Adiponectin and Resistin Gene Polymorphisms in Patients with Anorexia Nervosa and Obesity and its influence on metabolic phenotype

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Running title: adipokine polymorphisms in obesity and anorexia nervosa

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Summary:

Genes for adiponectin and resistin are candidate genes of insulin resistance and type 2 diabetes mellitus. The aim of our study was to determine the frequency of single nucleotide polymorphisms **(SNP)** 45T>G and 276G>T of the adiponectin gene and 62G>A and -180C>G of the resistin gene in patients with obesity (OB), anorexia nervosa (AN) and in control **healthy normal-weight women (NW)** and to study the influence of particular genotypes on serum concentrations of these hormones and on insulin sensitivity.

Serum adiponectin, resistin, tumor necrosis factor alpha (TNF-alpha), insulin, cholesterol, glycated hemoglobin (HbA1c) and blood glucose levels were measured in 77 patients with OB, 28 with AN and 38 NW. DNA analysis was carried out by polymerase chain reaction with restriction analysis of PCR product.

The presence of SNP ADP+276 G>T allele was accompanied by higher cholesterol levels in AN patients, higher adiponectin concentrations in OB patients and lower HbA1c levels in NW. SNP of the resistin gene 62G>A was associated with lower HbA1c in NW and higher cholesterol concentrations in OB group. The carriers of the minor G allele in the position -180 of the resistin gene within AN group had significantly higher BMI relative to non-carriers.

We conclude that polymorphisms in adiponectin and resistin genes can contribute to metabolic phenotype of patients with obesity and anorexia nervosa.

Key words: adiponectin, resistin, polymorphism, obesity, anorexia nervosa

Introduction:

Adipose tissue is now considered an important endocrine organ that produces numerous factors affecting food intake, metabolism of lipids and carbohydrates and numerous other processes in human body (Havel 2002). Some of adipose tissue-derived hormones such as adiponectin and resistin can represent a possible connection between obesity and insulin resistance (Haluzik *et al.* 2004b; Steppan *et al.* 2001). Adiponectin (ADP) is a protein hormone produced almost exclusively in adipose tissue (Scherer *et al.* 1995). It is considered one of the few insulin-sensitizing factors produced by adipose tissue. Its circulating levels are decreased in patients with obesity and type 2 diabetes (Arita *et al.* 1999; Hotta *et al.* 2000). Furthermore, it has been demonstrated, that patients with reduced adipose tissue mass (e.g. patients with anorexia nervosa or endurance-trained sportsmen) have markedly increased adiponectin circulating levels (Delporte *et al.* 2003; Haluzik *et al.* 1999; Housova *et al.* 2005). Recombinant adiponectin treatment decreases blood glucose in normal and diabetic rats predominantly due to increased liver insulin sensitivity (Berg *et al.* 2001). Mice with the knock-out of the adiponectin gene are characterized by insulin resistance and accelerated atherosclerosis which is both normalized by recombinant adiponectin substitution (Kubota *et al.* 2002).

Resistin (RETN) is a protein hormone produced both by adipocytes and immunocompetent cells including those residing in adipose tissue. Some studies found increased circulating resistin levels and its mRNA expression in adipose tissue in patients with obesity (Degawa-Yamauchi *et al.* 2003) while other studies failed to do so (Anderlova *et al.* 2006; Lee *et al.* 2003).

Circulating resistin levels are higher in genetic experimental models of obesity as well as in rodents with diet-induced obesity (Haluzik *et al.* 2004a). Resistin administration impairs

glucose tolerance and insulin action, while its neutralization by anti-resistin antibody improves glucose tolerance and insulin sensitivity (Steppan *et al.* 2001). Furthermore, resistin mRNA expression decreases during starvation (Kim *et al.* 2001) and resistin administration stimulates glucose production in the liver as demonstrated in both normal and resistin knock-out mice (Rajala *et al.* 2003).

