tanaka I. Improvement of high fat-diet-induced insulin resistance in dipeptidyl peptidase IV-deficient Fischer rats. Life Sci 2002 May 31;71(2):227-38.
(46) Frerker N, Raber K, Bode F, Skripuletz T, Nave H, Klemann C, et al. Phenotyping of congenic dipeptidyl peptidase 4 (DP4) deficient Dark Agouti (DA) rats suggests involvement of DP4 in neuro-, endocrine, and immune functions. Clin Chem Lab Med 2009;47(3):275-87.
(47) Ben-Shlomo S, Zvibel I, Varol C, Spektor L, Shlomai A, Santo EM, et al. Role of glucose-dependent insulinotropic polypeptide in adipose tissue inflammation of dipeptidylpeptidase 4-deficient rats. Obesity (Silver Spring) 2013 Nov;21(11):2331-41.
(48) Ben-Shlomo S, Zvibel I, Rabinowich L, Goldiner I, Shlomai A, Santo EM, et al. Dipeptidyl peptidase 4-deficient rats have improved bile secretory function in high fat diet-induced steatosis. Dig Dis Sci 2013 Jan;58(1):172-8.
(49) Karl T, Hoffmann T, Pabst R, von HS. Extreme reduction of dipeptidyl peptidase IV activity in F344 rat substrains is associated with various behavioral differences. Physiol Behav 2003 Oct;80(1):123-34.
(50) Stephan M, Radicke A, Leutloff S, Schmiedl A, Pabst R, von HS, et al. Dipeptidyl peptidase IV (DPP4)-deficiency attenuates diet-induced obesity in rats: possible implications for the hypothalamic

neuropeptidergic system. Behav Brain Res 2011 Jan;%20;216(2):712-8.
(51) Canneva F, Golub Y, Distler J, Dobner J, Meyer S, von HS. DPP4-deficient congenic rats display blunted stress, improved fear extinction and increased central NPY. Psychoneuroendocrinology 2015 Mar;53:195-206.
(52) Kirino Y, Sato Y, Kamimoto T, Kawazoe K, Minakuchi K, Nakahori Y. Interrelationship of dipeptidyl peptidase IV (DPP4) with the development of diabetes, dyslipidaemia and nephropathy: a streptozotocin-induced model using wild-type and DPP4-deficient rats. J Endocrinol 2009 Jan;200(1):53-61.
(53) Sato Y, Koshioka S, Kirino Y, Kamimoto T, Kawazoe K, Abe S, et al. Role of dipeptidyl peptidase IV (DPP4) in the development of dyslipidemia: DPP4 contributes to the steroid metabolism pathway. Life Sci 2011 Jan 3;88(1-2):43-9.
(54) Romacho T, Indrakusuma I, Rohrborn D, Castaneda TR, Jelenik T, Weiss J, et al. Adipose-tissue Specific Deletion Of Dipeptidyl Peptidase 4 (DPP4) Enhances M2 Macrophage Markers And Results In Smaller Adipocytes Under HFD. Diabetes, in press, Poster session presented at: 75th Annual meeting of the American Diabetes Association 2015.
(55) Sell H, Rohrborn D, Indrakusuma I, Jelenik T, Castaneda TR, Al-Hasani H, et al. Adipose-specific Dipeptidyl Peptidase 4 (DPP4) Knockout Mice Display Improved Fasting Insulin And Cholesterol Levels Despite Increased Weight Gain On HFD. Diabetes, in press, Poster session presented at: 75th Annual meeting of the American Diabetes Association 2015.
(56) Marguet D, Baggio L, Kobayashi T, Bernard AM, Pierres M, Nielsen PF, et al. Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proc Natl Acad Sci U S A 2000 Jun 6;97(12):6874-9.
(57) Conarello SL, Li Z, Ronan J, Roy RS, Zhu L, Jiang G, et al. Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. Proc Natl Acad Sci U S A 2003 May 27;100(11):6825-30.
(58) Yan S, Marguet D, Dobers J, Reutter W, Fan H. Deficiency of CD26 results in a change of cytokine and immunoglobulin secretion after stimulation by pokeweed mitogen. Eur J Immunol 2003 Jun;33(6):1519-27.
(59) Engel M, Hoffmann T, Manhart S, Heiser U, Chambre S, Huber R, et al. Rigidity and flexibility of dipeptidyl peptidase IV: crystal structures of and docking experiments with DPIV. J Mol Biol 2006 Jan 27;355(4):768-83.
(60) Longenecker KL, Stewart KD, Madar DJ, Jakob CG, Fry EH, Wilk S, et al. Crystal structures of DPP-IV (CD26) from rat kidney exhibit flexible accommodation of peptidase-selective inhibitors. Biochemistry 2006 Jun;%20;45(24):7474-82.
(61) Hoffmann T, Faust J, Neubert K, Ansorge S. Dipeptidyl peptidase IV (CD 26) and aminopeptidase N (CD 13) catalyzed hydrolysis of cytokines and peptides with N-terminal cytokine sequences. FEBS Lett 1993 Dec;%20;336(1):61-4.
(62) Lambeir AM, Proost P, Durinx C, Bal G, Senten K, Augustyns K, et al. Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. J Biol Chem 2001 Aug 10;276(32):29839-45.
(63) van BL, Ten Kulve JS, la Fleur SE, Ijzerman RG, Diamant M. Effects of glucagon-like peptide 1 on appetite and body weight: focus on the CNS. J Endocrinol 2014 Mar 7;221(1):T1-16.
(64) Drucker DJ. Enhancing incretin action for the treatment of type 2 diabetes. Diabetes Care 2003 Oct;26(10):2929-40.
(65) Rask E, Olsson T, Soderberg S, Holst JJ, Tura A, Pacini G, et al. Insulin secretion and incretin hormones after oral glucose in non-obese subjects with impaired glucose tolerance. Metabolism 2004 May;53(5):624-31.
(66) Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ. Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. Diabetes 2001 Mar;50(3):609-13.
(67) Gautier JF, Fetita S, Sobngwi E, Salaun-Martin C. Biological actions of the incretins GIP and GLP-1 and therapeutic perspectives in patients with type 2 diabetes. Diabetes Metab 2005 Jun;31(3 Pt 1):233-42.
(68) Wang W, Choi BK, Li W, Lao Z, Lee AY, Souza SC, et al. Quantification of intact and truncated stromal cell-derived factor-1alpha in circulation by immunoaffinity enrichment and tandem mass spectrometry. J Am Soc Mass Spectrom 2014 Apr;25(4):614-25.
(69) Busso N, Wagtmann N, Herling C, Chobaz-Peclat V, Bischof-Delaloye A, So A, et al. Circulating CD26 is negatively associated with inflammation in human and experimental arthritis. Am J Pathol 2005 Feb;166(2):433-42.
(70) Tariq M, Masoud MS, Mehmood A, Khan SN, Riazuddin S. Stromal cell derived factor-1alpha protects stem cell derived insulin-producing cells from glucotoxicity under high glucose conditions in-vitro and ameliorates drug induced diabetes in rats. J Transl Med 2013 May 6;11:115-11.
(71) Yano T, Liu Z, Donovan J, Thomas MK, Habener JF. Stromal cell derived factor-1 (SDF-1)/CXCL12 attenuates diabetes in mice and promotes pancreatic beta-cell survival by activation of the prosurvival kinase Akt. Diabetes 2007 Dec;56(12):2946-57.
(72) Humpert PM, Battista MJ, Lammert A, Reismann P, Djuric Z, Rudofsky G, Jr., et al.

Association of stromal cell-derived factor 1 genotype with diabetic foot syndrome and macrovascular disease in patients with type 2 diabetes. Clin Chem 2006 Jun;52(6):1206-8.
(73) Karimabad MN, Hassanshahi G. Significance of CXCL12 in type 2 diabetes mellitus and its associated complications. Inflammation 2015 Apr;38(2):710-7.
(74) Persaud SJ, Bewick GA. Peptide YY: more than just an appetite regulator. Diabetologia 2014 Sep;57(9):1762-9.
(75) Whim MD. Pancreatic beta cells synthesize neuropeptide Y and can rapidly release peptide co-transmitters. PLoS One 2011 Apr 29;6(4):e19478.
(76) Kos K, Baker AR, Jernas M, Harte AL, Clapham JC, O'Hare JP, et al. DPP-IV inhibition enhances the antilipolytic action of NPY in human adipose tissue. Diabetes Obes Metab 2009 Apr;11(4):285-92.
(77) Rosmaninho-Salgado J, Marques AP, Estrada M, Santana M, Cortez V, Grouzmann E, et al. Dipeptidyl-peptidase-IV by cleaving neuropeptide Y induces lipid accumulation and PPAR-gamma expression. Peptides 2012 Sep;37(1):49-54.
(78) Ahmad S, Wang L, Ward PE. Dipeptidyl(amino)peptidase IV and aminopeptidase M metabolize circulating substance P in vivo. J Pharmacol Exp Ther 1992 Mar;260(3):1257-61.
(79) Wang LH, Zhou SX, Li RC, Zheng LR, Zhu JH, Hu SJ, et al. Serum levels of calcitonin gene-related peptide and substance P are decreased in patients with diabetes mellitus and coronary artery disease. J Int Med Res 2012;40(1):134-40.
(80) Fu J, Liu B, Liu P, Liu L, Li G, Wu B, et al. Substance P is associated with the development of obesity, chronic inflammation and type 2 diabetes mellitus. Exp Clin Endocrinol Diabetes 2011 Mar;119(3):177-81.
(81) Karagiannides I, Bakirtzi K, Kokkotou E, Stavrakis D, Margolis KG, Thomou T, et al. Role of substance P in the regulation of glucose metabolism via insulin signaling-associated pathways. Endocrinology 2011 Dec;152(12):4571-80.
(82) Yang L, Di G, Qi X, Qu M, Wang Y, Duan H, et al. Substance P promotes diabetic corneal epithelial wound healing through molecular mechanisms mediated via the neurokinin-1 receptor. Diabetes 2014 Dec;63(12):4262-74.
(83) Brandt I, Lambeir AM, Ketelslegers JM, Vanderheyden M, Scharpe S, De M, I. Dipeptidyl-peptidase IV converts intact B-type natriuretic peptide into its des-SerPro form. Clin Chem 2006 Jan;52(1):82-7.
(84) dos SL, Salles TA, Arruda-Junior DF, Campos LC, Pereira AC, Barreto AL, et al. Circulating dipeptidyl peptidase IV activity correlates with cardiac dysfunction in human and experimental heart failure. Circ Heart Fail 2013 Sep 1;6(5):1029-38.
(85) Green BD, Irwin N, Flatt PR. Pituitary adenylate cyclase-activating peptide (PACAP): assessment of dipeptidyl peptidase IV degradation, insulin-releasing activity and antidiabetic potential. Peptides 2006 Jun;27(6):1349-58.
(86) Yada T, Nakata M, Shioda S. Insulinotropin PACAP potentiates insulin action. Stimulation of glucose uptake in 3T3-LI adipocytes. Ann N Y Acad Sci 2000;921:473-7.
(87) Akesson L, Ahren B, Manganiello VC, Holst LS, Edgren G, Degerman E. Dual effects of pituitary adenylate cyclase-activating polypeptide and isoproterenol on lipid metabolism and signaling in primary rat adipocytes. Endocrinology 2003 Dec;144(12):5293-9.
(88) Ahren B, Hughes TE. Inhibition of dipeptidyl peptidase-4 augments insulin secretion in response to exogenously administered glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide, pituitary adenylate cyclase-activating polypeptide, and gastrin-releasing peptide in mice. Endocrinology 2005 Apr;146(4):2055-9.
(89) Mentlein R. Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regul Pept 1999 Nov 30;85(1):9-24.
(90) Dworacka M, Krzyzagorska E, Iskakova S, Bekmukhambetov Y, Urazayev O, Dworacki G. Increased circulating RANTES in type 2 diabetes. Eur Cytokine Netw 2014 Jul;25(3):46-51.
(91) Kitade H, Sawamoto K, Nagashimada M, Inoue H, Yamamoto Y, Sai Y, et al. CCR5 plays a critical role in obesity-induced adipose tissue inflammation and insulin resistance by regulating both macrophage recruitment and M1/M2 status. Diabetes 2012 Jul;61(7):1680-90.
(92) Baturcam E, Abubaker J, Tiss A, Abu-Farha M, Khadir A, Al-Ghimlas F, et al. Physical exercise reduces the expression of RANTES and its CCR5 receptor in the adipose tissue of obese humans. Mediators Inflamm 2014;2014:627150.
(93) Pais R, Zietek T, Hauner H, Daniel H, Skurk T. RANTES (CCL5) reduces glucose-dependent secretion of glucagon-like peptides 1 and 2 and impairs glucose-induced insulin secretion in mice. Am J Physiol Gastrointest Liver Physiol 2014 Aug 1;307(3):G330-G337.
(94) Forssmann U, Stoetzer C, Stephan M, Kruschinski C, Skripuletz T, Schade J, et al. Inhibition of CD26/dipeptidyl peptidase IV enhances CCL11/eotaxin-mediated recruitment of eosinophils in vivo. J Immunol 2008 Jul 15;181(2):1120-7.
(95) Herder C, Haastert B, Muller-Scholze S, Koenig W, Thorand B, Holle R, et al. Association of systemic chemokine concentrations with impaired glucose tolerance and type 2 diabetes: results from the Cooperative Health Research in the Region of Augsburg Survey S4 (KORA S4). Diabetes 2005 Dec;54 Suppl 2:S11-S17.

(96) Baetta R, Corsini A. Pharmacology of dipeptidyl peptidase-4 inhibitors: similarities and differences. Drugs 2011 Jul 30;71(11):1441-67.
(97) Morishita R, Nakagami H. Teneligliptin : expectations for its pleiotropic action. Expert Opin Pharmacother 2015 Feb;16(3):417-26.
(98) Makdissi A, Ghanim H, Vora M, Green K, Abuaysheh S, Chaudhuri A, et al. Sitagliptin exerts an antinflammatory action. J Clin Endocrinol Metab 2012 Sep;97(9):3333-41.
(99) Dobrian AD, Ma Q, Lindsay JW, Leone KA, Ma K, Coben J, et al. Dipeptidyl peptidase IV inhibitor sitagliptin reduces local inflammation in adipose tissue and in pancreatic islets of obese mice. Am J Physiol Endocrinol Metab 2011 Feb;300(2):E410-E421.
(100) Ommen ES, Xu L, O'Neill EA, Goldstein BJ, Kaufman KD, Engel SS. Comparison of treatment with sitagliptin or sulfonylurea in patients with type 2 diabetes mellitus and mild renal impairment: a post hoc analysis of clinical trials. Diabetes Ther 2015 Mar;6(1):29-40.
(101) Kumar KV, Gupta AK. Clinical audit of patients using DPP4 inhibitors in longstanding type 2 diabetes. Diabetes Metab Syndr 2014 May 23;S1871-S4021.
(102) Aroor AR, Sowers JR, Jia G, DeMarco VG. Pleiotropic effects of the dipeptidylpeptidase-4 inhibitors on the cardiovascular system. Am J Physiol Heart Circ Physiol 2014 Aug 15;307(4):H477-H492.
(103) Pang Z, Nakagami H, Osako MK, Koriyama H, Nakagami F, Tomioka H, et al. Therapeutic vaccine against DPP4 improves glucose metabolism in mice. Proc Natl Acad Sci U S A 2014 Apr 1;111(13):E1256-E1263.
(104) Pratley RE, Salsali A. Inhibition of DPP-4: a new therapeutic approach for the treatment of type 2 diabetes. Curr Med Res Opin 2007 Apr;23(4):919-31.
(105) Stonehouse AH, Darsow T, Maggs DG. Incretin-based therapies. J Diabetes 2012 Mar;4(1):55-67.
(106) Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 2004 Jun;89(6):2548-56.
(107) Gustafson B, Hammarstedt A, Andersson CX, Smith U. Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. Arterioscler Thromb Vasc Biol 2007 Nov;27(11):2276-83.
(108) Pacheco R, Martinez-Navio JM, Lejeune M, Climent N, Oliva H, Gatell JM, et al. CD26, adenosine deaminase, and adenosine receptors mediate costimulatory signals in the immunological synapse. Proc Natl Acad Sci U S A 2005 Jul 5;102(27):9583-8.
(109) Schrader WP, West CA, Miczek AD, Norton EK. Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. J Biol Chem 1990 Nov 5;265(31):19312-8.
(110) Focosi D, Kast RE, Galimberti S, Petrini M. Conditioning response to granulocyte colony-stimulating factor via the dipeptidyl peptidase IV-adenosine deaminase complex. J Leukoc Biol 2008 Aug;84(2):331-7.
(111) Lessard J, Pelletier M, Biertho L, Biron S, Marceau S, Hould FS, et al. Characterization of dedifferentiating human mature adipocytes from the visceral and subcutaneous fat compartments: fibroblast-activation protein alpha and dipeptidyl peptidase 4 as major components of matrix remodeling. PLoS One 2015;10(3):e0122065.
(112) Badeanlou L, Furlan-Freguia C, Yang G, Ruf W, Samad F. Tissue factor-protease-activated receptor 2 signaling promotes diet-induced obesity and adipose inflammation. Nat Med 2011;17(11):1490-7.
(113) Lim J, Iyer A, Liu L, Suen JY, Lohman RJ, Seow V, et al. Diet-induced obesity, adipose inflammation, and metabolic dysfunction correlating with PAR2 expression are attenuated by PAR2 antagonism. FASEB J 2013 Dec;27(12):4757-67.
(114) Shirakawa J, Fujii H, Ohnuma K, Sato K, Ito Y, Kaji M, et al. Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. Diabetes 2011 Apr;60(4):1246-57.
(115) Shimasaki T, Masaki T, Mitsutomi K, Ueno D, Gotoh K, Chiba S, et al. The dipeptidyl peptidase-4 inhibitor des-fluoro-sitagliptin regulates brown adipose tissue uncoupling protein levels in mice with diet-induced obesity. PLoS One 2013;8(5):e63626.
(116) Fukuda-Tsuru S, Kakimoto T, Utsumi H, Kiuchi S, Ishii S. The novel dipeptidyl peptidase-4 inhibitor teneligliptin prevents high-fat diet-induced obesity accompanied with increased energy expenditure in mice. Eur J Pharmacol 2014 Jan 15;723:207-15.
(117) Liu L, Omar B, Marchetti P, Ahren B. Dipeptidyl peptidase-4 (DPP-4): Localization and activity in human and rodent islets. Biochem Biophys Res Commun 2014 Oct 24;453(3):398-404.
(118) Omar BA, Liehua L, Yamada Y, Seino Y, Marchetti P, Ahren B. Dipeptidyl peptidase 4 (DPP-4) is expressed in mouse and human islets and its activity is decreased in human islets from individuals with type 2 diabetes. Diabetologia 2014 Sep;57(9):1876-83.
(119) Shah P, Ardestani A, Dharmadhikari G, Laue S, Schumann DM, Kerr-Conte J, et al. The DPP-4 inhibitor linagliptin restores beta-cell function and survival in human isolated islets through GLP-1 stabilization. J Clin Endocrinol Metab 2013 Jul;98(7):E1163-E1172.

(120) Nagamatsu S, Ohara-Imaizumi M, Nakamichi Y, Aoyagi K, Nishiwaki C. DPP-4 inhibitor des-F-sitagliptin treatment increased insulin exocytosis from db/db mice beta cells. Biochem Biophys Res Commun 2011 Sep 9;412(4):556-60.
(121) Duttaroy A, Voelker F, Merriam K, Zhang X, Ren X, Subramanian K, et al. The DPP-4 inhibitor vildagliptin increases pancreatic beta cell mass in neonatal rats. Eur J Pharmacol 2011 Jan 15;650(2-3):703-7.
(122) Mu J, Woods J, Zhou YP, Roy RS, Li Z, Zycband E, et al. Chronic inhibition of dipeptidyl peptidase-4 with a sitagliptin analog preserves pancreatic beta-cell mass and function in a rodent model of type 2 diabetes. Diabetes 2006 Jun;55(6):1695-704.
(123) Takeda Y, Fujita Y, Honjo J, Yanagimachi T, Sakagami H, Takiyama Y, et al. Reduction of both beta cell death and alpha cell proliferation by dipeptidyl peptidase-4 inhibition in a streptozotocin-induced model of diabetes in mice. Diabetologia 2012 Feb;55(2):404-12.
(124) Han SJ, Choi SE, Kang Y, Jung JG, Yi SA, Kim HJ, et al. Effect of sitagliptin plus metformin on beta-cell function, islet integrity and islet gene expression in Zucker diabetic fatty rats. Diabetes Res Clin Pract 2011 May;92(2):213-22.
(125) Akarte AS, Srinivasan BP, Gandhi S. Vildagliptin selectively ameliorates GLP-1, GLUT4, SREBP-1c mRNA levels and stimulates beta-cell proliferation resulting in improved glucose homeostasis in rats with streptozotocin-induced diabetes. J Diabetes Complications 2012 Jul;26(4):266-74.
(126) D'Alessio DA, Denney AM, Hermiller LM, Prigeon RL, Martin JM, Tharp WG, et al. Treatment with the dipeptidyl peptidase-4 inhibitor vildagliptin improves fasting islet-cell function in subjects with type 2 diabetes. J Clin Endocrinol Metab 2009 Jan;94(1):81-8.
(127) Foley JE, Bunck MC, Moller-Goede DL, Poelma M, Nijpels G, Eekhoff EM, et al. Beta cell function following 1 year vildagliptin or placebo treatment and after 12 week washout in drug-naive patients with type 2 diabetes and mild hyperglycaemia: a randomised controlled trial. Diabetologia 2011 Aug;54(8):1985-91.
(128) Mari A, Scherbaum WA, Nilsson PM, Lalanne G, Schweizer A, Dunning BE, et al. Characterization of the influence of vildagliptin on model-assessed -cell function in patients with type 2 diabetes and mild hyperglycemia. J Clin Endocrinol Metab 2008 Jan;93(1):103-9.
(129) Leibowitz G, Cahn A, Bhatt DL, Hirshberg B, Mosenzon O, Wei C, et al. Impact of treatment with saxagliptin on glycaemic stability and beta-cell function in the SAVOR-TIMI 53 study. Diabetes Obes Metab 2015 May;17(5):487-94.
(130) Krawczyk M, Bonfrate L, Portincasa P. Nonalcoholic fatty liver disease. Best Pract Res Clin Gastroenterol 2010 Oct;24(5):695-708.
(131) Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. Hepatology 2010 May;51(5):1820-32.
(132) Mazza A, Fruci B, Garinis GA, Giuliano S, Malaguarnera R, Belfiore A. The role of metformin in the management of NAFLD. Exp Diabetes Res 2012;2012:716404.
(133) Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008 May;134(6):1655-69.
(134) Guo J, Friedman SL. Hepatic fibrogenesis. Semin Liver Dis 2007 Nov;27(4):413-26.
(135) Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005 Feb;115(2):209-18.
(136) Mentzel S, Dijkman HB, Van Son JP, Koene RA, Assmann KJ. Organ distribution of aminopeptidase A and dipeptidyl peptidase IV in normal mice. J Histochem Cytochem 1996 May;44(5):445-61.
(137) Itou M, Kawaguchi T, Taniguchi E, Sata M. Dipeptidyl peptidase-4: a key player in chronic liver disease. World J Gastroenterol 2013 Apr 21;19(15):2298-306.
(138) Miyazaki M, Kato M, Tanaka K, Tanaka M, Kohjima M, Nakamura K, et al. Increased hepatic expression of dipeptidyl peptidase-4 in non-alcoholic fatty liver disease and its association with insulin resistance and glucose metabolism. Mol Med Rep 2012 Mar;5(3):729-33.
(139) Gorrell MD, Wang XM, Park J, Ajami K, Yu DM, Knott H, et al. Structure and function in dipeptidyl peptidase IV and related proteins. Adv Exp Med Biol 2006;575:45-54.
(140) Eggstein S, Kreisel W, Gerok W, Eggstein M. [Dipeptidyl aminopeptidase IV in hospitalized patients and in galactosamine hepatitis of the rat: Activity and lectin affinity chromatography in serum and hepatic plasma membranes]. J Clin Chem Clin Biochem 1989 Sep;27(9):547-54.
(141) Matsumoto Y, Bishop GA, McCaughan GW. Altered zonal expression of the CD26 antigen (dipeptidyl peptidase IV) in human cirrhotic liver. Hepatology 1992 Jun;15(6):1048-53.
(142) Nilius R, Stuhec K, Dietrich R. Changes of dipeptidylpeptidase IV as a membrane marker of lymphocytes in acute and chronic liver diseases--biochemical and cytochemical investigations. Physiol Res 1991;40(1):95-102.
(143) Balaban YH, Korkusuz P, Simsek H, Gokcan H, Gedikoglu G, Pinar A, et al. Dipeptidyl peptidase IV (DDP IV) in NASH patients. Ann Hepatol 2007 Oct;6(4):242-50.
(144) Firneisz G, Varga T, Lengyel G, Feher J, Ghyczy D, Wichmann B, et al. Serum dipeptidyl peptidase-4 activity in insulin resistant patients with non-alcoholic fatty liver disease: a novel liver disease biomarker. PLoS One 2010;5(8):e12226.
(145) Kaji K, Yoshiji H, Ikenaka Y, Noguchi R, Aihara Y, Douhara A, et al. Dipeptidyl peptidase-4

inhibitor attenuates hepatic fibrosis via suppression of activated hepatic stellate cell in rats. J Gastroenterol 2014 Mar;49(3):481-91.
(146) Akaslan SB, Degertekin CK, Yilmaz G, Cakir N, Arslan M, Toruner FB. Effects of sitagliptin on nonalcoholic fatty liver disease in diet-induced obese rats. Metab Syndr Relat Disord 2013 Aug;11(4):243-50.
(147) Maiztegui B, Borelli MI, Madrid VG, Del ZH, Raschia MA, Francini F, et al. Sitagliptin prevents the development of metabolic and hormonal disturbances, increased beta-cell apoptosis and liver steatosis induced by a fructose-rich diet in normal rats. Clin Sci (Lond) 2011 Jan;120(2):73-80.
(148) Kern M, Kloting N, Niessen HG, Thomas L, Stiller D, Mark M, et al. Linagliptin improves insulin sensitivity and hepatic steatosis in diet-induced obesity. PLoS One 2012;7(6):e38744.
(149) Klein T, Fujii M, Sandel J, Shibazaki Y, Wakamatsu K, Mark M, et al. Linagliptin alleviates hepatic steatosis and inflammation in a mouse model of non-alcoholic steatohepatitis. Med Mol Morphol 2014 Sep;47(3):137-49.
(150) Ohyama T, Sato K, Yamazaki Y, Hashizume H, Horiguchi N, Kakizaki S, et al. MK-0626, a selective DPP-4 inhibitor, attenuates hepatic steatosis in ob/ob mice. World J Gastroenterol 2014 Nov 21;20(43):16227-35.
(151) Itou M, Kawaguchi T, Taniguchi E, Oriishi T, Sata M. Dipeptidyl Peptidase IV Inhibitor Improves Insulin Resistance and Steatosis in a Refractory Nonalcoholic Fatty Liver Disease Patient: A Case Report. Case Rep Gastroenterol 2012 May;6(2):538-44.
(152) Iwasaki T, Yoneda M, Inamori M, Shirakawa J, Higurashi T, Maeda S, et al. Sitagliptin as a novel treatment agent for non-alcoholic Fatty liver disease patients with type 2 diabetes mellitus. Hepatogastroenterology 2011 Nov;58(112):2103-5.
(153) Yilmaz Y, Yonal O, Deyneli O, Celikel CA, Kalayci C, Duman DG. Effects of sitagliptin in diabetic patients with nonalcoholic steatohepatitis. Acta Gastroenterol Belg 2012 Jun;75(2):240-4.
(154) Kanazawa I, Tanaka K, Sugimoto T. DPP-4 inhibitors improve liver dysfunction in type 2 diabetes mellitus. Med Sci Monit 2014;20:1662-7.
(155) Panjwani N, Mulvihill EE, Longuet C, Yusta B, Campbell JE, Brown TJ, et al. GLP-1 receptor activation indirectly reduces hepatic lipid accumulation but does not attenuate development of atherosclerosis in diabetic male ApoE(-/-) mice. Endocrinology 2013 Jan;154(1):127-39.
(156) Grundy SM. Obesity, metabolic syndrome, and cardiovascular disease. J Clin Endocrinol Metab 2004 Jun;89(6):2595-600.
(157) Chou E, Suzuma I, Way KJ, Opland D, Clermont AC, Naruse K, et al. Decreased cardiac expression of vascular endothelial growth factor and its receptors in insulin-resistant and diabetic States: a possible explanation for impaired collateral formation in cardiac tissue. Circulation 2002 Jan 22;105(3):373-9.
(158) Matsushita K, Blecker S, Pazin-Filho A, Bertoni A, Chang PP, Coresh J, et al. The association of hemoglobin a1c with incident heart failure among people without diabetes: the atherosclerosis risk in communities study. Diabetes 2010 Aug;59(8):2020-6.
(159) Yoon YS, Uchida S, Masuo O, Cejna M, Park JS, Gwon HC, et al. Progressive attenuation of myocardial vascular endothelial growth factor expression is a seminal event in diabetic cardiomyopathy: restoration of microvascular homeostasis and recovery of cardiac function in diabetic cardiomyopathy after replenishment of local vascular endothelial growth factor. Circulation 2005 Apr 26;111(16):2073-85.
(160) Pala L, Mannucci E, Pezzatini A, Ciani S, Sardi J, Raimondi L, et al. Dipeptidyl peptidase-IV expression and activity in human glomerular endothelial cells. Biochem Biophys Res Commun 2003 Oct 10;310(1):28-31.
(161) Shigeta T, Aoyama M, Bando YK, Monji A, Mitsui T, Takatsu M, et al. Dipeptidyl peptidase-4 modulates left ventricular dysfunction in chronic heart failure via angiogenesis-dependent and -independent actions. Circulation 2012 Oct 9;126(15):1838-51.
(162) Eltzschig HK, Faigle M, Knapp S, Karhausen J, Ibla J, Rosenberger P, et al. Endothelial catabolism of extracellular adenosine during hypoxia: the role of surface adenosine deaminase and CD26. Blood 2006 Sep 1;108(5):1602-10.
(163) Jin X, Shepherd RK, Duling BR, Linden J. Inosine binds to A3 adenosine receptors and stimulates mast cell degranulation. J Clin Invest 1997 Dec 1;100(11):2849-57.
(164) Chinda K, Palee S, Surinkaew S, Phornphutkul M, Chattipakorn S, Chattipakorn N. Cardioprotective effect of dipeptidyl peptidase-4 inhibitor during ischemia-reperfusion injury. Int J Cardiol 2013 Jul 31;167(2):451-7.
(165) Ye Y, Keyes KT, Zhang C, Perez-Polo JR, Lin Y, Birnbaum Y. The myocardial infarct size-limiting effect of sitagliptin is PKA-dependent, whereas the protective effect of pioglitazone is partially dependent on PKA. Am J Physiol Heart Circ Physiol 2010 May;298(5):H1454-H1465.
(166) Matsubara J, Sugiyama S, Sugamura K, Nakamura T, Fujiwara Y, Akiyama E, et al. A dipeptidyl peptidase-4 inhibitor, des-fluoro-sitagliptin, improves endothelial function and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice. J Am Coll Cardiol 2012 Jan 17;59(3):265-76.
(167) Shah Z, Kampfrath T, Deiuliis JA, Zhong J, Pineda C, Ying Z, et al. Long-term dipeptidyl-peptidase 4 inhibition reduces atherosclerosis and inflammation via effects on monocyte recruitment

and chemotaxis. Circulation 2011 Nov 22;124(21):2338-49.
(168) Bhatt DL, Cavender MA. Do dipeptidyl peptidase-4 inhibitors increase the risk of heart failure? JACC Heart Fail 2014 Dec;2(6):583-5.
(169) Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, et al. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. N Engl J Med 2013 Oct 3;369(14):1317-26.
(170) White WB, Cannon CP, Heller SR, Nissen SE, Bergenstal RM, Bakris GL, et al. Alogliptin after acute coronary syndrome in patients with type 2 diabetes. N Engl J Med 2013 Oct 3;369(14):1327-35.
(171) Ban K, Noyan-Ashraf MH, Hoefer J, Bolz SS, Drucker DJ, Husain M. Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. Circulation 2008 May 6;117(18):2340-50.
(172) Bullock BP, Heller RS, Habener JF. Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. Endocrinology 1996 Jul;137(7):2968-78.
(173) Wei Y, Mojsov S. Distribution of GLP-1 and PACAP receptors in human tissues. Acta Physiol Scand 1996 Jul;157(3):355-7.
(174) Arakawa M, Mita T, Azuma K, Ebato C, Goto H, Nomiyama T, et al. Inhibition of monocyte adhesion to endothelial cells and attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4. Diabetes 2010 Apr;59(4):1030-7.
(175) Ku HC, Chen WP, Su MJ. DPP4 deficiency preserves cardiac function via GLP-1 signaling in rats subjected to myocardial ischemia/reperfusion. Naunyn Schmiedebergs Arch Pharmacol 2011 Aug;384(2):197-207.
(176) Chang G, Zhang P, Ye L, Lu K, Wang Y, Duan Q, et al. Protective effects of sitagliptin on myocardial injury and cardiac function in an ischemia/reperfusion rat model. Eur J Pharmacol 2013 Oct 15;718(1-3):105-13.
(177) Liu Q, Adams L, Broyde A, Fernandez R, Baron AD, Parkes DG. The exenatide analogue AC3174 attenuates hypertension, insulin resistance, and renal dysfunction in Dahl salt-sensitive rats. Cardiovasc Diabetol 2010;9:32.
(178) Erdogdu O, Nathanson D, Sjoholm A, Nystrom T, Zhang Q. Exendin-4 stimulates proliferation of human coronary artery endothelial cells through eNOS-, PKA- and PI3K/Akt-dependent pathways and requires GLP-1 receptor. Mol Cell Endocrinol 2010 Aug 30;325(1-2):26-35.
(179) Fadini GP, Avogaro A. Cardiovascular effects of DPP-4 inhibition: beyond GLP-1. Vascul Pharmacol 2011 Jul;55(1-3):10-6.
(180) Ceriello A, Esposito K, Testa R, Bonfigli AR, Marra M, Giugliano D. The possible protective role of glucagon-like peptide 1 on endothelium during the meal and evidence for an "endothelial resistance" to glucagon-like peptide 1 in diabetes. Diabetes Care 2011 Mar;34(3):697-702.
(181) Goto H, Nomiyama T, Mita T, Yasunari E, Azuma K, Komiya K, et al. Exendin-4, a glucagon-like peptide-1 receptor agonist, reduces intimal thickening after vascular injury. Biochem Biophys Res Commun 2011 Feb 4;405(1):79-84.
(182) Nikolaidis LA, Mankad S, Sokos GG, Miske G, Shah A, Elahi D, et al. Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion. Circulation 2004 Mar 2;109(8):962-5.
(183) Sokos GG, Nikolaidis LA, Mankad S, Elahi D, Shannon RP. Glucagon-like peptide-1 infusion improves left ventricular ejection fraction and functional status in patients with chronic heart failure. J Card Fail 2006 Dec;12(9):694-9.
(184) Best JH, Hoogwerf BJ, Herman WH, Pelletier EM, Smith DB, Wenten M, et al. Risk of cardiovascular disease events in patients with type 2 diabetes prescribed the glucagon-like peptide 1 (GLP-1) receptor agonist exenatide twice daily or other glucose-lowering therapies: a retrospective analysis of the LifeLink database. Diabetes Care 2011 Jan;34(1):90-5.
(185) Fadini GP, Boscaro E, Albiero M, Menegazzo L, Frison V, de KS, et al. The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1alpha. Diabetes Care 2010 Jul;33(7):1607-9.
(186) Zaruba MM, Theiss HD, Vallaster M, Mehl U, Brunner S, David R, et al. Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction. Cell Stem Cell 2009 Apr 3;4(4):313-23.
(187) Segers VF, Revin V, Wu W, Qiu H, Yan Z, Lee RT, et al. Protease-resistant stromal cell-derived factor-1 for the treatment of experimental peripheral artery disease. Circulation 2011 Mar 29;123(12):1306-15.
(188) Boerrigter G, Costello-Boerrigter LC, Harty GJ, Lapp H, Burnett JC, Jr. Des-serine-proline brain natriuretic peptide 3-32 in cardiorenal regulation. Am J Physiol Regul Integr Comp Physiol 2007 Feb;292(2):R897-R901.
(189) Kawakami R, Saito Y, Kishimoto I, Harada M, Kuwahara K, Takahashi N, et al. Overexpression of brain natriuretic peptide facilitates neutrophil infiltration and cardiac matrix metalloproteinase-9 expression after acute myocardial infarction. Circulation 2004 Nov 23;110(21):3306-12.

(190) Kuhn M. Endothelial actions of atrial and B-type natriuretic peptides. Br J Pharmacol 2012 May;166(2):522-31.
(191) Palazzuoli A, Gallotta M, Quatrini I, Nuti R. Natriuretic peptides (BNP and NT-proBNP): measurement and relevance in heart failure. Vasc Health Risk Manag 2010;6:411-8.
(192) Ikushima H, Munakata Y, Iwata S, Ohnuma K, Kobayashi S, Dang NH, et al. Soluble CD26/dipeptidyl peptidase IV enhances transendothelial migration via its interaction with mannose 6-phosphate/insulin-like growth factor II receptor. Cell Immunol 2002 Jan;215(1):106-10.
(193) Ishibashi Y, Matsui T, Maeda S, Higashimoto Y, Yamagishi S. Advanced glycation end products evoke endothelial cell damage by stimulating soluble dipeptidyl peptidase-4 production and its interaction with mannose 6-phosphate/insulin-like growth factor II receptor. Cardiovasc Diabetol 2013;12:125.
(194) Sanchez-Ferrer C, Vallejo S, Romacho T, Villalobos L, Wronkowitz N, Sell H, et al. Dipeptidyl Peptidase-4 impairs microvascular endothelial-dependent relaxation : the role of cyclooxygenase. Diabetes 2013;62 Suppl.1:A128.
(195) Avogaro A, Fadini GP. The effects of dipeptidyl peptidase-4 inhibition on microvascular diabetes complications. Diabetes Care 2014 Oct;37(10):2884-94.

2.2. STUDY 2: SHEDDING OF DPP4 IS MEDIATED BY MMPS

SHEDDING OF DIPEPTIDYL PEPTIDASE 4 IS MEDIATED BY METALLOPROTEASES AND UP-REGULATED BY HYPOXIA IN HUMAN ADIPOCYTES AND SMOOTH MUSCLE CELLS

Diana Röhrborn[1], Jürgen Eckel[1, 2], Henrike Sell[1]

[1] Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Düsseldorf,Germany

[2] German Center for Diabetes Research (DZD e.V.), Düsseldorf, Germany

Published in: FEBS Lett**. 2014 Nov 3;588(21):3870-7.**

Key words: CD26/DPP4, hypoxia, non-classical secretion, proteases

Abstract

Dipeptidyl peptidase 4 is an important drug target for diabetes and a novel adipokine. However, it is unknown how soluble DPP4 (sDPP4) is cleaved from the cell membrane and released into the circulation. We show here that MMP1, MMP2 and MMP14 are involved in DPP4 shedding from human vascular smooth muscle cells (SMC) and MMP9 from adipocytes. Hypoxia increased DPP4 shedding from SMC which is associated with increased mRNA expression of MMP1. Our data suggest that constitutive as well as hypoxia-induced DPP4 shedding occurs due to a complex interplay between different MMPs in cell type-specific manner.

Introduction

Dipeptidyl peptidase 4 (DPP4) is a glycoprotein of 110 kDa, which is ubiquitously expressed on different cell types. The extracellular part of this type II cell surface protein is substantially glycosylated, which plays an important role in the interaction with different proteins (1). As an exopeptidase of the serine protease type, DPP4 cleaves numerous substrates at the penultimate position and thereby mostly inactivates them. Among these are peptides (e.g. stromal cell-derived factor 1 alpha (SDF1α), eotaxin) and cytokines (monocyte chemotactic protein-1 (MCP-1), interleukin 2(IL-2)) as well as the incretin hormones (1). The incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are major regulators of the postprandial insulin release (2). Therefore gliptins, a class of specific DPP4 inhibitors, are now widely used as a monotherapy or combination therapy for type 2 diabetes. By inhibition of DPP4 activity, these drugs prolong the half-life of GLP-1 and GIP which then stimulate pancreatic insulin secretion, suppress glucagon production and thereby contribute to an improved glycemic control (2;3).

DPP4 is not only present on the surface of cells, but can also be found in the circulation (4). By comprehensive proteomic profiling of the adipocyte secretome, we could identify soluble DPP4 (sDPP4) as a novel adipokine, with an upregulated release throughout the differentiation of adipocytes (5;6). We confirmed that mature adipocytes in comparison to macrophages and preadipocytes are a major source of DPP4 (5). Elevated serum levels of DPP4 were found in obese patients and correlate with the size of adipocytes and risk factors for the metabolic syndrome.

It has been reported, that DPP4 as a type II transmembrane protein is cleaved of the cell membrane in a process called shedding (7). The nature of enzymes contributing to the shedding of DPP4 and the regulation of this process is largely unknown. The aims of our study were to elucidate the underlying shedding mechanism and to explore the regulation of sDPP4 release *in vitro*. We show here for the first time that members of the metalloprotease family are involved in the release of sDPP4 from different primary human cells.

Material and methods

Material

Complete protease inhibitor (04693116001), and PhosStop phosphatase (04906837001) inhibitor cocktail were provided by Roche. Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech and by Sigma. DPP4 rabbit polyclonal antibody (H00001803-D01P) was obtained from Abnova. Beta actin mouse antibody (ab6276) was supplied by Abcam. HRP-conjugated goat anti-rabbit (W4011) and goat anti-mouse (W4021) IgG antibodies where purchased from Promega.

Collagenase NB4 (17465.02) was obtained from Serva. FCS (10270-106), Dulbecco's modified Eagles/HAM F12 (DMEM/F12) medium (42400-010), α-modified Eagle's (α MEM) medium (11900-016) and Ham's F-12 medium (21700-026) was supplied by Gibco (Invitrogen). Troglitazone was obtained from Sigma Aldrich.

The Protease inhibitors AEBSF (ALX-270-022), E64 (ALX-260-007) were dissolved in water. BB-94 (196440), MMP9 Inhibitor I (444278) and MMP2 Inhibitor III (444288) were purchased from Calbiochem and dissolved in sterile DMSO. Human Protease Array Kit (ARY021) was obtained from R&D Systems.

