The Effect of Very-Low-Calorie Diet on Mitochondrial Dysfunction in Subcutaneous Adipose Tissue and Peripheral Monocytes of Obese Subjects with Type 2 Diabetes Mellitus

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Short title: Mitochondrial dysfunction in obesity and T2DM

Summary

Mitochondrial dysfunction is a potentially important player in the development of insulin resistance and type 2 diabetes mellitus (T2DM). We investigated the changes of mRNA expression of genes encoding main enzymatic complexes of mitochondrial respiratory chain in subcutaneous adipose tissue (SCAT) and peripheral monocytes (PM) of 11 subjects with simple obesity (OB), 16 obese patients with T2DM and 17 healthy lean subjects (C) before and after very low-calorie diet (VLCD) using quantitative real time PCR. At baseline in SCAT, both T2DM and OB group had decreased mRNA expression of all investigated mitochondrial genes with the exception of 2 complex I (NDUFA12) and complex IV (COX4/1) enzymes in OB subjects. In contrast, in PM only the expression of complex I enzymes NDUFA12 and MT-ND5 was reduced in both T2DM and OB subjects along with decreased expression of citrate synthase (CS) in T2DM group. Additionally, T2DM subjects showed reduced activity of pyruvate dehydrogenase and complex IV in peripheral blood elements. VLCD further decreased mRNA expression of CS and complex I (NT-ND5) and II (SDHA) enzymes in SCAT and complex IV (COX4/1) and ATP synthase in PM of T2DM group, while increasing the activity of complex IV in their peripheral blood elements. We conclude that impaired mitochondrial biogenesis and decreased activity of respiratory chain enzymatic complexes was present in SCAT and PM of obese and diabetic patients. VLCD improved metabolic parameters and ameliorated mitochondrial oxidative function in peripheral blood elements of T2DM subjects but had only

minor and inconsistent effect on mitochondrial gene mRNA expression in SCAT and PM.

Key words: mitochondrial dysfunction, obesity, type 2 diabetes mellitus, very low-calorie diet, peripheral monocytes.

Introduction

Obesity and type 2 diabetes mellitus (T2DM), frequently interconnected within the metabolic syndrome, are among the leading causes of morbidity worldwide (Alberti and Zimmet 2013). Currently, the number of diabetic patients has reached pandemic levels; approximately 382 million people worldwide, or 8.3% of adults, suffer from diabetes, out of which 80 - 95% account for T2DM (IDF, 6th Diabetes Atlas, 2013). Type 2 diabetes is a well-known risk factor for atherosclerosis and subsequent cardiovascular and cerebrovascular diseases as well as for specific types of cancer (Paneni et al. 2014, Laakso and Kuusisto 2014, Vigneri et al. 2009, Matloch et al. 2016).

The main pathophysiological features of T2DM are insulin resistance and hyperglycemia (Stumvoll and Gerich 2001). Despite extensive research, the exact cause of insulin resistance is still not known, although a number of mechanisms are being considered (Wajchenberg 2000, Indulekha et al. 2011, Rasouli and Kern 2008). Particularly, endocrine dysfunction of adipose tissue, mainly the visceral adipose tissue (VAT), and chronic subclinical inflammation are the primary suspects contributing to decreased insulin sensitivity and impaired insulin production in pancreatic ß-cells (Scrapellini 2012). Moreover, recent studies have revealed a possible connection between pancreatic ß-cell dysfunction, insulin resistance and defects in mitochondrial metabolic processes (Sivitz and Yorek 2010).

Mitochondria are evolutionary ancient organelles located in the cytosol of eukaryotic cells. Described as “cellular power plants”, they play a central role in cell metabolism by generating

energy in the form of ATP (adenosine-tri-phosphate) from substrates such as saccharides and fatty acids in the process of oxidative phosphorylation (Duchen 2004). The oxidative capacity of mitochondria is determined by the expression levels of enzymatic complexes involved in oxidative phosphorylation, although the size and number of mitochondria are also of importance (Ritz and Berrut 2005). In simple obesity, the expression of genes encoding components of mitochondrial oxidative phosphorylation as well as mitochondrial oxidative phosphorylation capacity in white adipose tissue were found to be reduced along with an inverse correlation of this capacity with BMI (Mustelin et al. 2008, Fischer et al. 2015). In females, obesity and insulin resistance were also associated with decreased expression of mitochondrial complex I, III and IV components in both subcutaneous and visceral adipose tissue (Soronen et al. 2012). Furthermore, subjects with T2DM were shown to have reduced amount and smaller-sized mitochondria as well as impaired mitochondrial oxidative activity due to decreased expression of the mitochondrial respiratory chain enzymes in skeletal muscle and pancreatic ß-cells, resulting in the development of insulin resistance and ß-cell dysfunction (Morino et al. 2005, Kelley et al. 2002, Petersen et al. 2004, Kraunsoe et al. 2010, Mootha et al. 2003). However, little is known about mitochondrial function in white adipose tissue of obese diabetic patients. Moreover, very limited data currently exist regarding the mitochondria in peripheral monocytes, which are considered one of the main contributors to the development of local as well as systemic low-grade inflammation associated with obesity and insulin resistance (Hotamisligil 2006, Suganami et al. 2005, Del Pozo et al. 2011). To this end, we assessed the mRNA gene expression and enzymatic activity of components of the mitochondrial respiratory chain in subcutaneous adipose tissue (SCAT) and peripheral monocytes (PM) of subjects with T2DM and obesity. Furthermore we hypothesized that the improvement of metabolic parameters after short-term diet intervention could be in part mediated by changes in mitochondrial oxidative function and gene expression in SCAT and PM.

Materials and Methods

Study subjects

Eleven subjects with simple obesity (9 females, 2 males - OB group), 16 obese patients with type 2 diabetes mellitus (13 females, 3 males - T2DM group) and 17 healthy lean age- matched control subjects (12 females, 5 males - C group) were included in the study. Six subjects in T2DM group were treated with oral antidiabetic drugs (OAD), 5 were using a combination of OAD and insulin and 5 were on diet only. Fourteen out of the 16 subjects received antihypertensive treatment, 5 were on lipid-lowering therapy (4 on a statin, 1 on a combination of statin and fibrate) and 3 had thyroid hormone replacement therapy. In OB group 5 subjects were treated with antihypertensives, 2 with a statin and 3 with thyroid hormone replacement agents. All treatment remained unchanged for at least three months prior to the start of the study. Control subjects had no history of obesity and/or diabetes mellitus, arterial hypertension, or lipid metabolism disturbances and received no medication. Blood tests confirmed normal blood count, biochemical and hormonal parameters. None of the study subjects had active malignancy or acute infectious disease. The body weight of all study participants remained stable for at least three months before enrollment into the study.

All subjects in T2DM group underwent a routinely used 3-week very low-calorie diet (VLCD) with energy intake of 2500kJ per day (600 kcal per day) (Grams and Garvey 2015) at the Third Department of Medicine, General University Hospital in Prague. The diet was composed of 55 g of proteins, 50 g of saccharides and 20 g of fats divided into three main meals and was supervised by a dietitian nurse.

Written informed consent was signed by all participants before the beginning of the study. The study was approved by Human Ethics Review Board, First Faculty of Medicine and General University Hospital, Prague, Czech Republic and was performed in accordance with the

guidelines proposed in Declaration of Helsinki.

Anthropometric examination, blood and adipose tissue sampling

All patients with T2DM were examined twice; at baseline before the beginning of any intervention and after 3 weeks of VLCD, while obese and normal-weight healthy subjects were examined only once. All subjects were measured and weighed, and their body mass index (BMI) was calculated. Blood samples for biochemical and hormonal measurements were withdrawn between 07.00h and 08.00h after 12 h of overnight fasting. Blood samples were separated by centrifugation for 10 min at 1000 x g within 30 min from blood collection. Serum was subsequently stored in aliquots at -80°C until further analysis. Blood samples for monocyte isolation were collected in Na-EDTA anticoagulant from all subjects and processed within 1-2 h. Samples of subcutaneous adipose tissue for mRNA expression analysis were obtained from abdominal region using subcutaneous needle aspiration biopsy. Approximately 100 mg of adipose tissue was collected to 1 ml of RNA stabilization reagent (RNAlater, Qiagen, Hilden, Germany) and stored at - 80°C until further analysis.

Hormonal and biochemical assays

Serum insulin concentrations were measured by commercial RIA kit (Cis Bio International, Gif-sur-Yvette, France). Biochemical parameters (glucose, total and HDL-cholesterol, triglycerides) were measured at the Department of Biochemistry of General University Hospital in Prague by standard laboratory methods. The value of LDL-cholesterol was calculated according to Friedewald formula (Friedewald et al., 1972). The homeostasis model assessment (HOMA) was calculated as HOMA-IR index using the following formula: fasting serum insulin (mIU/l) x fasting serum glucose (mmol/l)/22.5. Glycated hemoglobin was analyzed by high performance liquid chromatography (HPLC) on Variant II BioRad analyzer (BioRad).

