Aus dem Deutschen Diabetes-Zentrum, Leibniz-Zentrum für Diabetes-Forschung
an der Heinrich-Heine-Universität Düsseldorf
(Direktor: Univ.-Prof. Dr. med. univ. Dr. h. c. Michael Roden)

Analysis of molecular crosstalk mechanisms between adipose tissue, skeletal muscle and the vascular wall

Habilitationsschrift

zur Erlangung der Venia Legendi im Fach Experimentelle Medizin an der Hohen Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

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Düsseldorf 2017

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Zusammenfassung

Adipositas bedingt ein erhöhtes Risiko einen Typ 2 Diabetes und kardiovaskulären Erkrankungen zu entwickeln. Dieser Zusammenhang hat die Erforschung möglicher zirkulierender Proteine angestoßen, die eine Verbindung zwischen einer vermehrten Fettspeicherung, einer subklinischen chronischen Entzündung im vergrößerten Fettgewebe und Stoffwechselstörungen wie Insulinresistenz und Typ 2 Diabetes sowie kardiovaskuläre Erkrankungen darstellen können. Das Ziel dieser Arbeiten war es, molekulare Mechanismen aufzuzeigen, wie freigesetzte Proteine aus dem vergrößerten Fettgewebe, sogenannte Adipokine, bei Adipositas den Skelettmuskel und die Gefäßwand negativ beeinflussen. Dabei wurden speziell die neuen Adipokine Chemerin, Heat shock protein 60 (Hsp60) und Dipeptidylpeptidase 4 (DPP4) untersucht. Ein weiteres Ziel der Arbeiten war die zirkulierenden Konzentrationen dieser Adipokine bei Adipositas und Adipositas-assoziierten Erkrankungen in humanen Serumproben sowie die Freisetzung dieser Adipokine aus dem vergrößerten Fettgewebe *in vivo* und *ex vivo* zu untersuchen.

Adipozyten-konditionierte Medien (CM), die alle von Fettzellen freigesetzten Adipokine enthalten, erzeugen muskuläre Insulinresistenz. Desweiteren bewirkt CM in diesen insulinresistenten Skelettmuskelzellen erhöhten oxidativer Stress, vermehrte Einlagerung von intrazellulären Lipiden, verminderte Expression von myogenen Markerproteinen und eine veränderte Freisetzung von zirkulierenden Proteinen, sogenannten Myokinen. Diese Befunde gleichen jenen aus humanen Skelettmuskelbiopsien von insulinresistenten Patienten. Gewichtsabnahme oder körperliche Betätigung der Patienten können zu einer Verbesserung der Insulinsensitivität des Muskels, einer Reduktion der oxidativen Stressantwort und einer Verminderung der Speicherung von intramuskulären Lipiden führen. Ähnliche Effekte sind auch *in vitro* durch Entzug des CM zu sehen, wobei die verminderte Expression von myogene Markern und die veränderte Freisetzung von Myokinen teilweise nicht reversibel sind. Einzelne Adipokine wie Chemerin und Hsp60 sind

alleine in der Lage Insulinresistenz in Skelettmuskelzellen zu erzeugen, wobei die Aktivierung inflammatorischer Signalwege eine entscheidende Rolle spielen. Chemerin und Hsp60 sind im Serum von adipösen Patienten erhöht und können auch im Fettgewebe eine Entzündung unterhalten.

Dem neuentdeckten Adipokin DPP4 kommt eine besondere Bedeutung zu, da schon länger DPP4 Inhibitoren erfolgreich in der Diabetestherapie eingesetzt werden. Serumkonzentrationen von DPP4 sind bei adipösen und insulin-resistenten Patienten sowie bei Patienten mit Metabolischem Syndrom erhöht. Außerdem wird DPP4 im subkutanen und viszeralen Fettgewebe von adipösen und insulin-resistenten Probanden verstärkt exprimiert. Parallel dazu wird DPP4 vom subkutanen Fettgewebe *in vivo* freigesetzt. *Ex vivo* sezerniert das viszerale Fettgewebe adipöser Patienten im Vergleich zu schlanken Kontrollen und zum subkutanen Fettgewebe erhöhte Mengen von DPP4. *In vitro* induziert DPP4 Insulinresistenz in Muskelzellen und Fettzelle. Diese Resultate unterstreichen die wichtige Rolle von DPP4 als Enzym für die Inaktivierung von Inkretinhormonen und anderer Proteine und illustriert eine mögliche neue Rolle von DPP4 im Fettgewebe.

Adipokine spielen auch eine Rolle für Veränderungen der Gefäßwand bei der Entwicklung einer Arteriosklerose. Mediatoren aus dem vergrößerten Fettgewebe wie DPP4 sind dazu in der Lage, Entzündung und Proliferation von glatten Muskelzellen zu induzieren. Dabei wird DPP4 von Fettzellen und auch glatten Muskelzellen über ein Shedding freigesetzt. An der Freisetzung von DPP4 sind zelltypabhängig verschiedene Metalloproteasen beteiligt, die durch Hypoxie reguliert werden können. DPP4 stimuliert dann in der Gefäßwand pro-inflammatorische Signalwege, was zur Sekretion von pro-inflammatorischen Zytokinen führt. Hemmung von DPP4 kann diese Effekte komplett aufheben. Basierend auf Strukturanalysen wurde DPP4 als ein Agonist des Protease-aktivierten Rezeptors 2 (PAR2) identifiziert. Eine durch siRNA reduzierte Expression von PAR2 sowie der Einsatz eines PAR2 Antagonisten können alle DPP4-induzierten Effekte an den glatten Muskelzellen aufheben.

Zusammenfassend spielen Adipokine wie Chemerin, Hsp60 und DPP4 eine wichtige Rolle in der Kommunikation zwischen dem Fettgewebe, dem Skelettmuskel und der Gefäßwand. Die Deregulation der Adipokinfreisetzung bei Adipositas trägt zur Entstehung von Insulinresistenz im Skelettmuskel und eine Entzündung und Dysfunktion der Gefäßwand bei. Die untersuchten Adipokine können als Biomarker für Adipositas-assoziierten Erkrankungen dienen und sind daher mögliche Ansatzpunkte für eine Therapie dieser Adipositas-assoziierten Erkrankungen.

Diese kumulative Habilitationsschrift beruht auf 9 begutachteten Originalarbeiten, von denen 6 Erstautorenschaften und 2 Letztautorschaften sind.

List of publications in this thesis

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2. **Sell H**, Laurencikiene J, Taube A, Eckardt K, Cramer A, Horrighs A, Arner P, Eckel J: Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. Diabetes 58:2731-2740, 2009 (IF: 8.505)

3. **Sell H**, Divoux A, Poitou C, Basdevant A, Bouillot JL, Bedossa P, Tordjman J, Eckel J, Clement K: Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery. J Clin Endocrinol Metab 95:2892-2896, 2010 (IF: 6.495)

4. **Sell H**, Habich C, Eckel J: Adaptive immunity in obesity and insulin resistance. Nat Rev Endocrinol 8:709-16, 2012 (IF: 11.025)

5. Märker T*, **Sell H***, Zillessen P, Glöde A, Kriebel J, Ouwens DM, Pattyn P, Ruige J, Famulla S, Roden M, Eckel J, Habich C: Heat shock protein 60 as a mediator of adipose tissue inflammation and insulin resistance. Diabetes 61:615-25, 2012 (IF: 7.895) * equal contribution

6. 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: Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 60:1917-1925, 2011 (IF: 8.206)

7. **Sell H**, Blüher M, Klöting N, Schlich R, Willems M, Ruppe F, Knoefel WT, Dietrich A, Fielding BA, Arner P, Frayn KN, Eckel J: Adipose DPP4 and obesity: correlation with insulin resistance and depot-specific release from adipose tissue *in vivo* and *in vitro*. Diabetes Care, 36:4083-90, 2013 (IF: 7.735)

8. Röhrborn 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 Letters, 588:3870-7, 2014 (IF: 3.341)

9. Wronkowitz N, Görgens SW, Romacho T, Villalobos LA, Sánchez-Ferrer CF, Peiró C, **Sell H***, Eckel J*. Soluble DPP4 induces inflammation and proliferation of human smooth muscle cells via protease-activated receptor 2. BBA Mol Bas Dis, 1842:1613-21, 2014 (IF: 4.910)
* equal contribution

1. Introduction

Obesity as defined by a body mass index (BMI) higher than 30 kg/m^2 is increasing at an alarming rate in many parts of the world and is becoming a global problem. According to the World Obesity Federation (former International Association for the Study of Obesity), the highest prevalence is observed in the Western Pacific Region with Nauru having 55.7 % obesity in males and Tonga having 70.3 % obesity in females. Furthermore, the prevalence of obesity has increased by more than 3-times in most European countries since 1980 (European Commission, 2010). According to the World Obesity Federation, the highestes obesity rates in Europe are 27.7 % in males Greek and 36 % in female Albanians. Germany is also amongst the countries with very high prevalence of overweight and obesity with 43.8 % of males being overweight and 23.3 % obese, and 29 % of females being overweight and 23.9 % obese (National survey from 2008-2011).

Obesity is an evitable disease and represents a serious threat to population health. It is not only causing physical disability and concomitant psychological problems, but it is dramatically increasing the risk of developing other serious non-communicable diseases including cancer, cardiovascular disease and type 2 diabetes. Worldwide, the contribution of different risk factors to disease burden has shifted towards non-communicable diseases, with overweight being the third most important risk factor in Western Europe for mortality and disability-adjusted life years (Lim, Vos et al. 2012). Furthermore, two third of the world population is now living in countries, where overweight and obesity causes more death than undernutrition (World Health Organization Global status report on non-communicable diseases. Geneva: WHO 2013). The cost for the direct treatment of obesity plus the treatment of obesity-associated diseases amounts globally to several billions of dollars yearly. Only for the U.S., the estimated annual cost of treating obesity and its related complications amounts to $168.4 billion dollars reflecting 16.5% of national spending on medical care (Cawley and Meyerhoefer 2011).

Obesity is the most important risk factor for developing type 2 diabetes (Wild and Byrne 2006). Indeed, 50% of diabetic men and 70% of diabetic women are obese. Type 2

diabetes is also becoming a serious health issue in obese adolescents (Goran, Ball et al. 2003). While only a small portion of patients develop diabetes because of maturity-onset diabetes of the young (MODY) (Fajans and Bell 2011), latent autoimmune diabetes of the adult (LADA) or rare genetic syndromes (Ola, Gigante et al. 2006), environmental components such as obesity strongly influence disease development. Chronic hyperglycemia in type 2 diabetes in turn causes microangiopathy, macroangiopathy and neuropathy as complications (Haas 1993). The most important pathophysiological feature of type 2 diabetes is the development of insulin resistance, which occurs mainly in liver, skeletal muscle and adipose tissue (Tripathy and Chavez 2010). Insulin resistance in these organs is present even before the onset of overt type 2 diabetes and in healthy relatives of patients with type 2 diabetes (Beck-Nielsen, Vaag et al. 2003, Bergman 2012). In this context, skeletal muscle is particularly important for insulin resistance as it accounts for about 80 % of insulin-stimulated glucose disposal and more than 90 % of insulin-stimulated non-oxidative glucose disposal (DeFronzo, Jacot et al. 1981). Therefore, the mechanisms of disturbed insulin action in patients with type 2 diabetes or model organisms in relation to obesity represent a key area of research in diabetes (Roden and Shulman 1999).

Obese patients do not only frequently develop type 2 diabetes, but also exhibit several features of the so-called metabolic syndrome. The metabolic syndrome (formally also called syndrome X or insulin resistance syndrome) is defined as a cluster of conditions that increase the risk of developing cardiovascular disease and type 2 diabetes (Sarafidis and Nilsson 2006). According to the International Diabetes Federation, the metabolic syndrome comprises the most dangerous heart attack risk factors: diabetes and prediabetes, abdominal obesity, high cholesterol and high blood pressure (BP). The following criteria are used: 1. Central obesity (waist circumference ≥ 94 cm for Caucasian men and ≥ 80 cm for Caucasian women; other values for waist circumference depending on ethnicity) plus any two of the following four criteria: 2. raised circulating fasting triglyceride level: ≥ 150 mg/dL (1.7 mmol/L), or pharmacotherapy for dyslipidemia; 3. reduced high density lipoprotein (HDL) cholesterol: < 40 mg/dL (1.03 mmol/L) in males and < 50 mg/dL (1.29 mmol/L) in females, or

pharmacotherapy for dyslipidemia; 4. raised BP: systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg, or pharmacotherapy for hypertension); and 5. raised fasting plasma glucose (FPG) ≥ 100 mg/dL (5.6 mmol/L), or overt type 2 diabetes. Recent statistical analysis by the American Heart Association confirmed that obesity is highly associated with the development of cardiovascular disease including atherosclerosis (Go, Mozaffarian et al. 2013). Numerous animal studies support these findings (Fuster, Castillo et al. 2011). Despite accumulating published data, it is not yet precisely clear how the expanded adipose tissue contributes to the pathogenesis of cardiovascular disease.

The association between the epidemics of obesity, type 2 diabetes and cardiovascular disease has advanced research on the endocrine link between lipid and glucose homeostasis and inflammation (Krebs and Roden 2005). It is recognized that elevated circulating plasma levels of triglycerides and free fatty acids (FFA) released in the obese state contribute to skeletal muscle insulin resistance (Samuel, Petersen et al. 2010) and vascular dysfunction (Steinberg and Baron 2002). By lipid overflow to tissues that are normally not storing lipids, formation of intracellular lipid metabolites that are involved in the development of insulin resistance and vascular inflammation is stimulated. These lipid mediators are lipotoxic agents that are not only impairing insulin signalling but also stimulated inflammatory processes in different tissues (Szendroedi, Yoshimura et al. 2014).

Adipose tissue is the major organ in mammals where energy can be stored in form of triglycerides, and until the 1980ies, the function of body fat was described to be restricted to energy storage in white adipose tissue and energy expenditure for non-shivering thermogenesis in brown adipose tissue. However, research in the last 20 years has demonstrated that adipocytes are also active secretory cells capable of releasing not only FFA by lipolysis but are also able to release a huge number of protein hormones, the so-called adipokines (Lehr, Hartwig et al. 2012). Among all adipokines (so far around 1000 proteins have been identified as factors released by adipocytes and adipose tissue using proteomic approaches), a number of adipokines have been identified to negatively influence muscle insulin sensitivity (Sell, Dietze-Schroeder et al. 2006, Famulla, Lamers et al. 2010)

potentially turning them into regulators of insulin sensitivity. Furthermore, adipokines are regulators of vascular function demonstrating their possible participation in the pathogenesis of cardiovascular disease (Ouwens, Sell et al. 2010). Adipokines such as chemerin, dipeptidyl peptidase 4 (DPP4) and other still unknown factors might constitute the missing link between adipose tissue, peripheral insulin resistance and vascular dysfunction (Ouwens, Sell et al. 2010, Taube, Schlich et al. 2012, Bluher 2014). Adipocyte-derived factors are significantly overexpressed and oversecreted in the obese state and are good predictors for a later development of atherosclerosis and type 2 diabetes (Pradhan, Manson et al. 2001, Festa, D'Agostino et al. 2002).

2. Hypotheses

Overall, this work aims to present novel insight into how organs communicate with each other with a focus on pathophysiological relevant organ crosstalk in the field of obesity, insulin resistance, type 2 diabetes and vascular dysfunction. We aim to elucidate molecular mechanisms how obesity and, more precisely, excess of adipose tissue negatively influences insulin sensitivity of skeletal muscle and vascular function via inflammatory endocrine pathways. Thereby, we explored novel endocrine mediators of insulin resistance and vascular dysfunction to be targets for prevention and therapy as well as potential biomarker of obesity-associated metabolic diseases. The following hypotheses were specifically tested:

I. Skeletal muscle cells treated with adipokines reflect multiple pathophysiological features of skeletal muscle metabolism in patients with insulin resistance and type 2 diabetes.

I.I. Adipokine-induced insulin resistance in skeletal muscle cells is reversible after removal of adipokines.

I.II. Chemotactic proteins such as chemerin are increased in obesity and able to induce insulin resistance in skeletal muscle cells.

I.III. An altered heat shock response including increased release of the adipokine Hsp60 in obesity is related to skeletal muscle insulin resistance.

II. The novel adipokine DPP4 is an adipokine that is linked to obesity, insulin resistance and the metabolic syndrome

II.I. Circulating DPP4 is increased in the obese state, in insulin resistance and the metabolic syndrome.

II.II. Adipose DPP4 particularly in the visceral fat depot is related to impaired glucose tolerance, insulin resistance and type 2 diabetes.

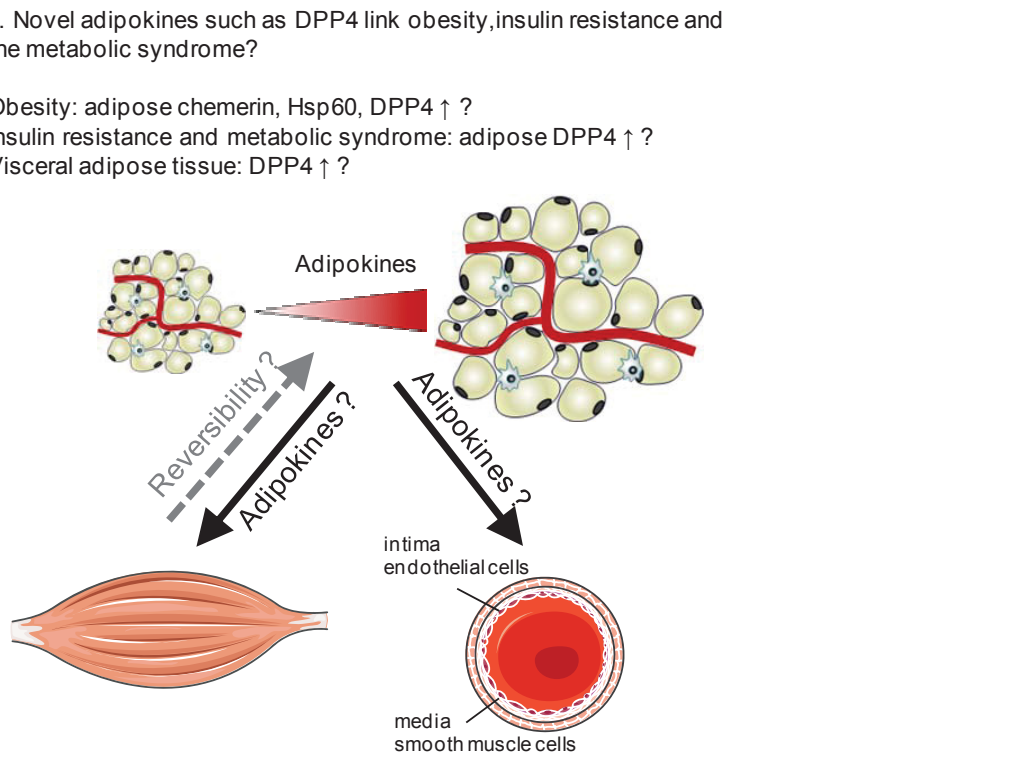
III. Adipokines such as DPP4 are negative regulators of vascular function by stimulating inflammatory signaling pathways.

III.I. The release of DPP4 is regulated by inflammatory stimuli and by hypoxia in adipocytes and in smooth muscle cells providing a link between adipose tissue inflammation, hypoxia and vascular dysfunction.

III.II. Increased circulating DPP4 from adipose tissue in the obese state induces inflammation, insulin resistance and vascular dysfunction in vascular cells such as smooth muscle cells and endothelial cells.

Figure 1

Schematic presentation of hypotheses tested in this work that aim to elucidate novel mechanisms of the development of insulin resistance and vascular dysfunction.



3. Methods

In the presented studies, culture of different primary human cells was used to mimic organ crosstalk *in vitro* in a setting that is the closest to the human situation. Cell culture methods were combined with *ex vivo* and *in vivo* measurements if possible in order to assess the relevance of our findings for patients with obesity and/or type 2 diabetes. In the following section, the most relevant methods are described for better understanding the experimental findings.