Adipocyte isolation and culture

Preadipocytes of human subcutaneous adipose tissue were obtained from lean or moderately overweight subjects undergoing plastic surgery. Isolation was performed as previously described (8). Cells from up to passage 4 were grown to confluence in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) supplemented with 10% FCS with medium change every 2-3 days. Passaging of the preadipocytes was performed according to Skurk et al.(9). Differentiation was started by adding 5 µmol/L troglitazone for 3 days to adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l d-panthothenic-acid, 66 nM insulin, 1 nM triiodo-l-thyronine, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 15 mmol/l HEPES, 14 nmol/l $NaHCO_3$, pH 7.4) as described previously (5). After 14 days cells where treated with the indicated substances diluted in α-modified DMEM and incubated for the indicated periods.

Smooth muscle cell culture

Primary human smooth muscle cells (SMC) from three different donors (Caucasian, one male, two females) were supplied as proliferating cells from Lonza, TebuBio and PromoCell and kept in culture according to the manufacturer's protocol. For all experiments subconfluent cells of passage three were used. SMC were characterized by morphologic criteria and by immunostaining with smooth muscle α-actin. For the experiments 100.000 cells/mL were seeded and grown for 24 hours in Growth medium (Promocell) with appropriate supplements. After washing with PBS and serum starvation for 24 hours cells were treated as indicated.

Hypoxia

For hypoxic experiments, cells were exposed to 1% O_2 supplemented with 5% CO_2 and respective concentrations of nitrogen in an Xvivo hypoxia chamber system (Biospherix) for 24 or 48 hours.

ELISA

DPP4 release to the cell culture medium was measured by human DPP4 DuoSet ELISA (R&D Systems, DY1180) according to the manufacturer's instructions.

Silencing of target genes

SMC were seeded in 6-well plates and grown until 60-80% confluence. Silencing experiments were performed by using 40 nM FlexiTube siRNA (Qiagen, MMP1 (SI03021802), MMP14 (SI03648841)) and 12 µL HiPerfect (Qiagen, 301705) according to the manufacturers' instructions. At day 10-12 of differentiation adipocytes were treated with 40 nM of the respective siRNA and 9 µL of HiPerfect. Optimal transfection conditions were tested by separate titration experiments. To control for unspecific effects, control cells were treated with AllStars Negative Control siRNA (QIAGEN, 1027280). After 24 hours mRNA was isolated to check for silencing efficiency. Supernatants were collected after 24 or 48 hours respectively.

qRT-PCR

Total RNA was isolated and reverse transcribed using the RNeasy Mini Kit (Qiagen, 74106) and Omniscript Reverse Transcription kit (Qiagen, 205113) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR (qRT PCR) using QuantiTect primer assays (Qiagen, ACTB, QT00057428, QT00001533, QT00040040, QT00088396, QT00014581, QT00055580) and GoTaq qPCR Master Mix (Promega, A6002) with 0.04-0.4 ng of generated cDNA on a Step One Plus Cycler (Applied Biosystems). Beta-actin was used as a reference gene and expression levels of investigated genes were normalized to beta-actin. Gene expression was analyzed via the ΔΔCt method and compared with the designated control.

Statistical analysis

Data are expressed as mean ± SEM. Unpaired two-tailed Student's ***t*** test or one-way ANOVA (post hoc test: Bonferroni's multiple comparison test) were used to determine statistical significance. All statistical analysis was done using Prism (GraphPad, La Jolla, CA, USA) considering a P value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

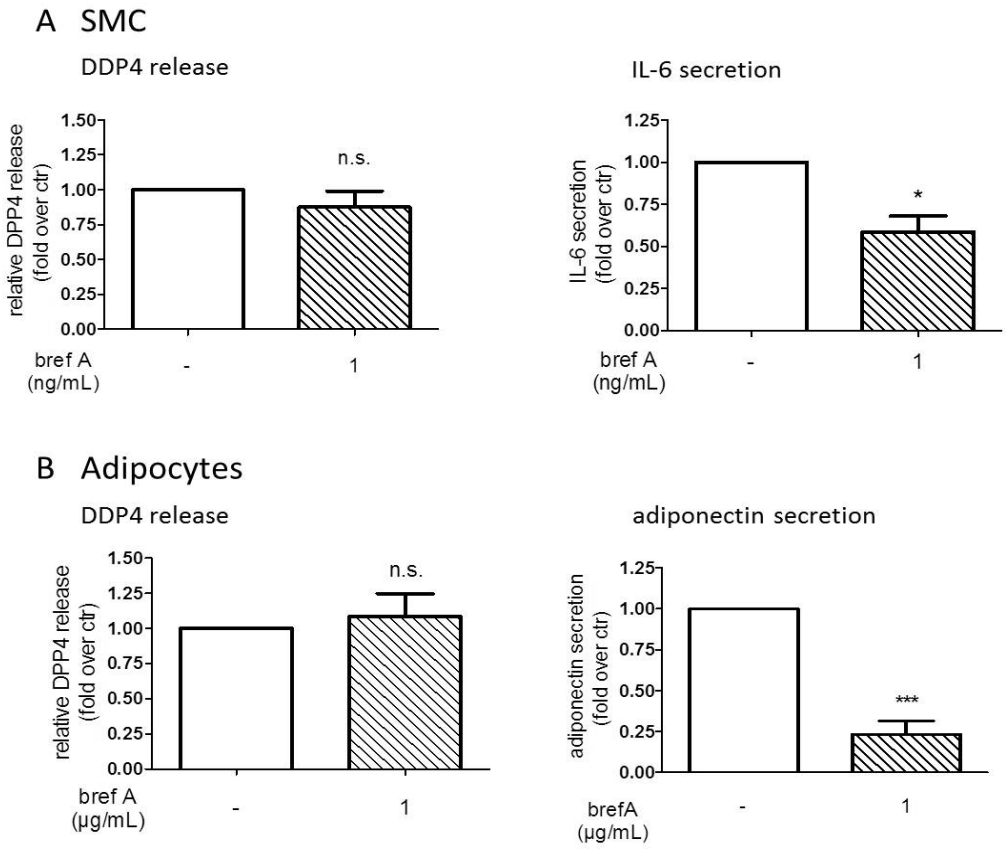


Fig. 1: Influence of brefA treatment on sDPP4 release in SMC (A) and adipocytes (B). Cells were treated for 24 hours with indicated concentrations of bref A. sDPP4 release and IL-6 or adiponectin secretion respectively to the culture medium was measured by ELISA. Data are mean values ± SEM, n = 3-4; *P < 0.05,***P < 0.001 vs. non-treated control; n.s. not significant; brefA, brefeldin A

Results

sDPP4 release is insensitive to brefeldin A treatment

In accordance with previously published data on sDPP4 release from skeletal muscle cells (10), sDPP4 release did not follow the classical ER/Golgi-dependant pathway in SMC and adipocytes, because it was insensitive to brefeldin A (bref A) treatment (Fig.1A, B). The applied concentrations of bref A were effective in blocking IL-6 secretion from SMC (Fig.1 A) and adiponectin secretion from adipocytes (Fig 1B).

Protease profile differs between SMC and adipocytes

To find out what types of proteases are released from different cell types and if there is a difference in the release profile of proteases, we used supernatants of adipocytes and SMC in a Protease Profiler Array. This array enables us to assess the release of 34 different proteases of the four main types of proteases, namely matrix metalloproteases (MMP), serine proteases, cysteine proteases and aspartyl proteases. A huge panel of proteases spotted on thismembrane belongs to the Cathepsins, which are serine, cysteine or aspartyl proteases. The comparison of different members of the Cathepsin family showed, that the detectable subtypes were more or less identical in the different cell types (Data not shown). In SMC, MMP1 and MMP3 signals were very prominent (Fig. 2A), whereas in adipocytes MMP2 and MMP9 signals were the strongest for all MMPs tested in the array (Fig. 2 B).

sDPP4 release can be reduced by general broad spectrum protease inhibitors

To identify the proteolytic enzymes involved in the DPP4 shedding, different

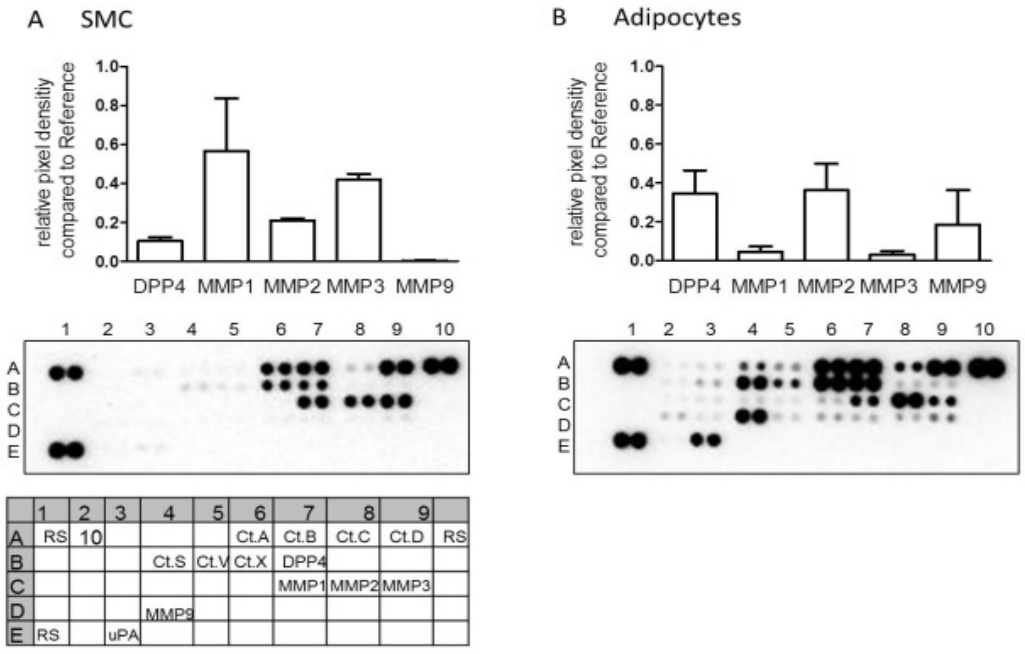


Fig. 2: Supernatants from SMC (A) and adipocytes (B) under control conditions were used to perform human Protease Profiler array. Data are depicted as relative pixel density compared to the reference spots. Representative arrays are presented and labeled with a grid pattern to identify corresponding spots. Data are mean values ± SEM, n = 2; RS reference spot; Ct, cathepsin; uPA, urokinase-type plasminogen activator.

classes of protease inhibitors were screened for their ability to block sDPP4 release. In SMC, sDPP4 release was impaired by the general broad spectrum MMP inhibitor BB-94, the general cysteine protease inhibitor E64 and the general serine protease inhibitor AEBSF to the same extent (Fig.3 A). To elucidate if a combination of these inhibitors could further diminish sDPP4 release, E64 and AEBSF, E64 and BB-94 as well as AEBSF and BB-94 were combined. None of these combinations showed additive effects on sDPP4 release (Fig. 3B). Furthermore, none of the treatments affected DPP4 protein expression (data not shown).

In adipocytes, only BB-94 showed a significant effect on sDPP4 release (Fig. 3 A). In the combination of the different classes of inhibitors no additive effects were observed (Fig. 3B). The protein expression of DPP4 remained unchanged after treatment with the different classes of protease inhibitors (data not shown).

sDPP4 release is mediated by metalloproteases

To correlate the shown effects of the general MMP inhibitor to a subset of MMPs, specific inhibitors or gene silencing were used. The IC_{50} values of BB-94 are lowest

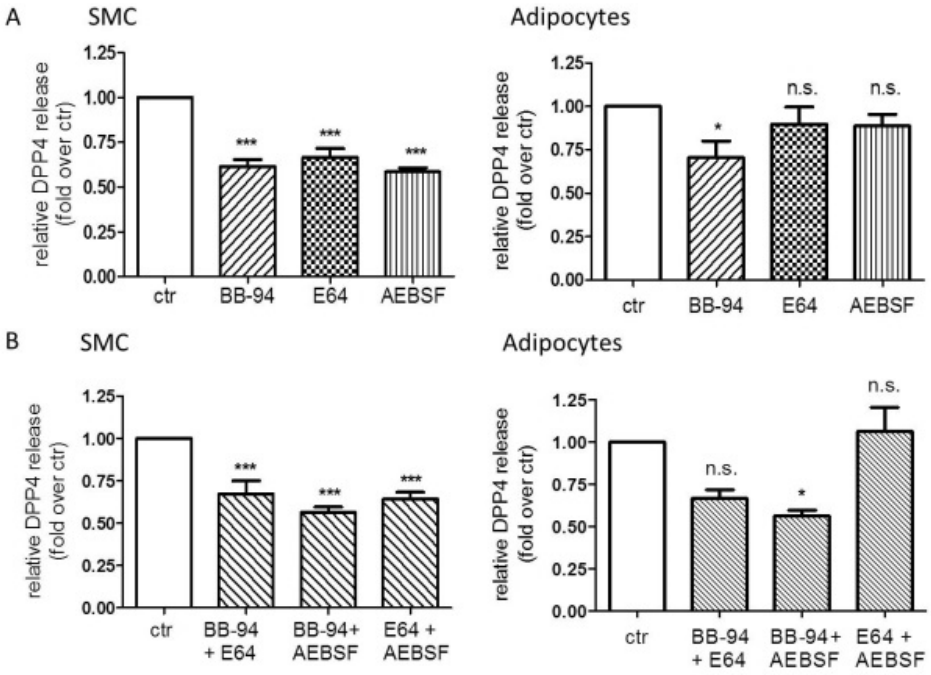


Fig 3: sDPP4 release after stimulation with different classes of Protease inhibitors in SMC and adipocytes. A: sDPP4 after treatment with 50 µM BB-94, 20 µM E64 and 20 µM AEBSF for 24 hours. sDPP4 release was measured by ELISA. Data were normalized to the respective control and are shown as fold over control. Data are mean values ± SEM, n > 3, *** P < 0.001 vs. respective control. B: sDPP4 after combined treatment with 20 µM E64, 20 µM AEBSF and 20 µM BB-94 as depicted for 24 hours. Data are mean values ± SEM, n > 5, ***P < 0.001 vs. respective control; n.s., not significant; ctr, control

for MMP14 (2 nM), MMP1 (3 nM), MMP2 and MMP9 (4 nM) and some of these MMPs are elevated in mouse models of obesity (11;12). To deduce our findings with BB-94 to a specific MMP, we used specific inhibitors if available or gene silencing if no specific inhibitors could be used. Expression of the respective MMP could be reduced by about 70 to 80 % using specific siRNAs (Supl. Fig.1). In SMC, inhibition of MMP2 and silencing of MMP1and MMP14 showed the most prominent effects on sDPP4 release, which was blocked by 20-30%, respectively (Fig. 4A). Inhibition of MMP9 in SMC showed only a slight effect on sDPP4 release (Fig. 4A). In contrast to SMC, MMP2 inhibition did not affect sDPP4 release in adipocytes, but MMP9 inhibition significantly reduced DPP4 shedding (Fig. 4B). Silencing of MMP1 or MMP14 in adipocytes had no effect on DPP4 shedding (Fig. 4B).

Hypoxia increases DPP4 shedding from SMC

To test whether lower oxygen levels can influence DPP4 shedding, SMC were incubated in an Xvivo hypoxia chamber system at 1% and 21% O_2 for 24 or 48 hours. After 24 hours as well as after 48 hours sDPP4 release was upregulated between 40 and 50%, respectively (Fig. 5A). However, DPP4 mRNA levels were not significantly elevated compared to normoxic conditions (Fig. 5B).

In addition to DPP4 shedding, mRNA expression of selected MMPs was tested after challenging of the SMC with 1% O_2. The most prominent effect of hypoxia could be shown for the expression of MMP1,

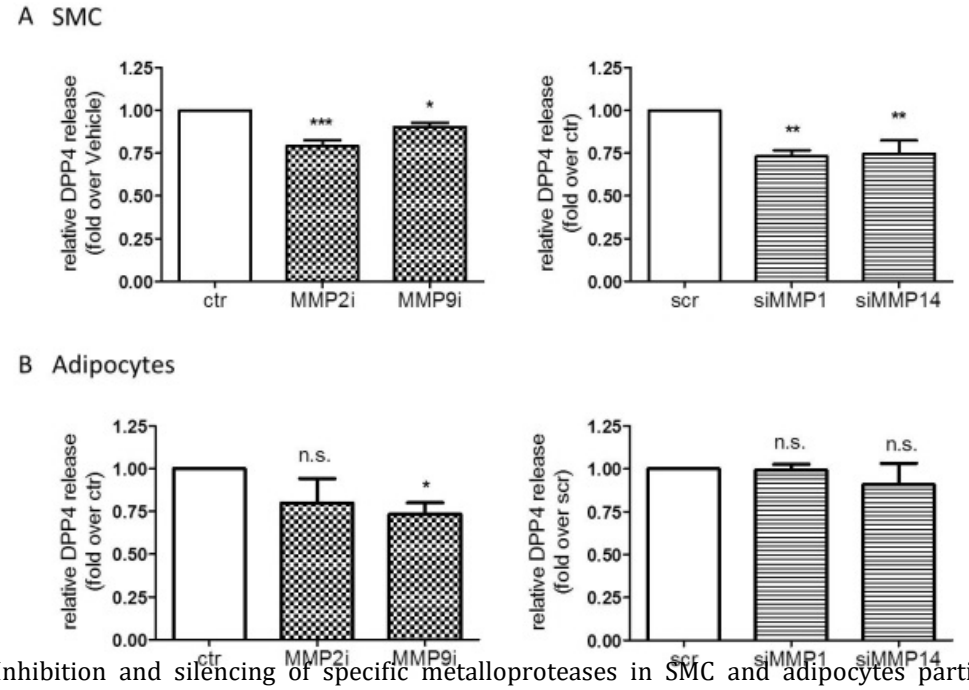


Fig. 4: Inhibition and silencing of specific metalloproteases in SMC and adipocytes partially prevented sDPP4 release. Treatment of SMC (A) and adipocytes (B) with specific MMP2 and MMP9 inhibitors in the indicated concentrations for 24 hours or with 40 nM MMP1 siRNA and 40 nM MMP14 siRNA respectively or with 40 nM scrambled siRNA for 48 hours. sDPP4 release is measured by ELISA. Data are presented as fold over respective control. Data are mean values ± SEM, n = 4-5, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. respective control; n.s., not significant;ctr, control; scr, scrambled

which was upregulated more than 3-times by hypoxia-treatment after 48 hours in SMC. Even after 24 hours, mRNA levels increased 2-fold in the hypoxic situation (Fig 5C). MMP9 mRNA expression showed at least a 1.5-fold increase in this setting (Fig. 5C). A slight but significant increase was also detected after 48 hours for the expression of ADAM17/TACE (Fig. 5C). In contrast, expression of MMP2 and MMP14 remained comparable to normoxic conditions (Data not shown).

Additional protease arrays were performed using supernatants from SMC cultured under hypoxic and normoxic conditions. Protease array results confirmed that hypoxia increased relative sDPP4 release and indicate that the increased expression of MMP1 mRNA is paralleled by increased MMP1 release (Data not shown).

Discussion

DPP4 is a type II transmembrane glycoprotein that is released from the membrane in a non-classical secretion mechanism. This is evidenced by insensitivity to brefeldin A treatment and the lack of a cleavable signal sequence at the N-terminus (13;14). Type II single-pass transmembrane proteins possess a signal anchor sequence which is positioned

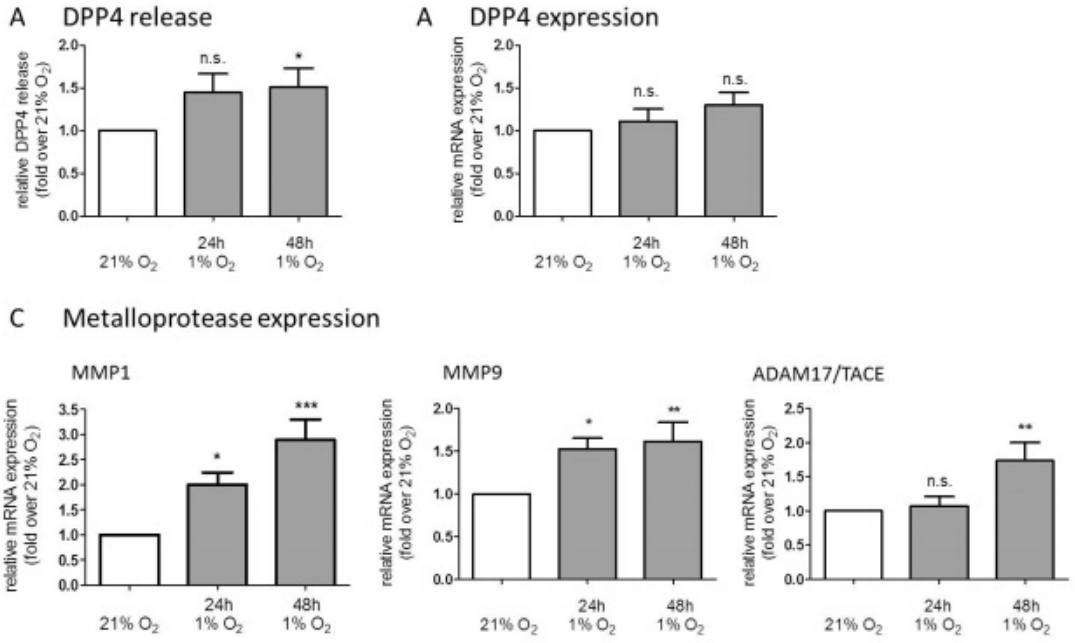


Fig 5: Influence of hypoxia on sDPP4 release and mRNA expression of selected genes in SMC. A: SMC were cultivated in parallel under normoxic (21% O_2) and under hypoxic (1% O_2) conditions for 24 and 48 hours DPP4 release to the culture medium was measured by ELISA. Data are depicted as fold over normoxic control. Data are mean values ± SEM, n = 7,* P < 0.05 vs. normoxic control B: DPP4 mRNA expression from the above mentioned treatment was measured by qRT PCR. Data were normalized to the mRNA expression level of actin and expressed relative to the normoxic control. Data are mean values ± SEM, n = 7 C: MMP1, MMP9 and ADAM17/TACE mRNA expression from the above mentioned treatment was measured by qRT PCR using specific primer sets for the indicated targets. Data were normalized to the mRNA expression level of actin and expressed relative to the normoxic control. Data are mean values ± SEM, n > 4, * P < 0.05; ** P < 0.01; *** P < 0.001 vs. normoxic control. Grey bars indicate treatment with 1% O2; n.s., not significant

within the membrane-spanning domain and which targets these proteins to the rough endoplasmatic reticulum. But in contrast to a classical secreted protein, the signal for the peptidase which cleaves off the signal sequence is missing (15). We were able to confirm, that DPP4 is insensitive to brefeldin A treatment not only in skeletal muscle cells (10), but also in SMC and adipocytes. Endogenous proteolytic release of transmembrane proteins is limited to type I or type II transmembrane proteins. This occurs by post-translational hydrolysis, the so called shedding (7). Bioinformatic analysis with Secretome 2.0 predicted DPP4 as non-classically secreted protein with a score of 0.719 (exceeding the threshold of 0.5). The cleavage of sDPP4 most likely occurs in the spacer region spanning amino acid 29 to 39 of the full length DPP4 according to the protein annotation at UniProt. However, the responsible enzymes are unknown so far.

Our data suggest that various types of proteases are able to induce sDPP4 release, because DPP4 shedding was significantly reduced by general serine-(AEBSF), cysteine-(E64) and metalloprotease (BB-94) inhibitors in SMC. Because combined treatment with the effective broad spectrum protease inhibitors lead to no additive effects we speculate, that in case of sDPP4 release a proteolytic cascade which Our results suggest that MMP1, MMP2 and MMP14 play a role in constitutive DPP4 shedding in SMC. One may keep in mind that MMP14 is able to activate MMP2 (17) and therefore might not only directly contribute to DPP4 shedding *in vitro*. Proteome profiler array data confirmed that MMP2 and MMP1 are released by SMC, whereas MMP9 was below the detection level in the supernatants of unstimulated

involves cathepsins and MMPs could be supposed. This goes in line with the rather complex activation of MMPs. MMPs belong to the family of zinc-dependent enzymes which are synthesized as an inactive proenzyme released into the extracellular space. Propeptides of MMPs are covalently bound to the zinc ion in the active center of these enzymes and thereby suppress the enzymatic activity until the propeptide is cleaved off (16). Different factors are able to activate MMPs like urokinase-type plasminogen activator (uPA), coagulation factors, phorbol esters or cytokines (16). But even MMPs can activate each other, which is known in case of MMP2 activation by MMP14 (17) or MMP9 activation by MMP2 and MMP3 (18). Also serine proteases like plasmin and kallikreins or cysteine proteases like Cathepsin G were shown to directly activate MMP1, MMP2 and MMP9 *in vitro* or *in vivo* (19).

For MMP2, MMP9 and MMP14 it has already been reported, that their expression levels are elevated in obese mice (11;12). Furthermore MMP1, MMP2, MMP9 and MMP14 play a role in the shedding of a large variety of substrates (20-24). These studies also emphasize that one MMP can shed several substrates and shedding of a specific target might not be limited to a specific MMP, but is a rather complex interplay between different enzymes.

SMC. Although also MMP3 was released from SMC, we did not further investigate this MMP due to the following reasons. First, MMP3 is described as a protective MMP in the context of atherosclerosis and it is not clear if this MMP is involved in shedding processes (25;26). Furthermore the IC_{50} of BB-94 for MMP3 is significantly higher in comparison to the other candidates. In adipocytes only the specific

inhibition of MMP9 could decrease DPP4 shedding, whereas MMP2 inhibition, MMP1 or MMP14 silencing respectively showed no effect. MMP1, MMP2 and MMP9 were detectable in the unstimulated supernatants of adipocytes. This result suggests, that constitutive DPP4 shedding is cell-type specific. None of the investigated mechanisms to block MMPs where able to reduce DDP4 release below 50%, which indicates, that DPP4 shedding is not only regulated by a single MMP.

We were able to demonstrate that low oxygen levels of 1% O_2 increase DPP4 shedding in SMC. In developing lesions during atherogenesis the oxygen supply is often exhausted, which is partly due to the highly oxygen-consuming foam cells and the relatively low diffusion rate of oxygen through the cell environment (27). This can result in severe hypoxia (< 1% O_2) in some areas. Hypoxia affects transcription of genes, which are for example responsible for matrix remodeling (28). For some MMPs like MMP1 and MMP9, it is already known that hypoxia stimulates their release and expression (29;30). In our setting, we were able to show an increased expression of MMP1, MMP9 and ADAM17/TACE under low oxygen supply. MMP1 release was relatively high in SMC compared to other MMPs, whereas it was relatively low in adipocytes. Therefore, MMP1 might be an important player in DPP4 shedding both under normoxic and particularly under hypoxic conditions in SMC. Though MMP9 mRNA expression is also upregulated due to hypoxia, this MMP could only play a minor role in DPP4 shedding in SMC, because it is barely detectable in supernatants of these cells compared to supernatants from adipocytes as shown by the Protease Array Analysis. Furthermore, MMP9 inhibition had only a very slight effect on DPP4 shedding. MMP2, which showed an impact on sDPP4 release after treatment with a specific inhibitor, was not influenced by hypoxia. ADAM17/TACE, cannot be ruled out to be involved in DPP4 shedding. From the literature it is known that it is involved in the processing of different substrates despite its eponymous target TNFα (31). Although we were able to silence ADAM17/TACE in SMC using different siRNAs, we always observed a concomitant upregulation of MMP1 thus preventing us from clarifying the role of ADAM17/TACE in DPP4 shedding. As no specific ADAM17/TACE inhibitor is available, it is unfortunately impossible to further study ADAM17/TACE at the moment. In the end, DPP4 expression itself is not influenced by hypoxia which shows, that the increased release of sDPP4 is not due to a higher DPP4 expression, but seems to be mediated at the level of DPP4 processing. In adipocytes, we could already show that culture at 1% O_2 for 24 hours had no effect on DPP4 release (5).

Adipose tissue and immune cells are recognized sources of DPP4. Circulating DPP4 concentrations are not only higher in obese patients but also in various inflammatory diseases (4;32). In a previous study, we were already able to show a significantly decreased DPP4 release from adipose tissue explants and in serum from obese patients who underwent bariatric surgery (5). MMP2 and MMP9 are key elements of extracellular matrix modulation in adipose tissue and both MMPs are significantly higher in serum of obese patients (33) and MMP2 is

significantly downregulated in serum of patients after bariatric surgery (34). High serum and mRNA expression levels of MMP1 and MMP9 are also associated with carotid atherosclerosis and plaque stability in patients (35-37). Accordingly, patients with a higher risk for cardiovascular disease are characterized by both higher circulating DPP4 and increased serum levels of MMP1 (38;39). In vitro, we have previously shown that sDPP4 in concentrations similar to circulating levels induces SMC proliferation (5). In parallel, sDPP4 induces inflammatory and stress pathways in vascular cells (40;41). Accordingly, sDPP4 generated in ischemic tissue or derived from perivascular and visceral adipose tissue might have similar effects in vivo (32;42). The multiple targets and functions of DPP4 and its inhibition by gliptins suggest that it could also play an important role beyond its effects on the incretin axis (43). As for the relation of DPP4 and potential shedding enzymes of DPP4 to obesity and atherosclerosis, it should be noted that only correlative data is available at the moment and no causal relationship can be established based on our current knowledge. In the future, research should focus on the molecular mechanisms of DPP4 shedding in the context of atherosclerosis and obesity which might finally help to better understand the role of sDPP4 in physiological and pathophysiological conditions.

In conclusion, we could show that several MMPs are involved in the constitutive DPP4 shedding as well as under hypoxic conditions *in vitro*. Our data suggest, that not a single MMP is involved in sDPP4 release, but it is rather an interplay between different shedding enzymes in a cell type-specific manner.

Acknowledgement

This work was supported by the Ministry of Science and Research of the State of North Rhine-Westphalia (MIWF NRW) and the Federal Ministry of Health (BMG). This study was supported in part by a grant from the Deutsche Forschungs-gemeinschaft (SE-1922 2-2), a grant from the German Diabetes Association (DDG) and a fellowship to HS from the European Foundation for the Study of Diabetes (EFSD).

We wish to thank the Dept. of Plastic Surgery, Florence-Nightingale-Hospital Düsseldorf, for support in obtaining adipose tissue samples. The technical assistance of Andrea Cramer, the scientific support from Dr. Nina Wronkowitz and the secretarial assistance of Birgit Hurow is gratefully acknowledged.

Reference List

1. **Lambeir AM, Durinx C, Scharpe S, De M, I** 2003 Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci 40:209-294

2. **Kazafeos K** 2011 Incretin effect: GLP-1, GIP, DPP4. Diabetes Res Clin Pract 93 Suppl 1:S32-S36

3. **Aschner P, Kipnes MS, Lunceford JK, Sanchez M, Mickel C, Williams-Herman DE** 2006 Effect of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy on glycemic control in patients with type 2 diabetes. Diabetes Care 29:2632-2637

4. **Cordero OJ, Salgado FJ, Nogueira M** 2009 On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 58:1723-1747

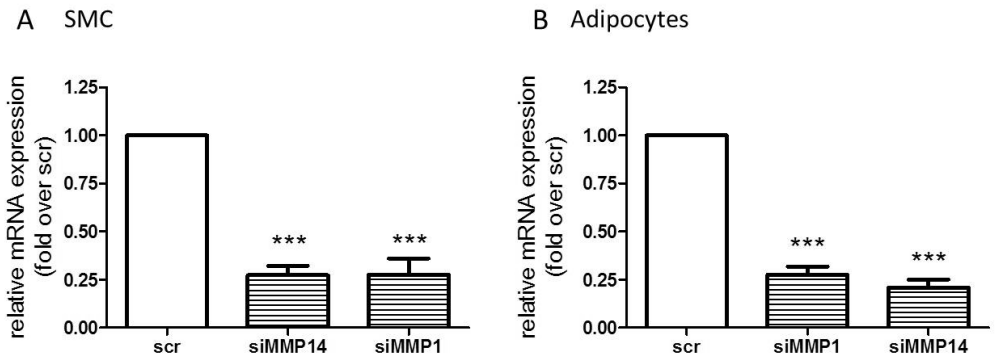
5. **Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, Eckardt K, Kaufman JM, Ryden M, Müller S, Hanisch F-G, Ruige J, Arner P, Sell H, Eckel J** 2011 Dipeptidyl Peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 60:1917-1925

6. **Lehr S, Hartwig S, Lamers D, Famulla S, Muller S, Hanisch FG, Cuvelier C, Ruige J, Eckardt K, Ouwens**

DM, Sell H, Eckel J 2012 Identification and validation of novel adipokines released from primary human adipocytes. Mol Cell Proteomics 11:M111

7. **Hooper NM, Karran EH, Turner AJ** 1997 Membrane protein secretases. Biochem J 321 (Pt 2):265-279
8. **Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J** 2005 Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. Diabetes 54:2003-2011
9. **Skurk T, Ecklebe S, Hauner H** 2007 A novel technique to propagate primary human preadipocytes without loss of differentiation capacity. Obesity (Silver Spring) 15:2925-2931
10. **Raschke S, Eckardt K, Bjorklund HK, Jensen J, Eckel J** 2013 Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells. PLoS One 8:e62008
11. **Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van OE, Tartare-Deckert S** 2003 Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. J Biol Chem 278:11888-11896
12. **Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR** 2002 Modulation of adiposetissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. Diabetes 51:1093-1101
13. **Nickel W** 2003 The mystery of nonclassical protein secretion. A current view on cargo proteins and ptential export routes. Eur J Biochem 270:2109-2119
14. **Caccia D, Dugo M, Callari M, Bongarzone I** 2013 Bioinformatics tools for secretome analysis. Biochim Biophys Acta
15. **Lodish H, Berk A, Zipursky SL, et al** 2000 Molecular Cell Biology. Insertion of Membrane Proteins into the ER Membrane. 4th ed. New York: W. H. Freeman
16. **Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T** 2003 Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253:269-285
17. **Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M** 1994 A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 370:61-65
18. **Toth M, Chvyrkova I, Bernardo MM, Hernandez-Barrantes S, Fridman R** 2003 Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes. Biochem Biophys Res Commun 308:386-395
19. **Saunders WB, Bayless KJ, Davis GE** 2005 MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. J Cell Sci 118:2325-2340
20. **Lu X, Wang Q, Hu G, Van PC, Fleisher M, Reiss M, Massague J, Kang Y** 2009 ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. Genes Dev 23:1882-1894
21. **Reinboldt S, Wenzel F, Rauch BH, Hohlfeld T, Grandoch M, Fischer JW, Weber AA** 2009 Preliminary evidence for a matrix metalloproteinase-2 (MMP-2)-dependent shedding of soluble CD40 ligand (sCD40L) from activated platelets. Platelets 20:441-444
22. **Ribeiro AS, Albergaria A, Sousa B, Correia AL, Bracke M, Seruca R, Schmitt FC, Paredes J** 2010 Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. Oncogene 29:392-402
23. **Fiore E, Fusco C, Romero P, Stamenkovic I** 2002 Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell-mediated cytotoxicity. Oncogene 21:5213-5223
24. **Sabbota AL, Kim HR, Zhe X, Fridman R, Bonfil RD, Cher ML** 2010 Shedding of RANKL by tumor-associated MT1-MMP activates Src-dependent prostate cancer cell migration. Cancer Res 70:5558-5566
25. **Preece G, Murphy G, Ager A** 1996 Metalloproteinase-mediated regulation of L-selectin levels on leucocytes. J Biol Chem 271:11634-11640
26. **Alexander MR, Moehle CW, Johnson JL, Yang Z, Lee JK, Jackson CL, Owens GK** 2012 Genetic inactivation of IL-1 signaling enhances atherosclerotic plaque instability and reduces outward vessel remodeling in advanced atherosclerosis in mice. J Clin Invest 122:70-79
27. **Hulten LM, Levin M** 2009 The role of hypoxia in atherosclerosis. Curr Opin Lipidol 20:409-414
28. **Faller DV** 1999 Endothelial cell responses to hypoxic stress. Clin Exp Pharmacol Physiol 26:74-84
29. **Nakayama K** 2013 cAMP-response Element-binding Protein (CREB) and NF-kappaB Transcription Factors Are Activated during Prolonged Hypoxia and Cooperatively Regulate the Induction of Matrix Metalloproteinase MMP1. J Biol Chem 288:22584-22595
30. **Yamamoto Y, Osanai T, Nishizaki F, Sukekawa T, Izumiyama K, Sagara S, Okumura K** 2012 Matrix metalloprotein-9 activation under cell-to-cell interaction between endothelial cells and monocytes: possible role of hypoxia and tumor necrosis factor-alpha. Heart Vessels 27:624-633
31. **Saftig P, Reiss K** 2011 The "A Disintegrin And Metalloproteases" ADAM10 and ADAM17: novel drug targets with therapeutic potential? Eur J Cell Biol 90:527-535
32. **Sell H, Bluher M, Kloting N, Schlich R, Willems M, Ruppe F, Knoefel WT, Dietrich A, Fielding BA, Arner P, Frayn KN, Eckel J** 2013 Adipose Dipeptidyl Peptidase-4 and Obesity: Correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro. Diabetes Care

33. **Derosa G, Ferrari I, D'Angelo A, Tinelli C, Salvadeo SA, Ciccarelli L, Piccinni MN, Gravina A, Ramondetti F, Maffioli P, Cicero AF** 2008 Matrix metalloproteinase-2 and -9 levels in obese patients. Endothelium 15(4):219-224
34. **Lee YJ, Heo YS, Park HS, Lee SH, Lee SK, Jang YJ** 2014 Serum SPARC and matrix metalloproteinase-2 and metalloproteinase-9 concentrations after bariatric surgery in obese adults. Obes Surg 24(4):604-610
35. **Muller A, Kramer SD, Meletta R, Beck K, Selivanova SV, Rancic Z, Kaufmann PA, Vos B, Meding J, Stellfeld T, Heinrich TK, Bauser M, Hutter J, Dinkelborg LM, Schibli R, Ametamey SM** 2014 Gene expression levels of matrix metalloproteinases in human atherosclerotic plaques and evaluation of radiolabeled inhibitors as imaging agents for plaque vulnerability. Nucl Med Biol 41(7):562-569
36. **Tan C, Liu Y, Li W, Deng F, Liu X, Wang X, Gui Y, Qin L, Hu C, Chen L** 2014 Associations of matrix metalloproteinase-9 and monocyte chemoattractant protein-1 concentrations with carotid atherosclerosis, based on measurements of plaque and intima-media thickness. Atherosclerosis 232(1):199-203
37. **Silvello D, Narvaes LB, Albuquerque LC, Forgiarini LF, Meurer L, Martinelli NC, Andrades ME, Clausell N, dos Santos KG, Rohde LE** 2014 Serum levels and polymorphisms of matrix metalloproteinases (MMPs) in carotid artery atherosclerosis: higher MMP-9 levels are associated with plaque vulnerability. Biomarkers 19(1):49-55
38. **Lehrke M, Greif M, Broedl UC, Lebherz C, Laubender RP, Becker A, von ZF, Tittus J, Reiser M, Becker C, Goke B, Steinbeck G, Leber AW, Parhofer KG** 2009 MMP-1 serum levels predict coronary atherosclerosis in humans. Cardiovasc Diabetol 8:50-58
39. **Zheng TP, Yang F, Gao Y, Baskota A, Chen T, Tian HM, Ran XW** 2014 Increased plasma DPP4 activities predict new-onset atherosclerosis in association with its proinflammatory effects in Chinese over a four year period: A prospective study. Atherosclerosis 235(2):619-624
40. **Wronkowitz N, Villalobos LA, Sanchez-Ferrer CF, Peiro C, Sell H, Eckel J** 2013 Dipeptidyl Peptidase 4 (DPP4) induces inflammation and proliferation of human smooth muscle cells by activation of the ERK-signalling pathway. Diabetes 62 (Suppl 1) A52
41. **Ervinna N, Mita T, Yasunari E, Azuma K, Tanaka R, Fujimura S, Sukmawati D, Nomiyama T, Kanazawa A, Kawamori R, Fujitani Y, Watada H** 2013 Anagliptin, a DPP-4 inhibitor, suppresses proliferation of vascular smooth muscles and monocyte inflammatory reaction and attenuates atherosclerosis in male apo E-deficient mice. Endocrinology 154:1260-1270
42. **Pala L, Rotella CM** 2013 The role of DPP4 activity in cardiovascular districts: in vivo and in vitro evidence. J Diabetes Res 2013:590456
43. **Fadini GP, Avogaro A** 2011 Cardiovascular effects of DPP-4 inhibition: beyond GLP-1. Vascul Pharmacol 55(1-3):10-16



Supl. Fig. 1: Silencing efficiency of siRNA in SMC (A) and adipocytes (B). Cells were treated with siRNA as described in Fig. 4. Expression of the depicted target gene was measured by qRT PCR. Data are presented as fold over scrambled siRNA. Data are mean values ± SEM, n = 3, *** $P < 0.001$ vs. scr; n.s., not significant; ctr, control; scr, scrambled

2.3. STUDY 3: REDUCED DPP4 ACTIVITY IMPROVES INSULIN SIGNALING

REDUCED DPP4 ACTIVITY IMPROVES INSULIN SIGNALING IN PRIMARY HUMAN ADIPOCYTES

Diana Röhrborn [1], Julia Brückner [1], Henrike Sell [1,2], Jürgen Eckel [1,2]

[1] Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Düsseldorf,Germany

[2] German Center for Diabetes Research (DZD e.V.), Düsseldorf, Germany

Published in: Biochem Biophys Res Commun. 2016 Feb 10. pii: S0006-291X(16)30209-1. doi: 10.1016/j.bbrc.2016.02.019.

Keywords: DPP4, siRNA-mediated silencing, DPP4 inhibition, primary human adipocytes, insulin signaling

Abstract

DPP4 is a ubiquitously expressed cell surface protease which is also released to the circulation as soluble DPP4 (sDPP4). Recently, we identified DPP4 as a novel adipokine oversecreted in obesity and thus potentially linking obesity to the metabolic syndrome. Furthermore, sDPP4 impairs insulin signaling in an autocrine and paracrine fashion in different cell types. However, it is still unknown which functional role DPP4 might play in adipocytes. Therefore, primary human adipocytes were treated with a specific DPP4 siRNA. Adipocyte differentiation was not affected by DPP4 silencing. Interestingly, DPP4 reduction improved insulin responsiveness of adipocytes at the level of insulin receptor, proteinkinase B (Akt) and Akt substrate of 160 kDa. To investigate whether the observed effects could be attributed to the enzymatic activity of DPP4, human adipocytes were treated with the DPP4 inhibitors sitagliptin and saxagliptin. Our data show that insulin-stimulated activation of Akt is augmented by DPP4 inhibitor treatment. Based on our previous observation that sDPP4 induces insulin resistance in adipocytes, and that adipose DPP4 levels are higher in obese insulin-resistant patients, we now suggest that the abundance of DPP4 might be a regulator of adipocyte insulin signaling.