PM separation and total RNA isolation from monocytes and adipose tissue

Peripheral blood leukocytes were obtained from blood samples using Ficoll-Paque™ Plus (Amersham Biosciences AB, Sweden). For each blood sample, 3.5 ml of Ficoll-Paque™ Plus was placed in a Falcon tube, and then 5 ml of blood sample was slowly added. Immediately after this preparation, tubes were centrifuged. After centrifugation, leukocyte agglomerates were placed in a tube containing 10 ml of PBS (0.01 M PBS), pH 7.4. Tubes were centrifuged again, the supernatant was discarded, and the cell pellet was dissolved in PBS. After centrifugation the supernatant was discarded and the pellet was dissolved in DE-GAS buffer (0.01 M PBS with O.5 M EDTA, pH 8 and 1% BSA). Monocytes were isolated from the cell pellet with magnetic activated cell sorting technique (MiniMacs Miltenyi Biotec, Bergisch Gladbach, Germany) using microbeads coated with CD14 antibody (MACS CD14 MicroBeads; Miltenyi Biotec). Total RNA was extracted from CD14+ monocyte samples on MagNA Pure instrument using MagNA Pure Compact RNA Isolation kit (Roche Diagnostics GmbH, Germany). Samples of SCAT were homogenized on a MagNA Lyser Instrument using MagNA Lyser Green Beads (Roche Diagnostics GmbH). Total RNA was extracted from the homogenized sample using RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden,Germany), and cDNA was prepared as described in detail previously (Dolezalova et al. 2007).

Mitochondrial enzyme activity assay

Platelets were isolated from 9 ml of blood with citrate by differential centrifugation according to Fox et al. (Fox et al. 1992) without addition of prostacyclin. Platelet protein was determined by the method of Lowry et al. (Lowry et al. 1951). The activities of respiratory chain complexes NADH-coenzyme Q_1o reductase (NQR, complex I), succinate-coenzyme Q_1o reductase (SQR, complex II), cytochrome *c* oxidase (COX, complex IV), NADH-cytochrome *c* reductase (NCCR, complex I+III) and the activity of citrate synthase (CS) serving as control enzyme were measured spectrophotometrically (Bohm et al. 2003; Srere 1969). All spectrophotometric measurements were performed in 1 ml cuvettes (1 cm, 37°C) using double beam

spectrophotometer Shimadzu UV-160. 100 pg of platelet protein was used for each enzyme assay. Each value represents the mean of 2 measurements on each platelet sample. The ratio between activity of individual respiratory chain complexes and CS was calculated to eliminate a possible effect of changes in the number of mitochondria in patient cells (Gellerich et al. 2002). Lymphocytes were isolated from EDTA-treated peripheral blood on a Ficoll medium using lymphopack tubes (Sigma) by centrifugation for 15 min. at 800g. Isolated lymphocytes were resuspended in phosphorus buffer saline, rapidly frozen and stored in -80 ^{o}C before the measurements. The activity of the pyruvate dehydrogenase complex (PDH) was estimated as the production of $^{14}CO_2$ generated by decarboxylation of [1-^{14}C]- pyruvate (Constantin-Teodosiu et al. 1991). All investigated genes and enzymes are summarized in Table 1.

Quantitative real-time PCR

mRNA expression of selected genes was determined as described in detail elsewhere (Dolezalova et al. 2007).

Statistical analysis

Statistical analysis was performed on SigmaStat software (Systat Inc., Chicago, IL). Anthropometric, biochemical, and hormonal results are expressed as means ± SEM. Differences in gene expression and serum parameters between study groups were evaluated using one-way ANOVA, or ANOVA on Ranks, as appropriate. Differences between T2DM subjects before and after VLCD were evaluated using paired t-test or Wilcoxon Signed-Rank test as appropriate. Statistical significance was assigned to $P<0.05$. The Benjamini-Hochberg correction for false discovery rate was used for multiple testing of mRNA gene expression profiles in both PM and SCAT (Benjamini and Hochberg 1995).

Results

Biochemical, anthropometric and hormonal characteristics of study subjects

a) at baseline

Anthropometric, biochemical and hormonal characteristics of all study groups are summarized in Table 1. As expected, both OB and T2DM groups had significantly higher body weight, BMI and waist circumference as well as elevated fasting insulin, HOMA index and triglyceride levels relative to control group, with BMI and waist circumference being even more increased in T2DM compared with non-diabetic OB subjects. T2DM patients had also markedly elevated fasting glucose and glycated hemoglobin relative to both control and OB groups. There was no significant difference in total and LDL-cholesterol in obese nondiabetic or T2DM patients compared with healthy lean controls, while HDL cholesterol was lower in T2DM than in control subjects.

b) influence of VLCD

After 3 weeks of very low-calorie diet (VLCD) there was an overall improvement in anthropometric and biochemical parameters in the T2DM group with decreased body weight, BMI and waist circumference and a reduction in serum fasting glucose and HOMA-IR. Total and LDL cholesterol were also markedly decreased relative to baseline levels, while no significant change in HDL cholesterol and triglycerides could be seen after dietary intervention. Glycated hemoglobin was not assessed after VLCD due to its relatively short duration (Table 2).

mRNA expression of respiratory chain enzyme complexes in SCAT and PM of OB and T2DM subjects

a) at baseline

mRNA expression of selected respiratory chain enzymes in SCAT and PM of OB and T2DM groups is summarized in Table 2. In SCAT of T2DM subjects mRNA expression of all studied enzymes was markedly decreased at baseline relative to control group. Similarly, OB subjects had significantly reduced mRNA expression of almost all enzymes compared with lean healthy controls except of cytochrome c oxidase subunit IV isoform 1 (COX 4/1) and NADH

dehydrogenase 1 alpha subcomplex 12 (NDUFA 12), where the tendency to lower values did not reach statistical significance. In contrast, no major difference between diabetic and non-diabetic obese patients could be seen at baseline (data not shown).

Peripheral monocytes showed significantly decreased mRNA expression of mitochondrially encoded NADH dehydrogenase 5 (MT-ND 5) and NDUFA 12 in both T2DM and OB groups relative to control subjects, while the expression of CS was reduced only in obese diabetic patients (Table 2). Other than that, no differences in mRNA expression in any of the studied genes could be seen between T2DM or OB group and healthy control subjects (Table 3), as well as in direct comparison between T2DM and OB group (data not shown).

b) T2DM subjects: influence of VLCD

There was a significant decrease in mRNA expression of CS, MTND 5, dyhydrolipoate-S-acetyltransferase (DLAT) and succinate dehydrogenase subunit A (SDHA) in SCAT of T2DM patients after VLCD while the expression of other genes remained unchanged (Figure 1). In contrast, only the expression of ATP synthase and COX 4/1 were reduced after VLCD in peripheral monocytes, whereas other studied genes showed no difference relative to baseline (Figure 2).

Activity of mitochondrial enzymatic complexes in T2DM subjects

a) at baseline

In addition to mRNA expression, in T2DM subjects we also measured the activity of key enzymes and enzymatic complexes involved in the mitochondrial respiratory chain in peripheral blood elements. At baseline, the activity of the PDH complex and complex IV represented by cytochrome c oxidase (COX) was markedly decreased in T2DM relative to control group,

whereas the activity of other enzymes did not show any difference between both cohorts. Interestingly, in contrast to all other analyzed enzymatic complexes T2DM patients had at baseline a non-significant increase in the activity of NADH-coenzyme Q_{10} reductase (NQR).

b) influence of VLCD

Activity of NQR that was, albeit non-significantly, increased at baseline showed marked decrease after 3 weeks of VLCD. Other than that, VLCD only increased the activity of COX, while having no effect on other respiratory chain enzymes (Figure 3).

Discussion

The most important finding of our study is that patients with obesity and T2DM had decreased mRNA expression of main enzymes involved in mitochondrial respiratory chain in SCAT and, to a lesser extent, also in PM relative to healthy lean subjects. The decreased expression of mitochondrial genes was even more pronounced in patients with T2DM relative to obese non-diabetic subjects. We have also demonstrated that patients with T2DM have partially impaired mitochondrial respiratory chain enzyme activity in peripheral blood elements. Short-term diet intervention had rather inconsistent effect on mitochondrial enzyme gene expression in SCAT and PM as well as their enzymatic activity, despite its overall positive influence on anthropometric, biochemical and hormonal parameters.

There is growing evidence that insulin resistance and the development of type 2 diabetes mellitus are closely related to mitochondrial function, total mitochondrial count and their abnormal morphology in skeletal muscle and adipose tissue, mainly its visceral compartment (Heinonen et al. 2015, Kim et al. 2008, Mitchell and Darley-Usmar 2012). High energy intake leads to electron overload of the mitochondrial respiratory chain complexes. Excess electrons are carried to oxygen that is subsequently converted to superoxide and other reactive oxygen species (ROS). ROS contribute to free fatty acid accumulation, insulinsensitive tissue damage

and possibly to the development of insulin resistance and P-cell failure (Meza-Miranda et al. 2014, Lowell and Shulman 2005). The main sites for ROS production in mitochondria are electron transport chain enzymatic complexes I and III (Kim et al. 2008). Here we show that the expression of almost all respiratory chain enzyme genes is markedly reduced already in subjects with simple obesity, which is in agreement with previously published works (Chattopadhyay et al. 2011). The presence of T2DM further enhances this dysfunction by decreasing the expression of the remaining genes (NDUFA12 and COX4/1). Interestingly, the 2 genes outside the respiratory chain - DLAT and CS - were reduced in a similar fashion indicating that preceding parts of the aerobic glycolytic pathway might be impaired as well. Moreover, as CS is considered a marker of mitochondrial mass (Civitarese et al. 2007, Merz et al. 2015), its decrease suggests that one of the main causes for the reduced respiratory chain enzyme mRNA expression might be the reduction in the amount of mitochondria. Collectively, these data further support the association between obesity and T2DM and impaired mitochondrial activity in subcutaneous white adipose tissue (Rieusset 2015, Brands et al. 2012, Dahlman et al. 2006, Gianotti et al. 2008).

