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ISSN: 0423-104X

e-ISSN: 2299-8306

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DOI: 10.5603/EP.a2021.0065

Article type: Original paper

Submitted: 2021-03-20

Accepted: 2021-03-23

Published online: 2021-07-12

Evaluation of the frequency of *RETN* c.62G>A and *RETN* c.-180C>G polymorphisms in the resistin coding gene in girls with *anorexia nervosa*

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Abstract

Introduction: *Anorexia nervosa* (AN) is a serious psychosomatic syndrome, classified as an eating disorder. AN patients strive to lose weight below the normal limits defined for a specific age and height, achieving their goal even at the expense of extreme emaciation. AN has a multifactorial aetiology. Genetic factors are believed to be significant in the predisposition to the development of AN. In girls suffering from AN significantly lower levels of resistin (RES) in blood serum are observed as compared to healthy girls. These differences may lead to a thesis that functional genetic polymorphisms in RES coding genes can be responsible for this phenomenon. In our pilot study we demonstrated significant differences in the distribution of genotypes in the locus *RETN* c.-180C>G of the RES gene in 67 girls with AN and 38 healthy girls. It seems reasonable to compare the frequency of polymorphisms of *RETN* c.62G>A and *RETN* c.-180C>G in the RES gene in girls with AN and in healthy subjects in a bigger cohort and to analyse correlations between individual variants of the polymorphisms referred to above and the RES levels in blood plasma.

Material and methods: The study covered 308 girls with the restrictive form of AN (AN) and 164 healthy girls (C) (aged 11–19 years). The RES levels in blood serum were determined by means of the ELISA method on a Bio-Vendor machine from LLC (Asheville, North Carolina, USA). The DNA isolation was carried out by means of Genomic Mini AX BLOOD (SPIN). The PCR reaction was carried out on a ThermoCycle T100 thermocycler. 80–150 ng of the studied DNA and relevant F and R starters were added to the reaction mixture. The reaction products were subjected to digestion by restriction enzymes and separated on agarose gels (RFLP).

Results: The average RES level in blood serum in the AN group was significantly lower ($p < 0.0001$) than in the C group. The distribution of genotypes in the locus *RETN c.62* of the RES gene was similar in both groups. A significant difference was demonstrated in the distribution of genotypes in the polymorphic site *RETN c.-180* of the RES gene between AN and C ($p = 0.0145$) and in the distribution of the C and G alleles in the locus *RETN c.-180* ($p < 0.0001$). The C allele occurred significantly more frequently than the G allele in the C group as compared to the AN

group. In all the study subjects jointly (AN and C) a significant positive correlation between the blood RES levels on one hand and the body mass ($r = 0.42$; $p < 0.0001$) and BMI ($r = 0.61$; $p < 0.0001$) on the other was observed. There was no correlation between the concentration of RES in blood serum and the distribution of genotypes in the loci of the resistin gene referred to above.

Conclusions: The CG genotype in the locus *RETN* c.-180 C>G of the RES gene may constitute one of the factors predisposing to the development of AN in girls. The genotype in the loci *RETN* c.62 G>A and *RETN* c.-180 C>G of the resistin gene has no influence on the levels of this hormone in blood in AN patients.

Key words: anorexia nervosa; polymorphism; resistin

Introduction

Anorexia nervosa (AN) is a psychosomatic syndrome classified as one of an eating disorder, characterised by excessive concentration on one's body weight and appearance, as well as abnormal eating patterns and energy expenditure [1]. *Anorexia nervosa* patients strive to lose weight below normal limits defined for a specific age and height, achieving their goal even at the expense of extreme emaciation [2]. *Anorexia nervosa* diagnostic criteria are determined in the International Statistical Classification of Diseases and Related Health Problems (ICD-10 — F-50) [3]. The criteria most often applied for the purposes of scientific research are the ones contained in the chapter Feeding and Eating Disorders of the fifth edition of Diagnostic and Statistical Manual of Mental Disorders 5, (DSM-5). DSM-5 distinguishes two forms of AN: restrictive AN and bulimic-purging AN [4]. This disorder affects ca. 1% of the population of girls and women aged 15–24 years, and the incidence is estimated at 0.7–1/10,000 women annually [5].

Despite numerous studies devoted to AN, its aetiology remains unclear, and the significance of specific pathogenetic factors is controversial. Authors agree, however, on the multifactorial theory of the disease development. Both individual factors predisposing to the disease (genetic factors and some personality traits), as well as environmental ones (sociocultural, family-related, and socioeconomic) may play a role in the aetiopathogenesis of AN.

The relationship between sociocultural factors and the risk of AN can be suggested by the higher incidence of AN in western countries, where the cult of the body and health, the model of a slim figure, and physical fitness are promoted, and at the same time obesity is excessively stigmatised.

In recent years these countries have observed an increase in the incidence of eating disorders, AN included, by ca. 20–30%. Transcultural studies, however, do not allow us to confirm the thesis that the effects of western culture are sufficient for AN to develop [6].

The theory of the passive correlation of environmental and genetic factors has been raised. Parents of patients suffering from AN often demonstrate certain common personality traits, such as excessive ambitions, perfectionism, and perseverance. These qualities, perhaps genetically determined, are passed on to their offspring. Nevertheless, the significance of family-related factors in AN has not been fully proven; most analyses conducted thus far constitute correlative studies, not allowing us to draw certain cause and effect conclusions [6].

Research carried out in the last three decades have demonstrated that genetic factors contribute to the development of AN. Twin and family studies have allowed us to determine the heritability of eating disorders, i.e. to assess what part of the inter-individual phenotypic variability is dependent on genotypic variation. According to those studies the heritability of AN is high; it is estimated at the level of 48–88% [7], which indicates the legitimacy of searching for genes predisposing to AN. A large study of families, covering 1831 relatives of 504 probands with AN, 177 patients with *bulimia nervosa*, and 181 subjects from a control group, demonstrated that first-degree female relatives have an at least ten-fold higher risk of developing full-blown AN than the general population [7].