Introduction

During obesity, adipose tissue (AT) expansion and a low-grade chronic inflammation occur, leading to an altered secretory profile in AT with a shift towards a more pro-inflammatory secretome (1). Although several studies confirmed that there is a link between dysregulated AT secretome and obesity-associated diseases like whole-body insulin resistance (IR) and type 2 diabetes mellitus (T2DM), the exact underlying molecular mechanisms remain mostly unknown. By comprehensive proteomic profiling of the adipocyte secretome our group identified novel adipokines including dipeptidylpeptidase 4 (DPP4). DPP4 is a serine protease, which was already discovered in 1966 by Hopsu-Havu and Glenner (2) and gained considerable interest because of its ability to cleave and thereby inactivate the incretin hormones, which are the main regulators of postprandial insulin secretion (3). Beyond this incretin-inactivation, DPP4 can cleave numerous substrates at the penultimate position and therefore alters the activity of cytokines or hormones (4;5). Furthermore, DPP4 is ubiquitously expressed on various cell types. It is able to interact with different binding partners and is thereby involved in signaling processes (5-8). DPP4 is not only a transmembrane protein, but is also released into the circulation as a fully functional enzyme by a process called shedding, which is regulated by matrix metalloproteases (MMP) (9). Therefore DPP4 is also able to act in a paracrine and endocrine way on different cells. DPP4 is upregulated in visceral versus subcutaneous adipose tissue (AT) and it is higher in obese than in lean patients (10;11). Furthermore, insulin-resistant obese patients have elevated levels of circulating DPP4 (11). Since DPP4 is upregulated during differentiation of human *in vitro* cultured adipocytes, and its expression is higher in mature adipocytes than in preadipocytes or macrophages, the role of DPP4 in AT is especially interesting in the context of obesity (10).

We were able to show here for the first time that knock-down of DPP4 improves insulin signaling in adipocytes. This seems to be linked to the enzymatic function of DPP4. Taken together with our previously published observation that sDPP4 impairs insulin signaling in adipocytes (10) we postulate that DPP4 abundance might be a regulator of AT insulin signaling.

Material and methods

Material

Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech and by Sigma. Antibodies were supplied as following: Adiponectin (Abcam, ab22554), DPP4 (abcam, ab129060), HSL (Cell signaling technologies, 4107),PPARγ (CST, 2435), IRS-1 (upstate, 06-248), total Insulin receptor (Calbiochem, GR36), phospho-Insulinreceptor (Tyr1150/1151) (C.S.T., 3024), total Akt (CST, 9272), phospho-Akt (Ser473) (CST, 9271), total ERK (p42/44) (CST, 9102), phospho-ERK (Thr202/Tyr204) (CST, 9101), total NFκB (CST, 8242), phospho-NFκB (CST, 3033), phospho-AS160 (Thr642) (CST, 2997). Protein levels were normalized to beta-Actin protein levels (abcam, ab6276). HRP-conjugated goat anti-rabbit (W4011) and goat anti-mouse (W4021) IgG antibodies where purchased from Promega.

Collagenase NB4 (17465.02) was obtained from Serva. FCS (10270-106), Dulbecco's modified Eagles/HAM F12 (DMEM/F12) medium (42400-010), α-modified Eagle's (α MEM) medium (11900-016) and Ham's F-12 medium (21700-026) were supplied by Gibco (Invitrogen). Troglitazone, IBMX (I5879_1G)

and Dexametheasone (D4902-25 mg) were obtained from Sigma Aldrich. Adipokine profiler arrays were supplied by R&D systems (ARY024).

Adipocyte isolation and culture

Subcutaneous adipose tissue was obtained from lean or moderately overweight women undergoing plastic surgery. Primary human adipocytes were isolated by collagenase digestion as previously described (12). Cells were proliferated and frozen after passage 1. Passaging of the preadipocytes was performed according to Skurk et al.(13). Frozen cells were seeded in basal medium (BM; DMEM/F12, 14 nmol/l $NaHCO_3$, 33 mmol/l biotin, and 17 mmol/l d-panthothenic-acid) supplemented with 10% FCS and 1% antibiotic-antimycotic-mix in six-well plates at a density of 70.000 cells per mL. One day prior to reaching confluence cells were silenced. 24h after silencing, differentiation was induced by adding an adipocyte differentiation medium (BM supplemented with 3% FCS, 100nM human insulin, 1µM Dexamethasone, 0,2mM IBMX, 0,25µM troglitazone and 0,5% gentamycin) which was used until day 7 of differentiation and was then changed to a maintenance medium (BM supplemented with 3% FCS, 100nM human insulin, 1µM Dexamethasone and 0,5% gentamycin) for additional 7 days. Medium was changed every 2-3 days. After 14 days, cells were treated with the indicated substances diluted in α-modified DMEM and incubated for the indicated periods.

Silencing

Frozen preadipocytes were seeded and cultured in 6-well-plates according to the above mentioned instructions. One day prior to reaching confluence (usually 4 days after seeding), silencing experiments were performed by using 20nM FlexiTube siRNA targeted against DPP4 (Qiagen Hs_DPP4_1 Cat. No: SI00030212) or 20nM AllStars Negative Control siRNA (Qiagen Cat No: 1027280) and 6µL HiPerfect (Qiagen, 301705) per well according to manufacturers' instructions. Optimal transfection conditions for high silencing efficiencies were elucidated by separate titration experiments. Silencing efficiency was assessed by qRT-PCR at different time-points during differentiation.

qRT-PCR

Total RNA was isolated and reverse transcribed using the RNeasy Mini Kit (Qiagen, 74106) and Omniscript Reverse Transcription kit (Qiagen, 205113) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR (qRT PCR) using QuantiTect primer assays (Qiagen) and QuantiTect SYBR Green PCR Kit (Qiagen, 204145) on a Step One Plus Cycler (Applied Biosystems). Optimal cDNA amount for each primer was separately tested. Beta-actin was used as a reference gene and expression levels of investigated genes were normalized to beta-actin. Gene expression was analyzed via the ΔΔCt method and compared with the designated control.

Isolation of total protein

Total cellular proteins were isolated at the indicated time-points during adipocyte culture by aspirating the culture medium and washing with ice-cold PBS. The Kinexus lysis buffer consisted of 20mM MOPS, 2mM EGTA, 5mM EDTA and 1% Triton X-100. After pH was adjusted to 7.2 1mM DTT, 40µL Complete protease inhibitor cocktail (Roche, 04693116001) and 100µL PhosStop (Roche, 04906837001) per mL lysis buffer were freshly added and cells were scratched from the plates. After 1-2h overhead shaking at 4°C lysates were centrifuged for 15min at 4°C and 10.000 *g*. Protein amount was determined by using the QuickStart Bradford Protein Assay (Bio-Rad, 500-0205).

Western Blot

5 µg of the total cellular proteins were separated by SDS-PAGE using 10% horizontal gels. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) in a semidry blotting system. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween for 1-2 h at room temperature and probed with the indicated primary antibodies. After being washed, membranes were incubated with a secondary horseradish peroxidase (HRP)-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore). Signals were visualized and analyzed on a Bio-Rad VersaDoc 4000 MP work station.

ELISA

DPP4 release and adiponectin secretion to the cell culture medium were measured by human DuoSet

ELISA (R&D Systems, DY1180 and DY1065) according to the manufacturer's instructions. IL6 and MCP-1 secretion was measured by ELISA (Hölzel Diagnostika 950030192 and 873030096) according to the manufacturer's instructions.

Oil Red O staining

After 14 days of differentiation and the indicated treatments, adipocyteswere washed with PBS and fixed overnight with a solution containing 71% picric acid (vol/vol), 24% acetic acid (vol/vol), and 5% formaldehyde (wt/vol). Afterwards, cells were washed three times with PBS and lipids were subsequently stained with 0.3% Oil Red O (Sigma-Aldrich, O0625-25G) dissolved in 60% isopropanol for 10 min. The staining was quantified by dissolving Oil Red O with 100% isopropanol and measuring absorbance at 500 nm.

DPP4 activity assay

The efficiency of DPP4 inhibition in primary human adipocytes cultures was measured with the DPP-4 Activity Assay Kit (sigma Aldrich, MAK088-1KT) according to the manufacturers' instructions. Undiluted supernatants of cells treated with vehicle (DMSO), saxagliptin or sitagliptin (100nM) were examined and the resulting fluorescent signal detected with a Tecan microplate reader (Infinite M200, Tecan).

Statistical analysis

Data are expressed as mean ± SEM. Unpaired two-tailed Student's *t* test was used to determine statistical significance between selected pairs of treatments. All statistical analysis was done using Prism (GraphPad, La Jolla, CA, USA) considering a *P* value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

siRNA-mediated silencing of DPP4 does not affect adipocyte differentiation

To elucidate the role of DPP4 within primary human adipocytes, DPP4 was silenced one day prior to the start of adipocyte differentiation via siRNA. DPP4 silencing was very stable at mRNA level until the end of differentiation, and also resulted in significant down-regulation of DPP4 protein level and approximately 50%

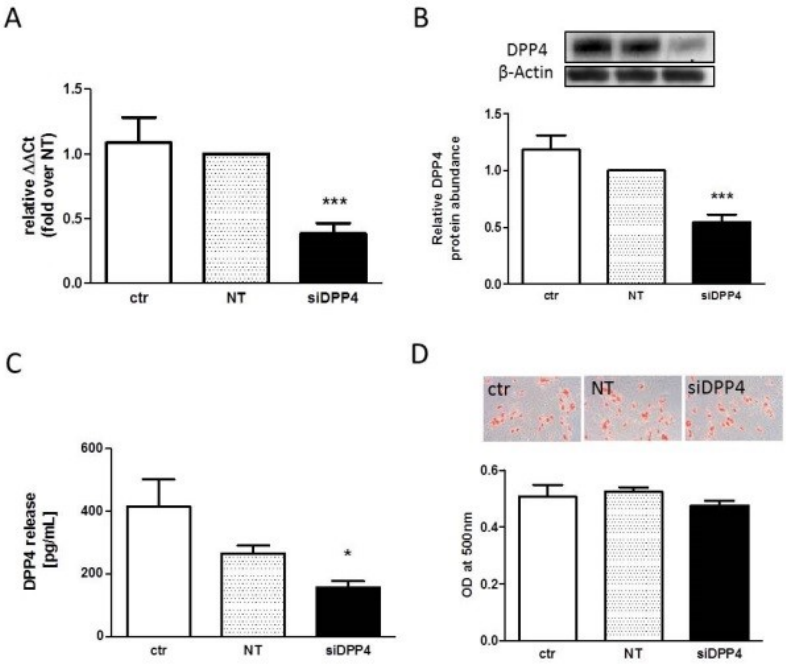


Fig. 1: Effect of siRNA-mediated silencing of DPP4 on primary human adipocyte differentiation. Human primary adipocytes were silenced one day prior to start of differentiation and silencing efficiency was validated on day 14 of differentiation on mRNA (A), protein (B) and release level (C). To assess accumulation of lipids an Oil Red staining was performed on day 14 of differentiation (D). Data are mean values ± SEM, n=3-4, *P < 0.05,***P < 0.001 vs. NT; Ctr control; NT non-target control siRNA; siDPP4 siRNA against DPP4; representative Western Blots for (B) are presented.

Tab.1 Silencing does not affect the expression level of differentiation markers
The level of protein or mRNA expression of the indicated markers on d14 of differentiation was assessed. Expression levels are presented in percent of non-target control siRNA on d14 of differentiation ± SEM. n.d. not determined

Expression level on d14 in % of NT	adiponectin	PPARγ	HSL	Glut4
mRNA	84 ± 3.6	98.2 ± 1.5	n.d.	71.9 ± 9.6
protein	86.4 ± 8.1	88.4 ± 5.1	80.6 ± 1.5	104.4 ± 10.3

reduction of DPP4 release (Fig. 1A-C). To assess the impact of DPP4 silencing on adipocyte differentiation, both expression of classical differentiation markers at mRNA

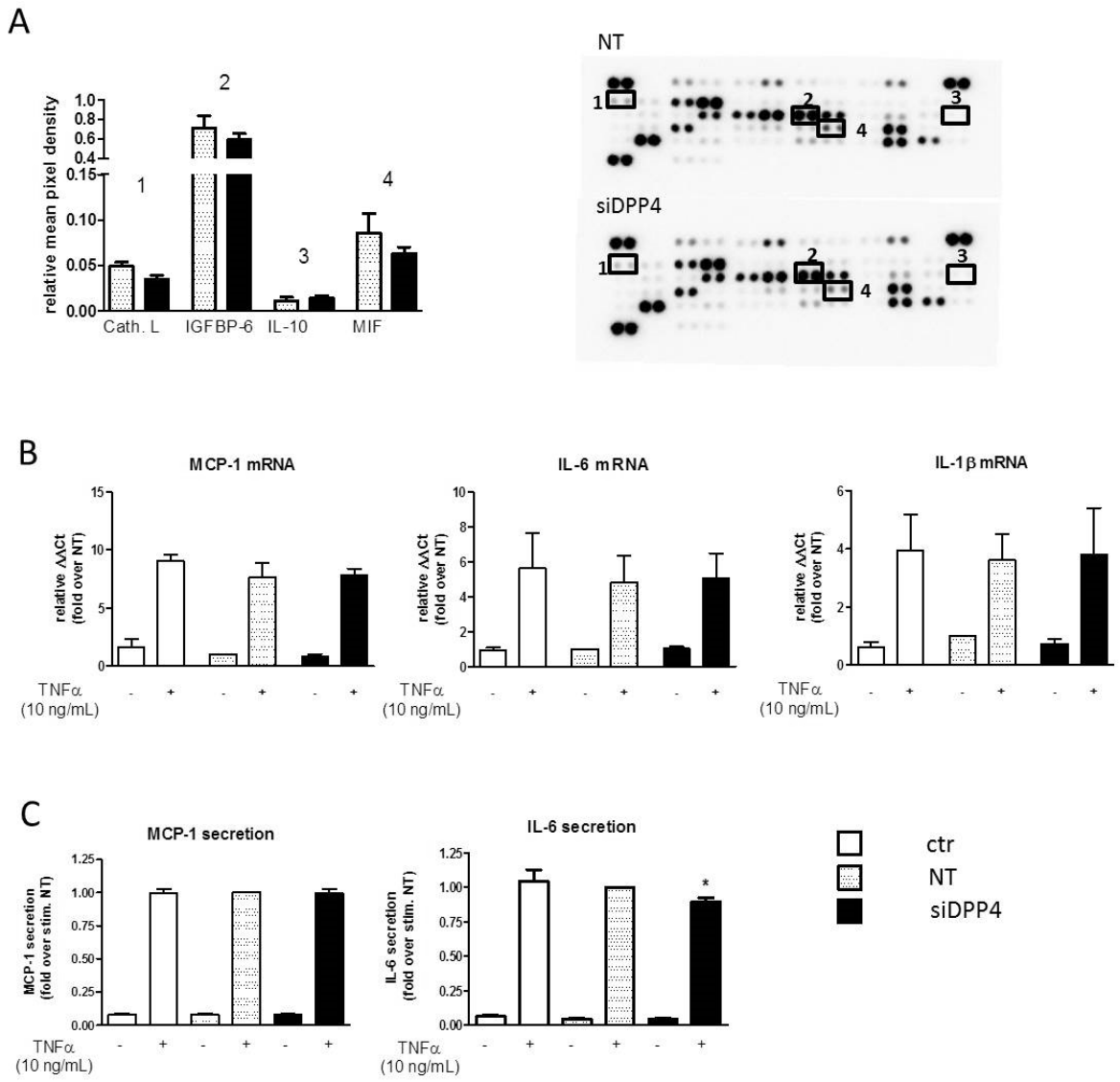


Fig. 2: Influence on secretory output or inflammation by DPP4 silencing. Supernatants of adipocytes on day 14 of differentiation were used to perform human adipokine profiler arrays (A). Data are depicted as relative pixel density normalized to the intensity of the reference spots. Representative arrays are presented and spots corresponding to the blot are highlighted. To assess whether inflammation induced by inflammatory stimuli differs between silenced and control cells mRNA expression (B) and secretion (C) of selected NFκB target genes was assessed after 24h treatment with 10 ng/mL TNFα by qRT-PCR and ELISA. Data are mean values ± SEM, n = 4-5, *P < 0.05 vs NT; Ctr control; NT non-target control siRNA; siDPP4 siRNA against DPP4;

and protein level (Tab. 1), as well as lipid droplet formation at the end of differentiation were analyzed (Fig. 1 D). None of these investigated parameters showed significant changes when compared to the non-target control siRNA (NT).

Dpp4 silencing does not affect adipocyte secretory function

To elaborate whether DPP4 silencing has an impact on the adipocyte secretome, adipokine profiler arrays were performed. Of the spotted adipokines, 31 adipokines could be detected in the adipocyte conditioned media. However none of the detected adipokines was significantly altered upon DPP4 silencing (Fig. 2A).

To analyze whether DPP4 knock-down has an effect on the responsiveness to inflammation, silenced cells were treated with the pro-inflammatory cytokine TNFα. This stimulation of adipocytes resulted in a strong induction of NFκB target genes MCP-1, IL-1β and IL-6 at the level of mRNA expression (Fig 2B) as well as secretion (Fig 2C). After DPP4 silencing, a slight but significant reduction in TNFα-stimulated IL-6 secretion can be observed whereas MCP-1 levels remained unaltered (Fig 2C).

siRNA-mediated DPP4 silencing and DPP4 inhibition improves insulin signaling

An important functional parameter of adipocytes is the insulin sensitivity. To

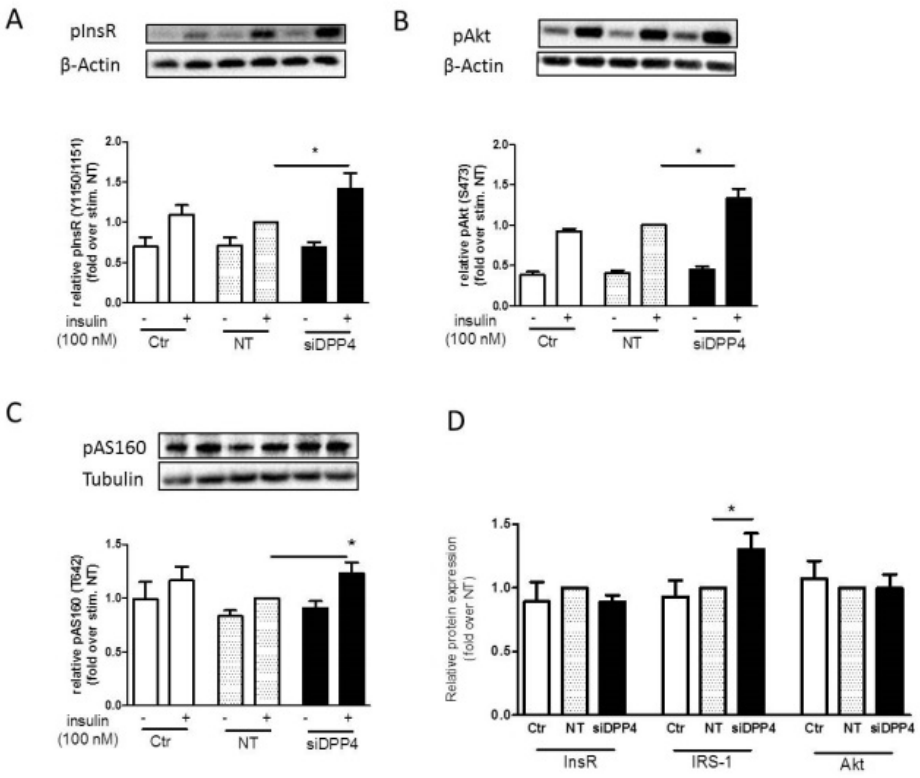


Fig. 3: Expression of proteins within the insulin signaling pathway after siRNA-mediated DPP4 silencing. On day 14 of differentiation, primary adipocytes were stimulated with 100 nM of insulin for 10 min and proteins of the insulin signaling cascade were analyzed by Western Blot. Levels of phospho InsR (A) phospho-Akt (B) and phospho-AS160 (C) were analyzed. Furthermore total protein levels of the unstimulated proteins were assessed (D).Data were normalized to the indicated housekeepers and are presented as fold over stimulated scr. Data are mean values ± SEM, n =7-9; * P < 0.05vs. NT; Ctr control; NT non-target control siRNA; siDPP4 siRNA against DPP4; representative Western Blots for are presented.

assess whether knock-down of DPP4 has a potential impact on this function we analyzed activation and expression of different proteins within the insulin signaling cascade.

We could observe a significant upregulation of insulin-stimulated insulin receptor (InsR), proteinkinase B (Akt) and Akt substrate of 160 kDa (AS160) phosphorylation (Fig 3 A-C) with most prominent effects at the receptor level. The basal phosphorylation levels of the investigated proteins remained unaltered. Interestingly, the total protein level of IRS-1 was significantly upregulated upon DPP4 silencing, whereas the total protein expression of the InsR and Akt were unaltered compared to control (Fig 3D).

To clarify if these effects could be explained by the enzymatic activity of DPP4, the well-established DPP4 inhibitors sitagliptin and saxagliptin were used to block DPP4 activity using *in vitro* differentiated mature adipocytes. Both inhibitors were able to almost completely block DPP4 activity measured in adipocyte supernatants (Fig 4A). In agreement with our silencing data, we could observe a significantly increased insulin-stimulated Akt phosphorylation with both inhibitors (Fig 4B) by about 30%. Also in this setting total protein levels of Akt and InsR remained unaltered by the treatment (data not shown).

Discussion

During obesity which is a major health risk in Western society, AT expansion and inflammation lead to a dysregulated release of adipokines. Recently we were able to show that DPP4 is upregulated during obesity and might link this adipokine to the metabolic syndrome (10;11). However the role of DPP4 within AT and how it affects adipocyte function is not known so far.

From a study by Rosmaninho-Salgado and colleagues it could be speculated that sDPP4 might stimulate lipid accumulation and PPARγ expression through degradation of neuropeptide Y (NPY), which is well-known for its anti-lipolytic activity (14). However, the concentrations of sDPP4 used in this study are beyond the physiological levels of sDPP4. Furthermore studies on the

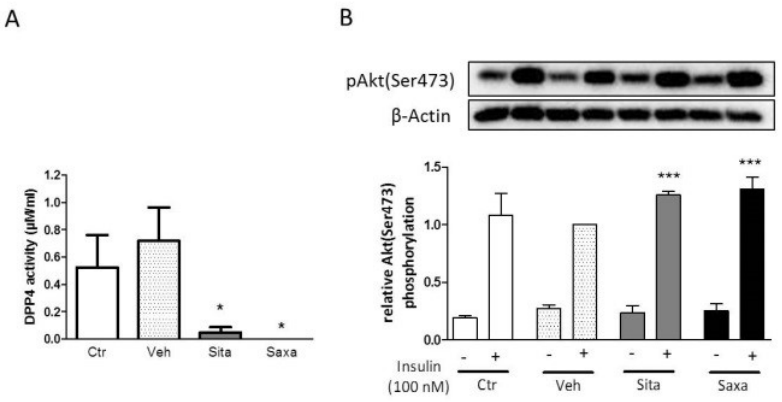


Fig. 4: Impact of DPP4i treatment on DPP4 activity and the level of Akt activation. Human primary adipocytes were incubated from day 10 of differentiation until day 14 of differentiation with 100 nM of the DPP4i sitagliptin or saxagliptin. On day 14 of differentiation, supernatants were collected and remaining DPP4 activity was measured with an activity assay (A). Furthermore cells were treated with 100 nM insulin for 10 min and insulin signaling was assessed by Western Blot. DMSO served as vehicle control. Representative blots for (B) are depicted. Data are mean values ± SEM, n =4 *P < 0.05, ***P < 0.001 vs Veh; Ctr control; Veh DMSO vehicle; Sita sitagliptin; Saxa saxagliptin

physiological role of DPP4 on adipocyte differentiation are missing. We could show here that DPP4 depletion by siRNA-mediated silencing does not affect adipocyte differentiation illustrated by differentiation markers such as adiponectin, PPARγ or HSL and by amount of incorporated lipids determined by OilRed staining.

Recently, several groups observed beneficial anti-inflammatory effects of DPP4i. Cytokine production and mRNA expression of inflammatory markers like IL6 or TNFα could be reduced by DPP4i both *in vitro* (20;21) and *in vivo* (15;16;22). Although the adipokine array data indicate an unaltered secretory output from adipocytes after silencing, this could be misleading since DPP4 only clips its substrates at the penultimate position resulting in only slightly altered proteins, which might still be detectable by the spotted antibodies. This problem was extensively summarized in a recent review by Mulvihill and Drucker (6). However, our data indicate that DPP4 silencing seems not to primarily target adipocyte inflammation or secretory output.

Interestingly, DPP4 silencing mediates a prominent effect on insulin signaling in the adipocytes with reduced DPP4 being associated with improved signaling. This is in line with our previous observations that increasing DPP4 levels by addition of recombinant protein impairs insulin signaling in adipocytes (10). One point which could explain the improved responsiveness towards insulin in general is the elevated protein level of total IRS-1. Low levels of IRS-1 are often observed in T2D and higher amounts of IRS-1 protein are associated with improved insulin signaling (23). However, how DPP4 affects IRS-1 protein level in the absence of insulin remains elusive and needs to be addressed in the future.

There are some other possible mechanisms by which DPP4 depletion might lead to insulin sensitizing in adipocytes. First, it seems plausible that the clipping of DPP4 substrates could influence insulin sensitivity. This could be either by altering the binding mode of the substrates to insulin target proteins and hence positively influence InsR responsiveness towards insulin. Or the altered activity of substrates could influence regulators of insulin signaling. In this scenario DPP4i would directly affect clipping of DPP4 substrates and therefore impair insulin signaling. Yet unidentified secreted factors and other mechanisms could potentially explain how reduced amount and activity of DPP4 improves insulin signaling. This needs to be further addressed in the future.

A second conceivable explanation despite affecting DPP4 substrates is that DPP4 and the insulin receptor could interact with each other and thereby DPP4 somehow reduces the activation of the insulin receptor. That the InsR is able to interact with other proteins which could alter its responsiveness towards insulin is well known. Glycoprotein-1, ectonucleotide pyrophosphatase/phosphodiesterase and alpha 2-HS glycoprotein were proposed as negative regulators, since they interact with the extracellular domain of the InsR and thereby negatively affect insulin binding (24;25). This mode of action is also conceivable in case of DPP4. A similar mechanism with opposite function is proposed for Gpc4 by Ussar and colleagues, who could show that both membrane-

bound as well as the free form of Gpc4 enhance insulin signaling (26). Gpc4 seems to interact with the InsR in the unstimulated state and upon insulin stimulation the receptor is unbound to Gpc4. In contrast to our results Gpc4 is also pro-adipogenic (26). Since we also observed sensitizing effects when DPP4 enzymatic activity is blocked (8), the binding of the inhibitor somehow seems to affect the binding of DPP4 to InsR and /or other partners. This could be because of a conformational change of DPP4 upon inhibitor binding which was proposed by Wronkowitz et al in case of the binding to protease activated receptor 2 (8). Another example that DPP4i treatment affects DPP4 interaction with binding partners is reported for Fibronectin as shown by Piazza et al (27).

In conclusion, we could show here for the first time that knock-down of DPP4 in human adipocytes leads to an improved insulin signaling response despite a similar rate of differentiation. This effect is at least partly mediated by DPP4 enzymatic activity. This finding is of importance since AT is a key source of DPP4 in obese patients and links obesity to the metabolic syndrome (10;11). Taken together with our previously published results on sDPP4 in adipocytes (10), the amount of DPP4 might be a regulator for insulin signaling in AT, especially in the obese state and might also open up new explanations for the beneficial effects of DPP4i on insulin sensitivity beyond the incretin hormones.

Acknowledgement

This work was supported by the Ministry of Science and Research of the State of North Rhine-Westphalia (MIWF NRW) and the Federal Ministry of Health (BMG). We wish to thank the Dept. of Plastic Surgery, Florence-Nightingale-Hospital Düsseldorf, for support in obtaining adipose tissue samples. The technical assistance of Andrea Cramer, the scientific support from Dr. Nina Wronkowitz and the secretarial assistance of Birgit Hurow is gratefully acknowledged.

Reference List

1. Romacho T, Elsen M, Rohrborn D, Eckel J 2014 Adipose tissue and its role in organ crosstalk. Acta Physiol (Oxf) 210(4):733-753
2. Hopsu-Havu VK, Glenner GG 1966 A new dipeptide naphthylamidase hydrolyzing glycyl-prolyl-beta-naphthylamide. Histochemie 7(3):197-201
3. Mest HJ, Mentlein R 2005 Dipeptidyl peptidase inhibitors as new drugs for the treatment of type 2 diabetes. Diabetologia 48(4):616-620
4. Mentlein R 1999 Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regul Pept 85:9-24
5. Lambeir AM, Durinx C, Scharpe S, De M, I 2003 Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci 40:209-294
6. Mulvihill EE, Drucker DJ 2014 Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. Endocr Rev 35(6):992-1019
7. Cordero OJ, Salgado FJ, Nogueira M 2009 On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 58:1723-1747
8. Wronkowitz N, Gorgens SW, Romacho T, Villalobos LA, Sanchez-Ferrer CF, Peiro C, Sell H, Eckel J 2014 Soluble DPP4 induces inflammation and proliferation of human smooth muscle cells via protease-activated receptor 2. Biochim Biophys Acta 1842:1613-1621
9. Rohrborn D, Eckel J, Sell H 2014 Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by

hypoxia in human adipocytes and smooth muscle cells. FEBS Lett 588:3870-3877
10. Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, Eckardt K, Kaufman JM, Ryden M, Muller S, Hanisch FG, Ruige J, Arner P, Sell H, Eckel J 2011 Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 60:1917-1925
11. Sell H, Bluher M, Kloting N, Schlich R, Willems M, Ruppe F, Knoefel WT, Dietrich A, Fielding BA, Arner P, Frayn KN, Eckel J 2013 Adipose dipeptidyl peptidase-4 and obesity: correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro. Diabetes Care 36:4083-4090
12. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J 2005 Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. Diabetes 54(7):2003-2011
13. Skurk T, Ecklebe S, Hauner H 2007 A novel technique to propagate primary human preadipocytes without loss of differentiation capacity. Obesity (Silver Spring) 15(12):2925-2931
14. Rosmaninho-Salgado J, Marques AP, Estrada M, Santana M, Cortez V, Grouzmann E, Cavadas C 2012 Dipeptidyl-peptidase-IV by cleaving neuropeptide Y induces lipid accumulation and PPAR-gamma expression. Peptides 37:49-54
15. Dobrian AD, Ma Q, Lindsay JW, Leone KA, Ma K, Coben J, Galkina EV, Nadler JL 2011 Dipeptidyl peptidase IV inhibitor sitagliptin reduces local inflammation in adipose tissue and in pancreatic islets of obese mice. Am J Physiol Endocrinol Metab 300(2):E410-E421
16. Miyagawa K, Kondo T, Goto R, Matsuyama R, Ono K, Kitano S, Kawasaki S, Igata M, Kawashima J, Matsumura T, Motoshima H, Araki E 2013 Effects of combination therapy with vildagliptin and valsartan in a mouse model of type 2 diabetes. Cardiovasc Diabetol 12:160-12
17. Derosa G, Carbone A, Franzetti I, Querci F, Fogari E, Bianchi L, Bonaventura A, Romano D, Cicero AF, Maffioli P 2012 Effects of a combination of sitagliptin plus metformin vs metformin monotherapy on glycemic control, beta-cell function and insulin resistance in type 2 diabetic patients. Diabetes Res Clin Pract 98(1):51-60
18. Eriksson-Hogling D, Andersson DP, Backdahl J, Hoffstedt J, Rossner S, Thorell A, Arner E, Arner P, Ryden M 2015 Adipose tissue morphology predicts improved insulin sensitivity following moderate or pronounced weight loss. Int J Obes (Lond) 39(6):893-898
19. Yang J, Eliasson B, Smith U, Cushman SW, Sherman AS 2012 The size of large adipose cells is a predictor of insulin resistance in first-degree relatives of type 2 diabetic patients. Obesity (Silver Spring) 20(5):932-938
20. Ikeda T, Kumagai E, Iwata S, Yamakawa A 2013 Soluble CD26/Dipeptidyl Peptidase IV Enhances the Transcription of IL-6 and TNF-alpha in THP-1 Cells and Monocytes. PLoS One 8:e66520
21. Shinjo T, Nakatsu Y, Iwashita M, Sano T, Sakoda H, Ishihara H, Kushiyama A, Fujishiro M, Fukushima T, Tsuchiya Y, Kamata H, Nishimura F, Asano T 2015 DPP-4 inhibitor anagliptin exerts anti-inflammatory effects on macrophages, adipocytes, and mouse livers by suppressing NF-kappaB activation. Am J Physiol Endocrinol Metabajpendo
22. Makdissi A, Ghanim H, Vora M, Green K, Abuaysheh S, Chaudhuri A, Dhindsa S, Dandona P 2012 Sitagliptin exerts an antinflammatory action. J Clin Endocrinol Metab 97(9):3333-3341
23. Carvalho E, Jansson PA, Axelsen M, Eriksson JW, Huang X, Groop L, Rondinone C, Sjostrom L, Smith U 1999 Low cellular IRS 1 gene and protein expression predict insulin resistance and NIDDM. FASEB J 13(15):2173-2178
24. Srinivas PR, Wagner AS, Reddy LV, Deutsch DD, Leon MA, Goustin AS, Grunberger G 1993 Serum alpha 2-HS-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. Mol Endocrinol 7(11):1445-1455
25. Youngren JF 2007 Regulation of insulin receptor function. Cell Mol Life Sci 64(7-8):873-891
26. Ussar S, Bezy O, Bluher M, Kahn CR 2012 Glypican-4 enhances insulin signaling via interaction with the insulin receptor and serves as a novel adipokine. Diabetes 61(9):2289-2298
27. Piazza GA, Callanan HM, Mowery J, Hixson DC 1989 Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. Biochem J 262:327-3

2.4. STUDY 4: IMPACT OF ADIPOSE TISSUE-SPECIFIC DPP4 DELETION

DPP4 DELETION IN ADIPOSE TISSUE IMPROVES HEPATIC INSULIN RESISTANCE IN DIET-INDUCED OBESITY

Tania Romacho *[1], Henrike Sell *[1,5], Ira Indrakusuma [1], Diana Roehrborn [1], Tamara R. Castañeda [2], Tomas Jelenik [3], Sonja Hartwig [2], Jürgen Weiß [2,5], Hadi Al-Hasani [2], Michael Roden [3,4,5], Jürgen Eckel[1,5].

*Co-first author.

[1]Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Germany
[2] Institute for Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Germany
[3]Institute for Clinical Diabetology, Leibniz Center for Diabetes Research at Heinrich Heine University Düsseldorf, Germany
[4]Department of Endocrinology and Diabetology, Medical Faculty, Heinrich-Heine University Düsseldorf, Germany
[5]German Center for Diabetes Research (DZD), München-Neuherberg, Germany

Submitted to: Cell reports

Summary

Besides a therapeutic target for type 2 diabetes, dipeptidyl peptidase 4 (DPP4) is an adipokine potentially linked to human obesity. The role of adipose tissue (AT)-derived DPP4 was explored generating an AT-specific KO mouse (AT-DPP4-KO). Under high fat diet (HFD), AT-DPP4-KO displayed reduced circulating DPP4, proving AT as a relevant source. Independently of glucose-stimulated incretin hormones, glucose tolerance and suppression of endogenous glucose production were improved. AT-DPP4-KO displayed smaller adipocytes, increased M2 macrophages and decreased AT fibrosis. IGF binding protein 3 (IGFBP3) levels were lower in AT and serum, while free IGF1 was increased. In type 2 diabetes patients treated with the DPP4 inhibitor sitagliptin, both free IGF1 and free/total ratio increased. During HFD, lack of adipose DPP4 triggers beneficial remodeling and decreases production of IGFBP3, resulting in increased free IGF1 and improved glucose tolerance and hepatic insulin resistance, supporting a key role of DPP4 in obesity-related metabolic disorders.

Highlights

- AT-DPP4-KO is a novel model which demonstrates that AT is a major source of soluble DPP4 in obesity.
- Under HFD, AT-DPP4-KO mice display improved glucose tolerance and hepatic insulin resistance, independently of glucose-stimulated incretin hormones.
- Lack of adipose DPP4 triggers favorable AT remodeling during HFD with smaller adipocytes, reduced fibrosis and increased M2 macrophage-markers.
- IGF binding protein 3 (IGFBP3) levels were reduced in AT and serum of AT-DPP4-KO mice, resulting in increased free IGF1 potentially linked to the observed improved hepatic insulin resistance.
- Analogously, type 2 diabetes patients treated with the DPP4 inhibitor sitagliptin, displayed increased serum levels of free IGF1 and free/total IGF ratio.
- Our model underpins a key role of DPP4 in obesity-related metabolic disorders.

Introduction

Obesity is reaching an epidemic dimension nowadays (Ng et al., 2014) and is intimately related to the development of insulin resistance (Wellen and Hotamisligil, 2003), characterized by decreased insulin-stimulated glucose uptake in muscle (DeFronzo and Tripathy, 2009) and adipose tissue (AT) (Guilherme et al., 2008), as well as by impaired insulin-mediated suppression of endogenous (hepatic) glucose production (EGP) (Perry et al., 2014). Consequently, obesity represents a major risk factor for type 2 diabetes (Hardy et al., 2012). In search for the mechanisms underlying these diseases, AT has been recognized as an important endocrine organ releasing very diverse bioactive factors named adipokines (Romacho et al., 2014). By an in-depth proteomic profiling of the secretome of primary human adipocytes, we identified dipeptidyl peptidase 4 (DPP4) as an adipokine (Lehr et al., 2012). DPP4 is a ubiquitous transmembrane glycoprotein and exoprotease which cleaves N-terminal dipeptides from a wide range of substrates including growth factors, neuropeptides and chemokines (Yazbeck et al., 2009). Of note, DPP4 also cleaves and inactivates the members of the incretin hormone familiy glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), which account for approximately up to 70% of postprandial insulin secretion (Nauck et al., 1986). Thus, DPP4 inhibitors are currently used as glucose-lowering agents to prolong the insulinotropic effect of incretins in type 2 diabetes. Besides its enzymatic activity, DPP4 was originally identified as the CD26 antigen in lymphocytes involved in T cell activation (Fleischer, 1994). Both, the abundance of the membrane-bound form and the activity of circulating soluble DPP4 are altered in a variety of inflammatory diseases, metabolic syndrome and diabetes mellitus (Rohrborn et al., 2015). Although a fraction of soluble DPP4 most likely originates from immune cells (Cordero et al., 2009), the major source of circulating DPP4 remains unknown.

In vitro, DPP4 knockdown and pharmacological inhibition in human adipocytes improved insulin signaling without affecting adipogenesis (Rohrborn et al., 2016). Studies in animals show that circulating DPP4 activity is higher in C57/BL6 mice under high fat diet (HFD) (Nistala et al., 2014) and in ob/ob mice (Ruter et al., 2004). On the contrary, genetic DPP4 deletion in Fischer rats and congenital DPP4 depletion in Dark Agouti rats resulted in improved glucose tolerance under HFD (Frerker et al., 2009; Yasuda et al., 2002). Furthermore, whole body DPP4 knockout (KO) mice display improved glucose tolerance, increased GLP-1 levels and prevented insulin resistance during HFD (Conarello et al., 2003; Marguet et al., 2000). We previously identified DPP4 as an adipokine that may be a missing link between increased AT mass in obesity and obesity-associated metabolic diseases. Since previous studies suggested that DPP4 may affect AT adipogenesis, inflammation and insulin sensitivity (Rohrborn et al., 2015), we aimed to explore the role of DPP4 within AT in the context of metabolic diseases. However, DPP4 pharmacological

inhibition and genetic deletion affect both central regulation of food intake and energy expenditure. Therefore, we have generated an AT specific DPP4 KO mouse (AT-DPP4-KO) in order to characterize the impact of AT-derived DPP4 in obesity.

Results

The AT-DPP4-KO mouse model

Cre expression was only detectable in mature adipocytes from KO animals (Fig 1A), where DPP4 protein levels were significantly reduced by up to 65 % (Fig. 1B) with unaltered DPP4 expression in the stromal vascular fraction (SVF) within AT (Fig. 1C). DPP4 protein levels in immune cells isolated by peritoneal lavage from AT-DPP4-KO mice remained similar to WT mice (Fig. 1D). This specific AT KO resulted in significantly lower serum DPP4 in KO animals on HFD (Fig 1E). As DPP4 KO was restricted to AT, we did not observe differences in incretin levels. Circulating active GLP-1 and GIP were similar between the genotypes both in basal conditions and after 15 min oral glucose load (Suppl. fig. 1). To further prove the specificity of our animal model we explored DPP4 enzymatic activity in different organs and tissues such as liver, pancreas, kidney, lung, heart, spleen, brain, ingWAT, epiWAT and BAT. DPP4 was only decreased in WAT and BAT (Fig. 1F).

Lack of DPP4 in AT improves glucose tolerance in mice under HFD in spite of increased body weight

After 24 weeks of HFD, AT-DPP4-KO mice gained more body weight compared to WT (41.9 ± 3.6 g vs. 56.7 ± 4.3 g; Fig. 2A). This was paralleled by an increase in fat and lean mass (Suppl.Fig. 1A-B). Analogously, the genotype did not affect EE (volume of oxygen consumed ($VO_2/kg^{0.75}/h$), respiratory quotient (RQ) and SPA (Suppl. Fig. 2 A-C). These differences in body weight, fat and lean mass were not due to differences in food intake normalized to body weight (Suppl. Fig. 2d). Body length was similar in both genotypes (Suppl. Fig. 2E). Furthermore, BAT mass and RQ was not affected by the genotype (Suppl. Fig. 2B).