Previous studies indicated the existence of complex interactions between circulating monocytes and adipose tissue in the development of low-grade systemic inflammation, insulin resistance and type 2 diabetes mellitus (Mraz et al. 2011, Harford et al. 2011). However, there is currently limited knowledge on the relationship between mitochondrial dysfunction in circulating peripheral monocytes and the development of metabolic diseases, mainly T2DM. Previous data suggest that the amount of mitochondrial DNA in PM of T2DM patients is reduced and may be related to disease pathogenesis and earlier disease onset (Lee et al. 1998, Wong et al. 2009). Here we show for the first time that simple obesity is associated with decreased mRNA expression of Complex I enzymes (MT-ND5 and NDUFA 12) in PM. In addition, the presence of T2DM lowers the expression of CS suggesting also in PM a possible reduction of

mitochondrial amount relative to healthy individuals as well as obese nondiabetic subjects (Civitarese et al. 2007). Nevertheless, as the expression of other respiratory chain enzymes was not affected in either group, it seems that compared with SCAT the mitochondria of PM are much less prone to disturbances associated with obesity and T2DM. This is in contrast with the situation in healthy individuals, where the expression of mitochondria-related genes was reported to be comparable between mononuclear blood cells and white visceral adipose tissue (Fabricius et al. 2010). Interestingly, when assessing the activity of respiratory chain enzymes in peripheral blood elements, we found, except of decreased activity of Complex IV, an unexpected, albeit non-significant, increase in the activity of Complex I (NQR). Whether this was a result of substrate surplus for the respiratory chain or, in contrast, a compensatory reaction to decreased activity of Complex IV or PDH and whether it could be responsible for increased production of ROS remains questionable.

In our previous works, we have demonstrated beneficial effects of short-term calorie restriction and regular physical activity on the metabolic and proinflammatory profile of obese and diabetic patients (Mraz et al. 2011, Touskova et al. 2012, Klouckova et al. 2016, Trachta et al. 2014). In the present study 3 weeks of VLCD had a rather inconsistent effect on mRNA expression of respiratory chain enzymes with no significant improvement in any of the studied genes. Conversely, despite markedly improved overall metabolic parameters, several genes had even reduced mRNA expression including CS, DLAT and MT-ND5 in SCAT and COX 4/1 and ATP 50 in PM. These findings are in contrast with recent data that indicate a positive effect of long-term calorie restriction and large weight loss after bariatric surgery on mitochondrial biogenesis in patients with obesity (Lopez-Lluch et al. 2006, Vijgen et al. 2013, Nijhawan et al. 2013, Jahansouz et al. 2015, Coen et al. 2015). Whether this lack of effect of weight loss on mRNA expression of respiratory chain enzymes might be attributable to the different type or a much shorter duration of the intervention or to the fact, that acute weight reduction is *per se* a

stressful procedure associated e.g. with increased expression of proinflammatory cytokines in adipose tissue (Snel et al. 2011), remains to be further elucidated. However, the normalization of the increased activity of Complex I and reduced activity of Complex IV in peripheral blood elements after VLCD suggests that even short-term diet intervention can improve mitochondrial function regardless of its influence on the expression level.

The use of slightly different cell types (peripheral monocytes vs. blood platelets) to assess mRNA expression and activity of mitochondrial enzymes might to some extent limit the interpretation and extrapolation of our results, even though both PM and platelets are being routinely employed as a convenient source of mitochondria when evaluating their morphology and function (Zharikov and Siva 2013, Widlansky et al 2010). As the activity of citrate synthase rather than its mRNA expression is primarily used to quantify mitochondrial mass, the decrease in CS mRNA expression might not completely translate into similar reduction of mitochondrial amount. Analogously, mRNA expression of mitochondrial genes might not fully reflect the actual enzymatic activity of respiratory chain complexes. The relatively lower number of subjects in each group also constitutes a potential limitation of our study. In summary, obesity and type 2 diabetes mellitus were in our study associated with impaired mRNA expression of mitochondrial enzyme genes in subcutaneous adipose tissue and in part also in peripheral blood elements. Three weeks of strict caloric restriction lead to overall improvement of anthropometric and metabolic parameters in obese diabetic patients along with amelioration of mitochondrial oxidative function in peripheral blood elements, while having an inconsistent and rather non-significant effect on mitochondrial gene mRNA expression in both SCAT and PM.

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Table 1. Enzymes of the oxidative phosphorylation and their corresponding genes

Enzyme complex	**Abbreviation**	**mRNA expression**	**Abbreviation**	**DNA location**
Pyruvate dehydrogenase complex	PDH	**Dihydrolipoate-S-acetyltransferase**	*DLAT*	nDNA
Citrate synthase	CS	**Citrate synthase**	*CS*	nDNA
NADH - coenzyme Q_{10} reductase (complex I)	NQR	**NADH - ubiquinone dehydrogenase 1 alpha subcomplex 12**	*NDUFA 12*	nDNA
		Mitochondrially encoded NADH dehydrogenase 5	*MT-ND5*	mtDNA
Succinate - coenzyme Q_{10} reductase (complex II)	SQR	**Succinate dehydrogenase subunit A**	*SDHA*	nDNA
NADH - cytochrome c reductase (complex I-III)	NCCR	**Cytochrome c 1**	*CYC 1*	nDNA
Cytochrome c oxidase (complex IV)	COX	**Cytochrome c oxidase subunit IV isoform 1**	*COX 4/1*	nDNA
		ATP synthase	*ATP 50*	mtDNA

nDNA - nucelar DNA, mtDNA - mitochondrial DNA

Table 2. Clinical, hormonal, and metabolic characteristics of study subjects at baseline and after 3 weeks of VLCD

	C group n = 17	OB group n = 12	T2DM group		p-value
			Before VLCD n = 16	After VLCD n = 16	
Age (years)	46.1 ± 2.1	48.9 ± 3.8	51.6 ± 2.5	51.6 ± 2.5	0.342
Sex (females/males)	12/5	9/2	13/3	13/3	
Body weight (kg)	67.7 ± 1.7	**118.6 ± 5.6***	**141.6 ± 5.9***	**129.9 ± 5.3*^**	**<0.001**
BMI (kg/m^2)	22.8 ± 0.5	**40.2 ± 1.4***	**51.5 ± 2.0*°**	**47.3 ± 1.9*^**	**<0.001**
Waist circumference (cm)	80 ± 2	**122 ± 2***	**140±4*°**	**135 ± 4*°^**	**<0.001**
Serum fasting glucose (mmol/l)	4.74 ± 0.11	4.65 ± 0.15	**7.86 ± 0.95*°**	**5.94 ± 0.5C**	**<0.001**
HbA1c (% IFCC)	3.44 ± 0.09	3.84 ± 0.14	**6.03 ± 0.52°***	Not assessed	**<0.001**
Serum fasting insulin (mIU/l)	6.8 ± 0.8	**29.1 ± 4.0***	**34.2 ± 3.4***	**26.8 ± 3.7***	**<0.001**
HOMA-IR	1.45 ± 0.20	**6.16 ± 6.16***	**12.19±1.69***	**6.25 ± 0.91*^**	**<0.001**
Total-cholesterol (mmol/l)	4.79 ± 0.23	5.43 ± 0.42	4.67 ± 0.20	**3.98 ±0.20*°^**	**0.006**
HDL-cholesterol (mmol/l)	1.60 ± 0.11	1.25 ± 0.12	**1.02 ± 0.04***	**1.09 ± 0.19***	**<0.001**
LDL-cholesterol (mmol/l)	2.71 ± 0.20	3.17 ± 0.32	2.84 ± 0.18	**2.19 ± 0.20°^**	**0.037**
Triglycerides (mmol/l)	1.07 ± 0.12	**1.98 ± 0.31***	**1.81 ± 0.15***	1.55 ± 0.14	**<0.001**

Values are mean ± SEM. Statistical significance is from one-way ANOVA or ANOVA on Ranks. Differences between T2DM subjects before and after VLCD were evaluated using paired t- test or Wilcoxon Signed-Rank test as appropriate. * $p < 0.05$ vs. control subjects, ° $p<0.05$ vs. obese non-diabetic, * $p<0.05$ vs. T2DM group before VLCD.

HOMA-IR - homeostatic model assessment of insulin resistance; LDL - low density lipoprotein; HDL - high density lipoprotein; IFCC, International Federation of Clinical Chemistry.

Table 3. mRNA expression changes in PM and SCAT of OB and T2DM patients relative to control group at baseline

Gene symbols	Gene name	Peripheral monocytes				Subcutaneous adi		pose tissue	
		OB		T2DM		OB		T2DM	
		Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value
ATP 50	**ATP synthase**	0.86	NS	0.99	NS	**0.70**	**<0.001**	**0.69**	**<0.001**
COX 4/1	**Cytochrome c oxidase subunit IV isoform 1**	1.14	NS	1.04	NS	0.93	NS	**0.68**	**0.001**
CS	**Citrate synthase**	0.95	NS	**0.78**	**0.012**	**0.39**	**<0.001**	**0.43**	**<0.001**
CYC 1	**Cytochrome c-1**	0.75	NS	0.84	NS	**0.54**	**<0.001**	**0.58**	**<0.001**
DLAT	**Dihydrolipoate-S-acetyltransferase**	0.88	NS	1.02	NS	**0.39**	**<0.001**	**0.42**	**<0.001**
MT-ND 5	**Mitochondrially encoded NADH dehydrogenase 5**	**0.55**	**0.006**	**0.64**	**0.021**	**0.54**	**<0.001**	**0.46**	**<0.001**
NDUFA 12	**NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12**	**0.68**	**0.006**	**0.60**	**0.002**	0.73	NS	**0.63**	**0.009**
SDHA	**succinate dehydrogenase complex, subunit A**	0.92	NS	0.91	NS	**0.70**	**0.017**	**0.51**	**<0.001**

NS, nonsignificant. The mean value of the relative gene expression of control group was taken as 1.0. p value < 0.05 denotes statistical significance.