Two different strategies to clarify the molecular background of multifactorial diseases such as AN have been proposed. The first approach is based on candidate gene studies, and the other (the genome-wide association study — GWAS) is devoted to studying the entire genome, without making *a priori* hypotheses. Differences in the distribution of alleles or genotypes between the patients and the control group, carefully matched according to ethnicity, suggest a role of the specific locus in the pathogenesis of the disorder [7]. On the other hand, studies applying linkage analysis are not conducted on a large scale in AN patients at present due to high costs and logistic difficulties [6].

Numerous attempts to find genetic variants for AN using a candidate gene approach [8–11] failed to provide satisfactory results due to small sample sizes, methodological differences, and heterogeneity of the studied populations [8].

Duncan et al. [12] observed, on the basis of the largest cohort of AN patients studied thus far, that the strongest genetic correlation among all the studied metabolic and psychological phenotypes is a negative correlation between *anorexia nervosa* and insulin resistance.

The hormone of the adipose tissue participating in the modulation of insulin resistance is resistin (RES) [13–15]. The resistin coding gene *RETN* mapped in the region 19p13.2 contains 4 exons [16, 17]. In humans the RES expression is detected in preadipocytes, minor in the brown adipose tissue, but its main source are macrophages [17, 18]. In mice some minor expression is detected in the pituitary gland and the arcuate nucleus of the hypothalamus. RES is suggested to play a role in central mechanisms associated with food intake through inhibition of secretion of catecholamines in the hypothalamus [19]. It plays a direct role in causing insulin resistance (the word “resistin” derives from the phrase “insulin resistance”), as well as indirectly by blocking adipocyte differentiation — in causing ectopic fat storage in the liver and in the skeletal muscles [20].

The concentration of RES in blood serum positively correlates with body mass and BMI; it drops during starvation and returns to normal after resuming feeding [21–23]. In girls suffering from AN a significantly lower concentration of RES in blood serum is found as compared to healthy girls with normal body weight and obesity [24].

Differences in RES levels in blood in subjects with AN as compared to healthy subjects, observed by numerous authors, led to the hypothesis that functional genetic polymorphism in the RES coding gene could be responsible for this phenomenon.

The subject literature available contains only a few works on this topic. Křížova et al. [25], when studying single alleles in the loci +62G>A and -180C>G of the RES gene found that the presence of a less frequently occurring G allele in the polymorphic site *RETN* c.-180C>G may constitute a protective factor for certain metabolic abnormalities. The authors found a correlation between a higher BMI in the AN group and less frequent G allele in the site *RETN* c.-180C>G ($p < 0.05$).

In the pilot study we conducted in 2017 in 68 girls suffering from AN and 38 healthy girls [26], focusing on the incidence of polymorphisms in adiponectin and resistin genes, we obtained encouraging results in sites where functional polymorphisms can be located in the context of causing insulin resistance. We demonstrated significant differences in the distribution of

genotypes in the sites *RETN* c.-180C>G of the resistin gene. There were no significant differences in the locus *RETN* c.62G>A. Hence it seems legitimate to continue research in this scope in a bigger cohort, assuming that perhaps one of the genotypes in the locus *RETN* c.-180C>G of the resistin gene could constitute a factor predisposing to the development of *anorexia nervosa.*

The objective of the present study is to compare the frequency of *RETN* c.62G>A and *RETN* c.-180C>G polymorphisms in the resistin gene in girls with *anorexia nervosa*, and to analyse correlations between variants of these polymorphisms and the level of resistin in blood serum of the subjects.

Material and methods

The study covered 472 girls aged 11–19 years, including 308 girls with the restrictive form of *anorexia nervosa* (AN) and 164 healthy girls (C). All the study subjects came from the region of Silesia. The present study is complementary to our research (not published thus far) focusing on the assessment of the frequency of *ADIPOQ* c.45T>G and *ADIPOQ* c.276G>T polymorphisms in the adiponectin coding gene. It covers the same group of girls treated in Clinical Hospital No. 1 in Zabrze and a group of healthy subjects (Tab. 1). The AN group included patients suffering from the restrictive form of anorexia nervosa, diagnosed based on the ICD-10 [3] and DSM-5 [4] criteria after ruling out other somatic and mental disorders that could have been the cause of emaciation. Tests were carried out prior to the therapy onset, during the first three days after admission. The condition for taking blood samples for hormonal tests was a stable status of the patients and no features of dehydration. The preliminary results of additional tests (electrolytes, AspAT, AlAT, creatinine) allowed us to rule out acute and chronic liver and kidney diseases.

The control group (C) consisted of girls with normal body weight, healthy as of the time of the study, with no chronic diseases, who had not been on any medications in the previous month, and who had not been on any slimming diet and had not been using any other slimming method over the previous three months.

Informed consent for taking part in the study was obtained from the subject’s parents / legal guardians and by the subject herself if aged > 16 years.

The Bioethics Committee of the Medical University of Silesia in Katowice issued permission to conduct the study (Decision No. KNW/0022/KB1/108/1/11 dated 20 September 2011).

The present study used the data on the subjects' body mass, height, BMI, and BMI-SDS obtained in measurements performed during the previous study referred to above.

The molecular tests were carried out in the Department of Genetics (Institute of Psychiatry and Neurology, Warsaw) with the use of DNA isolated from frozen samples of peripheral blood. The isolation was obtained by means of a commercial Genomic Mini Ax Blood Spin kit (A&A Biotechnology, Gdynia, Poland).