Despite some controversies both resistin and adiponectin genes are considered new candidate genes of insulin resistance and type 2 diabetes mellitus. Polymorphisms for both adiponectin and resistin genes have been studied mostly in obese subjects and/or patients with type 2 diabetes, while to our best knowledge no data regarding single nucleotide polymorphisms in the adiponectin and the resistin genes in anorexia nervosa patients has been published yet.

A presence of G allele in locus 276 of ADP gene was accompanied by with lower and T alele with higher levels of ADP (Hara et al. 2002, Fredriksson et al. 2005). G/G genotype of SNP 276 was found to be associated with impaired glucose tolerance (Gonzalez-Sanchez et al. 2005). These authors also proved the connection between a presence of G alele in position 45 and impaired glucose tolerance. In contrast association between this polymorphism and insulin resistance was not described in another study (Filippi et al. 2004). Menzaghi found out that genotypes 45G and 276T of ADP gene tightly correlate with a lot of markers of insulin resistance (body weight, waist circumference, HOMA index, total to HDL cholesterol ratio) (Menzaghi et al. 2002).

Two articles about resistin gene 62G>A SNP were published. In Chinese population lower frequency of alele A was demonstrated in type 2 diabetic patients than in non diabetic persons. Diabetics with GG genotype had higher prevalence of arterial hypertension. Authors of this article consider this polymorphism as an independent factor of type 2 DM and arterial hypertension (Tan et al. 2003). Similar study was presented by German authors (Gouni-Berthold et al. 2005) that did not prove the

difference in appearance of alele A between diabetic and non-diabetic patients. Only one paper focused on -180C>G SNP in promotor region of resistin gene has been published so far. GG homozygotes had significantly higher levels of resistin mRNA in abdominal subcutaneous fat and these positively and independently correlated with insulin resistance and amount of fat in liver (Smith et al. 2003).

The aim of our study was to characterize the influence of polymorphisms +45T>G and +276T>G of the adiponectin gene and +62G>A and -180C>G of the resistin gene on the circulating levels of these hormones and metabolic phenotype of the carriers.

Methods:

Twenty-eight female patients with anorexia nervosa (BMI 15.72 ± 0.36 kg/m^2), 77 obese female patients (BMI 43.48 ± 1.12 kg/m2) and 38 age- and sex-matched healthy controls (BMI 22.32 ± 0.40 kg/m^2) were included into the study. **Control subjects had no family history of diabetes and/or obesity.** The diagnosis of eating disorder was based on the Diagnostic Statistical Manual IV diagnostic system. All subjects provided written informed consent and the study was approved by the ethics review committee of the General University Hospital.

All subjects included into the study underwent a single blood drawing after overnight fasting and anthropometric examination (height and weight were taken, and body mass index (BMI) was calculated).

Serum adiponectin, resistin and TNF-alpha levels were measured by ELISA kits (Linco Research, USA; BioVendor, CR; R&D Systems, USA) and insulin levels by RIA kit (Immunotech, CR). **Serum glycated hemoglobin (HbA1c) concentrations were assessed by high performance liquid chromatography, serum cholesterol and** blood glucose levels by standard laboratory methods at the Department of Clinical Biochemistry of the University

Hospital in Prague. Homeostasis model assessment (HOMA) index was also calculated as described previously (Matthews *et al.* 1985).

DNA was isolated from 500 μl of whole blood on the MagNA Pure Compact instrument using the MagNA Pure Nucleid Acid Isolation Kit (both Roche Diagnostics, GmbH, Germany). The average DNA concentration (127.49 ± 5.05 μg/ml) was determined from absorbance at 260 nm (BioPhotometer, Eppendorf AG, Germany). All samples had a 260/280 nm absorbance ratio between 1.6 and 1.79. The integrity of the DNA was checked by electrophoresis on 0.8 % agarose gel with an ethidium bromide.

Isolated DNA was used for determination of 2 single nucleotide polymorphisms (SNP) in the adiponectin gene (45 T>G a 276 G>T) and 2 SNP in the resistin gene (62 G>A, -180 C>G). All SNPs were detected by polymerase chain reaction (PCR) on the MyCycler^{TM}Thermo Cycler instrument (Bio-Rad, USA) with subsequent restriction analysis of PCR products (RFLP).