Figure Captions

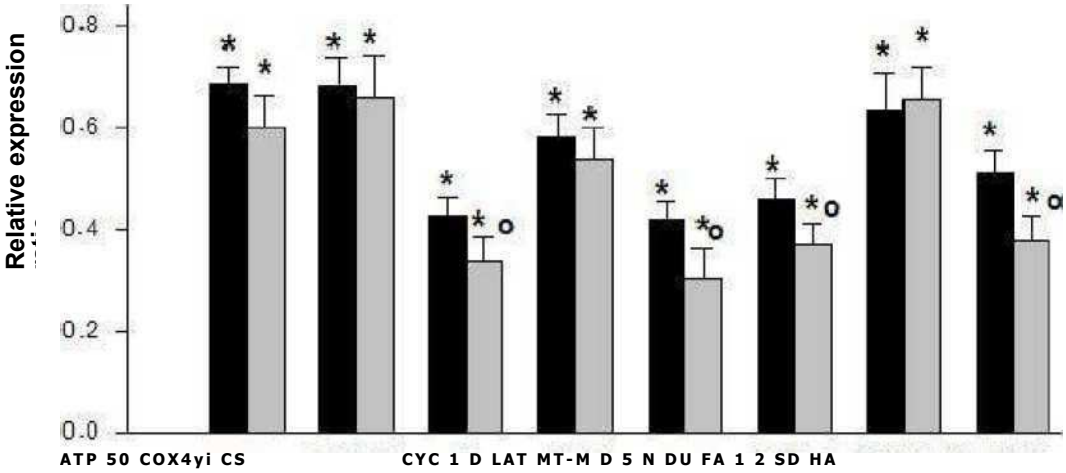
Figure 1. mRNA expression of mitochondrial genes in subcutaneous adipose tissue (SCAT) of obese T2DM subjects before (n=16, black bar) and after VLCD (n=16, grey bar). Values are means ± SEM. The mean value of the relative gene expression of control group was taken as 1.0. T2DM - type 2 diabetes mellitus; VLCD - very low-calorie diet

Figure 2. mRNA expression of mitochondrial genes in peripheral monocytes (PM) of obese T2DM subjects before (n=16, black bar) and after VLCD (n=16, grey bar). Values are means ± SEM. The mean value of the relative gene expression of control group was taken as 1.0. T2DM - type 2 diabetes mellitus; VLCD - very low-calorie diet

Figure 3. Activity of respiratory chain enzymatic complexes in peripheral blood elements of obese T2DM subjects before (n=16, black bar) and after VLCD (n=16, grey bar). Values are means ± SEM. The mean value of the relative gene expression of control group was taken as 1.0. T2DM - type 2 diabetes mellitus; VLCD - very low-calorie diet

1.2

1.0



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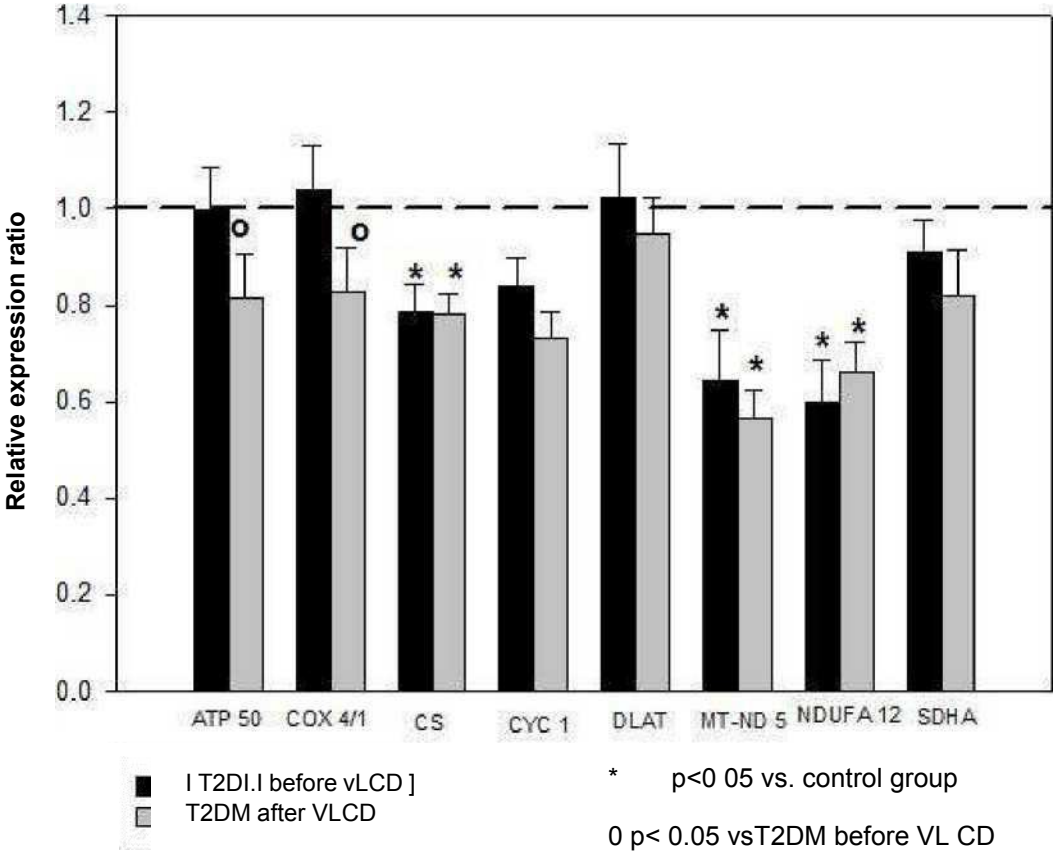
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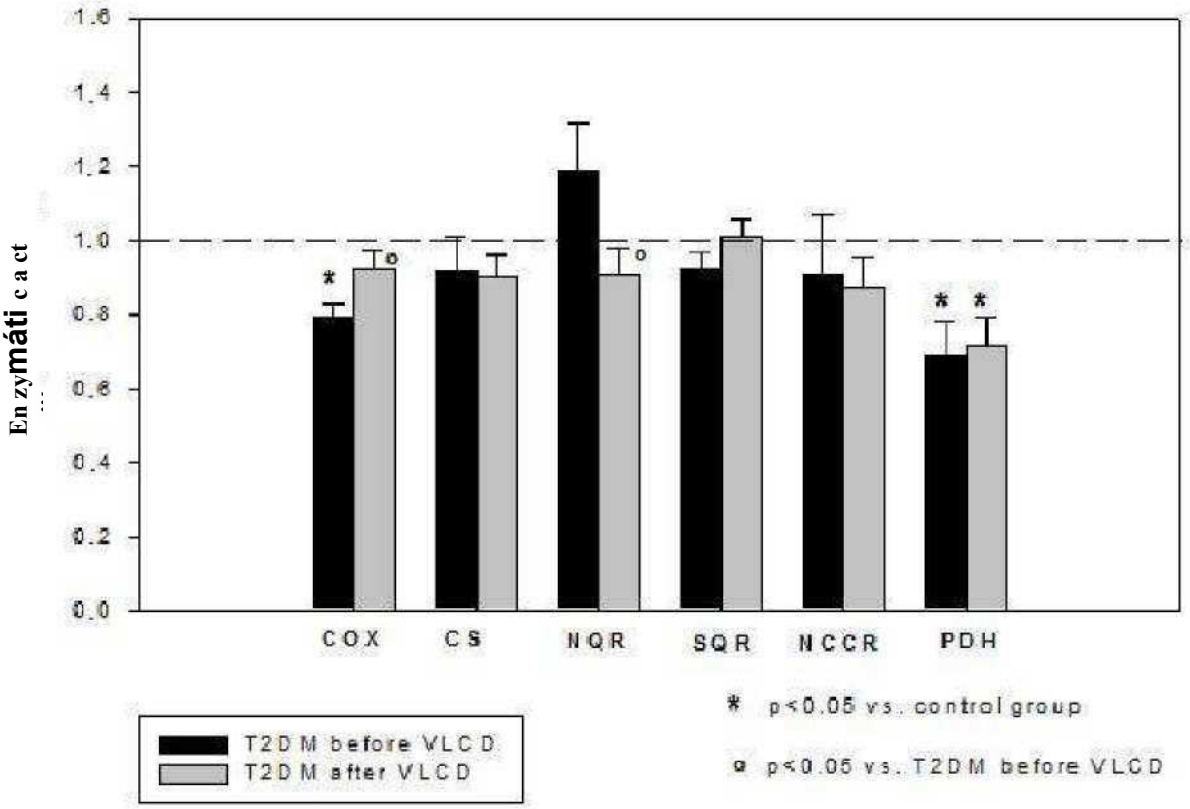
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PDH
T2DM before VLCD
T2DM after VLCD
* p<0.05 vs. control group
o p<0.05 vs. T2DM before VLCD

Serum concentrations and subcutaneous adipose tissue mRNA expression of omentin in morbid obesity and type 2 diabetes mellitus: the effect of very-low-calorie diet, physical activity and laparoscopic sleeve gastrectomy

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Short title: Omentin in obesity and T2DM

Summary

Omentin is a novel adipokine with insulin-sensitizing effects expressed predominantly in visceral fat. We investigated serum omentin levels and its mRNA expression in subcutaneous adipose tissue (SCAT) of 11 women with type 2 diabetes mellitus (T2DM), 37 obese non-diabetic women (OB) and 26 healthy lean women (C) before and after various weight loss interventions: 2-week very-low-calorie diet (VLCD), 3-month regular exercise and laparoscopic sleeve gastrectomy (LSG). At baseline, both T2DM and OB groups had decreased serum omentin concentrations compared with C group while omentin mRNA expression in SCAT did not significantly differ among the groups. Neither VLCD nor exercise significantly affected serum omentin concentrations and its mRNA expression in SCAT of OB or T2DM group. LSG significantly increased serum omentin levels in OB group. In contrast, omentin mRNA expression in SCAT was significantly reduced after LSG. Baseline fasting serum omentin levels in a combined group of the studied subjects (C, OB, T2DM) negatively correlated with BMI, CRP, insulin, LDL-cholesterol, triglycerides and leptin and were positively related to HDL-cholesterol. Reduced circulating omentin levels could play a role in the etiopathogenesis of obesity and T2DM. The increase in circulating omentin levels and the decrease in omentin mRNA expression in SCAT of obese women after LSG might contribute to surgery-induced metabolic improvements and sustained reduction of body weight.

