

Dysregulation of Zinc and Iron Balance in Adipose Tissue from Diabetic Sand Rats (*Psammomys obesus*)

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Abstract

Background: In obesity, the distribution and metabolic function of adipose tissue are of vast importance for the risk of type 2 diabetes development. The homeostasis of zinc and iron is believed to be disturbed in diabetic patients. Zinc dyshomeostasis could affect the metabolic function of adipose tissue as zinc is known to facilitate the functions of insulin within adipose tissue as well as take part in cell proliferation and apoptosis. Further, altered iron levels have been shown to affect insulin sensitivity. This study investigates the intracellular zinc regulation and total zinc and iron status in adipose tissues in obesity-linked, type 2 diabetes in the *Psammomys obesus* model.

Methods: Subcutaneous and visceral adipose tissue were collected from diabetic (n=6) and non-diabetic animals (n=6). Total zinc and iron levels were analyzed by induced-coupled plasma mass spectrometry. Gene expressions of zinc transporters of the SLC30A and SLC39A family, regulating the intracellular zinc distribution, as well as several metabolic markers were investigated by RealTime-PCR.

Results: Diabetic animals exhibited signs of an altered zinc homeostasis i.e. a re-distribution of total zinc within visceral adipose tissues and altered transcription of zinc regulatory proteins *ZIP6*, *ZIP8*, *ZIP9*, and *ZnT9*. Further, diabetic animals displayed an iron accumulation in visceral adipose tissue that was positively correlated with insulin degrading enzyme and peroxisome proliferator-activated receptor gamma.

Conclusion: *Psammomys obesus*, a complex animal model of diet-induced type 2 diabetes, exhibits changes in the mineral status of zinc and iron in visceral adipose tissue. These changes might be related to the altered insulin sensitivity and metabolic function of visceral adipose tissue seen in type 2 diabetes. This study warrants further investigations into the role minerals, especially zinc and iron, play in the pathophysiology of type 2 diabetes.

Keywords: Adipose tissue; Iron homeostasis; Type 2 diabetes; *Psammomys obesus*; Peroxisome proliferator-activated receptor gamma; Zinc homeostasis; ZIP 14

Abbreviations: T2D: Type 2 Diabetes; *P. obesus*: *Psammomys obesus*; HSL: Hormone Sensitive Lipase; LPL: Lipoprotein Lipase; IDE: Insulin Degrading Enzyme; PPARγ: Peroxisome Proliferator-activated Receptor Gamma

Introduction

Type 2 diabetes (T2D) is a potential consequence of obesity affecting up to 20% of all obese individuals [1]. The distribution of fat in the body is important for the incidence of T2D since subcutaneous adipose tissue and visceral adipose tissue have different structural and metabolic characteristics [2,3]. Visceral adipose tissue shows a higher level of basal lipolysis, an accelerated response to lipolytic hormones, and a decreased response to the inhibitory effect of insulin on lipolysis. This metabolic characteristic of visceral adipose tissue results in an enlarged flooding of free fatty acids directly to the liver, through the portal vein, and thereby an increased gluconeogenesis. Furthermore, ectopic triglyceride accumulation occurs in muscle and pancreas. The result is peripheral insulin resistance and beta-cell dysfunction [2].

Overweight and obese individuals show altered levels of many essential trace elements in the blood [4,5]. The same pattern is found in type 2 diabetic individuals [6]. These alterations in trace element status have been associated with diverse metabolic changes [4,6-8]. Especially the disturbances in iron and zinc homeostasis have been in focus.

Zinc is an important mineral as it mimics and amplifies the pancreatic and peripheral functions of insulin. Furthermore, alterations in zinc balance are related to T2D [9-13]. Zinc permits the formation of insulin-hexamers in beta-cells and in part regulates the beta-cell mass [14-16]. In other insulin responsive tissues i.e. muscle and fat, zinc shows many insulinomimetic properties by affecting the insulin-receptor signal transduction [13,17].

Zinc deficiency has been associated with increased levels of glycated hemoglobin in patients with T2D [6]. In obese individuals, zinc deficiency has also been associated with an aggravated lipid profile, increased glucose, and insulin levels [7,18]. Epidemiological studies indicate a relationship between zinc deficiency and an increased frequency of obesity [4]. In addition, obesity and T2D have been linked

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to alterations in the zinc transporters, which control intracellular zinc balance. During the last decade, thorough investigation of the insulin-granule localized ZnT8, has elucidated the importance of ZnT8 in the supply of zinc to the crystallization, processing, and secretion of insulin [19,20]. A common genetic variant of ZnT8 has then proven to increase the risk of developing T2D [21]. On the contrary, a recent study investigating around 150.000 people found that loss-of-function mutations in the gene coding for the zinc transporter ZnT8 protects against the development of T2D [22]. A potential explanation of this paradoxical finding is the complementary role of other zinc transporters such as ZnT3 and ZnT7 in diabetes development [23,24].

Two families of solute-linked zinc transporter families are known: The zinc transporters (ZnT or Slc30a) responsible for zinc efflux and the ZRT/IRT-related proteins (ZIP or Slc39a) facilitating zinc influx [25,26]. In addition, at least three members of the ZIP family are able to transport other minerals. Transport of Mn^{2+} has been shown for ZIP7, ZIP8 and ZIP14, Cd^{2+} transport for ZIP8 and ZIP14 [27-29]. Furthermore, ZIP14 has been shown to play an essential role in iron transport and homeostasis [30].

Although most research on the regulation of intracellular zinc homeostasis in obesity and diabetes has been focused on the role of zinc in beta-cells, we have previously shown that in adipose tissue, the gene expression of several zinc transporters differ in obese individuals compared to their lean counterparts. Consistent transcriptional down-regulations were seen in visceral adipose tissue compared with subcutaneous adipose tissue and in obese individuals compared with lean individuals. This suggests that both obesity and differences in metabolic activity of various adipose tissues regulate the zinc transporters and thereby the intracellular zinc environment [31]. To what extent, intracellular zinc regulation is affected in adipose tissue during diabetes is so far not known.

