**Adipose DPP4 and obesity: correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro**

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Abstract

**Objective:** To study expression of the recently identified adipokine dipeptidyl peptidase 4 (DPP4) in subcutaneous (SAT) and visceral adipose tissue (VAT) of patients with different BMI and insulin sensitivity as well as to assess circulating DPP4 in relation to obesity and insulin sensitivity.

**Research Design and Methods:** DPP4 expression was measured in SAT and VAT from 196 subjects with a wide range of BMI and insulin sensitivity. DPP4 release was measured *ex vivo* in paired biopsies from SAT and VAT as well as *in vivo* from SAT 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 SAT and VAT with VAT constantly displaying higher expression compared to SAT. *Ex vivo* release of DPP4 from adipose tissue explants was higher from VAT as compared to SAT 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 subjects 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 VAT, adipocyte size and adipose tissue inflammation.

**Conclusions:** DPP4 is a novel adipokine with higher release from VAT that is particularly pronounced in obese and insulin-resistant patients. Our data suggest that DPP4 might be a marker for visceral obesity and the metabolic syndrome.
**Introduction**

Obesity is a worldwide increasing health issue, economical burden and, as the hallmark of the metabolic syndrome, the obese state is frequently associated with the development of chronic diseases including type 2 diabetes (1, 2). The association between the epidemics of obesity and diabetes has promoted research on the endocrine link between lipid and glucose homeostasis, demonstrating that adipose tissue is an endocrine organ releasing various adipokines. A complex inter-organ crosstalk scenario between adipose tissue and other central and peripheral organs underlies the progression of obesity-related metabolic disorders with adipose tissue being a key player in this scenario (3). The current view of the role of expanded adipose tissue in obesity identifies adipokines as a potential link between obesity and insulin resistance (4). This link stimulated a further characterization of the adipocyte secretome utilizing diverse proteomic profiling approaches leading to the discovery of novel adipokines including dipeptidyl peptidase 4 (DPP4) (5).

DPP4 is a transmembrane glycoprotein and exoprotease cleaving N-terminal dipeptides from various substrates (6). Most importantly, DPP4 also cleaves and inactivates the incretins glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). In this context, DPP4-inhibitors are in clinical use as anti-diabetic drugs to improve glycaemic control by stimulating pancreatic insulin secretion and suppressing glucagon production (7). We recently demonstrated that adipocytes release DPP4 in a differentiation-dependent manner (5). Circulating DPP4 concentrations are increased in obese subjects and correlate 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. As circulating DPP4 is increased in obese patients with the metabolic syndrome (5), we hypothesize that DPP4 expression and release from visceral adipose tissue is more prominent as compared to subcutaneous fat and that visceral DPP4 might be a marker for insulin sensitivity.
Materials and Methods

Patients

For all studies, protocols were approved by local Ethics Committees and all participants gave written, informed consent.

**DPP4 expression in paired biopsies from subcutaneous and visceral adipose tissue:** Paired samples of visceral and subcutaneous adipose tissue were obtained from 196 Caucasians (97 men and 99 women) undergoing open visceral surgery for various reasons including gastric banding, cholecystectomy, appendectomy and weight reduction surgery (8). Patients with severe conditions including generalized inflammation or end-stage malignant diseases were not included. The age ranged from 24 to 86 years and BMI from 20.8 to 54.1 kg/m². 67 patients had impaired glucose tolerance or type 2 diabetes. Adipose tissue specimens were immediately frozen in liquid nitrogen after excision. Clinical parameters were assessed as described previously (9).

**In vitro release of DPP4 from paired biopsies from subcutaneous and visceral adipose tissue:** Subcutaneous and visceral adipose tissue biopsies were obtained during planned visceral surgery (hernia, gall bladder surgery and other non-inflammatory and non-malignant causes) from 12 lean and 11 obese patients (BMI 22±2 and 38±3 kg/m², respectively; age 62±6 and 58±6 years, respectively). Adipose tissue specimens were immediately transferred to medium and explants were generated as described earlier (10). Briefly, fat explants were cultured in serum-free medium. After 24 hrs, the conditioned medium was collected and stored in aliquots at –80°C until further use.