The analysis of selected polymorphisms in the gene of resistin (*RETN*) was performed according to the following schedule:

- amplification in the PCR reaction of a section of the gene containing a polymorphic locus. The PCR reaction was conducted in the Thermo Cycle thermal cycler by Bio-Rad with the application of the NXT Taq PCR kit by EURx, containing the following: hot start NXT TaqDNA Polymerase, reaction buffer, $MgCl_2$, and dNTPs, and with the application of relevant F and R starters. 80–150 ng of the tested DNA was added to the reaction mixture. The reaction was conducted in 10 μL in the following conditions: 3 minutes at 95°C, and then 35 cycles: 30 seconds at 95°C, 30 seconds at 58°C, and 1 minute at 72°C. The final stage was a 5-minute incubation of the product of the PCR reaction at 72°C;
- digesting the product of the PCR reaction with a restriction enzyme. The reaction was conducted for 1 hour at a temperature of 37°C for both enzymes, *BseRI* and *BpiI*;
- electrophoretic division of the digestion products obtained in the agarose gel containing ethidium bromide;
- preparation of photographic documentation;
- statistical analysis of the results obtained.

The starters applied and the selected restriction enzymes identifying the polymorphic loci examined are listed below:

RETN gene polymorphism c.62 G > A

Starter F 5'– GCC GAG ACC ACA TGT CAC T – 3'

Starter R 5' – CCT CCG GGC CTA CTA AAG AA – 3'

Restrictive enzyme *BseRI*

RETN gene polymorphism c.-180 C > G

Starter F 5’– TTTT GT CAT GTTT GCA TCA GC – 3’

Starter R 5’ –GGG CTG AGC TAA CCA AAT– 3’

Restrictive enzyme *BpiI*

The Bioethics Committee of the Medical University of Silesia in Katowice issued permission to conduct the study (Decision No. KNW/0022/KB1/108/1/11 dated 20 September 2011).

Statistical analysis

The database was drawn up in an Excel spreadsheet by Microsoft (Redmond, Washington, USA). Statistical calculations were performed using MedCalc software ver. 19.1.3 (MedCalc, Ostend, Belgium). The level of $\alpha = 0.05$ was considered significant. The following parameters of the descriptive statistics were determined: arithmetic mean, median, minimum and maximum value, lower and upper quartile, standard deviation (SD), standard error (SE), and 95% confidence interval around the mean value. For all parameters the conformity of their distribution with the normal distribution was verified. The conformity assessment applied the Shapiro-Wilk test. The assessment of differences between the means was performed by means of Student’s t-test with a separate evaluation of variances and the U Mann-Whitney test. A one-way analysis of variance ANOVA was also performed. Homogeneity of variances was evaluated by means of Levene’s test.

The tests results were presented graphically as a box plot with a mean value and a 95% confidence interval for the mean value marked as an error bar. An analysis of contingent tables was performed for qualitative variables. Descriptive statistics were calculated: Pearson’s chi-square, the significance level p, and the C contingency coefficient. The results are presented in graphic form by means of categorised histograms.

Results

The average age in the AN and C groups was similar (15.06 ± 1.57 years and 15.16 ± 2.12 years, respectively). The average body weight in the AN group was significantly lower ($p < 0.05$) than in the C group (40.21 ± 6.01 kg and 49.87 ± 7.93 kg, respectively). The average BMI and BMI-

SDS in girls with AN were significantly lower ($p < 0.05$) than in the healthy girls (BMI: 15.19 ± 1.67 kg/m^2 vs. 20.12 ± 2.46 kg/m^2, BMI-SDS: –2.53 ± 0.96 vs. 0.12 ± 1.06, respectively) (Tab. 1).

The average RES level in blood serum in the AN group was significantly lower ($p<0.0001$) than in the C group (2.60 ± 0.62 ng/mL and 5.65 ± 2.42 ng/mL, respectively) (Tab. 1; Fig. 1).

The frequency of genotypes in the *RETN c.62* and *RETN c.-180* polymorphic site of the resistin gene in both groups (AN and C) is depicted in Table 2 and Figures 2 and 3.

The most frequent genotype in the *RETN c.62* polymorphic site of the resistin gene in both groups was the GG genotype. The GA genotype in this gene was more frequent in girls with AN as compared to the healthy subjects (Tab. 2; Fig. 2). The most frequent genotype in the *RETN c.-180* polymorphic site in the AN patients was the CG genotype, then the CC genotype, and the least frequent was the GG genotype; whereas, in the healthy subjects the CC genotype was the most often observed, then the CG genotype, and the least frequent was the GG genotype (Tab. 2, Fig. 3).

The distribution of genotypes in the *RETN c.62* polymorphic site of the resistin gene was similar in both groups (AN and C) and did not differ statistically significantly ($p = 0.059$). However, a statistically significant difference was observed in the distribution of genotypes in the *RETN c.-180* polymorphic site of the resistin gene between the AN and C groups ($p = 0.0145$) (Fig. 3).

The frequency of single alleles in *RETN c.62* polymorphic sites in both groups was similar, and no statistically significant differences were observed in this respect. The G allele occurred more frequently than the A allele in the *RETN c.62* polymorphic site (Tab. 3, Fig. 4).

Statistically significant differences were demonstrated in the distribution of C and G alleles in the *RETN c.-180* polymorphic site between the AN group and the C group ($p < 0.0001$). The C allele occurred significantly more frequently than the G allele in the group of healthy girls than in the subjects with AN (Tab. 3, Fig. 5).

In the entire group of the studied girls (AN and C) a statistically significant positive correlation between the RES level in blood serum and the body weight ($r = 0.42$; $p < 0.0001$) and BMI ($r = 0.61$; $p < 0.0001$) was observed (Fig. 6 and 7). No statistically significant relationships were observed between these parameters in individual groups (AN and C).

No relationship between the RES concentration in blood serum and the distribution of genotypes in the investigated polymorphic sites of the resistin gene were observed (Tab. 4).

Discussion

Following the data from literature pertaining to the influence of polymorphisms located in the 3`UTR region, as well as in the sequence preceding the gene upon the expression of the resistin gene, we selected two polymorphic sites for our study: *RETN* c.62 G>A and *RETN* c.-180 C>G.