During the oral glucose tolerance test, plasma glucose was lower during the first 60 minutes in KO animals compared to the diet-matched controls (Fig. 2B-C), while plasma insulin levels were significantly increased during the initial 30 minutes (Fig. 2D). HOMA-IR index was significantly lower in KO mice after 24 weeks HFD (Fig. 2E). The hyperinsulinemic-euglycemic clamps revealed increased suppression of endogenous glucose production (EGP) in KO animals after HFD (Fig. 2F). Whole body glucose disposal rate (Rd) and NEFA suppression were not different from WT (Suppl Fig. 3), indicating that AT-DPP4-KO mice exhibit selective improvement of hepatic insulin senstivity upon HFD. Of note, circulating DPP4 levels correlated negatively with hepatic insulin sensitivity, as assessed from suppression of EGP in WT animals (Fig 2G). Under chow diet, insulin sensitivity in AT-DPP4-KO mice was not different from KO.

Liver lipid content as assessed histologically (Supl. Fig 4A) or colorimetrically after extraction (Supl. Fig 4B) did not differ between the WT and KO animals. The KO animals under HFD had similar plasma triglycerides (Suppl. Fig. 4D) but significantly reduced cholesterol

plasma levels compared to their matched WT controls (Suppl. Fig. 4C).

DPP4 deletion in AT promotes remodeling

The 24-week HFD resulted in alterations of the morphology of epiWAT in both genotypes. Within the HFD group, the KO mice displayed a marked shift in the adipocyte size distribution towards smaller adipocytes in epiWAT (Fig. 3A). A comparison between AT-DPP4-KO animals and WT animals under HFD showed decreased mean adipocyte size in AT-DPP4-KO mice (Fig. 3B). Adipocyte size was positively correlated with circulating DPP4 levels (Fig. 3C), as previously shown for humans (Sell et al., 2013). In order to determine if the changes observed in adipocyte size were related to fibrosis, we investigated the expression of several fibrosis markers, namely *Col1A1*, *Col3A1* and *Col6A6*. All fibrosis markers were reduced in the epiWAT of KO animals (Fig. 3D-F). Adipocyte size was also substantially reduced in the ingWAT of KO under HFD and circulating DPP4 levels significantly correlated with adipocyte size in this depot (Suppl. Fig. 5C). However, the lack of DPP4 in adipocytes did not affect the expression of fibrosis markers in the subcutaneous fat depot (Suppl. Fig. 5D-F).

Since AT inflammation is involved in the development of insulin resistance, we also assessed macrophage infiltration in AT and the expression of the M2 anti-inflammatory macrophage markers vs. M1 pro-inflammatory markers. Crown-like structures (CLS) are formed as a result of macrophages infiltrating into AT to reabsorb dead adipocytes. Macrophage infiltration within AT was analyzed by counting galectin-3 positive CLS. Under HFD, the KO animals displayed significantly increased number of CLS only in the epiWAT (Fig 3G-H). In line with this observation, in the epiWAT, M1 pro-inflammatory markers IL-6 and chemokine (C-C Motif) ligand 2 (CCL2) were significantly increased in KO mice compared to WT animals under HFD (Fig. 3i). On the other hand, the anti-inflammatory M2 macrophage markers mannose receptor 1 (Mrc1) and interleukin (IL)-10 were also significantly upregulated in KO animals under HFD (Fig. 3J). Regarding the subcutaneous depot an increase of the M1 marker galectin-3 was observed although no differences in CLS were found in this depot (Suppl. Fig. 6A-B). Markers of adipogenesis such as adiponectin, PPARγ and GLUT4 were simultaneously increased in epiWAT (Fig. 3I) but remained unaltered in ingWAT (Suppl. Fig. 6D).

DPP4 deletion results in reduced circulating IGFBP3 and increased free IGF1 levels

In order to characterize how the lack of DPP4 in adipocytes affects adipokine release, we screened the adipokine profile in conditioned medium (CM) obtained from AT explants of animals after 24 weeks of HFD (data not shown) and gained preliminary evidence that IGFBP3 was a factor downregulated in the CM from epiWAT of KO animals. We observed that IGFBP3 release was reduced in the CM from explants of epiWAT of KO animals under HFD in parallel to reduced IGFBP3 gene expression (Fig. 4A-B). This was paralleled by reduced circulating levels in the KO animals which underwent HFD (Fig. 4C). Furthermore, KO animals after HFD displayed increased free IGF1

circulating levels (Fig. 4D), although no changes were observed among total IGF1 levels (Fig. 4E).

IGFBP3 impairs insulin signaling and insulin-stimulated suppression of glucose production in HepG2 cells

In order to further study the effects IGFBP3 on insulin sensitivity in hepatocytes, HepG2 cells were treated with growing concentrations of IGFBP3, which resulted in increasing impairment of insulin-stimulated Akt phosphorylation at Thr308 but not at Ser473 (Fig. 5A). IGFBP3 (300 ng/ml) reduced insulin stimulated Akt phosphorylation at Thr308 by approximately 20% (Fig. 5B) while Akt phosphorylation at Ser473 remained unaltered (Fig. 5C). Finally, IGFBP3 (300 ng/ml and 1 μg/ml) resulted in complete abrogation of insulin-stimulated suppression of glucose production by hepatocytes (Fig. 5D).

DPP4 inhibition in patients with type 2 diabetes increases free IGF1 circulating concentrations

IGFBP3, free IGF1, total IGF1, DPP4 protein and DPP4 activity were measured in serum samples obtained from recent onset metformin-treated type 2 diabetes patients with or without treatment with the DPP4 inhibitor sitagliptin. Both groups of patients had comparable anthropometric and and metabolic parameters. However, patients on sitagliptin treatment displayed significantly lower DPP4 activity in serum with comparable amounts of DPP4 protein in circulation (Table 1). Circulating IGFBP3 concentrations were similar in both groups (Fig. 6A). However, patients treated with sitagliptin had significantly higher serum levels of free IGF1 and total IGF1 (Fig. 6B-C). In addition, the free IGF1/total IGF1 ratio was higher in patients receiving sitagliptin (Fig. 6D). Total IGF1 correlated positively with free IGF1 ($r=0.50$, $p=0.0002$) and negatively with hs-CRP ($r=-0.31$, $p=0.02$). The free IGF1/total IGF1 ratio declined with aging ($r=0.30$, $p=0.04$). Of note, IGFBP3 correlated with cholesterol ($r=0.56$, $p<0.0001$), LDL-cholesterol ($r=0.45$, $p=0.001$), serum triglycerides ($r=0.44$, $p=0.002$) and γGT ($r=0.31$, $p=0.03$). DPP4 was positively related to liver transaminases (ALT $r=0.54$, $p<0.0001$; AST $r=0.40$, $p=0.004$; γGT $r=0.40$, $p=0.004$).

Discussion

The novel mouse model of adipose-specific knockout of DPP4-KO demonstrates that AT is a major source of circulating DPP4 in obesity neither affecting basal nor glucose-stimulated incretin hormone secretion. When fed a HFD, AT-DPP4-KO mice display improved glucose tolerance and hepatic insulin resistance. DPP4 depletion affects AT itself through a favorable remodeling during HFD with smaller adipocytes, reduced fibrosis and increased M2 macrophage-mediated inflammation.

Our group has previously provided the first evidence for DPP4 being an adipokine (Lamers et al., 2011) and demonstrated that DPP4 is upregulated in human obesity and the metabolic syndrome (Sell et al., 2013). AT-DPP4-KO mice prove that AT is an important source of circulating DPP4 in the obese state. Here, we provide the first comparison of DPP4 enzymatic activity in AT in relation to other organs such as the liver, pancreas or the kidney, which may represent sources of circulating DPP4 (Hildebrandt

et al., 1991; Kirino et al., 2009; Shinjo et al., 2015). Of note, except from liver, all other tested tissues are probably only minor contributors to circulating DPP4 due to their relative low mass compared to enlarged AT. The tissue specificity of our model compared to previously described global DPP4 KO models (Conarello et al., 2003; Marguet et al., 2000) allows us to detect the impact of DPP4 deletion within AT and dissect these effects from incretin-mediated actions, since GIP and active GLP-1 did not differ among the genotypes. Therefore, the effects of DPP4 deletion in AT under HFD on body composition, glucose tolerance and hepatic insulin sensitivity occur independent of the incretin system which is not affected by adipose KO of DPP4. In global DPP4 KO mice, active GLP-1 and insulin are significantly increased after an oral glucose load (Marguet et al., 2000). Furthermore, global KO mice display significantly lower food intake resulting in almost complete resistance to diet-induced obesity (Conarello et al., 2003). But not only food intake is responsible for this resistance to HFD, as demonstrated by pair feeding experiments, global KO mice are also characterized by higher oxygen consumption and increased expression of UCP-1 in BAT. Thus, central effects of global DPP4 KO on food intake and increased BAT activity can be attributed to DPP4 depletion in combination with a boosted incretin system. DPP4 ablation only in AT did not affect food intake nor oxygen consumption and occurred without affecting the incretin system which makes our novel model independent from central effects of DPP4 and incretin actions.

AT-DPP4-KO mice display improved glucose tolerance under HFD compared to controls despite higher body weight and fat mass. Furthermore, insulin secretion in the initial phase of oGTTs and HOMA-IR was improved in AT-DPP4 KO mice on HFD compared to WT mice. In fact, deletion of DPP4 in AT seems to play a role specifically for hepatic insulin sensitivity. Compared to WT mice on HFD, AT-DPP4-KO mice displayed significantly increased insulin-mediated suppression of EGP, but no difference in basal EGP, insulin-mediated suppression of NEFA and insulin-stimulated whole body glucose disposal. These data indicate that improved insulin action in the liver is the key metabolic feature of AT-DPP4-KO mice on HFD. Impaired insulin-mediated suppression of EGP is an early feature of insulin resistant states and determines the progression to impaired glucose metabolism in obese humans (Ter Horst et al., 2016). Abnormal suppression of EGP further contributes to hyperglycemia in type 2 diabetes mellitus (Mittelman et al., 1997). One may speculate that DPP4 overexpression could contribute to the pathogenesis of hepatic insulin resistance and type 2 diabetes and that its deletion in AT may therefore delay the development of type 2 diabetes. The mechanism by which AT-DPP4-KO affects hepatic insulin sensitivity is still unknown. We can exclude a contribution of NEFA and circulating adiponectin (data not shown) as these parameters were not affected by the KO, while IGFBP3 and IGF1 may be the mediators for improved hepatic insulin

sensitivity. Release from AT and circulating levels of IGFBP3 were reduced in KO mice under HFD. In addition to decreased IGFBP3, free IGF1 was specifically increased in serum of KO mice under HFD. IGFBP3 is one of the main circulating IGFBP species found in serum and binds approximately 90% of circulating IGF1 (Jogie-Brahim et al., 2009). Both IGFBP3 and IGF1 are increased in patients with impaired glucose tolerance and type 2 diabetes (Kim and Lee, 2015). Additionally, IGFBP3 overexpression in transgenic mice impairs insulin secretion resulting in fasting hyperglycemia, impaired glucose tolerance, and insulin resistance (Nguyen et al., 2011; Silha et al., 2002). Differently, whole body KO of IGFBP3 results in a complex metabolic phenotype with higher body growth and weight due to increased growth hormone concentrations and decreased resting metabolic rate compared to controls (Yamada et al., 2010). Furthermore, IGFBP3 KO animals display increased basal glucose turnover but preserved insulin sensitivity and reduced WAT mass. However, it should be noted that complete absence of IGFBP3 is not comparable with our model where IGFBP3 is only reduced, total IGF1 unaltered and animal growth normal. In our KO model, reduced IGFBP3 levels occur in parallel to higher free IGF1 which is known to suppress hepatic glucose production (Laager et al., 1993). Alternatively, IGFBP3 could also regulate hepatic glucose production independently from IGF1 as previously shown (Muzumdar et al., 2006). In fact, surface association of IGFBP3 on hepatocytes has been demonstrated by immunohistochemistry (Hallak et al., 2002). Furthermore, treatment of adipocytes with IGFBP3 resulted in impaired insulin signaling and reduced insulin-stimulated glucose uptake (Chan et al., 2005). Here, we also provide evidence that IGFBP3 also impairs insulin signaling and insulin-stimulated suppression of glucose production by hepatocytes. In this study, reduced IGFBP3 levels could therefore contribute to improved hepatic insulin sensitivity in two different ways, first by regulating the bioavailability of IGF1 in the form of increased free IGF1, and second by direct hepatic effects of IGFBP3 independent of IGF1. Taken together, we propose that the reduced release of IGFBP3 from visceral AT might mediate a protective crosstalk between AT and liver in AT-DPP4-KO mice on HFD.

Adipocyte size, a surrogate marker of insulin resistance (Cotillard et al., 2014), was reduced in both subcutaneous and visceral fat of AT-DPP4-KO mice on HFD. Our results are in line with our previous report in humans, where circulating DPP4 positively correlated with adipocyte size (Sell et al., 2013). In diet-induced obesity, DPP4 inhibitors have been proven to reduce adipocyte size (Chae et al., 2015). However, solely in the epiWAT of KO mice under HFD, we observed a trend towards increased markers of adipogenesis potentially suggesting hyperplasia. This is in line with our findings reported in human subcutaneous adipocytes, where DPP4 knockdown did not affect adipogenesis (Rohrborn et al., 2016). We discarded AT fibrosis as the cause of the reduced adipocyte size. Thus, fibrosis was also solely reduced in epiWAT from AT-DPP4-KO mice under HFD. Since AT fibrosis has been linked to insulin resistance (Sun et al., 2013), we hypothesize that reduced fibrosis in visceral fat may represent an

additional mechanism for DPP4 deficiency to promote a protective remodeling in AT during diet-induced obesity. Although chronic inflammation in AT links obesity to its complications, there is growing evidence that pro-inflammatory signaling is a prerequisite for AT remodeling and expansion (Wernstedt et al., 2014). We employed galectin-3 to quantify CLS in AT, which besides being a M1 macrophage marker, has been recently proposed as an indicator of phagocytosis of apoptotic cells (MacKinnon et al., 2008). Thus, galectin-3 KO mice exhibit impaired expansion of AT under diet-induced obesity resulting in increased hyperglycemia and glucose intolerance under HFD (Darrow and Shohet, 2015). Therefore, increased galectin-3 positive macrophages in epiWAT from KO mice may contribute to a beneficial AT expansion under HFD. As mentioned, IGFBP3 levels were reduced in both CM from epiWAT and serum from KO animals. IGFBP3 deletion has been shown to improve differentiation in adipocytes via direct interaction with PPARγ, while both administration of exogenous IGFBP3 and overexpression inhibits adipogenesis in 3T3-L1 (Jogie-Brahim et al., 2009). Therefore, we propose that reduced IGFBP3 levels in AT-DPP4 KO mice promotes a beneficial remodeling during HFD, characterized by reduced hypertrophy, fibrosis and inflammation while adipogenesis is increased.

Of note, also in humans with type 2 diabetes DPP4 inhibition increases free and total IGF1 concentrations in serum. Although not directly comparable to our mouse model, where circulating DPP4 is reduced and incretin levels unaltered, these data provide additional evidence for a link between DPP4 and the IGF1/IGFBP3 axis. Recombinant IGF1 and combinations of recombinant IGF1 with IGFBP3 have been previously discussed as treatment options for type 2 diabetes (Ranke, 2005). However, the long term potential of this therapy has not been further developed due to various side effects and the association of very high IGF1 levels with prostate and breast cancer (Renehan et al., 2004). Nevertheless, IGF1 has been reported to exert vasoprotective effects not only in type 2 diabetes (Ezzat et al., 2008) and lower IGF1 serum concentrations are associated with diabetes (Teppala and Shankar, 2010). Since pharmacological interventions increasing circulating IGF1 have not been reported so far, the observed increase in serum free IGF1 and total IGF1 exerted by the DPP4 inhibitor sitagliptin may represent a novel therapeutic target in type 2 diabetes. It can be speculated that increased IGF1 concentrations could be linked to altered hepatic glucose production in patients treated with DPP4 inhibitors. In fact, sitagliptin improves the suppression of EGP in dogs (Edgerton et al., 2009) and in humans (Muscelli et al., 2012). Also vildagliptin triggers a better suppression of EGP (Balas et al., 2007) or lower basal EGP in patients (Vella et al., 2007).

Future studies will help to clarify how DPP4 deletion in AT can be translated into a therapeutic tool in metabolic diseases. This is of special interest in the case of obese type 2 diabetic patients which are currently treated with DPP4 inhibitors. How exactly DPP4 inhibitors

might also affect AT biology and hepatic insulin sensitivity in addition to their incretin-mediated effects remains fully unexplored. Since DPP4 is an adipokine upregulated in adipose tissue in obesity both in human and rodents, AT arises as an important and novel source of DPP4 and as a new therapeutic target for DPP4 inhibitors.

Experimental procedures

Materials

Reagents for SDS-PAGE were supplied by GE Healthcare (Freiburg, Germany) and Sigma-Aldrich (Munich, Germany). Antibodies against murine DPP4, CD11b and β-actin were supplied from Abcam (Cambridge, U.K.) and pAkt Ser473 and Thr308 from New England Biolabs (Frankfurt, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG anti-bodies were supplied by Promega (Mannheim, Germany). Complete protease inhibitor cocktail and PhosStop phosphatase inhibitor cocktail were provided by Roche (Mannheim, Germany). Unless stated elsewhere, all reagents were purchased from Sigma-Aldrich.

Mouse model

The adipose-specific DPP4 knockout (AT-DPP4-KO) mice have been generated by a Cre-lox strategy based on deletion of exons 1 and 2 of the DPP4 gene and crossbreeding with B6.Cg-Tg(Fabp4 cre)1Rev/J mice (Jackson Laboratories), expressing Cre-recombinase under the control of the Fabp4 promoter. Genetic deletion of DPP4 was confirmed at the DNA level by gel electrophoresis and also at the RNA and protein level by real-time PCR and Western blotting.

C57BL/6J floxed mice and adipose-specific DPP4 knockout male mice were housed in mixed groups of maximum 5 animals per cage under a light/dark cycle (12 h/12 h), in a temperature controlled room (22 ºC) with standard food and water *ad libitum*. At 12 weeks of age animals were randomly allocated to standard chow diet or HFD (60% fat; Research diets D12492) for 24 weeks. All procedures followed the principles outlined in the European Commission Council Directive for the care and use of laboratory animals (86/609/EEC). Animal experiments were approved by the Ethics Committee of the State Agency for Nature, Environment and Consumer Protection (LANUV, North Rhine-Westphalia, Germany) and conducted at the animal facility of the German Diabetes Center.

DPP4 activity in serum, organs and tissues

DPP4 activity was measured with a fluorometric kit from Sigma Aldrich. 10 µl of serum was assayed as detailed by the manufacturer. 10 mg of frozen tissues were homogenized in cold assay buffer. Following homogenization, the samples were centrifuged at 13000 *rpm* for 15 min at 4°C. The resulting supernatants were collected, diluted 1:10 and were immediately used for DPP4 activity fluorometric assay following manufacturer's instructions. The resulting fluorescent signal was detected with a microplate reader (Infinite M200, Tecan). DPP4 enzymatic activity in each tissue was expressed as the amount of cleaved AMC per minute per total tissue (µU/ tissue) at 37°C.

Western blot

5 µg of proteins were separated by SDS-PAGE using 10% horizontal gels. The proteins were then transferred to a polyvinylidene difluoride membrane (Millipore) in a semidry blotting system. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween for 1 h at room temperature and later incubated with the indicated primary antibodies. After washing with TBST, membranes were incubated with a secondary horseradish peroxidase (HRP)-coupled antibody and processed for chemiluminescence detection using Immobilon HRP substrate (Millipore). Signals were visualized and analyzed on a Bio-Rad VersaDoc 4000 MP work station.

Peritoneal lavage

Mice were sacrificed by cervical dislocation and the peritoneum was flushed with cold PBS with 5% FCS in order to harvest peritoneal cells. The resulting peritoneal lavage was resuspended in 0.4 ml MACS buffer (1× PBS, 2% FCS, 5 mM EDTA). For Western blot analysis about 3×10^6 cells were resuspended in lysis buffer and stained with anti-CD11b (ab53187 Abcam) or a DPP4 antibody.

Body composition

Longitudinal changes in body composition of all animals were assessed using quantitative nuclear magnetic resonance imaging (Whole Body Composition Analyzer; Echo MRI, Texas, USA).

Body composition was measured at the beginning of the diet and at every 4 weeks.

Hyperinsulinemic-euglycemic clamp test

At 4-6 days before the clamp, an indwelling silicon catheter (Silastic Laboratory Tubing, Dow Corning, Midland, MI) was placed in the right-side jugular vein of the animals under isoflurane anesthesia (CP Pharma, Burgdorf, Germany), as previously described (Jelenik et al., 2014). After recovering from surgery, the mice were fasted during the 6 h prior to the clamp, with free access to drinking water. In order to assess basal glucose disposal, D-[6,6-$^2H_2$]glucose (98% enriched) (Cambridge Isotope Laboratories, Andover, MA) was infused at a rate of 4 μmol/kg/min for 120 min. The hyperinsulinemic-euglycemic clamp was performed with a primed (40 mU/kg), continuous infusion of insulin (10 mU/kg/min) (Huminsulin; Lilly, Giessen, Germany) for 180 min. Euglycemia was maintained by periodically adjusting a variable 20% glucose infusate. D-[6,6-$^2H_2$] glucose was co-infused together with insulin (0.4 μmol/kg/min) and variable glucose infusion to obtain stable tracer concentrations during varying glucose infusion rates (GIR expressed as "mg glucose/ (kg body weight x minute)). Blood samples were taken at 10 min intervals during the basal period and during the last 30 min of the hyperinsulinemic-euglycemic clamps. After the clamps, mice were exsanguinated through cervical incision and killed by cervical dislocation, serum and organs were collected for future analysis.

Glucose tolerance tests

For oral glucose tolerance tests (oGTT), a glucose solution (2 g/kg body weight, 40% solution in 0.9% wt/vol. NaCl) was given by gavage into 16 h fasted animals. Tail blood glucose levels were measured with a glucometer (Free Style Precision Abbot, Wiesbaden, Germany) at 0, 15, 30, 45, 60, and 120 minutes after gavage.

Adipose tissue immunohistochemistry analysis

Pieces of AT were collected from WT and AT-DPP4-KO mice after 24 weeks of HFD or chow diet. The tissues were fixed in 10% formalin and then dehydrated in ethanol followed by paraffin embedding. A total of 200 adipocytes from 3 different levels of the tissue (100 μm distance between levels) were analyzed per animal and depot, respectively.

a) Adipocyte size

Adipocyte area was measured by a blinded observer at a 10-fold magnification with Cellsense software (Olympus SIS). Adipocyte sizes were clustered in corresponding cell size groups and represented as percentage of cells in each size class.

b) Crown-like structures (CLS)

10-μm dewaxed serial sections were incubated with anti-mouse Mac-2/galectin-3 (Cedarlane Laboratories, Canada) followed by incubation with a biotinylated HRP-conjugated secondary antibody goat anti-rat IgG (Mac-2/galectin-3; Vector Laboratories; Burlingame, CA, USA). Histochemical reactions were performed using Vector's Vectastain ABC Kit and diaminobenzidine as substrate. Sections were counterstained with hematoxylin-eosin and mounted in coverslips. CLS structures per 1000 cells were counted by a blinded observer with Image J software in microphotographs obtained at a 40-fold magnification.

Generation of conditioned media from AT explants

Explants from ingWAT and epiWAT from animals fed HFD for 24 weeks were collected in sterile cold PBS. Connective tissue and vessels were removed and fat pads were cut into pieces of 10 mg. After three times washing and centrifugation in fresh PBS supplemented with an antibiotic and antimycotic mix (Life Technologies) explants were weighted and 100 mg of adipose tissue were incubated in in low-glucose DMEM smooth muscle cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 50 mg/ml gentamycin for 24h at 37°C and 5%CO_2 to generate 1 ml conditioned medium.

Real-time quantitative PCR (RT-qPCR).

Total RNA was extracted from frozen tissue samples using TRIzol Reagent (Ambion Life Technologies, Darmstadt, Germany) and RNeasy purification kit (Qiagen, Hilden, Germany) and cDNA was transcribed using 1 μg of total RNA (Omniscript reverse transcription kit, Qiagen).

qPCR was performed using QuantiFast SYBR Green PCR kit (Qiagen). mRNA expression was measured by RT-qPCR with a Step One Plus Real-Time PCR System (AB Applied Biosystems). Values were normalized to the concentration of the housekeeping gene 18S in each sample. mRNA expression was measured in epiWAT and ingWAT from WT and AT-DPP4-KO mice as previously described (Schmittgen and Livak, 2008). The following predesigned primers were used: *Mm_Dpp4_1_SG, Mm_Il10_1_SG, Mm_Adipoq_1_SG, Mm_Ccl2_1_SG, Mm_Il6_1_SG, Mm_Col1a1_1_SG, Mm_Col3a1_2_SG Mm_Col6a6_1_SG, Mm_Lgals_3_SG, Mm_Loxl1_2_SG Mm_Mrc_1_SG, Mm_Pparg_1_SG, Mm_Slc2a4_1_SG, Mm_Tnf_1_SG* (Applied Biosystems). Customized primers were purchased from Eurofins and had the following sequences *Cre fwd*: GATTCGACCAGGTTCGTTC *Cre rev*: GCTAACCAGCGTTTTCGT. *UCP-1 rev*: CTTTGCCTCACTCAGGAT; *UCP-1 fwd:* ACTGCCACACCTCCAGTC

Hormone and biochemical assays

Plasma insulin was measured with a mouse insulin ELISA immunoassay (Mercodia, Uppsala, Sweden). Mouse DPP4 (Boster), IGBP3, total IGF1 (R&D systems) and free IGF1 (abcam, United Kingdom) were measured by ELISA. Human IGFBP3, free and total IGF1 were measured using ELISA kits from R&D Systems. Human DPP4 was monitored by ELISA (Abnova).

HepG2 culture and glucose production assay

HepG2 cells were obtained from ATCC to study hepatocytes and checked for mycoplasma contamination. Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 1x Antibiotic-Antimycotic mix (Gibco). Cells were maintained at 37 °C with humidified air and CO2 (5%). At subconfluence, cells were treated with 300 ng/ml human recombinant IGFBP3 (Biotechne) for 18 h. Cells were washed two times with PBS to remove glucose and incubated for 3 h in glucose production medium (glucose- and phenol red-free DMEM, 20 mM sodium lactate, 2 mM sodium pyruvate) in the presence or absence of 100 nM insulin (Sigma) during 10 min. A quantity of 150 µl of medium was sampled for measurement of glucose concentration using a colorimetric glucose assay kit (Sigma). Glucose concentration was normalized with cellular protein concentration.

Patients

The study was approved by the ethics committee of the Heinrich Heine University Düsseldorf and was conducted in accordance with the Declaration of Helsinki. All participants provided a written informed consent. All patients were participants of the prospective German Diabetes Study (GDS), which evaluates the long-term course of diabetes (Szendroedi et al., 2016). Inclusion criteria for entry into the GDS were type 2 diabetes, known diabetes duration ≤1 year, and 18–69 years of age. Exclusion criteria were: secondary diabetes, pregnancy, severe diseases (cancer), psychiatric disorders, immunosuppressive therapy and limited cooperation ability. Fasting serum samples were obtained from patients treated with sitagliptin in addition to metformin (n=17) and patients treated only with metformin (n=33) matched for age, body max index (BMI) and sex.

Statistics

Statistical analysis was performed with the GraphPad Prism software (La Jolla, CA, USA). *p* values were calculated using the two-tailed Student's *t*-test. For statistical comparisons between experimental groups, two-way ANOVA was used, followed by Bonferroni *post hoc* test. A *p* value below 0.05 was considered statistically significant. Data are shown as the mean ± S.E.M or mean ± S.D if specified. Normal distribution and equal variances were trested for each analysis. Animals were randomly allocated to the different diets and at least three animals were included in each experimental group. Groups size was calculated by Simple Interactive Statistical Analysis.

Acknowledgments

The authors thank Alexander Strom from the Institute for Clinical Diabetology, German Diabetes Center, Düsseldorf, for his help and technical advice. The secretarial assistance of Birgit Hurow and the technical help of Andrea Cramer, Waltraud Paßlack and Kay Jeruschke are gratefully acknowledged. We would also like to thank Pavel Bobrov for matching of the patients. This work was supported by the German Federal Ministry of Health

(Germany) and the Ministry of Innovation, Science, and Research of the State North Rhine-Westphalia (Germany), an EFSD-MSD grant, the Commission of the European Communities (Collaborative Project ADAPT, Contract No. HEALTH-F2-2008-201100), the Helmholtz Alliance to Universities: Imaging and Curing Environmental Metabolic Diseases (ICEMED), the German Research Foundation (DFG, SFB 1116), German Diabetes Association (DDG), Schmutzler Stiftung and the German Center for Diabetes Research (DZD e.V.). TR was the recipient of a Marie Curie Intra-European Fellowship (2012-IEF- 328793ADDIO).

Author contributions

TR wrote the manuscript; TR, HS, MR and JE designed experiments; TR, HS, DR and II performed experiments; TRC collaborated in setting oGTT and performed the analysis of the data of the metabolic cages; TJ performed hyperinsulinemic-euglycemic clamps; TR, HS, DR, II, TRC, TJ, HA-H, JE and MR discussed the manuscript, SH performed Luminex experiments, JW trained for immunohistochemistry.

References

1. Balas, B., Baig, M.R., Watson, C., Dunning, B.E., Ligueros-Saylan, M., Wang, Y., He, Y.L., Darland, C., Holst, J.J., Deacon, C.F., et al. (2007). The dipeptidyl peptidase IV inhibitor vildagliptin suppresses endogenous glucose production and enhances islet function after single-dose administration in type 2 diabetic patients. J Clin Endocrinol Metab *92*, 1249-1255.
2. Chae, Y.N., Kim, T.H., Kim, M.K., Shin, C.Y., Jung, I.H., Sohn, Y.S., and Son, M.H. (2015). Beneficial Effects of Evogliptin, a Novel Dipeptidyl Peptidase 4 Inhibitor, on Adiposity with Increased Ppargc1a in White Adipose Tissue in Obese Mice. PLoS. One *10*, e0144064.
3. Chan, S.S., Twigg, S.M., Firth, S.M., and Baxter, R.C. (2005). Insulin-like growth factor binding protein-3 leads to insulin resistance in adipocytes. J. Clin. Endocrinol. Metab *90*, 6588-6595.
4. Conarello, S.L., Li, Z., Ronan, J., Roy, R.S., Zhu, L., Jiang, G., Liu, F., Woods, J., Zycband, E., Moller, D.E., et al. (2003). Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. Proc. Natl. Acad. Sci. U. S. A *100*, 6825-6830.
5. Cordero, O.J., Salgado, F.J., and Nogueira, M. (2009). On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol. Immunother *58*, 1723-1747.
6. Cotillard, A., Poitou, C., Torcivia, A., Bouillot, J.L., Dietrich, A., Kloting, N., Gregoire, C., Lolmede, K., Bluher, M., and Clement, K. (2014). Adipocyte size threshold matters: link with risk of type 2 diabetes and improved insulin resistance after gastric bypass. J. Clin. Endocrinol. Metab *99*, E1466-E1470.
7. Darrow, A.L., and Shohet, R.V. (2015). Galectin-3 deficiency exacerbates hyperglycemia and the endothelial response to diabetes. Cardiovasc. Diabetol *14*, 73.
8. DeFronzo, R.A., and Tripathy, D. (2009). Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care *32 Suppl 2*, S157-S163.
9. Edgerton, D.S., Johnson, K.M., Neal, D.W., Scott, M., Hobbs, C.H., Zhang, X., Duttaroy, A., and Cherrington, A.D. (2009). Inhibition of dipeptidyl peptidase-4 by vildagliptin during glucagon-like Peptide 1 infusion increases liver glucose uptake in the conscious dog. Diabetes *58*, 243-249.
10. Ezzat, V.A., Duncan, E.R., Wheatcroft, S.B., and Kearney, M.T. (2008). The role of IGF-I and its binding proteins in the

development of type 2 diabetes and cardiovascular disease. Diabetes Obes Metab *10*, 198-211.

11. Fleischer, B. (1994). CD26: a surface protease involved in T-cell activation. Immunol. Today *15*, 180-184.
12. Frerker, N., Raber, K., Bode, F., Skripuletz, T., Nave, H., Klemann, C., Pabst, R., Stephan, M., Schade, J., Brabant, G., et al. (2009). Phenotyping of congenic dipeptidyl peptidase 4 (DP4) deficient Dark Agouti (DA) rats suggests involvement of DP4 in neuro-, endocrine, and immune functions. Clin. Chem. Lab Med *47*, 275-287.
13. Guilherme, A., Virbasius, J.V., Puri, V., and Czech, M.P. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat. Rev. Mol. Cell Biol *9*, 367-377.
14. Hallak, H., Moehren, G., Tang, J., Kaou, M., Addas, M., Hoek, J.B., and Rubin, R. (2002). Epidermal growth factor-induced activation of the insulin-like growth factor I receptor in rat hepatocytes. Hepatology *36*, 1509-1518.
15. Hardy, O.T., Czech, M.P., and Corvera, S. (2012). What causes the insulin resistance underlying obesity? Curr. Opin. Endocrinol. Diabetes Obes *19*, 81-87.
16. Hildebrandt, M., Reutter, W., and Gitlin, J.D. (1991). Tissue-specific regulation of dipeptidyl peptidase IV expression during development. Biochem. J *277 (Pt 2)*, 331-334.
17. Jelenik, T., Sequaris, G., Kaul, K., Ouwens, D.M., Phielix, E., Kotzka, J., Knebel, B., Weiss, J., Reinbeck, A.L., Janke, L., et al. (2014). Tissue-specific differences in the development of insulin resistance in a mouse model for type 1 diabetes. Diabetes *63*, 3856-3867.
18. Jogie-Brahim, S., Feldman, D., and Oh, Y. (2009). Unraveling insulin-like growth factor binding protein-3 actions in human disease. Endocr. Rev *30*, 417-437.
19. Kim, M.S., and Lee, D.Y. (2015). Insulin-like growth factor (IGF)-I and IGF binding proteins axis in diabetes mellitus. Ann. Pediatr. Endocrinol. Metab *20*, 69-73.
20. Kirino, Y., Kamimoto, T., Sato, Y., Kawazoe, K., Minakuchi, K., and Nakahori, Y. (2009). Increased plasma dipeptidyl peptidase IV (DPP IV) activity and decreased DPP IV activity of visceral but not subcutaneous adipose tissue in impaired glucose tolerance rats induced by high-fat or high-sucrose diet. Biol. Pharm. Bull *32*, 463-467.
21. Laager, R., Ninnis, R., and Keller, U. (1993). Comparison of the effects of recombinant human insulin-like growth factor-I and insulin on glucose and leucine kinetics in humans. J. Clin. Invest *92*, 1903-1909.
22. Lamers, D., Famulla, S., Wronkowitz, N., Hartwig, S., Lehr, S., Ouwens, D.M., Eckardt, K., Kaufman, J.M., Ryden, M., Muller, S., et al. (2011). Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes *60*, 1917-1925.
23. Lehr, S., Hartwig, S., Lamers, D., Famulla, S., Muller, S., Hanisch, F.G., Cuvelier, C., Ruige, J., Eckardt, K., Ouwens, D.M., et al. (2012). Identification and validation of novel adipokines released from primary human adipocytes. Mol. Cell Proteomics *11*, M111.
24. MacKinnon, A.C., Farnworth, S.L., Hodkinson, P.S., Henderson, N.C., Atkinson, K.M., Leffler, H., Nilsson, U.J., Haslett, C., Forbes, S.J., and Sethi, T. (2008). Regulation of alternative macrophage activation by galectin-3. J. Immunol *180*, 2650-2658.
25. Marguet, D., Baggio, L., Kobayashi, T., Bernard, A.M., Pierres, M., Nielsen, P.F., Ribel, U., Watanabe, T., Drucker, D.J., and Wagtmann, N. (2000). Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proc. Natl. Acad. Sci. U. S. A *97*, 6874-6879.
26. Mittelman, S.D., Fu, Y.Y., Rebrin, K., Steil, G., and Bergman, R.N. (1997). Indirect effect of insulin to suppress endogenous glucose production is dominant, even with hyperglucagonemia. J. Clin. Invest *100*, 3121-3130.
27. Muscelli, E., Casolaro, A., Gastaldelli, A., Mari, A., Seghieri, G., Astiarraga, B., Chen, Y., Alba, M., Holst, J., and Ferrannini, E. (2012). Mechanisms for the antihyperglycemic effect of sitagliptin in patients with type 2 diabetes. J Clin Endocrinol Metab *97*, 2818-2826.
28. Muzumdar, R.H., Ma, X., Fishman, S., Yang, X., Atzmon, G., Vuguin, P., Einstein, F.H.,

Hwang, D., Cohen, P., and Barzilai, N. (2006). Central and opposing effects of IGF-I and IGF-binding protein-3 on systemic insulin action. Diabetes *55*, 2788-2796.

29. Nauck, M.A., Homberger, E., Siegel, E.G., Allen, R.C., Eaton, R.P., Ebert, R., and Creutzfeldt, W. (1986). Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses. J. Clin. Endocrinol. Metab *63*, 492-498.
30. Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E.C., Biryukov, S., Abbafati, C., Abera, S.F., et al. (2014). Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet *384*, 766-781.
31. Nguyen, K.H., Yao, X.H., Moulik, S., Mishra, S., and Nyomba, B.L. (2011). Human IGF binding protein-3 overexpression impairs glucose regulation in mice via an inhibition of insulin secretion. Endocrinology *152*, 2184-2196.
32. Nistala, R., Habibi, J., Lastra, G., Manrique, C., Aroor, A.R., Hayden, M.R., Garro, M., Meuth, A., Johnson, M., Whaley-Connell, A., et al. (2014). Prevention of obesity-induced renal injury in male mice by DPP4 inhibition. Endocrinology *155*, 2266-2276.
33. Perry, R.J., Samuel, V.T., Petersen, K.F., and Shulman, G.I. (2014). The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. Nature *510*, 84-91.
34. Ranke, M.B. (2005). Insulin-like growth factor-I treatment of growth disorders, diabetes mellitus and insulin resistance. Trends Endocrinol Metab *16*, 190-197.
35. Renehan, A.G., Zwahlen, M., Minder, C., O'Dwyer, S.T., Shalet, S.M., and Egger, M. (2004). Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet *363*, 1346-1353.
36. Rohrborn, D., Bruckner, J., Sell, H., and Eckel, J. (2016). Reduced DPP4 activity improves insulin signaling in primary human adipocytes. Biochem. Biophys. Res. Commun *471*, 348-354.
37. Rohrborn, D., Wronkowitz, N., and Eckel, J. (2015). DPP4 in Diabetes. Front Immunol *6*, 386.
38. Romacho, T., Elsen, M., Rohrborn, D., and Eckel, J. (2014). Adipose tissue and its role in organ crosstalk. Acta Physiol (Oxf) *210*, 733-753.
39. Ruter, J., Hoffmann, T., Demuth, H.U., Moschansky, P., Klapp, B.F., and Hildebrandt, M. (2004). Evidence for an interaction between leptin, T cell costimulatory antigens CD28, CTLA-4 and CD26 (dipeptidyl peptidase IV) in BCG-induced immune responses of leptin- and leptin receptor-deficient mice. Biol. Chem *385*, 537-541.
40. Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc *3*, 1101-1108.
41. Sell, H., Bluher, M., Kloting, N., Schlich, R., Willems, M., Ruppe, F., Knoefel, W.T., Dietrich, A., Fielding, B.A., Arner, P., et al. (2013). Adipose Dipeptidyl Peptidase-4 and Obesity: Correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro. Diabetes Care.
42. Shinjo, T., Nakatsu, Y., Iwashita, M., Sano, T., Sakoda, H., Ishihara, H., Kushiyama, A., Fujishiro, M., Fukushima, T., Tsuchiya, Y., et al. (2015). DPP-IV inhibitor anagliptin exerts anti-inflammatory effects on macrophages, adipocytes, and mouse livers by suppressing NF-kappaB activation. Am. J. Physiol Endocrinol. Metab *309*, E214-E223.
43. Silha, J.V., Gui, Y., and Murphy, L.J. (2002). Impaired glucose homeostasis in insulin-like growth factor-binding protein-3-transgenic mice. Am. J. Physiol Endocrinol. Metab *283*, E937-E945.
44. Sun, K., Tordjman, J., Clement, K., and Scherer, P.E. (2013). Fibrosis and adipose tissue dysfunction. Cell Metab *18*, 470-477.

45. Szendroedi, J., Saxena, A., Weber, K.S., Strassburger, K., Herder, C., Burkart, V., Nowotny, B., Icks, A., Kuss, O., Ziegler, D., et al. (2016). Cohort profile: the German Diabetes Study (GDS). Cardiovasc Diabetol *15*, 59.
46. Teppala, S., and Shankar, A. (2010). Association between serum IGF-1 and diabetes among U.S. adults. Diabetes Care *33*, 2257-2259.
47. Ter Horst, K.W., Gilijamse, P.W., Ackermans, M.T., Soeters, M.R., Nieuwdorp, M., Romijn, J.A., and Serlie, M.J. (2016). Impaired insulin action in the liver, but not in adipose tissue or muscle, is a distinct metabolic feature of impaired fasting glucose in obese humans. Metabolism *65*, 757-763.
48. Vella, A., Bock, G., Giesler, P.D., Burton, D.B., Serra, D.B., Saylan, M.L., Dunning, B.E., Foley, J.E., Rizza, R.A., and Camilleri, M. (2007). Effects of dipeptidyl peptidase-4 inhibition on gastrointestinal function, meal appearance, and glucose metabolism in type 2 diabetes. Diabetes *56*, 1475-1480.
49. Wellen, K.E., and Hotamisligil, G.S. (2003). Obesity-induced inflammatory changes in adipose tissue. J. Clin. Invest *112*, 1785-1788.
50. Wernstedt, A., I, Tao, C., Morley, T.S., Wang, Q.A., Delgado-Lopez, F., Wang, Z.V., and Scherer, P.E. (2014). Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. Cell Metab *20*, 103-118.
51. Yamada, P.M., Mehta, H.H., Hwang, D., Roos, K.P., Hevener, A.L., and Lee, K.W. (2010). Evidence of a role for insulin-like growth factor binding protein (IGFBP)-3 in metabolic regulation. Endocrinology *151*, 5741-5750.
52. Yasuda, N., Nagakura, T., Yamazaki, K., Inoue, T., and Tanaka, I. (2002). Improvement of high fat-diet-induced insulin resistance in dipeptidyl peptidase IV-deficient Fischer rats. Life Sci *71*, 227-238.
53. Yazbeck, R., Howarth, G.S., and Abbott, C.A. (2009). Dipeptidyl peptidase inhibitors, an emerging drug class for inflammatory disease? Trends Pharmacol. Sci *30*, 600-607.

Table 1

Antropometric and clinical data obtained from type 2 diabetic patients treated with metformin alone or in combination with sitagliptin.