3.1. Culture of human primary skeletal muscle cells, adipocytes and smooth muscle cells

3.1.1. Human skeletal muscle cells

Human skeletal myotubes are not taken into culture directly from biopsies, but are differentiated *in vitro* from satellite cells isolated from muscle biopsies (Dietze, Koenen et al. 2002). This procedure is associated with several advantages but also disadvantages. First, satellite cells can be expanded over several (up to five) passages which dramatically increases the starting material for cell culture studies and minimizes the amount of biopsy necessary for meaningful experiments. Second, satellite cells adhere to culture dishes and differentiate into myotubes that are also adherent, which makes it possible to stimulate cell culture over differentiation and at the end of differentiation with various stimuli. On the other hand, primary myotubes cannot be cultured and stimulated once isolated from the biopsy. But culture of satellite cells also have disadvantages as the milieu of the skeletal muscle within a patient with type 2 diabetes and obesity cannot be reproduced and satellite cells might lose their diabetic phenotype over the long culturing periods. However, we and others have shown that primary human myotubes in cell culture are a powerful tool to study mechanisms of insulin resistance as insulin resistance can be induced by many different means and studied in detail (Henry, Abrams et al. 1995, Thompson, Pratley et al. 1996, Vollenweider, Menard et al. 2002, Brozinick, Roberts et al. 2003, Eckardt, Sell et al. 2009). For all our experiments, satellite cells were isolated from M. rectus abdominis by trypsin

digestion followed by a purification step with fibroblast-specific magnetic beads in order to eliminate contaminating fibroblasts. After two passages, all myoblasts are characterized by the manufacturer (PromoCell) using immunohistochemical detection of sarcomeric myosin in differentiated cultures at 100 % confluence. Primary human satellite cells were used from healthy Caucasian donors with an age minimum of 16 years. For individual experiments, myoblasts are seeded at a density of 10.000 cells/cm2 and cultured in a-modified Eagles/Hams F-12 medium containing Skeletal Muscle Cell Growth Medium Supplement (5 % Fetal Calf Serum, 50 µg/ml bovine fetuin, 100 ng/ml recombinant human Epidermal Growth Factor, 1 ng/ml recombinant human Basic Fibroblast Growth Factor, 10 µg/ml recombinant human insulin, 0,4 µg/ml dexamethasone, from Promocell) up to near confluence. The cells were then differentiated and fused by culture in a-modified Eagles medium for 6 days. Culture are regularly checked for increased expression of myogenic marker such as the glucose transporter GLUT4, myogenin and myosin heavy chain. Furthermore, myotube fusion can be monitored by microscopy as multiple nuclei are visible within one myotube.

3.1.2. Human adipocytes

Adipocytes can be cultured in two different ways. First, mature adipocytes can be used as floating cultures directly after taking a biopsy and immediate collagenase digestion (Skurk, Alberti-Huber et al. 2009). The second type of adipocyte culture uses preadipocytes isolated from adipose tissue that are differentiated to mature adipocytes in adherent cultures *in vitro* (Liu, Spelleken et al. 1998). The advantage of this adherent culture is the possibility to manipulate adipocytes differentiation, to analyse adipocyte differentiation over time and many different options to treat the cells in culture. In addition, floating adipocytes are extremely fragile so that apoptosis and cell leakage has to be checked for every experiment. Thus, all experiments presented in this work were performed with adipocytes that were differentiated from preadipocytes. For the isolation of preadipocytes, adipose tissue samples were obtained from the mammary fat or subcutaneous fat from the abdominal region of normal weight or moderate overweight women undergoing elective surgery such as

mammary reduction or other types of plastic surgery. The procedure to obtain adipose tissue was approved by the ethical committee of Heinrich-Heine-University Düsseldorf and Sana-Klinikum Gerresheim. None of the individuals was on medication or had acute infection. Adipose tissue samples were dissected from connective tissues and larger vessels and minced in pieces of about 10 mg in weight. Preadipocytes were isolated by collagenase digestion using NB4 standard grade collagenase from Serva at 250 mU/ml. Isolated cell pelleted after digestion were filtered through a 40 µm mesh and resuspended in Dulbecco's modified Eagles/Hams F12 medium supplemented with 10 % fetal calf serum and antibiotic-antimycotic mix (Gibco). Preadipocytes were then seeded at a density of 6500 cells/cm2. Cells are expanded over 2 passages in Dulbecco's modified Eagles/Hams F12 medium containing 33 µM biotin, 17 µM d-pantothenic acid, 2.5 % fetal calf serum, 8.7 µM insulin, 10 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast growth factor and 0.5 % gentamycin. For experiments, preadipocytes of passage 3 were seeded at a density of 35.000 cells/cm2 and differentiated in Dulbecco's modified Eagles/Hams F12 medium containing 33 µM biotin, 17 µM d-pantothenic acid, 3 % fetal calf serum, 100 nM insulin, 1 µM dexamethasone, and 0.5 % gentamycin with the addition of 200 µM 3-Isobutyl-1-methylxanthin and 250 nM troglitazone for the first 7 days. After 14 days, around 80 % of all seeded preadipocytes developed to differentiated adipocytes, as defined by cytoplasm completely filled with small or large lipid droplets. These cells are directly included in experiments or used for the generation of adipocyte-conditioned medium (CM). For the generation of CM, differentiated adipocytes are incubated for 24 h in skeletal muscle cell differentiation medium or smooth muscle cell hunger medium.

3.1.3. Human smooth muscle cells

Human vascular smooth muscle cells in culture are an important tool in understanding their functionality and contribution to contraction of the vascular wall and the development of cardiovascular disease such as atherosclerosis. Smooth muscle cells in culture lose many of their contractile properties that they possess *in vivo* which represents both an advantage and a disadvantage. By having a rather proliferative phenotype, these cells ideally model

disease-associated smooth muscle with proliferative capacity (Lamers, Schlich et al. 2011).. The disadvantage of this remodeling of smooth muscle cells can be overcome by different means such as differentiation into contractile cells *in vitro* (Blumensatt, Wronkowitz et al. 2014) or by culture of intact vessel rings (Proudfoot and Shanahan 2012). Then, functional contraction can be studied in detail. In our studies, we used smooth muscle cells from the proliferative phenotype as we aimed to study the proliferation of smooth muscle cells as a hallmark of the development of vascular dysfunction and atherosclerosis.

3.2. Analysis of insulin signalling and glucose uptake

Insulin sensitivity of an organ or of isolated cells is defined as the half-maximal concentration of insulin required to achieve glucose uptake or glycogen synthesis. *In vivo*, glucose disposal is very complex, involves different organs and is best quantified by the hyperinsulinemic-euglycemic clamp (Roden 2006). In insulin resistance, the maximal response and/or insulin sensitivity of the response can be reduced. Upstream of glucose uptake and glycogen synthesis, insulin stimulates a cascade of signalling molecules including the insulin receptor, insulin receptor substrates and many kinases downstream of the latter. The serine/threonine kinase Akt plays a key factor in the insulin signalling cascade widely used to monitor insulin sensitivity in organs and cultured cells. In skeletal muscle cells, adipocytes and smooth muscle cells, Akt phosphorylation was measured after stimulation with 100 nM insulin for 10 min. Although, the insulin concentration used is higher than peak insulin concentrations observed *in vivo*, testing of different insulin concentrations revealed that this is a concentration achieving approximately half-maximal response with a good dynamic range. In addition to Akt phosphorylation, substrates of Akt such as AS160 and glycogen synthase kinase (GSK) 3 can be measured.

As insulin-stimulated Akt phosphorylation is not always correlated with the observed glucose or glycogen synthesis (Zierath, Krook et al. 2000), the direct measurement of glucose uptake should be performed for key experimental settings as the “gold standard” of *in vitro* analysis of insulin sensitivity (Sell, Jensen et al. 2012). Differentiated skeletal muscle

cells can be treated with the desired compound or protein overnight so that direct medium change before the glucose uptake can be avoided to minimize interference with stress signalling. 30 min prior to the uptake experiment, cells are stimulated with 100 nM insulin. Cells are then incubated either with L-glucose or deoxy D-glucose at 0.25 µCi/ml. L-glucose is a control to measure any nonspecific uptake and measurement of its uptake should be repeated in each single experiment to be substracted from deoxy-D-glucose uptake. Deoxy-D-glucose cannot be metabolized and its intracellular accumulation after insulin stimulation is measured. Uptake of 2-desoxy-glucose was measured for 2 hours after insulin stimulation. After washing with cytochalasin B to inhibit any further glucose transport, cells are lyzed in NaOH. A scintillation cocktail is added to the lysates and the samples analysed by liquid scintillation counting on a beta-counter. Insulin can normally increase glucose uptake in untreated skeletal muscle cells by 50 to 100 %.

3.3. Adipokine release *in vivo*, *ex vivo* and *in vitro*

In vitro, adipokine release can be studied in detail from adipocytes in culture. The advantage of adipocyte cultures are the versatile experimental setups during differentiation and at the end of differentiation. Inflammatory stimuli or other forms of stress such as hypoxia can be used to stimulate an obesity-like environment (Famulla, Horrighs et al. 2011). Furthermore, adipokine release can be analyzed after silencing of specific targets (see **Publication 8**). While adipokines in culture can be used to study adipokine release in a very controlled and reproducible setting, it should be noted that it is not clear if these cells behave in the same way in their natural environment in the patient, which is a general drawback of all cell cultures. During the last years, methods to overcome this drawback have been elaborated such as adipocyte cultures in 3D using matrigel and decellularized material from adipose tissue (Pellegrinelli, Heuvingh et al. 2014). In addition, adipocytes can be differentiated and maintained at their usual oxygen concentrations found within the tissue (5-10% oxygen compared to widely used 21 % oxygen) (Famulla, Schlich et al. 2012). Future

use of these novel cell culture techniques will demonstrate if adipocytes behave differently in more advanced cell culture settings.

Adipokine release can also be measured directly from adipose tissue *ex vivo*. For this purpose, adipose tissue explants are generated from adipocytes tissue biopsies. For our experiments, paired biopsies from subcutaneous and visceral adipose tissue were immediately transferred to medium. Explants were generated after removal of connective tissue and visible vessel. Adipose tissue was cut into pieces of approximately 10 mg each, washed with PBS for 3 times to remove cellular debris and contaminating blood. Afterwards, explants were weighted and cultured in serum-free medium (100 mg fat explants in 1 ml medium). The medium was collected at different time points to measure linearity of adipokine release and after 24 hours for cumulative adipokine release. Aliquots were stored at –80 C until further use. The release of adipokine *ex vivo* is the sum of the release of all cells present within adipose tissue and potentially closer to the situation *in vivo*. However, it is not possible to distinguish the contributions of a single cell type. It should be noted that adipokine release is very different from mature adipocytes compared to immune cells within adipose tissue and preadipocytes (Fain, Madan et al. 2004). *Ex vivo* measurements are also limited in a way that explants can only be kept for a short period without signs of necrosis such as lactate dehydrogenase release. Therefore, regular measurements to prove the intactness of explants are necessary.

Although adipose tissue mass dramatically increases and this increase can be correlated with many adipokines in serum in the obese state, the origin of the measured factor is not clear. One method to overcome this limitation of adipokine measurements in the circulation is the measurement of the arteriovenous difference across the tissue. The relative simple principle behind this technique is that the difference in the composition of a blood sample from the arterial supply to a tissue and from the venous drainage from the same tissue is measured and reflects the release or uptake of the measured factor (Frayn and Coppack 2001). In humans, the veins draining from subcutaneous anterior abdominal adipose tissue are the only accessible vessels that have adipose tissue as an almost unique

contributing tissue. Technically, a superficial epigastric vein and an arterialized dorsal hand vein, which is kept in a warming box at 60°C, are cannulated. The cannulae are kept patent with an intravenous infusion of 0.9 % saline. Adipose tissue blood flow is measured in abdominal subcutaneous adipose tissue in the fasting state (Samra, Frayn et al. 1995) and calculated from the washout of 133Xe (Jansson and Lonnroth 1995). After resting for 45 min, blood samples are taken simultaneously from the two sites. This technique can be applied to any adipokine or to metabolites such as glucose or fatty acids also in combination with tracers.

Other methods to measure adipose tissue secretory output *in vivo* are microperfusion (Trajanoski, Brunner et al. 1997) or microdialysis of adipose tissue (Murdolo, Herder et al. 2008). Here, adipose tissue is targeted with a permeable microtube that is inserted into subcutaneous adipose tissue through the skin. Adipose tissue release of adipokines is then measured in a buffer that is perfused in the tissue. Although the sample is taken directly within adipose tissue, not all adipokines can be measured properly for their release. Tissue injury introduced by this method is strongly influencing the release of mostly inflammatory adipokines such as IL-6 or IL-8 over time rather reflecting acute inflammatory processes at sites of insertion than adipokine release.

4. Adipose tissue inflammation in obesity and its impact on skeletal muscle insulin resistance

The first part of these studies is focusing on molecular mechanisms by which obesity and increased adipose tissue mass negatively influences insulin sensitivity of skeletal muscle via inflammatory endocrine pathways (**publication 1-5**). Cell culture studies using a crosstalk model of adipocytes and skeletal myocytes are able to reproduce various physiological features of defective skeletal muscle metabolism in patients. One important aspect here is the fact that muscle insulin resistance is at partly reversible with many but not all defects normalizing after intervention. Furthermore, this approach is ideal to study single isolated factors which would be impossible in the complex *in vivo* setting. Here, I present and discuss data on the novel adipokines chemerin and heat shock protein 60 (Hsp60) which are both dysregulated in the obese state and inducers of skeletal muscle insulin resistance involving inflammatory pathways. Mechanisms to explain dysregulated adipokine production in obese adipose tissue with respect to chronic low-grade inflammation will also be discussed. In addition to detailed *in vitro* work, clinical data are presented to translate the function of these adipokines to patients with obesity, type 2 diabetes and the metabolic syndrome

4.1. Mechanisms of adipokine-induced insulin resistance in skeletal muscle

Publication 1: Adipokine-induce insulin resistance in skeletal muscle cells is reversible

Background: Insulin resistance in skeletal muscle is an early event in the development of diabetes with obesity being one of the major contributing factors. In this context, the negative crosstalk between adipose tissue and skeletal muscle is involved in early metabolic disturbances leading to insulin resistance, and adipokines might be major contributors to the development of insulin resistance. Reduction of adipose tissue mass by weight loss is a validated approach to reverse insulin resistance (Petersen, Dufour et al. 2005). *In vitro*, insulin resistance disappears in cultured skeletal muscle biopsies from obese patients (Brozinick, Roberts et al. 2003, Pender, Goldfine et al. 2005) but can be retained in muscle biopsies from obese and diabetic patients as shown in other studies (Henry, Abrams et al.

1995, Thompson, Pratley et al. 1996, Vollenweider, Menard et al. 2002, Brozinick, Roberts et al. 2003).

Based on the experimental approach using CM from differentiated human adipocytes to impair insulin signalling in human skeletal muscle cells, this study was aimed at analyzing reversibility of adipocyte-induced insulin resistance in skeletal muscle cells and underlying mechanisms.

Methods: Human skeletal muscle cells were treated with adipocyte-CM to induce insulin resistance followed by a recovery period of 24 to 48 h after CM withdrawal. Insulin signaling, differentiation marker, myokine release and oxidative stress were measured in the presence of CM and/or after CM withdrawal.

Results: CM-induced insulin resistance was paralleled by enhanced production of reactive oxygen species (ROS) and ceramide as well as a downregulation of myogenic transcription factors such as myogenin and myoD. Regeneration of myotubes for 24 or 48 h after induction of insulin resistance restored normal insulin signalling. However, the expression level of myogenin could not be re-established. In addition to decreasing myogenin expression, CM also decreased the release of IL-6 and IL-8, and increased monocyte chemotactic protein-1 (MCP-1) secretion from skeletal muscle cells. While regeneration of myotubes re-established normal secretion of IL-6, the release of IL-8 and MCP-1 remained impaired over 48 h after withdrawal of CM.

Conclusions: This study showed that adipocyte-induced insulin resistance is a reversible process in skeletal muscle cells, at least at the level of insulin signaling. However, some alterations are not fully reversible and may illustrate longer lasting damage to the myotubes by one time treatment with CM. Skeletal muscle cells display long-lasting myogenin downregulation and secretory defects of IL-8 and MCP-1. In our model, we observe a loss of myogenin expression with preservation of muscle phenotype such as insulin signalling, myotube morphology and glucose transporter 4 (GLUT4) expression.

Limitations: In vitro differentiated skeletal muscle cells and adipocytes were obtained from healthy donors thus limiting the translation of our findings to obesity-related insulin resistance

in vivo. Further limitations include the relative short time of recovery from CM related to the maximal survival of myotubes in culture and the omitted measurement of glucose uptake in addition to insulin signalling.

Obesity, particularly visceral accumulation of fat leading to a high waist circumference, is related to peripheral insulin resistance in several organs, which includes adipose tissue itself (Ruan and Lodish 2003) and most importantly skeletal muscle (Brozinick, Roberts et al. 2003). Whether myotubes from obese insulin-resistant patients retain defects in cell culture is still a controversial issue. On one hand, studies demonstrate that insulin resistance dissolve through culture using biopsies of skeletal muscle from obese patients (Brozinick, Roberts et al. 2003, Pender, Goldfine et al. 2005) which indicate that insulin resistance might be a abolishable phenotypic trait which is acquired with obesity and only present when cells are exposed to the insulin resistance-inducing environment. However, other studies using skeletal muscle biopsies originating from obese and diabetic patients demonstrate that insulin resistance can be preserved in culture (Henry, Abrams et al. 1995, Thompson, Pratley et al. 1996, Vollenweider, Menard et al. 2002, Brozinick, Roberts et al. 2003) even over several passages (Wolf, Chen et al. 2013).

Using adipocyte-CM from primary human adipocytes, the most important features of insulin-resistant skeletal muscle, namely defective insulin signalling on various levels of the insulin signalling cascade as well as impaired translocation of GLUT4 and reduced insulin-stimulated glucose uptake can be reproduced in primary human skeletal muscle cells (Dietze, Koenen et al. 2002, Dietze, Ramrath et al. 2004, Sell, Dietze-Schroeder et al. 2006) (**Publication 1**). In addition, it has been shown that the insulin-resistance inducing capacity of adipocyte-CM is adipocyte size- and BMI-dependent with hypertrophy and high BMI being associated with stronger induction of insulin resistance (Skurk, Alberti-Huber et al. 2009). This induction of insulin resistance *in vitro* is comparable to the human situation where insulin resistance is characterized by reduced insulin-stimulated phosphorylation of tyrosine residues in insulin receptor substrate 1 (IRS-1) (Krook, Bjornholm et al. 2000), PI-3 kinase

signalling including Akt phosphorylation (Cusi, Maezono et al. 2000), defective GLUT4 transport activity (Krook, Bjornholm et al. 2000) and finally reduced glucose uptake into muscle after insulin stimulation (Kelley, Mokan et al. 1992). As heat inactivation of adipocyte-CM results in a complete loss of effects on insulin signalling (Dietze, Ramrath et al. 2004) and furthermore several single adipokines including MCP-1 and chemerin function as inducers of insulin resistance, it is highly probable that proteins are the responsible factors within adipocyte-CM.

Adipocyte-CM also alters differentiation marker and muscle regulatory factors in skeletal myocytes. Namely, myogenin, myosin heavy chain (MHC) and MyoD are significantly reduced in insulin-resistant myotubes (Taube, Lambernd et al. 2012) (**Publication 1**). Similarly, myogenin and MyoD are decreased in skeletal muscle in type 2 diabetes and obesity (Ashwal, Hemi et al. 2011, Watts, McAinch et al. 2013). *In vitro* modulation of myogenin in myogenic cells demonstrated that diversification towards expression of fast or slow skeletal muscle fiber types can be determined by the activity and amount of myogenin with high myogenin being related to an increased presence of the slow phenotype (Alapat, Chaudhry et al. 2009). In this respect, it has been demonstrated already a long time ago that insulin sensitivity positively correlates with the amount of slow-twitch oxidative fibers (Lillioja, Young et al. 1987). However, it is not the case that the loss of muscle regulators like myogenin or MyoD are related to altered fibre type of skeletal muscle (Aguiar, Vechetti-Junior et al. 2012). In patients, it is also not clear if reduced muscle regulatory factors are related to muscle insulin resistance and obesity as alterations in fibre type or markers of fibre type could be observed in obese and insulin-resistant patients in some studies (Lillioja, Young et al. 1987, Giebelstein, Poschmann et al. 2012) but not in others (Zierath, He et al. 1996). Whether these defects are directly related to insulin resistance is not clear and should be further investigated.

Insulin-resistant skeletal muscle cells treated with adipocyte-CM show reduced mitochondrial capacity, higher oxidative stress and no apoptosis (**Publication 1**). In the insulin-resistant state, adipokine-treated skeletal muscle cells produce more ROS and nitric

oxide (NO) while lowering succinate dehydrogenase (SDH) activity. ROS are proposed to be causal agent for insulin resistance being elevated before insulin resistance develops (Fridlyand and Philipson 2006). In terms of organ crosstalk, tumour necrosis factor (TNF) α is an adipokine that is for example able to induce ROS directly in adipocytes (Chen, Zhao et al. 2010) and indirectly via myostatin in skeletal muscle cells (Sriram, Subramanian et al. 2011). An additional prominent feature of insulin-resistant skeletal muscle is an altered mitochondrial morphology and function which might be an underlying mechanism of or secondary to the development of skeletal muscle insulin resistance (Szendroedi, Phielix et al. 2011). Although mitochondrial function has not been measured directly in our study, activity of SDH is reduced in adipokine-treated skeletal muscle cells, which is similar to what occurs in skeletal muscle of patients with type 2 diabetes compared to controls (Oberbach, Bossenz et al. 2006). Triglyceride accumulation in skeletal muscle in the form of intramyocellular lipids (IMCL) and accumulation as intrahepatic lipid in the liver are relevant markers for insulin resistance (Krssak, Falk Petersen et al. 1999, Anderwald, Bernroider et al. 2002). Moreover, certain lipid metabolites have been shown to impair insulin signalling. Most importantly, ceramide and diacylglycerol (DAG) relate to insulin resistance in humans (Adams, Pratipanawatr et al. 2004, Szendroedi, Yoshimura et al. 2014). Ceramide is able to directly impair insulin action in skeletal muscle *in vitro* (Hajduch, Balendran et al. 2001, Levin, Monetti et al. 2007) while the involvement of DAG is not clearly demonstrated *in vitro* (Chavez, Knotts et al. 2003). Ceramide is induced by adipokines in our model but we have not analyzed if insulin-resistant human skeletal muscle cells directly increase DAG content after treatment with adipocyte-CM. However, skeletal muscle cells pretreated with adipocyte-CM accumulate significantly higher amounts of DAG compared to control cells when challenged with palmitic acid (Taube, Lambernd et al. 2012).