Like zinc, disturbances in iron homeostasis have been connected to the pathogenesis of T2D. Dietary iron deficiency has been linked to obesity, whereas various forms of iron overload have been linked to the development of diabetes [32,33]. High fat diet can reduce iron uptake in the gut [34]. It has also been speculated that obesity-induced inflammation increases the level of hepcidin in this way leading to reduced dietary iron uptake [8]. Furthermore - as described in a recent review by Becker and co-workers [35] - an increased hepcidin expression could result in reduced iron absorption on the cellular level. In macrophages, such sequestering of iron could lead to anemia and other adverse physiological effects seen in obesity [4,35]. Paradoxically, hepcidin levels also rise in dietary iron overload. This means that cellular or tissue specific iron deficiency can be present despite a total body over-load potentially participating in the development of disturbances in adipocytes or beta-cells [8,36,37]. In disease related iron accumulation such as hemochromatosis and dietary iron overload have been linked to a higher risk of diabetes and beta-cell failure. In rodent studies, reduction of iron levels through phlebotomy or low iron diet has been shown to improve insulin secretion and insulin levels in both hemochromatosis and dietary overload [8].

Several studies focus on the role of micronutrients, e.g. zinc and iron, in relation to glucose metabolism and diabetes. Less is known about how micronutrient status affects adipocytes. It has been shown that iron status can affect adipokine regulation hereby potentially affecting lipid metabolism [8]. At present, the micronutrient status and regulation in adipose tissue are thus far from unraveled. Iron and

zinc share several co-transport mechanisms including DMT and ZIP14 [38,39] but are seldom investigated together.

The aims of this study were thus: to establish zinc and iron status in adipose tissue of diabetic Israeli sand rats, *Psammomys obesus* (*P. obesus*), and investigate if intracellular zinc regulation in adipose tissue is changed by the development of high energy diet- induced T2D, correlating gene expression of zinc regulatory proteins - including the zinc and iron transporter *ZIP14*- with mineral status and gene expression of key markers of adipocyte proliferation and glucose metabolism.

Materials and Methods

Animals

P. obesus were purchased from Harlan Laboratories, Jerusalem, Israel. The gerbils were ten weeks of age at the time of the acclimatization start. The animals were supplied with water ad libitum and maintained on a 12-h dark-light cycle in a 25°C environment. Principles of laboratory animal care were followed and study approval was obtained from the Animal Experiments Inspectorate, Ministry of Justice. Diabetes prone animals, confirmed by their blood glucose levels on high- energy diet (Formulab Diet 5008, 3.5 kcal/g, Harlan Teklad), were chosen for this experiment and put on high-energy diet for 5 weeks in order to develop T2D (n=6). Diabetic animals were compared with diabetes resistant control animals on low-energy diet (3084-111507, 2.5 kcal/g, Harlan Teklad) (n=6). The following tissues were dissected immediately after cervical dislocation in isoflurane anesthesia: Subcutaneous adipose tissue, retroperitoneal adipose tissue and epididymal adipose tissue. Both retroperitoneal and epididymal adipose tissue were considered visceral adipose tissue.

Measurements of trace elements

20-200 mg of adipose tissue was used for analysis. Samples were processed and mineralized in a Multiwave 3000 (Anton Paar) by a standard method (Bovine Liver) using 5.5 ml nitric acid and 0.5 ml hydrochloric acid. The samples were transferred to plastic tubes by several rinsing procedures in de-ionized pure water. Total zinc (Zn), iron (Fe), and copper (Cu) levels as well as the Cu/Zn ratio were determined in all three adipose tissues by Inductively coupled plasma mass spectrometry (ICP-MS) on an X series^{II} ICP-MS equipped with a conventional Mainhard nebulizer and a Peltier cooled quartz impact bead spray chamber operated at 3°C (Thermo Electron Cooperation, Bremen, Germany) equipped with a CETAC autosampler model ASX-520. Two samples from subcutaneous adipose tissue were excluded due to technical failure (n=5 for both diabetic and control animals of subcutaneous adipose tissue, n=6 for both diabetic and non-diabetic animals of retroperitoneal and epididymal adipose tissue). The accuracy and precision of the analytical procedures for metal levels in adipose tissue were assessed with a simultaneous analysis of a standard reference.

RNA extraction and cDNA synthesis

RNA purification of all tissue was performed using TRIzol (Invitrogen) according to the manufacturer's instructions. 100-200 mg of adipose tissue was used. The integrity of the RNA was confirmed by visual inspection of the two ribosomal RNAs on an ethidium bromide stained agarose gel. The concentration of the purified total RNA was determined spectrophotometrically by the A260/A280 ratio. 450 ng of RNA were reverse transcribed into cDNA using the ImProm-IITM Reverse Transcription System (Promega, Denmark). The cDNA was

checked for genomic DNA contamination by PCR analysis using the Qiagen HotStarTaq Master Mix Kit (VWR, Denmark) with an intron-spanning primer-set of β -actin (TAC, Copenhagen).

Primer design and selected genes

The genome of *P. obesus* is still to be sequenced, but by aligning homologous cDNA sequences from rat, mice, and human we managed to design specific primers for *P. obesus*. The specific primers were designed for *P. obesus* using www.ncbi.nlm.nih.gov. The specific genes of *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens* were compared and similar regions were identified to predict the gene sequence of *P. obesus* (Table 1).

We were able to construct primers for the investigation of the gene expression of *ZnT1*, *ZnT6*, *ZnT7*, *ZnT9*, *ZIP3*, *ZIP6*, *ZIP8*, *ZIP9*, *ZIP11*, *ZIP13*, and *ZIP14*. In addition, the gene expression of *Hormone sensitive lipase (HSL)* and *lipoprotein lipase (LPL)* were investigated as indicators of lipid metabolism. The gene expression of *insulin degrading enzyme (IDE)* was measured as representative of the glucose metabolism alongside the transcription of two main adipocyte markers, *peroxisome proliferator-activated receptor gamma (PPAR γ)* and *leptin*.

Real-time PCR

Quantitative real-time PCR was performed in duplicate with IQ Sybr Green supermix (Bio-rad, Denmark) in a MyiQ Single-Color Real-time PCR detection system (Bio-Rad, Denmark). For all reactions a melting curve was included. The results were analysed with iQTM5 Optical System Software, Version 2.0. Starting quantities were calculated from a standard curve. Values were normalised to the geometric of three house-keeping genes [40,41].