**Serum DPP4 and adipose DPP4 expression in insulin-sensitive (IS) and insulin-resistant (IR) obese subjects:** 60 morbidly obese men and women with a BMI of 45± 1.3 kg/m² scheduled for elective cholecystectomy, explorative laparotomy, or gastric sleeve resection were selected and distributed into two experimental groups of IS and IR obesity with 30 subjects each as described previously (11). On the basis of the glucose infusion rate (GIR) in euglycemic hyperinsulinemic clamp, patients were defined as either IS (GIR > 70 µmol/kg·min) or IR (GIR ≤ 60 < mol/kg·min). Both groups were matched for gender, age and BMI. Clinical parameters were assessed as described previously (11).
In vivo release of DPP4 from subcutaneous adipose tissue: 27 healthy volunteers (15 women, 12 men) (BMI 21 – 41.5 kg/m2, age 32 – 56 years) were recruited from the greater Oxford community by advertisement or from the Oxford BioBank (12). None of the subjects was taking medication known to affect lipid metabolism and all were normoglycemic. Arterio-venous differences were measured across subcutaneous abdominal adipose tissue. A superficial epigastric vein draining subcutaneous abdominal fat (13), and an arterialized dorsal hand vein (with the hand kept in a warming box at 60°C), were cannulated. The cannulae were kept patent with an intravenous infusion of 0.9% saline. Adipose tissue blood flow (ATBF) was measured in abdominal subcutaneous adipose tissue in the fasting state as described previously (14) and calculated from the washout of 133Xe assuming a partition coefficient of 10 ml/g (15). After the subjects had rested for 45 min, blood samples were taken simultaneously from the two sites. Samples were stored at -80°C until analyzed.

Measurement of DPP4 expression

Human DPP4 mRNA expression was measured by quantitative real-time RT-PCR in a fluorescent temperature cycler using the TaqMan assay, and fluorescence was detected on an ABI PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany). Total RNA was isolated using TRIzol (Life technologies, Grand Island, NY), and 1 µg RNA was reverse transcribed with standard reagents (Life technologies). From each RT-PCR, 2 µl were amplified and in a 26 µl PCR using the Brilliant SYBR green QPCR Core reagent kit from stratagene (La Jolla, CA) according to the manufacturer’s instructions. Samples were incubated in the ABI PRISM 7000 sequence detector for an initial denaturation at 95C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95C for 15 s, 60C for 1 min and 72C for 1 min. Human DPP4 mRNA expression was calculated relative to the mRNA expression of 18s rRNA, all determined by premixed assays on demand for DPP4 and 18s rRNA (Applied Biosystems, Darmstadt, Germany). Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 68C and heating slowly to 95C with measurement of fluorescence) at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.
Measurement of DPP4 in serum and conditioned medium

DPP4 release by adipose tissue explants and serum concentration was determined by ELISA (R&D Systems, Wiesbaden, Germany). The assay was performed in duplicates according to the manufacturer’s instructions. The intra- and inter-assay variation was 5.4 % and 8.1 %, respectively.

Statistical analysis

Data are expressed as mean ± SEM. The Shapiro–Wilcoxon test was used to test the Gaussian distribution of biological parameters. Student t test were used for comparison between two groups and ANOVA followed by P for linear trend post-test for comparison between multiple groups. Mann-Whitney was used for variable that were not normally distributed. Correlations were performed by Pearson or as indicated in the graphs. All statistical analyses were done using JMP statistics software (SAS Institute Inc., Cary, NC) or Prism (GraphPad Software, Inc., La Jolla, CA) considering a P value < 0.05 as statistically significant.
Results

*DPP4 expression correlates with BMI, is higher in visceral adipose tissue and is increased in visceral adipose tissue of lean patients with impaired glucose tolerance*

*DPP4* expression in subcutaneous and visceral adipose tissue was measured in 196 individuals of various BMI that were characterized by impaired or normal glucose tolerance, as assessed by OGTT. *DPP4* expression in both depots correlated positively with BMI (Fig. 1A-B) and correlated with each other (r=0.28, p<0.0001). *DPP4* expression was significantly higher in visceral adipose tissue as compared to subcutaneous adipose tissue. Furthermore, *DPP4* expression significantly increased in both depots with increasing BMI from lean to obese subgroups (Fig. 1C). In lean individuals with impaired glucose tolerance *DPP4* was significantly increased in visceral adipose tissue but not in the subcutaneous depot (Fig. 1D). This difference was not related to BMI or gender in both groups. In overweight and obese subjects there is no difference between *DPP4* expression in the visceral adipose tissue according to glucose tolerance (Fig. 1E). *DPP4* mRNA in both depots correlated with various clinical parameters and measures of adipose tissue inflammation (Table 1). *DPP4* expression in subcutaneous and visceral adipose tissue of normoglycemic patients was positively associated with several measures of obesity including body fat, visceral and subcutaneous fat area, circulating leptin and IL-6 and the amount of macrophages in adipose tissue, and negatively associated with circulating adiponectin. As for the metabolic state of normoglycemic patients, *DPP4* expression in adipose tissue was positively correlated with fasting plasma insulin, HbA1c and negatively correlated with glucose infusion rate obtained during euglycemic-hyperinsulinemic clamp. In addition, *DPP4* expression was associated with total cholesterol, free fatty acids (FFA) and triglycerides. In both normoglycemic patients and patients with impaired glucose tolerance, *DPP4* expression in visceral fat positively correlated with visceral fat area and FFA while a negative correlation was found for glucose infusion rate and circulating adiponectin.