The tests we performed demonstrated that girls suffering from *anorexia nervosa* are different from healthy subjects in terms of the frequency of genotypes in the *RETN* c.-180 C>G polymorphic site of the resistin gene. The most common genotype in AN patients is the CG genotype, and in healthy subjects — the CC genotype. Also, the single C allele occurs significantly more frequently than the G allele in healthy girls than in girls with AN in this polymorphic site. Other authors [25] have also observed that the C allele is more common in this locus in healthy women than in women suffering from AN.

In our pilot study [26] we observed similar differences in terms of the frequency of CC and GG homozygotes and CG heterozygotes in the *RETN* c.-180 C>G polymorphic site of the resistin gene between the group of healthy subjects and the AN patients. The CG genotype ($p < 0.05$) occurred significantly more frequently in the AN group than in healthy subjects (47.69% vs. 28.95%) ($p < 0.05$). The control group was dominated by the CC genotype as compared to the AN group (57.89% vs. 28.95% ($p < 0.05$). Therefore, the CG genotype in this site of the resistin gene seems to constitute one of the factors predisposing to *anorexia nervosa*.

Smith et al. [20] investigated the functionality of the -180 C>G polymorphism in the resistin gene. They demonstrated higher levels of mRNA resistin in the subcutaneous adipose tissue of the abdomen in GG homozygotes in the *RETN* c.-180 C>G polymorphic site as compared to CG and CC homozygotes. Furthermore, they demonstrated a positive correlation between the expression of mRNA resistin on one hand and insulin resistance and fat content in the liver on the other. This could be indicative of the possible significance of this variant of the *RETN* gene in the pathogenesis of AN.

Engert et al. [27] claimed that variant 5' of human resistin, g.−420 C> G (the same polymorphism as c.−180 C> G named with a different numbering system), is associated with obesity. In Canadian populations of patients with diabetes, obesity, and a control group they observed significantly higher BMI values ($p = 0.03$) in individuals with the CG and GG genotypes in comparison to CC homozygotes in this polymorphic site of the resistin gene. They did not confirm, however, the described relationship for this promotor variant in the Scandinavian population.

The *RETN* c.−180 polymorphic site has been investigated by other authors; they did not, however, detect any relationship between this single nucleotide polymorphism (SNP) with obesity [28, 29]. Summing up, there is a consensus that SNP -180 C>G is not related to obesity (when investigating each genotype separately), but in certain populations or in other circumstances, which have not been determined yet, the GG genotype may predispose to a higher content of the adipose tissue.

When choosing the *RETN* c.62 G>A polymorphic site for our study, we took into account the associations of c.62AA and c.62AG with a reduced risk and of c.62GG with an increased risk of the development of the type 2 diabetes and hypertension, discovered by Tan et al. [30]. Tan et al. studied 1102 Chinese patients with type 2 diabetes and 743 patients without diabetes. Subjects suffering from type 2 diabetes exhibited a lower frequency of the occurrence of the A allele of the resistin gene in the *RETN* c. 62 site (the frequency of the GG and GA/AA genotypes was 83.5% and 16.5%, respectively) than subjects from the control group (GG:GA/GA, 75.1% vs. 24.9%; $p < 0.001$). In patients with diabetes and the GG genotype hypertension was more frequently observed (GG GA/AA, 49.8% vs. 36.2%; $p = 0.001$). Further analyses confirmed that the presence of the G allele in the *RETN* c.62 polymorphic site acts as an independent factor contributing to type 2 diabetes and hypertension. These results were suggestive of a possible role of resistin in the pathogenesis of type 2 diabetes and hypertension associated with insulin resistance [30].

Observations of these authors have not been confirmed in any other studies. For example, Gouni-Berthold et al. [31] investigated 384 patients with type 2 diabetes (224 males and 160 females) with 434 individuals without diabetes constituting a control group (248 males and 186 females) from the German Caucasian population, in terms of the relationship between this polymorphism

and type 2 diabetes, hypertension, the level of lipoproteins, and the RES concentration in blood, as well as atherosclerosis. The A allele in the + 62G > A polymorphic site in the RES gene occurred in 34 subjects from the control group and 24 subjects with diabetes (frequencies of the alleles: 4% and 3.2%, respectively). The frequency of genotypes was similar in subjects with type 2 diabetes (93.75% and 6.25%, respectively, for GG:GA/AA) and without diabetes (92.2% and 7.8%, respectively for GG:GA/AA) ($p = 0.31$). In carriers of the A allele in the control group and in the entire investigated cohort hypertension occurred significantly more frequently ($p = 0.039$ and $p = 0.005$, respectively). Such a relationship did not occur in subjects with diabetes. No relationship between the investigated polymorphism and BMI, the presence of atherosclerosis, the level of triglycerides, HDL, and LDL cholesterol was detected, both in the control group and the group of subjects with diabetes. Similarly, no difference in the RES concentration in blood between carriers and non-carriers of variant A in this polymorphic site was demonstrated. Summing up, the study demonstrated that in the German Caucasian population variant A occurring in the *RETN* c.62 polymorphic site of the RES gene is associated with hypertension but not with type 2 diabetes [31].

In the Czech population, authors [25] compared the frequency of single alleles in the *RETN* c.62 G>A polymorphic site of the resistin gene in obese subjects (BMI: 43.48 ± 1.12 kg/m^2), AN patients (BMI: 15.72 ± 0.36 kg/m^2), and in healthy females (BMI: 22.32 ± 0.40 kg/m^2). In patients with AN they demonstrated — as we did in our study — that the most frequent allele in the locus *RETN* c.62 G>A of the resistin gene is the G allele (100% and 93.8%, respectively).