Determination of stable housekeeping genes

The GeNorm method was used to determine the stability of the house keeping genes [41]. All housekeeping genes used in this study were stably expressed according to GeNorm analyses performed on our samples.

Statistical analysis

Real-time PCR data are given as mean starting quantity of (gene of

interest/geometric mean of three housekeeping genes) \pm SEM and trace element measurements as mean value \pm SD. The statistical package GraphPad prism 5 was used. Statistical analyses on the PCR results were done on log-transformed data to obtain normal distribution. Unpaired T-test was used to detect statistical significance when diabetic and non-diabetic *P. obesus* were compared followed by Welch's correction when needed. One-way repeated measurements analysis of variance (ANOVA) was used to detect statistically significant differences when comparing the three adipose tissues in diabetic *P. obesus* or non-diabetic *P. obesus*. Bonferroni's post-test was used following a significant one-way ANOVA analysis. The levels of trace elements were analysed non-parametrically as data were not normally distributed. Mann Whitney test was used when comparing diabetic animals with non-diabetic animals whereas Kruskal-Wallis test followed by Dunn's Multiple Comparison Test was used when comparing the three different kinds of adipose tissue. Mean differences (mean diff) are given with a 95% confidence interval. A standard pearson correlation was used as a measurement of a possible linear correlation between the investigated parameters. Pearsons r-value, indicating the strength of the correlation, as well as the p-value, are given. A p-value < 0.05 was considered significant.

Results

Blood glucose and HbA1c% levels confirmed that high-energy diet induced T2D in the diabetes prone animals

We investigated 6 diabetic animals (diabetes prone) after 5 weeks on high-energy diet. As controls we included 6 non-diabetic animals (diabetes resistant) that were kept on their natural low-energy diet throughout the experiment. The diabetic animals showed increased non-fasting blood glucose levels compared with non-diabetic animals [$p < 0.001$, mean diff: 20.67, 95%CI (14.46; 26.87)], mean non-diabetic: 3.45 ± 0.18 mmol/L, mean diabetic: 24.12 ± 2.78 mmol/L]. A blood glucose level of >10 mmol/L was considered indicative of diabetes. An increase in HbA1c% level was seen in the diabetic animals before and after the experiment [$p = 0.0234$, mean diff: 0.73, 95%CI (0.12; 1.34), mean before: $7.29 \pm 0.19\%$, mean after: $8.02 \pm 0.20\%$].

<i>Cyclophilin A</i>	F: 5' AGG TCC TGG CAT CTT GTC CA 3'. R: 5'CTT GCT GGT CTT GCC ATT CC 3'.
β actin	F: 5'CTA CAA TGA GCT GCG TGT GGC 3'. R: 5'ATC CAG ACG CAG GAT GGC ATG 3'.
<i>HSP</i>	F: 5' GAT TGA CAT CAT CCC CAA CC 3'.R: 5'CTG CTC ATC ATC GTT GTG CT 3'.
<i>ZnT1</i>	F: 5' TGC TGT GCA TGC TGC TGC TG 3'. R: 5'GCG GAT CCA GCC GAA CGT GTT 3'.
<i>ZnT6</i>	F: 5' GCC CTG ATG ACG TTT GGC ACC 3'. R: 5'TGC ACT GAC CCA GCC AAT GAG C 3'.
<i>ZnT7</i>	F: 5' TCC CCA GTG CTA TCA GAG GGT GC 3'. R: 5'GCT GTC TCA CTC CAG CCT GAG T 3'.
<i>ZnT9</i>	F: 5' AAG GGA CCA GGA AAA GTG GT 3'. R: 5'TAC GGG TGA GAA GGA TCT GG 3'.
<i>ZIP3</i>	F: 5' GAA GGC GCA CCG CTC CAA GA 3'. R: 5' TCA GCA CCA GCT GCT CCA CG 3'.
<i>ZIP6</i>	F: 5' GGG CGA CGG GCT GCA CAA TT 3'. R: 5' GGC CAA CAT GGC TGA CAG AGC 3'.
<i>ZIP8</i>	F: 5' GGG GCT CAG TAC GTC CAT AGC GA 3'. R: 5' ACA TGC CTC CAG CGA GTG CA 3'.
<i>ZIP9</i>	F: 5' GCT GGC CTG GAG CGG AAT CG 3'. R: 5' GGG AGG ACA TGG ACG GTG GC 3'.
<i>ZIP11</i>	F: 5' CAG AGG GTC TTG CTG TTG GCG T 3'. R: 5' CCA TGC CGC TCA GCT GTC CA 3'.
<i>ZIP13</i>	F: 5' ACT CAT GGG CTG GCT GTG GC 3'. R: 5' GGC AAA GCA GGC CCC CAG AA 3'.
<i>ZIP14</i>	F: 5' CCC TGA GCG ATG GGC TCC ACA 3'. R: 5' TAG CAG CAG CAG GCA GAG AGG A 3'.
<i>PPARγ</i>	F: 5' CGC ACT GGA ACT AGA TGA CA 3'. R: 5' GGT GAA GGC TCA TGT CCG TC 3'.
<i>LPL</i>	F: 5' CCA GCT GGA CCT AAC TTT GAG 3'. R: 5' CCT CCA TTG GGG TAA ATG TC 3'.
<i>HSL</i>	F: 5' GGC TTT GTG GCA CAG ACC TC 3'. R: 5' CCA GGA AGG AGT TGA GCC AT 3'.
<i>Leptin</i>	F: 5' TCG CGG TCT ACC AAC ACA TC 3'. R: 5' TCT CTA GGT CGT TGG ATA TTT GCA 3'.
<i>IDE</i>	F: 5' GCA GCC TCA CCA GCA TGC CA 3'. R: 5' ACT CCT AAG GCA GCC TGC TTG G 3'.

Table 1: Primers designed for *P. obesus*. Forward and reverse primer sequences.

In diabetic animals, an altered distribution of zinc was seen between visceral adipose tissues. The Zn/Cu ratio was however not affected

Comparing diabetic and non-diabetic animals, indications of a reduced zinc content ($p=0.09$) were seen only in epididymal adipose tissue, whereas no signs of differences were found in retroperitoneal or subcutaneous adipose tissue (Figure 1).