Key words: Omentin, subcutaneous adipose tissue, obesity, laparoscopic sleeve gastrectomy, very-low-calorie diet.

Introduction

Obesity has become a major contributor to the global burden of chronic diseases affecting virtually all ages and socioeconomic groups worldwide (Haslam and James 2005, Guh *et al.* 2009). It is now generally accepted that obesity increases the risk of multiple metabolic diseases, such as hyperlipidemia, insulin resistance, type 2 diabetes mellitus (T2DM), arterial hypertension, atherosclerosis and cardiovascular complications (Field *et al.* 2001, Stumvoll *et al.* 2005, Fujita *et al.* 2006).

Endocrine function of adipose tissue and its changes in obesity and T2DM have drawn a lot of research interest over the last two decades. It has been demonstrated in numerous studies that obesity markedly changes adipose tissue endocrine production (Blüher 2009, Batra and Siegmund 2012, Blüher 2012). Furthermore, functional differences of adipose tissue are associated with its anatomic distribution in subcutaneous (SCAT) and visceral omental (VAT) depots with VAT being considered more closely related to metabolic complications (Wajchenberg 2000, Després *et al.* 2008, Indulekha *et al.* 2011, Baglioni *et al.* 2012). In obese patients, adipose tissue secrets excessive amounts of proinflammatory cytokines and adipokines (e.g., resistin, leptin, TNF-α, IL-1, IL-6) that in turn contribute to chronic low-grade inflammation and the development of insulin resistance, T2DM and increased rate of cardiovascular complications (Shoelson *et al.* 2006, Hotamisligil 2006, Zeyda and Stulnig 2009, Sell *et al.* 2012).

Omentin (or intelectin-1) is a novel adipokine that is predominantly secreted by stromal vascular cells in VAT (Schäffler *et al.* 2005, Yang *et al.* 2006). Lower omentin expression levels are detectable also in SCAT (Kralisch *et al.* 2005, Barth *et al.* 2010). Serum concentrations of omentin-1, the major circulating isoform in human plasma, and mRNA expression of omentin in VAT are decreased in obese and T2DM patients (Auguet *et al.* 2011,

Pan *et al.* 2010, Jialal et al. 2013). Reduced circulating omentin-1 levels are associated with low plasma adiponectin and high-density lipoprotein (HDL-cholesterol) levels (de Souza Batista *et al.* 2007). In addition, circulating omentin-1 levels negatively correlate with serum leptin, resistin and insulin levels, body mass index (BMI), and HOMA index (de Souza Batista *et al.* 2007). *In vitro* studies have revealed that omentin has an insulin sensitizing effect on adipocytes in both visceral and subcutaneous adipose depot through increased insulin signal transduction by activation of Akt/protein kinase B (Akt/PKB). It also enhances insulin-stimulated glucose uptake in human adipocytes (Yang *et al.* 2006). These results indicate that omentin might provide a new potential target for treatment of insulin resistance/T2DM.

Little information is available with respect to changes of serum omentin levels after various weight-reducing interventions. To our best knowledge, only one study focusing on the influence of calorie restriction and weight loss on serum omentin levels has been published so far (Moreno-Navarrete *et al.* 2010). The results indicate that hypocaloric diet-induced weight loss accompanied by improvement of insulin sensitivity was associated with increased serum omentin levels. Serum omentin concentrations after regular physical exercise were explored by Saremi and colleagues who found a significant increase of serum omentin concentrations in overweight and obese men after 12 weeks of aerobic training (Saremi *et. al* 2010). Studies aiming at the effect of bariatric surgery are presently lacking.

We hypothesized that improvement of metabolic parameters after selected weight-reducing interventions could be associated with the changes in serum omentin levels or its gene expression in SCAT. To this end, we investigated the effects of three types of intervention (2-week very-low calorie diet (VLCD), 3-month regular physical activity and bariatric surgery - laparoscopic sleeve gastrectomy (LSG)) on serum omentin levels and its

mRNA expression in SCAT of obese women. Furthermore, we explored whether the presence of T2DM affects the response of serum omentin and its SCAT mRNA expression to VLCD.

Methods

Study subjects

Eleven obese women with type 2 diabetes mellitus (T2DM group), thirty-seven obese non-diabetic females (OB group) and twenty-six lean healthy women (C group) were included in the study. Twenty-three out of thirty-seven obese non-diabetic patients were on antihypertensive treatment, ten patients were treated with statins and two of them were on combined therapy with ezetimib. Eleven OB patients received thyroid hormone substitution therapy. All T2DM patients were treated either with oral antidiabetic drugs, insulin, or its combination. The antidiabetic treatment remained unchanged for at least three months prior to the start of the study. During the 2-week VLCD period, insulin and sulphonylurea doses were decreased to avoid hypoglycemia resulting from improved insulin sensitivity, decreased energy intake and body weight. Decrease in insulin/sulphonylurea doses was necessary in all patients treated with insulin, sulphonylurea or its combination. The doses of metformin were not changed throughout the VLCD period.

All of the diabetic patients were treated with antihypertensive drugs; four patients were treated with statins and one with a fibrate. Three T2DM patients were on thyroid hormone substitution therapy. Control subjects had no history of obesity and/or diabetes mellitus, arterial hypertension, or lipid metabolism disturbances and received no medication. Blood tests confirmed normal blood count, biochemical and hormonal parameters.

Ten out of thirty-seven obese and all of the diabetic patients underwent a 2-week VLCD with energy intake 2500kJ per day (600 kcal per day). During the reduction program all

patients were hospitalized at the Third Department of Medicine, General University Hospital in Prague. Thirteen obese non-diabetic patients took part in a 3-month exercise program at the Recondition Center in Prague. The patients underwent 30 minutes of aerobic exercise three times a week under the supervision of a certified coach, patients kept their usual eating habits during the exercise program. The remaining thirteen obese patients underwent LSG at the Surgical Clinic, Military University Hospital in Prague. The body weight of all study participants remained stable for at least three months before the enrollment into the study. Written informed consent was signed by all participants before beginning of the study. The study was approved by Human Ethical Review Committee, First Faculty of Medicine and General University Hospital, Prague, Czech Republic and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

Anthropometric examination, blood and adipose tissue sampling

All patients included in the reduction program (T2DM and obese non-diabetic subjects) were examined twice; at basal state before beginning of any intervention and after 2 weeks of VLCD. Obese non-diabetic patients enrolled in the physical activity program were examined at basal state before the start of regular exercise and after 3 months of the exercise program. Patients undergoing bariatric surgery intervention were examined four times – at basal state before surgical intervention and then 6, 12 and 24 months after the surgery, respectively. Normal-weight healthy subjects were examined only once. All subjects were measured and weighted, and their BMI was calculated. Blood samples for biochemical and hormonal parameters measurement were withdrawn between 07.00h and 08.00h after 12 h of overnight fasting. Blood samples were separated by centrifugation for 10min at 1000 x g within 30 min from blood collection. Serum was subsequently stored in aliquots at -80°C until further analysis. Samples of subcutaneous adipose tissue for mRNA expression analysis were

obtained from abdominal region with subcutaneous needle aspiration biopsy. Approximately 100 mg of adipose tissue was collected to 1 ml of RNA stabilization Reagent (RNAlater, Qiagen, Hilden, Germany) and stored at - 80°C until further analysis.