As well as studying the frequency of single alleles in these polymorphic sites, we also analysed the frequency of genotypes. We demonstrated that the most frequent genotype was the GG genotype in both groups; we did not detect the AA genotype in any of the subjects. The distribution of genotypes in the *RETN* c.62 G>A polymorphic sites of the resistin gene in the AN patients examined by us does not differ from the distribution of these genotypes in healthy subjects. We made similar observations in our pilot study. This could confirm that the genotype in this locus does not have any influence on the predisposition to develop AN.

We demonstrated significantly lower concentrations of RES in blood serum in girls with AN than in healthy subjects ($p < 0.0001$). The RES concentration correlates positively with the body mass and BMI in all the study subjects jointly ($p < 0.0001$). Similar observations have been made by

other authors [32], as well as by us in our pilot study [26].

Ziora et al. [24] observed that the group of girls with AN demonstrated a significantly lower RES concentration in blood serum (3.87 ng/mL) as compared to healthy subjects. In 87 girls with the restrictive form of AN, 17 girls with “nonspecific eating disorders”, 30 obese, and 61 healthy girls with a normal body weight, it was demonstrated that the average concentrations of resistin in blood of the girls with AN (2.8 ng/mL) were nearly two-fold lower as compared to healthy subjects (4.1 ng/mL) and obese girls (4.8 ng/mL). In all the study subjects jointly, the RES concentration correlated positively with BMI. Conversion of blood resistin levels into BMI indicated that in girls suffering from AN the values of resistin levels in blood/BMI were higher than in obese girls (unlike the absolute values), albeit close to the values obtained for healthy girls.

Křížova et al. [25] obtained similar results when comparing blood resistin levels in 28 females with AN and selecting — besides healthy subjects — an additional group of 77 obese women. They observed significantly lower resistin concentrations in blood in AN patients quoted in absolute values (3.99 ± 0.33 ng/mL) as compared to obese subjects (8.11 ± 0.60 ng/mL; $p < 0.001$) and healthy women (6.27 ± 0.50 ng/mL; $p < 0.05$).

Dolezalova et al. [33], who investigated the influence of chronic malnutrition on the endocrine function of the adipose tissue in 12 females with the restrictive form of *anorexia nervosa*, did not detect any differences in blood RES levels in the AN group as compared to the group of healthy women. They did, however, demonstrate a significantly higher expression of mRNA resistin and a lower expression of IL-6 and the immunocompetent cells marker (CD 68) in the subcutaneous adipose tissue in AN patients as compared to healthy subjects. On the basis of these observations, the authors conclude that local changes in the mRNA resistin expression in the adipose tissue may not reflect the blood levels of this hormone in AN.

On the other hand, other authors [34] did not detect any significant differences between average RES levels in patients with the restrictive (3.44 ng/mL) and bulimic-purgatory form of AN (5.12 ng/mL) nor in AN female patients (4.36 ng/mL) as compared to healthy subjects (4.38 ng/mL). In none of the investigated groups researchers observed any correlations between the blood RES levels on one hand and BMI and the adipose tissue content on the other. Hence they concluded that the concentration of resistin in blood is not related to the nutritional status.

In healthy subjects a positive relationship between the RES concentration in blood on one hand and the quantity of the adipose tissue and BMI on the other was demonstrated [32]. In our studies [24, 35] we also observed a strong positive relationship between the RES level in blood and BMI jointly in all the study subjects ($r = 0.66$; $p < 0.0001$); there was, however, no correlations in individual groups, regarded separately. This could have been associated — like in the study by Housova et al. [34] — with a small spread of the values of resistin levels and BMI within an individual group.

A drop in the blood resistin level can be a consequence not only of malnutrition, but also of a reduced production of this hormone in the bone marrow, or an increase of the resistin clearance, or it could be an effect of a disfunction of mononuclears/macrophages and impaired production of such cytokines as TNF and IL-6, which enhance the RES expression in macrophages [16, 36–38]. Our previous study [24] demonstrated that the elimination of the influence of the body mass factor on the values of determined resistin levels in blood by converting the blood RES concentrations into BMI indicates that the quantity of this hormone in blood in girls suffering from AN is in fact comparable with healthy subjects and definitely higher than in obese subjects. This may be connected with an enhanced production of this hormone in other organs and tissues (e.g. macrophages), and not only in the adipose tissue, in AN patients.

The relationship between resistin and body mass in humans still remains controversial [39, 40], since the relationships between the level of this hormone in blood and the body mass in obese subjects in the opinions of the authors cited above are divergent. Perhaps the polymorphism of the RES gene and the carrier-state for the G allele in the site -180 of the RES gene has a positive influence on the increase of the BMI. In our studies we did not observe any statistically significant relationships between the blood plasma resistin level and the genotype in both *loci*: *RETN* c.62 G>A and *RETN* c.-180 of the RES gene in girls suffering from *anorexia nervosa* and in healthy subjects, similarly to the pilot study; we did not, however, examine obese girls.

We are aware of certain limitations. Association studies of psychiatric disorders are usually underpowered due to the small sample sizes. As the access to AN patients is lower than in other population studies, hence the study spanned several years. We decided on the final sizes of the studied groups having taken into account the subject literature devoted to association studies, adjusting our technical and financial abilities as much as possible. Another limitation is the fact that the blood plasma resistin levels in AN patients were not determined in all patients, and if

they were, it was carried out in the acute phase of the illness, caused by starvation and a low content of the adipose tissue. Nevertheless, from the statistical perspective a group of this size is sufficient for hormonal studies. One needs to bear in mind also that it is the acute phase of the illness that constitutes the most distinct feature of differentiating AN patients and healthy subjects. Numerous adaptive mechanisms of the body can be observed in such cases, including hormonal ones, which is a very interesting phenomenon.

Based on the results obtained in our study, we conclude that the CG genotype in the *RETN* c.-180 C>G polymorphic site of the resistin gene may constitute one of the factors predisposing to the development of AN in girls.