Skeletal muscle is the heaviest organ in non-obese humans representing an important producer of endocrine mediators. Many studies have analysed how the secretory output from skeletal muscle is regulated by exercise and how in this scenario so-called myokines exert beneficial effects on glucose metabolism in healthy and insulin-resistant

volunteers (Eckardt, Gorgens et al. 2014). Less is known about myokines in the insulin-resistant state without contraction. One of the most studied myokines is IL-6 which is highly induced by exercise (Steensberg, van Hall et al. 2000). In our experiments, IL-6 secretion is significantly downregulated in skeletal myotubes after treatment with adipocyte-CM (**Publication 1**). In a similar approach, the regulation of IL-6 secretion was analysed after incubation with high concentrations of TNFα in skeletal muscle cells which showed a significant upregulation of IL-6 mRNA and release directly after stimulation (Bouzakri, Plomgaard et al. 2011). However, TNFα is not highly present in adipocyte-CM due to limited release by adipocytes in culture and instability of the protein so that both conditions are difficult to compare. Furthermore, IL-6 is present in adipocyte-CM itself making it necessary to first withdraw the CM before measuring IL-6 release by skeletal muscle cells. The observed downregulation of IL-6 after treatment with adipokines however goes in line with the reduction in myogenic marker with IL-6 being a known myogenic marker itself (Baeza-Raja and Munoz-Canoves 2004). *In vivo*, IL-6 expression is not altered in insulin-resistant patients in skeletal muscle (Andreasen, Kelly et al. 2011). While healthy controls benefit from IL-6 infusion in terms of fatty acid oxidation and insulin-stimulated glucose uptake, patients with type 2 diabetes are resistant to these positive metabolic effects (Carey, Steinberg et al. 2006, Jiang, Duque-Guimaraes et al. 2012). MCP-1 is also a known myokine to be increased after inflammatory stimuli or lipid treatment in skeletal muscle cells (Patsouris, Cao et al. 2014), which is similar to the increased release after adipokine treatment as observed in our study (**Publication 1**). *In vivo*, MCP-1 expression is not different in skeletal muscle from obese or insulin-resistant donors (Tantiwong, Shanmugasundaram et al. 2010) but increased in obese type 2 diabetic patients (Patsouris, Cao et al. 2014). From the later study, it can be suggested that MCP-1 is a myokine that is induced in muscle in a state of chronic inflammation which is associated with macrophage infiltration although to a lesser extent than observed in expanded adipose tissue as discussed later.

In our study, several features of insulin resistance were reversible after CM withdrawal. While insulin signalling recovered rapidly on the level of insulin-stimulated Akt

and GSK3 phosphorylation, myotube differentiation marker only partially normalized. Also the release of the myokines IL-8 and MCP-1 was more constantly disturbed. Interestingly, the classical exercise-induced myokine IL-6 normalized rapidly after CM withdrawal and normalization of insulin sensitivity. Limitations of our study include the lack of information on glucose uptake after normalization of insulin signalling. Further studies are also needed to test if normal metabolic flexibility to switch between substrates can be restored in the myotubes. *In vivo*, insulin resistance and type 2 diabetes are not a one way street but can be reversed if treated early or aggressively. Different types of intervention such as weight loss and lifestyle intervention by strict dieting and exercise can lead to improvement of insulin resistance and remission of type 2 diabetes. Established type 2 diabetes can also be reversed by obesity surgery such as Roux-en-Y bypass (Perugini and Malkani 2011). Mechanistic studies show that for example bariatric surgery can slightly improve mitochondrial function in parallel to weight loss (Nijhawan, Richards et al. 2013). In blood monocytes, basal and maximal mitochondrial respiration is improved significantly while maximal respiration in skeletal muscle tended to improve as well. Diet-induced weight loss in obese patients is however not very efficient in increasing mitochondrial mass (Snel, Gastaldelli et al. 2012) while adding exercise induces mitochondrial biogenesis (Toledo, Menshikova et al. 2008). A very-low calorie intervention in obese insulin-resistant women demonstrated increased insulin sensitivity but decreased mitochondrial function (Rabol, Svendsen et al. 2009). Interestingly, weight loss in lean, insulin-resistant offspring of patients with type 2 diabetes can improve both mitochondrial capacity and insulin resistance without affecting circulating adipokines but intramyocellular lipids (IMCL) (Petersen, Dufour et al. 2012). In contrast to diet intervention, exercise alone is very effective in increasing mitochondrial capacity (Toledo and Goodpaster 2013) with additional effects in patients undergoing bariatric surgery (Coen, Menshikova et al. 2015). Oxidative stress markers can be effectively reduced by exercise training in parallel to muscle insulin resistance (Ludzki, Paglialunga et al. 2015). Exercise intervention but not diet-induce weight loss is able to reduce ceramide concentrations in skeletal muscle while DAG in skeletal muscle can be

decreased by both types of intervention (Dube, Amati et al. 2011). Although the constant positive effect of exercise to improve mitochondrial capacity, oxidative stress and accumulation of different lipid species is accepted, the mechanistical link to reduced insulin resistance, reduced intramyocellular lipids or enhancement of skeletal muscle metabolism is still not fully conclusive. Other features of muscle insulin resistance in humans and in cell culture are not reversible such as the reduced expression of myogenic marker. Myogenin and MyoD are not increasing in parallel to improved glucose utilization after exercise and diet intervention in obese patients (Ryan, Li et al. 2013). Furthermore, it is not known if regulation of myokine expression and release in the insulin-resistant skeletal muscle can be reversed by interventions restoring insulin sensitivity in this organ. All pathophysiological features of insulin resistance as assessed in our model of adipokine-induced insulin resistance in human primary skeletal myotubes are summarized in Table 1 and compared to known traits of skeletal muscle insulin resistance *in vivo*. The aspect of reversibility has also been summarized to highlight yet unanswered questions.

Table 1

Reversible features of insulin-resistant skeletal muscle in vivo versus adipokine-induced insulin-resistant skeletal muscle cells in vitro

Feature	*In vitro*	Reversibility *in vitro*	*In vivo*	Reversibility *in vivo*
Insulin signalling (insulin-stimulated Akt phosphorylation)	reduced	yes	reduced	yes
Insulin-stimulated glucose uptake	reduced	unknown	reduced	yes
Myogenic marker	reduced	partially	reduced	no
Oxidative stress	increased	unknown	increased	yes
Mitochondrial function	altered	unknown	altered	yes
IMCL	increased	unknown	increased	yes
Ceramide	increased	unknown	increased	yes
DAG	increased	unknown	increased	yes
Myokine expression/release	altered	partially	altered	unknown

DAG diacylglycerols

IMCL intramyocellular lipids

4.2. Underlying mechanisms of adipose tissue inflammation in obesity leading to increased release of pro-inflammatory and chemotactic adipokines

Publication 2: Chemerin induces insulin resistance in human skeletal muscle cells

Background: Chemerin is a secreted chemoattractant protein and a novel adipokine necessary for adipogenesis (Goralski, McCarthy et al. 2007). Chemerin is elevated in adipose tissue of diabetic Psammomys obesus sand rats compared to non-diabetic controls (Bozaoglu, Bolton et al. 2007). In humans, no difference in chemerin levels could be found between patients with and without type 2 diabetes despite a correlation of chemerin levels with BMI, blood triglycerides and blood pressure (Bozaoglu, Bolton et al. 2007). The objective of this study was to analyze effects of chemerin on skeletal muscle insulin sensitivity and to clarify if this adipokine is a player in the negative crosstalk between adipose tissue and skeletal muscle.

Methods: Human skeletal muscle cells were treated with chemerin to study insulin signaling, glucose uptake and activation of stress kinases. The release of chemerin was analyzed from *in vitro* differentiated human adipocytes and adipose tissue explants from lean and obese patients.

Results: Human adipocytes express chemerin and Chemokine like receptor 1 (CMKLR1) differentiation-dependently and secrete chemerin. Chemerin release is slightly but significantly increased by TNFα and markedly inhibited by over 80 % by peroxisome proliferator-activated receptor (PPAR) γ activation. Adipose tissue explants from obese patients are characterized by significantly higher chemerin secretion as compared to lean controls (21 ng and 8 ng from 10^7 cells, respectively). Chemerin release is correlated with BMI, waist-hip-ratio and adipocyte volume. Furthermore, higher chemerin release is associated with insulin resistance at the level of lipogenesis and insulin-induced anti-lipolysis in adipocytes. Chemerin induces insulin resistance in human skeletal muscle cells at the level of IRS1, Akt and GSK3 phosphorylation and reduces insulin-stimulated glucose uptake. Moreover, chemerin activates p38 Mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and extracellular signal–regulated

kinase (ERK) 1/2 signalling in a dose-dependent manner. Accordingly, inhibition of ERK signalling prevents chemerin-induced insulin resistance on the level of insulin signalling and also glucose uptake.

Conclusions: Our data show that skeletal muscle is a target tissue for chemerin where this chemotactic protein induces inflammation and insulin resistance. As chemerin release is significantly higher from enlarged adipocytes, it might be involved in the negative crosstalk between adipose tissue and skeletal muscle. The possible role of chemerin as a connecting link between obesity and diabetes needs however to be established by further studies, since cell types other than adipocytes secrete this cytokine and may contribute to its effect on skeletal muscle cells.

Limitations: This *in vitro* study can only illustrate a small part of the complex pathophysiological effects of chemerin *in vivo*.

Originality: This is the first study on direct effects of chemerin on insulin sensitivity in skeletal muscle cells.

Publication 3: Chemerin correlates with markers for fatty liver and decreases after surgery-induced weight loss in morbidly obese patients

Background: Chemerin is a new adipokine involved in *in vitro* adipogenesis and insulin resistance and associates with BMI *in vivo*. In humans chemerin plasma concentrations correlate with BMI and are elevated in patients with the metabolic syndrome being associated with FBG, HDL-cholesterol, triglycerides and FBG (Bozaoglu, Bolton et al. 2007, Bozaoglu, Segal et al. 2009). The relationship between chemerin and the metabolic state in morbid obesity in humans as well as the effect of weight loss has not yet been studied. Therefore, this study aims at analyzing chemerin levels in morbidly obese patients before and during surgery-induced weight loss and the relationship between chemerin concentration, anthropometric measurements, biochemical parameters and markers of inflammation and hepatosteatosis in a longitudinal study at baseline and 12 to 24 months after surgery.

Methods: 60 obese female patients (BMI 50.0±1.0) being candidates for gastric bypass were included in a prospective study and examined before and 3, 6, 12 and 24 months after bariatric surgery. Serum concentrations of chemerin were measured in addition to anthropometric parameters, homeostatic model assessment (HOMA)-IR, total cholesterol, HDL-cholesterol, triglycerides, C-reactive protein (CRP) and several other adipokines at all time points. Liver histology and macrophage content in adipose tissue were assessed at baseline.

Results: Chemerin serum concentrations were higher in obese patients compared to non-obese persons (353.8±18.0 ng/ml versus 191±14 ng/ml, $p<0.001$). At baseline, chemerin concentrations correlated positively with BMI, CRP, IL-6, HOMA-IR and the amount of omental macrophages, and negatively with HDL-cholesterol levels. Furthermore, chemerin was elevated in patients with a significant activity score for Non-alcoholic fatty liver disease (NAFLD), portal inflammation, fibrosis and fibroinflammation. After surgery, chemerin decreased significantly to 253.0±14.9 ng/ml after 1 year and pursued its decrease in patients studied for 2 years. The strong decrease of chemerin in the 3 months after surgery was significantly associated with the decrease in HOMA-IR and FBG.

Conclusions: Chemerin concentrations are elevated in morbidly obese patients and correlated with insulin resistance and markers of liver pathology. Chemerin plasma concentrations decreased after bariatric surgery. This study suggests that chemerin might mediate metabolic alterations in obesity which improves after gastric bypass.

Limitations: Chemerin expression in liver and its contribution to circulating chemerin have not been studied.

Originality: This study is the first report on chemerin changes in a longitudinal follow-up of morbidly obese patients before and after surgery-induced weight loss.

Publication 4 (Review): Adaptive immunity in obesity and insulin resistance

Obesity is the hallmark of the metabolic syndrome and predisposes patients to the development of major chronic metabolic diseases including type 2 diabetes. Adipose tissue

expansion in obesity is characterized by increasing infiltration of pro-inflammatory immune cells into adipose tissue causing chronic, low-grade inflammation. Phenotypic switching of macrophages is an important mechanism of adipose tissue inflammation, and there is a crucial involvement of cells from the adaptive immune system in this process. T-cell phenotype changes and recruitment of B and T lymphocytes precedes macrophage infiltration. Cytokines and chemokines produced by immune cells influence local and systemic inflammation, which is a pathogenic link between obesity and insulin resistance. Antigens absorbed from the gut might contribute to T-cell activation and recruitment into visceral adipose tissue in obesity. This review summarizes the evidence for infiltration of obesity-associated adipose tissue by cells of the adaptive immune system, how they affect innate cell populations and the influence of adaptive immune system cells on the development of insulin resistance.

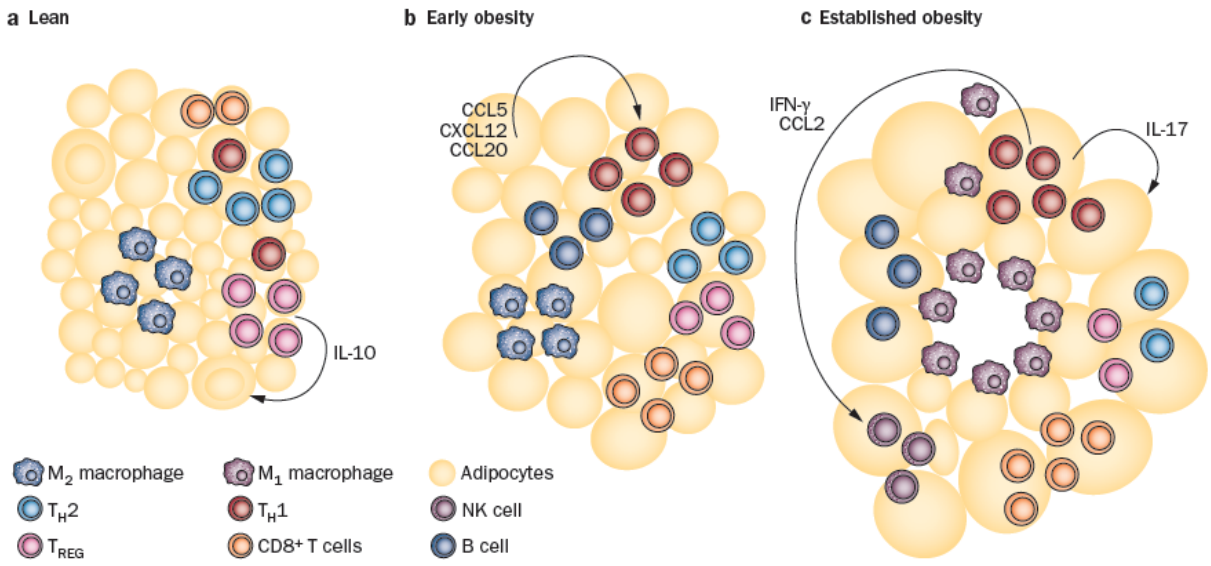
During expansion of adipose tissue, adipocytes start to display stress when hypertrophy and mechanical constraints are reaching a critical level. These signs include altered lipid composition, hypoxia, altered mitochondrial function and oxidative stress with overproduction of ROS, endoplasmic reticulum (ER) stress, pro-apoptotic and pro-inflammatory signaling, increased lipolysis and pro-inflammatory adipokine release. Increased secretion of adipokines with pro-inflammatory and chemotactic properties in obesity has been recognized as a molecular link to the development of insulin resistance (Sell, Dietze-Schroeder et al. 2006, Sell and Eckel 2009, Sell and Eckel 2010) (**Publication 4**). In this context, adipose tissue inflammation has been recognized as one crucial step in the development of obesity-associated complications. In 2003, the important discovery of macrophage infiltration in expanding adipose tissue (Weisberg, McCann et al. 2003, Xu, Barnes et al. 2003) linked adipose tissue inflammation to both obesity and insulin resistance. Since this feature of obese adipose tissue has been revealed, a number of mechanisms potentially leading to the infiltration of macrophages into adipose tissue have been described, which include adipocyte hypoxia, fibrosis, ER stress and adipocyte death leading to

increased secretion of chemotactic adipokines. Based on detailed microscopic studies, crown-like structures of macrophages around necrotic-like enlarged adipocytes have been identified that stimulate macrophage recruitment in obese individuals (Cinti, Mitchell et al. 2005) and in obese mice, particularly in visceral adipose tissue (Murano, Barbatelli et al. 2008). It has been put forward that apoptosis is not very likely to be an underlying mechanism of adipocyte death. Apoptosis is a physiological process in normal adipocyte turnover, which is rather associated with accumulation of M2 macrophages within adipose tissue that are anti-inflammatory and related to tissue repair (Fischer-Posovszky, Wang et al. 2011). In obesity however, more M1 macrophages are present in enlarged and inflamed adipose tissue (Lumeng, Bodzin et al. 2007). Data on adipose tissue inflammation in the course of obesity development is mostly obtained in obese animal models and demonstrate that this chronic inflammation of adipose tissue results from a multifaceted interplay of adipocytes, macrophages and different other immune cells of both the innate and the adaptive immune system. The infiltration of pro-inflammatory M1 macrophages is likely to be only the last step of immune action potentially starting with dysfunctional hypertrophied adipocytes. These adipocytes display immune functions leading to CD4+ T cell activation (Meijer, de Vries et al. 2011), potentially prior to macrophage infiltration. Initially, B and TH1 lymphocytes are recruited to enlarging adipose tissue by factors such as CC chemokine (CCL) 5 and CXC chemokine (CXCL) 5 produced by preadipocytes and adipocytes (Kintscher, Hartge et al. 2008, Winer, Winer et al. 2011). Local inflammation triggers release of TH1 cytokines such as interferon (INF) γ that itself stimulates chemokine release by adipocytes. The final step in adipose tissue inflammation consists of the recruitment of M1 macrophages by these chemotactic adipokines such as MCP-1 (Takahashi, Mizuarai et al. 2003). The time course of these processes is presented in Figure 2. Most importantly, the epidemiological evidence that visceral obesity is associated with a greater risk for obesity-related diseases when compared with subcutaneous obesity (Despres and Lemieux 2006) goes in line with differential increase in inflammation in the two depots (Kloting, Fasshauer et al. 2010). Adipokine release in adipose tissue inflammation are different in the visceral and

the subcutaneous depots (Caspar-Bauguil, Cousin et al. 2005). Macrophage, T cell and natural killer (NK) cell infiltration are increased in visceral adipose tissue as compared to the subcutaneous depot in obese donors (Cancello, Henegar et al. 2005, O'Rourke, Metcalf et al. 2009). Here also, IFNγ expression is increased in NK cells from visceral fat as compared to NK cells from the subcutaneous depot. In addition, the downregulation of regulatory T cells (T_{reg}) in obesity is more pronounced in visceral adipose tissue (Deiuliis, Shah et al. 2011).

Figure 2

(A) M2 polarized macrophages, T_{reg} and T_H2 polarized T cells predominate in adipose tissue in the lean state and inflammation is limited by T-cell secreted IL-10. (B) Early in obesity, there is a shift in the T cell populations towards CD8+ and T_H1 polarized lymphocytes and B cells are recruited to adipose tissue. Increased release of chemotactic adipokines by enlarged adipocytes contributes to this modulation of the adaptive immune system within adipose tissue. (C) Later in obesity, an infiltration of NK cells and proinflammatory M1 polarized macrophages may be triggered by increased INFγ and chemokines such as CCL2 released from CD8+ and T_H1 polarized lymphocytes. Other factors released from these T cells such as IL-17 could contribute to insulin resistance of adipocytes.