Hormonal and biochemical assays

Serum omentin levels were measured by a commercial ELISA kit (BioVendor, Brno, Czech Republic). Sensitivity was 0.5 ng/ml. Serum adiponectin, CRP, leptin, resistin, and insulin concentrations were measured by commercial ELISA a RIA kits as described previously (Dolinkova *et al.* 2008). The intra- and interassay variabilities for all methods were less than 5.0 and 10.0%, respectively. Biochemical parameters (glucose; total and HDL-cholesterol, triglycerides) were measured at the Department of Biochemistry of General University Hospital by standard laboratory methods. The value of LDL-cholesterol was calculated according to Friedewald formula. The homeostasis model assessment (HOMA) was calculated as HOMA-IR index using the following formula: fasting serum insulin (mIU/l) x fasting serum glucose (mmol/l)/22.5. Glycated hemoglobin was analyzed by high performance liquid chromatography (HPLC) on Variant II BioRad analyzer (BioRad).

mRNA expression determination by quantitative real-time PCR (qRT-PCR)

Samples of subcutaneous adipose tissue were homogenized on MagNA Lyser Instrument with MagNA Lyser Green beads (Roche Diagnostics GmbH, Germany). Total RNA from homogenized tissue was extracted on MagNA Pure instrument using Magna Pure Compact RNA Isolation kit (tissue) (Roche Diagnostics GmbH, Germany). The RNA concentration was determined from absorbance at 260 nm on a NanoPhotometer (Implen, Munchen, Germany). Reverse transcription was performed using 0.25 μg of total RNA to synthesize the first strand cDNA using the random primers as per the instructions of the High-Capacity

cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Gene expression of omentin -1 was performed on a 7500 Real-Time PCR System using TaqMan ® gene Expression Assays (Applied Biosystems, Foster City, CA, USA). For reaction a mix of TaqMan® Universal PCR Master Mix II, NO AmpErase® UNG (Applied Biosystems, Foster City, CA, USA), nuclease-free water (Fermentas Life Science, Lithuania) and specific TaqManGene expression Assays (Applied Biosystems, Foster City, CA) was used.

Controls with no template cDNA were performed with each assay and all samples were run at least in duplicate. The increase in fluorescence was measured in real time and threshold cycle (Ct) values were obtained. To compensate for variations in RNA amount and efficiency of reverse transcription, beta-2-microglobulin was used as endogenous reference and results were normalized to the mean of these values. The formula $2^{-\Delta\Delta Ct}$ was used to calculate relative gene expression.

Statistical analysis

Statistical analysis was performed on SigmaStat software (Systat Inc., Chicago, IL). Anthropometric, hormonal and biochemical results are expressed as means ± SEM (standard error of the means). Unpaired t-test or Mann-Whitney *U* test was used for group comparison as appropriate. Differences between T2DM and obese patients before and after VLCD, and obese non-diabetics before and after physical activity and LSG were evaluated using paired t-test or Wilcoxon Signed –Rank test as appropriate. Statistical significance was assigned to $p< 0.05$. The correlations between the values were estimated by Spearman correlation test. Multiple linear regression analysis was used to show the independent relationships of other parameters with serum omentin levels. A p value < 0.05 denoted statistical significance.

Results

Anthropometric, biochemical and hormonal characteristics of study subjects

Anthropometric, biochemical and hormonal characteristics of all study groups are summarized in Table 1. As expected both OB and T2DM patients had markedly increased BMI and circulating CRP levels relative to control group. Serum concentrations of leptin, insulin, glucose, HbA1c, LDL-cholesterol, triglycerides, and HOMA-index were significantly increased in both OB and T2DM patients relative to control group. On the contrary, circulating adiponectin and serum HDL-cholesterol levels were significantly decreased in both OB and T2DM patients compared with control group. Serum total cholesterol and circulating resistin levels did not significantly differ among the groups. In T2DM subjects, BMI and circulating CRP levels were significantly elevated as compared to OB patients. Serum concentrations of glucose, HbA1c and leptin were markedly elevated, whereas HDL-cholesterol levels were significantly decreased in T2DM group relative to OB group.

Serum concentrations of omentin were significantly decreased in both OB and T2DM patients relative to C subjects. Serum omentin levels did not significantly differ between T2DM and OB group (Table 1). mRNA expression of omentin (ITLN1) did not differ among the studied groups (data not shown).

The influence of VLCD on hormonal and biochemical parameters, serum omentin and its mRNA expression in SCAT in T2DM and OB patients

The influence of VLCD on hormonal and biochemical parameters in T2DM and OB patients is summarized in Table 2. Two weeks of VLCD significantly reduced BMI, circulating levels of CRP, glucose, HDL- and total-cholesterol in T2DM patients. Serum insulin, LDL-cholesterol, triglycerides, leptin, resistin, and adiponectin levels, and HOMA

index of diabetic patients were not significantly affected by VLCD. In obese non-diabetic subjects, a significant decrease of BMI, serum total cholesterol, LDL- and HDL-cholesterol was found after VLCD. The diet intervention did not significantly affect CRP, serum insulin, glucose, HOMA index, triglycerides, leptin, resistin and adiponectin levels in OB group. HbA1c was not assessed after VLCD.

VLCD had no significant effect on serum omentin levels in either T2DM or obese patients (Table 2). mRNA expression of omentin in SCAT was not affected by VLCD in any group studied (data not shown).

The effect of a 3-month regular physical activity on hormonal and biochemical parameters, serum omentin and its SCAT mRNA expression in obese non-diabetic women

Changes of the studied parameters after physical activity program are summarized in Table 3. Three months of regular exercise significantly decreased BMI, HOMA index, serum insulin, glucose and leptin levels. CRP, HbA1c, total-cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, adiponectin and resistin levels were not significantly affected by physical activity. 3-months regular exercise did not significantly affect either serum omentin levels (Table 3) or its gene expression in SCAT of OB group (data not shown).

The effect of LSG on hormonal and biochemical parameters, serum omentin and its SCAT mRNA expression in obese non-diabetic patients

The changes of hormonal and biochemical parameters in obese women after LSG are summarized in Table 4. Overall, LSG had no significant effect on blood glucose levels during the 2-year follow up. At month 6 after the surgery BMI, insulin levels, HbA1c and HOMA index, triglycerides and leptin levels were significantly decreased relative to baseline levels.

On the contrary, adiponectin levels markedly increased at month 6 after LSG. CRP, total-cholesterol, LDL-cholesterol, HDL-cholesterol and resistin levels were not significantly affected at month 6 after LSG as compared to baseline values. One year after the surgery, BMI was further decreased, as were LDL-cholesterol levels. CRP, fasting insulin, HbA1c, HOMA index, triglycerides and leptin levels were significantly reduced at month 12 after LSG relative to baseline values. HDL-cholesterol and adiponectin levels were markedly increased, whereas total-cholesterol and resistin levels did not significantly change 1 year after surgery. At month 24 after LSG, BMI, HbA1c, leptin and adiponectin levels significantly increased compared with those values measured at month 12 after LSG. Circulating CRP and LDL-cholesterol levels were markedly decreased at month 24 relative to pre-surgery values. On the contrary, serum HDL-cholesterol levels were significantly increased 2 years after LSG as compared to its pre-surgery levels. Serum insulin, HOMA index, total cholesterol, triglycerides and resistin levels were not significantly different from baseline values at month 24 after LSG.

Serum omentin levels significantly increased 6 months after LSG, and a sustained increase of circulating omentin levels remained significant during the 2-year follow up (Table 4). In contrast, LSG decreased omentin gene expression in SCAT. This effect was at first detected at month 6 after LSG and was most pronounced at month 12 after LSG (Figure 1). At month 24 after LSG we observed a slight non-significant increase of omentin mRNA expression in SCAT when compared with month 12, but the expression still remained markedly reduced relative to baseline values.

Relationship of serum omentin levels and its gene expression in SCAT to other studied parameters

The relationship of serum omentin levels and its mRNA expression in SCAT to other studied parameters was calculated in a combined population of healthy controls, OB and T2DM subjects at baseline (before the interventions). Serum omentin concentrations were inversely associated with BMI, CRP, serum insulin, LDL-cholesterol, triglycerides and leptin levels, whereas it positively correlated with HDL-cholesterol levels (Figure 2). We failed to find any significant relationship of baseline serum omentin to fasting blood glucose, HbA1c, HOMA-IR index, total cholesterol, adiponectin, and resistin (data not shown). Multiple regression analysis was performed with baseline serum omentin levels as dependent and other anthropometrical and biochemical parameters as independent variables. None of the factors included was identified as statistically significant independent predictor of serum omentin concentrations (data not shown). We failed to find any significant relationship of omentin gene expression in SCAT to other studied parameters including BMI, CRP, serum insulin, blood glucose, HbA1c, HOMA index, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, leptin, adiponectin and resistin levels (data not shown). Baseline serum omentin levels in the group of obese patients who underwent LSG significantly positively correlated with blood glucose ($r = 0.56$, $p = 0.046$) while no other significant relationships of serum omentin levels to other studied parameters in OB patients before LSG were found (data not shown). At month 12 after LSG, omentin mRNA expression significantly inversely correlated with CRP ($r = -0.63$, $p = 0.03$). Two years after the surgery, omentin mRNA expression positively correlated with fasting blood glucose levels ($r = 0.55$, $p = 0.049$). At month 6 after LSG, the difference in serum omentin levels (serum omentin) inversely correlated with the difference in serum insulin levels (serum insulin) ($r = -0.68$, $p = 0.019$) and with the difference in HOMA index (HOMA) ($r = -0.71$, $p = 0.013$), respectively. 24 months after the surgery, the difference in serum omentin levels (serum omentin) negatively correlated with the difference in serum CRP levels (serum CRP) ($r = -0.75$, $p = 0.038$) and it was

positively related to the difference in serum LDL-cholesterol levels (serum LDL) ($r = 0.71$, $p = 0.013$). Multiple linear regression analyses were performed with serum omentin as dependent and the other anthropometrical and biochemical parameters as independent variables. BMI ($\beta = 17.32$, $p = 0.049$), insulin levels ($\beta = -7.08$, $p = 0.005$) and HOMA index ($\beta = -29.62$, $p = 0.034$) were found to be the independent predictors of changes of serum omentin levels after LSG. The results obtained from multiple linear regression analyses are summarized in Table 5.