Furthermore, we believe that the genotype of the resistin gene in the *RETN* c.62 G>A and *RETN* c.-180 C>G polymorphic site has no influence on the levels of resistin in blood of girls suffering from AN.

Funding sources

KNW-1-092/K/8/K

KNW-1-091/K/9/K

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Table 1. Characteristics of examined girls with *anorexia nervosa* (AN) and healthy girls (C) and the results of serum resistin assessment

	AN (n = 308)	**C (n = 164)**
	Mean ± SD (range)	**Mean ± SD (range)**
Age [years]	15.06 ± 1.57 (11–19)	15.16 ± 2.12 (11–19)
Height [cm]	162.47 ± 6.47 (143.0–177.0)	160.31 ± 7.06 (142.0–175.0)

Body weight [kg]	40.21 ± 6.01 (18.7–54.8)	49.87* ± 7.93 (30.2–65.0)
BMI [kg/m^2]	15.19 ± 1.67 (9.11–18.6)	20.12* ± 2.46 (13.9–26.13)
BMI-SDS	–2.53 ± 0.96 (–5.49– –1.94)	0.12* ± 1.06 (–2.17– 2.0)
Resistin [ng/mL]	2.60 ± 0.62 (1.09–3.99)	5.65** ± 2.42 (2.56–11.46)

SD — standard deviation; BMI —body mass index; BMI-SDS — body mass index-standard deviation score (subjects' BMI from the mean BMI for age and gender); AN vs. C * $p < 0.05$; AN vs. C ** $p < 0.0001$

Table 2. The genotype frequency in the polymorphic sites of the resistin gene (*RETN c.62* and *RETN c.-180*) in the examined girls with *anorexia nervosa* (AN) and healthy controls (C)

Examined gene **Genotype**	**AN** **Number (%)**	**C** **Number (%)**
	n = 308	n = 163
	p = 0.059; c = 0.087	
RETN c.62		
GA	38 (12.3%)	11 (6.7%)
GG	270 (87.7%)	152 (93.3%)
AA	0 (0%)	0 (0%)
	n = 306	n = 155
	p = 0.0145; c = 0.134	
RETN c.-180		
CC	119 (38.9%)	78 (50.3%)*
CG	136 (44.4%)	64 (41.3%)*
GG	51 (16.7%)	13 (8.4%)*

C *vs.* AN * $p < 0.05$

Table 3. The allele frequency in the polymorphic sites of the resistin gene (*RETN c.62* and *RETN c.-180*) in the examined girls with *anorexia nervosa* (AN) and healthy controls (C)

Examined gene **Allele**	**AN** **Number (%)**	**C** **Number (%)**
	n = 308	n = 163
	p = 0.059; c = 0.087	
RETN c.62		
G	578 (93.8%)	315 (96.65%)
A	38 (6.2%)	11 (3.4%)
	n = 306	n = 155
	p < 0.0001; c = 0.133	
RETN c.-180		
C	375 (61.4%)	232 (74.8%)*
G	236 (38.6%)	78 (25.2%)*

C vs. AN *__p < 0.0001__

Table 4. Mean concentration of resistin in the serum depending on the genotype in the polymorphic site of the resistin gene (*RETN c.62* and *RETN c.-180*) in examined girls with *anorexia nervosa* (AN) and healthy controls (C)

Examined gene **Genotype**	**Resistin in serum [ng/mL]**	
	AN (n = 172)	**C** (n = 129)
	p = 0.687	p= 0.680

RETN c.62		
GA GG AA	2.74 2.65 –	4.24 4.46 –
	AN (n = 170)	**C** (n = 127)
	p = 0.398	p = 0.117
RETN c.-180		
CC CG GG	2.65 2.5 2.83	4.56 4.18 6.04

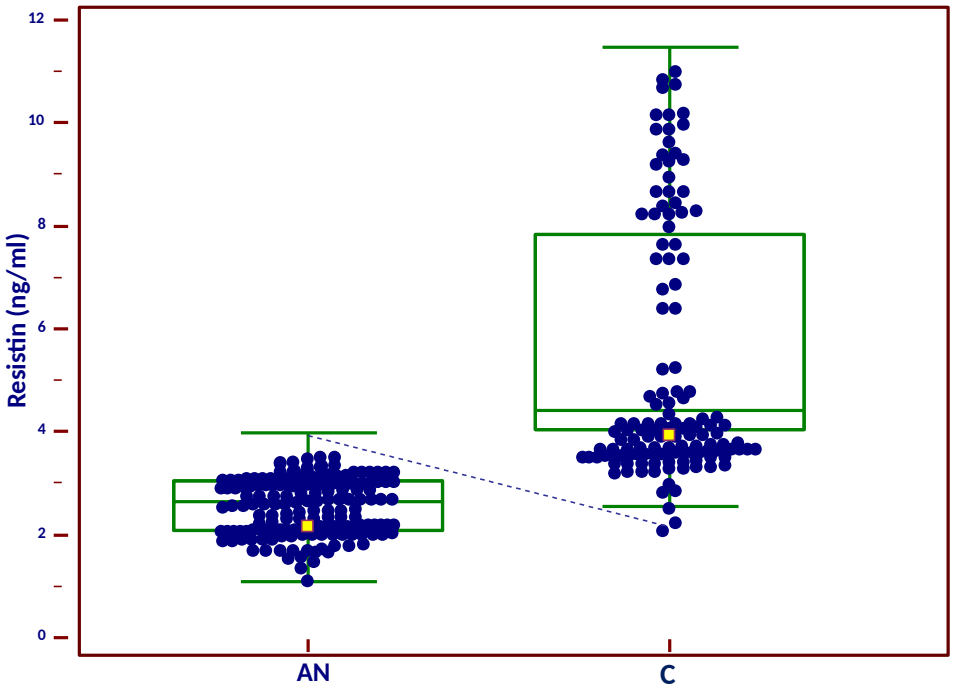

Figure 1. Serum resistin concentrations in girls with *anorexia nervosa* (AN) and healthy controls (C). AN vs. C $p < 0.0001$