Adipocyte hypertrophy-related hypoxia and ER stress are two prominent mechanisms discussed to initiate inflammation in adipocytes and thereby the recruitment of immune cells to adipose tissue (Hummasti and Hotamisligil 2010, Trayhurn 2014). Hypoxia is indeed *in vitro* regulating adipokine production which has been shown for pro-inflammatory adipokines

(Wang, Wood et al. 2007, Famulla, Horrighs et al. 2011) and chemotactic adipokines such as CCL5 (Skurk, Mack et al. 2009). *In vivo*, hypoxia can be measured in adipose tissue of obese mice (Hosogai, Fukuhara et al. 2007) but the human data are very controversial at the moment with studies showing both hypoxia (Pasarica, Sereda et al. 2009) and hyperoxia (Goossens, Bizzarri et al. 2011) in obese human adipose tissue.

The ER is a rapid responder to changes in cellular nutrient and energy status. ER-mediated stress signalling and the related unfolded protein response have been shown to be activated in obesity mediating both metabolic and immune responses. ER stress may then represent a link between hypoxia and adipocyte death in obese adipose tissue as dysregulated redox state may increase unfolded and misfolded proteins and subsequently ER stress (Flamment, Hajduch et al. 2012). In fact, hypoxia *in vitro* increases ER stress (Hosogai, Fukuhara et al. 2007) and induces apoptosis, increases lipolysis and depresses insulin signalling along with glucose uptake in adipocytes (Yin, Gao et al. 2009). *In vivo*, ER stress is also increased in fat of obese study participants (Boden, Duan et al. 2008) with ER stress being related to the number of apoptotic adipocytes (Sharma, Das et al. 2008). As increased ER activity can be observed in cell types that are specialized in endocrine function such as β-cells and mature B lymphocytes when protein production is at high demand, it is very probable that ER stress is also related to adipocyte dysfunction in terms of adipokine secretion.

The discovery that adipose tissue is characterized by low-grade chronic inflammation in the obese state and that there is a regulated infiltration of various immune cells into adipose tissue in the course of tissue expansion has prompted research on mediators and molecular mechanisms of these processes. Soon, it became evident that chemokines such as MCP-1 are crucial initiators and mediators of adipose tissue inflammation (Sell and Eckel 2007). Per definition, chemokines are released proteins that attract different types of immune cells including monocytes, T lymphocytes, neutrophils, basophils or eosinophils with each factor activating a single or more immune cell types (Balkwill 1998). In a narrow classification, chemokines are a subgroup of cytokines that display chemotactic properties

towards immune cells and possess four highly conserved cysteins. CXC chemokines are characterized two N-terminal cysteines separated by one amino acid. CC chemokines have two N-terminal cysteines that are adjacent. MCP-1, also called CCL2, is a prominent example from this group of around 30 members. In addition to these classical structurally defined chemokines, many other protein hormones and also adipokines display chemotactic properties and participate in adipose tissue inflammation. As an example, the newly discovered adipokine chemerin has chemotactic abilities towards dendritic cells and macrophages. However, the structure of chemerin shows no homology to known chemokine families. Chemerin is an interesting candidate for metabolic research as it has various tissue specific functions and is related to various obesity-related metabolic diseases.

As MCP-1 has been described as one of the first candidates for being a mediator of adipose tissue inflammation, *in vitro* and *in vivo* evidence for a causal link between this chemokine/adipokine and obesity-related insulin resistance has been collected. MCP-1 is secreted from adipocytes (Gerhardt, Romero et al. 2001) with large adipocytes secreting significantly higher amounts of MCP-1 in parallel to other pro-inflammatory adipokines (Skurk, Alberti-Huber et al. 2007). In addition, insulin and inflammatory stimuli regulate MCP-1 release (Sartipy and Loskutoff 2003). Within adipose tissue, the stroma-vascular fraction containing preadipocytes and immune cells highly contributes to MCP-1 release in addition to adipocytes (Fain and Madan 2005). Adipocytes treated with MCP-1 are characterized by a significantly impaired insulin-stimulated glucose uptake (Sartipy and Loskutoff 2003). In addition, we could show that MCP-1 is an inducer of insulin resistance in peripheral organs such as skeletal muscle in an endocrine way. MCP-1 concentrations below published circulating concentrations of this adipokine are sufficient to impair muscle insulin signalling via stress signalling (Sell, Dietze-Schroeder et al. 2006). Obese and type 2 diabetic study participants reach higher MCP-1 serum concentrations and are characterized by increased expression of this chemokine in human adipose tissue (Sell and Eckel 2009). Mouse models with manipulated expression of MCP-1 and its receptor C-C motif receptor (CCR) 2 could not help to establish a causal link between MCP-1 and adipose tissue inflammation as well as

the development of insulin resistance which is based on conflicting data (Chen, Mumick et al. 2005, Kanda, Tateya et al. 2006, Inouye, Shi et al. 2007, Lumeng, Deyoung et al. 2007).

Chemerin is a chemotactic protein that was first described in the context of psoriasis (Albanesi, Scarponi et al. 2009) but was afterwards discovered to be essential for adipogenesis (Bozaoglu, Bolton et al. 2007, Goralski, McCarthy et al. 2007). Chemerin and its receptors CMKLR1/ChemR23, G protein-coupled receptor (GPR) 1 and C-C chemokine receptor-like (CCRL) 2 are ubiquitously expressed and exert tissue specific actions. Due to highest expression of chemerin, adipose tissue and liver have been postulated to be the major contributors to circulating chemerin (Bozaoglu, Bolton et al. 2007, Shin, Lee et al. 2012). Chemerin expression as well as expression of its receptors dramatically increases during adipogenesis *in vitro and in vivo* while ablation of chemerin completely abolishes adipogenesis (Goralski, McCarthy et al. 2007). Within adipose tissue, chemerin can function as a growth factor as it can stimulate angiogenesis via capillary outgrowth mediated by ERK activation (Bozaoglu, Curran et al. 2010, Kaur, Adya et al. 2010). Chemerin's role in both angiogenesis and adipogenesis suggests that this chemokine could be a mediator of adipose tissue enlargement by increasing the capillary blood flow within adipose tissue. A huge amount of data has been collected to show that chemerin can be regarded as an endocrine mediator of obesity and obesity-associated metabolic diseases. Chemerin serum levels are elevated in patients with type 2 diabetes and obesity as shown by us and others (Li, Shi et al. 2014). Our data published in **Publication 2** demonstrate that adipose tissue and isolated adipocytes from obese study participants release significantly more chemerin compared to lean controls. In this context, chemerin release positively correlates with adipocyte size and negatively with insulin sensitivity of adipose tissue on the level of insulin-stimulated lipogenesis and insulin-stimulated repression of lipolysis. Also in morbidly obese patients, chemerin serum concentrations are correlated to BMI and HOMA-IR (**Publication 3**). It has not been clarified so far if chemerin itself alters glucose uptake in adipocytes as the only two existing studies on this subject are describing completely opposite effects (Takahashi, Takahashi et al. 2008, Kralisch, Weise et al. 2009). But in an endocrine way, chemerin acts

as an adipokine that can induce insulin resistance in skeletal muscle cells (**Publication 2**). This notion is supported by other studies demonstrating that chemerin induces altered mitochondrial function in skeletal muscle underlying insulin resistance (Xie, Deng et al. 2015) and that overexpression of human chemerin in mice specifically impairs muscle insulin sensitivity (Becker, Rabe et al. 2010).

As adipokines are seen as a link between NAFLD and insulin resistance (Tilg, Moschen et al. 2017) and chemerin is also originating from liver, chemerin has also been studied intensively in the context of liver diseases. In fact, hepatocytes release similar amounts of chemerin compared to adipocytes but chemerin expression in hepatocytes *in vitro* is not significantly regulate by any of the stimuli that regulate chemerin in adipocytes (Krautbauer, Wanninger et al. 2013). While chemerin induction in liver could not been shown for all studied obese mouse models with fatty liver (Ernst, Issa et al. 2010, Rourke, Muruganandan et al. 2014), most human data indicate that circulating chemerin and hepatic chemerin is increased in NAFLD (Kukla, Zwirska-Korczala et al. 2010, Yilmaz, Yonal et al. 2011, Docke, Lock et al. 2013). In our own study on serum chemerin in morbid obesity (**Publication 3**), chemerin is increased in patients with hepatic fibrosis, portal inflammation and a high score for fibroinflammation (NAFIN). Most interestingly, we observed a long-term reduction of chemerin after surgery-induced weight loss even after cessation of weight loss, which might be attributed to a hepatic contribution to circulating chemerin. Further studies should address the question of hepatic output of chemerin and mechanistic insight if chemerin acts as a causal factor in the development of NAFLD.

Chemerin is also related to inflammatory diseases with metabolic disturbances in the gastrointestinal tract. In fact, enlargement of mesenteric fat is usually found in patients with inflammatory bowel disease and more specifically in Crohn's disease (Weigert, Obermeier et al. 2010). Chemerin serum concentrations are elevated in inflammatory bowel disease (Weigert, Obermeier et al. 2010) where chemerin could be involved in the recruitment of tissue macrophages (Buechler 2014). Although data in humans are insufficient to suggest a causal role of chemerin in humans, data obtained in experimental animal models of

inflammatory bowel disease indicate a potential involvement of this chemokine in the development of this disease. Chemerin application is able to worsen inflammation and outcome in mouse models of inflammatory bowel disease whereas antibody treatment is reducing inflammation in epithelial cells and can improve histological scores *in vivo* (Lin, Yang et al. 2014).

Recent publications extend our knowledge on organ-specific actions of chemerin to the vascular wall (Ferland and Watts 2015). Chemerin is associated both with the incidence of coronary artery disease (Dong, Ji et al. 2011) and with the risk for cardiovascular disease (Dessein, Tsang et al. 2014). In type 2 diabetic patients, chemerin is increased in relation to endothelial dysfunction and early atherosclerosis (Gu, Cheng et al. 2015, Lu, Zhao et al. 2015). The chemerin receptor ChemR23 is present on smooth muscle cells and foam cells in atherosclerotic lesions (Kostopoulos, Spiroglou et al. 2014). Furthermore, chemerin is produced by perivascular adipose tissue and exerts vasoactive effects through ChemR23 adding a completely new function to this chemerin receptor (Watts, Dorrance et al. 2013, Neves, Nguyen Dinh Cat et al. 2015). Chemerin also activates ERK signalling leading to increased contractile response to endothelin-1 in aortic rings (Lobato, Neves et al. 2012). Additionally, vasorelaxation is decreased after chemerin treatment with reduced NO production and increased ROS generation being mechanistically involved (Neves, Lobato et al. 2014).

The global chemerin knockout mouse has elucidated a surprising function of chemerin in the pancreas (Takahashi, Okimura et al. 2011). Despite the fact that knockout animals display fewer macrophages in adipose tissue underlying its chemotactic properties, these animals are less glucose tolerant than wildtype controls due to increased hepatic glucose production and impaired insulin secretion. Chemerin deficiency in islets leads to decreased expression of the β-cell specific transcription factor MafA which impairs glucose-stimulated insulin secretion. Concordantly, transgenic overexpression of chemerin in mice increased glucose-stimulated insulin secretion and enhanced glucose tolerance. This study demonstrates that chemerin is a crucial factor for β-cell function in mice. In humans, serum

chemerin is positively associated with β-cell function as assessed by fasting β-cell function and an insulinogenic index derived from the oral glucose tolerance test (Hatziagelaki, Herder et al. 2015). Overall, chemerin is differentially regulated in different organs under various physiological and pathophysiological conditions acting as an adipokine, chemotactic factor and growth factor.

4.3. Heat shock response as a marker of cellular stress in obesity in relation to inflammation and associated metabolic complications

Publication 5: Hsp60 is released from adipocytes upon inflammation and induces insulin resistance in skeletal muscle cells

Background: The stress protein heat shock protein 60 (Hsp60) induces secretion of pro-inflammatory mediators from immune cells and adipocytes. Furthermore, elevated Hsp60 concentrations have been measured in the circulation of individuals with type 2 diabetes (Dasu, Devaraj et al. 2010). Thus, Hsp60 could be a potential trigger of human adipocyte inflammation. As insulin resistance is typical for obesity emerging early in the development of the metabolic syndrome and is highly associated with increased visceral adipose tissue inflammation, we hypothesized that Hsp60 could be a mediator of insulin resistance in relation to adipose tissue inflammation. This study aimed to analyze the effects of Hsp60 on the release of inflammatory mediators from human adipocytes and skeletal muscle cells on insulin sensitivity and to quantify plasma Hsp60 concentrations in lean and obese individuals with and without type 2 diabetes.

Methods: Human adipocytes and skeletal muscle cells were treated with Hsp60 followed by measurements of secretory function and signalling. Hsp60 serum concentrations and expression in adipose tissue were assessed in lean and obese participants with and without type 2 diabetes.

Results: Human adipocytes released Hsp60 in higher amounts compared to cells from the stroma-vascular fraction while Hsp60 release from skeletal muscle cells could not be detected. Hsp60 induced stress and inflammatory signalling and concentration-dependent insulin resistance at the level of Akt phosphorylation in human adipocytes. Furthermore, Hsp60 stimulated human adipocytes to secrete pro-inflammatory cytokines. In skeletal muscle cells, Hsp60 also activated pro-inflammatory signalling and stress pathways. Furthermore, Hsp60 inhibited insulin signalling and glucose uptake. Human skeletal muscle cells released IL-6, IL-8 and MCP-1 upon Hsp60-stimulation. Plasma Hsp60 concentrations

were 61 % higher in obese males with and without type 2 diabetes than in lean males and correlated positively with BMI, BP, plasma leptin and HOMA-IR.

Conclusions: Inflammatory stress induces the release of Hsp60 by human adipocytes that exerts autocrine/paracrine effects on adipocytes characterized by an increased release of pro-inflammatory adipokines, increased inflammatory signalling and insulin resistance. Furthermore, Hsp60 has endocrine effects on skeletal muscle cells inducing insulin resistance. Clinical data reveal positive associations of circulating Hsp60 concentrations with BMI, leptin, HOMA-IR and blood pressure. Therefore, Hsp60 might represent a novel adipokine involved in adipose tissue inflammation contributing to the development of insulin resistance.

Limitations: Although visceral adipose tissue is a predominant source of Hsp60 in obese patients with type 2 diabetes, *in vitro* experiments with adipocytes were performed using preadipocytes from subcutaneous adipose tissue.

Originality: This study is the first report on a clear link between adipose-derived Hsp60 and skeletal muscle insulin resistance.

Members of the Hsp family also belong to the adipokine family and are released upon cellular stress from adipocytes *in vitro* and potentially also *in vivo* (Lehr, Hartwig et al. 2012). In obesity, skeletal muscle, liver and adipose tissue display an altered intracellular stress defence system (Kondo, Koga et al. 2011). In this context, a dysbalance of the heat shock response (HSR) occurs in addition to the already described chronic low-grade inflammation, altered mitochondrial function, uncontrolled oxidative and ER stress. HSR is a key cellular instrument to cope with different stressors including metabolic stress (Richter, Haslbeck et al. 2010) and Hsps represent the most important highly conserved components (Lanneau, Brunet et al. 2008). Hsp60 is a mitochondrial chaperone but can also be located extramitochondrially and extracellularly. Whether Hsp60 is protective or harmful is related to its location. Under normal physiological conditions, Hsp60 is mainly localized in mitochondria but also in the cytosol and to a lower extend membrane-associated. Intracellularly, Hsp60

binds to misfolded, aggregated and nascent proteins and assists in proper folding, dissolving or translocation. Finally, it eliminates damaged proteins by marking them for degradation thereby exerting its cytoprotective effects. Under various forms of stress however, Hsp60 can be converted into a harmful released molecule. Extracellularly, Hsps are binding partners and ligands for Toll-like receptors thereby activating inflammatory processes (Asea 2008). Hsp60 release occurs from intact cells including immune and vascular cells to the circulation (Ireland, Leoni et al. 2007). Our data on Hsp60 demonstrate active release of this Hsp from adipocytes in a differentiation-dependent way (**Publication 5**).

Although Hsps have been identified as adipokines, only a few studies have analyzed the regulation and possible additional functions of Hsps in adipocytes and in adipose tissue. Protein expression of Hsp60, Hsp72 and Hsp90 is upregulated in adipose tissue of obese humans (Tiss, Khadir et al. 2014). In our study, freshly isolated stroma-vascular cells from adipose tissue express less Hsp60 than isolated mature adipocytes indicating that *in vivo* Hsp60 is more abundant in differentiated fat cells. Cultured adipocytes however express similar amounts of Hsp60 irrespective of their state of differentiation while release is higher from mature adipocytes compared to preadipocytes. Hsp60 is closely related to inflammation in adipocytes as Hsp60 stimulates release of inflammatory cytokines and adipokines on one hand, and inflammatory stress induces the secretion of Hsp60 on the other hand which creates a vicious cycle. As the physiological signals triggering the secretion of pro-inflammatory mediators from adipocytes in the obese state remain largely unknown, Hsp60 is an interesting candidate in this context. The notion of Hsp60 as a link to inflammation in adipose tissue is further supported by the observation that obese patients are characterized by higher circulating Hsp60 levels. Interestingly, Hsp60 expression is lower in the brain in obesity and type 2 diabetes (Kleinridders, Lauritzen et al. 2013). Mechanistically, reduced leptin signalling in the brain due to leptin resistance is causal for reduced Hsp60 while leptin treatment induces Hsp60 expression. Thus, increased leptin production of adipose tissue could trigger increased Hsp60 protein abundance in the obese state.

Our observation that circulating Hsp60 is elevated in obesity is particularly relevant in the context of obesity-associated metabolic diseases including insulin resistance, type 2 diabetes and cardiovascular disease. We could show that HSP60 impairs insulin signalling in human skeletal myotubes where it also stimulates inflammatory signalling as well as secretion of myokines. As Hsp60 release from myotubes itself is not detectable even in concentrated medium, it can be envisaged that adipose-derived Hsp60 could stimulate insulin resistance in skeletal muscle. It is of note that Hsp60 expression is particularly high in visceral adipose tissue of obese patients which have the metabolic syndrome and Hsp60 serum level are also elevated in type 2 diabetic patients (Dasu, Devaraj et al. 2010). The new finding that Hsp60 is an adipokine and might relate to adipose tissue inflammation is further supported by unpublished data on Hsp60 concentrations in morbidly obese patients that undergo bariatric surgery. Hsp60 concentrations are dramatically higher in morbidly obese patients when compared to lean volunteers (31.6 ± 4.7 ng/ml versus 12.6 ± 2.6 ng/ml, $p<0.05$). Patients in the highest quartile of serum Hsp60 are characterized by significantly elevated CRP and IL-6 independently of BMI, glycemia and insulinemia ($p<0.05$ by analysis of variance compared to all lower quartiles). After weight-reducing surgery, serum Hsp60 decreased significantly from 31.6 ± 4.7 ng/ml at baseline to 22.3 ± 3.0 ng/ml (3 months), 26.5 ± 5.5 (6 months) and 21.1 ± 3.3 ng/ml (12 months), ($p=0.017$ for trend by multivariate analysis of variance). Thus, circulating Hsp60 concentrations decrease with weight loss in parallel to the reduction of other inflammatory cytokines including chemerin and IL-6 (see **Publication 3**). Another interesting fact is the observation that both at baseline and 12 months after surgery, Hsp60 is correlated to the ApoB/ApoA1 ratio ($r=0.43$; $p=0.014$ and $r=0.58$; $p=0.002$, respectively) and the cholesterol/HDL ratio ($r=0.34$; $p=0.05$ and $r=0.46$; $p=0.008$, respectively). These two parameters are powerful predictors for cardiovascular disease in obesity (Lamarche, Moorjani et al. 1996, Yusuf, Hawken et al. 2004). In fact, the link between Hsp60 and cardiovascular disease is known since many years (Wick, Jakic et al. 2014) and could here be extended to the notion that Hsp60 is related to cardiovascular risk in morbid obesity. Increased Hsp60 levels in the circulation of obese patients could also

reach and stimulate the vascular wall where it serves as a danger signal and activator of innate and adaptive immunity (Grundtman, Kreutmayer et al. 2011). Overall, Hsp60 can be seen as an interesting new molecular link between adipose tissue inflammation and obesity-associated metabolic diseases.

5. Novel adipokines linking obesity and the metabolic syndrome: Dipeptidyl peptidase 4 as an example

The second part of this work focuses on a specific adipokine, namely DPP4 that is not only a prominent drug target for type 2 diabetes but also a recently discovered adipokine by our group (**Publication 6-7**). Beyond the established view that DPP4 is only an important enzyme for incretin deactivation, we established DPP4 as a up-and-coming marker for expanded adipose tissue in obesity. Circulating and adipose DPP4 is not only associated with insulin resistance and type 2 diabetes in study participants but it is also linked to the metabolic syndrome.