The mix for all PCRs contained 2x PPP Master Mix (Top-Bio s.r.o., Czech Republic), forward and reverse primers, PCR H_20 (Top-Bio s.r.o., Czech Republic) and approximately 150ng of DNA. All primer sequences as well as PCR and RFLP conditions are shown in Table 1 - 4.

PCR and RFLP products were detected by electrophoresis on 2% agarose gel with 1% ethidium bromide. PCR and RFLP products were verified by sequence-based typing on the ABI PRISM 310 instrument (Applied Biosystems, Ca, USA).

Statistical analysis was performed using the SigmaStat software (Jandel Scientific, USA). Means and standard errors were calculated from obtained data. Significant differences between groups were confirmed by non-paired t-tests or Mann-Whitney Rank Sum tests. The differences in frequencies of individual polymorphisms were assessed by frequent analysis, Fisher exact tests and chi-square tests.

Results:

Anthropometric, biochemical and hormonal parameters

Anthropometric, biochemical and hormonal parameters are shown in Table 5. As expected BMI was markedly increased in obese and decreased in anorexia nervosa group relative to control group. Blood glucose levels, insulin levels and HOMA index were significantly higher in obese group relative to both anorexia nervosa patients as well as control subjects. Glycated hemoglobin (HbA1c) concentrations were significantly higher in obese group relative to control subjects, while it was significantly lower in anorexia nervosa patients relative to both other groups. No differences in cholesterol levels were found between groups. Adiponectin concentrations were significantly lower in obese women compared to the control group. In contrast, anorexia nervosa patients had significantly higher adiponectin levels compared to healthy women. Resistin concentrations were significantly higher in obese and lower in anorexia nervosa patients relative to control group. No differences in TNF-α serum concentrations were found between groups. (Table 5)

Adiponectin and resistin gene polymorphism frequency and their influence on the metabolic phenotype

The frequency of studied genotypes did not differ between anorexia nervosa, obese and control group, respectively. Overall, the G allele in locus 62 and C allele in locus –180 of the resistin gene as well as the T allele in locus 45 and the G allele in locus 276 of the adiponectin gene were all present more frequently than minor alleles A, G, G and T in corresponding loci (RETN 62: $p<0.0001$, RETN -180: $p<0.0002$, ADP 45: $p<0.0001$, ADP 276: $p<0.0001$). **The frequency of G allele in locus 62 of RETN gene was 94% in NW, 100% in AN and 96 % in OB, frequency of C allele in locus -180 of RETN gene was 81% in NW, 65% in AN and 65% in OB. T allele in position 45 od ADP gene was presented 95% in NW, 100% in**

AN and 94% in OB group and G allele in position 276 of ADP gene 83% in NW, 71% in AN and 72% in OB group (Table 6).

For further analysis, subjects in each group were divided into subgroups based on the genotype. In anorexia nervosa patients, serum cholesterol levels were higher in carriers of the minor T allele in position 276 of the adiponectin gene relative to G/G genotype (5.85±0.33 vs. 4.56±0.20, $p<0.05$). Furthermore, AN subjects with the minor G allele in position -180 of the resistin gene had higher BMI in comparison with carriers of the C/C genotype (16.12±0.41 vs. 14.54±0.59, $p<0.05$.).

In obese patients, carriers of the minor T allele had higher adiponectin levels than the G/G homozygotes for ADP+276 (18.95±1.94 vs. 14.67±1.37, $p<0.05$). Obese patients with the minor A allele in position 62 of the resistin gene had higher cholesterol levels than OB patients with the G/G genotype in the same position (6.15±0.47 vs. 4.77±0.44, $p<0.001$).

In the control group, persons with the minor T allele of the ADP 276 possessed lower levels of HbA1c than subjects with the G/G genotype (3.58±0.10 vs. 3.85±0.06, $p<0.05$). The presence of minor allele A in SNP RETN+62 showed lower levels of HbA1c compared to the G/G genotype (3.26±0.13 vs. 3.80±0.05, $p<0.001$).