Comparing the distribution of zinc among different adipose tissues, differences were seen comparing diabetic and non-diabetic animals. A significant difference was thus seen between epididymal adipose tissue and retroperitoneal adipose tissue ($p=0.006$) in the diabetic animals, with a higher level of zinc in retroperitoneal adipose tissue. The non-diabetic animals on the other hand, displayed no significant differences in the zinc levels when comparing the three different adipose tissues.

Calculations of the Cu/Zn ratio showed no alterations between diabetic and non-diabetic animals or between the different kinds of adipose tissue.

In conclusion, T2D induction affected the distribution of zinc between the two visceral adipose depots: epididymal adipose tissue and retroperitoneal adipose tissue.

An accumulation of iron was found in epididymal adipose tissue in diabetic animals

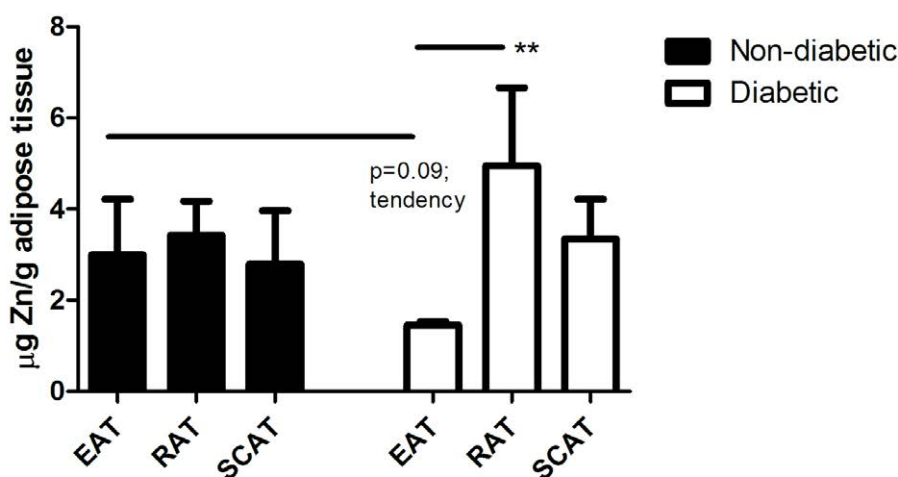
An accumulation of iron was found in epididymal adipose tissue upon T2D development as diabetic animals had a significantly higher Fe content in epididymal adipose tissue compared to non-diabetic animals ($p=0.0152$).

Quantities of the Zn, Fe and the Cu/Zn ratio are shown in Table 2.

Altered transcriptional regulations of zinc transporters were found in visceral adipose tissue from diabetic animals

Distinct T2D-induced changes in the gene expression of zinc transporters of the ZnT and ZIP families were seen in visceral adipose tissue. In retroperitoneal adipose tissue, *ZnT9* ($p=0.0161$), *ZIP8* ($p<0.0001$), and *ZIP9* ($p=0.0036$) were all transcriptionally down-regulated in diabetic animals compared with non-diabetic counterparts, whereas *ZIP6* gene expression was up-regulated in both retroperitoneal adipose tissue ($p=0.0400$) and epididymal adipose tissue ($p=0.0442$). The gene expression of the only known extra-cellular efflux transporter *ZnT1* as well as the gene expression of *ZnT6* and *ZnT7* were unaffected by the induction of diabetes as were *ZIP3*, *ZIP11*, *ZIP13*, and *ZIP14*.

No significant differences were seen when comparing the three



*= P value < 0.05; **= P value < 0.01, ***= P value < 0.001.

Figure 1: Zinc content in adipose tissue. The content of zinc in epididymal adipose tissue (EAT), retroperitoneal adipose tissue (RAT) and subcutaneous adipose tissue (SCAT) from non-diabetic animals (non-diabetic) and diabetic animals (diabetic). Results are expressed as µg zinc/g adipose tissue (µg Zn/g adipose tissue).

Trace element	Epididymal adipose tissue		Retroperitoneal adipose tissue		Subcutaneous adipose tissue	
	ND	D	ND	D	ND	D
Zn	3.0 (± 1.2)	1.4 (± 0.1)	3.4 (± 0.7)	5.0 (± 1.7) ** Compared with epididymal adipose tissue from D.	2.8 (± 1.2)	3.3 (± 0.9)
Cu	2.7 (± 4.9)	0.4 (± 0.2)	0.9 (± 0.6)	1.0 (± 0.6)	1.4 (± 1.8)	2.7 (± 3.5)
Fe	8.9 (± 2.2)	12.4 (± 1.6) * Compared with epididymal adipose tissue from ND.	26.8 (± 23.8)	19.6 (± 12.6)	21.6 (± 15.4)	19.4 (± 15.7)
Cu/Zn ratio	0.57 (± 0.45)	0.27 (± 0.09)	0.31 (± 0.22)	0.21 (± 0.09)	0.41 (± 0.14)	0.60 (± 0.47)

ND= Non-diabetic animals. D= Diabetic animals.

*= P value < 0.05; **= P value < 0.01, ***= P value < 0.001.

Table 2: Content of zinc (Zn), iron (Fe), copper (Cu) and the calculated Cu/Zn ratio in adipose tissue from *P. obesus*. Contents are shown as µg trace element/g adipose tissue ± std. deviation.

adipose tissues in diabetic animals. In non-diabetic animals, however, a higher gene expression of *ZIP6* was found in subcutaneous adipose tissue compared with epididymal adipose tissue ($p=0.0247$).

The gene expression patterns of the measured *ZnTs* are shown in Figure 2 and the gene expression patterns of the measured *ZIPs* in Figure 3.

In conclusion, altered gene expression of zinc transporters as a result of T2D was found only in visceral adipose tissue, especially retroperitoneal adipose tissue. Of the investigated zinc transporters; three zinc transporters were transcriptionally down-regulated in retroperitoneal adipose tissue (*ZnT9*, *ZIP8*, *ZIP9*), whereas one (*ZIP6*) was up-regulated in both retroperitoneal adipose tissue and epididymal adipose tissue.