5.1. Dipeptidyl peptidase 4 as an adipokine

Publication 6: Dipeptidyl peptidase 4 is a novel adipokine linking obesity and the metabolic syndrome

Background: Comprehensive proteomic profiling of the human adipocyte secretome identified DPP4 as a novel adipokine. DPP4 is an ubiquitously expressed transmembrane glycoprotein, which cleaves N-terminal dipeptides from a variety of substrates including the incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). Since GLP-1 remains active under hyperglycemic conditions in type 2 diabetes, DPP4 has gained considerable interest as a therapeutic target, and DPP4 inhibitors that prolong the insulinotrophic effect of GLP1 are now in clinical use as anti-diabetic drugs (Ahren 2007). The circulating amount and activity of DPP4 have been found to be altered in inflammatory diseases (Yazbeck, Howarth et al. 2009). Although a fraction of soluble DPP4 most likely originates from cells of the immune system (Cordero, Salgado et al. 2009), the major source of circulating DPP4 and its regulation remain unknown. In addition, no data are available regarding the potential effects of soluble DPP4 on insulin target tissues including muscle and fat. In the present investigation, we combined *in vitro* experiments with two independent clinical studies, aiming to validate DPP4 as a novel adipokine and to characterize the association of DPP4 with different parameters of the metabolic syndrome.

Methods: Human adipocytes, skeletal and smooth muscle cells were used to monitor DPP4 release and to assess the effects of soluble DPP4 on insulin signalling. In lean and obese volunteers, depot-specific expression of DPP4 and its release from adipose tissue explants was determined and correlated to parameters of the metabolic syndrome.

Results: DPP4 is a novel adipokine released from differentiated human adipocytes in higher amounts than from macrophages or preadipocytes. Direct addition of DPP4 to fat, skeletal and smooth muscle cells impairs insulin signalling. DPP4 expression is substantially elevated in visceral fat of obese patients and serum DPP4 correlates with adipocyte size and all parameters of the metabolic syndrome. Furthermore, adipose tissue explants from obese study participants release significantly more DPP4 with a considerable decrease after weight reduction.

Conclusions: DPP4 is a novel adipokine that is substantially overexpressed in visceral fat from obese patients and exhibits an augmented release in obesity. Soluble DPP4 exerts auto- and paracrine effects and impairs insulin signalling. The tight correlation of DPP4 release to adipocyte cell size and the risk of having the metabolic syndrome suggest that DPP4 might be a novel biomarker and a potential link between obesity and the metabolic syndrome.

Limitations: The clinical data do not provide a proof of a causal role for DPP4 in the metabolic syndrome.

Originality: This report is the first description of DPP4 as an adipokine and its incretin-independent effects on insulin signalling in different human primary cells.

DPP4 is a type II transmembrane protein. It has peptidase activity and belongs to the serine peptidase subfamily S9B together with fibroblast activation protein α, the cytoplasmic DPP8 and DPP9 as well DPP6 and DPP10 that both have no enzymatic activity (Rohrborn, Wronkowitz et al. 2015). DPP4 acts as an exopeptidase cleaving dipeptides from the N-terminus of substrates containing proline at the penultimate position, which leads to inactivation of these substrates or generation of new bioactive proteins (Cordero, Salgado et

al. 2009). DPP4 protein contains 4 domains: a large extracellular domain, a flexible stalk region, a transmembrane domain and a cytoplasmic domain. The extracellular part of DPP4 contains the catalytic domain and additionally a cysteine-rich region and a highly glycosylated region. 20 % of the molecular mass of DPP4 originate from glycosylations that affect folding, stability and trafficking of the protein (Fan, Meng et al. 1997). In addition, the highly conserved Glu 205 and Glu 206 in the glycosylated region are vital for serine peptidase activity (Abbott, McCaughan et al. 1999). The cysteine-rich region of DPP4 mediates interactions of DPP4 with other proteins and is essential for the formation of DPP4 homodimers as a prerequisite for its enzymatic activity (Abbott, McCaughan et al. 1999). Interaction of DPP4 with various other proteins through the cysteine-rich region mediates effects of DPP4 in the immune response and in tumour invasion that are independent of its enzymatic action (Lambeir, Durinx et al. 2003). The peptidase activity of DPP4 depends on the catalytic triad (Ser 630, Asp 708, His 740) within the catalytic region. This catalytic center is located in an internal cavity and encased by the β-propeller domain in the glycosylation region. Substrates as well as inhibitors can penetrate and leave the catalytical center by inducing a conformational change called “side opening” and “propeller opening” (Aertgeerts, Ye et al. 2004).

DPP4 cleaves many substrates but it is best known for its peptidase activity towards incretin hormones. Most importantly, DPP4 inactivates the incretin hormones GLP-1 and GIP which are potent glucose-lowering proteins. In fact, these incretins account for nearly half of the insulin secretion postprandially. DPP4 reduces the half-life of these incretins to a few minutes. GLP-1 is released from L-cells to the circulation within a few minutes after meal intake and stimulates G-protein coupled GLP-1 receptors on β-cells to increase insulin secretion (Meier and Nauck 2005). In addition to this insulinotropic action, GLP-1 also augments β-cell mass by inducing proliferation and differentiation on one hand and by inhibiting apoptosis on the other hand (Drucker 2006). Directly in the gastrointestinal tract, GLP-1 delays gastric emptying while it also regulates satiety centrally. GIP is released from K-cells in the gut (Seino, Fukushima et al. 2010). GIP acts similar to GLP-1 when it comes to

stimulation of insulin secretion and β-cell proliferation as well as the prevention of β-cell apoptosis. In addition, it also reduces gastric acid secretion and upregulates lipid storage in adipose tissue (Seino, Fukushima et al. 2010).

DPP4 inhibitors improve glucose-stimulated insulin secretion from β-cell by preventing DPP4-mediated degradation of endogenous incretin hormones, thereby representing an important pharmacotherapeutic approach for type 2 diabetes. The DPP4 inhibitors in clinical use (sitagliptin, vildagliptin, saxagliptin, linagliptin and alogliptin; teneligliptin, anagliptin and trelagliptin for the Japanese and Korean market) depress DPP4 activity to 10-30 % (Baetta and Corsini 2011). Although all DPP4 inhibitors share the same general mechanism of inhibition, they differ in their pharmacodynamic and pharmacokinetic characteristics due to different chemical structures and distinct ways of insertion into the catalytic pocket (Baetta and Corsini 2011). While vildagliptin and saxagliptin display peptide structures, sitagliptin, linagliptin and alogliptin were identified by compound library screening and do not possess substrate-mimicking structures (β-amino acid like, xanthine and modified pyremidinedione structures, respectively). This diversity of chemical structures relates to the unique binding mode of each inhibitor to the active site of DPP4 (Nabeno, Akahoshi et al. 2013).

DPP4 inhibitors are used as second-line treatment for patients already taking metformin but not reaching adequate haemoglobin A1c (HbA1c) levels (according to “Nationale Versorgungsleitlinie: Therapie des Typ 2 Diabetes). Here, DPP4 inhibitors have several advantages such as oral application, low incidence of hypoglycaemia and neutral effects on body weight (Scheen 2015). Additional benefits include strong anti-inflammatory properties described for sitagliptin (Makdissi, Ghanim et al. 2012, Satoh-Asahara, Sasaki et al. 2013, Tremblay, Lamarche et al. 2014) and potential lipid-lowering action described for anagliptin (Aoki, Ijima et al. 2015, Nishio, Abe et al. 2015).

Research in the last 10 years has not only focused on preclinical and clinical effects of DPP4 inhibitors but has also established a role of DPP4 independent of its role in the degradation of incretin hormones (Zhong, Rao et al. 2013). In this respect, other sources of

DPP4 in addition to the gut have been explored. DPP4 is ubiquitously expressed in various tissues and cell types such as vascular cells, epithelial cells, fibroblasts and immune cells (Cordero, Salgado et al. 2009). Even though part of the circulating DPP4 probably stems from immune cells (Cordero, Salgado et al. 2009), the exact source of soluble DPP4 and the regulation of its release remained largely unknown. We discovered DPP4 by a proteomic profiling of CM from human primary adipocytes. Two complementary methods, 1-dimensional SDS-PAGE/LC-ESI-MS/MS and 2-dimensional SDS-PAGE/MALDI-MS, were used to profile the CM that was collected from five individual donors to account for any biological variance (Lehr, Hartwig et al. 2012). Due to low protein abundance in CM from most cells types, the material was concentrated by a factor of 1,000. DPP4 was finally identified by LC-MS. Subsequent analysis of DPP4 in cell culture validated DPP4 as an adipokine as it was expressed in human adipocytes and released from these cells (**Publication 6**). Interestingly, DPP4 expression and release increases significantly from preadipocytes to differentiated adipocytes with mature adipocytes also releasing about three times more DPP4 compared to macrophages residing in adipose tissue. These data point to a large contribution of fat cells to the overall release of DPP4 from adipose tissue. As nothing is known about regulation of DPP4 in adipocytes, we also tested several metabolic and inflammatory stimuli for their effect on DPP4 expression and release. It is known that the promoter of DPP4 contains consensus sites for various transcription factors including hypoxia-inducible factor (HIF-1) α, hepatocyte nuclear factor (HNF-1) α and NF-κB (Bohm, Gum et al. 1995, Senkel, Lucas et al. 2005, Dang, Chun et al. 2008, Gu, Tsuda et al. 2008). DPP4 gene transcription can also be stimulated by INFα, β and γ (Bauvois, Djavaheri-Mergny et al. 2000). DPP4 release from human adipocytes is upregulated by short-term stimulation with insulin and TNFα but not by hypoxia. Differently, adipocyte differentiation at lower oxygen concentrations (5 and 10 % oxygen) induces higher DPP4 expression and release compared to differentiation at 21 % oxygen pointing to a prolonged activation of HIF-1α which might be necessary to regulate DPP4 (Famulla, Schlich et al. 2012).

It is known from animal experiments that adipose tissue exhibits relative high DPP4 activity that is only higher in liver (Hildebrandt, Reutter et al. 1991, Shinjo, Nakatsu et al. 2015). Data on DPP4 sources from human tissues are not available but the fact that circulating DPP4 levels are higher in obese compared to lean study participants points to adipose tissue as a potential source of DPP4 (**Publication 6**). This observation is further supported by experiments using adipose tissue biopsies and isolated adipocytes from lean and obese patients which demonstrate that adipocytes from obese donors release almost twice as much DPP4 compared to lean donors (**Publication 6**). Both circulating DPP4 concentration and direct release of DPP4 from adipocytes *ex vivo* correlate with adipocyte size, BMI and leptin. In addition, circulating DPP4 is positively correlated to insulin and negatively correlated to adiponectin. A correlation of circulating DPP4 with BMI could also be reproduced in young and healthy Japanese men (Kirino, Sei et al. 2012). Further support of adipose tissue as a potential source of DPP4 originates from the analysis of serum DPP4 before and after weight loss. In **Publication 6**, we could show that surgery induced-weight loss is able to reduce adipose tissue-derived DPP4 as measured *ex vivo* which is reflected by reduced circulating concentrations of DPP4 as well. In addition to our resulst, weight loss induces a significant reduction in DPP4 serum concentrations in children (Reinehr, Roth et al. 2010). In fact, the decrease in DPP4 was related to changes whole body fat and the child-specific BMI Standard Deviation Score.

5.2. Dipeptidyl peptidase 4 in obesity and obesity-associated metabolic diseases

Publication 7: Adipose DPP4 in obesity and insulin resistance

Background: Adipocytes release DPP4 in a differentiation-dependent manner and circulating DPP4 concentrations are increased in obesity correlating with fasting plasma insulin, leptin and the adipocyte size in subcutaneous adipose tissue. However, the tissue source of circulating DPP4 is not known. This study aimed to assess DPP4 expression and release in paired biopsies of subcutaneous and visceral adipose tissue of lean and obese patients, and patients with or without impaired glucose tolerance as well as DPP4 release from adipose tissue *in vivo*.

Methods: DPP4 expression was measured in paired biopsies from volunteers with a wide range of BMI and insulin sensitivity. DPP4 release was measured *ex vivo* in paired biopsies from subcutaneous and visceral adipose tissue as well as *in vivo* from subcutaneous fat of lean and obese patients. Circulating DPP4 was measured in insulin-sensitive and insulin-resistant BMI-matched obese patients.

Results: DPP4 expression positively correlated to BMI both in subcutaneous and visceral adipose tissue with visceral fat persistently displaying higher expression compared to subcutaneous fat. Interestingly, DPP4 was significantly increased in visceral adipose tissue but not in the subcutaneous depot in lean individuals with impaired glucose tolerance. *Ex vivo* release of DPP4 from adipose tissue explants was higher from visceral as compared to subcutaneous adipose tissue in lean and obese patients with obese patients displaying higher DPP4 release compared to lean controls. Net release of DPP4 from adipose tissue was also demonstrated *in vivo* with greater release in obese patients compared with lean, and women compared with men. Insulin-sensitive obese patients had significantly lower circulating DPP4 as compared to obesity-matched insulin-resistant patients. Here, DPP4 positively correlated with the amount of visceral adipose tissue, adipocyte size and adipose tissue inflammation.

Conclusions: DPP4 is a novel adipokine with higher release from visceral adipose tissue that is particularly pronounced in obese and insulin-resistant patients. Our data suggest that DPP4 might be a marker for visceral obesity, insulin resistance and the metabolic syndrome.
Limitations: There is a net uptake of DPP4 in adipose tissue of a few volunteers, especially when arterial concentrations of soluble DPP4 are relatively low. These data demonstrate that subcutaneous abdominal adipose tissue is not the only source of circulating DPP4.
Originality: This study combines data from several unique clinical cohorts such as BMI-matched insulin-sensitive and insulin-resistant obese patients to provide evidence for a relationship between adipose DPP4 and insulin resistance on a systemic and adipose tissue level.

DPP4 is increased in obesity both in circulation and within adipose tissue (**Publication 6-7**). Studying DPP4 in different groups of patients with varying BMI provides consistent evidence for DPP4 being predominantly expressed in visceral adipose tissue. Protein abundance is almost two-times higher in visceral compared to subcutaneous adipose tissue in lean and also in obese patients (**Publication 6**). On the level of gene expression, DPP4 is three-times higher in visceral adipose tissue compared to subcutaneous adipose tissue in lean persons while DPP4 is almost seven-times higher in visceral compared to subcutaneous fat of obese patients (**Publication 7**). In agreement with DPP4 expression, adipose tissue explants from visceral adipose tissue biopsies release more DPP4 compared to paired subcutaneous adipose tissue samples which is indicative of a potential higher relative contribution of visceral adipose tissue to circulating DPP4 levels. Comparing lean controls to obese patients, DPP4 release from visceral adipose tissue is significantly elevated from the later. It should however been noted that subcutaneous depots are predominant in humans and might therefore be the most important source of adipose-derived DPP4. It could be envisaged that visceral adipose tissue contributes to higher DPP4 serum concentrations in morbidly obese patients with central obesity and insulin resistance as DPP4 levels were higher in obese volunteers with insulin resistance compared to BMI-

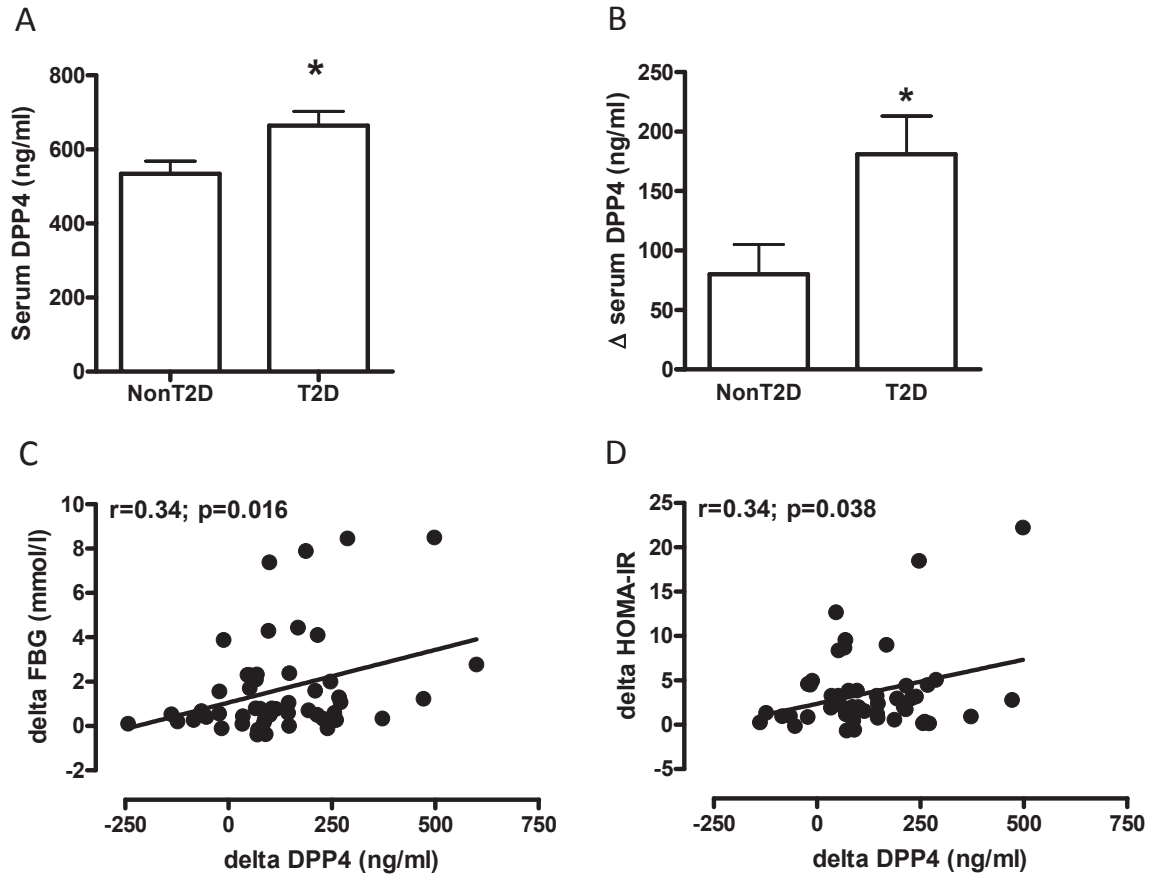
matched insulin-sensitive obese controls (**Publication 7**). The two groups of obese humans differ only in a few aspects namely visceral adipose tissue mass and DPP4 expression in visceral fat which is significantly elevated in the insulin-resistant patients. In the context of prescription of DPP4 inhibitors to obese patients with type 2 diabetes, it might be speculated that elevated serum DPP4 concentrations might affect treatment efficacy. In multivariate analyses, success of DPP4 inhibitor treatment was associated with a lower baseline HbA1c and duration of diabetes, and a higher BMI and comorbidity (Kozawa, Kitamura et al. 2013, Monami, Ragghianti et al. 2016). Conversely, increased serum DPP4 concentrations are related to inferior response to sitagliptin in type 2 diabetic patients that are weakly controlled by sulfonylurea or metformin (Aso, Inukai et al. 2012). Another study found that higher BMI predicts less HbA1c lowering by sitagliptin (Bando, Aoki et al. 2012). However, it is not clear how exactly treatment with DPP4 inhibitors is attenuated in detail.

We could show that circulating DPP4 is higher in obese humans with the metabolic syndrome (**Publication 6-7**). It is very likely that higher DPP4 originates from enlarged visceral adipose tissue as this depot releases significantly more DPP4 *ex vivo* in patients with the metabolic syndrome compared to patients without the metabolic syndrome despite similar BMI (**Publication 7**). In addition, we have measured serum DPP4 in 53 morbidly obese females scheduled for gastric bypass (n=47) and gastric banding (n=6) (unpublished data). Serum DPP4 is significantly higher in morbidly obese patients with type 2 diabetes compared to non-diabetic patients (Figure 3A). After adjustment for BMI, this difference was still significant. Serum concentrations of DPP4 significantly decreased in the first year after surgery similarly to data already published in **Publication 6**. However, the reduction of DPP4 was significantly higher in patients with type 2 diabetes (Figure 3B). The decrease in DPP4 concentrations over 1 year post-surgery significantly correlated with a decrease in FBG and HOMA-IR (Figure 3C-D). In summary, morbid obese patients with type 2 diabetes are characterized by higher serum DPP4 but surgery-induced weight loss also results in a significantly stronger drop in circulating DPP4 compared to morbid obese patients without type 2 diabetes.