	Metformin	Metformin + Sitagliptin
n (male/female)	33 (25/8)	17 (13/4)
Age (years)	52 ± 11	52 ± 12
BMI (kg/m^2)	29.0 ± 4.3	28.3 ± 4.5
Waist circumference (cm)	110 ± 11	96 ± 13
WHR	0.96 ± 0.06	0.93 ± 0.08
M-value (mg/kg*min)	6.18 ± 2.11	6.65 ± 2.53
Fasting blood glucose (mg/dl)	127 ± 25	140 ± 36
Fasting insulin (mU/L)	14.8 ± 6.6	12.8 ± 7.2
C-peptide (ng/ml)	3.07 ± 1.05	2.79 ± 1.02
HbA1c (%)	6.56 ± 1.04	6.42 ± 1.12

hs-CRP (mg/dl)	0.46 ± 0.64	0.25 ± 0.20
Triglyceride (mg/dl)	159 ± 98	131 ± 75
Cholesterol (mg/dl)	208 ± 46	199 ± 42
HDL-cholesterol (mg/dl)	46.6 ± 12.6	54.5 ± 15.8
LDL-cholesterol (mg/dl)	133 ± 39	122 ± 38
AST (U/L)	23.5 ± 9.3	24.0 ± 6.6
ALT (U/L)	32.4 ± 18.3	32.6 ± 19.5
γGT (U/L)	37.1 ± 27.1	33.7 ± 16.4
estimated glomerular filtration rate (ml/min*1.73m^2)	87.6 ± 15.2	90.7 ± 14.8
DPP4 activity (µU/ml)	1.03 ± 0.25	0.51 ± 0.16*
DPP4 protein (ng/ml)	314 ± 23	337 ± 31

BMI: Body mass Index, WHR: waist to hip ratio. Data expressed as mean ± SD, *$p<0.05$ vs. patients treated with metformin alone.

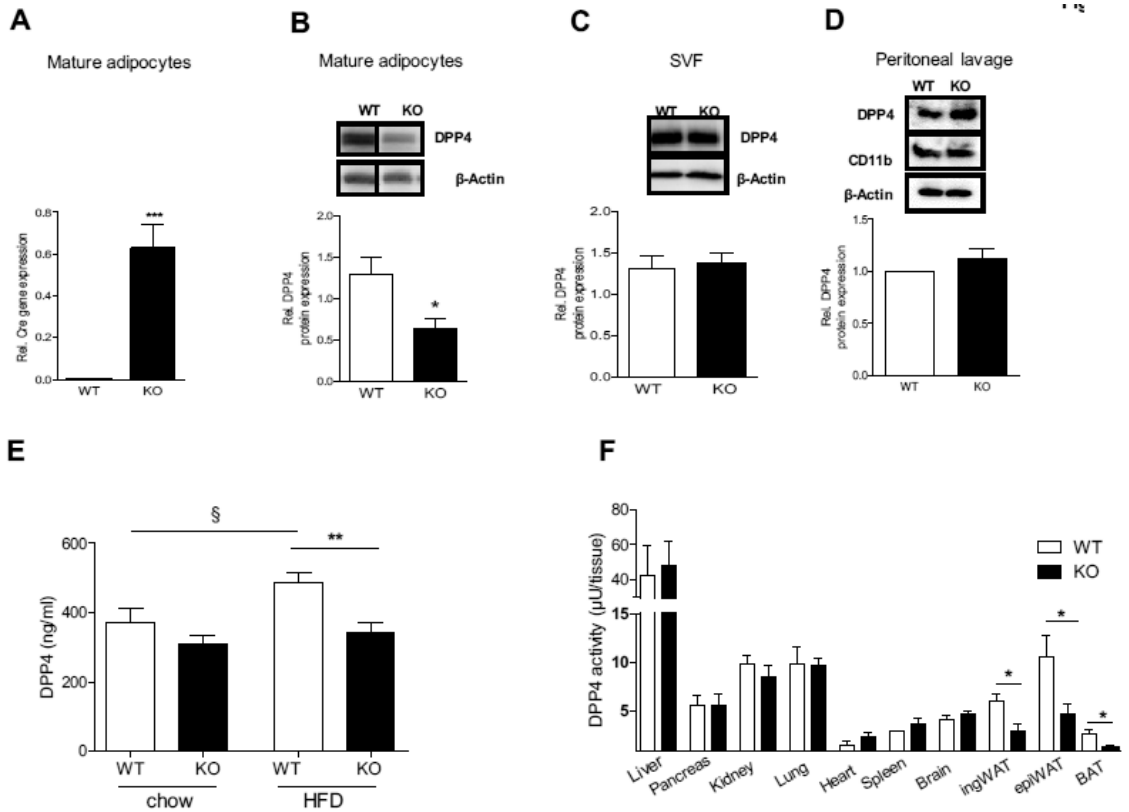


Figure 1. **DPP4 deletion is restricted to adipocytes in AT-DPP4-KO mice**. **(A)** Cre mRNA levels in mature adipocytes isolated from wild type (WT) or their corresponding DPP4 adipose tissue–specific

knockout littermates (KO) (n=12 animals per group). mRNA levels were normalized to 18S levels. (**B**) DPP4 protein levels in mature adipocytes and in stroma vascular fraction (**C**) isolated from the inguinal subcutaneous AT depot, from WT and KO animals (n=4-6 animals per group). Corresponding representative blots are shown on top. (**D**) DPP4 and CD11b expression in resident immune cells isolated from peritoneal lavage (n=3 animals per group). Corresponding representative blot is shown on top. (**E**) Fasting circulating DPP4 levels assessed by ELISA in WT and KO mice after 24 week of chow and HFD (n=11 animals per group in ND and n=22 animals per group in HFD). (**E**) DPP4 enzymatic activity in different tissues and organs (n=4 animals per group). Data expressed as mean ± S.E.M. ****p<0.001;* ***p<0.01;* **p<0.05* vs. WT littermates on the same diet; §*p<0.05* vs corresponding genotype on different diet.

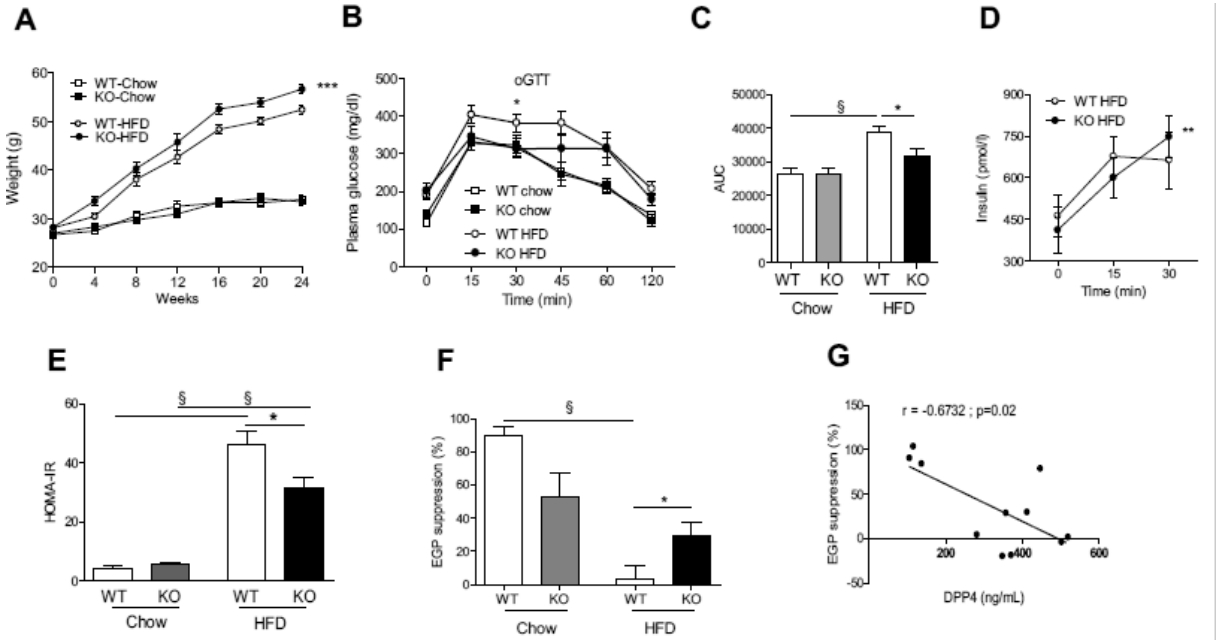


Figure 2. AT-DPP4-KO animals gain more body weight but display improved glucose tolerance and hepatic insulin sensitivity on HFD. (**A**) Body weight was assessed every 4 weeks during chow and HFD (n=11-16 animals per group on chow, n= 26-31 animals per group on HFD). Data expressed as mean ± S.E.M. ****p<0.01* vs. diet-matched WT littermates. (**B**) oGTTs were performed after 18-20 weeks of chow or HFD (WT chow n=8; KO chow n=6; WT HFD n=9; KO HFD n=6). (**C**) Corresponding AUC of oGTT. (**D**) Plasma insulin levels at 0, 15 and 30 minutes after glucose gavage (2 mg/g body weight) (WT HFD n=9; KO HFD n=7). (**E**) HOMA-IR after 24 weeks of dietary intervention (WT chow n=7; KO chow n=7; WT HFD n=17, KO HFD n=15). (**F**) Percentage of suppression of endogenous glucose production (EGP) during hyperinsulinemic-euglycemic clamps, after 22 weeks of dietary intervention (WT chow n=4; KO chow n=9; WT HFD n=7; KO HFD n=9). (**G**) Correlation between EGP suppression and DPP4 circulating levels (WT chow n=4; WT HFD n=7). Data expressed as mean ± S.E.M. **p<0.05; **p<0.01; ***p<0.001* vs. WT littermates on the same diet, §*p<0.05* vs corresponding genotype on different diet.

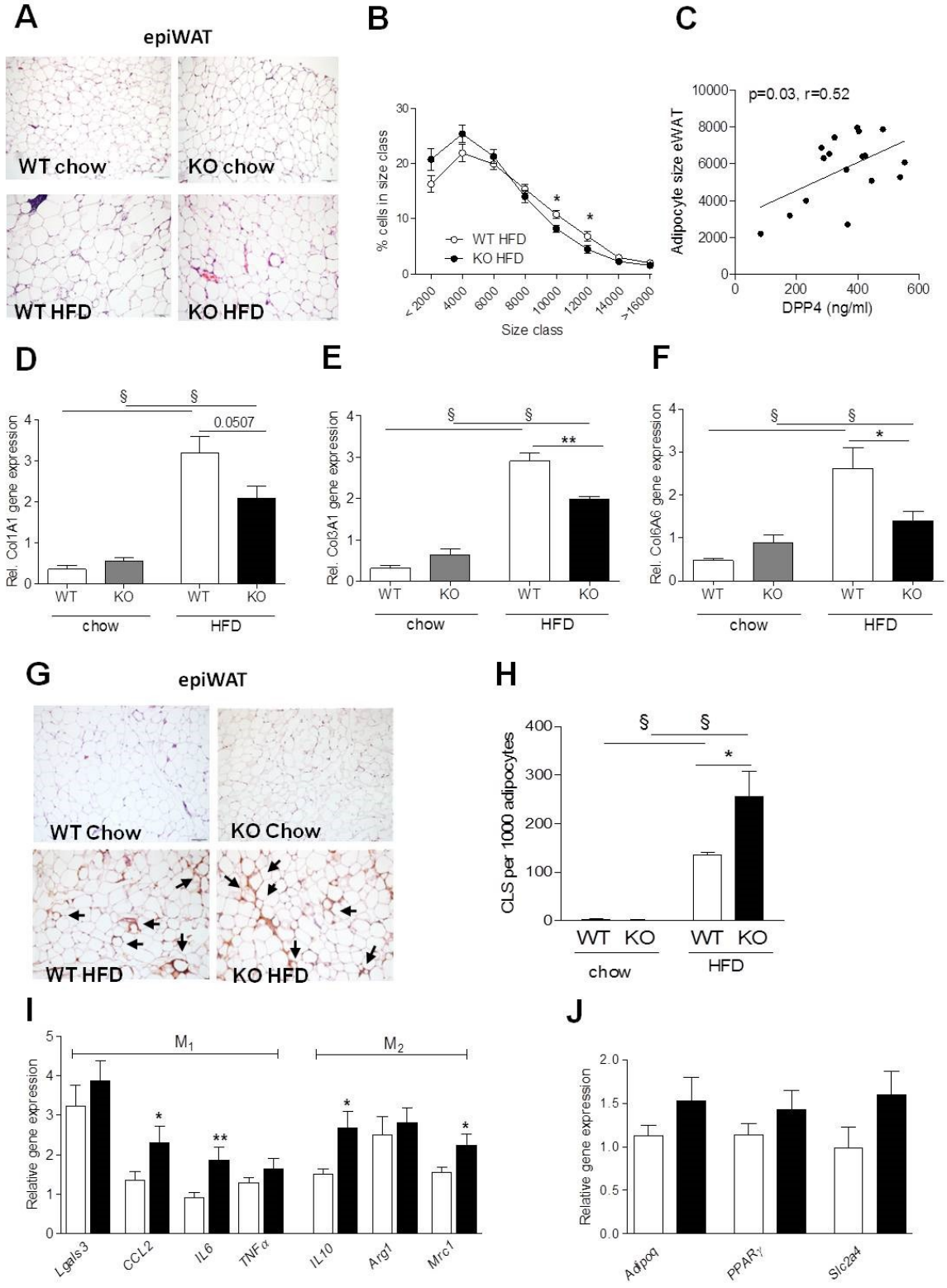


Figure 3. Adipocyte morphology, fibrosis markers and galectin-3 positive macrophage infiltration in epiWAT. (**A**) Representative microphotographs of hematoxylin-eosin stained adipocytes from epiWÁT obtained from WT and KO animals after 24 weeks under chow or HFD. (**B**) Percentage of cells per adipocyte size class (μm²) in epiWAT obtained from WT and KO animals after 24 weeks under chow or

HFD (WT HFD n=16; KO HFD n=16). (**C**) Correlation between adipocyte size and DPP4 circulating levels (n=33). mRNA levels of the fibrosis markers collagen 1A1 (**D**), collagen 3A1 (**E**) and collagen 6A6 (**F**), respectively (n=5-8 animals per group). (**G**) Representative microphotographs of immunohistochemistry staining for galectin-3, showing CLS in epiWAT. (**H**) Number of CLS per every 1000 adipocytes calculated after 24 weeks of dietary intervention (WT chow n=4; KO chow n=4; WT HFD n=7; KO HFD n=6). (**I**) mRNA levels of M1 macrophage markers: Lgals3, CCL2, IL-6, TNF-α, and M2 macrophage markers: IL-10, Arg10 and Mrc1 in epiWAT from WT and KO animals after 24 weeks of HFD (WT HFD n= 24; KO HFD n=25). (**J**) mRNA levels of the adipogenic markers: adiponectin, PPARγ (WT HFD n=24; KO HFD n=25) and GLUT4 (WT HFD n=6; KO HFD n=6). Data are expressed as ± S.E.M. **p<0.05; **p<0.01* vs. WT littermates under HFD. All mRNA levels normalized to 18S.

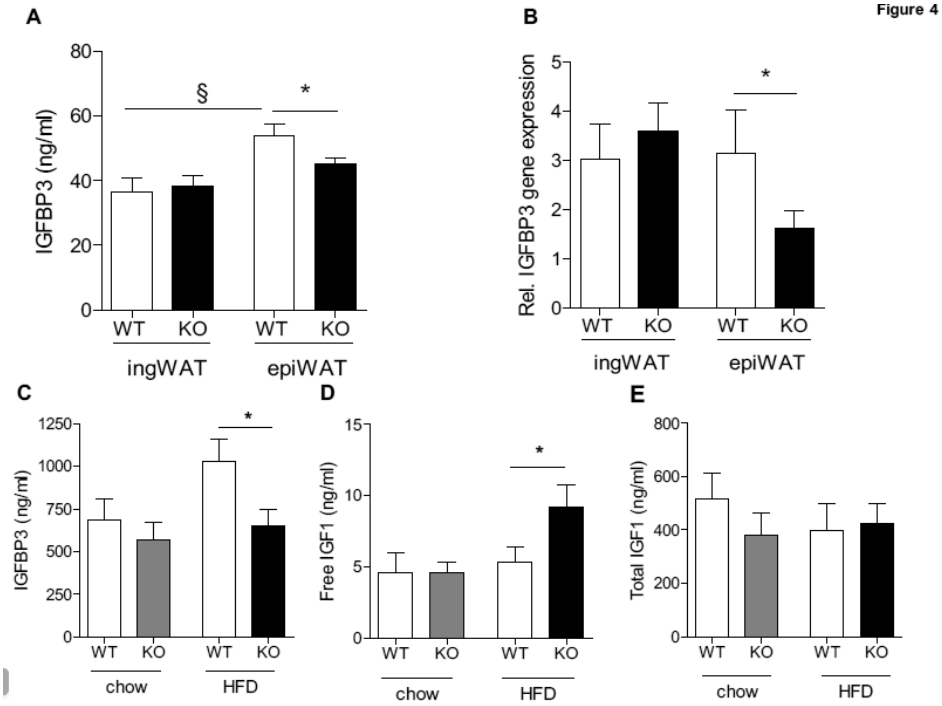


Figure 4. IGFBP3 is downregulated in epiWAT from KO animals under HFD and reduced in serum in parallel to increased concentrations of free IGF1. (**A**) IGFBP3 levels in CM from explants obtained from ingWAT or epiWAT isolated from WT and KO animals after 24 weeks on HFD (ingWAT: WT HFD n=3, KO HFD n=7; epiWAT: WT HFD n=3; KO HFD n=6). (**B**) mRNA levels of IGFBP3 in ingWAT and epiWAT of animals on HFD (ingWAT: WT HFD n=6, KO HFD n=6; epiWAT: WT HFD n=5; KO HFD n=6). **(C)** Plasma IGFBP3 levels in WT and KO animals after 24 weeks dietary intervention (WT chow n=14; KO chow n=13; WT HFD n=8; KO HFD n=18). (**D**) Plasma free IGF1 levels in WT and KO animals after 24 weeks dietary intervention (WT chow n=7; KO chow n=11; WT HFD n=16; KO HFD n=19). (**E**) Plasma total IGF1 levels in WT and KO animals after 24 weeks dietary intervention (WT chow n=8; KO chow n=11; WT HFD n=14; KO HFD n=18). Data are expressed as ± S.E.M. **p<0.05;* vs. WT littermates on HFD, *§p<0.05* vs different fat depot in corresponding genotype.

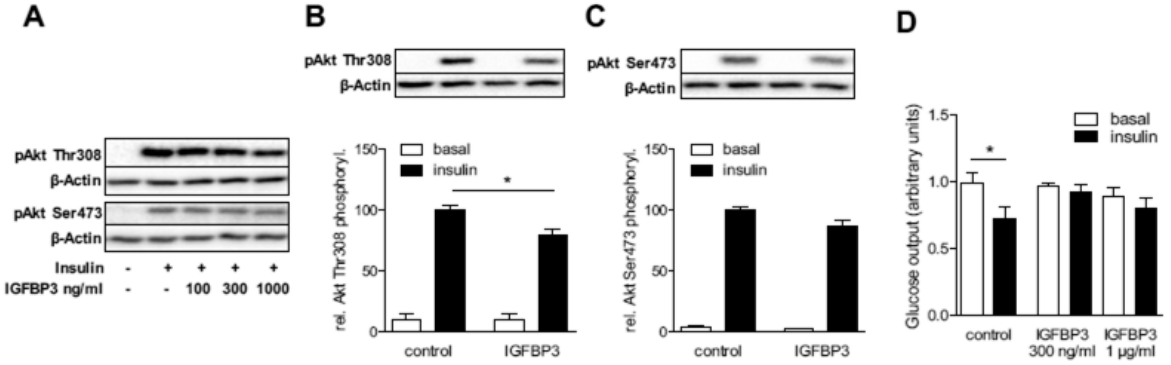


Figure 5. IGFBP3 impairs insulin signaling and insulin-induced suppression of glucose production in HepG2 cells. (**A**) HepG2 cells were treated with 100-1000 ng/ml IGFBP3 for 18 h and stimulated with 100 nM insulin for 10 min. Representative Western Blots are presented. (**B-C**) HepG2 cells were treated with 300 ng/ml IGFBP3 for 18 h and stimulated with 100 nM insulin for 10 min. Data are expressed as ± S.E.M (n=4). **p<0.05;* vs. insulin-stimulated control. Representative Western Blots are presented. (**D**) HepG2 cells were treated with IGFBP3 for 18 h and glucose production was measured as detailed in Materials and Methods. Forskolin was used as a positive control and induced glucose production (from 1.0 ± 0.1 to 1.9 ± 0.7, p=0.01). Data are expressed as ± S.E.M (n = 4). **p<0.05;* vs. basal control.

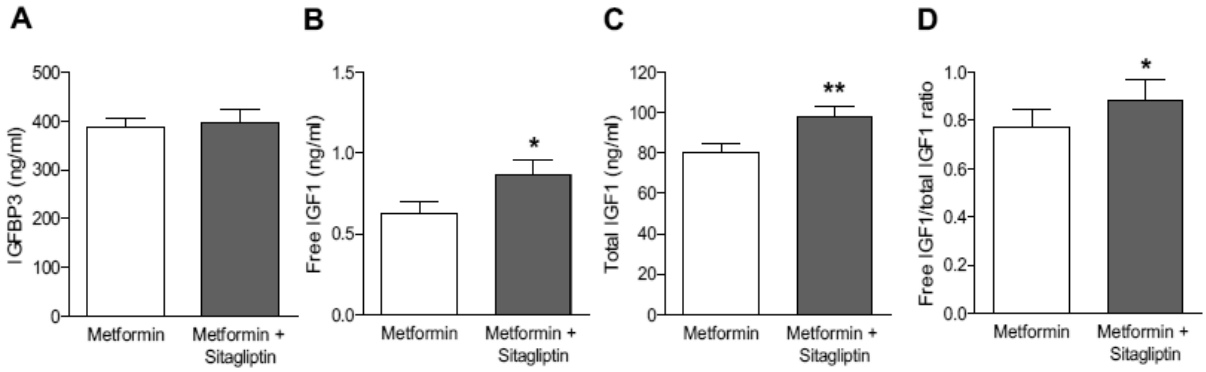
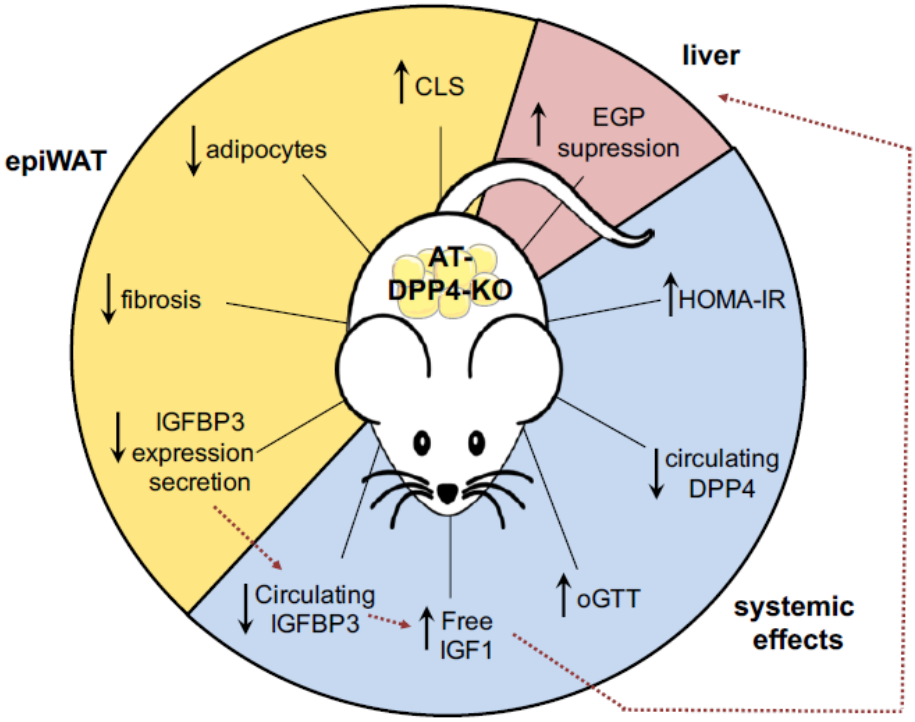


Figure 6. Type 2 diabetic patients treated with metformin alone or in combination with sitagliptin are characterized by increased serum free IGF1, serum total IGF1 and free IGF1/total IGF1 ratio compared to patients with type 2 diabetes treated with metformin alone. IGFBP3 (**A**), free IGF1 (**B**) and total IGF1 (**C**) were measured by ELISA. Free IGF1/total IGF1 ratio was calculated. **p<0.05; **p<0.01* vs. patients treated with metformin alone

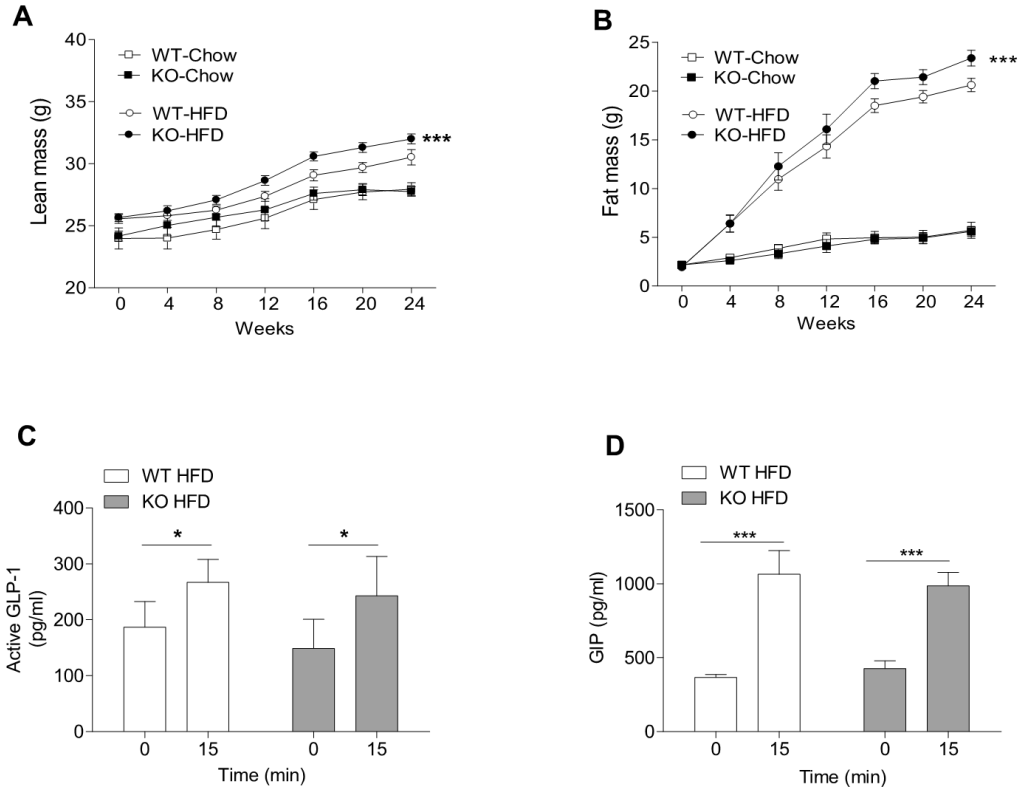
Graphical abstract



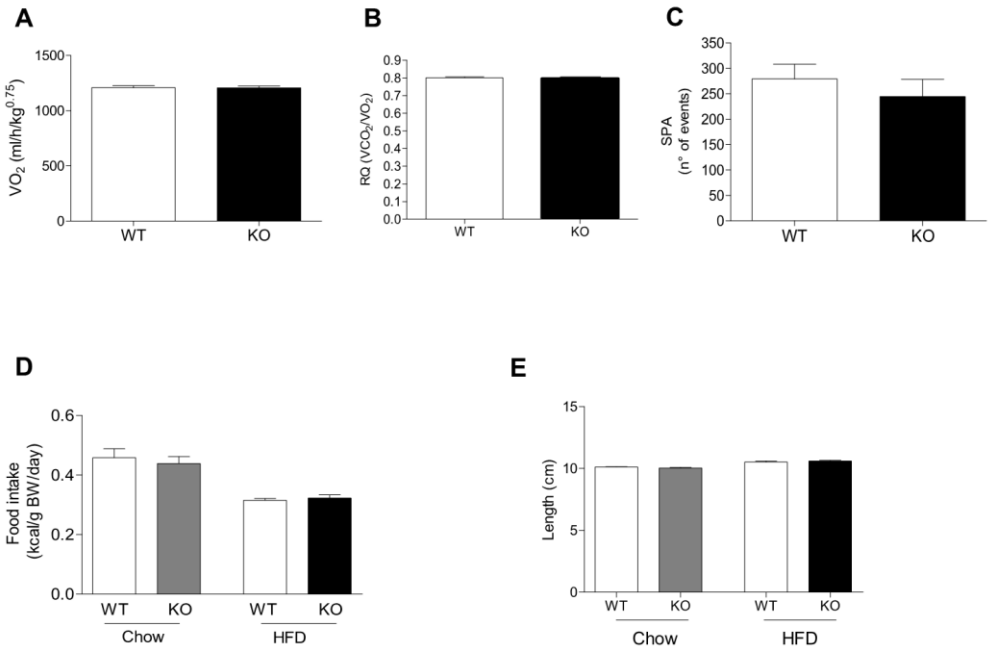
Supplemental Information

DPP4 deletion in adipose tissue improves hepatic insulin resistance in diet-induced obesity

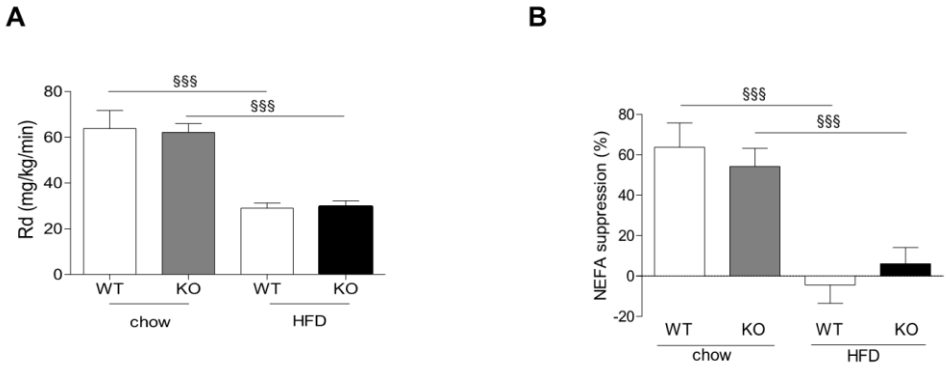
Tania Romacho, Henrike Sell, Ira Indrakusuma, Diana Roehrborn, Tamara R. Castañeda, Tomas Jelenik, Sonja Hartwig, Jürgen Weiß, Hadi Al-Hasani, Michael Roden, Jürgen Eckel.



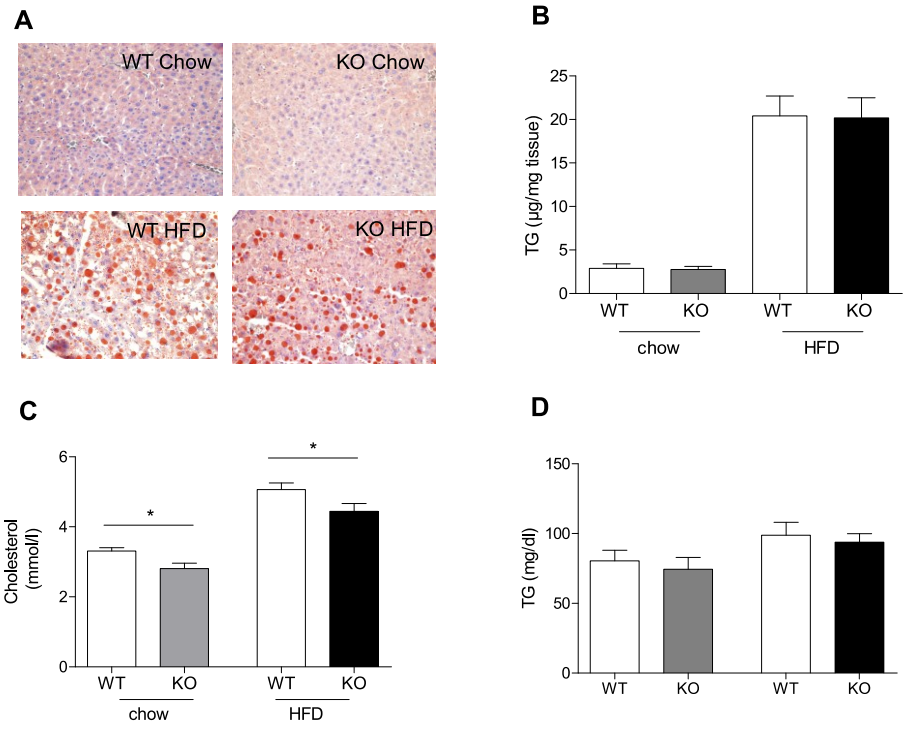
Supplementary Figure 1. Increased lean and fat mass but unaltered plasma incretins in AT-DPP4-KO animals under HFD. Lean mass (**A**) and fat mass (**B**) was assessed every 4 weeks during dietary intervention (n=11-16 animals per group on chow, n= 26-31 animals per group on HFD). Data expressed as mean ± S.E.M. ***p<0.01 vs. vs. diet-matched WT littermates. Plasma active GLP-1 (**C**) and GIP (**D**) levels at 0 and 15 min after glucose gavage (2mg/g body weight) in animals at week 20 of HFD (WT HFD n=4; KO HFD n=4-5). **p<0.05; ***p<0.001* vs. levels at time 0.



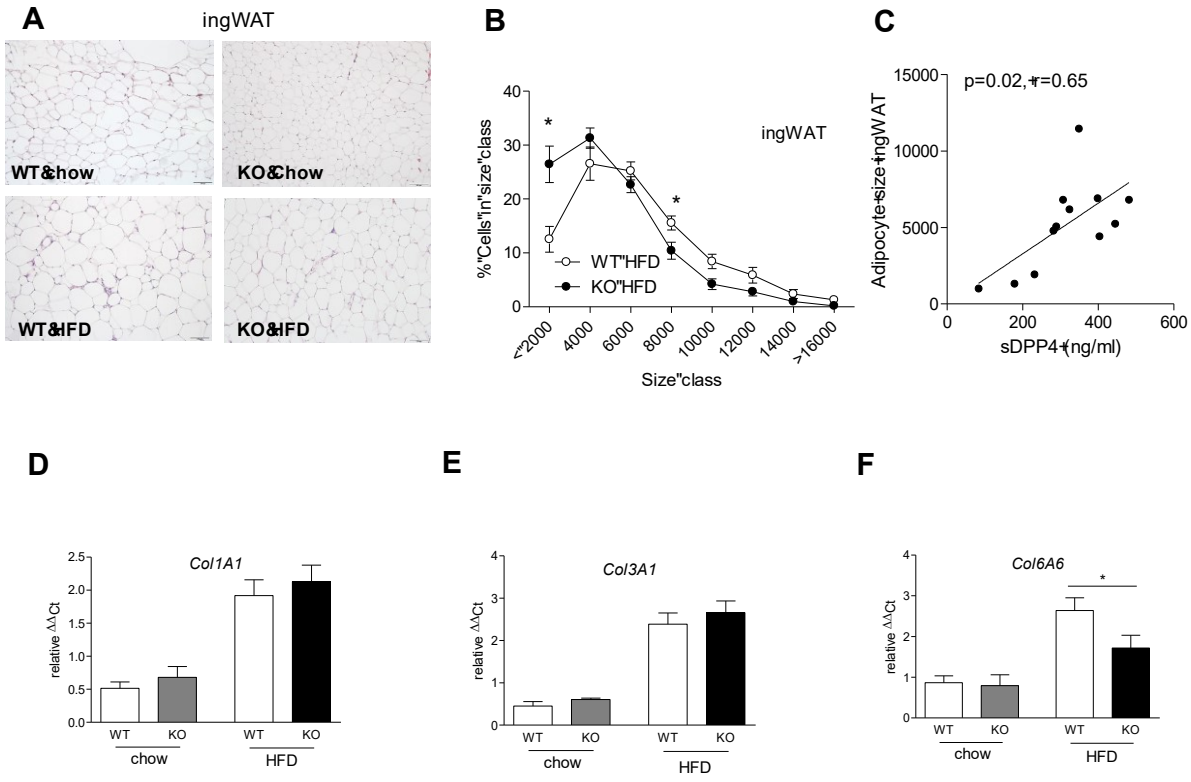
Supplementary Figure 2. Metabolic phenotyping of AT-DPP4-KO mice. Measurements were carried out for 4 days (48 h adaptation + 48h follow up) at week 20 after dietary intervention. (**A**) Energy expenditure corrected per body surface (WT HFD n=19; KO HFD n=16). (**B**) Respiratory quotient (RQ; WT HFD n=15; KO HFD n=13) (**c**) Spontaneous physical activity (SPA; WT HFD n=20; KO HFD n= 18. (**C**) Daily food intake (WT HFD n=23; KO HFD n=21) (**D**) Nose-tail length was measured in isofluorane-anesthetised animals at week 30 of age (WT chow n=8; KO chow n=8; WT HFD n=14; KO HFD n=14). Data are expressed as mean ± S.E.M.



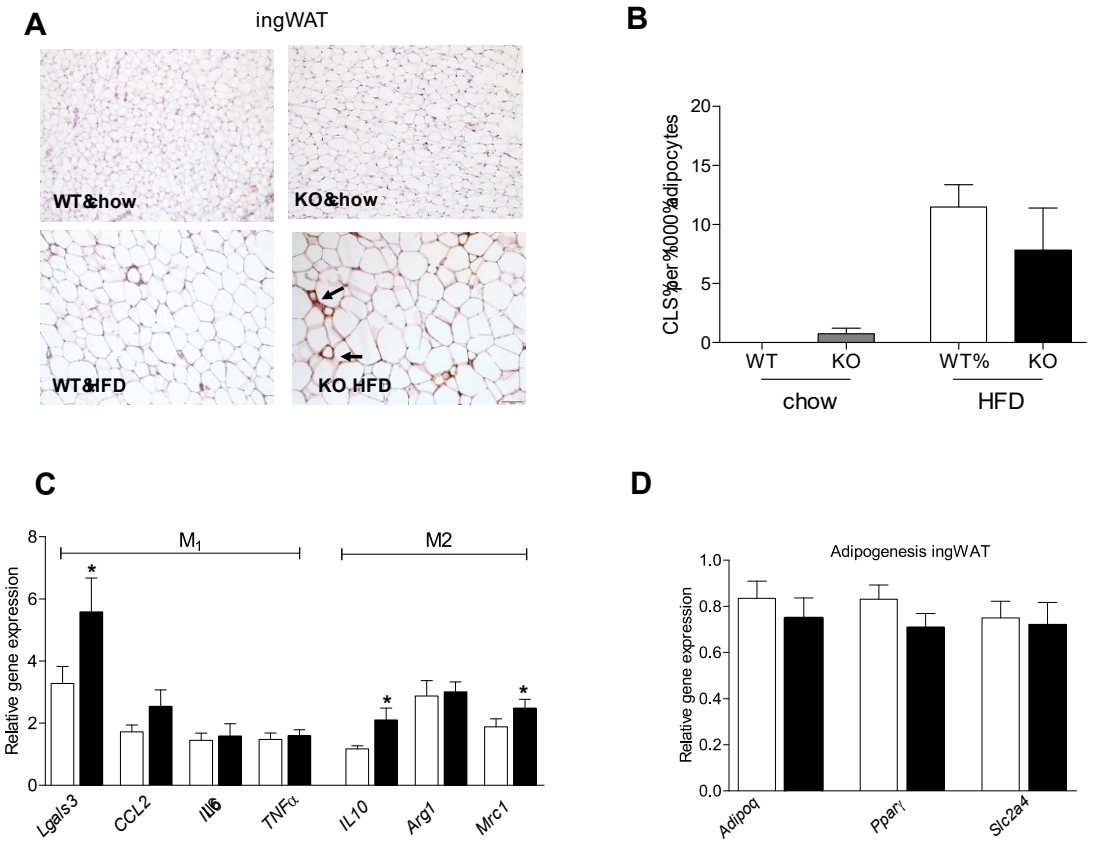
Supplementary Figure 3. **Insulin sensitivity *in vivo*.** Hyperinsulinemic-euglycemic clamps were performed after 22 weeks of dietary intervention. (**A**) Glucose disposal rate (Rd) and (**B**) suppression of non-esterified fatty acids (NEFA) were assessed (WT chow n=4; KO chow n= 9; WT HFD n= 7; KO HFD n=9). Data are expressed as mean ± S.E.M. *§§§p<0.001* vs. same genotype under chow diet.



Supplementary Figure 4. Lipidic profile and hepatic steatosis in AT-DPP4-KO mice.
(**A**) Representative microphotographs of Oil Red-stained liver sections adipocytes from WT and KO animals under chow or HFD. (**B**) Hepatic triglyceride content in WT and KO animals under HFD for 24 weeks (WT chow n=7; KO chow n=8; WT HFD n=8; KO HFD n=11). (**C**) Plasma cholesterol and triglycerides (**D**) in WT and KO animals under chow or HFD for 24 weeks (WT chow n=8; KO chow n=8; WT HFD n= 14; KO HFD n=14). **p<0.05* vs. matched-WT littermates under same diet.



Supplementary Figure 5. Improved adipose tissue remodeling in ingWAT. (**A**) Representative microphotographs of hematoxylin-eosin stained adipocytes from WT and KO animals under chow or HFD. (**B**) Percentage of cells per adipocyte size class (μm^2) in ingWAT obtained from WT and KO animals after 24 weeks under chow or HFD (WT HFD n=11; KO HFD n=11). (**C**) Correlation between adipocyte size and DPP4 circulating levels in WT animals (n=33). Expression of the fibrosis markers collagen 1A1 (**D**), collagen 3A1 (**E**) and collagen 6A6 (**F**) (WT chow n=5-6; KO chow n=6-8, WT HFD n=6; KO HFD n=6). mRNA levels were normalized to 18S. Data are expressed as mean ± S.E.M. **p<0.05* HFD-matched littermates. **p<0.05* vs. matched-WT littermates under same diet.