Discussion:

The aim of our study was to determine the frequencies of 45T>G and 276T>G adiponectin and 62G>A and -180C>G resistin gene polymorphisms in the patients with obesity, anorexia nervosa and a control group of healthy normal-weight women. Furthermore, we focused on the influence of particular genotypes on serum levels of adiponectin and resistin and on its influence on metabolic parameters including HOMA index as a rough measure of insulin sensitivity.

The presence of the minor T allele in the locus 276 of the adiponectin gene increased adiponectin levels in obese patients and decreased HbA1c concentrations in healthy women, respectively. These results are in accordance with previously published data. It has been described that subjects with the G/G genotype in position 276 of the adiponectin gene had lower adiponectin concentrations and impaired glucose tolerance, respectively (Gonzalez-Sanchez *et al.* 2005; Hara *et al.* 2002). Carriers of the minor T allele in position 276 of the adiponectin gene possessed higher adiponectin mRNA expression in visceral fat which positively correlated with adiponectin levels (Fredriksson *et al.* 2006). Interestingly, the presence of the minor T allele of the position 276 in the adiponectin gene in anorexia nervosa patients was accompanied by increased cholesterol levels while no effect on glycated hemoglobin or circulating adiponectin levels was noted. Taken together our data suggest that the effect of the presence of the minor T allele in ADP 276 is significantly modified by nutritional status that can probably overcome some of modifying effects of this polymorphism on serum adiponectin levels.

Another polymorphism studied in our cohort of patients was 62G>A polymorphism of resistin gene. Obese carriers of the minor A allele in the locus 62 of the resistin gene had significantly higher cholesterol levels than obese G/G homozygotes while the presence of the minor A allele in healthy **normal-weight women** was accompanied by lower HbA1c levels. In study by Tan et al. a lower frequency of allele A in this SNP was found in type 2 diabetic patients relative to non-diabetic persons (Tan *et al.* 2003).

Connection between the presence of the minor G allele in position -180 of the resistin gene and higher BMI in anorexia nervosa patients has not been described yet. It is possible, that this SNP can be, as well as presence of the minor T allele in SNP ADP +276, the protective factor of some metabolic anomalies.

With the exception of the above mentioned connection between SNP 276G>T of the adiponectin gene and serum adiponectin levels, no other influences of the studied polymorphisms on adiponectin or resistin levels were found in our study.

It has to be noted that our study is limited by relatively low number of subjects; therefore some minor genotype-induced differences in metabolic factors may have been missed due to lower statistical power. Despite this limitation our data **together with previously published studies** indicate that both adiponectin and resistin gene polymorphisms can significantly influence the metabolic phenotype of patients with nutritional disorders such as obesity and anorexia nervosa. We suggest that the manifestations of respective genotypes differ in patients with different nutritional status.

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Table 1:

Detection details of SNP adiponectin 45 T>G

forward primer*	5‘ – GAA GTA GAC TCT GCT GAG ATG G – 3‘
reverse primer*	5‘ – TAT CAG TGT AGG AGG TCT GTG ATG - 3‘
PCR conditions	95°C/5min 1x 95°C/60s 35x 58°C/45s 35x 72°C/45s 35x 72°C/5min 1x
PCR product length	372bp
restriction enzyme	*Sma I* °
RFLP conditions	30°C/24h
RFLP products length	in presence G allele 219 bp a 153 bp in presence T allele 372 bp

* primer sequences from (Xita *et al.* 2004)

° Fermentas Life Sciences, Lithuania

Table 2:

Detection details of SNP adiponectin 276 G>T

forward primer*	5‘ – GGC CTC TTT CAT CAC AGA CC - 3‘
reverse primer*	5‘ – AGA TGC AGC AAA GCC AAA GT - 3‘
PCR conditions	95°C/5min 1x 95°C/60s 35x 58°C/45s 35x 72°C/45s 35x 72°C/5min 1x
PCR product length	196 bp
restriction enzyme	*Mva 1269I* °
RFLP conditions	37°C/24h
RFLP products length	in presence G allele 148 a 48 bp in presence T allele 196 bp