Figure 3

DPP4 serum concentrations in morbidly obese patients before and after surgery-induced weight loss in relation to the presence of type 2 diabetes (unpublished data)

*(A) Serum DPP4 concentrations were measured at baseline. Patients were grouped according to the absence or presence of type 2 diabetes. (B) The decrease in serum DPP4 was significantly higher in patients with type 2 diabetes. * p<0.05 compared to designated control. (C) Linear regression analysis of the reduction in DPP4 serum concentration and FBG as well as HOMA-IR. Statistical evaluation is indicated in each graph.*



Similar to human data, serum DPP4 activity and DPP4 expression in visceral fat increases with the development of streptozotocin-induced diabetes in rats (Kirino, Sato et al. 2009). So far, it is not known why DPP4 is overexpressed in adipose tissue of obese patients and particularly in patients with the metabolic syndrome. There are hints for an epigenetic regulation of DPP4 expression as methylation levels of the DPP4 promoter are negatively

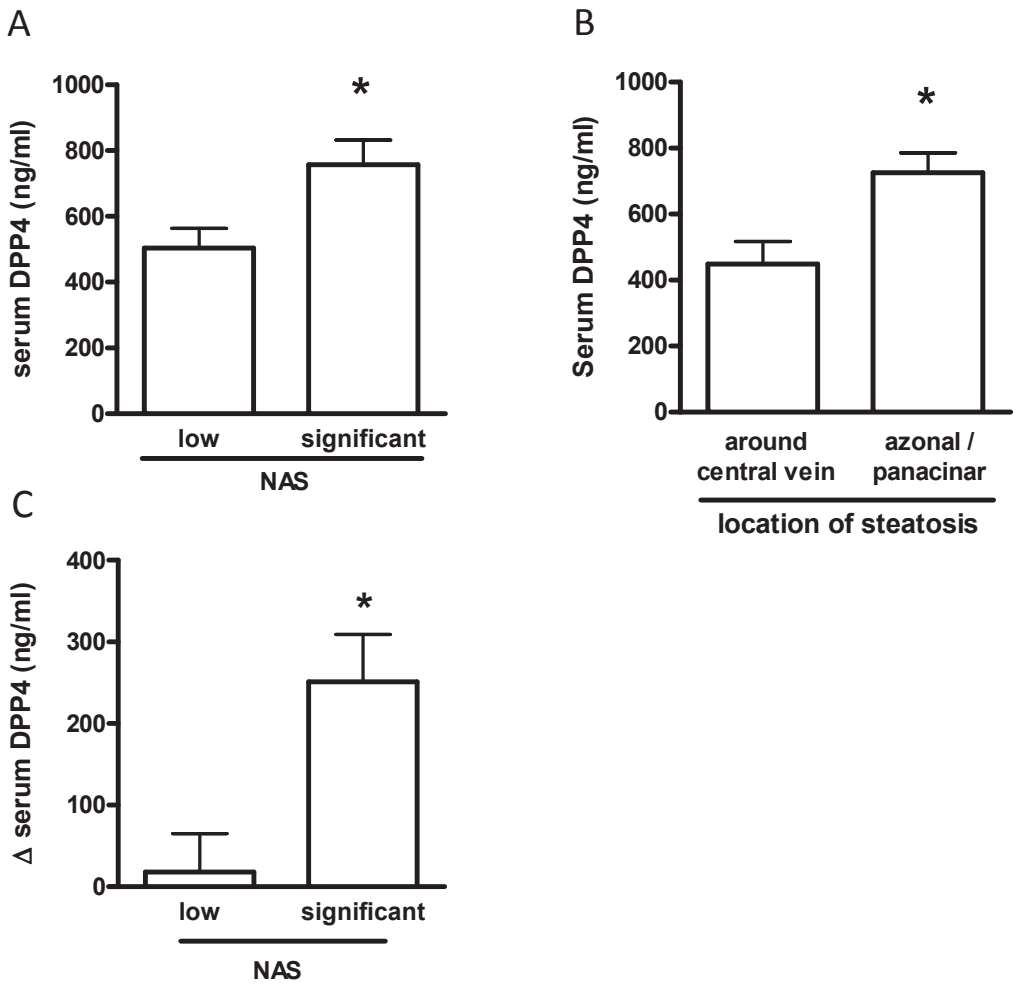
associated with DPP4 mRNA expression in visceral adipose tissue in obese women with and without the metabolic syndrome and are additionally associated with HDL-cholesterol (Turcot, Bouchard et al. 2011). Furthermore, DPP4 is already upregulated in visceral adipose tissue in lean patients without type 2 diabetes but with impaired glucose tolerance compared to participants with normal glucose tolerance (**Publication 7**). It might be speculated that mechanisms including epigenetic regulation leading to higher DPP4 expression in obese subject with the metabolic syndrome could also be translated to lean patients that have not yet fully developed insulin resistance. *In vitro* experiments further support the idea of DPP4 being associated to insulin resistance in obesity as reduction of DPP4 expression by silencing improves adipocyte insulin signalling (Rohrborn, Bruckner et al. 2016). Here, DPP4 reduction improved insulin responsiveness of adipocytes at the level of insulin receptor, Akt and Akt substrate of 160 kDa. Similar effects can be obtained by long-term DPP4 inhibition in the same cells. Taken together with the observation that DPP4 induces insulin resistance in adipocytes (**Publication 6**) and the finding that adipose tissue of morbid obese patients with insulin resistance is characterized by higher DPP4 expression, it could be speculated that the adipose level of DPP4 might be a regulator of adipocyte insulin signalling.

In obesity, other organs such as the liver are discussed as sources of DPP4 in relation to metabolic diseases. Unpublished data from morbidly obese patients further demonstrates that a significant NAFLD Activity Score (NAS) (3 and higher) is related to elevated serum DPP4 concentrations (Figure 4A) independent of BMI. In this cohort, one patient was characterized by Non-alcoholic steatohepatitis (NASH) (NAS=5) and also highest DPP4 concentrations (1064 ng/ml) (NAS=4 (n=4) with 643 ± 79 ng/ml). Furthermore, DPP4 was elevated in patients with azonal/panacinar location of steatosis compared to steatosis around central veins (Figure 4B). Surgery-induced decrease in DPP4 was significantly higher in patients with significant NAS (Figure 4C) but weight loss and decrease in leptin serum concentrations were not different between the groups. Thus, the liver might also contribute to circulating DPP4 in addition to adipose tissue.

Figure 4

DPP4 serum concentrations in morbidly obese patients before and after surgery-induced weight loss in relation to hepatic histology

*Serum DPP4 was measured at baseline. Patients were grouped according to low (0-2) or significant (>2) NAS (A) and location of steatosis (B). (C) The decrease in serum DPP4 was significantly higher in patients with significant NAS. * p<0.05 compared to designated control.*



More and more studies focus on effects of DPP4 inhibitors in patient with NAFLD and other liver diseases. Inhibition of DPP4 improved serum transaminases (Iwasaki, Yoneda et al. 2011, Yilmaz, Yonal et al. 2012, Kanazawa, Tanaka et al. 2014) and NAFLD features such as ballooning and elevated NAS (Yilmaz, Yonal et al. 2012). Otherwise, a very recently published randomized, double-blind, placebo-controlled trial could not find any significant

effect of sitagliptin treatment on transaminases and liver stiffness (Cui, Philo et al. 2016). As the number of patients included in this trial was relatively small, more clinical trials are necessary to clarify if DPP4 inhibitors are effective in terms of liver disease in patients with type 2 diabetes and NAFLD.

Although DPP4 in adipose tissue and in the circulation is clearly related to obesity, insulin resistance and type 2 diabetes as well as potentially NAFLD, a causal role for DPP4 in the development and progression of these diseases could not be established so far.

6. Mechanisms of crosstalk between adipose tissue and smooth muscle cells in obesity-associated vascular disease

In the last part of this cumulative work, I will extend the view that endocrine mediators from enlarged adipose tissue are mechanistically linked to components of the metabolic syndrome from skeletal muscle to the vascular wall. More specifically, molecular mechanisms of crosstalk between adipose tissue and smooth muscle in obesity-associated vascular disease will be discussed and the role of different specialized fat depots for vascular disease will be elucidated (Ouwens, Sell et al. 2010, Schlich, Willems et al. 2013). With the completely new role of DPP4 in adipokine-driven proliferation and inflammation, we have identified a novel mediator of smooth muscle-cell associated vascular dysfunction (**Publication 9**). Above all, we identified a potential receptor for DPP4 which will facilitate a better analysis of incretin-independent effects of DPP4 and its putative contribution to the development of metabolic diseases. In addition to cellular work on DPP4 in crosstalk with smooth muscle, molecular work on basic biochemical mechanisms of DPP4 release by shedding are also discussed (**Publication 8**).

6.1. Role of different fat depots in vascular disease

Almost all arteries are surrounded by so-called perivascular adipose tissue in a way that no fascial layer separates this fat depot from the vascular wall (Ouwens, Sell et al. 2010). Adipocytes in perivascular adipose tissue have been compared to subcutaneous and visceral adipocytes in humans. Similar to other fat depots, perivascular adipose tissue expands during the development of obesity (Ding, Hsu et al. 2009). Perivascular adipocytes are smaller and of more heterogeneous shape with higher uncoupling protein-1 expression (Chatterjee, Stoll et al. 2009). Also similar to other adipose tissue depots, perivascular adipose tissue is a source of adipokines that can directly reach the vascular wall. In general, perivascular adipose tissue is characterized by a pro-inflammatory phenotype (Omar, Chatterjee et al. 2014). Secretion of adiponectin is significantly reduced, whereas pro-inflammatory adipokines such as IL-6, IL-8, and MCP-1 are more abundantly released from

perivascular adipocytes, which has been shown for both freshly isolated adipose tissue and *in vitro*-differentiated adipocytes (Chatterjee, Stoll et al. 2009). In parallel, gene expression is altered in perivascular adipose tissue compared to subcutaneous fat with an enrichment of regulated genes related to angiogenesis, vascular morphology, inflammation, and blood clotting (Chatterjee, Aronow et al. 2013). Human perivascular adipose tissue displays chemotactic properties towards different types of immune cells by a marked release of different chemokines, so that it can be speculated that this particular fat depot might increase inflammation during the progression of obesity-associated atherosclerosis (Henrichot, Juge-Aubry et al. 2005). Perivascular adipocytes also produce more angiogenic factors such as hepatocyte growth factor (Rittig, Dolderer et al. 2012) and vascular endothelial growth factor (VEGF) (Schlich, Willems et al. 2013) compared to subcutaneous adipocytes.

Adipokines secreted from perivascular adipose tissue can directly regulate vascular function through paracrine and endocrine effects on the vascular wall regulating vasoreactivity both via vasorelaxation and vasoconstriction (Gao, Zeng et al. 2005). *Ex vivo* experiments using human arteries have demonstrated that expansion and inflammation of perivascular adipose tissue contributes to the development of endothelial dysfunction. Perivascular adipose tissue from healthy donors releases more adiponectin that is critical for normal vasodilation via upregulated bioavailability of NO (Greenstein, Khavandi et al. 2009). This effect is lost in obesity due to adipocyte hypertrophy and lower adiponectin availability coupled to hypoxia, inflammation, and oxidative stress. A second study could demonstrate that perivascular adipose tissue from lean volunteers improves insulin-induced vasodilation in isolated skeletal muscle resistance arteries and favoured microvascular recruitment. In contrast, perivascular adipose tissue from obese study participants caused insulin-induced vasoconstriction and reduced microvascular recruitment to skeletal muscle potentially contributing to the development of insulin resistance (Meijer, Serne et al. 2015). Another study could not relate perivascular fat mass to local endothelial function but rather a correlation between perivascular adipose tissue, insulin resistance and local blood flow supporting the hypothesis that vascular modulation of blood flow within muscle might

contribute to insulin resistance (Rittig, Staib et al. 2008). Obese patient characterized by anti-contractile activity of perivascular adipose tissue compared with lean controls display restored contractile activity of perivascular fat as soon as 6 months after surgery-induced weight loss (Aghamohammadzadeh, Greenstein et al. 2013). The improvement in vascular function occurred in parallel to increased adiponectin expression and NO bioavailability in perivascular adipose tissue. The important role of hypoadiponectinemia in endothelial dysfunction is further highlighted by a strong negative correlation between serum adiponectin and endothelial dysfunction (Shimabukuro, Higa et al. 2003).

The use of CM from perivascular adipose tissue for stimulation of vascular cells has provided insight how inflammatory stimuli affect the physiology of the vascular wall. Secretory production from perivascular adipocytes enhanced endothelial cell tubulogenesis and monocyte migration compared with subcutaneous adipocytes demonstrating that perivascular adipokines could play a role in inflammatory cellular crosstalk in atherosclerosis (Chatterjee, Aronow et al. 2013). Similarly, adipocyte-CM from different adipose tissue depots including perivascular, epicardial, visceral and subcutaneous depots induce proliferation and inflammation of smooth muscle cells as critical steps in arterial wall thickening (Lamers, Schlich et al. 2011, Schlich, Willems et al. 2013). In line with the anti-inflammatory and anti-atherogenic function of adiponectin, generation of adipocyte-CM in the presence of excess adiponectin can abrogate adipokine-induced smooth muscle cell proliferation (Lamers, Schlich et al. 2011). The precise nature of adipokines released from perivascular adipose tissue that stimulate proliferation and migration of smooth muscle cells is not known. We could show that VEGF is a strong candidate in this respect as VEGF secretion is higher from perivascular adipose tissue particularly from patients with type 2 diabetes and VEGF release correlates with the proliferative effects of adipocyte-CM (Schlich, Willems et al. 2013).

6.2. Molecular mechanisms of dipeptidyl peptidase 4 release from adipose tissue and vascular cells

Publication 8: DPP4 shedding is regulated by metalloproteases and hypoxia

Background: Dipeptidyl peptidase 4 (DPP4) is a glycoprotein of 110 kDa, which is ubiquitously expressed on different cell types. As a type II transmembrane protein, DPP4 is cleaved of the cell membrane in a process called shedding (Hooper, Karran et al. 1997). The specific enzymes contributing to the shedding of DPP4 and the regulation of this process is largely unknown. Therefore, this study focused on the identification of shedding enzymes for DPP4 and to explore the regulation of DPP4 release *in vitro*.

Our data suggest that constitutive as well as hypoxia-induced DPP4 shedding occurs due to a complex interplay between different matrix metalloproteases (MMP) in a cell type-specific manner.

Methods: Human adipocytes and smooth muscle cells were treated with broad range inhibitors of proteases and specific MMP inhibitors. In addition, MMPs were silenced. DPP4 release was also measured in cells cultured in a hypoxic environment.

Results: Adipocytes and smooth muscle cells are characterized by a different pattern of released proteases. MMP1, MMP2 and MMP14 are involved in DPP4 shedding from human vascular smooth muscle cells while MMP9 highly contributes to DPP4 shedding from adipocytes. Hypoxia increased DPP4 shedding from smooth muscle cells which is associated with increased mRNA expression of MMP1.

Conclusions: MMPs are important enzymes for DPP4 shedding accounting for about 50 % of its release. Several MMPs are involved in DPP4 release, in a cell type-specific manner. Upregulation of MMP1 is a mechanism how hypoxia induces increased DPP4 release.

Limitations: Expression of MMPs has not been measured in adipose tissue from lean and obese humans so that the increased release of DPP4 from enlarged adipose tissue cannot be linked to altered shedding.

Originality: This study identifies shedding enzymes for DPP4 providing first evidence for a regulated shedding process of this target.

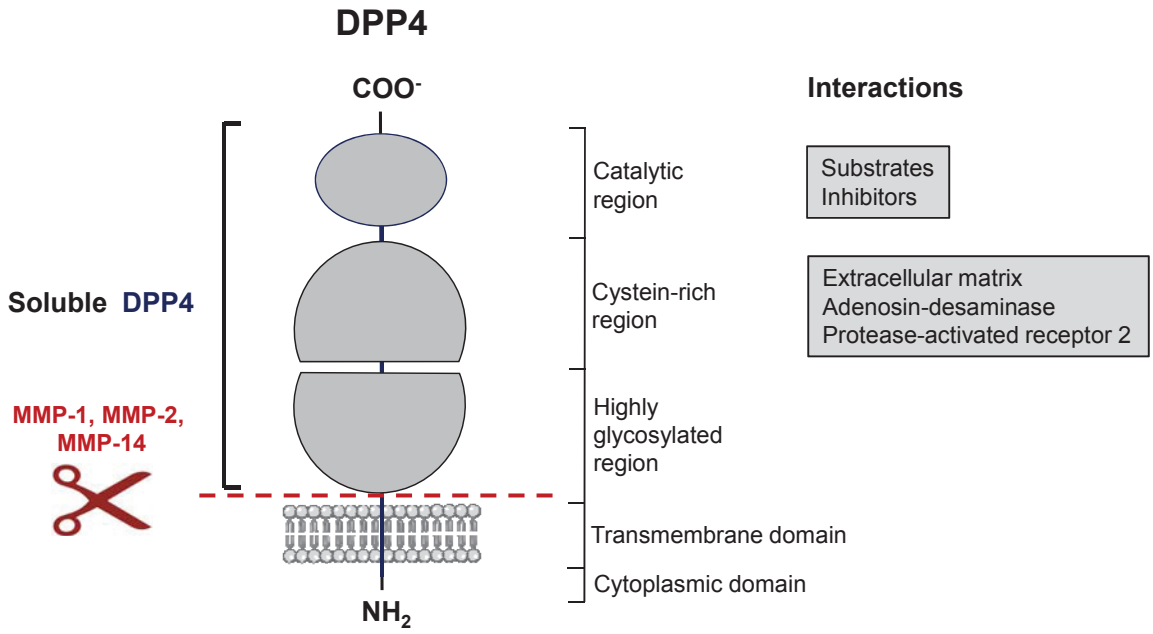
DPP4 is a member of the type II transmembrane protein family and possesses a signal peptide for targeting to the ER followed by translocation across the cell membrane. However, DPP4 is not classically secreted but its signal peptide functions as a membrane anchor. DPP4 can then be released from the cell surface into the circulation by a process called ectodomain shedding (**Publication 8**). Shedding is a process that is highly regulated by specific agonists, antagonists and intracellular signalling leading to the release of around 2 % of cell surface proteins (Hayashida, Bartlett et al. 2010). The small proportion of proteins shedded from cells indicates that this is a very selective process. Indeed, only few proteins are known to undergo ectodomain shedding including known proteins such as hepatocyte growth factor (HGF) (Mizuno, Takehara et al. 1992) and the IL-6 receptor (Croucher, Wang et al. 1999). Also adipokines are released by shedding as exemplified by TNFα (Black, Rauch et al. 1997). TNFα shedding can be stimulated by classical signalling pathways involved in shedding of many proteins such as stimulation of different PKC isoforms by phorbol esters (Wheeler, Ness et al. 2003). Furthermore, TNFα is very specifically targeted by the shedding enzyme ADAM17 (or TNFα-converting enzyme - TACE) (Black, Rauch et al. 1997). In general, shedding can be regulated by intracellular signalling also involving the intracellular part of shedded proteins, by activation and trafficking of shedding enzymes and by recruitment and modifications of substrates all of which are involved in TNFα shedding.

Our study on DPP4 shedding is the first to analyse the constitutive and stimulated release of DPP4 from the cell surface of human cells. The proof that DPP4 is not classically released was obtained by treatment with brefeldin A, a lactone antibiotic that inhibits the classical ER/Golgi-dependant secretion pathway indirectly by preventing formation of transport vesicles (**Publication 8**). DPP4 release is insensitive to brefeldin A in three different primary human cell types tested by us. However, DPP4 release is sensitive to broad range inhibitors of MMPs. Unlike for TNFα, DPP4 is not shedded predominantly by a single shedding enzyme (Figure 5). Shedding seems to be cell type dependent as DPP4 shedding predominantly occurs via MMP9 in adipocytes and via MMP1, MMP2 and MMP14 in smooth

muscle cells. In smooth muscle cells, we observed a regulation of DPP4 shedding by hypoxia (1 % oxygen) that was not involving regulation of DPP4 expression but rather expression of the shedding enzyme MMP1. Short-term hypoxia does not seem to alter DPP4 release by adipocytes (unpublished data) but rather long-term hypoxia increases DPP4 release in parallel to increased protein abundance of DPP4 (Famulla, Schlich et al. 2012). Thus, shedding in adipocytes seems not to be affected by hypoxia but DPP4 expression. In humans, it is unknown so far if higher DPP4 release from visceral adipose tissue in obesity rather reflects higher DPP4 expression or if DPP4 shedding is dysregulated. MMP9 is upregulated in obese humans and particularly in obese patients with type 2 diabetes compare to lean controls (Dandona, Ghanim et al. 2014, Das, Ma et al. 2015). Thus, increased MMP9 in expanding adipose tissue in obesity might mediate an increased DPP4 release and potentially also in a depot-specific way.

Figure 5

Schematic overview of the DPP4 domain structure and DPP4 shedding (adapted from (Rohrborn, Wronkowitz et al. 2015))



6.3. Adipokine-driven proliferation and inflammation as mediators of smooth muscle cell -associated vascular dysfunction

Publication 9: DPP4 induces inflammation and proliferation in human smooth muscle cells via PAR2

Background: Clinical data suggest that DPP4 inhibitors are a safe treatment for patients with type 2 diabetes and prior cardiovascular disease (Son and Kim 2015) while it even may hold promise for cardiovascular protection *in vitro* and in animal models (Wronkowitz, Romacho et al. 2014). Since GLP-1 itself has favourable cardiovascular effects (Anagnostis, Athyros et al. 2011, Saraiva and Sposito 2014), most of these protective effects of DPP4 inhibition are attributed to the increased bioavailability of GLP-1. Nevertheless, there are accumulating *in vitro* and animal data suggesting that DPP4 inhibitors mediate vascular protection independent of GLP-1, involving endothelial repair (Fadini, Boscaro et al. 2010), anti-inflammatory effects (Dobrian, Ma et al. 2010, Fadini, Boscaro et al. 2010, Ta, Li et al. 2010) and blunting of ischemic injury (Zhang, Huang et al. 2010). In addition, we were the first investigating direct effects of DPP4 on human adipocytes, human skeletal muscle cells and smooth muscle cells showing a DPP4-induced insulin resistance and an increased smooth muscle cell proliferation (Lamers, Famulla et al. 2011).Therefore, the aim of this study was to investigate direct effects of DPP4 on human vascular smooth muscle cells and to identify responsible signalling pathways.