Supplementary Figure 6. Macrophage infiltration in ingWAT. (**A**) Representative microphotographs of immunohistochemistry staining for galectin-3, showing crown-like structures (CLS) in epiWAT. (**B**) Number of CLS per every 1000 adipocytes was calculated by a blind observer in WT and KO under chow or HFD after 24 weeks of dietary intervention. (**C**) mRNA levels of M1 macrophage markers: Lgals3, CCL2, IL-6, TNF-α (WT HFD n=21-24; KO HFD n=15-24) and M2 macrophage markers: IL-10, Arg10 and Mrc1(WT HFD 12-18; KO HFD n=16 animals) in epiWAT from WT and KO animals after 24 weeks of HFD. (**D**) mRNA levels of the adipogenic markers: adiponectin, PPARγ and GLUT4 (WT HFD n=8; KO HFD n=7 animals). mRNA levels were normalized to 18S. Data are expressed as mean ± S.E.M. **p<0.05* vs. matched WT littermates under HFD.

EXPERIMENTAL PROCEDURES

Energy balance

Age-matched AT-DPP4-KO and WT control mice were singly housed in an indirect calorimetry system (TSE Phenomaster system, Bad Homburg, Germany) for four light/dark cycles (12 h/12h), at week 20 of dietary intervention. After an adaptation period of 48 h, the data obtained during the last 2 light/dark cycles were used to quantify spontaneous physical activity (SPA), energy expenditure (EE; ml/h/kg $^{0.75}$) and the respiratory quotient (RQ, VCO2/VO2), as previously described (Castaneda et al., 2011). Measurements of food intake were taken manually every day in the morning.

Hormone and biochemical assays

Total GIP and GLP-1 were measured with a DuoPlex assay (BioRad, Hercules, California). Active GLP-1 was measured with a Singleplex kit (Millipore, Billerica, California).
Cholesterol and triglycerides were obtained by standard peroxidase-based clinical assays (ErbaLachema, Czech Republic). Non-esterified fatty acids were quantified using colorimetric assay (NEFA C; Wako, Neuss, Germany).

Liver fat content

Livers were dissected and fixed in 10% formalin, and then dehydrated in ethanol followed by cryopreservation and further sectioning for oil red O stainings. Representative images from at least 5 animals per group were taken with a Leica DM6000 B microscope (Jelenik et al., 2014).
Triglycerides content in liver was assessed by a colorimetric kit (RANDOX) and normalized per protein content as estimated by BCA (Thermo) (Baumeier et al., 2015).

Statistics

Statistical analysis was performed with the GraphPad Prism software (La Jolla, CA, USA). *p* values were calculated using the two-tailed Student's *t*-test. For statistical comparisons between experimental groups, two-way ANOVA was used, followed by Bonferroni *post hoc* test. A *p* value below 0.05 was considered statistically significant. Data are shown as the mean ± S.E.M or mean ± S.D if specified. Normal distribution and equal variances were trested for each analysis. Animals were randomly allocated to the different diets and at least three animals were included in each experimental group. Groups size was calculated by Simple Interactive Statistical Analysis.

References

Baumeier, C., Kaiser, D., Heeren, J., Scheja, L., John, C., Weise, C., Eravci, M., Lagerpusch, M., Schulze, G., Joost, H.G., et al. (2015). Caloric restriction and intermittent fasting alter hepatic lipid droplet proteome and diacylglycerol species and prevent diabetes in NZO mice. Biochim. Biophys. Acta *1851*, 566-576.

Castañeda, T.R., Nogueiras, R., Muller, T.D., Krishna, R., Grant, E., Jones, A., Ottaway, N., Ananthakrishnan, G., Pfluger, P.T., Chaudhary, N., et al. (2011). Decreased glucose tolerance and plasma adiponectin:resistin ratio in a mouse model of post-traumatic stress disorder. Diabetologia *54*, 900-909.

Jelenik, T., Sequaris, G., Kaul, K., Ouwens, D.M., Phielix, E., Kotzka, J., Knebel, B., Weiss, J., Reinbeck, A.L., Janke, L., et al. (2014). Tissue-specific differences in the development of insulin resistance in a mouse model for type 1 diabetes. Diabetes *63*, 3856-3867.

2.5. AUTHOR CONTRIBUTIONS

„DPP4 in Diabetes“
Röhrborn D, Wronkowitz N, Eckel J
Front Immunol. 2015 Jul 27;6:386. doi: 10.3389/fimmu.2015.00386.

Total contribution:	53%
Conceived/designed experiments:	-
Performed experiments:	-
Analysed data:	-
Contribution to discussion:	50%
Wrote manuscript:	50%
Reviewed/edited manuscript:	60%
Author:	1st

„Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells”
Röhrborn D, Eckel J, Sell H.
FEBS Lett. 2014 Nov 3;588(21):3870-7. doi: 10.1016/j.febslet.2014.08.029. Epub 2014 Sep 12.

Total contribution:	90%
Conceived/designed experiments:	80%
Performed experiments:	100%
Analysed data:	100%
Contribution to discussion:	80%
Wrote manuscript:	100%
Reviewed/edited manuscript:	80%
Author:	1st
Impact factor:	3,169

„Reduced DPP4 activity improves insulin signaling in primary human adipocytes”
Röhrborn D, Brückner J, Sell H, Eckel J.
Biochem Biophys Res Commun. 2016 Mar 11;471(3):348-54. doi:10.1016/j.bbrc.2016.02.019

Total contribution:	91%
Conceived/designed experiments:	90%

Performed experiments:	90%
Analysed data:	90%
Contribution to discussion:	85%
Wrote manuscript:	100%
Reviewed/edited manuscript:	90%
Author:	1st
Impact factor:	2,297

„ DPP4 deletion in adipose tissue improves hepatic insulin resistance in diet-induced obesity “ Tania Romacho , Henrike Sell , Ira Indrakusuma, Diana Roehrborn, Tamara R. Castañeda, Tomas Jelenik, Sonja Hartwig, Jürgen Weiß, Hadi Al-Hasani, Michael Roden, Jürgen Eckel”
Submitted to Cell Reports

Total contribution:	22%
Conceived/designed experiments:	20%
Performed experiments:	30%
Analysed data:	30%
Contribution to discussion:	30%
Wrote manuscript:	10%
Reviewed/edited manuscript:	10%
Author:	2nd
Impact factor:	7.870

3. DISCUSSION

3.1. RELEASE OF SOLUBLE DPP4

3.1.1. MECHANISM AND REGULATION OF DPP4 RELEASE

Additionally to being expressed as a membrane-located protease, DPP4 is also found in high amounts in the circulation with full enzymatic potential (211). However, controverse reports about the mechanism of release can be found in the literature. On the one hand, some researchers found DPP4 in granules or secretory lysosomes (212), whereas, on the other hand, Andrieu et al proposed an active cleavage mechanism from the cell surface (213). I was able to show that DPP4 release is not mediated through the classical ER/Golgi-dependent release mechanism, since *in vitro* cultured primary human adipocytes and human SMC are insensitive to brefeldin A treatment. This also supports an earlier finding of our group, where DPP4 release from SkMc is also insensitive to brefeldin A treatment (108). Since DPP4 belongs to the group of type II transmembrane proteins and endogenous proteolytic release is limited to type I or type II transmembrane proteins, it is very likely, that DPP4 is released via this mechanism. This post-translational hydrolysis mechanism is called shedding (100).

By using different broad spectrum protease inhibitors, I found out, that various types of proteases are involved in DPP4 release. Especially in human adipocytes only with the broad spectrum metalloprotease inhibitor batimastat significant differences could be obeserved, meaning that at least in this cell type MMPs are the major sheddases for DPP4. Although I saw significant changes of DPP4 release with broad spectrum inhibitors against serine-, cysteine- and metalloproteases, I could not see additive effects of these inhibitors on DPP4 shedding. Analysis of supernatants from SMC and adipocytes revealed a relatively high release of certain MMPs and cathepsins in a cell-type specific manner. It is well-known that activation of MMPs is rather complex. MMPs are released into the extracellular space as inactive proenzymes. Only upon cleavage of the propeptides, which block the active center of the MMPs, they become active (214). Interestingly a lot of different factors are able to activate MMPs. In addition to serine proteases like plasmin and kallikreins, also cysteine proteases like cathepsin G are able

to activate MMPs (215). Furthermore, MMPs are also able to activate one another, as it is reported in case of MMP2 activation via MMP14 (216), or MMP9 activation via MMP2 or MMP3 (217). Therefore, I postulate that DPP4 shedding involves a catalytic cascade which involves MMPs and cathepsins. The fact that some MMPs are reported to be dysregulated in obesity further supports the involvement of these MMPs in DPP4 shedding, since it is known that DPP4 release is higher under obese conditions (186). Especially in case of MMP2, MMP9 and MMP14, levels are upregulated in obese mice (218;219). Particularly in AT, MMPs play an important role in tissue remodeling, which is necessary to keep up normal AT function during expansion of this tissue in obesity (37). In this work I was able to show that MMP1, MMP2 and MMP14 seem to be involved in constitutive DPP4 shedding from SMC, whereas in adipocytes, only specific inhibition of MMP9 resulted in a decreased DPP4 shedding. All of these four candidates are known to be involved in the shedding of various substrates (220-224). This also underpins the complex interplay of different enzymes in the shedding process, since one MMP is not limited to a specific target and one target might be released by several MMPs depending on experimental conditions or investigated cell-type.

In addition to elucidating the mechanism how DPP4 is released, it is also of great importance to understand how this release is regulated in order to understand the dysregulation of DPP4 under physiopathologic conditions like T2DM. DPP4 serum levels are elevated in obese subjects and can be reduced to normal levels after bariatric surgery (185). Furthermore, DPP4 serum levels and release from AT is elevated in patients with diagnosed metabolic syndrome (185). The most obvious regulator of DPP4 in the context of T2DM seems to be glucose levels. Therefore several groups addressed the impact of high and low glucose levels on DPP4 *in vitro* (225;226) and *in vivo* (225). However, the results are rather controversial and dependent on the investigated cell type. In Caco-2 cells, which are a model resembling the enterocytes lining the small intestine (227), high glucose levels inhibited DPP4 gene expression and DPP4 activity which seems to be mediated by HNF-1α (226). Das et al reported lower intracellular DPP4 content and release under high glucose conditions in differentiated 3T3-L1 cells, which is regulated at the transcriptional level (225). However, this is only true in non-diabetic conditions, whereas when they induced diabetic conditions by streptozotocin treatment, no decrease of DPP4 was observed (225). Furthermore, it is quite interesting that the authors only observed a positive correlation of DPP4 levels and blood glucose later than 4 weeks of high-fat diet feeding. So they postulated that in the physiological

state DPP4 is negatively regulated by high glucose levels, thereby generating more active incretins, which improve glucose disposal. This regulation is lost in T2DM, where a reduced glucose uptake into adipocytes occurs. Therefore in adipocytes, isolated from diabetic, streptozotocin-treated animals, the conditions reflect low glucose levels and DPP4 is upregulated (225). However, I found no regulation of DPP4 release in human SMC by high glucose levels (Fig. 4). This might be either due to the fact that Das and coworkers used a cell-line for their experiments wereas I used primary cells or due to the difference between mice and human.

During the development of obesity, alterations in the secretory profile of AT towards more pro-inflammatory cytokines occur. This also leads to an elevated number of classically activated M1 macrophages either via recruiting them from the circulation or via a phenotypic switch from M2 to M1 macrophages (228). So another plausible mechanism of DPP4 regulation might be the induction via cytokines present during the local chronic low-grade inflammation of AT. Indeed, Das et al were able to show that TNFα markedly increased DPP4 release from *in vitro* cultured 3T3-L1 cells. This seems to be mediated at the release level since neither mRNA nor intracellular DPP4 levels are affected (225). Furthermore, TNFα-induced DPP4 shedding seems to be uncoupled from glucose levels, meaning that inflammation is an independent regulator of DPP4 release from adipocytes (225). This observation is in accordance with our previously published data on primary human adipocytes, where we also see an induction of DPP4 release by TNFα (185). This regulation seems to be independent of the source of adipocytes, whether mouse or human. However, when I repeated this experiment in SMC I saw no regulation of DPP4 release by TNFα (Fig 4), which points towards an adipocyte specific mode of DPP4 regulation. Therefore, induction of DPP4 release seems to be cell type specific and TNFα-induced DPP4 release might be limited to adipocytes underlining the role of adipose tissue inflammation in the development of obesity. Bauvois and colleagues were also able to show that interferon γ (IFNγ) is able to induce DPP4 gene expression in B lymphatic leukemia cells by increasing tyrosine phosphorylation of Stat1α. Stat1α is thereby able to accumulate in the nucleus and binds to GAS response elements in the promoter region of DPP4, leading to an upregulation in mRNA expression (229). In my case, IFNγ had no influence on DPP4 release neither from

human primary adipocytes nor human SMC (Fig 4), which might be due to the difference in the used cell types.

While increasing in size during obese AT expansion, adipocytes reach a critical size for oxygen diffusion at some point (42). Whereas efficient oxygen diffusion only occurs approximately 100µm apart from blood vessels, adipocytes can increase to around 180µm in diameter (230). So at some point hypoxia occurs within AT, leading to an altered gene expression. We were able to show in adipocytes which were differentiated at 10% as well as 5% O_2 significantly upregulated DPP4 expression and release (231), whereas 1% O_2 for 24h seems to have no effect on DPP4 release from adipocytes (185). In SMC, 1% O_2 significantly increases DPP4 release after 24h and 48h. However, this seems to be independent from Hif-1α binding to the promoter region of DPP4, since mRNA levels are unaffected. Hypoxia seems to rather influence sheddase levels or activity since I could show increased mRNA levels of MMP1 and MMP9 in SMC .

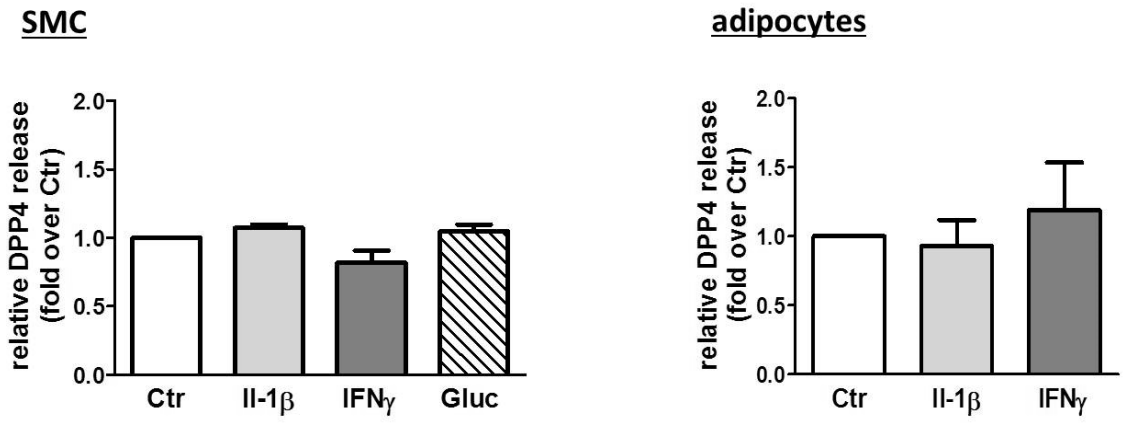


FIGURE 4 REGULATION OF DPP4 RELEASE.
Human SMC and differentiated adipocytes were cultured *in vitro* and treated with 100ng/mL IL-1β or IFNγ, as well as 20mM glucose respectively for 24h. Supernatants were collected and DPP4 release was monitored via DuoSet DPP4 ELISA from R&D systems (DY1180). Assays were performed according to manufacturers instructions. Data are depicted as mean ±SEM n=3-4; Ctr control; Gluc glucose.

3.1.2. IMPACT OF SOLUBLE DPP4 ON THE PHYSIOLOGY OF DIFFERENT CELLS

To date not much is known regarding the impact of soluble DPP4 on the physiology of different cells, and most studies published so far have concentrated on the effects of DPP4 inhibitors. However, the knowledge on how DPP4 is released and how this release is regulated helps to understand the role of circulating DPP4 independent from its

source. Most of the studies on DPP4 inhibitors have attributed the effects to an impact on the incretin axis. However, there are also direct effects of sDPP4 independent of GLP-1. We were the first to show that soluble DPP4 impairs insulin signaling in different cell types namely SkMc, SMC and adipocytes on the level of Akt activation (185). Furthermore sDPP4 induces SMC proliferation and migration (158). Ishibashi and coworkers showed that sDPP4 elevates reactive oxygen species (ROS) production and receptor for advanced glycation end-products (RAGE) expression in endothelial cells (EC) (232). Upon binding to mannose-6-phosphat/insulin-like growth factor-II receptor (M6P/IGF-IIR) sDPP4 potentiates the effects of advanced glycation end products in EC, which was prevented by the DPP4i linagliptin. Our group also pointed out, that circulating sDPP4 might be involved in the progression of atherosclerosis, since it initiates stress and inflammatory signaling cascades in SMC and elevates the release of inflammatory cytokines like IL-6 and MCP-1 (184). The observed effects could be prevented by DPP4i and seem to be mediated through the PAR2 receptor (184). These observations might also help to understand the beneficial effects of DPP4i on the cardiovascular system and further strengthen the role of DPP4 beyond the incretin axis (233). These data are also supported by another study, where Ervinna and colleagues could show an increased ERK1/2 activation in rat SMC after short-term treatment with sDPP4, which was partially blocked by DPP4i (234). Very recently, we were also able to show that sDPP4 triggers thromboxan release from endothelial cells through a PAR2-mediated pathway (235). Since thromboxan acts as a vasoconstrictor prostanoid, sDPP4 impairs endothelium-dependent relaxation. Thereby we were able to show that sDPP4 is a direct mediator of endothelial dysfunction. All these data indicate that dysregulated DPP4 release, as it occurs during the development of obesity, has an impact on distinct target tissues in a para- or endocrine way, like e.g. acting on the vascular wall and inducing inflammation in this setting.

3.2. Adipose tissue-derived DPP4

3.2.1. Role of DPP4 within adipose tissue

3.2.1.1. DPP4 and AT remodeling

Our group already described DPP4 as an adipokine in 2011 (185) and we were able to show that especially during obesity and in T2DM DPP4 levels are upregulated (186). This observation points towards an important role of AT in systemic levels of DPP4, but the knowledge about the role of DPP4 within AT is very limited. Therefore, one aim of this thesis was to elucidate the role of AT-derived DPP4 during the development of obesity by a unique AT-specific KO mouse model. Furthermore, it was aimed to dissect the role of DPP4 in AT by *in vitro* silencing of DPP4 or treatment with well characterized DPP4 inhibitors of primary human adipocytes.

We were already able to show that DPP4 levels positively correlate with adipocyte diameter in human biopsies (185). This correlation could also be shown in the investigated mice. Furthermore, a size shift towards more small adipocytes and less big adipocytes was observed in KO animals under HFD feeding. It can be speculated that adipocyte size could be related to enzymatic activity of DPP4, since adipocyte size is reduced upon DPP4 inhibitor treatment in animal models with diet-induced obesity (DIO) (236). However, the authors also observed metabolic alterations in white AT which is paralleled by increased energy expenditure. This was not the case in the AT specific KO model and seems to be due to the systemic impact of orally applied DPP4i. Recently, it was also reported that DPP4 might be involved in dedifferentiation of mature adipocytes, since expression of MMP1, FAP and DPP4 was upregulated during a dedifferentiation protocol. Furthermore, this altered expression leads to an upregulation of inflammatory markers like IL-6 and Il-8 (237). However, no evidence for alterations in dedifferentiation was found in AT specific KO mouse model.

The occurance of smaller adipocytes in AT can also be explained by increased AT fibrosis, which increases the stiffness of this tissue and is upregulated during obesity. That means that due to AT fibrosis the adipocytes are not able to further increase their size by lipid uptake (238). Fibrosis seems to be a key factor for stimulating local inflammatory responses with upregulated levels of lysyl oxidase (LOX) and collagens I and III, as was shown by a degradation-resistant overexpression model of HIF-1α in AT (68). The size of the adipocytes is not further increasing since the ECM is unable to remodel and accommodate the need for increased adipocyte size due to higher lipid accumulation. The ECM is translating adipocyte stress into a pro-inflammatory signal with fibrosis being a key hallmark of metabolically dysfunctional AT (64). Khan and coworkers showed upon crossing a Col6a1-deficient mouse into the ob/ob background that hypertrophy per se is not sufficient to induce an inflammatory response (238). So

reducing collagen VI improved metabolic parameters and glucose tolerance, increased survival rates and reduced CLS in AT (238). In this thesis in the KO model, Col6a6 and Col3a1 were significantly downregulated under HFD compared to their WT littermates. This observation further strengthens the hypothesis that DPP4 depletion in AT leads to a beneficial AT remodeling.

DPP4 levels also negatively correlate with adiponectin levels in humans (185) as well as in AT specific DPP4 KO mouse model. However, no significant changes in adipogenesis markers within AT were observed neither in chow nor in HFD challenged animals. Furthermore, no differences in the differentiation of *in vitro* cultured human adipocytes with reduced DPP4 content in respect of lipid droplet accumulation or expression of differentiation markers (Fig. 5) were found. In conclusion, the observed data indicate that DPP4 seems to play no critical role in adipocyte differentiation per se.

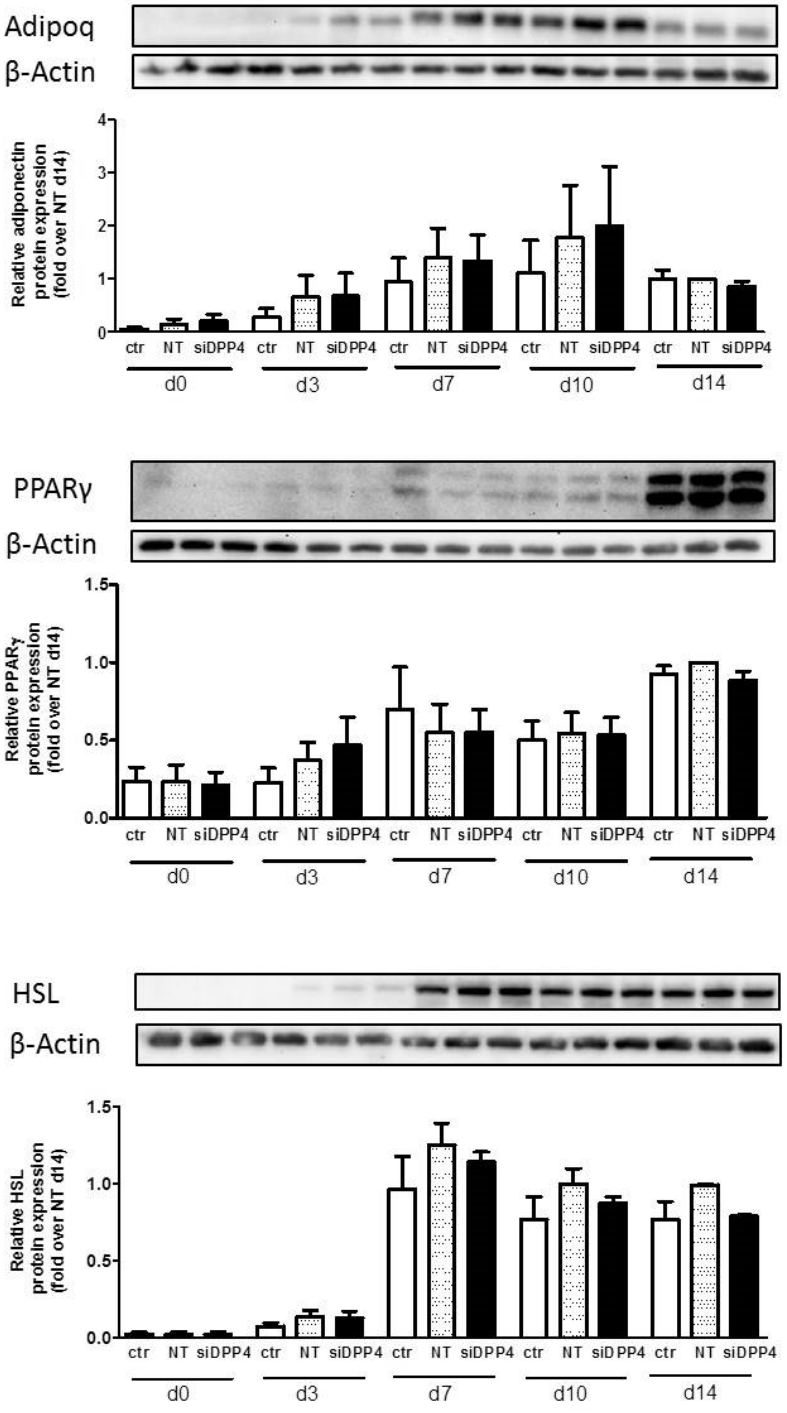


FIGURE 5: EFFECT OF SIRNA-MEDIATED SILENCING OF DPP4 ON ADIPOCYTE DIFFERENTIATION.
Protein expression of differentiation markers was assessed after siRNA-mediated silencing of DPP4 via Western Blot analysis. Data are expressed as mean ±SEM, n =4. Ctr control; NT non-target siRNA, siDPP4 DPP4-specific siRNA

3.2.1.2. DPP4 AND ITS ROLE IN AT MACROPHAGE PHENOTYPE AND INFLAMMATION

Macrophages and other immune cells also play an important role in normal AT function. During obesity the number of pro-inflammatory M1 macrophages is increasing via recruitment of novel macrophages and via a phenotypic switch from resident anti-inflammatory M2 macrophages (37). The expression of M1 macrophage markers compared to M2 markers is markedly increased in obese T2DM patients (239). Co-culture experiments of macrophages and adipocytes showed that the production of pro-inflammatory cytokines and FFA creates a paracrine vicious cycle with further upregulated release of macrophage-attracting cytokines (240). M2 polarized macrophages may help to preserve normal adipocyte function. Patsouris and colleagues were able to show that a specific ablation of $CD11c^+$ macrophages leads to a normalization of systemic insulin sensitivity (241). Improving the imbalance between M1 and M2 macrophages within AT might be a novel strategy to treat or prevent obesity-induced disorders by improving insulin sensitivity (242;243). To preserve adequate adipocyte function and insulin action during obesity it may be fruitful to identify novel factors that retain M2 polarisation or trigger a phenotypic switch from M1 towards M2 macrophages. In the adipose-specific DPP4 KO model both in VAT and SAT a significant upregulation of the M2 macrophage markers IL-10 and mannose receptor, C type 1 (Mrc1) was observed in HFD compared to wildtype. However, I also saw an upregulation of pro-inflammatory markers like IL-6 and MCP-1 and a higher number of crown-like structures (CLS) in VAT. It should be noted that inflammation might be beneficial for normal adipocyte function to some extent and that macrophages could help to remove necrotic adipocytes by surrounding them in CLS (85). Acute inflammation of white AT in contrast to chronic inflammation seems to sustain healthy AT expansion and prevents lipotoxicity and chronic inflammation (244). The AT specific DPP4 KO model at least partially fulfills the definition of healthy AT expansion characterized by enlargement of fat pad mass due to accumulation of smaller mature adipocytes, recruitment of macrophages in an appropriate ratio (M2 vs M1) and a minimal induction of ECM and inflammation (64). The notion that DPP4 might somehow be involved in inflammatory processes is also supported by studies on DPP4i. Shinjo and colleagues for example could show a weak but significant reduction in LPS- or TNFα-

induced expression of IL-6 and MCP-1 in 3T3-L1 cells (245). In the *in vitro* model of DPP4 depletion in primary human adipocytes I could observe no marked effects on TNFα-induced inflammation in respect of NFκB activation or expression and release of inflammatory markers. This difference might be due to the lack of interaction between adipocytes and macrophages as present in whole AT or it might reflect the difference of primary human cells vs. a mouse cell line as it was used in the study of Shinjo.

3.2.1.3. DPP4 AND ALTERATIONS IN AT SECRETOME

In addition to alterations in AT composition with respect to immune cell phenotype and remodeling of AT during obesity, another feature is the alteration of the AT secretome. Already in 2011, Xu and colleagues provided first evidence that DPP4 inhibition regulates adipokine secretion (246). DPP4 as an exopeptidase is able to inactivate cytokines secreted within AT or to generate novel bioactive compounds upon cleavage. Theoretically, it has a broad substrate specificity and can act on numerous different targets which might be involved in the progression or severity of obesity-associated diseases (107). However, as extensively reviewed by Mulvihill and Drucker it turned out to be very difficult to identify physiological targets of DPP4 (187). One reason is limitations in assay sensitivity making it rather difficult to differentiate between intact vs. cleaved substrates *in vivo*. Furthermore, there are no KO mouse models established or selective antagonists available for most of the potential physiological targets (187). I aimed to elucidate the role of siRNA-mediated DPP4 silencing on adipocyte secretome *in vitro* by the use of adipokine profiler arrays. I could observe no significant changes in the secretome of primary human adipocytes and very high donor variability. On the one hand it could mean, that DPP4 reduction plays no key role in secretion of the investigated adipokines, but it might on the other hand also mean that the assay is not sensitive enough to find changes in cleaved vs. intact peptides. Depending on where the spotted antibodies bind their substrates it is conceivable that also the cleaved forms are still recognized. In addition, it might be speculated that the ratio of cleaved versus intact substrates of DPP4 seems not to affect overall release of these factors. In conclusion, with the obtained data, the possibility that DPP4 alters the secretory output of adipocytes can not completely be ruled out. In the AT-specific KO mouse model significant changes in the secretion level of resistin and insulin-like growth factor binding protein 3 (IGFBP-3) from AT biopsies were found. The difference towards using human primary cells is that the secretome of the whole AT is obtained by the use of

biopsies in a more physiological setting. But this might also be a disadvantage since one can't ascribe the changes in the secretome directly to the adipocytes. It might as well be a change in the secretome of immune cells via cross-talk to adipocytes.

3.2.1.4. DPP4 AND AT FUNCTION

AT accounts for approximately 10% of postprandial glucose disposal. Therefore, insulin sensitivity is an important feature of normal adipocyte function. We were able to previously show that sDPP4 directly impairs insulin-stimulated Akt phosphorylation in human adipocytes (185). Furthermore, circulating DPP4 levels are significantly higher in obese individuals with insulin resistance than in their insulin sensitive counterparts (186). Upon siRNA-mediated silencing, I could show improved insulin signaling on the level of insulin receptor (InsR), proteinkinase b (Akt) as well as akt-substrate of 160kDa (AS160) phosphorylation. This phenomenon might be due to elevated basal levels of insulin receptor substrate 1 (IRS-1). However, it is also possible that DPP4 and the InsR interact with each other, either directly upon binding or indirectly via a substrate of DPP4. It has already been reported that the InsR is able to interact with different molecules which alter the responsiveness towards insulin in a positive (247) or negative way (248;249). The effects of DPP4 on insulin signaling seem to be partially mediated through DPP4 enzymatic activity since upon DPP4 inhibition via sitaglitpin or saxagliptin treatment I found a consistent upregulation of Akt phosphorylation. It was also shown that deleterious effects of recombinant sDPP4 on insulin signaling are prevented upon DPP4 inhibitor treatment (185). These results might point towards an involvement of DPP4 substrates, which might be present at higher levels due to DPP4 ablation. These substrates might thereby be able to improve insulin signaling. On the other hand, it is also possible that the seen effects of DPP4i might be mediated by a conformational change of DPP4 upon inhibitor binding. The conformational change may result in an inability to impair InsR responsiveness towards insulin. This mechanism was proposed in case of DPP4 activation of PAR2 which results in a downstream signaling event. The activation of downstream signaling pathways was abrogated upon DPP4i treatment (184). Also the binding of DPP4 to other partners can be altered upon DPP4i treatment, as it is the case for fibronectin (250).

DPP4 might also be a regulator of lipolysis since it could be shown that NPY is regulated by AT-derived DPP4 (251). Furthermore, this study revealed an augmented antilipolytic effect of NPY via DPP4i treatment. DPP4 might also regulate lipolysis through ADA interaction and alteration in adenosine levels. It is well known that adenosine levels affect lipolysis (252). However, I could observe no effects of siRNA-mediated silencing on adipocyte lipolysis (Fig. 6), neither on basal glycerol release nor on isoproterenol-stimulated lipolysis. Furthermore, the suppression of lipolysis via insulin was unaffected upon DPP4 silencing. In contrast to my approach, Kos and colleagues directly applied recombinant human NPY with or without DPP4 inhibitor on adipocytes (251). NPY is mainly produced in the brain and reaches adipose tissue via the blood stream (253). It is well known that NPY inhibits lipolysis in human AT (254). Upon treatment of adipocytes with recombinant NPY directly, Kos and colleagues rather mimick a cross-talk scenario of hypothalamus and AT which is not the case in my model (251). Therefore it is not possible to elucidate the role of DPP4 inhibition on adipocyte lipolysis directly. In addition to that Kos et al directly isolated mature adipocytes whereas in this thesis I differentiated isolated preadipocytes *in vitro*. Furthermore, it might be possible that the concentrations of insulin and isoproteronol I used in the study are not optimal to observe differences in glycerol release. To finally evaluate the role of DPP4 inhibition on lipolysis further experiments are necessary.

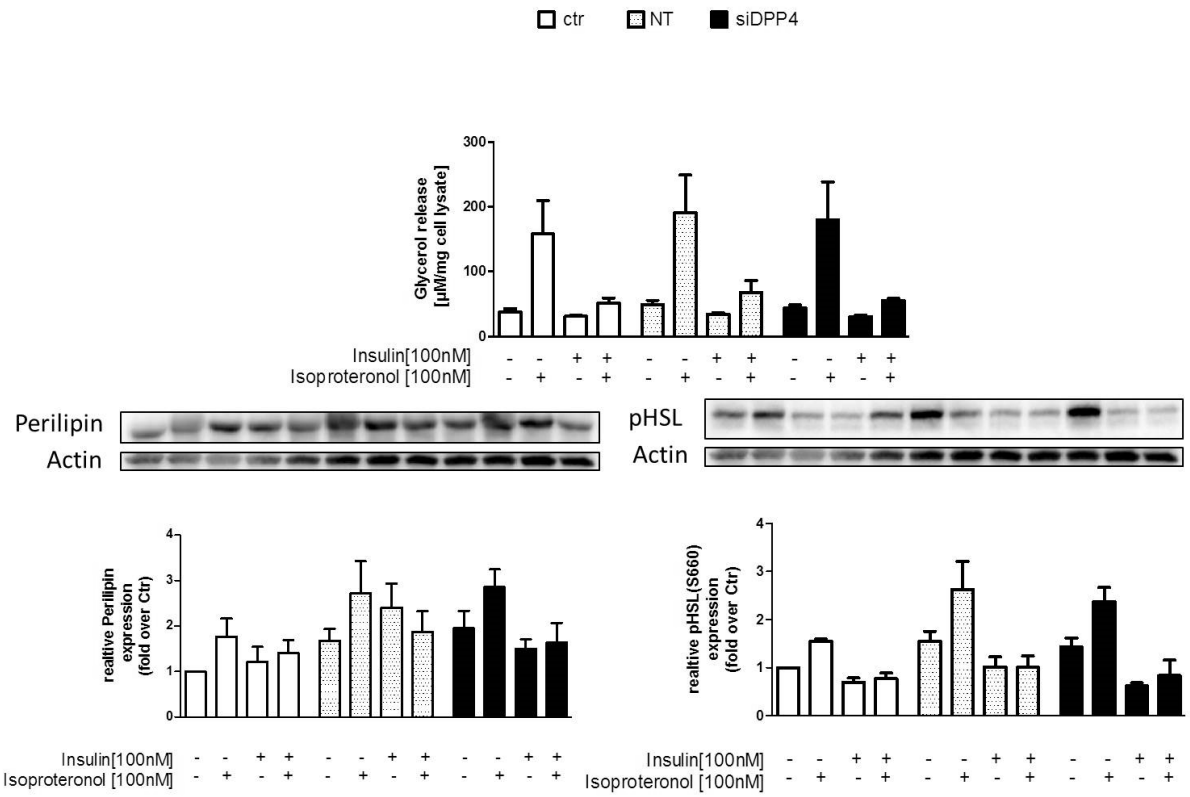


FIGURE 6: IMPACT OF SIRNA-MEDIATED DPP4 SILENCING ON LIPOLYSIS.
In vitro differentiated adipocytes were stimulated with 100nM insulin or isoproterenol as indicated and glycerol release to the cell culture medium was measured as well as intracellular protein expression of key mediators of lipolysis. Data are expressed as mean ± SEM. n=4. Ctr control; NT non-target siRNA; siDPP4 DPP4-specific siRNA

3.2.2. SYSTEMIC ROLE OF AT-DERIVED DPP4

3.2.2.1. SOURCE OF CIRCULATING DPP4

DPP4 is ubiquitously expressed on numerous cell types so that the main source of circulating DPP4 is difficult to define. In the AT-specific DPP4-KO mouse model it was shown, that DPP4 activity is very high in liver, AT, kidney and lung. Although it looks as if liver has the highest DPP4 activity in chow animal, one has to keep in mind that only DPP4 activity in two white AT depots and in one brown AT depot has been measured. There are many more AT depots within the body whose contribution to DPP4 activity could not be assessed. Furthermore, the measured activities of the three AT depots put together already display half the activity measured in the liver. During the progression

of obesity mice mainly gain fat mass and the body composition is shifted towards almost 50% fat content after 24weeks of HFD challenge in contrast to around 20% fat mass in animals fed with a chow diet. Therefore, AT might especially contribute to DPP4 circulating levels in the obese state. Circulating levels of DPP4 are significantly higher in WT-HFD mice in comparison to their littermates on chow diet. The serum level of DPP4 is lower in AT-specific KO animals irrespective of the diet but only reaches significance in HFD animals. This shows that the increase of DPP4 circulating levels is mainly coming from AT during the progression of obesity and also proofs that the mouse model has a systemic impact on DPP4 levels.

3.2.2.2. THE ROLE OF DPP4 ON METABOLIC PARAMETERS AND BODY WEIGHT

To date there have only been studies on whole body KO mice (208;209). DPP4 serum levels in homozygous KO mice are below detection level and thus levels of the incretin hormone GLP-1 are significantly upregulated (208). In the whole body KO animals, an improved glucose tolerance and a protection from DIO and IR were observed (208;209). In line with these data, AT specific DPP4 KO mice on HFD showed an improved glucose tolerance in in an oral glucose tolerance test, which might be due to alleviated incretin action and thus pointing towards an involvement of the incretin system. However, no difference in the level of active GLP-1 or GIP was observed in the circulation in the AT specific KO mouse. So one can speculate that the improvements on glucose tolerance observed in the generated mouse model might be independent from the incretin system. No differences in systemic insulin sensitivity or glucose tolerance have been observed after bypassing the incretin system by hyperinsulinemic-euglycemic clamps or intraperitoneal glucose / insulin tolerance tests. Interestingly, KO animals showed even significantly elevated body weight under HFD and thus opposing the data in the total KO mice. The authors observed a significantly lower body weight under HFD which is due to an effect of the KO on food intake and body length (209). This also shows one of the disadvantages of the whole body KO, since the seen beneficial effects on insulin sensitivity and AT phenotype can not directly be addressed to lower DPP4 levels, but might rather be due to the lower body weight per se. It is well known that DPP4 serves as a central regulator of satiety upon regulating food intake. NPY is one of the physiological substrates of DPP4, which was shown to have an altered receptor affinity in rats with DPP4 loss-of-function mutation thus altering food intake and feeding motivation (255;256). In the AT-specific KO model, effects on the central regulation of

food intake can be excluded and no differences in food intake have been observed. Data on clinical use of DPP4 inhibitors have repeatedly shown, that they act weight neutral (257). Therefore the impact of DPP4 on body weight seems rather complex and might be dependent on the primary organ targeted by the DPP4 inhibition.

3.2.2.3. AT-DERIVED DPP4 AND LIVER FUNCTION

It is well known that elevated serum levels of DPP4 are associated with various liver diseases like liver cirrhosis (258), hepatitis C infection (259) and NAFLD (260). Recently it could also be shown that in the diabetic and/or obese state circulating levels of DPP4 are associated with apoptosis and liver fibrosis (261). Therefore, one of the most important targets for circulating DPP4 might be the liver. Interestingly, a significant difference in endogenous glucose production (EGP) was observed in the generated mouse model in KO animals under HFD challenge. Especially during fasting, glucose is removed from the circulation in a constant rate and therefore, EGP by the liver is necessary to counteract the disappearance of glucose (262). As a key regulatory hormone for glucose disposal insulin immediately post-feeding removes glucose from the circulation upon triggering glucose uptake into SkMc and AT. Furthermore, it directly suppresses EGP in the liver. EGP is also suppressed indirectly via suppression of glucagon production in the pancreas (262). Therefore, EGP is a key diagnostic parameter of hepatic insulin sensitivity. During the development of T2DM, insulin action is impaired and effective glucose disposal is lowered (263). Under metabolically healthy conditions, glucose and insulin are in tight cross-talk to promote glucose disposal and to suppress EGP, which is abrogated in T2DM (264). In the AT specific DPP4 KO mouse model, EGP suppression is significantly elevated in KO mice under HFD challenge. Although, it is still significantly different from the chow control group, this points towards a selective improvement in liver insulin sensitivity upon AT-specific DPP4 KO. DPP4 inhibitors have been shown to be beneficial for liver steatosis and fibrosis via different mechanisms. Sitagliptin treated rats showed improved liver function via suppression of stellate cell proliferation and collagen synthesis (265). Diet-induced liver steatosis in a β-cell-specific glucokinase haploinsufficient mouse model was prevented by des-fluoro-sitagliptin treatment via decreasing the expression of e.g. SREBP-1c and

fatty acid synthase (FAS) (266). In AT specific DPP4 KO mice, no significant differences in respect to lipid content within the liver were seen. Also circulating levels of triglyceride or FFA were unaltered. However, all these measurements were performed after 24 weeks of HFD challenge and it might well be that progression of steatosis is slower in KO animals thus preserving insulin sensitivity also for a longer period.

A key question arising from the observations is which factor is mediating the beneficial effect on EGP suppression. It is reported that DPP4 enzymatic inhibition via vildagliptin treatment also enhances EGP suppression in T2DM patients in comparison to placebo (267). So there are two possible explanations. Either lower DPP4 levels in the circulation are directly contributing to improved liver sensitivity or DPP4 substrates are altered and thus mediating the seen effect. Via adipokine profiler arrays it could be shown that resistin as well as IGFBP-3 were significantly lower in the secretome of biopsies generated from visceral AT of KO animals on HFD. In addition, IGFBP-3 levels turned out to be significantly reduced in the circulation while free IGF-1 levels are elevated in KO after HFD. Among circulating total IGF-1 levels there were no changes observed. These differences in circulating levels of IGFBP3 and free IGF-1 might have an impact on peripheral organs like the liver.