Reduced gene expression of metabolic parameters in diabetic animals vs. non-diabetic animals – different patterns in visceral and subcutaneous adipose tissue

LPL, *IDE*, *PPAR γ* , *HSL*, and *leptin* were uniformly expressed in subcutaneous and visceral (retroperitoneal and epididymal) adipose

tissue in both diabetic and non-diabetic *P. obesus*. However, comparing diabetic and non-diabetic animals – several differences in metabolic parameters were observed.

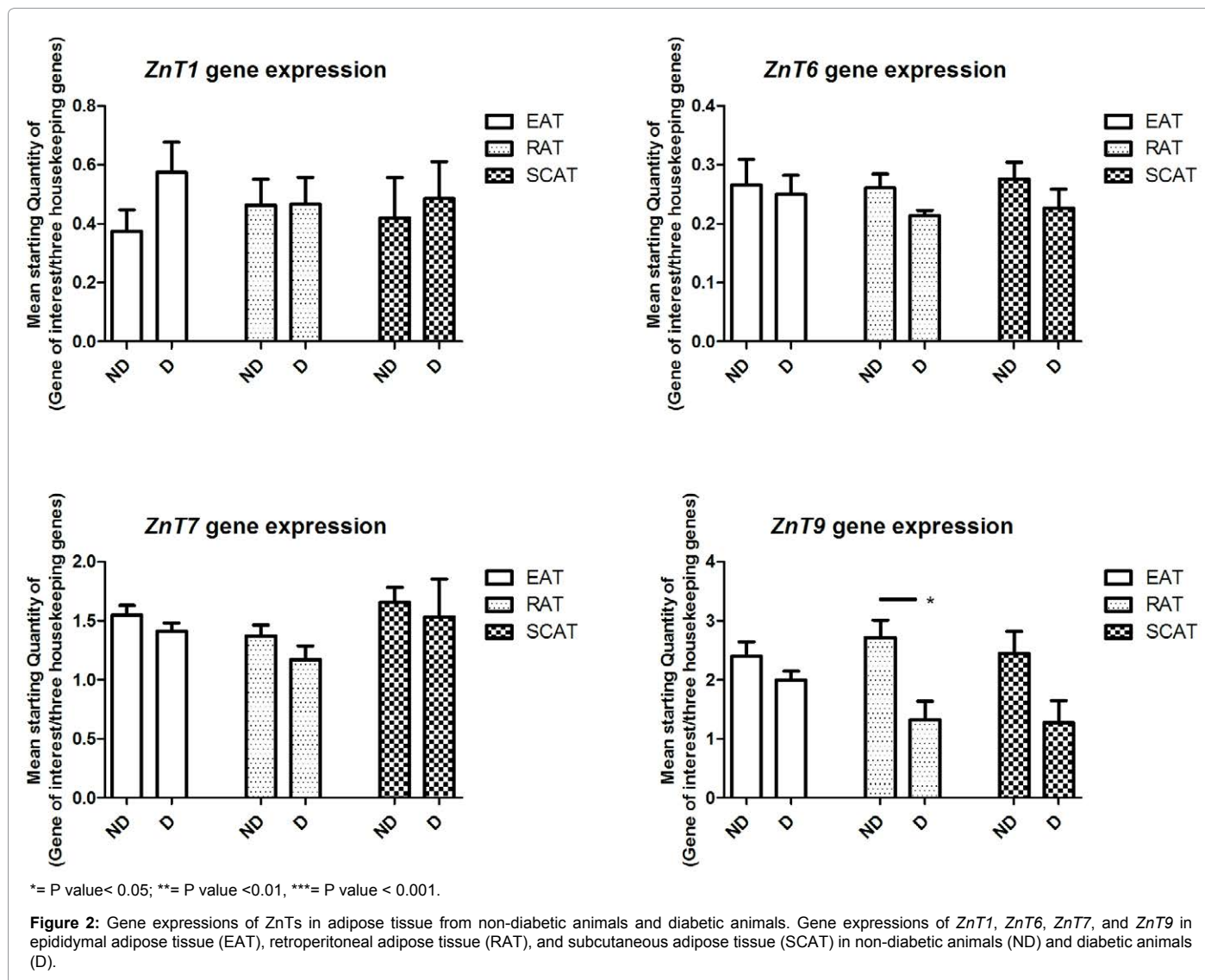
Compared to non-diabetic controls, induction of diabetes resulted in a reduced gene expression of *LPL* in all 3 types of adipose tissue; epididymal adipose tissue ($p=0.0169$), retroperitoneal adipose tissue ($p=0.0061$), and subcutaneous adipose tissue ($p=0.0178$).