*In vitro DPP4 release is most prominent in visceral adipose tissue from obese patients*

Paired biopsies of subcutaneous and visceral adipose tissue from lean and obese subjects were used to study *DPP4* release *in vitro*. *DPP4* release was significantly higher from visceral fat in both lean and
obese subjects (Fig. 2A). The highest DPP4 release was measured from visceral adipose tissue of obese subjects. Dividing the group of obese subjects in those with type 2 diabetes and those without, we observed a significant increase of DPP4 release from visceral adipose tissue in those obese patients with type 2 diabetes (BMI and age not different between obese subgroups) (Fig. 2B). DPP4 release from subcutaneous adipose tissue only tended to be higher in obese patients irrespective of their diabetes state (data not shown).

In vivo release of DPP4 is higher in obese patients and in females

Arterio-venous differences of DPP4 were measured across subcutaneous abdominal adipose tissue in lean and obese subjects as described before (13). In 16 out of all 27 subjects studied there was net release of DPP4 from adipose tissue (Fig. 2C). This was related to the arterial DPP4 concentration, as the net release of DPP4 was negatively correlated with the arterial level such that the greatest net release of DPP4 was associated with the lowest arterial concentration (Fig. 2C). In patients with lower arterial DPP4 (< 288 ng/ml, n=14), obese subjects were characterized by a significantly higher net release of DPP4 as compared to lean patients (Fig. 2D) and there was a correlation between DPP4 release and BMI (Spearman’s rank correlation 0.72, p = 0.01, data not shown). Women (n=16) showed net release (p = 0.014 for difference from no net release as assessed by Wilcoxon Signed Rank Test), and showed significantly more release than men (Fig. 2E). Amongst the women, DPP4 release was significantly related to BMI (r= 0.59, p = 0.021, data not shown).

Circulating DPP4 is lower in BMI-matched healthy obese patients and correlates with insulin resistance and adipose tissue inflammation

DPP4 expression and circulating levels were also measured in healthy, insulin-sensitive obese patients (IS) and compared to BMI-, age- and gender-matched insulin-resistant obese patients (IR). DPP4 expression was significantly elevated in both subcutaneous and visceral fat of IS patients as compared to IR subjects (Fig. 3A). DPP4 circulating concentrations were significantly elevated in IR as compared to IS (Fig. 3B). There was no difference in circulating DPP4 in females compared to males (data not shown). Serum DPP4 correlated with the amount of visceral fat as well as adipocyte size and adipose tissue inflammation expressed as percentage of macrophages in visceral adipose tissue (Fig.
Furthermore, DPP4 correlated with insulin resistance as it was negatively related to GDR and positively associated with fasting insulin and HbA1c (Fig. 4F-H).
Discussion

We recently identified DPP4 as a new adipokine that might be a missing link between increased adipose tissue mass in obesity and obesity-associated metabolic diseases (5). Although much attention has focused on the role of DPP4 in the degradation of GLP-1, our earlier data suggest that DPP4 also exerts direct effects, as it is able to induce insulin resistance in adipocytes and skeletal muscle cells in concentrations that can be found in the circulation of overweight and obese subjects (5). Therefore, DPP4 might also have local effects within adipose tissue and systemic effects via the blood circulation. To better understand the regulation of DPP4 in humans with different degrees of obesity and insulin sensitivity, we presently measured DPP4 mRNA expression in adipose tissue and correlated it with clinical parameters and adipose tissue measures. DPP4 expression is constantly lower in subcutaneous adipose tissue irrespective of the body fat level suggesting that there is a depot-specific control of DPP4 expression. The fact that circulating DPP4 and DPP4 expression in adipose tissue correlates with adipocyte size and adipose tissue inflammation might also suggest that pro-inflammatory adipokines released from enlarged adipocytes could regulate DPP4 release.