Figure 2. Distribution of genotypes in locus *RETN* c.62 in girls with *anorexia nervosa* (AN) and healthy controls (C)

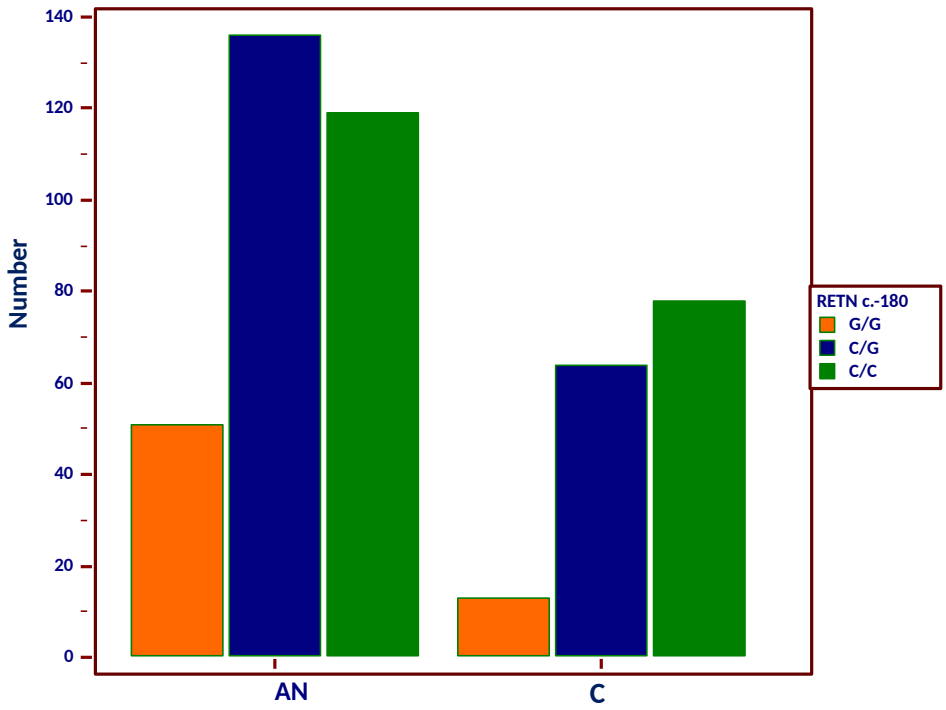

Figure 3. Distribution of genotypes in locus *RETN c.-180* in girls with *anorexia nervosa* (AN) and healthy controls (C). AN vs. C p = 0.0145

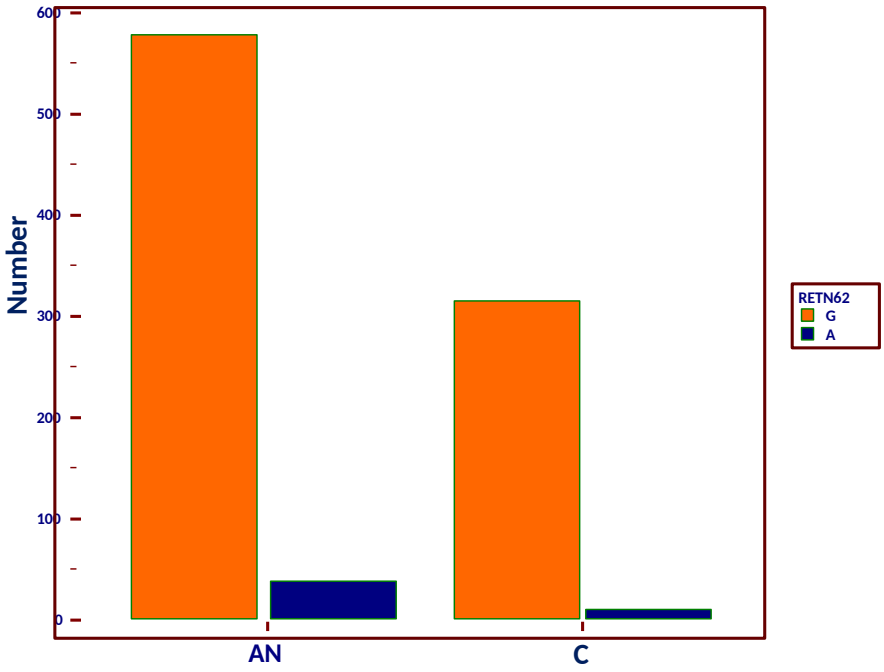

Figure 4. Distribution of G and A alleles in *RETN* c.62 polymorphic site in girls with *anorexia nervosa* (AN) and healthy controls (C)