Discussion

The most important finding of the present study is that serum omentin levels were significantly increased after LSG, whereas its mRNA expression in SCAT was significantly reduced after the surgery. 3 months of regular physical activity and 2 weeks of VLCD had no significant effect on serum omentin levels and its mRNA expression in SCAT despite its overall positive effects on anthropometric, hormonal and biochemical parameters. Furthermore, serum omentin concentrations negatively correlated with BMI, CRP, insulin, LDL-cholesterol, triglycerides and leptin levels and were positively related to serum HDL cholesterol. In contrast, omentin mRNA expression in SCAT did not correlate with any of the anthropometric and biochemical parameters studied.

In our study, we found, in agreement with other previously published papers (Auget *et al.* 2011, de Souza Batista *et al.* 2007, Yan *et al.* 2011, Jialal *et al.* 2013), markedly reduced serum omentin concentrations in both obese and T2DM patients as compared to healthy normal-weight subjects. Although we failed to find any significant differences in serum omentin levels between obese non-diabetic and T2DM patients, we cannot exclude the possibility that our findings might have been influenced by a relatively low number of T2DM

patients (11 vs. 37 in obese group) and the heterogeneity of a group of non-diabetic obese patients. Inverse correlations of circulating omentin with BMI, CRP, fasting insulin, LDL-cholesterol, triglycerides and leptin and its positive relation to HDL-cholesterol are in accordance with previous reports (de Souza Batista *et al.* 2007) and indicate that serum omentin might represent a potential marker of metabolic syndrome and endothelial dysfunction (Moreno-Navarette *et al.* 2010, Zhou *et al.* 2012). The exact factors contributing to markedly reduced circulating omentin levels in obesity and diabetes still remain to be determined. Bearing in mind the results of previous studies (de Souza Batista *et al.* 2007, Choi *et al.* 2011, Zhou *et al.* 2012), increased insulin levels typically found in patients with obesity and T2DM might be an important contributor preceding decreased omentin levels. Another possible player contributing to decreased omentin levels could be excessive adiposity and obesity-associated metabolic complications. This possibility is supported by the finding of increased circulating levels of omentin in patients with anorexia nervosa with severely reduced body fat content (Guo *et al.* 2012). Although the multiple linear regression analyses performed in our study did not lead to the identification of any from the tested parameters as the independent predictors of pre-interventions fasting serum omentin levels, we revealed that BMI, insulin and HOMA index are independent predictors of the difference in serum omentin levels (serum omentin) after LSG. Both OB and T2DM groups in our study displayed markedly increased BMI, CRP, insulin, glucose, HOMA, LDL-cholesterol, triglycerides and leptin, whereas serum adiponectin and HDL-cholesterol were reduced in both groups. Such metabolic and hormonal status reflects long-term disturbances in the function of metabolically active tissues with a prominent role of endocrine dysfunction of adipose tissue. Reduced circulating omentin levels may thus reflect the overall metabolic phenotype rather than a single altered hormonal or biochemical parameter. In contrast, the increase in serum omentin

levels after LSG may be connected mainly to the reduction of body weight and fasting serum insulin levels after the surgery.

Omentin is predominantly expressed in visceral adipose tissue and its expression levels in SCAT are significantly lower (Schäffler *et al.* 2005, Kralisch *et al.* 2005, Yang *et al.* 2006). The lack of statistically significant difference in gene expression of omentin in SCAT of obese and T2DM patients as compared to lean women is in agreement with findings of Auget *et al.* (Auget *et al.* 2011). Although the study of Jialal et al. (2013) revealed significantly decreased SCAT secreted omentin protein in subjects with metabolic syndrome without T2DM, we did not examine SCAT secreted protein in our study. It is generally well-known that mRNA expression may not accurately reflect protein expression or secretion. However, it is noteworthy that a clear trend towards increased omentin mRNA expression in both OB and T2DM groups was observed in our study. It has been previously reported that decreased omentin gene expression in VAT of obese and insulin resistant or T2DM patients correlates with systemic metabolic parameters (de Souza Batista *et al.* 2011). This finding suggests that omentin produced by VAT could be a direct contributor to systemic metabolic regulations. Our results show that omentin mRNA expression in SCAT is not significantly related to systemic metabolic parameters and that omentin mRNA expression in SCAT is independent of the serum levels. Further studies focused on omentin expression in different fat depots are needed to dissect the potentially distinct roles of SCAT- and VAT-produced omentin.

In our previous works, we have demonstrated the beneficial effect of short-term calorie restriction on metabolic and proinflammatory profile of obese and diabetic patients (Mraz *et al.* 2011, Touskova *et al.* 2012). In the present study we have investigated the effect of various obesity treatment methods (VLCD, exercise, LSG) on serum omentin levels and its mRNA expression in SCAT. Moreno-Navarrete *et al.* (2010) reported that serum omentin levels significantly increased after a 4-month hypocaloric diet. Contrary to this report, in our

study 2-week VLCD and regular 3-month physical activity had no significant impact on serum omentin levels and its gene expression in SCAT. The differences between our and Moreno-Navarretes´ results can be explained by shorter duration of dietary intervention in our study.

Similarly, although the 3-month exercise explored in our study reduced both BMI and circulating insulin levels, the changes were probably not profound enough to significantly affect serum omentin concentrations. This hypothesis is further supported by the fact that more profound weight loss induced by LSG markedly increased serum omentin levels and significantly decreased omentin mRNA expression in SCAT during the 2-year follow-up. However, our results from exercise branch are in disagreement with the findings of Saremi et al., who observed a significant increase of serum omentin concentrations in overweight and obese men after 12 weeks of aerobic training (Saremi *et al.* 2010). Such a discrepancy in the results obtained by Saremi et al. and ours could be explained by the major differences in the study design; mainly the intensity of aerobic training was much higher in the study of Saremi et al. (50-60 minutes 5 times a week), which led to a more marked improvement of lipid profile compared with our study. In contrast, we observed a more profound decrease in BMI, fasting insulin levels, and HOMA IR index in our study. This difference may have been due to distinct characteristics of subjects included in both studies (males vs. females, average pre-exercise BMI 29.1 vs. 38.2).

In conclusion, we have demonstrated that obese and T2DM patients have decreased circulating omentin levels, but unchanged mRNA expression of omentin in SCAT. Our results suggest that the increase of circulating omentin levels together with the reduction of its expression in SCAT after LSG could contribute to surgery-induced metabolic improvements and sustained reduction of body weight.

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Table 1. Clinical, hormonal and metabolic characteristics of the study subjects.

	Controls	**OB**	**T2DM**
Number (n)	26	37	11
Age (years)	42.8 ± 2.17	49.2 ± 1.87	56.9 ± 2.83
BMI (kg/m2)	22.5 ± 0.35	**41.5± 0.82***	**52.6 ± 2.59*°**
CRP (mg/l)	0.2 ± 0.07	**1.1 ± 0.15***	**2.3 ± 0.50*°**
Fasting insulin (mIU/l)	18.1 ± 1.71	**38.7± 2.76***	**37.8± 4.43***
Fasting blood glucose (mmol/l)	4.8 ± 0.07	**6.1 ± 0.39***	**9.3 ± 1.10*°**
HbA1c (% IFCC)	3.7 ± 0.08	**4.7 ± 0.31***	**7.3 ± 0.55*°**
HOMA-IR index	3.9 ± 0.44	**10.4± 1.17***	**11.6± 2.19***
Total cholesterol (mmol/l)	4.9 ± 0.14	5.1± 0.21	4.5± 0.23
LDL-cholesterol (mmol/l)	2.1 ± 0.13	**2.9 ± 0.19***	**2.6 ± 0.22***
HDL-cholesterol (mmol/l)	2.4 ± 0.12	**1.4 ± 0.07***	**0.9 ± 0.04*°**
Triglycerides (mmol/l)	0.9 ± 0.08	**1.9 ± 0.20***	**1.9 ± 0.19***
Leptin (ng/ml)	18.8 ± 3.99	**47.7± 2.72***	**64.5 ± 6.95*°**
Adiponectin (µg/l)	27.8 ± 2.44	**13.6± 1.08***	**14 ± 2.63***
Resistin (ng/ml)	5.7 ± 0.28	6.5± 0.43	10.6 ± 2.26
Serum omentin (ng/ml)	565.5 ± 27.74	**397.6± 30.36***	**474.9± 44.61***

OB – obese non-diabetic, T2DM – type 2 diabetes mellitus.