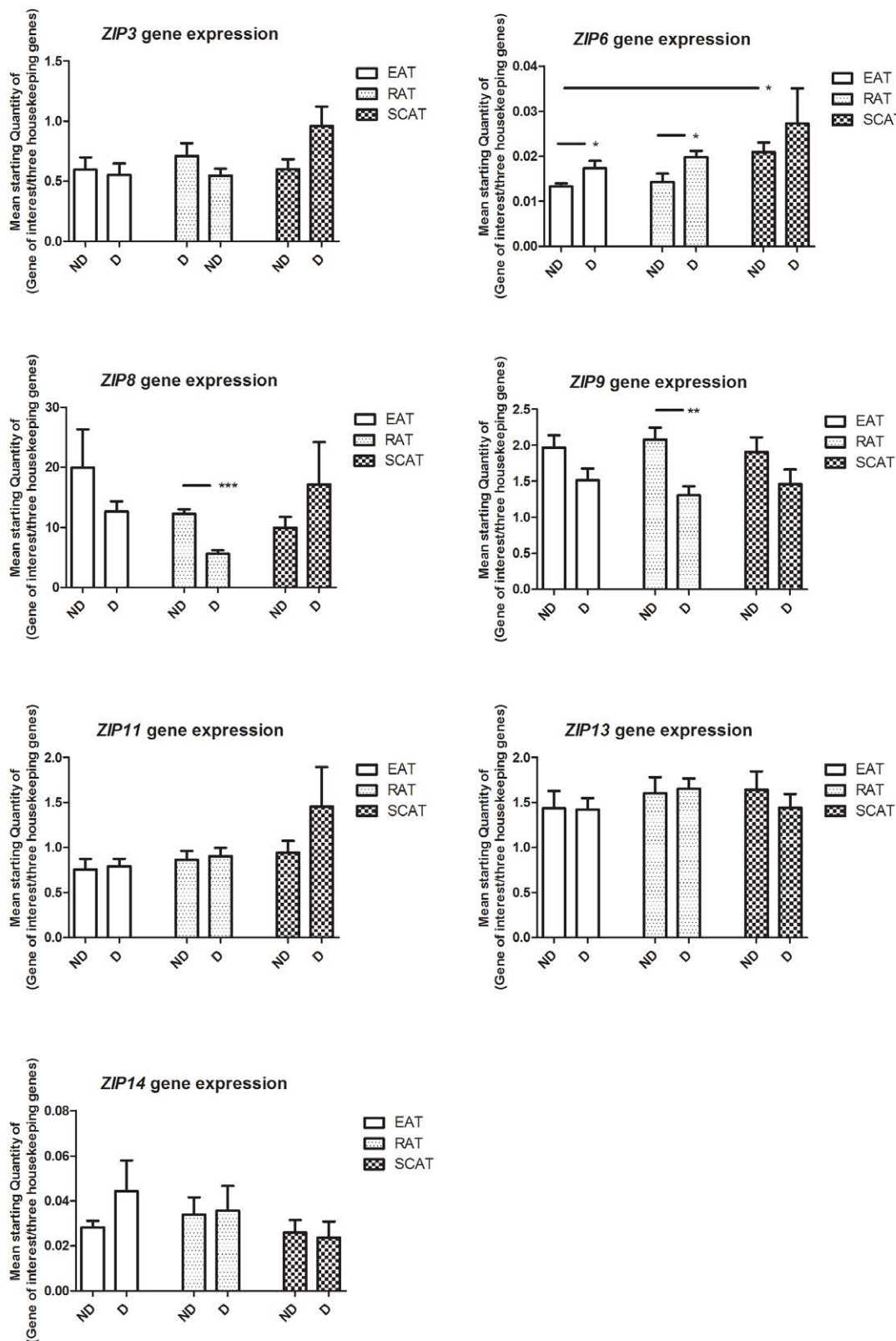
In addition, the gene expression of *IDE* was reduced in retroperitoneal adipose tissue ($p=0.0098$), whereas *PPAR γ* was reduced in subcutaneous adipose tissue ($p=0.0041$) but not in other types of adipose tissue. No changes were observed for *HSL* or *leptin*.

The gene expressions of the various metabolic markers are shown in Figure 4.

Zinc concentrations correlated with gene expression of zinc transporter protein *ZIP6* in diabetic animals

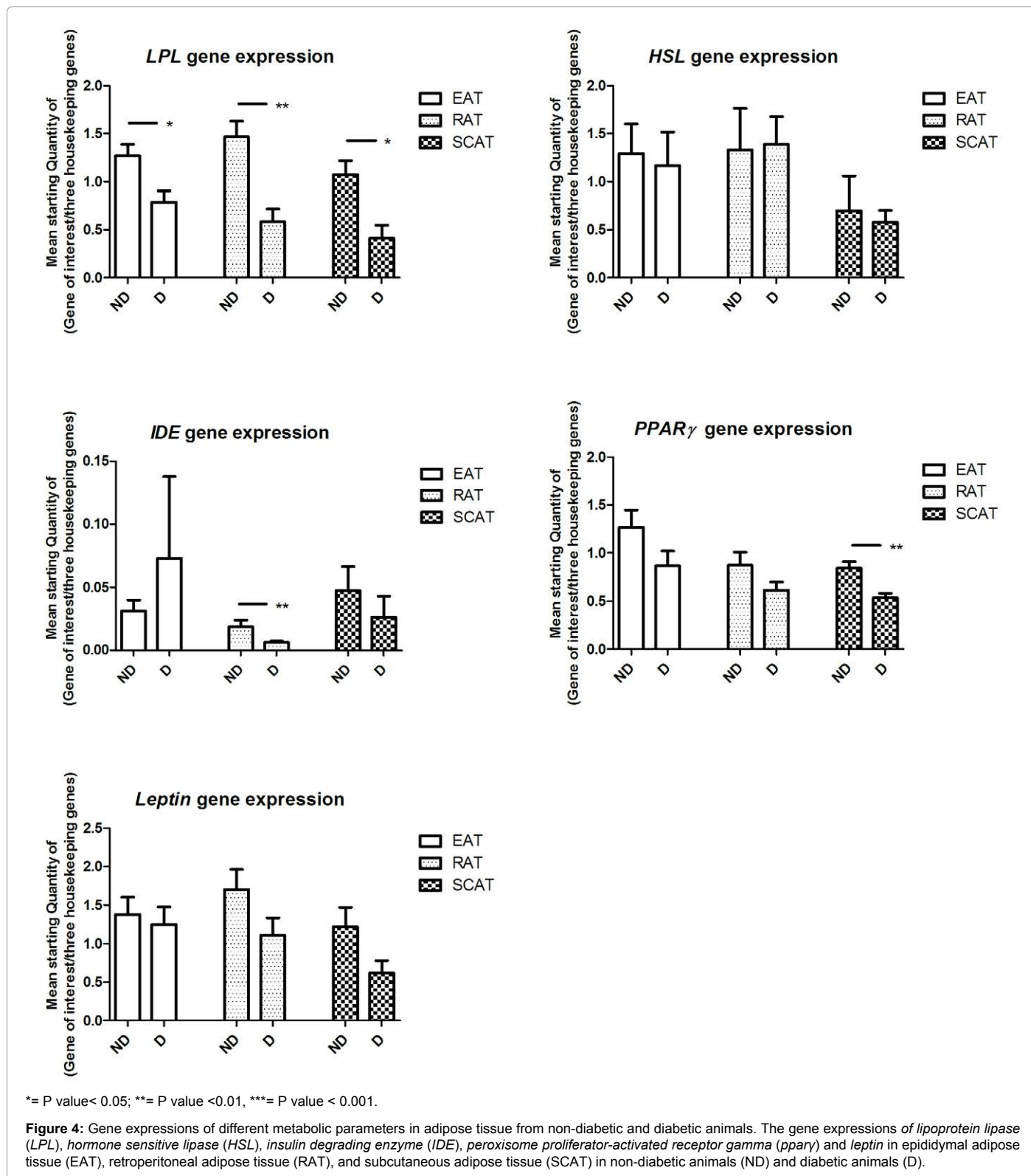
As the zinc transporters showed a high degree of regulation in visceral adipose tissue, we correlated the Zn levels with the gene expression level of *ZIP6*, a plasma membrane located zinc transporter.