Methods: Human smooth muscle cells were treated with physiological concentrations of DPP4 to assess effects on signalling, proliferation and inflammation. Using various inhibitors for DPP4 and signalling pathways as well as Protease-activated receptor (PAR) 2 silencing and antagonist GB83, underlying mechanisms were dissected.

Results: DPP4 induces a concentration-dependent activation of ERK1/2 lasting for up to 24 h. Differently, phosphorylation of the NF-κB subunit p65 could be transiently increased by DPP4 with a maximum after 6 h. Furthermore, we could observe an increased expression and secretion of pro-inflammatory cytokines such as IL-6, IL-8 and MCP-1 (2.5-, 2.4- and 1.5-fold, respectively) as well as an induction of inducible nitric oxide synthase (iNOS) by

DPP4 treatment. All direct effects of DPP4 on signalling, proliferation and inflammation could completely be blocked by DPP4 inhibition. Bioinformatic analysis and signalling signature induced by DPP4 suggest that DPP4 might be an agonist for PAR2. After silencing of PAR2, DPP4-induced ERK activation as well as proliferation was totally abolished. Additionally, DPP4-induced upregulation of IL-6 and IL-8 could completely be prevented by the PAR2 silencing. The PAR2 antagonist GB83 also impeded DPP4-induced signalling and proliferation.

Conclusions: In human smooth muscle cells, DPP4 directly activates the MAPK and NF-κB signalling in a PAR2-dependent way and results in the induction of inflammation and proliferation. DPP4 inhibition was able to block all observed effects which extends the current view on incretin-independent effects of DPP4 and shed light on cardiovascular effects of DPP4 inhibitors.

Limitations: No proof for a direct binding of DPP4 to PAR2 has been found. Further studies are needed to elucidate mechanisms how DPP4 inhibition prevents binding of DPP4 to potential binding partners such as PAR2.

Originality: PAR2 was identified as a potential receptor for DPP4 and DPP4-induced activation of downstream signalling pathways detailed as direct effects of DPP4.

There is increasing evidence from *in vitro* and *in vivo* studies that DPP4 inhibitors exert effects in the cardiovascular system that are independent from GLP-1 (Fadini and Avogaro 2011). This concept has triggered several studies analysing *in vitro* effects of DPP4 in vascular cells including our own work (**Publication 6** and **9**). We demonstrate that DPP4 activates MAPK and NF-κB leading to increased proliferation, induction of iNOS and elevated expression and secretion of pro-inflammatory cytokines. All observed effects were completely prevented by co-treatment with a DPP4 inhibitor illustrating the importance of DPP4 activity to mediate pro-inflammatory and pro-atherogenic changes in smooth muscle cells. Other studies have corroborated our findings in smooth muscle cells from different origin namely human airway smooth muscle cells (Shiobara, Chibana et al. 2016) and

primary murine smooth muscle cells (Ervinna, Mita et al. 2013). DPP4 also affects endothelial cells as it stimulates ROS generation and the expression of the receptor for advanced glycation end products (AGE) (Ishibashi, Matsui et al. 2013). In the light of additional AGE-induced upregulating of DPP4 expression and production, a viscous cycle leads to endothelial cell damage and expression of pro-inflammatory factors. In addition to cells of the vascular wall, DPP4 also mediates immunmodulatory effects of immune cells that infiltrate the vascular wall at early or late atherosclerotic lesions. Injection with recombinant DPP4 in mice results in increased monocyte migration similar to that stimulated by TNFα which is completely abolished by DPP4 inhibition (Shah, Kampfrath et al. 2011). Recombinant DPP4 stimulates transendothelial T cell migration (Iwata, Yamaguchi et al. 1999). In THP-1 monocytes, combined treatment of lipopolysaccharide and DPP4 activates transcriptional activity of TNFα and IL-6 promoters thus potentiating inflammation beyond each treatment alone (Ikeda, Kumagai et al. 2013).

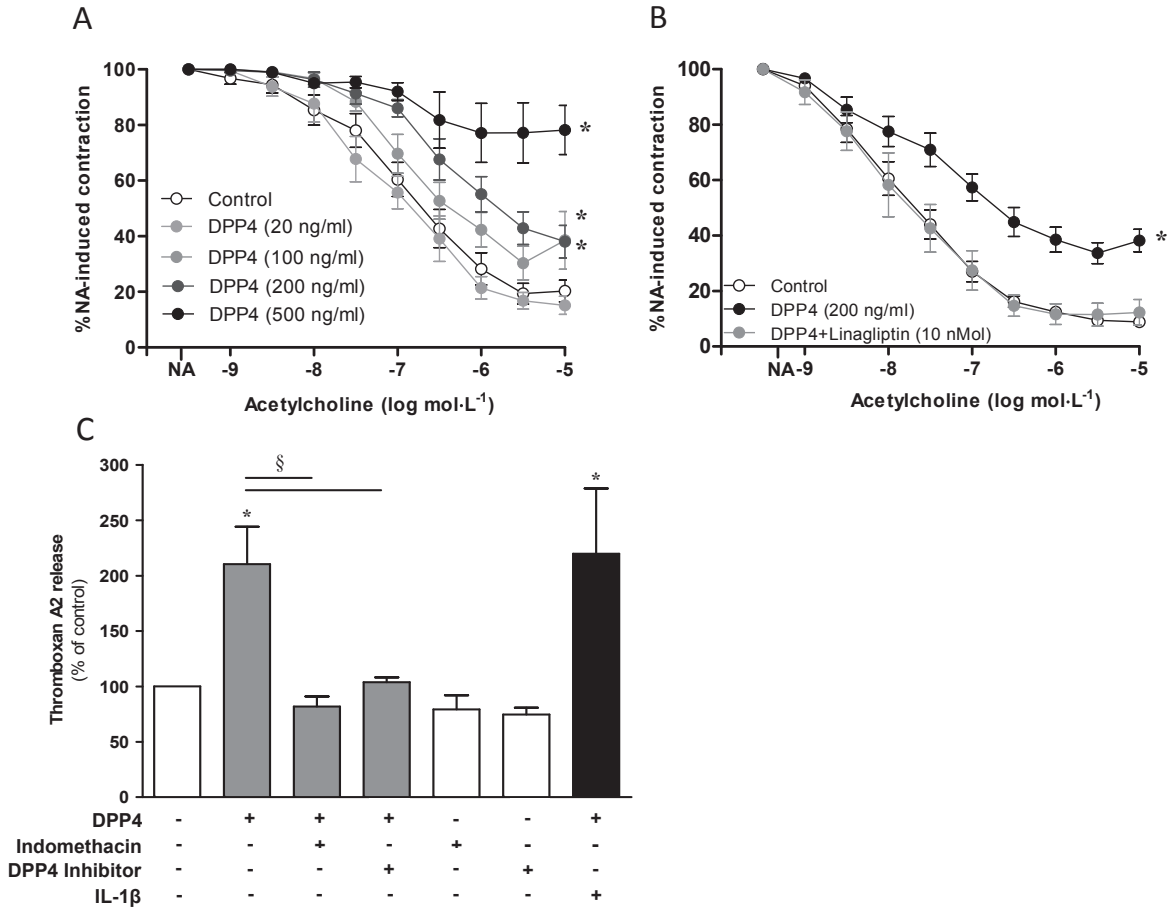
DPP4 is not only stimulating inflammation and cell damage but it also influences vascular function *ex vivo*. We measured effects of recombinant DPP4 on vascular reactivity of murine mesenteric arteries (Romacho, Vallejo et al. 2016). DPP4 impaired the endothelium-dependent relaxation to acetylcholine in a concentration-dependent manner by up to 75 % (Figure 6A). Blocking DPP4 activity with linagliptin completely prevented this effect (Figure 6B). In human endothelial cells, DPP4 accordingly stimulates the release of thromboxane A_2 (TXA_2) which can be abolished by treatment with a DPP4 inhibitor and the cyclooxygenase (COX) inhibitor indomethacin (Figure 6C). Thus, our data point to a direct impairment of endothelium-dependent relaxation by DPP4 cyclooxygenase activation and vasoconstrictor prostanoids.

Figure 6

Direct effects of DPP4 on vascular reactivity

*(A) Concentration-dependent relaxations induced by acetylcholine in isolated mesenteric microvessels either with or without increasing concentrations of DPP4. Results are expressed as mean ± SEM of 37 segments obtained from 7 animals. *p<0.05 compared to*

*control. (B) Effects of the DPP4 inhibitor linagliptin on the impaired relaxation to acetylcholine induced by DPP4. Results are expressed as mean ± SEM of 25 segments obtained from 6 animals. *p<0.05 compared to control. (C) DPP4 stimulates* TXA_2 *release in cultured human coronary artery endothelial cells. The COX inhibitor indomethacin and the DP4 inhibitor K579 were added to the experimental setting 30 minutes prior to the challenge with DPP4. IL-1β was used as a positive control. Results were expressed as mean ± SEM of at least 4 experiments. * p<0.05 compared to untreated cells, § p<0.05 compared to DPP4 alone. Figure adapted from* (Romacho, Vallejo et al. 2016).



Several receptors are involved in the observed effects of DPP4 on immune and vascular cells. An association between DPP4 mannose-6 phosphat/Insulin-like growth factor II receptor (M6P/IGFIIR) could be shown first (Ikushima, Munakata et al. 2000, Ikushima, Munakata et al. 2002). Binding to M6P/IGFIIR occurs via M6P in the glycosylated regions of DPP4. The complex is internalized to mediate T cell costimulatory signaling. Furthermore, DPP4 activates M6P/IGFIIR on endothelial cells where it mediates transendothelial T cell

migration (Ikushima, Munakata et al. 2002) and stimulates ROS formation (Ishibashi, Matsui et al. 2013). Also TLR4 has been discussed as a potential receptor for DPP4 by one study analysing macrophage inflammation (Ta, Li et al. 2010). We could show that PAR2 might act as a receptor for DPP4 particularly in vascular cells. PAR2 can potentially bind to a short sequence in the cysteine-rich region of DPP4 that shares high homology with the auto-activating tethered ligand of PAR2. All DPP4-mediated effects on proliferation and inflammatory signalling can be prevented by silencing of DPP4 and DPP4 antagonism in smooth muscle cells (**Publication 9**). Additionally, PAR2 agonism abolishes endothelial dysfunction and prostanoid release induced by DPP4 (Romacho, Vallejo et al. 2016). Although binding studies are lacking to substantiate our claim of PAR2 being a receptor of DPP4, it is clear that all observed vascular effects of DPP4 observed in our studies depend on PAR2 presence and activation.

Most effects of DPP4 described in the literature are related to the cleavage of numerous peptide substrates. However, the proposed mechanism of direct effects of DPP4 as observed *in vitro* is not contrary but rather an addition to the current view of DPP4 action. Ideally, all evidence from *in vitro* data should be transferred to the *in vivo* situation. Although, suggested signalling pathways for DPP4 have never been studied in the context of DPP4 knockout or inhibition, descriptive data in rodent models of cardiovascular diseases clearly demonstrates that both DPP4 knockout and inhibition improves cardiovascular outcome after myocardial infarction (Sauve, Ban et al. 2010) and atherosclerosis development in ApoE-/- mice (Ta, Schuyler et al. 2011, Ervinna, Mita et al. 2013, Zeng, Li et al. 2014). Technically, any researcher would be unable to distinguish between the direct effects of DPP4 or effects of DPP4 substrates including incretins. Thus, *in vitro* models as used in our studies are at the moment the only possible way to study effects of DPP4 independent from the classical action of DPP4 on incretin hormones or other substrates. Nevertheless, the evidence that DPP4 contributes to monocyte migration, macrophage-mediated inflammation, smooth muscle cells proliferation and endothelial dysfunction qualifies DPP4 as a risk factor for the development of atherosclerosis.

A bundle of data obtained *in vitro* and in pre-clinical studies using different DPP4 inhibitors first pointed to a potential beneficial effect of these drugs on the cardiovascular system (Scheen 2013, Rohrborn, Wronkowitz et al. 2015). Most importantly, DPP4 inhibitors increase survival after myocardial infarction while they decrease myocardial infarct size, reduce ischemia/reperfusion injury and attenuate adverse remodeling after myocardial infarction in animal models (Sauve, Ban et al. 2010, Ye, Keyes et al. 2010, Connelly, Zhang et al. 2013, Zeng, Li et al. 2014). In addition, DPP4 inhibitors display anti-atherosclerotic effects and beneficial effects on endothelial function in mice prone to the development of atherosclerosis (Shah, Kampfrath et al. 2011, Matsubara, Sugiyama et al. 2012). In humans, several meta-analyses performed in type 2 diabetic patients without selection for prior cardiovascular events demonstrated beneficial cardiovascular effects of DPP4 inhibitors (Johansen, Neubacher et al. 2012, Patil, Al Badarin et al. 2012, Monami, Ahren et al. 2013). It should be noted, however, that individuals included in these analyses were recruited in phases II and III studies and were mostly younger with a short duration of diabetes, which is not the ideal population to assess cardiovascular outcomes. Therefore, several safety outcome trials were performed for the different DPP4 inhibitors: SAVOR-TIMI 53 for saxagliptin, EXAMINE for aloglitin and TECOS for sitagliptin (Scirica, Bhatt et al. 2013, White, Cannon et al. 2013, Green, Bethel et al. 2015). In summary, these trials demonstrated the safety of DPP4 inhibitors for cardiovascular outcomes in patients with type 2 diabetes and cardiovascular risk. However, some issues with saxagliptin related to higher risk of hypoglycaemia and hospitalization due to heart failure are still discussed. As DPP4 inhibitors seem to display cardioprotective effects in younger patients prior to the development of cardiovascular disease, it might be speculated that the beneficial effects of DPP4 inhibitors might be higher in patients with a shorter duration of type 2 diabetes. In these patients, DPP4 is already increased and could contribute to endothelial dysfunction and early inflammation in the vascular wall. Whether particularly obese patients with high circulating DPP4 would benefit from treatment with a DPP4 inhibitor in a cardioprotective way is a completely open question that needs to be addressed in the future.

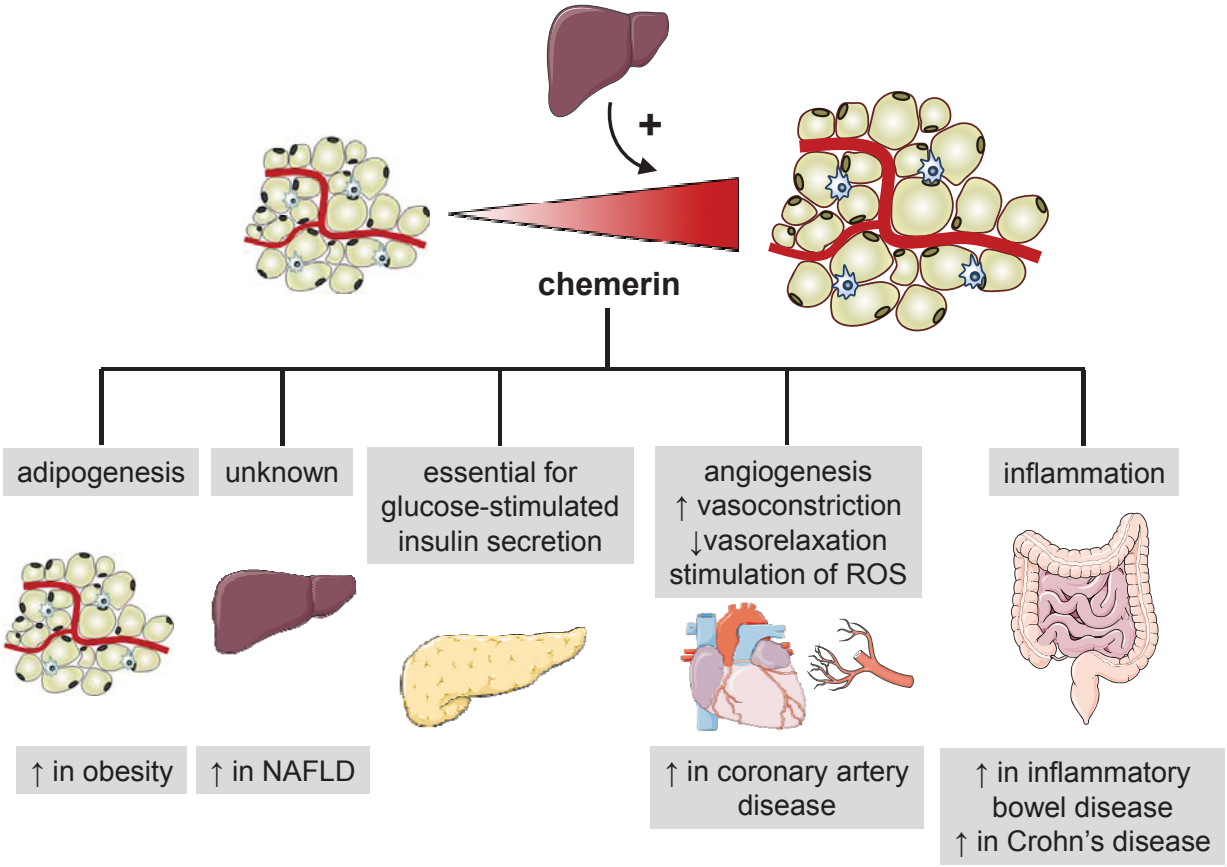
7. Conclusions

Adipokines present in adipocyte-CM induce alterations in human skeletal muscle cells, which reflect the key features of insulin-resistant skeletal muscle, namely defective insulin signalling at various levels as well as impaired translocation of GLUT4 and subsequently reduced insulin-stimulated glucose uptake (Dietze, Koenen et al. 2002, Dietze, Ramrath et al. 2004, Sell, Dietze-Schroeder et al. 2006). The induction of insulin resistance *in vitro* is comparable to the human situation where insulin resistance is characterized by impaired insulin signalling and glucose uptake. Further alterations in skeletal muscle of insulin-resistant and type 2 diabetic patients include altered differentiation marker and muscle regulatory factors, increased ROS, reduced SDH activity, increased IMCL content and more specifically increased DAG and ceramide. All these features can also been found in human skeletal muscle cells treated with adipokines *in vitro* (Taube, Lambernd et al. 2012) illustrating that adipokines most probably contribute to skeletal muscle insulin resistance *in vivo* in addition to other circulating factors such as lipids and regulatory micro RNAs. We can also show that a following withdrawal of adipokines from already insulin-resistant myotubes results in a recovery of some of these features including a return of insulin signalling to normal levels compared to myotubes never challenged with adipokines. Thus, improvement of insulin resistance by weight reducing interventions such as bariatric surgery (Perugini and Malkani 2011) is potentially mediated by a reduction of circulating adipokines. However, myotube differentiation marker only partially normalized and release of the myokines IL-8 and MCP-1 was more constantly disturbed following adipokine withdrawal from insulin-resistant myotubes. Thus, skeletal muscle cells with reversed insulin resistance retain certain defects similarly to skeletal muscle *in vivo* (Ryan, Li et al. 2013). We conclude that adipokine-induced insulin resistance is relevant in skeletal muscle of patients with adipokines being potential targets for the treatment of insulin resistance. Modulation of adipokine release from enlarged adipose tissue either by weight reduction or tissue remodelling reducing inflammation in adipose tissue could help to restore normal insulin sensitivity in patients.

We identified the chemotactic protein chemerin as an adipokine being involved in the induction of insulin resistance in skeletal muscle. In an endocrine way, chemerin can induce insulin resistance in skeletal muscle cells. This notion is supported by other studies where chemerin induces altered mitochondrial function in skeletal muscle underlying insulin resistance (Xie, Deng et al. 2015) and overexpression of human chemerin in mice specifically impairs muscle insulin sensitivity (Becker, Rabe et al. 2010). Chemerin serum levels are elevated in patients with type 2 diabetes and obesity. In addition to skeletal muscle, chemerin is also linked to the liver where it is increased in NAFLD (Kukla, Zwirska-Korczala et al. 2010, Yilmaz, Yonal et al. 2011, Docke, Lock et al. 2013). We could show that chemerin is increased in morbid obesity patients with hepatic fibrosis, portal inflammation and a high score for NAFIN. A long-term reduction of chemerin after surgery-induced weight loss even after cessation of weight loss might be attributed to a hepatic contribution to circulating chemerin in these patients. Further studies should address the question of hepatic output of chemerin and mechanistic insight if chemerin acts as a causal factor in the development of NAFLD. Chemerin is also related to inflammatory diseases with metabolic disturbances in the gastrointestinal tract such as Crohn's disease (Weigert, Obermeier et al. 2010) and inflammatory bowel disease (Weigert, Obermeier et al. 2010) where chemerin could be involved in the recruitment of tissue macrophages (Buechler 2014). Furthermore, chemerin is associated both with the incidence of coronary artery disease (Dong, Ji et al. 2011) and with the risk for cardiovascular disease (Dessein, Tsang et al. 2014). In type 2 diabetic patients, chemerin is increased in relation to endothelial dysfunction and early atherosclerosis (Gu, Cheng et al. 2015, Lu, Zhao et al. 2015). Differently, chemerin has been shown to exert a protective function in the pancreas as chemerin knockout mice shown impaired glucose-stimulated insulin secretion (Takahashi, Okimura et al. 2011) and serum chemerin is positively associated with β-cell function in humans (Hatziagelaki, Herder et al. 2015). In summary, our current knowledge on the regulation of chemerin in different organs and its relation to function and dysfunction of different organs demonstrates that chemerin

has a multifunctional role as chemokine, adipokine, and possibly also as a growth factor as summarized in Figure 7 (Ferland and Watts 2015).