The IGF system comprises two IGFs, two IGF receptors and six IGF binding proteins. Upon linkage to IGF, the IGFBPs escape degradation and the transport through body compartments is facilitated (268). The most abundant IGFBP in human serum is IGFBP-3, which binds about 90% of the circulating IGFs without a clear preference for one IGF (269). Thereby IGFBPs lower the amount of free IGFs and thus may act anti-proliferative, anti-mitotic and pro-apoptotic (270). IGFBPs consist of three domains, whereupon the central region contains not only IGF binding domains but also binding domains for cells, acid labile subunit (ALS) and heparin (271;272). Thus, IGFBP-3 is also able to act in an IGF-independent way. *In vitro* studies could show IGFBP-3 receptors are present on the surface of different cells (273). Furthermore, IGFBP-3 can be internalized and, upon linkage with the retinoic acid receptor, is able to induce gene expression (274). It has been shown that IGFBP-3 is able to interact with the nuclear receptor PPARγ, thus acting on the regulation of glucose and lipid metabolism (275). Several studies have shown that IGF-1 and IGFBP-3 are linked to the development of T2DM. Sesti and coworkers showed a positive correlation of endogenous IGF-1 levels and the degree of glucose intolerance in patients (276). The increased serum levels of IGF-1 might be counter-regulating the detrimental effects of IGFBP-3, since IGF-1 seems to be

acting in a protective way on insulin sensitivity. Higher production of IGF-1 seems to be a counterregulatory mechanism to combat the high levels of IGFBP-3 seen in T2DM. IGFBP-3 inhibits the biologic activity of IGF-1, contributing to less free IGF-1 and thereby increasing the risk of diabetes (277). Furthermore, IGF-1 is involved in lipid clearance from the circulation via increasing FFA uptake into adipocytes (278;279). Additionally, IGF-1 is able to suppress hepatic glucose production (280;281), thereby enhancing hepatic insulin sensitivity. An inverse correlation of serum IGFBP-3 and insulin sensitivity and an association of higher levels with BMI and fasting insulin further strengthens the detrimental role of IGFBP-3 in T2DM (282). This was also confirmed by *in vitro* studies in 3T3-L1 adipocytes, where IGFBP-3 induces IR via inhibition of insulin-stimulated Glut4 translocation and impaired glucose uptake (283). In this thesis it was shown, that IGFBP3-treated HepG2 cells display impaired insulin signaling and responsiveness towards insulin-stimulated glucose production. This is in line with the above mentioned studies in 3T3-L1 adipocytes and points towards a direct effect of IGFBP3 on liver physiology. In IGFBP-3 overexpressing mice it was shown that plasma IGFBP-3 levels mediate peripheral IR independent of IGF-1 (284). Furthermore, ER stress in the liver is associated with elF2α signaling via IGFBP-3. Transgenic mice, overexpressing a constitutively active regulatory subunit of the phosphatase that terminates ER stress-signaling by phospho-eIF2α, showed higher hepatic mRNA expression and circulating levels of IGFBP-3, which are transducing the ER stress to peripheral organs like AT (285). Mass spectrometry analysis revealed that IGF-1 is a substrate of DPP4. Upon cleavage IGF-1 has a lowered receptor binding affinity, but an increased binding to IGFBP-3 than the full length form (286). However, treatment of pigs for 72h with sitagliptin and also treatment of healthy non-diabetic human subjects for 10 days revealed no impact on circulating levels of IGF-1 or IGFBP-3 (287;288). In this thesis serum levels of free IGF-1 and also total IGF-1 were increased in recent onset metformin-trated type 2 diabetes patients additionally treated with the DPP4i sitagliptin. This highlights how difficult it is to predict changes of DPP4i in downstream pathways.

Finally it seems plausible that the lowered IGFBP-3 release in the AT-specific KO mouse model may contribute to the selective hepatic insulin sensitizing effects first upon

increased free IGF-1 levels and second via directly mediating insulin sensitivity of hepatocytes. So in AT specific DPP4 KO mice under HFD, reduced release of IGFBP3 from visceral AT might contribute to a protective cross-talk between AT and liver. However, the concrete mechanism how DPP4 is linked to IGFBP-3 remains open and needs to be addressed in the future.

3.2.2.4. ADVANTAGE OF TISSUE-SPECIFIC KO APPROACHES

Another drawback of the whole body KO animals in addition to the already mentioned effect on feeding behaviour, is that the immune cells are also affected by the KO. Immune cell infiltration and activation is a crucial parameter of the progression of AT dysfunction during obesity. Furthermore, low DPP4 levels might also be contributing to chronic inflammatory diseases or cancer. Low circulating levels are e.g. associated with different types of cancer (105;289), rheumatoid arthritis (290) and chronic obstructive pulmonary disease (291). DPP4 is interacting with numerous binding partners and is thereby involved in various intracellular downstream signaling processes (107). Therefore the role of DPP4 within the body is multidimensional and tissue-specific KO approaches are more fruitful in dissecting the role of DPP4 in different organs. However, there are some concerns upcoming for the use of aP2 as AT-specific promoter, as used in this mouse model (162). For example, it has been reported that also macrophages are expressing aP2 and might thereby be affected by the KO. To rule out that the model affects DPP4 expression in macrophages, AT has been fractionated into mature adipocytes and the stroma-vascular fraction. The major cellular components of the stroma-vascular fraction are preadipocytes, immune cells and fibroblasts. No difference in DPP4 protein expression in WT or KO preparations was observed. Furthermore, it also has been confirmed that CD11c positive fractions from peritoneal lavage display no difference in DPP4 expression. Thereby, it could be ruled out that the KO affects macrophages and that the shown effects are overlapped by macrophage contribution.

3.3. DPP4 AS AN IMPORTANT ADIPOKINE IN THE CONTEXT OF OBESITY AND T2DM

As already highlighted before, DPP4 displays a wide-spread role in diabetes. This is mediated through its incretin-dependent, but also through its incretin-independent functions. Most of the so far published studies have allocated the beneficial effects of

DPP4i to prolonging the half-life of the incretin hormones like GLP-1 (190;205;292). However, more and more studies also revealed that DPP4 exerts its role in diabetes in an incretin-independent way for example via regulating T-cell activation directly (293) or indirectly via facilitating adenosine clearance upon ADA binding (294). Thereby DPP4 is involved in inflammation, which is a major contributor of IR and T2DM during the progression of obesity. But DPP4 may also exert GLP-1-independent effects via the regulation of other substrates (266;295). Furthermore, we confirmed that DPP4 plays a role in insulin signaling via impairing pAkt activation in an endocrine and paracrine manner (185). This was also supported by the fact that siRNA-mediated silencing or treatment with specific DPP4i improved insulin signaling in adipocytes as presented in this thesis. DPP4 also plays a role in glucose homeostasis via adenosine, which is involved in maintaining high efficiency of insulin signaling and insulin-stimulated glucose transport (181). Additionally, it could be shown by an AT-specific KO mouse approach that AT is one important contributor to circulating DPP4. Furthermore, elevated DPP4 release into the circulation, which is observed during HFD-feeding, is prevented in KO animals. Since DPP4 is an adipokine and AT mass is mainly expanding during obesity, it is important to understand the regulation and mechanism of DPP4 release. In this context I was able show an involvement of MMPs in DPP4 shedding, which are known to be dysregulated in obesity (218;219). On the other way round, it is also reported that DPP4 itself mediates MMP activation (179). MMPs might also be the reason for the observed effects on healthy AT remodeling under HFD challenge. They could be one link of lowered fibrosis and the occurring adipocyte size shift and altered DPP4 levels within AT in obesity. The influence of DPP4 on the AT secretory output could be another mediating mechanism, since altered resistin and IGFBP-3 levels in KO animals under HFD were observed. DPP4 release could be regulated by inflammatory markers like TNFα, as well as metabolic parameters like insulin (185), both of which are important players in T2DM. Interestingly I could show that AT-derived DPP4 has a selective impact on hepatic insulin sensitivity most likely via regulating circulating levels of IGFBP3.

Although questions on the exact molecular mechanisms underlying these observations still remain open, I was able to highlight in this work the role of DPP4 in obesity especially beyond the incretin axis. Thereby, I contributed significantly to a better understanding of this complex molecule as summarized in Fig 7.

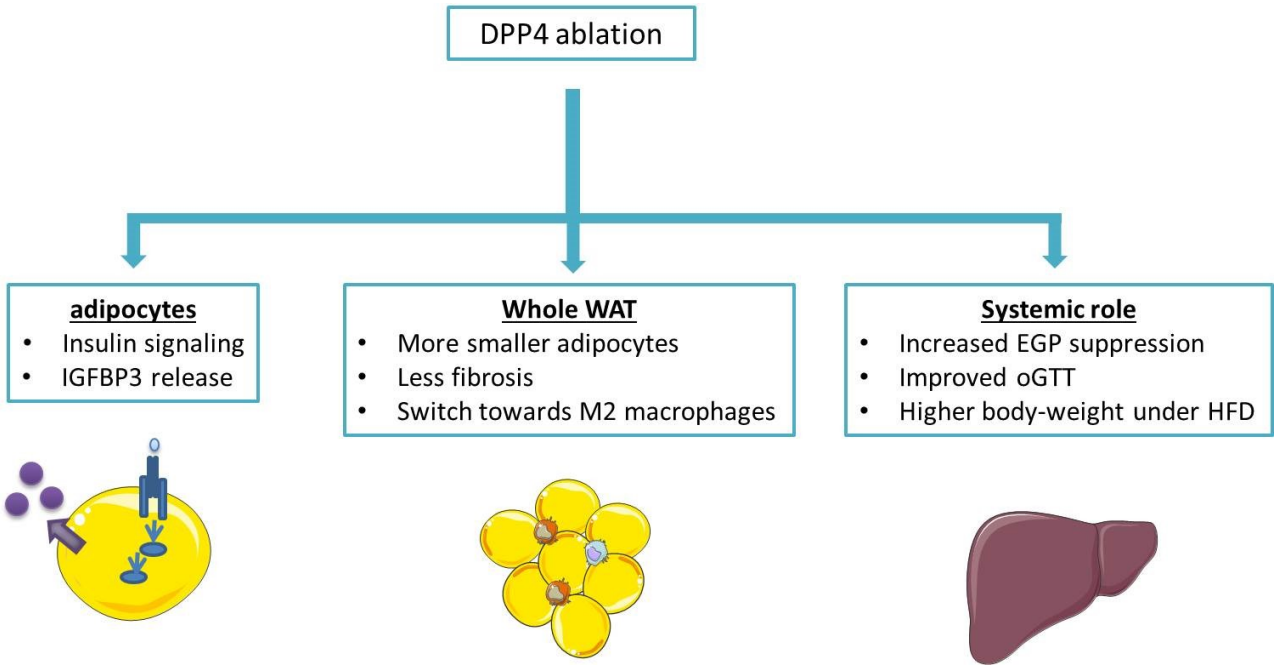


FIGURE 7: IMPACT OF DPP4 ABLATION.
The outcome of this thesis is summarized in this figure. Downregulation of DPP4 has been shown to have a beneficicial effect on adipocytes themself, AT in total as well as systemically via e.g. improving hepatic insulin sensitivity. IGFBP3 insulin-like growth factor binding protein 3; EGP endogenous glucose production; oGTT oral glucose tolerance test; HFD high fat diet

3.4. PERSPECTIVES

DPP4 is not only expressed as a cell surface protease, but is also released into the circulation. It is well known that circulating levels of DPP4 correlate with various obesity-associated complications like MetS (185;186), cardiovascular diseases (294), and cancer (105). However, the mechanism of DPP4 release and its regulation is mainly unknown. Therefore, the first objective of this thesis was to identify the sheddases and regulatory factors involved in DPP4 release from SMC and adipocytes. I confirmed that DPP4 release occurs through a non-classical secretory pathway. Constitutive as well as hypoxia-induced DPP4 shedding from SMC is mediated via MMPs like MMP1, MMP2 and MMP14. Furthermore, I could show that DPP4 release via MMPs is cell-type specific and involves MMP9 in adipocytes. I also confirmed that DPP4 shedding is a complex interplay between different shedding enzymes and might involve other unidentified factors, since I was only able to block DPP4 release by about 50% in the investigated cell types. A causal relationship between DPP4 and the involved shedding enzymes in obesity is lacking so far. Therefore, future research should focus on the molecular mechanisms to better understand the dysregulated release of sDPP4 in pathophysiological conditions.

Although we already described DPP4 as an adipokine potentially linking obesity to the MetS (185), it was unclear which role DPP4 plays within AT. The second objective of this work was to assess the role of DPP4 within adipocytes *in vitro* via siRNA-mediated silencing or the use of DPP4i. I demonstrated that DPP4 plays no central role in adipocyte differentiation or TNFα-induced inflammation. However, I provide clear evidence that reduction of DPP4 improves insulin signaling in adipocytes and seems to be at least partly mediated via its enzymatic activity. This is in line with previously published data were sDPP4 directly impairs insulin-stimulated Akt phosphorylation in adipocytes which could be prevented by DPP4i (185). However, the physiological role of DPP4 in adipocyte function downstream of AS160 phosphorylation remains elusive. Future studies should therefore investigate the impact of siRNA-mediated silencing on Glut4 translocation or glucose uptake in adipocytes. Furthermore, a role of DPP4 in altering the adipocyte secretome could not be ruled out by the generated data and needs

to be addressed in future experiments with assays bypassing the limitations of the used adipokine arrays.

Data on global DPP4 KO mice demonstrated a high impact of DPP4 on metabolic health (208;209). These results are difficult to interpret, because of the impaired food intake, the KO in every organ and most importantly also in immune cells. From the already published data we hypothesized that, especially during obesity, AT is a major source of sDPP4. The third objective of this thesis was to elucidate the role of AT-derived DPP4 during obesity on AT itself and in the cross-talk to other organs. In this thesis, a unique AT-specific KO mouse model was established which was challenged with HFD feeding for 24 weeks. It could be demonstrated that DPP4 depletion in AT plays a beneficial role in visceral AT remodeling under diet-induced obesity. This was mediated through elevated expression of M2 macrophage markers, lowered expression of fibrosis markers, and a shift towards smaller adipocytes. On the systemic level, it could be shown that DPP4 depletion, despite elevated body-weight, leads to an improved oral glucose tolerance and selective improvement of hepatic insulin sensitivity possibly in an incretin-independent way. Although, IGFBP-3 was suggested as a potential mediator of the seen effects, the molecular link between DPP4 and IGFBP-3 remains open and needs to be addressed by future experiments. Although IGFBP3 directly affects insulin signaling in HepG2 cells these experiments are only a hint for a potential interaction of IGFBP3 and liver. The exact underlying mechanism is still unknown and further experiments need to address the impact of IGFBP3 on liver cells. Additionally, *ex vivo* insulin signaling in the liver of AT specific DPP4 KO animals in comparison to control animals should be analysed in the future. It was also demonstrated that liver has a high impact on circulating active levels of sDPP4. So via a liver-specific KO approach the impact of sDPP4 derived from liver in the context of obesity should be addressed and compared to the AT-specific data.

BIBLIOGRAPHY

1. **Fonseca-Alaniz MH, Takada J, Alonso-Vale MI, Lima FB** 2007 Adipose tissue as an endocrine organ: from theory to practice. J Pediatr (Rio J) 83:S192-S203

2. **Ahima RS, Flier JS** 2000 Adipose tissue as an endocrine organ. Trends Endocrinol Metab 11:327-333

3. **Cannon B, Nedergaard J** 2004 Brown adipose tissue: function and physiological significance. Physiol Rev 84:277-359

4. **Pasco JA, Holloway KL, Dobbins AG, Kotowicz MA, Williams LJ, Brennan SL** 2014 Body mass index and measures of body fat for defining obesity and underweight: a cross-sectional, population-based study. BMC Obes 1:9

5. **Goran MI, Ball GD, Cruz ML** 2003 Obesity and risk of type 2 diabetes and cardiovascular disease in children and adolescents. J Clin Endocrinol Metab 88:1417-1427

6. **Murphy NF, MacIntyre K, Stewart S, Hart CL, Hole D, McMurray JJ** 2006 Long-term cardiovascular consequences of obesity: 20-year follow-up of more than 15 000 middle-aged men and women (the Renfrew-Paisley study). Eur Heart J 27:96-106

7. **Xu L, Kitade H, Ni Y, Ota T** 2015 Roles of Chemokines and Chemokine Receptors in Obesity-Associated Insulin Resistance and Nonalcoholic Fatty Liver Disease. Biomolecules 5:1563-1579

8. **Bhatt HB, Smith RJ** 2015 Fatty liver disease in diabetes mellitus. Hepatobiliary Surg Nutr 4:101-108

9. **Polednak AP** 2008 Estimating the number of U.S. incident cancers attributable to obesity and the impact on temporal trends in incidence rates for obesity-related cancers. Cancer Detect Prev 32:190-199

10. **Burgering BM, Coffer PJ** 1995 Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376:599-602

11. **Laakso M, Edelman SV, Brechtel G, Baron AD** 1990 Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. J Clin Invest 85:1844-1852

12. **Baron AD, Brechtel G, Johnson A, Fineberg N, Henry DP, Steinberg HO** 1994 Interactions between insulin and norepinephrine on blood pressure and insulin sensitivity. Studies in lean and obese men. J Clin Invest 93:2453-2462

13. **Karpe F, Fielding BA, Ilic V, Macdonald IA, Summers LK, Frayn KN** 2002 Impaired postprandial adipose tissue blood flow response is related to aspects of insulin sensitivity. Diabetes 51:2467-2473

14. **Girousse A, Tavernier G, Valle C, Moro C, Mejhert N, Dinel AL, Houssier M, Roussel B, Besse-Patin A, Combes M, Mir L, Monbrun L, Bezaire V, Prunet-Marcassus B, Waget A, Vila I, Caspar-Bauguil S, Louche K, Marques MA, Mairal A, Renoud ML, Galitzky J, Holm C, Mouisel E, Thalamas C, Viguerie N, Sulpice T, Burcelin R, Arner P, Langin D** 2013 Partial inhibition of adipose tissue lipolysis

improves glucose metabolism and insulin sensitivity without alteration of fat mass. PLoS Biol 11:e1001485

15. **Perry RJ, Camporez JP, Kursawe R, Titchenell PM, Zhang D, Perry CJ, Jurczak MJ, Abudukadier A, Han MS, Zhang XM, Ruan HB, Yang X, Caprio S, Kaech SM, Sul HS, Birnbaum MJ, Davis RJ, Cline GW, Petersen KF, Shulman GI** 2015 Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes. Cell 160:745-758

16. **RANDLE PJ, GARLAND PB, HALES CN, NEWSHOLME EA** 1963 The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1:785-789

17. **Arner P, Langin D** 2014 Lipolysis in lipid turnover, cancer cachexia, and obesity-induced insulin resistance. Trends Endocrinol Metab 25:255-262

18. **Samuel VT, Shulman GI** 2012 Mechanisms for insulin resistance: common threads and missing links. Cell 148:852-871

19. **Boden G, Chen X, Rosner J, Barton M** 1995 Effects of a 48-h fat infusion on insulin secretion and glucose utilization. Diabetes 44:1239-1242

20. **Kitamura T, Kitamura Y, Kuroda S, Hino Y, Ando M, Kotani K, Konishi H, Matsuzaki H, Kikkawa U, Ogawa W, Kasuga M** 1999 Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt. Mol Cell Biol 19:6286-6296

21. **Choi SM, Tucker DF, Gross DN, Easton RM, DiPilato LM, Dean AS, Monks BR, Birnbaum MJ** 2010 Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. Mol Cell Biol 30:5009-5020

22. **Ahmed K, Tunaru S, Tang C, Muller M, Gille A, Sassmann A, Hanson J, Offermanns S** 2010 An autocrine lactate loop mediates insulin-dependent inhibition of lipolysis through GPR81. Cell Metab 11:311-319

23. **Morigny P, Houssier M, Mouisel E, Langin D** 2015 Adipocyte lipolysis and insulin resistance. Biochimie

24. **Goldrick RB, McLoughlin GM** 1970 Lipolysis and lipogenesis from glucose in human fat cells of different sizes. Effects of insulin, epinephrine, and theophylline. J Clin Invest 49:1213-1223

25. **Jacobsson B, Smith U** 1972 Effect of cell size on lipolysis and antilipolytic action of insulin in human fat cells. J Lipid Res 13:651-656

26. **Ostman J, Backman L, Hallberg D** 1975 Cell size and the antilipolytic effect of insulin in human subcutaneous adipose tissue. Diabetologia 11:159-164

27. **Pedersen O, Hjollund E, Sorensen NS** 1982 Insulin receptor binding and insulin action in human fat cells: effects of obesity and fasting. Metabolism 31:884-895

28. **Reynisdottir S, Ellerfeldt K, Wahrenberg H, Lithell H, Arner P** 1994 Multiple lipolysis defects in the insulin resistance (metabolic) syndrome. J Clin Invest 93:2590-2599

29. **Gustafson B, Hedjazifar S, Gogg S, Hammarstedt A, Smith U** 2015 Insulin resistance and impaired adipogenesis. Trends Endocrinol Metab 26:193-200

30. **Herman MA, Peroni OD, Villoria J, Schon MR, Abumrad NA, Bluher M, Klein S, Kahn BB** 2012 A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. Nature 484:333-338

31. **Graham TE, Yang Q, Bluher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB** 2006 Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. N Engl J Med 354:2552-2563

32. **Hauner H, Rohrig K, Spelleken M, Liu LS, Eckel J** 1998 Development of insulin-responsive glucose uptake and GLUT4 expression in differentiating human adipocyte precursor cells. Int J Obes Relat Metab Disord 22:448-453

33. **Kahn BB, Charron MJ, Lodish HF, Cushman SW, Flier JS** 1989 Differential regulation of two glucose transporters in adipose cells from diabetic and insulin-treated diabetic rats. J Clin Invest 84:404-411

34. **Garvey WT, Maianu L, Hueckstead TP, Birnbaum MJ, Molina JM, Ciaraldi TP** 1991 Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. J Clin Invest 87:1072-1081

35. **Mullins GR, Wang L, Raje V, Sherwood SG, Grande RC, Boroda S, Eaton JM, Blancquaert S, Roger PP, Leitinger N, Harris TE** 2014 Catecholamine-induced lipolysis causes mTOR complex dissociation and inhibits glucose uptake in adipocytes. Proc Natl Acad Sci U S A 111:17450-17455

36. **Fayard E, Xue G, Parcellier A, Bozulic L, Hemmings BA** 2010 Protein kinase B (PKB/Akt), a key mediator of the PI3K signaling pathway. Curr Top Microbiol Immunol 346:31-56

37. **Badoud F, Perreault M, Zulyniak MA, Mutch DM** 2015 Molecular insights into the role of white adipose tissue in metabolically unhealthy normal weight and metabolically healthy obese individuals. FASEB J 29:748-758

38. **Crossno JT, Jr., Majka SM, Grazia T, Gill RG, Klemm DJ** 2006 Rosiglitazone promotes development of a novel adipocyte population from bone marrow-derived circulating progenitor cells. J Clin Invest 116:3220-3228

39. **Primeau V, Coderre L, Karelis AD, Brochu M, Lavoie ME, Messier V, Sladek R, Rabasa-Lhoret R** 2011 Characterizing the profile of obese patients who are metabolically healthy. Int J Obes (Lond) 35:971-981

40. **Marques-Vidal P, Pecoud A, Hayoz D, Paccaud F, Mooser V, Waeber G, Vollenweider P** 2010 Normal weight obesity: relationship with lipids, glycaemic status, liver enzymes and inflammation. Nutr Metab Cardiovasc Dis 20:669-675

41. **Bjorntorp P, Berchtold P, Holm J, Larsson B** 1971 The glucose uptake of human adipose tissue in obesity. Eur J Clin Invest 1:480-485

42. **Kloting N, Bluher M** 2014 Adipocyte dysfunction, inflammation and metabolic syndrome. Rev Endocr Metab Disord 15:277-287

43. **Hoffstedt J, Arner E, Wahrenberg H, Andersson DP, Qvisth V, Lofgren P, Ryden M, Thorne A, Wiren M, Palmer M, Thorell A, Toft E, Arner P** 2010 Regional impact of adipose tissue morphology on the metabolic profile in morbid obesity. Diabetologia 53:2496-2503

44. **Majithia AR, Flannick J, Shahinian P, Guo M, Bray MA, Fontanillas P, Gabriel SB, Rosen ED, Altshuler D** 2014 Rare variants in PPARG with decreased activity in adipocyte differentiation are associated with increased risk of type 2 diabetes. Proc Natl Acad Sci U S A 111:13127-13132

45. **Pajvani UB, Scherer PE** 2003 Adiponectin: systemic contributor to insulin sensitivity. Curr Diab Rep 3:207-213

46. **Laurencikiene J, Skurk T, Kulyte A, Heden P, Astrom G, Sjolin E, Ryden M, Hauner H, Arner P** 2011 Regulation of lipolysis in small and large fat cells of the same subject. J Clin Endocrinol Metab 96:E2045-E2049

47. **Cotillard A, Poitou C, Torcivia A, Bouillot JL, Dietrich A, Kloting N, Gregoire C, Lolmede K, Bluher M, Clement K** 2014 Adipocyte size threshold matters: link with risk of type 2 diabetes and improved insulin resistance after gastric bypass. J Clin Endocrinol Metab 99:E1466-E1470

48. **Rodriguez A, Catalan V, Gomez-Ambrosi J, Garcia-Navarro S, Rotellar F, Valenti V, Silva C, Gil MJ, Salvador J, Burrell MA, Calamita G, Malagon MM, Fruhbeck G** 2011 Insulin- and leptin-mediated control of aquaglyceroporins in human adipocytes and hepatocytes is mediated via the PI3K/Akt/mTOR signaling cascade. J Clin Endocrinol Metab 96:E586-E597

49. **Kloting N, Fasshauer M, Dietrich A, Kovacs P, Schon MR, Kern M, Stumvoll M, Bluher M** 2010 Insulin-sensitive obesity. Am J Physiol Endocrinol Metab 299:E506-E515

50. **McLaughlin T, Sherman A, Tsao P, Gonzalez O, Yee G, Lamendola C, Reaven GM, Cushman SW** 2007 Enhanced proportion of small adipose cells in insulin-resistant vs insulin-sensitive obese individuals implicates impaired adipogenesis. Diabetologia 50:1707-1715

51. **Stefan N, Kantartzis K, Machann J, Schick F, Thamer C, Rittig K, Balletshofer B, Machicao F, Fritsche A, Haring HU** 2008 Identification and characterization of metabolically benign obesity in humans. Arch Intern Med 168:1609-1616

52. **O'Connell J, Lynch L, Cawood TJ, Kwasnik A, Nolan N, Geoghegan J, McCormick A, O'Farrelly C, O'Shea D** 2010 The relationship of omental and subcutaneous adipocyte size to metabolic disease in severe obesity. PLoS One 5:e9997

53. **Xu XJ, Gauthier MS, Hess DT, Apovian CM, Cacicedo JM, Gokce N, Farb M, Valentine RJ, Ruderman NB** 2012 Insulin sensitive and resistant obesity in humans: AMPK activity, oxidative stress, and depot-specific changes in gene expression in adipose tissue. J Lipid Res 53:792-801

54. **Bost F, Aouadi M, Caron L, Binetruy B** 2005 The role of MAPKs in adipocyte differentiation and obesity. Biochimie 87:51-56

55. **Johnson GL, Lapadat R** 2002 Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298:1911-1912

56. **Ruderman N, Chisholm D, Pi-Sunyer X, Schneider S** 1998 The metabolically obese, normal-weight individual revisited. Diabetes 47:699-713

57. **Dvorak RV, DeNino WF, Ades PA, Poehlman ET** 1999 Phenotypic characteristics associated with insulin resistance in metabolically obese but normal-weight young women. Diabetes 48:2210-2214

58. **Messier V, Karelis AD, Prud'homme D, Primeau V, Brochu M, Rabasa-Lhoret R** 2010 Identifying metabolically healthy but obese individuals in sedentary postmenopausal women. Obesity (Silver Spring) 18:911-917

59. **Brochu M, Tchernof A, Dionne IJ, Sites CK, Eltabbakh GH, Sims EA, Poehlman ET** 2001 What are the physical characteristics associated with a normal metabolic profile despite a high level of obesity in postmenopausal women? J Clin Endocrinol Metab 86:1020-1025

60. **Shin MJ, Lee JH, Jang Y, Park E, Oh J, Chung JH, Chung N** 2006 Insulin resistance, adipokines, and oxidative stress in nondiabetic, hypercholesterolemic patients: leptin as an 8-epi-prostaglandin F2alpha determinant. Metabolism 55:918-922

61. **Karelis AD, Faraj M, Bastard JP, St-Pierre DH, Brochu M, Prud'homme D, Rabasa-Lhoret R** 2005 The metabolically healthy but obese individual presents a favorable inflammation profile. J Clin Endocrinol Metab 90:4145-4150

62. **Lackey DE, Burk DH, Ali MR, Mostaedi R, Smith WH, Park J, Scherer PE, Seay SA, McCoin CS, Bonaldo P, Adams SH** 2014 Contributions of adipose tissue architectural and tensile properties toward defining healthy and unhealthy obesity. Am J Physiol Endocrinol Metab 306:E233-E246

63. **Tang W, Zeve D, Suh JM, Bosnakovski D, Kyba M, Hammer RE, Tallquist MD, Graff JM** 2008 White fat progenitor cells reside in the adipose vasculature. Science 322:583-586

64. **Sun K, Wernstedt A, I, Kusminski CM, Bueno AC, Wang ZV, Pollard JW, Brekken RA, Scherer PE** 2012 Dichotomous effects of VEGF-A on adipose tissue dysfunction. Proc Natl Acad Sci U S A 109:5874-5879

65. **Pessin JE, Kwon H** 2012 How does high-fat diet induce adipose tissue fibrosis? J Investig Med 60:1147-1150

66. **Brook CG, Lloyd JK, Wolf OH** 1972 Relation between age of onset of obesity and size and number of adipose cells. Br Med J 2:25-27

67. **Helmlinger G, Yuan F, Dellian M, Jain RK** 1997 Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 3:177-182

68. **Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, Wang ZV, Landskroner-Eiger S, Dineen S, Magalang UJ, Brekken RA, Scherer PE** 2009 Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. Mol Cell Biol 29:4467-4483

69. **Henegar C, Tordjman J, Achard V, Lacasa D, Cremer I, Guerre-Millo M, Poitou C, Basdevant A, Stich V, Viguerie N, Langin D, Bedossa P, Zucker JD, Clement K** 2008 Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity. Genome Biol 9:R14

70. **Keophiphath M, Achard V, Henegar C, Rouault C, Clement K, Lacasa D** 2009 Macrophage-secreted factors promote a profibrotic phenotype in human preadipocytes. Mol Endocrinol 23:11-24

71. **Divoux A, Tordjman J, Lacasa D, Veyrie N, Hugol D, Aissat A, Basdevant A, Guerre-Millo M, Poitou C, Zucker JD, Bedossa P, Clement K** 2010 Fibrosis in human adipose tissue: composition, distribution, and link with lipid metabolism and fat mass loss. Diabetes 59:2817-2825

72. **Alligier M, Meugnier E, Debard C, Lambert-Porcheron S, Chanseaume E, Sothier M, Loizon E, Hssain AA, Brozek J, Scoazec JY, Morio B, Vidal H, Laville M** 2012 Subcutaneous adipose tissue remodeling during the initial phase of weight gain induced by overfeeding in humans. J Clin Endocrinol Metab 97:E183-E192

73. **Spencer M, Unal R, Zhu B, Rasouli N, McGehee RE, Jr., Peterson CA, Kern PA** 2011 Adipose tissue extracellular matrix and vascular abnormalities in obesity and insulin resistance. J Clin Endocrinol Metab 96:E1990-E1998

74. **Patel P, Abate N** 2013 Role of subcutaneous adipose tissue in the pathogenesis of insulin resistance. J Obes 2013:489187

75. **Wellen KE, Hotamisligil GS** 2005 Inflammation, stress, and diabetes. J Clin Invest 115:1111-1119

76. **Lacasa D, Taleb S, Keophiphath M, Miranville A, Clement K** 2007 Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes. Endocrinology 148:868-877

77. **Zhang HH, Kumar S, Barnett AH, Eggo MC** 2001 Dexamethasone inhibits tumor necrosis factor-alpha-induced apoptosis and interleukin-1 beta release in human subcutaneous adipocytes and preadipocytes. J Clin Endocrinol Metab 86:2817-2825

78. **Keuper M, Bluher M, Schon MR, Moller P, Dzyakanchuk A, Amrein K, Debatin KM, Wabitsch M, Fischer-Posovszky P** 2011 An inflammatory micro-environment promotes human adipocyte apoptosis. Mol Cell Endocrinol 339:105-113

79. **Goossens GH** 2008 The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav 94:206-218

80. **Dordevic AL, Konstantopoulos N, Cameron-Smith D** 2014 3T3-L1 preadipocytes exhibit heightened monocyte-chemoattractant protein-1 response to acute fatty acid exposure. PLoS One 9:e99382

81. **Chawla A, Nguyen KD, Goh YP** 2011 Macrophage-mediated inflammation in metabolic disease. Nat Rev Immunol 11:738-749

82. **Cancello R, Clement K** 2006 Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. BJOG 113:1141-1147

83. **Takahashi K, Mizuarai S, Araki H, Mashiko S, Ishihara A, Kanatani A, Itadani H, Kotani H** 2003 Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice. J Biol Chem 278:46654-46660

84. **Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M** 2006 MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest 116:1494-1505

85. **Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS** 2005 Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 46:2347-2355

86. **Fabbrini E, Cella M, McCartney SA, Fuchs A, Abumrad NA, Pietka TA, Chen Z, Finck BN, Han DH, Magkos F, Conte C, Bradley D, Fraterrigo G, Eagon JC, Patterson BW, Colonna M, Klein S** 2013 Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals. Gastroenterology 145:366-374

87. **Zhao R, Tang D, Yi S, Li W, Wu C, Lu Y, Hou X, Song J, Lin P, Chen L, Sun L** 2014 Elevated peripheral frequencies of Th22 cells: a novel potent participant in obesity and type 2 diabetes. PLoS One 9:e85770

88. **Apostolopoulos V, de Court, Stojanovska L, Blatch GL, Tangalakis K, de Court** 2016 The complex immunological and inflammatory network of adipose tissue in obesity. Mol Nutr Food Res 60:43-57

89. **Cook KS, Min HY, Johnson D, Chaplinsky RJ, Flier JS, Hunt CR, Spiegelman BM** 1987 Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. Science 237:402-405

90. **Maffei M, Fei H, Lee GH, Dani C, Leroy P, Zhang Y, Proenca R, Negrel R, Ailhaud G, Friedman JM** 1995 Increased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the db locus. Proc Natl Acad Sci U S A 92:6957-6960

91. **Lehr S, Hartwig S, Sell H** 2012 Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders. Proteomics Clin Appl 6:91-101

92. **Halberg N, Wernstedt-Asterholm I, Scherer PE** 2008 The adipocyte as an endocrine cell. Endocrinol Metab Clin North Am 37:753-7xi

93. **Pardo M, Roca-Rivada A, Seoane LM, Casanueva FF** 2012 Obesidomics: contribution of adipose tissue secretome analysis to obesity research. Endocrine 41:374-383

94. **Kershaw EE, Flier JS** 2004 Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89:2548-2556

95. **Dahlman I, Elsen M, Tennagels N, Korn M, Brockmann B, Sell H, Eckel J, Arner P** 2012 Functional annotation of the human fat cell secretome. Arch Physiol Biochem 118:84-91

96. **Halban PA, Irminger JC** 1994 Sorting and processing of secretory proteins. Biochem J 299 (Pt 1):1-18

97. **D'Arcangelo JG, Stahmer KR, Miller EA** 2013 Vesicle-mediated export from the ER: COPII coat function and regulation. Biochim Biophys Acta 1833:2464-2472

98. **Vitale A, Denecke J** 1999 The endoplasmic reticulum-gateway of the secretory pathway. Plant Cell 11:615-628

99. **Xie L, Boyle D, Sanford D, Scherer PE, Pessin JE, Mora S** 2006 Intracellular trafficking and secretion of adiponectin is dependent on GGA-coated vesicles. J Biol Chem 281:7253-7259

100. **Hooper NM, Karran EH, Turner AJ** 1997 Membrane protein secretases. Biochem J 321 (Pt 2):265-279

101. **Nickel W** 2003 The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur J Biochem 270:2109-2119

102. **Florkiewicz RZ, Majack RA, Buechler RD, Florkiewicz E** 1995 Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/Golgi pathway. J Cell Physiol 162:388-399

103. **Dahl JP, Binda A, Canfield VA, Levenson R** 2000 Participation of Na,K-ATPase in FGF-2 secretion: rescue of ouabain-inhibitable FGF-2 secretion by ouabain-resistant Na,K-ATPase alpha subunits. Biochemistry 39:14877-14883

104. **Eder C** 2009 Mechanisms of interleukin-1beta release. Immunobiology 214:543-553

105. **Cordero OJ, Salgado FJ, Nogueira M** 2009 On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 58:1723-1747

106. **Lemire I, Lazure C, Crine P, Boileau G** 1997 Secretion of a type II integral membrane protein induced by mutation of the transmembrane segment. Biochem J 322 (Pt 1):335-342

107. **Lambeir AM, Durinx C, Scharpe S, De M, I** 2003 Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci 40:209-294

108. **Raschke S, Eckardt K, Bjorklund HK, Jensen J, Eckel J** 2013 Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells. PLoS One 8:e62008

109. **Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP** 1997 A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385:729-733

110. **Ouchi N, Parker JL, Lugus JJ, Walsh K** 2011 Adipokines in inflammation and metabolic disease. Nat Rev Immunol 11:85-97

111. **Romacho T, Elsen M, Rohrborn D, Eckel J** 2014 Adipose tissue and its role in organ crosstalk. Acta Physiol (Oxf) 210:733-753

112. **Karastergiou K, Mohamed-Ali V** 2010 The autocrine and paracrine roles of adipokines. Mol Cell Endocrinol 318:69-78

113. **Cawthorn WP, Sethi JK** 2008 TNF-alpha and adipocyte biology. FEBS Lett 582:117-131

114. **Ryden M, Arner P** 2007 Tumour necrosis factor-alpha in human adipose tissue -- from signalling mechanisms to clinical implications. J Intern Med 262:431-438

115. **Fain JN, Bahouth SW, Madan AK** 2004 TNFalpha release by the nonfat cells of human adipose tissue. Int J Obes Relat Metab Disord 28:616-622

116. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr.** 2003 Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796-1808

117. **Cawthorn WP, Heyd F, Hegyi K, Sethi JK** 2007 Tumour necrosis factor-alpha inhibits adipogenesis via a beta-catenin/TCF4(TCF7L2)-dependent pathway. Cell Death Differ 14:1361-1373

118. **Lagathu C, Bastard JP, Auclair M, Maachi M, Capeau J, Caron M** 2003 Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. Biochem Biophys Res Commun 311:372-379

119. **Ruan H, Miles PD, Ladd CM, Ross K, Golub TR, Olefsky JM, Lodish HF** 2002 Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. Diabetes 51:3176-3188

120. **Sell H, Laurencikiene J, Taube A, Eckardt K, Cramer A, Horrighs A, Arner P, Eckel J** 2009 Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. Diabetes 58:2731-2740

121. **Sell H, Eckel J** 2007 Regulation of retinol binding protein 4 production in primary human adipocytes by adiponectin, troglitazone and TNF-alpha. Diabetologia 50:2221-2223

122. **Norseen J, Hosooka T, Hammarstedt A, Yore MM, Kant S, Aryal P, Kiernan UA, Phillips DA, Maruyama H, Kraus BJ, Usheva A, Davis RJ, Smith U, Kahn BB** 2012 Retinol-binding protein 4 inhibits insulin signaling in adipocytes by inducing proinflammatory cytokines in macrophages through a c-Jun N-

terminal kinase- and toll-like receptor 4-dependent and retinol-independent mechanism. Mol Cell Biol 32:2010-2019

123. **Fasshauer M, Klein J, Lossner U, Paschke R** 2003 Interleukin (IL)-6 mRNA expression is stimulated by insulin, isoproterenol, tumour necrosis factor alpha, growth hormone, and IL-6 in 3T3-L1 adipocytes. Horm Metab Res 35:147-152

124. **Stephens JM, Lee J, Pilch PF** 1997 Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem 272:971-976

125. **Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van OE** 2001 SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. J Biol Chem 276:47944-47949

126. **Peraldi P, Filloux C, Emanuelli B, Hilton DJ, Van OE** 2001 Insulin induces suppressor of cytokine signaling-3 tyrosine phosphorylation through janus-activated kinase. J Biol Chem 276:24614-24620

127. **Peraldi P, Hotamisligil GS, Buurman WA, White MF, Spiegelman BM** 1996 Tumor necrosis factor (TNF)-alpha inhibits insulin signaling through stimulation of the p55 TNF receptor and activation of sphingomyelinase. J Biol Chem 271:13018-13022

128. **Teruel T, Hernandez R, Lorenzo M** 2001 Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. Diabetes 50:2563-2571

129. **Path G, Bornstein SR, Gurniak M, Chrousos GP, Scherbaum WA, Hauner H** 2001 Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. J Clin Endocrinol Metab 86:2281-2288

130. **Fasshauer M, Paschke R, Stumvoll M** 2004 Adiponectin, obesity, and cardiovascular disease. Biochimie 86:779-784

131. **Gil-Campos M, Canete RR, Gil A** 2004 Adiponectin, the missing link in insulin resistance and obesity. Clin Nutr 23:963-974

132. **Fu Y, Luo N, Klein RL, Garvey WT** 2005 Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. J Lipid Res 46:1369-1379

133. **Muruganandan S, Parlee SD, Rourke JL, Ernst MC, Goralski KB, Sinal CJ** 2011 Chemerin, a novel peroxisome proliferator-activated receptor gamma (PPARgamma) target gene that promotes mesenchymal stem cell adipogenesis. J Biol Chem 286:23982-23995

134. **Cheng J, Song ZY, Pu L, Yang H, Zheng JM, Zhang ZY, Shi XE, Yang GS** 2013 Retinol binding protein 4 affects the adipogenesis of porcine preadipocytes through insulin signaling pathways. Biochem Cell Biol 91:236-243

135. **Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB** 2005 Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 436:356-362

136. **Cho YM, Youn BS, Lee H, Lee N, Min SS, Kwak SH, Lee HK, Park KS** 2006 Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. Diabetes Care 29:2457-2461

137. **Ost A, Danielsson A, Liden M, Eriksson U, Nystrom FH, Stralfors P** 2007 Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes. FASEB J 21:3696-3704