*= P value < 0.05; **= P value < 0.01, ***= P value < 0.001.

Figure 3: Gene expressions of ZIPs in adipose tissue from non-diabetic animals and diabetic animals. Gene expressions of ZIP3, ZIP6, ZIP8, ZIP9, ZIP11, ZIP13, and ZIP14 in epididymal adipose tissue (EAT), retroperitoneal adipose tissue (RAT), and subcutaneous adipose tissue (SCAT) in non-diabetic animals (ND) and diabetic animals (D).



A significant correlation was found between the gene expression of *ZIP6* and the Zn content in retroperitoneal adipose tissue in the diabetic animals ($r=0.91$, $p=0.01$). The correlation was not present in the non-diabetic animals neither in epididymal adipose tissue (Table 3).

Iron levels correlated with an altered gene expression of *IDE* and *PPAR γ*

A standard Pearson correlation was used as a measurement of a possible linear correlation between the investigated parameters i.e. the

	Epididymal adipose tissue		Retroperitoneal adipose tissue	
	ND	D	ND	D
Correlation between zinc transporter ZIP6 and Zn content	r=-0.22 p=0.68	r=0.37 p=0.47	r=-0.66 p=0.15	r=0.91 p=0.01 *

*= P value < 0.05; **= P value < 0.01, ***= P value < 0.001.

Table 3. Correlations between zinc (Zn) content and the gene expression of zinc transporter ZIP6 in adipose tissue from *P. obesus*. ND= Non-diabetic animals. D= Diabetic animals. Correlations were performed using Pearson correlations. Pearson's r value and p-value are indicated in the table.

	Fe content	Zn content
IDE	r=0.87 p=0.02 *	r=0.38 p=0.46
PPARγ	r=0.90 p=0.01*	r=0.32 p=0.53
LPL	r=0.22 p=0.67	r=-0.28 p=0.59
HSL	r=- 0.78 p=0.06	r=-0.50 p=0.31
Leptin	r=0.60 p=0.21	r=-0.54 p=0.27

*= P value < 0.05; **= P value < 0.01, ***= P value < 0.001.

Table 4: Correlations between metabolic markers and iron and zinc content in epididymal adipose tissue. Correlations of iron (Fe) and zinc (Zn) content with metabolic markers; *insulin degrading enzyme (IDE)*, *peroxisome proliferator-activated receptor gamma (PPAR γ)*, *lipoprotein lipase (LPL)*, *hormone sensitive lipase (HSL)*, and *leptin* in diabetic animals. Correlations were performed using Pearson correlations. Pearson's r value and p-value are indicated in the table.

gene expression of selected genes and the actual concentrations of Fe and Zn. In this way correlating the changes observed of Fe in epididymal adipose tissue from diabetic animals with the measured metabolic markers showed a significant positive correlation with *IDE* (r=0.87, p=0.02) and *PPAR γ* (r=0.90, p=0.01). No significant correlations were found in regard of *LPL*, *HSL*, or *leptin* and neither when correlating Zn with the metabolic markers (Table 4).

Discussion

In the present study we examined the multi-factorial animal model of T2D, *P. obesus*, for the first time investigating if the zinc and iron levels in adipose tissue of these animals respond to the development of metabolically elicited diabetes in the genetically susceptible animals. The development of T2D in *P. obesus* is comparable with human T2D in many ways: it is partly diet-induced but shows a genetic component, insulin resistance is present in muscle tissue, and many of the disease stages are similar, making this model of particular interest for the investigation of obesity-induced T2D [42,43].

We found evidence of changes within the total content of Fe. These changes correlated with an increased gene expression of the metabolic markers *IDE* and *PPAR γ* , supporting other reports that iron participates in adipose tissue metabolism [44]. Intracellular signs of an altered zinc homeostasis were seen, as gene expression patterns of several zinc transporters of both the ZnT and ZIP family were affected. In addition, significant differences in total zinc content were seen between various types of adipose tissue in the diabetic animals. The changes observed, were found in retroperitoneal and epididymal adipose tissue, thus indicating that intracellular zinc signalling could play a role in the development of metabolic disease in visceral fat [3].

The fact that intracellular zinc homeostasis is affected in adipose tissue upon weight gain has previously been shown by Smidt and co-workers. The study investigated human adipose tissue from obese

individuals, and a consistent transcriptional down-regulation of zinc transporters of both families (ZnTs and ZIPs) were found in adipose tissue from obese individuals compared to lean controls [31]. The present study shows that an altered regulation in the gene expression of zinc transporters is not only found in obesity but also in T2D.

We found a transcriptional down-regulation of *ZnT9*, *ZIP9*, and *ZIP8* in retroperitoneal adipose tissue. This expression pattern of *ZIP8* is consistent with the findings of Smidt and co-workers, whereas it is the first time that *ZnT9* and *ZIP9* have been investigated in adipose tissue.

Genetic variations of *ZIP8* have previously been associated with obesity, and the present study indicates that regulation of *ZIP8* gene expression could be an upstream event of zinc dyshomeostasis in metabolically-stressed adipocytes [45].

In addition to *ZIP8*, the gene expression of *ZIP6* also responds to metabolic changes in *P. Obesus*, being up-regulated in visceral adipose tissue from diabetic animals. As *ZIP6* is primarily located at the plasma membrane, we correlated the zinc level in visceral adipose tissue with *ZIP6* gene expression, and found a significant positive correlation in the retroperitoneal adipose tissue from diabetic animals, implying a physiological consequence of the diabetes-induced up-regulation of *ZIP6* gene expression on the Zn level. We have previously found *ZIP6* to be down-regulated in obese women compared to lean controls [31]. This study did however not include diabetic individuals. However, it cannot be excluded that differences between humans and other species also contribute to the difference in *ZIP6* gene regulation.