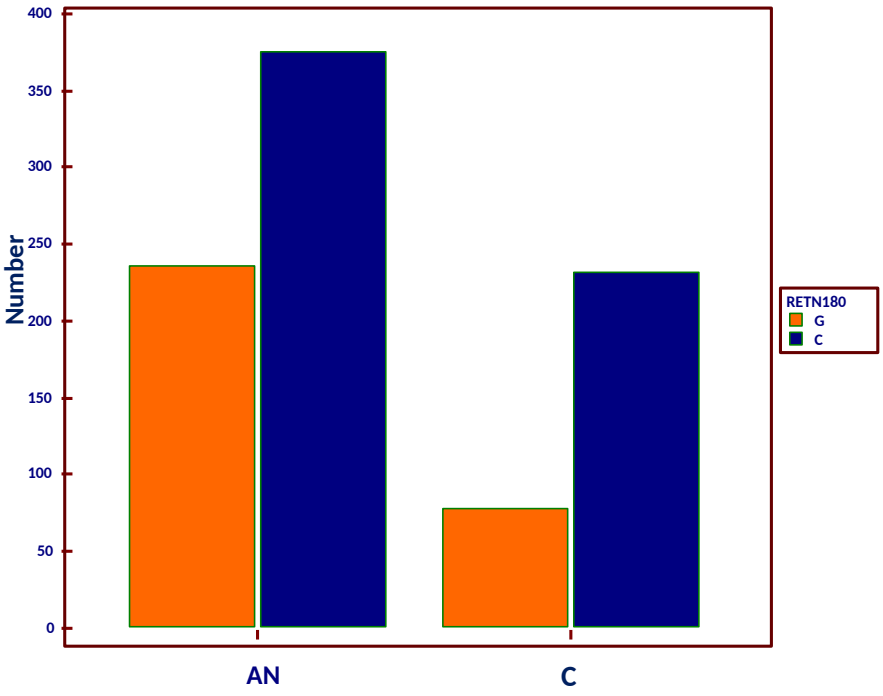

Figure 5. Distribution of G and C alleles in *RETN c.-180* polymorphic site in girls with *anorexia nervosa* (AN) and healthy controls (C). AN vs. C $p < 0.0001$

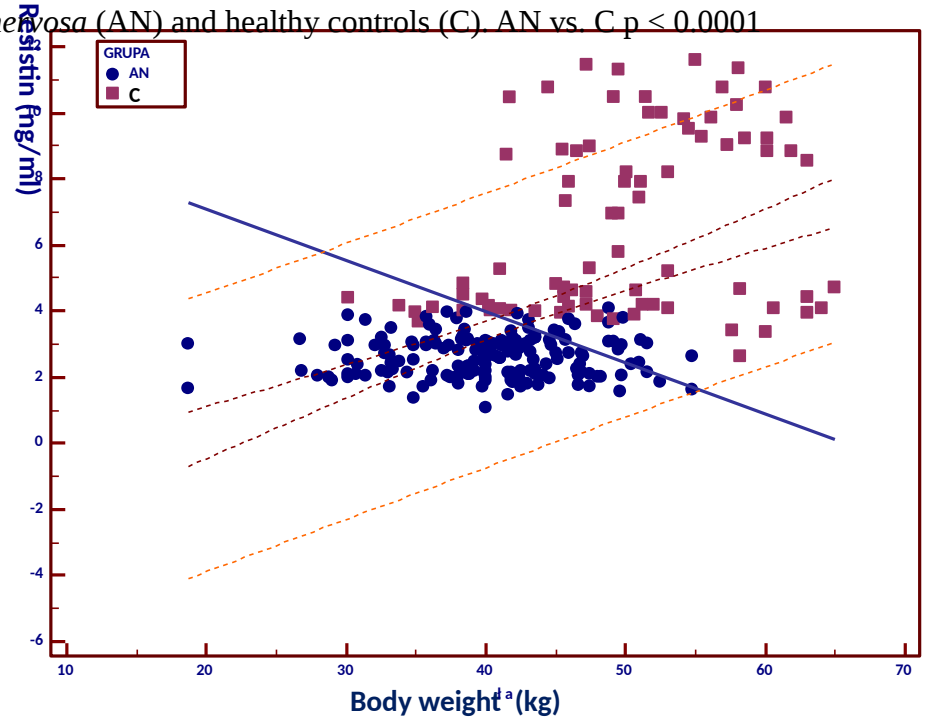

Figure 6. Correlation between serum resistin level and body weight in girls with *anorexia nervosa* (AN) and healthy controls (C)

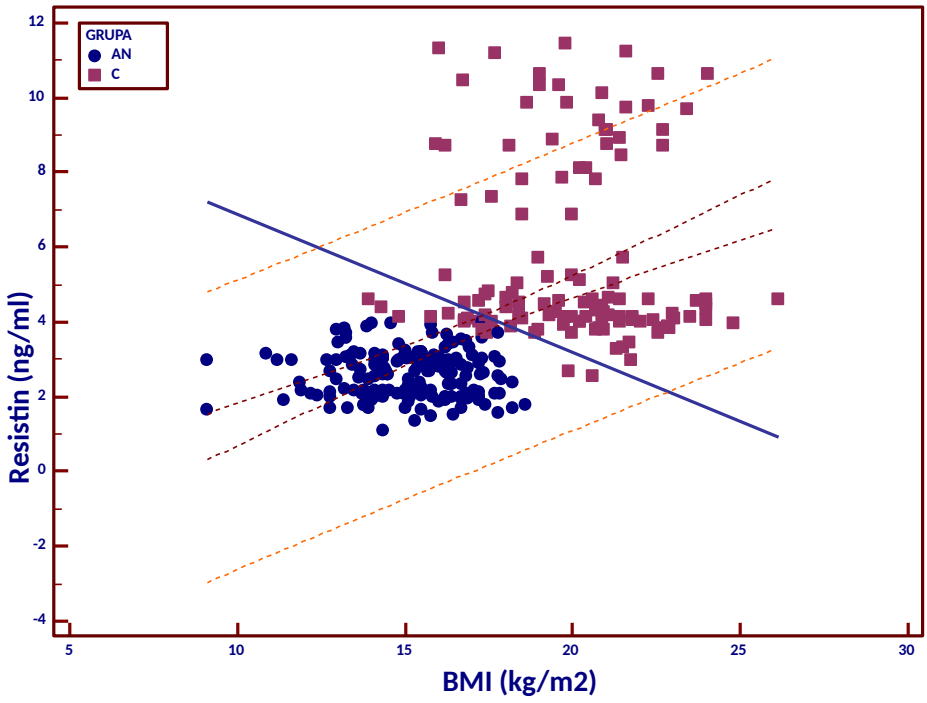

Figure 7. Correlation between serum resistin level and BMI in girls with *anorexia nervosa* (AN) and healthy controls (C)