Values are means ± SEM. Statistical significance is from unpaired t-test or Mann-Whitney *U* test as appropriate. * $p < 0.05$ vs. controls, ° $p < 0.05$ vs. obese non-diabetic patients

Table 2. Obese non-diabetic and type 2 diabetes mellitus patients: the effect of VLCD

	Obese non-diabetic		**T2DM**	
	Obese before VLCD	**Obese after VLCD**	**T2DM before VLCD**	**T2DM after VLCD**
Number (n)	10	10	11	11
Age (years)	60.3 ± 3.19	60.3 ± 3.19	56.9± 2.83	56.9 ± 2.83
BMI (kg/m2)	44.5 ± 1.75	**42.7± 1.63***	52.6± 2.59	**49.4 ± 2.34°**
CRP (mg/l)	1.4 ± 0.35	1.2± 0.27	2.3 ± 0.50	**1.4 ± 0.36°**
Fasting insulin (mIU/l)	35.7 ± 5.09	33.5 ± 4.19	37.8± 4.43	37.4 ± 6.78
Fasting blood glucose (mmol/l)	7.4 ± 1.19	6.0± 0.61	9.3 ± 1.09	**6.7 ± 0.75°**
HbA1c (% IFCC)	5.9 ± 0.90	Not assessed	7.3 ± 0.55	Not assessed
HOMA-IR index	11.5 ± 3.09	7.9± 1.90	11.6± 2.19	6.9 ± 1.76
Total cholesterol (mmol/l)	4.8 ± 0.32	**3.9 ± 0.28***	4.5 ± 0.23	**3.8 ± 0.19°**
LDL-cholesterol (mmol/l)	2.3 ± 0.39	**1.8 ± 0.28***	2.6 ± 0.22	2.2 ± 0.19
HDL-cholesterol (mmol/l)	1.5 ± 0.24	**1.3 ± 0.25***	1.0 ± 0.04	**0.9 ± 0.04°**
Triglycerides (mmol/l)	2.4 ± 0.64	1.8± 0.31	1.9 ± 0.19	1.7 ± 0.19
Leptin (ng/ml)	48.4 ± 4.89	41.0 ± 6.09	64.5± 6.95	53.6 ± 7.11
Adiponectin (µg/l)	15.0 ± 2.39	15.7 ± 2.51	14 ± 2.63	12.9 ± 1.77
Resistin (ng/ml)	6.9 ± 0.73	7.4± 1.10	10.6± 2.26	9.0 ± 1.20
Serum omentin (ng/ml)	403.8 ± 57.68	406.9 ± 54.20	474.9± 44.61	485.7 ± 42.88

Values are means ± SEM. Statistical significance is from paired t-test or Wilcoxon Signed-Rank test as appropriate. * $p< 0.05$ vs. OB before VLCD ° $p< 0.05$ vs. T2DM before VLCD

Table 3. Obese non-diabetic: the effect of a 3-month physical activity.

	Obese before PA	**Obese after PA**
Number (n)	13	13
Age (years)	49.5 ± 2.21	49.5 ± 2.21
BMI (kg/m2)	38.2 ± 1.02	**35.4 ± 1.03°**
CRP (mg/l)	0.7 ± 0.19	0.5 ± 0.14
Fasting insulin (mIU/l)	46.7 ± 5.14	**38.9 ± 4.54°**
Fasting blood glucose (mmol/l)	5.8 ± 0.18	**5.4 ± 0.13°**
HbA1c (% IFCC)	4.1 ± 0.14	3.9 ± 0.11
HOMA-IR index	12.1 ± 1.41	**8.0 ± 1.47°**
Total cholesterol (mmol/l)	5.4 ± 0.36	5,4 ± 0.29
LDL-cholesterol (mmol/l)	3.3 ± 0.33	3.29 ± 0.27
HDL-cholesterol (mmol/l)	1.3 ± 0.07	1.4 ± 0.07
Triglycerides (mmol/l)	1.7 ± 0.18	1.5 ± 0.19
Leptin (ng/ml)	39.9 ± 4.99	**31.0 ± 3.86°**
Adiponectin (µg/l)	10.9 ± 1.71	10.9 ± 2.06
Resistin (ng/ml)	4.9 ± 0.44	5.9 ± 0.46
Serum omentin (ng/ml)	432.8 ± 56.46	412.1 ± 58.25

Values are means ± SEM. Statistical significance is from paired t-test or Wilcoxon Signed-Rank test as appropriate. ° $p < 0.05$ vs. OB before PA

Table 4. Obese non-diabetic subjects: the effect of laparoscopic sleeve gastrectomy.

	Obese non-diabetic			
	Obese before LSG	**Obese after LSG_6m**	**Obese after LSG_12m**	**Obese after LSG_24m**
Number (n)	13	13	13	13
Age (years)	41 ± 2.1			
BMI (kg/m2)	42.5 ± 1.08	**33.2± 1.09***	**31.6 ± 1.14*°**	**33.2 ± 1.68*×**
CRP (mg/l)	1.3 ± 0.24	1.0± 0.25	**0.9± 0.2***	**0.7 ± 0.10***
Fasting insulin (mIU/l)	32.8 ± 3.31	**24.3± 2.45***	**25.7± 4.12***	30.5 ± 6.52
Fasting blood glucose (mmol/l)	5.4 ± 0.48	4.9± 0.21	4.9± 0.21	**5.2 ± 0.19×**
HbA1c (% IFCC)	4.2 ± 0.31	**3.7 ± 0.16***	**3.7 ± 0.17***	**4.0 ± 0.19×**
HOMA-IR index	7.9 ± 1.69	**5.2 ± 0.81***	**5.5 ± 1.35***	**7.1 ± 1.56 ×**
Total cholesterol (mmol/l)	5.1 ± 0.4	4.7± 0.39	4.9± 0.34	4.8 ± 0.36
LDL-cholesterol (mmol/l)	3.1 ± 0.3	2.9± 0.33	**2.7± 0.3***	**2.4 ± 0.27***
HDL-cholesterol (mmol/l)	1.3 ± 0.08	1.4± 0.08	**1.6 ± 0.09*°**	**1.7 ± 0.12***
Triglycerides (mmol/l)	1.7 ± 0.22	**1.4 ± 0.16***	**1.3 ± 0.16***	1.4 ± 0.29
Leptin (ng/ml)	54.8 ± 3.33	**21.9± 3.01***	**21.8± 3.93***	**28.2 ± 5.95*×**
Adiponectin (µg/l)	15.3 ± 1.5	**19.0± 1.86***	**21.9 ± 2.14*°**	**26.1 ± 3.12*×**
Resistin (ng/ml)	7.8 ± 0.78	8.2± 0.73	7.3± 2.78	6.6 ± 0.5
Serum omentin (ng/ml)	358.1 ± 45.9	**455.7± 34.79***	**419.9 ± 41.2***	**449.7 ± 44.75***

Values are means ± SEM. Statistical significance is from paired t-test or Wilcoxon Signed-Rank test as appropriate. * P < 0.05 vs. OB before LSG ° p< 0.05 vs. OB after LSG_6m × p< 0.05 vs. OB after LSG_12m

Table 5. Multiple linear regression analyses performed with serum omentin as dependent and the other anthropometrical and biochemical parameters as independent variables in the group of obese non-diabetic patients undergoing LSG.

	Obese after LSG_6m		**Obese after LSG_12m**		**Obese after LSG_24m**	
	β	p	β	p	β	p
BMI (kg/m^2)	**17.3**	**0.049**	2.1	0.60	-19.4	0.33
CRP (mg/l)	13.5	0.71	-13.6	0.72	-131.8	0.19
Fasting insulin (mIU/l)	**-7.1**	**0.01**	0.9	0.97	**11.6**	**0.03**
Fasting blood glucose (mmol/l)	16.7	0.77	68.3	0.84	-68.2	0.6
HbA1c (% IFCC)	6.5	0.63	-21.4	0.28	-51.6	0.63
HOMA-IR index	**-29.6**	**0.03**	-14.7	0.91	-16.7	0.57
Total cholesterol (mmol/l)	-224.4	0.12	120.3	0.61	-295.8	0.07
LDL-cholesterol (mmol/l)	210.5	0.17	-137.5	0.59	328.8	0.09
HDL-cholesterol (mmol/l)	299.3	0.19	-173.2	0.64	109.7	0.65
Triglycerides (mmol/l)	-99.5	0.09	30.5	0.59	-276.7	0.06
Leptin (ng/ml)	1.4	0.44	-0.4	0.88	-1.6	0.58
Adiponectin (µg/l)	9.8	0.3	-4.3	0.7	-1.9	0.92
Resistin (ng/ml)	-11.3	0.2	-14.5	0.72	3.0	0.95

Figure Captions

Figure 1. The effect of laparoscopic sleeve gastrectomy (LSG) on mRNA expression of omentin (ITLN1) in subcutaneous adipose tissue (SCAT) of obese non-diabetic women (n = 13). The patients were examined before surgical intervention and 6, 12 and 24 months after the surgery, respectively. *p < 0.001 vs. before LSG; $^{+}$p < 0.05 vs. at month 6 after LSG.

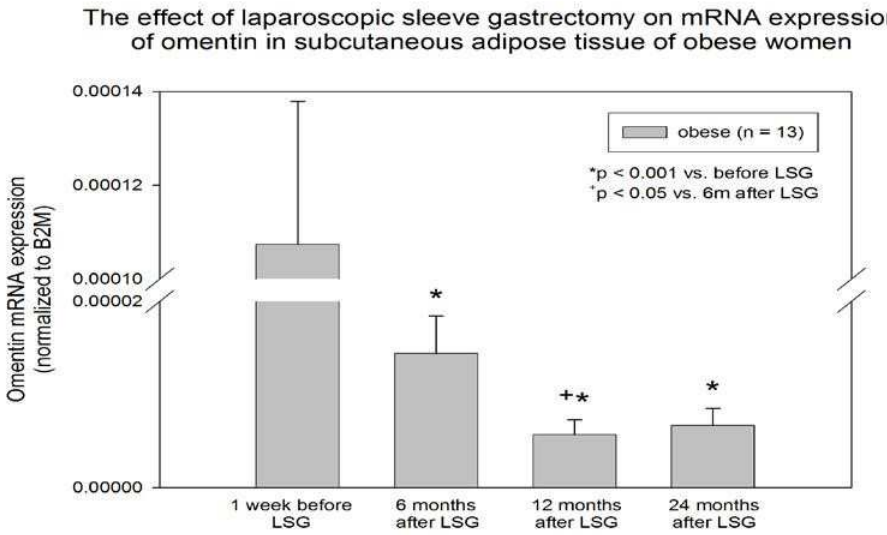

Figure 2. Significant relationships of serum omentin levels with anthropometric and hormonal parameters calculated in a combined population of normal-weight healthy women, obese non-diabetic and diabetic patients