In this study, we found that whereas the non-diabetic animals showed equal zinc distribution between the three fat depots, the diabetic animals had a significantly lower zinc level in epididymal adipose tissue compared to retroperitoneal adipose tissue. Retroperitoneal and epididymal adipose tissue are however metabolically different visceral adipose depots, as epididymal adipose tissue is likely to be more affected by reproductive hormones.

The intracellular zinc pool is a mixture of both free and protein bound zinc. The total amount of intracellular zinc reaches micro-molar concentration levels whereas free intracellular zinc is found only in pico-molar concentrations. This reflects the high amount of protein-bound zinc. Accordingly, alterations in protein-bound zinc might camouflage small alterations in free zinc when comparing diabetic and non-diabetic sand rats [46]. Similarly, as many proteins harbour zinc, quantitative measurements of protein-bound zinc might disguise different patterns of protein expression. In general, zinc deficiency needs to be quite severe before any actual changes in mineral content are detected in various tissues [47]. As the free zinc is as low as pico-molar concentrations, even small alterations in ambient zinc content could be reflected by intracellular zinc dys-homeostasis and hence dysfunctional intracellular zinc signalling, and at least *in vitro* changes in ambient zinc are directly reflected in the intracellular zinc regulation [48].

Supporting this, we found that a type 2 diabetic environment leads to a transcriptional down-regulation of *ZnT9*, *ZIP8*, and *ZIP9* in visceral adipose tissue, as well as a transcriptional up-regulation of *ZIP6*. Whether this pattern constitutes the complete intracellular response to the altered mineral status of the diabetes prone *P. Obesus* remains to be seen. As the genome of this animal model is currently not sequenced, all primers for the present study had to be designed from scratch based on primers from other animal models and we still need to establish if any of the primers currently not functioning does in fact

reflect that one or more zinc transporters of either the ZnT or the ZIP family are not expressed in *P. obesus*.

An altered cellular zinc homeostasis due to alterations in the expression of zinc transporters in T2D could have several adverse metabolic consequences if reflected by altered protein expression. Both zinc accumulation and zinc deprivation may result in apoptosis and hence loss of beta cell mass. Moreover zinc deficiency within the cell is known to cause oxidative damage [49] which could further accelerate beta-cell death.

Metabolic intracellular regulation is also zinc dependent, as zinc shows many insulinomimetic properties in adipose tissue. Zinc thus stimulates glucose uptake and lipogenesis, and inhibits the release of free fatty acids through its stimulation of the insulin signalling pathway [9]. Changes in the gene expression of ZIP and ZnT transporters could thereby induce oxidative or apoptotic damage within the cell as well as induce an altered insulin signalling affecting the metabolic regulation of the adipocyte.

We also saw a significant down-regulation of ZIP9 gene expression in diabetic animals compared to non-diabetic animals. At present, little is known about ZIP9. However, as ZIP9 is localized in the trans-Golgi network supposedly transporting zinc from Golgi to the cytosol, it could be speculated that the down-regulation of this transporter could serve as a way of ensuring sufficient presence of zinc in the Golgi apparatus [50-55].

Induction of diabetes was also reflected in a down-regulation of ZnT9 gene expression in retroperitoneal adipose tissue. Not much is known about the zinc transporting functions of ZnT9 besides its localization in the cytoplasm in human cells [56].

The altered metabolic activity in the high-calorie challenged diabetes-prone *P. obesus* was also reflected in signs of a reduced insulin sensitivity of the adipose tissue. The gene expression of *IDE* was hence reduced in retroperitoneal adipose tissue in diabetic animals compared to control animals. Down-regulation of *IDE* indicates elevated insulin levels signifying an insulin resistance in the tissue [57]. Although, *IDE* belongs to a family of zinc-metalloproteases and is regulated by the Zn level present in the tissue, *IDE* gene expression was not directly correlated to zinc concentrations in this study [18]. Instead, we found a significant correlation between *IDE* and iron homeostasis i.e. high iron concentration correlated with a high level of *IDE* gene expression, indicating an increased insulin metabolism in iron-rich environments in the diabetic animals.

The high iron content found in the present study is in line with recent discovery done by Orr and co-workers also finding an accumulation of iron in epididymal adipose tissue from mice fed a high-energy diet [58]. Our study is the first to show that this iron accumulation seems to be specific for epididymal adipose tissue when comparing epididymal, retroperitoneal, and subcutaneous adipose tissues. The iron accumulation in epididymal adipose tissue likely induces impaired insulin signalling and insulin resistance as shown in C57BL/6 mice fed an iron-enriched diet [44].

The content of iron in epididymal adipose tissue was also positively correlated with the gene expression of *PPAR γ* [18]. *PPAR γ* is the main regulator of adipogenesis and serves as a transcription factor controlling genes involved in both lipid metabolism and adipogenesis [59,60]. The low *PPAR γ* gene expression level in subcutaneous fat seen in the diabetic animals could be an indication of decreased adipogenesis in these depots. The ability to form new adipocytes in

response to excess energy intake is hypothesized to be important for a healthy adipose tissue expansion, as expansion which is primarily driven by enlargement of the individual adipocytes results in insulin resistance and hypoxia [61].

In conclusion; this study supports the hypothesis that dysregulation of mineral homeostasis is part of the pathology of T2D, at least when induced by a combination of genetic and dietary factors, emphasizing the role of zinc and iron in the function of adipose tissue in general and in visceral adipose depots in particular. Diabetic animals displayed increased iron concentration in adipose tissue which directly correlated with altered expression of *IDE* and *PPAR γ* . Alterations in *PPAR γ* gene expression also support the hypothesis that a down-regulation of subcutaneous adipogenesis could participate in metabolic stress in the *P. obesus* model. Moreover, shifts in total zinc between epididymal and retroperitoneal visceral adipose depots, accompanied by changes in expression of zinc transporters, indicate that changes in intracellular zinc homeostasis participate in altered adipocyte functioning during metabolic stress.

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