The findings with DPP4 expression in adipose tissue in relation to BMI have been divergent as a first report on this subject demonstrated higher DPP4 expression in adipose tissue from obese patients compared to lean controls (16) while data from a second study described higher DPP4 expression in lean subjects compared to obese ones (17). Together with our previous publication describing DPP4 as a novel adipokine (5), we now show in different groups of patients that both DPP4 mRNA expression and DPP4 protein levels are increased in both subcutaneous and visceral adipose tissue from obese subjects. Also using different groups of patients such as consecutive patients with a continuous spectrum of different BMI as well as profoundly characterized insulin-sensitive and insulin-resistant morbidly obese patients, we can furthermore demonstrate that DPP4 expression, especially in visceral adipose tissue, is negatively associated to insulin sensitivity both in lean as well as in obese subjects.

In order to extend our understanding of how DPP4 is not only expressed in adipose tissue but also released from the tissue, we also studied DPP4 release from adipose tissue explants ex vivo.
Subcutaneous adipose tissue biopsies from obese patients are characterized by higher DPP4 release as compared to lean controls. This set of data corroborates our earlier study showing that enlarged subcutaneous adipocytes from obese patients release higher amounts of DPP4 compared to adipocytes from lean controls (5). Additionally, we now show that adipose tissue explants from visceral adipose tissue release more DPP4 compared to subcutaneous fat pointing to a possible higher contribution of visceral adipose tissue to circulating DPP4 levels. DPP4 release from visceral adipose tissue of obese patients is again higher as compared to lean controls. Until now, it is not known if higher release of DPP4 from visceral adipose tissue in obesity only reflects higher DPP4 expression and therefore higher presence at the plasma membrane or if enzymes involved in DPP4 shedding from the membrane are also regulated in obesity. Mechanisms how DPP4 is released from the cell membrane are unknown and involve enzymes not yet identified. It might be speculated that metalloproteinases (MMPs) and A Disintegrin And Metalloproteinases (ADAMs) could be shedding partners for DPP4. In fact, the first so-called sheddase ADAM17 is involved in TNFα processing at the cell surface thereby leading to TNFα release into the circulation (18). Dysregulation of MMPs and ADAMs in association with fibrosis in expanding adipose tissue in obesity might contribute to increased DPP4 release and its depot-specific differences (19, 20).

DPP4 expression and release is higher in obese patients with the metabolic syndrome and type 2 diabetes. Animal data support the notion that DPP4 expression in adipose tissue increases with developing type 2 diabetes as streptozotocin-induced diabetic rats display significantly increased DPP4 activity in epididymal fat (21). How DPP4 is regulated in this context is not known. We have demonstrated previously higher DPP4 release from obese patients with the metabolic syndrome and higher circulating DPP4 levels in obese patients with the metabolic syndrome as compared to obese controls (5). In this study, we could further refine these findings in a well-characterized cohort of insulin-sensitive morbidly obese patients compared to insulin-resistant BMI-matched obese subjects, showing that despite similar adiposity circulating DPP4 levels and DPP4 expression in visceral adipose tissue are higher in the insulin-resistant patients. Some studies have already attempted to explain why DPP4 is overexpressed in adipose tissue of patients with the metabolic syndrome. A first
study analyzing SNPs revealed that DPP4 polymorphisms are probably not modulating DPP4 expression and the association of DPP4 expression with cardiovascular risk (22). Conversely, DPP4 expression seems to be mediated by epigenetic effects. 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 (23). Furthermore, three DPP4 polymorphisms were significantly associated with methylation levels. Interestingly, we observe a significant increased DPP4 expression in visceral adipose tissue of lean patients with impaired glucose tolerance which might suggest that mechanisms explaining 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. It should be noted that this phenomenon was only restricted to visceral adipose tissue which again illustrates the major difference between both the subcutaneous and the visceral depot in conferring an increased metabolic risk.

Data on in vivo DPP4 release measuring aterio-venous differences suggest that subcutaneous abdominal adipose tissue is not the only source of circulating DPP4, although there is net release in most of the subjects. In fact, net release is seen when the arterial concentrations of soluble DPP4 are relatively low. When concentrations are high, this fat depot can extract DPP4 from the circulation. The data are similar to those seen for steroid hormones (24) for both estradiol and testosterone, uptake by subcutaneous abdominal adipose tissue is seen when arterial concentrations are high, but release when arterial concentrations are low. The data show a strong effect of obesity on net release. Interestingly but unexplained, DPP4 net release is higher in women although circulating DPP4 and adipose DPP4 expression is not different between males and females. In animals, DPP4 expression and activity in streptozotocin-induced diabetic rats is not only significantly increased in epididymal fat but also in liver (21). Data from humans also indicate that liver might be a primary source of DPP4 in addition to adipose tissue as patients with non-alcoholic liver disease (NAFLD) have higher circulating DPP4 activity as compared to controls (25). However, it should be noted that NAFLD patient had also significantly higher BMI and DPP4 was not adjusted for BMI in this study. Additionally, the authors describe that patients without NAFLD but with type 2 diabetes had similar circulating DPP4 activity.
as compared to controls, which would not agree with our data. However, patients with type 2 diabetes were about 30 years older in average than controls and we know from our previous study that circulating DPP4 levels decline with age (5). Additional studies are needed to clarify if DPP4 concentrations in the circulation are predictive of NAFLD independently from age and BMI.