Figure 7
Summary of chemerin action in different organs and its regulation in related diseases

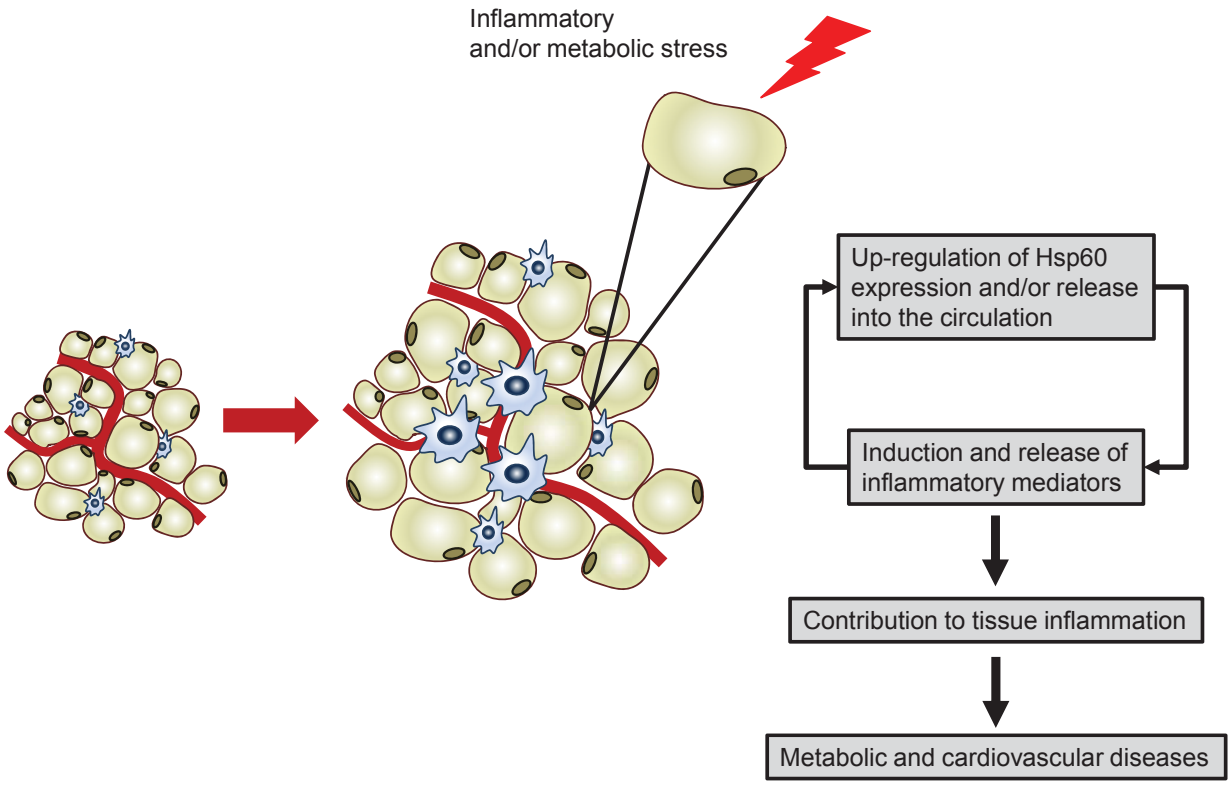


Hsp60 is a second novel adipokine that was studied in relation to expansion of adipose tissue and inflammation in obesity. We demonstrated that Hsp60 is closely related to inflammation in adipocytes as Hsp60 stimulates release of inflammatory cytokines and adipokines on one hand and inflammatory stress induces the secretion of Hsp60 on the other hand which creates a vicious cycle as presented in Figure 8. Therefore, Hsp60 as part of the heat shock response is a novel interesting signal triggering the secretion of pro-inflammatory mediators from adipocytes in the obese state in addition to hypoxia and ER stress. The notion of Hsp60 as a link to inflammation in adipose tissue is further supported by the observation that obese patients are characterized by higher circulating Hsp60 levels. This later observation is particularly relevant in the context of insulin resistance, type 2 diabetes and cardiovascular disease. Our data show that HSP60 impairs insulin signalling in human skeletal myotubes where it also stimulates inflammatory signalling as well as secretion of

myokines. As Hsp60 is not released from myotubes, adipose-derived Hsp60 could stimulate insulin resistance in skeletal muscle. Overall, Hsp60 can be seen as an interesting new molecular link between adipose tissue inflammation and obesity-associated metabolic diseases as summarized in Figure 7.

Figure 8

Hsp60 in obesity and related metabolic diseases (adapted from (Habich and Sell 2015))



Adipose tissue and more precisely adipocytes could be established as a source of DPP4 in humans by our work. DPP4 expression is highest in adipocytes compared to preadipocytes and immune cells from adipose tissue. In adipocytes, DPP4 is acutely regulated by metabolic stimuli such as insulin and inflammatory stimuli such TNFα. In addition, chronic hypoxia upregulates DPP4 expression and release (Famulla, Schlich et al. 2012). Taken together, these *in vitro* observations are in line with our observed upregulation of DPP4 expression in adipose tissue in the obese state where hypoxia and inflammation occur. In addition to higher DPP4 expression in adipose tissue in obesity, we observed higher circulating levels of DPP4 in obese patients. Unpublished data using adipose tissue-specific DPP4 knockout mice demonstrates that knockout leads to lower circulating DPP4

when animals are fed a high-fat diet. Thus, adipose tissue is an important source of circulating DPP4 in obese rodents. *Ex vivo* experiments performed with human adipose tissue further support this notion as adipocytes and adipose tissue explants from obese study volunteers release more DPP4. Furthermore, serum DPP4 is lower after weight loss.

DPP4 in circulation and in adipose tissue is also related to insulin resistance and the metabolic syndrome. Consistently, DPP4 is predominantly expressed in visceral adipose tissue which is known to confer a high metabolic risk (Despres and Lemieux 2006). Particularly in obese patients, DPP4 release from visceral adipose tissue is significantly elevated. Consequently, visceral adipose tissue contributes to higher DPP4 serum concentrations in morbidly obese patients with central obesity and insulin resistance compared to BMI-matched insulin-sensitive obese controls. In fact, these two groups of obese humans differ only in visceral adipose tissue mass and DPP4 expression in visceral fat which is significantly elevated in the insulin-resistant patients. In obesity, other organs such as the liver are discussed as sources of DPP4 in relation to metabolic diseases. Increased expression of DPP4 can be found in liver of patients with NAFLD (Firneisz, Varga et al. 2010, Miyazaki, Kato et al. 2012). Thus, one might speculate that the liver also contributes to circulating DPP4 in addition to adipose tissue, which is relevant in lean and obese patients with NAFLD.

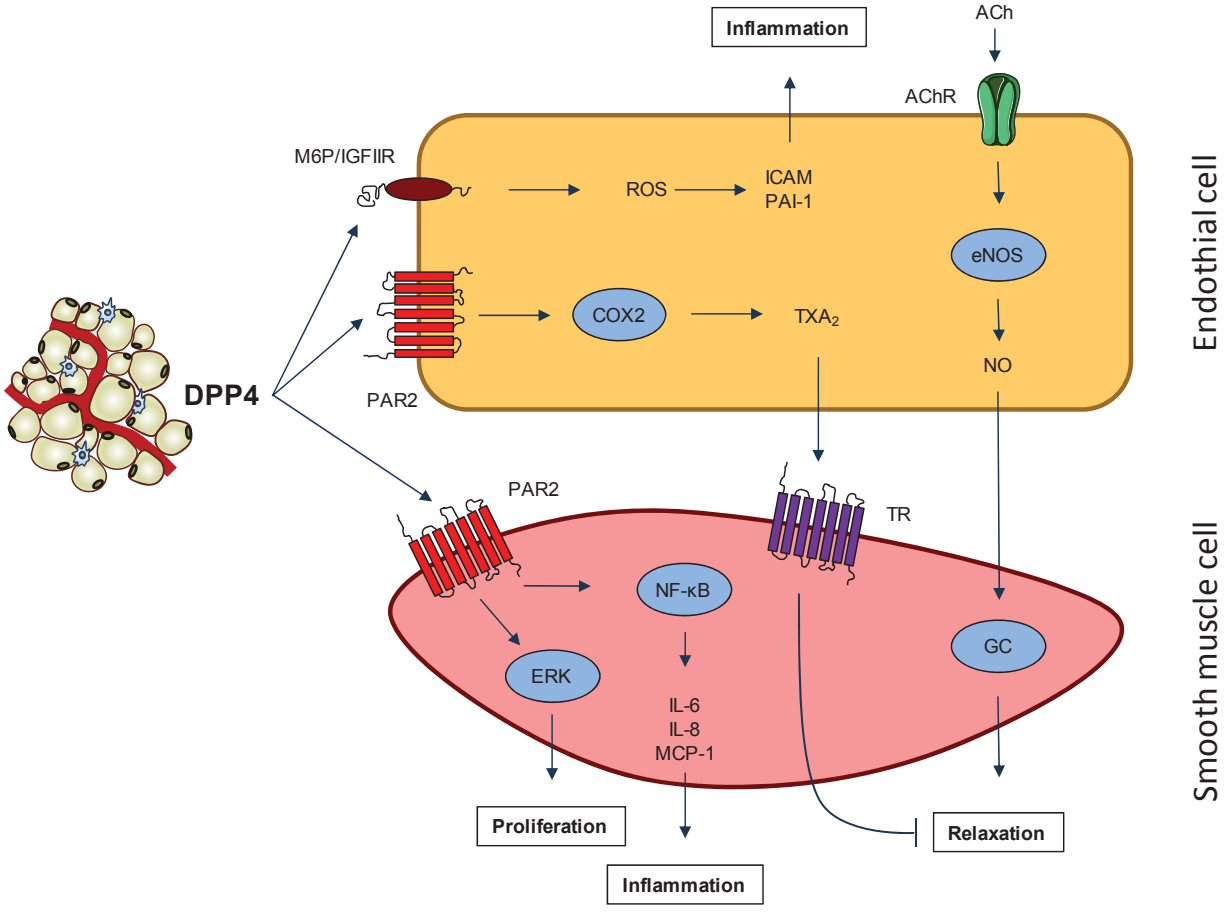
Circulating DPP4 exerts direct receptor-mediated effects on vascular cells that are incretin-independent. It is known that DPP4 signals via M6P/IGFIIR (Ikushima, Munakata et al. 2000, Ikushima, Munakata et al. 2002) and TLR4 (Ta, Li et al. 2010). We identified PAR2 as a receptor for DPP4 particularly in vascular endothelial and smooth muscle cells. Downstream effects of DPP4 in endothelial cells include ROS generation and expression of inflammatory markers such as ICAM and PAI-1 (Ishibashi, Matsui et al. 2013). We could show that also endothelial function is impaired by stimulation of COX expression and the release of TXA_2 which translates into reduced endothelium-dependen relaxation by acetylcholine (Romacho, Vallejo et al. 2016). In addition to endothelial cells, DPP4 induces proliferation and inflammation in smooth muscle cells via activation of MAPK and NF-κB. All

observed effects were completely prevented by co-treatment with a DPP4 inhibitor illustrating the importance of DPP4 activity to mediate pro-inflammatory and pro-atherogenic changes in endothelial and smooth muscle cells. In addition, all observed vascular effects of DPP4 in our studies depend on PAR2 presence and activation. As DPP4 also mediates immunomodulatory effects of immune cells that infiltrate the vascular wall at early and late atherosclerotic lesions (Iwata, Yamaguchi et al. 1999, Shah, Kampfrath et al. 2011), it can be speculated that increased circulating DPP4 in the obese state can contribute to various stages of the development of atherosclerosis. Thus, our data also illustrates mechanisms how DPP4 inhibitors exert beneficial cardiovascular effects independent from GLP-1which has been observed in many *in vitro* and *in vivo* studies (Fadini and Avogaro 2011).

Figure 9

Schematic presentation of known molecular effects of DPP4 on vascular cells

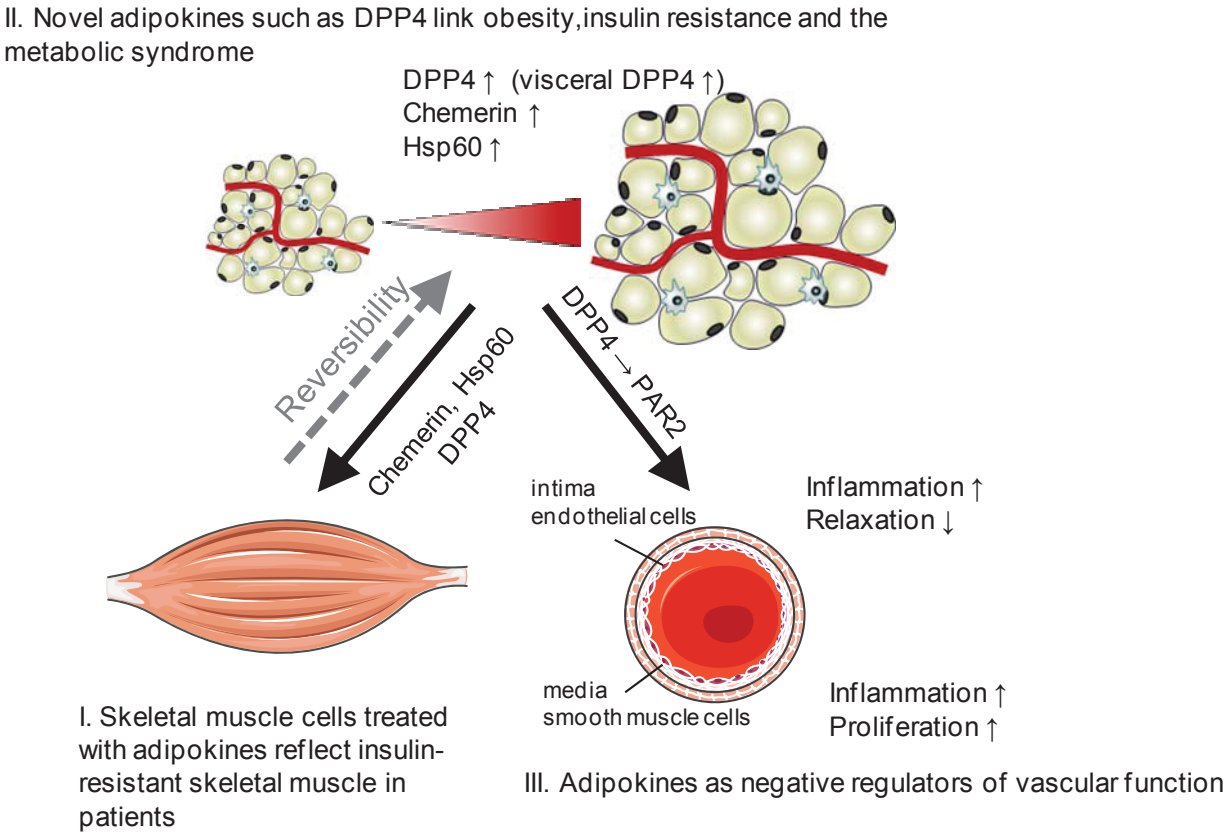
ACh, acetylcholine; AChR, acetylcholine receptor; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclase; ICAM, intercellular adhesion molecule; PAI-1, Plasminogen activator inhibitor-1; TR, thromboxane receptor



In summary, our results provide new insights into molecular mechanisms how obesity and more specifically excess of adipose tissue negatively influences skeletal muscle insulin sensitivity via inflammatory adipokines. We showed that insulin resistance in skeletal muscle is at least partly reversible with many defects but not all normalizing after intervention also *in vivo*. Chemerin and Hsp60 are candidates for adipokines that could contribute to muscle insulin resistance as both are dysregulated in the obese state and normalized after weight reduction. Furthermore, both adipokines induce insulin resistance in human skeletal muscle cells via inflammatory pathways. A causal role for many adipokines in obesity-associated metabolic diseases has not been established so far which is also the case for chemerin and Hsp60. In several of our studies, we provide a novel role for DPP4 besides being a prominent drug target for type 2 diabetes based on its importance as an enzyme for incretin deactivation. We established DPP4 as a promising target for adipose tissue in obesity. However, a causal role for DPP4 in obesity-associated metabolic disease is not yet defined. *In vitro* and *ex vivo*, DPP4 is also a mediator of vascular inflammation and endothelial dysfunction. In this context, we identified novel pathways stimulated by DPP4 and PAR2 as a novel potential receptor for DPP4. We propose that these direct effects of DPP4 *in vitro* are in addition to the current view of enzymatic DPP4 action. Our recent knowledge that DPP4 contributes to monocyte migration, macrophage-mediated inflammation, smooth muscle cell proliferation and endothelial dysfunction suggests that DPP4 can be seen as a risk factor for atherosclerosis.Taken together, there is a huge amount of evidence that adipokines released from enlarged adipose tissue in the obese state are active player in the development of obesity-associated metabolic diseases. Nevertheless, better understanding of how adipokines and adipose tissue inflammation interact with different organs will help to develop novel strategies to target factors such as adipose DPP4 and thus to improve therapy of patients with type 2 diabetes.

Figure 10

Summary of results obtained in the presented studies describing novel mechanisms of insulin resistance and vascular dysfunction.



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Acknowledgments

First, I would like to thank Prof. Michael Roden for his constant encouragement over many years to pursue my work, his interest in my research and his support in preparing this work. My special acknowledgment goes to my mentor Prof. Jürgen Eckel. I would like to sincerely thank him for sharing his passion for science with me and for supporting my work for many years not only by providing additional funds and opening doors to many labs all over Europe but also by constant encouragement to continue to find answers to the most interesting open questions. Among the great scientists I met in the last couple of years, I would like to particularly thank Prof. Karine Clément and Prof. Peter Arner who inspired my work and supported my career in many different ways. Dr. Kristin Eckardt and Dr. Christiane Habich were not only important collaborators but are also friends. Most impact came from our PhD students by bringing in fresh ideas, enthusiasm and dedication to their work: Susanne Famulla, Daniela Lamers, Raphaela Schlich, Nina Wronkowitz and Diana Röhrborn. Last but not least, I would like to express infinite gratitude to my husband and my parents for taking care of my well-being and for their love.

Abbreviations

Ach	acetylcholine
AChR	acetylcholine receptor
AGE	advanced glycation end products
BP	blood pressure
BMI	body mass index
CCL	CC chemokine
CCR	C-C motif receptor
CCRL	C-C chemokine receptor-like
CM	conditioned medium
CMKLR1	Chemokine like receptor 1
COX	cyclooxygenase
CRP	C-reactive protein
CXCL	CXC chemokine
DAG	diacylglycerols
DPP4	dipeptidyl peptidase 4
eNOS	endothelial nitric oxide synthase
FFA	free fatty acids
FPG	fasting plasma glucose
GC	guanylate cyclise
GIP	gastric inhibitory polypeptide
GLP-1	glucagon-like peptide-1
GPR	G protein-coupled receptor
GSK	glycogen synthase kinase
HbA1c	haemoglobin A1c
HDL	high density lipoprotein
HIF	hypoxia-inducible factor
HNF	hepatocyte nuclear factor

HOMA	homeostatic model assessment
Hsp60	heat shock protein 60
HSR	heat shock response
ICAM	intercellular adhesion molecule
IMCL	intramyocellular lipids
iNOS	inducible nitric oxide synthase
IRS-1	insulin receptor substrate 1
LADA	latent autoimmune diabetes of the adult
M6P/IGFIIR	mannose-6 phosphat/Insulin-like growth factor II receptor
MAPK	Mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MHC	myosin heavy chain
MMP	matrix metalloprotease
MODY	maturity-onset diabetes of the young
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD Activity Score
NASH	Non-alcoholic steatohepatitis
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species
SDH	succinate dehydrogenase
TNF	tumour necrosis factor
TR	thromboxane receptor
T_{reg}	regulatory T cell

TXA_2	thromboxane A_2
VEGF	vascular endothelial growth factor