138. **Schaffler A, Scholmerich J, Buchler C** 2005 Mechanisms of disease: adipocytokines and visceral adipose tissue--emerging role in nonalcoholic fatty liver disease. Nat Clin Pract Gastroenterol Hepatol 2:273-280

139. **Pouliot MC, Despres JP, Nadeau A, Moorjani S, Prud'homme D, Lupien PJ, Tremblay A, Bouchard C** 1992 Visceral obesity in men. Associations with glucose tolerance, plasma insulin, and lipoprotein levels. Diabetes 41:826-834

140. **Walsh K** 2009 Adipokines, myokines and cardiovascular disease. Circ J 73:13-18

141. **Stefan N, Haring HU** 2013 The role of hepatokines in metabolism. Nat Rev Endocrinol 9:144-152

142. **Shimano M, Ouchi N, Walsh K** 2012 Cardiokines: recent progress in elucidating the cardiac secretome. Circulation 126:e327-e332

143. **Villarroya J, Cereijo R, Villarroya F** 2013 An endocrine role for brown adipose tissue? Am J Physiol Endocrinol Metab 305:E567-E572

144. **Caro JF, Dohm LG, Pories WJ, Sinha MK** 1989 Cellular alterations in liver, skeletal muscle, and adipose tissue responsible for insulin resistance in obesity and type II diabetes. Diabetes Metab Rev 5:665-689

145. **Dietze D, Koenen M, Rohrig K, Horikoshi H, Hauner H, Eckel J** 2002 Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. Diabetes 51:2369-2376

146. **Sell H, Eckardt K, Taube A, Tews D, Gurgui M, Van Echten-Deckert G, Eckel J** 2008 Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility. Am J Physiol Endocrinol Metab 294:E1070-E1077

147. **Sell H, Dietze-Schroeder D, Eckel J** 2006 The adipocyte-myocyte axis in insulin resistance. Trends Endocrinol Metab 17:416-422

148. **Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J** 2005 Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. Diabetes 54:2003-2011

149. **Huang X, Yang Z** 2015 Resistin's, obesity and insulin resistance: the continuing disconnect between rodents and humans. J Endocrinol Invest

150. **Palanivel R, Maida A, Liu Y, Sweeney G** 2006 Regulation of insulin signalling, glucose uptake and metabolism in rat skeletal muscle cells upon prolonged exposure to resistin. Diabetologia 49:183-190

151. **Aragones G, Saavedra P, Heras M, Cabre A, Girona J, Masana L** 2012 Fatty acid-binding protein 4 impairs the insulin-dependent nitric oxide pathway in vascular endothelial cells. Cardiovasc Diabetol 11:72

152. **Xu A, Tso AW, Cheung BM, Wang Y, Wat NM, Fong CH, Yeung DC, Janus ED, Sham PC, Lam KS** 2007 Circulating adipocyte-fatty acid binding protein levels predict the development of the metabolic syndrome: a 5-year prospective study. Circulation 115:1537-1543

153. **Choi KM, Lee JS, Kim EJ, Baik SH, Seo HS, Choi DS, Oh DJ, Park CG** 2008 Implication of lipocalin-2 and visfatin levels in patients with coronary heart disease. Eur J Endocrinol 158:203-207

154. **Liu JT, Song E, Xu A, Berger T, Mak TW, Tse HF, Law IK, Huang B, Liang Y, Vanhoutte PM, Wang Y** 2012 Lipocalin-2 deficiency prevents endothelial dysfunction associated with dietary obesity: role of cytochrome P450 2C inhibition. Br J Pharmacol 165:520-531

155. **Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S** 2005 Adipokines: molecular links between obesity and atheroslcerosis. Am J Physiol Heart Circ Physiol 288:H2031-H2041

156. **Guzik TJ, Mangalat D, Korbut R** 2006 Adipocytokines - novel link between inflammation and vascular function? J Physiol Pharmacol 57:505-528

157. **Blumensatt M, Wronkowitz N, Wiza C, Cramer A, Mueller H, Rabelink MJ, Hoeben RC, Eckel J, Sell H, Ouwens DM** 2014 Adipocyte-derived factors impair insulin signaling in differentiated human vascular smooth muscle cells via the upregulation of miR-143. Biochim Biophys Acta 1842:275-283

158. **Lamers D, Schlich R, Horrighs A, Cramer A, Sell H, Eckel J** 2012 Differential impact of oleate, palmitate, and adipokines on expression of NF-kappaB target genes in human vascular smooth muscle cells. Mol Cell Endocrinol 362:194-201

159. **Wajchenberg BL** 2007 beta-cell failure in diabetes and preservation by clinical treatment. Endocr Rev 28:187-218

160. **Rakatzi I, Mueller H, Ritzeler O, Tennagels N, Eckel J** 2004 Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1. Diabetologia 47:249-258

161. **Lin P, Chen L, Li D, Liu J, Yang N, Sun Y, Xu Y, Fu Y, Hou X** 2009 Adiponectin reduces glucotoxicity-induced apoptosis of INS-1 rat insulin-secreting cells on a microfluidic chip. Tohoku J Exp Med 217:59-65

162. **Lee KY, Russell SJ, Ussar S, Boucher J, Vernochet C, Mori MA, Smyth G, Rourk M, Cederquist C, Rosen ED, Kahn BB, Kahn CR** 2013 Lessons on conditional gene targeting in mouse adipose tissue. Diabetes 62:864-874

163. **Schinner S, Ulgen F, Papewalis C, Schott M, Woelk A, Vidal-Puig A, Scherbaum WA** 2008 Regulation of insulin secretion, glucokinase gene transcription and beta cell proliferation by adipocyte-derived Wnt signalling molecules. Diabetologia 51:147-154

164. **Gerner RR, Wieser V, Moschen AR, Tilg H** 2013 Metabolic inflammation: role of cytokines in the crosstalk between adipose tissue and liver. Can J Physiol Pharmacol 91:867-872

165. **Zhou L, Sell H, Eckardt K, Yang Z, Eckel J** 2007 Conditioned medium obtained from in vitro differentiated adipocytes and resistin induce insulin resistance in human hepatocytes. FEBS Lett 581:4303-4308

166. **Clement S, Juge-Aubry C, Sgroi A, Conzelmann S, Pazienza V, Pittet-Cuenod B, Meier CA, Negro F** 2008 Monocyte chemoattractant protein-1 secreted by adipose tissue induces direct lipid accumulation in hepatocytes. Hepatology 48:799-807

167. **Berg AH, Combs TP, Du X, Brownlee M, Scherer PE** 2001 The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat Med 7:947-953

168. **Liu Q, Yuan B, Lo KA, Patterson HC, Sun Y, Lodish HF** 2012 Adiponectin regulates expression of hepatic genes critical for glucose and lipid metabolism. Proc Natl Acad Sci U S A 109:14568-14573

169. **Nishijima-Matsunobu A, Aoki S, Uchihashi K, Fujimoto K, Toda S** 2013 Three-dimensional culture model for analyzing crosstalk between adipose tissue and hepatocytes. Cell Tissue Res 352:611-621

170. **Deacon CF, Ahren B, Holst JJ** 2004 Inhibitors of dipeptidyl peptidase IV: a novel approach for the prevention and treatment of Type 2 diabetes? Expert Opin Investig Drugs 13:1091-1102

171. **Herman GA, Bergman A, Stevens C, Kotey P, Yi B, Zhao P, Dietrich B, Golor G, Schrodter A, Keymeulen B, Lasseter KC, Kipnes MS, Snyder K, Hilliard D, Tanen M, Cilissen C, De SM, de L, I, Van DK, Wang AQ, Zeng W, Davies MJ, Tanaka W, Holst JJ, Deacon CF, Gottesdiener KM, Wagner JA** 2006 Effect of single oral doses of sitagliptin, a dipeptidyl peptidase-4 inhibitor, on incretin and plasma glucose levels after an oral glucose tolerance test in patients with type 2 diabetes. J Clin Endocrinol Metab 91:4612-4619

172. **Karasik A, Aschner P, Katzeff H, Davies MJ, Stein PP** 2008 Sitagliptin, a DPP-4 inhibitor for the treatment of patients with type 2 diabetes: a review of recent clinical trials. Curr Med Res Opin 24:489-496

173. **Hopsu-Havu VK, Glenner GG** 1966 A new dipeptide naphthylamidase hydrolyzing glycyl-prolyl-beta-naphthylamide. Histochemie 7:197-201

174. **Abbott CA, McCaughan GW, Gorrell MD** 1999 Two highly conserved glutamic acid residues in the predicted beta propeller domain of dipeptidyl peptidase IV are required for its enzyme activity. FEBS Lett 458:278-284

175. **Bohm SK, Gum JR, Jr., Erickson RH, Hicks JW, Kim YS** 1995 Human dipeptidyl peptidase IV gene promoter: tissue-specific regulation from a TATA-less GC-rich sequence characteristic of a housekeeping gene promoter. Biochem J 311 (Pt 3):835-843

176. **Chien CH, Huang LH, Chou CY, Chen YS, Han YS, Chang GG, Liang PH, Chen X** 2004 One site mutation disrupts dimer formation in human DPP-IV proteins. J Biol Chem 279:52338-52345

177. **Scanlan MJ, Raj BK, Calvo B, Garin-Chesa P, Sanz-Moncasi MP, Healey JH, Old LJ, Rettig WJ** 1994 Molecular cloning of fibroblast activation protein alpha, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. Proc Natl Acad Sci U S A 91:5657-5661

178. **Ghersi G, Dong H, Goldstein LA, Yeh Y, Hakkinen L, Larjava HS, Chen WT** 2003 Seprase-dPPIV association and prolyl peptidase and gelatinase activities of the protease complex. Adv Exp Med Biol 524:87-94

179. **Gorrell MD** 2005 Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. Clin Sci (Lond) 108:277-292

180. **Zhong J, Rao X, Deiuliis J, Braunstein Z, Narula V, Hazey J, Mikami D, Needleman B, Satoskar AR, Rajagopalan S** 2013 A potential role for dendritic cell/macrophage-expressing DPP4 in obesity-induced visceral inflammation. Diabetes 62:149-157

181. **Ciaraldi TP** 1988 The role of adenosine in insulin action coupling in rat adipocytes. Mol Cell Endocrinol 60:31-41

182. **Heseltine L, Webster JM, Taylor R** 1995 Adenosine effects upon insulin action on lipolysis and glucose transport in human adipocytes. Mol Cell Biochem 144:147-151

183. **Ikushima H, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, Schlossman SF, Morimoto C** 2000 Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation. Proc Natl Acad Sci U S A 97:8439-8444

184. **Wronkowitz N, Gorgens SW, Romacho T, Villalobos LA, Sanchez-Ferrer CF, Peiro C, Sell H, Eckel J** 2014 Soluble DPP4 induces inflammation and proliferation of human smooth muscle cells via protease-activated receptor 2. Biochim Biophys Acta 1842:1613-1621

185. **Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, Eckardt K, Kaufman JM, Ryden M, Müller S, Hanisch F-G, Ruige J, Arner P, Sell H, Eckel J** 2011 Dipeptidyl Peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 60:1917-1925

186. **Sell H, Bluher M, Kloting N, Schlich R, Willems M, Ruppe F, Knoefel WT, Dietrich A, Fielding BA, Arner P, Frayn KN, Eckel J** 2013 Adipose Dipeptidyl Peptidase-4 and Obesity: Correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro. Diabetes Care

187. **Mulvihill EE, Drucker DJ** 2014 Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. Endocr Rev 35:992-1019

188. **Mentlein R, Gallwitz B, Schmidt WE** 1993 Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem 214:829-835

189. **Green BD, Flatt PR, Bailey CJ** 2006 Dipeptidyl peptidase IV (DPP IV) inhibitors: A newly emerging drug class for the treatment of type 2 diabetes. Diab Vasc Dis Res 3:159-165

190. **Pratley RE, Salsali A** 2007 Inhibition of DPP-4: a new therapeutic approach for the treatment of type 2 diabetes. Curr Med Res Opin 23:919-931

191. **Chen XW, He ZX, Zhou ZW, Yang T, Zhang X, Yang YX, Duan W, Zhou SF** 2015 Clinical pharmacology of dipeptidyl peptidase 4 inhibitors indicated for the treatment of type 2 diabetes mellitus. Clin Exp Pharmacol Physiol 42:999-1024

192. **Rummey C, Metz G** 2007 Homology models of dipeptidyl peptidases 8 and 9 with a focus on loop predictions near the active site. Proteins 66:160-171

193. **Knudsen LB, Pridal L** 1996 Glucagon-like peptide-1-(9-36) amide is a major metabolite of glucagon-like peptide-1-(7-36) amide after in vivo administration to dogs, and it acts as an antagonist on the pancreatic receptor. Eur J Pharmacol 318:429-435

194. **Deacon CF, Plamboeck A, Rosenkilde MM, de HJ, Holst JJ** 2006 GIP-(3-42) does not antagonize insulinotropic effects of GIP at physiological concentrations. Am J Physiol Endocrinol Metab 291:E468-E475

195. **Gault VA, Parker JC, Harriott P, Flatt PR, O'Harte FP** 2002 Evidence that the major degradation product of glucose-dependent insulinotropic polypeptide, GIP(3-42), is a GIP receptor antagonist in vivo. J Endocrinol 175:525-533

196. **Capuano A, Sportiello L, Maiorino MI, Rossi F, Giugliano D, Esposito K** 2013 Dipeptidyl peptidase-4 inhibitors in type 2 diabetes therapy--focus on alogliptin. Drug Des Devel Ther 7:989-1001

197. **Rohrborn D, Wronkowitz N, Eckel J** 2015 DPP4 in Diabetes. Front Immunol 6:386

198. **Shah Z, Pineda C, Kampfrath T, Maiseyeu A, Ying Z, Racoma I, Deiuliis J, Xu X, Sun Q, Moffatt-Bruce S, Villamena F, Rajagopalan S** 2011 Acute DPP-4 inhibition modulates vascular tone through GLP-1 independent pathways. Vascul Pharmacol 55:2-9

199. **Shirakawa J, Fujii H, Ohnuma K, Sato K, Ito Y, Kaji M, Sakamoto E, Koganei M, Sasaki H, Nagashima Y, Amo K, Aoki K, Morimoto C, Takeda E, Terauchi Y** 2011 Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. Diabetes 60:1246-1257

200. **Tariq M, Masoud MS, Mehmood A, Khan SN, Riazuddin S** 2013 Stromal cell derived factor-1alpha protects stem cell derived insulin-producing cells from glucotoxicity under high glucose conditions in-vitro and ameliorates drug induced diabetes in rats. J Transl Med 11:115

201. **Yano T, Liu Z, Donovan J, Thomas MK, Habener JF** 2007 Stromal cell derived factor-1 (SDF-1)/CXCL12 attenuates diabetes in mice and promotes pancreatic beta-cell survival by activation of the prosurvival kinase Akt. Diabetes 56:2946-2957

202. **Persaud SJ, Bewick GA** 2014 Peptide YY: more than just an appetite regulator. Diabetologia 57:1762-1769

203. **Whim MD** 2011 Pancreatic beta cells synthesize neuropeptide Y and can rapidly release peptide co-transmitters. PLoS One 6:e19478

204. **Karagiannides I, Bakirtzi K, Kokkotou E, Stavrakis D, Margolis KG, Thomou T, Giorgadze N, Kirkland JL, Pothoulakis C** 2011 Role of substance P in the regulation of glucose metabolism via insulin signaling-associated pathways. Endocrinology 152:4571-4580

205. **Frerker N, Raber K, Bode F, Skripuletz T, Nave H, Klemann C, Pabst R, Stephan M, Schade J, Brabant G, Wedekind D, Jacobs R, Jorns A, Forssmann U, Straub RH, Johannes S, Hoffmann T, Wagner L, Demuth HU, von HS** 2009 Phenotyping of congenic dipeptidyl peptidase 4 (DP4) deficient Dark Agouti (DA) rats suggests involvement of DP4 in neuro-, endocrine, and immune functions. Clin Chem Lab Med 47:275-287

206. **Ben-Shlomo S, Zvibel I, Varol C, Spektor L, Shlomai A, Santo EM, Halpern Z, Oren R, Fishman S** 2013 Role of glucose-dependent insulinotropic polypeptide in adipose tissue inflammation of dipeptidylpeptidase 4-deficient rats. Obesity (Silver Spring) 21:2331-2341

207. **Ben-Shlomo S, Zvibel I, Rabinowich L, Goldiner I, Shlomai A, Santo EM, Halpern Z, Oren R, Fishman S** 2013 Dipeptidyl peptidase 4-deficient rats have improved bile secretory function in high fat diet-induced steatosis. Dig Dis Sci 58:172-178

208. **Marguet D, Baggio L, Kobayashi T, Bernard AM, Pierres M, Nielsen PF, Ribel U, Watanabe T, Drucker DJ, Wagtmann N** 2000 Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proc Natl Acad Sci U S A 97:6874-6879

209. **Conarello SL, Li Z, Ronan J, Roy RS, Zhu L, Jiang G, Liu F, Woods J, Zycband E, Moller DE, Thornberry NA, Zhang BB** 2003 Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. Proc Natl Acad Sci U S A 100:6825-6830

210. **Greenberg AS, McDaniel ML** 2002 Identifying the links between obesity, insulin resistance and beta-cell function: potential role of adipocyte-derived cytokines in the pathogenesis of type 2 diabetes. Eur J Clin Invest 32 Suppl 3:24-34

211. **Durinx C, Lambeir AM, Bosmans E, Falmagne JB, Berghmans R, Haemers A, Scharpe S, De M, I** 2000 Molecular characterization of dipeptidyl peptidase activity in serum: soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides. Eur J Biochem 267:5608-5613

212. **Grondin G, Hooper NM, LeBel D** 1999 Specific localization of membrane dipeptidase and dipeptidyl peptidase IV in secretion granules of two different pancreatic islet cells. J Histochem Cytochem 47:489-498

213. **Andrieu T, Thibault V, Malet I, Laporte J, Bauvois B, Agut H, Cahour A** 2003 Similar increased serum dipeptidyl peptidase IV activity in chronic hepatitis C and other viral infections. J Clin Virol 27:59-68

214. **Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T** 2003 Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253:269-285

215. **Saunders WB, Bayless KJ, Davis GE** 2005 MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. J Cell Sci 118:2325-2340

216. **Sato H, Kinoshita T, Takino T, Nakayama K, Seiki M** 1996 Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. FEBS Lett 393:101-104

217. **Toth M, Chvyrkova I, Bernardo MM, Hernandez-Barrantes S, Fridman R** 2003 Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes. Biochem Biophys Res Commun 308:386-395

218. **Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van OE, Tartare-Deckert S** 2003 Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. J Biol Chem 278:11888-11896

219. **Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR** 2002 Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. Diabetes 51:1093-1101

220. **Fiore E, Fusco C, Romero P, Stamenkovic I** 2002 Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell-mediated cytotoxicity. Oncogene 21:5213-5223

221. **Lu X, Wang Q, Hu G, Van PC, Fleisher M, Reiss M, Massague J, Kang Y** 2009 ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. Genes Dev 23:1882-1894

222. **Reinboldt S, Wenzel F, Rauch BH, Hohlfeld T, Grandoch M, Fischer JW, Weber AA** 2009 Preliminary evidence for a matrix metalloproteinase-2 (MMP-2)-dependent shedding of soluble CD40 ligand (sCD40L) from activated platelets. Platelets 20:441-444

223. **Ribeiro AS, Albergaria A, Sousa B, Correia AL, Bracke M, Seruca R, Schmitt FC, Paredes J** 2010 Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. Oncogene 29:392-402

224. **Sabbota AL, Kim HR, Zhe X, Fridman R, Bonfil RD, Cher ML** 2010 Shedding of RANKL by tumor-associated MT1-MMP activates Src-dependent prostate cancer cell migration. Cancer Res 70:5558-5566

225. **Das SS, Hayashi H, Sato T, Yamada R, Hiratsuka M, Hirasawa N** 2014 Regulation of dipeptidyl peptidase 4 production in adipocytes by glucose. Diabetes Metab Syndr Obes 7:185-194

226. **Gu N, Tsuda M, Matsunaga T, Adachi T, Yasuda K, Ishihara A, Tsuda K** 2008 Glucose regulation of dipeptidyl peptidase IV gene expression is mediated by hepatocyte nuclear factor-1alpha in epithelial intestinal cells. Clin Exp Pharmacol Physiol 35:1433-1439

227. **Pinto M, Robine-Leon S, Appay M.D., Kedinger M., Triadou N., Dussaulx E., Lacroix B., Simon-Assmann P., Haffen K., Fogh J., Zweibaum A.** 1983 Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biology of the cell 47:323-330

228. **Lumeng CN, Bodzin JL, Saltiel AR** 2007 Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 117:175-184

229. **Bauvois B, Djavaheri-Mergny M, Rouillard D, Dumont J, Wietzerbin J** 2000 Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. Oncogene 19:265-272

230. **Trayhurn P, Wang B, Wood IS** 2008 Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? Br J Nutr 100:227-235

231. **Famulla S, Schlich R, Sell H, Eckel J** 2012 Differentiation of human adipocytes at physiological oxygen levels results in increased adiponectin secretion and isoproterenol-stimulated lipolysis. Adipocyte 1:132-181

232. **Ishibashi Y, Matsui T, Maeda S, Higashimoto Y, Yamagishi S** 2013 Advanced glycation end products evoke endothelial cell damage by stimulating soluble dipeptidyl peptidase-4 production and its interaction with mannose 6-phosphate/insulin-like growth factor II receptor. Cardiovasc Diabetol 12:125

233. **Ta NN, Li Y, Schuyler CA, Lopes-Virella MF, Huang Y** 2010 DPP-4 (CD26) inhibitor alogliptin inhibits TLR4-mediated ERK activation and ERK-dependent MMP-1 expression by U937 histiocytes. Atherosclerosis 213:429-435

234. **Ervinna N, Mita T, Yasunari E, Azuma K, Tanaka R, Fujimura S, Sukmawati D, Nomiyama T, Kanazawa A, Kawamori R, Fujitani Y, Watada H** 2013 Anagliptin, a DPP-4 inhibitor, suppresses proliferation of vascular smooth muscles and monocyte inflammatory reaction and attenuates atherosclerosis in male apo E-deficient mice. Endocrinology 154:1260-1270

235. **Romacho T, Vallejo S, Villalobos LA, Wronkowitz N, Indrakusuma I, Sell H, Eckel J, Sanchez-Ferrer CF, Peiro C** 2016 Soluble dipeptidyl peptidase-4 induces microvascular endothelial dysfunction through proteinase-activated receptor-2 and thromboxane A2 release. J Hypertens

236. **Chae YN, Kim TH, Kim MK, Shin CY, Jung IH, Sohn YS, Son MH** 2015 Beneficial Effects of Evogliptin, a Novel Dipeptidyl Peptidase 4 Inhibitor, on Adiposity with Increased Ppargc1a in White Adipose Tissue in Obese Mice. PLoS One 10:e0144064

237. **Lessard J, Pelletier M, Biertho L, Biron S, Marceau S, Hould FS, Lebel S, Moustarah F, Lescelleur O, Marceau P, Tchernof A** 2015 Characterization of dedifferentiating human mature adipocytes from the visceral and subcutaneous fat compartments: fibroblast-activation protein alpha and dipeptidyl peptidase 4 as major components of matrix remodeling. PLoS One 10:e0122065

238. **Khan T, Muise ES, Iyengar P, Wang ZV, Chandalia M, Abate N, Zhang BB, Bonaldo P, Chua S, Scherer PE** 2009 Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. Mol Cell Biol 29:1575-1591

239. **Satoh N, Shimatsu A, Himeno A, Sasaki Y, Yamakage H, Yamada K, Suganami T, Ogawa Y** 2010 Unbalanced M1/M2 phenotype of peripheral blood monocytes in obese diabetic patients: effect of pioglitazone. Diabetes Care 33:e7

240. **Suganami T, Nishida J, Ogawa Y** 2005 A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. Arterioscler Thromb Vasc Biol 25:2062-2068

241. **Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM, Neels JG** 2008 Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. Cell Metab 8:301-309

242. **Odegaard JI, Ricardo-Gonzalez RR, Red EA, Vats D, Morel CR, Goforth MH, Subramanian V, Mukundan L, Ferrante AW, Chawla A** 2008 Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. Cell Metab 7:496-507

243. **Kang K, Reilly SM, Karabacak V, Gangl MR, Fitzgerald K, Hatano B, Lee CH** 2008 Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. Cell Metab 7:485-495

244. **Stern JH, Scherer PE** 2015 Adipose tissue biology in 2014: Advances in our understanding of adipose tissue homeostasis. Nat Rev Endocrinol 11:71-72

245. **Shinjo T, Nakatsu Y, Iwashita M, Sano T, Sakoda H, Ishihara H, Kushiyama A, Fujishiro M, Fukushima T, Tsuchiya Y, Kamata H, Nishimura F, Asano T** 2015 DPP-IV inhibitor anagliptin exerts anti-inflammatory effects on macrophages, adipocytes, and mouse livers by suppressing NF-kappaB activation. Am J Physiol Endocrinol Metab 309:E214-E223

246. **Xu L, Spinas GA, Niessen M** 2011 Pro- or anti-inflammatory properties of the adipokine dipeptidyl peptidase-4? Gastroenterology 141:e17

247. **Ussar S, Bezy O, Bluher M, Kahn CR** 2012 Glypican-4 enhances insulin signaling via interaction with the insulin receptor and serves as a novel adipokine. Diabetes 61:2289-2298

248. **Srinivas PR, Wagner AS, Reddy LV, Deutsch DD, Leon MA, Goustin AS, Grunberger G** 1993 Serum alpha 2-HS-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. Mol Endocrinol 7:1445-1455

249. **Youngren JF** 2007 Regulation of insulin receptor function. Cell Mol Life Sci 64:873-891

250. **Piazza GA, Callanan HM, Mowery J, Hixson DC** 1989 Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. Biochem J 262:327-334

251. **Kos K, Baker AR, Jernas M, Harte AL, Clapham JC, O'Hare JP, Carlsson L, Kumar S, McTernan PG** 2009 DPP-IV inhibition enhances the antilipolytic action of NPY in human adipose tissue. Diabetes Obes Metab 11:285-292

252. **Fain JN, Shepherd RE** 1979 Hormonal regulation of lipolysis: role of cyclic nucleotides, adenosine, and free fatty acids. Adv Exp Med Biol 111:43-77

253. **Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, Crow TJ, Tatemoto K, Polak JM** 1983 Neuropeptide Y distribution in human brain. Nature 306:584-586

254. **Valet P, Berlan M, Beauville M, Crampes F, Montastruc JL, Lafontan M** 1990 Neuropeptide Y and peptide YY inhibit lipolysis in human and dog fat cells through a pertussis toxin-sensitive G protein. J Clin Invest 85:291-295

255. **Yasuda N, Nagakura T, Yamazaki K, Inoue T, Tanaka I** 2002 Improvement of high fat-diet-induced insulin resistance in dipeptidyl peptidase IV-deficient Fischer rats. Life Sci 71:227-238

256. **Karl T, Hoffmann T, Pabst R, von HS** 2003 Behavioral effects of neuropeptide Y in F344 rat substrains with a reduced dipeptidyl-peptidase IV activity. Pharmacol Biochem Behav 75:869-879

257. **Ahren B** 2008 Emerging dipeptidyl peptidase-4 inhibitors for the treatment of diabetes. Expert Opin Emerg Drugs 13:593-607

258. **Matsumoto Y, Bishop GA, McCaughan GW** 1992 Altered zonal expression of the CD26 antigen (dipeptidyl peptidase IV) in human cirrhotic liver. Hepatology 15:1048-1053

259. **Itou M, Kawaguchi T, Taniguchi E, Sumie S, Oriishi T, Mitsuyama K, Tsuruta O, Ueno T, Sata M** 2008 Altered expression of glucagon-like peptide-1 and dipeptidyl peptidase IV in patients with HCV-related glucose intolerance. J Gastroenterol Hepatol 23:244-251

260. **Miyazaki M, Kato M, Tanaka K, Tanaka M, Kohjima M, Nakamura K, Enjoji M, Nakamuta M, Kotoh K, Takayanagi R** 2012 Increased hepatic expression of dipeptidyl peptidase-4 in non-alcoholic fatty liver disease and its association with insulin resistance and glucose metabolism. Mol Med Rep 5:729-733

261. **Williams KH, Vieira De Ribeiro AJ, Prakoso E, Veillard AS, Shackel NA, Brooks B, Bu Y, Cavanagh E, Raleigh J, McLennan SV, McCaughan GW, Keane FM, Zekry A, Gorrell MD, Twigg SM** 2015 Circulating dipeptidyl peptidase-4 activity correlates with measures of hepatocyte apoptosis and fibrosis in non-alcoholic fatty liver disease in type 2 diabetes mellitus and obesity: A dual cohort cross-sectional study. J Diabetes 7:809-819

262. **Aronoff Stephen L., Berkowitz Kathy, Schreiner Barb, Want Laura** 2004 Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes Spectrum 17:183-190

263. **Mevorach M, Giacca A, Aharon Y, Hawkins M, Shamoon H, Rossetti L** 1998 Regulation of endogenous glucose production by glucose per se is impaired in type 2 diabetes mellitus. J Clin Invest 102:744-753

264. **Basu A, Caumo A, Bettini F, Gelisio A, Alzaid A, Cobelli C, Rizza RA** 1997 Impaired basal glucose effectiveness in NIDDM: contribution of defects in glucose disappearance and production, measured using an optimized minimal model independent protocol. Diabetes 46:421-432

265. **Kaji K, Yoshiji H, Ikenaka Y, Noguchi R, Aihara Y, Douhara A, Moriya K, Kawaratani H, Shirai Y, Yoshii J, Yanase K, Kitade M, Namisaki T, Fukui H** 2014 Dipeptidyl peptidase-4 inhibitor attenuates hepatic fibrosis via suppression of activated hepatic stellate cell in rats. J Gastroenterol 49:481-491

266. **Shirakawa J, Amo K, Ohminami H, Orime K, Togashi Y, Ito Y, Tajima K, Koganei M, Sasaki H, Takeda E, Terauchi Y** 2011 Protective effects of dipeptidyl peptidase-4 (DPP-4) inhibitor against increased beta cell apoptosis induced by dietary sucrose and linoleic acid in mice with diabetes. J Biol Chem 286:25467-25476

267. **Balas B, Baig MR, Watson C, Dunning BE, Ligueros-Saylan M, Wang Y, He YL, Darland C, Holst JJ, Deacon CF, Cusi K, Mari A, Foley JE, DeFronzo RA** 2007 The dipeptidyl peptidase IV inhibitor vildagliptin suppresses endogenous glucose production and enhances islet function after single-dose administration in type 2 diabetic patients. J Clin Endocrinol Metab 92:1249-1255

268. **Hwa V, Oh Y, Rosenfeld RG** 1999 The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20:761-787

269. **Jogie-Brahim S, Feldman D, Oh Y** 2009 Unraveling insulin-like growth factor binding protein-3 actions in human disease. Endocr Rev 30:417-437

270. **Juul A, Dalgaard P, Blum WF, Bang P, Hall K, Michaelsen KF, Muller J, Skakkebaek NE** 1995 Serum levels of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) in healthy infants, children, and adolescents: the relation to IGF-I, IGF-II, IGFBP-1, IGFBP-2, age, sex, body mass index, and pubertal maturation. J Clin Endocrinol Metab 80:2534-2542

271. **Ranke MB** 2015 Insulin-like growth factor binding-protein-3 (IGFBP-3). Best Pract Res Clin Endocrinol Metab 29:701-711

272. **Shimasaki S, Ling N** 1991 Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6). Prog Growth Factor Res 3:243-266

273. **Booth BA, Boes M, Dake BL, Knudtson KL, Bar RS** 2002 IGFBP-3 binding to endothelial cells inhibits plasmin and thrombin proteolysis. Am J Physiol Endocrinol Metab 282:E52-E58

274. **Lee KW, Liu B, Ma L, Li H, Bang P, Koeffler HP, Cohen P** 2004 Cellular internalization of insulin-like growth factor binding protein-3: distinct endocytic pathways facilitate re-uptake and nuclear localization. J Biol Chem 279:469-476

275. **Schedlich LJ, Graham LD** 2002 Role of insulin-like growth factor binding protein-3 in breast cancer cell growth. Microsc Res Tech 59:12-22

276. **Sesti G, Sciacqua A, Cardellini M, Marini MA, Maio R, Vatrano M, Succurro E, Lauro R, Federici M, Perticone F** 2005 Plasma concentration of IGF-I is independently associated with insulin sensitivity in subjects with different degrees of glucose tolerance. Diabetes Care 28:120-125

277. **Muzumdar RH, Ma X, Fishman S, Yang X, Atzmon G, Vuguin P, Einstein FH, Hwang D, Cohen P, Barzilai N** 2006 Central and opposing effects of IGF-I and IGF-binding protein-3 on systemic insulin action. Diabetes 55:2788-2796

278. **Scavo LM, Karas M, Murray M, Leroith D** 2004 Insulin-like growth factor-I stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes. J Clin Endocrinol Metab 89:3543-3553

279. **Pratipanawatr T, Pratipanawatr W, Rosen C, Berria R, Bajaj M, Cusi K, Mandarino L, Kashyap S, Belfort R, DeFronzo RA** 2002 Effect of IGF-I on FFA and glucose metabolism in control and type 2 diabetic subjects. Am J Physiol Endocrinol Metab 282:E1360-E1368

280. **Laager R, Keller U** 1993 Effects of recombinant human insulin-like growth factor I and insulin on counterregulation during acute plasma glucose decrements in normal and type 2 (non-insulin-dependent) diabetic subjects. Diabetologia 36:966-971

281. **Jacob R, Barrett E, Plewe G, Fagin KD, Sherwin RS** 1989 Acute effects of insulin-like growth factor I on glucose and amino acid metabolism in the awake fasted rat. Comparison with insulin. J Clin Invest 83:1717-1723

282. **Heald AH, Cade JE, Cruickshank JK, Anderson S, White A, Gibson JM** 2003 The influence of dietary intake on the insulin-like growth factor (IGF) system across three ethnic groups: a population-based study. Public Health Nutr 6:175-180

283. **Chan SS, Twigg SM, Firth SM, Baxter RC** 2005 Insulin-like growth factor binding protein-3 leads to insulin resistance in adipocytes. J Clin Endocrinol Metab 90:6588-6595

284. **Silha JV, Gui Y, Murphy LJ** 2002 Impaired glucose homeostasis in insulin-like growth factor-binding protein-3-transgenic mice. Am J Physiol Endocrinol Metab 283:E937-E945

285. **Birkenfeld AL, Lee HY, Majumdar S, Jurczak MJ, Camporez JP, Jornayvaz FR, Frederick DW, Guigni B, Kahn M, Zhang D, Weismann D, Arafat AM, Pfeiffer AF, Lieske S, Oyadomari S, Ron D, Samuel VT, Shulman GI** 2011 Influence of the hepatic eukaryotic initiation factor 2alpha (eIF2alpha) endoplasmic reticulum (ER) stress response pathway on insulin-mediated ER stress and hepatic and peripheral glucose metabolism. J Biol Chem 286:36163-36170

286. **Lin CT, Tang HY, Han YS, Liu HP, Huang SF, Chien CH, Shyy J, Chiu JJ, Chen X** 2010 Downregulation of Signaling-active IGF-1 by Dipeptidyl Peptidase IV (DPP-IV). Int J Biomed Sci 6:301-309

287. **Faidley TD, Leiting B, Pryor KD, Lyons K, Hickey GJ, Thompson DR** 2006 Inhibition of dipeptidyl-peptidase IV does not increase circulating IGF-1 concentrations in growing pigs. Exp Biol Med (Maywood) 231:1373-1378

288. **Bergman AJ, Stevens C, Zhou Y, Yi B, Laethem M, De SM, Snyder K, Hilliard D, Tanaka W, Zeng W, Tanen M, Wang AQ, Chen L, Winchell G, Davies MJ, Ramael S, Wagner JA, Herman GA** 2006 Pharmacokinetic and pharmacodynamic properties of multiple oral doses of sitagliptin, a dipeptidyl peptidase-IV inhibitor: a double-blind, randomized, placebo-controlled study in healthy male volunteers. Clin Ther 28:55-72

289. **Wesley UV, Albino AP, Tiwari S, Houghton AN** 1999 A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. J Exp Med 190:311-322

290. **Cordero OJ, Salgado FJ, Mera-Varela A, Nogueira M** 2001 Serum interleukin-12, interleukin-15, soluble CD26, and adenosine deaminase in patients with rheumatoid arthritis. Rheumatol Int 21:69-74

291. **Somborac-Bacura A, Buljevic S, Rumora L, Culic O, Detel D, Pancirov D, Popovic-Grle S, Varljen J, Cepelak I, Zanic-Grubisic T** 2012 Decreased soluble dipeptidyl peptidase IV activity as a potential serum biomarker for COPD. Clin Biochem 45:1245-1250

292. **Omar B, Ahren B** 2014 Pleiotropic mechanisms for the glucose-lowering action of DPP-4 inhibitors. Diabetes 63:2196-2202

293. **Yu DM, Slaitini L, Gysbers V, Riekhoff AG, Kahne T, Knott HM, De M, I, Abbott CA, McCaughan GW, Gorrell MD** 2011 Soluble CD26 / dipeptidyl peptidase IV enhances human lymphocyte proliferation in vitro independent of dipeptidyl peptidase enzyme activity and adenosine deaminase binding. Scand J Immunol 73:102-111

294. **Zhong J, Rao X, Rajagopalan S** 2013 An emerging role of dipeptidyl peptidase 4 (DPP4) beyond glucose control: potential implications in cardiovascular disease. Atherosclerosis 226:305-314

295. **Fadini GP, Boscaro E, Albiero M, Menegazzo L, Frison V, de KS, Agostini C, Tiengo A, Avogaro A** 2010 The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1alpha. Diabetes Care 33:1607-1609

DANKSAGUNG

Ein großes Dankeschön möchte ich vor allem an Prof. Jürgen Eckel richten, für die Bereitstellung des Themas und viele sehr gute fachliche Diskussionen, die es mir ermöglicht haben, Ergebnisse auch aus neuen Blickwinkeln zu beurteilen. Außerdem vielen Dank für das entgegengebrachte Vertrauen und das fortwährende Interesse an meiner Arbeit. Dank der bereitgestellten finanziellen Mittel hatte ich die Möglichkeit Ideen im Labor umzusetzen und an nationalen und internationalen Kongressen teilzunehmen. All das lieferte wertvolle Beiträge zum Voranschreiten der Arbeit und hat mich nicht nur fachlich sondern auch persönlich reifen lassen.

Herrn Prof. Ulrich Rüther danke ich für die Übernahme des Korreferats und die Möglichkeit an den Arbeitsgruppen Seminaren teilzunehmen, die ich dank der angenehmen Atmosphäre immer in Erinnerung behalten werde. Vielen Dank auch für die anregenden Diskussionen, die mir oft neue Möglichkeiten eröffnet haben.

Ein ganz besonderer Dank gilt allen derzeitigen und ehemaligen Mitgliedern der AG Eckel, die einem auch aus den tiefsten Motivationslöchern herausgeholfen haben. Ein besonderer Dank gilt hier Henrike für die fachliche Betreuung, das Korrekturlesen der Arbeit und für anregende Diskussionen. Vielen Dank auch an Manu für ein immer offenes Ohr für Fragen zu Differenzierung. Vielen Dank an Kristin für das Einarbeiten in das qRT-PCR Geheimnis und für deine nette und unkomplizierte Art. Vielen Dank an Birgit für das Überwinden vieler bürokratischer Hürden und die netten privaten Gespräche. Ein Riesendank auch an Andrea, für die tatkräftige Unterstützung beim Präparieren und Aufziehen. Vielen Dank für gefühlte 100.000 bradfords, die du immer ohne Murren übernommen hast. ;-) Ohne dich wäre ich verzweifelt! Und für die Hilfe bei Blots, die wie aus Zauberhand fertig zum Einlesen morgens bereit standen. :-) Auch vielen Dank für deine Lebensfreude mit der du uns alle oft angesteckt hast..
Danke an alle Doktoranden und Studenten für nette Mittagspläusche fernab vom Laboralltag, danke für unvergessliche Karnevalsbesuche und den ein oder anderen Weihnachtsmarktglühwein, sowie die Adrenalinkicks auf der Rheinkirmes und danke für die „Dönerstage“. ;-)
Danke an Ira, Thorsten und Sven für die nette Atmosphäre im Hamsterkäfig, für alle fachlichen und unfachlichen Gespräche. ;-) Danke für Kletterabende, gemeinsames Public viewing und die Geburtstagsfeiern. Dank euch verging die Zeit im Endeffekt doch wie im Flug.
Ein besonderer Dank gilt auch Nina, nicht nur für viele fachliche Hilfestellungen zu DPP4 und den Smoothies, sondern auch für ein immer offenes Ohr, für die netten Apfelpäuschen, die ich verdammt vermissen werde. Selbst ungeliebte Aufgaben wurden dank dir erträglich und lustig. Vielen Dank für die tolle Zusammenarbeit am „Monster-DPP4-Review“. Außerdem an dich und auch an Sven vielen Dank für die tollen privaten Abende und die tollen gemeinsamen Kongresszeiten! Ihr seid mir echt ans Herz gewachsen und habt mir die Zeit fernab von der Familie wahnsinnig erleichtert..

Abschließend bedanke ich mich von ganzem Herzen bei Mom und Paps, bei Franzi und Max für das Vertrauen, die liebevolle Unterstützung und ein immer offenes Ohr, wenn es mal wieder nicht so leicht war.. Die oft stundenlangen Telefonate haben mich stets aufgeheitert und wieder motiviert nach vorn zu schauen. Ich danke euch, dass ihr mir immer den Rücken gestärkt habt auch über die weite Distanz. Danke, dass ihr immer an mich geglaubt habt und mir das alles ermöglicht habt!

Und dir Steve gilt der größte Dank, deswegen kommst du zum Schluss.. Du warst und bist mein „Fels in der Brandung“, du hast mich zurück auf den Boden geholt und es immer verstanden mich aufzuheitern. Ohne dich hätte ich die Herausforderung niemals angenommen und gemeistert, weit weg von zu Hause eine Dr. Arbeit zu machen. In allem was ich tue gibst du mir den nötigen Halt und ich freue mich wahnsinnig auf die kommende kleine (oder große ;-)) Herausforderung, die wir uns geschenkt haben. ;-) Am Ende wird immer alles gut.. Danke, dass es dich gibt und dass du bei mir bist.

EIDESSTAATLICHE ERKLÄRUNG

Die vorliegende Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis“, die mir von der Heinrich-Heine-Universität Düsseldorf nahegelegt worden.. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

(Diana Röhrborn)

Düsseldorf, den