In conclusion, we could demonstrate that DPP4 is an adipokine with significantly higher expression in and release from visceral adipose tissue that is further increased in obese subjects. Circulating DPP4 is higher in insulin resistant versus insulin sensitive obese. Finally, DPP4 overexpression in visceral adipose tissue may be a marker of adipose tissue inflammation known to be associated with insulin resistance and the metabolic syndrome. Further work is needed to elucidate the functional role of DPP4 within adipose tissue and to define whether higher DPP4 expression and serum concentration may contribute to higher efficacy of DPP4 inhibitors in patients with a high proportion of visceral fat.
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The authors declare to have no conflict of interest.

Author Contributions: H.S. takes responsibility for this work and wrote the manuscript. H.S., M.B., N.K., R.S., M.W. and B.A.F. researched data. F.R., W.T.K. and A.D. contributed to study design. H.S., M.B., P.A., K.F. and J.E. contributed to discussion. All authors reviewed/edited the manuscript.
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Table 1 Pearson’s correlation coefficients of adipose DPP4 expression with clinical parameters and adipose tissue measures

<table>
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<tr>
<th>Variable</th>
<th>Normal glucose tolerance (n=129)</th>
<th>Impaired glucose tolerance (n=67)</th>
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<td>Subcutaneous DPP4 mRNA expression</td>
<td>Visceral DPP4 mRNA expression</td>
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<td>% macrophages in subcutaneous fat</td>
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* p<0.05, ** p<0.01, *** p<0.001
Figure Legends

Figure 1  DPP4 expression in paired subcutaneous and visceral adipose tissue of lean and obese patients in relation to obesity and glucose tolerance

DPP4 expression in adipose tissue was measured in 197 individuals. A-B Lineal regression analysis of DPP4 expression in subcutaneous and visceral adipose tissue and BMI. Statistical evaluation is indicated in each graph. C-E Comparison of subcutaneous and visceral DPP4 expression in lean, overweight and obese subjects (C), lean subjects with (igt) and without (ngt) impaired glucose tolerance (D) and overweight/obese subjects with and without impaired glucose tolerance (E). * p < 0.05 compared to subcutaneous, § p < 0.05 compared to visceral adipose tissue of ngt.

Figure 2  In vitro DPP4 release from paired subcutaneous and visceral adipose tissue of lean and obese patients and in vivo DPP4 release from abdominal subcutaneous adipose tissue of lean and obese men and women

(A-B) DPP4 release was measured from paired adipose tissue specimen of 12 lean and 11 obese (5 with type 2 diabetes) by ELISA. * p < 0.05 compared to respective subcutaneous adipose tissue or between groups as indicated. (C-E) DPP4 was measured in arterialized blood and adipose tissue venous blood in lean and obese patients and the net release of DPP4 calculated (n=27). (C) Correlation of arterial DPP4 with DPP4 net release from adipose tissue taking blood flow into account. Linear regression was performed using Spearman correlation. (D) Net release of DPP4 from adipose tissue in lean and obese subjects with low arterial DPP4 (< 288 ng/ml, n=14). *p=0.05 using Mann-Whitney test. (E) Net release of DPP4 from adipose tissue of men and women. *p=0.05 using Mann-Whitney test.

Figure 3  DPP4 expression in adipose tissue and circulating DPP4 in insulin-sensitive (IS) and insulin-resistant (IR) morbidly obese subjects

(A) DPP4 expression in adipose tissue was measured in IS (n=30) and IR (n=30) obese subjects. * p < 0.05 compared to respective subcutaneous adipose tissue or between groups as indicated. (B)
Circulating DPP4 concentration in IS and IR groups. * p < 0.05 compared to IS. (C-H) Linear regression analysis of DPP4 serum concentration and measures of adipose tissue morphology/inflammation or insulin sensitivity. Statistical evaluation is indicated in each graph.