* primer sequences from (Xita et al. 2004)

° Fermentas Life Sciences, Lithuania

Table 3:

Detection details of SNP resistin +62G>A

forward primer*	5‘ – GCC GAG ACC ACA TGT CAC T - 3‘
reverse primer*	5‘ – CCT CCG GGC CTA CTA AAG AA - 3‘
PCR conditions	96°C/2min 1x 94°C/30s 35x 54,3°C/30s 35x 72°C/30s 35x 72°C/5min 1x
PCR product length	233 bp
restriction enzyme	*BseR I* °
RFLP conditions	37°C/24h
RFLP products length	in presence A allele 151 bp a 82 bp in presence T allele 233 bp

* primer sequences from (Tan et al. 2003)

° New England Biolabs, USA

Table 4:

Detection details of SNP resistin -180 C>G

forward primer*	5‘ – TTT TGT CAT GTT TGC ATC AGC - 3‘
reverse primer*	5‘ – AGA TGC AGC AAA GCC AAA GT - 3‘
PCR conditions	96°C/2min 1x 94°C/30s 35x 58,2°C/30s 35x 72°C/30s 35x 72°C/5min 1x
PCR product length	330 bp
restriction enzyme	*BpiI* °
RFLP conditions	37°C/24h
RFLP products length	in presence C allele 202 bp a 128 bp in presence G allele 330 bp

* primer sequences from (Smith *et al.* 2003)

° Fermentas Life Sciences, Lithuania

Table 5:

Body mass index (BMI), concentrations of adiponectin, resistin, insulin, tumor necrosis factor-alpha (TNF-α), cholesterol, glycated hemoglobin (HbA1c), blood glucose and HOMA index in patients with anorexia nervosa, obesity and in a control group of **healthy normal-weight women.**

	Control group	**Anorexia nervosa**	**Obese women**
N	38	28	77
BMI (kg/m^2)	22.32±0.40	15.72±0.36**++	43.48±1.12** ^{oo}
Glycaemia (mmol/l)	4.14 ± 0.16	3.91 ± 0.05++	5.65±0.23** ^{oo}
Insulin (mIU/ml)	15.52 ±1.87	11.52 ± 1.08++	32.63±2.60** ^{oo}
HOMA index (mmol.IU/l^2)	2.97 ± 0.45	2.00 ± 0.18++	8.65±0.85** ^{oo}
HbA1c (%)	3.72±0.07	3.41±0.12*++	4.21±0.12* ^{oo}
Cholesterol (mmol/l)	4.86±0.14	4.80±0.17	4.88±0.11
Adiponectin (μg/ml)	33.24±4.41	58.44±7.17*++	17.02±1.19** ^{oo}
Resistin (ng/ml)	6.27±0.50	3.99±0.33*++	8.11±0.60* ^{oo}
TNF-alpha (ng/ml)	0.91±0.17	1.37±0.30	2.30±0.94

Results are expressed as means ± standard error means

* statistically significant difference compared to a control group ($p<0.05$)

** statistically significant difference compared to a control group ($p<0.001$)

++ statistically significant difference compared to obese women ($p<0.001$)

^{oo} **statistically significant difference compared to patients with anorexia nervosa ($p<0.001$)**

Table 6: The allele frequency of studied SNP in patients with anorexia nervosa, obesity and in a control group of healthy normal-weight women.

		Control group	**Anorexia nervosa**	**Obese women**
RETN 62	Allele G	94 %	100%	96%
	Allele A	6%	0%	4%
RETN -180	Allele C	81%	65%	65%
	Allele G	19%	35%	35%
ADP 45	Allele T	95%	100%	94%
	Allele G	5%	0%	6%
ADP 276	Allele G	83%	71%	72%
	Allele T	17%	29%	28%