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Brief Report—Endocrine Research

Serum Vaspin Concentrations Are Closely Related to Insulin Resistance, and rs77060950 at *SERPINA12* Genetically Defines Distinct Group with Higher Serum Levels in Japanese Population

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Context: Vaspin is an adipokine with insulin-sensitizing effects identified from visceral adipose tissues of genetically obese rats.

Objective: We investigated genetic and nongenetic factors that define serum concentrations of vaspin.

Design, Setting and Participants: Vaspin levels were measured with RIA in Japanese subjects with normal fasting plasma glucose (NFG; n = 259) and type 2 diabetes patients (T2D; n = 275). Single nucleotide polymorphisms (SNP) at *SERPINA12* (vaspin) gene locus were discovered, and five SNP were genotyped in the subjects with varied body mass index (n = 1138).

Results: The level of serum vaspin in 93% of the samples was found to vary from 0.2 to nearly 2 ng/ml in NFG subjects (n = 259) and from 0.2 to nearly 3 ng/ml in T2D patients (n = 275) ($Vaspin_{Low}$ group), whereas a significant subpopulation (7%) in both groups displayed much higher levels of 10–40 ng/ml ($Vaspin_{High}$ group). In the $Vaspin_{Low}$ group, serum vaspin levels in T2D were significantly higher than healthy subjects (0.99 ± 0.04 *vs.* 0.86 ± 0.02 ng/ml; $P < 0.01$). Both in T2D and genotyped Japanese population, serum vaspin levels closely correlated with homeostasis model of assessment for insulin resistance rather than anthropometric parameters. By genotyping, rs77060950 tightly linked to serum vaspin levels, *i.e.* CC (0.6 ± 0.4 ng/ml), CA (18.4 ± 9.6 ng/ml), and AA (30.5 ± 5.1 ng/ml) ($P < 2 \times 10^{-16}$). Putative GATA-2 and GATA-3 binding consensus site was found at rs77060950.

Conclusions: Serum vaspin levels were related to insulin resistance, and higher levels of serum vaspin in 7% of the Japanese population are closely linked to minor allele sequence (A) of rs77060950. (***J Clin Endocrinol Metab*** **97: E1202–E1207, 2012)**

Visceral adipose tissue-derived serine proteinase inhibitor (vaspin), *SERPINA12*, was identified from visceral adipose tissues of OLETF (Otsuka Long-Evans Tokushima Fatty) rats, which is an animal model for obesity and type 2 diabetes (T2D) (1). The mRNA expression of vaspin paralleled with the degree of obesity and insulin resistance in OLETF rats, and the injection of recombinant human vaspin into diet-induced

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Abbreviations: BMI, Body mass index; HOMA-IR, homeostasis model of assessment for insulin resistance; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; NFG, normal fasting plasma glucose; SFA, subdermal fat area; SNP, single nucleotide polymorphism; T2D, type 2 diabetes; vaspin, visceral adipose tissue-derived serine proteinase inhibitor; VFA, visceral fat area.

obese mice ameliorated insulin resistance, indicating that vaspin is a compensatory factor in the status of obesity (1, 2). In humans, serum vaspin levels correlated with body mass index (BMI) and insulin sensitivity in adults (3–5), children (6, 7), and obese women with polycystic ovary syndrome (8–10). Furthermore, serum vaspin levels were decreased by weight reduction and lifestyle modification in obese adults (11–13) and children (6). The line of evidence supported the notion that vaspin plays an important role in the progression of obesity and insulin resistance.

Although many clinical reports investigating serum vaspin levels in the patients with obesity and metabolic syndrome are available, there are few reports investigating the genetic effects on the serum vaspin levels. Here, we measured serum vaspin levels by a wide-range RIA system, and we found that approximately 7% of the population of both normal subjects and patients with T2D demonstrated more than approximately a 10-fold higher concentration of serum vaspin levels compared with the rest of the population. We discovered that a minor allele of rs77060950 in the *SERPINA12* gene was significantly associated with high serum levels of vaspin in the Japanese population and demonstrated that rs77060950 altered the transcriptional activity.

Subjects and Methods

Subjects and research design

Japanese subjects with normal fasting plasma glucose (NFG; n = 259; age, 36.6 ± 0.73 yr) and patients with T2D (n = 275; age, 61.6 ± 0.80 yr) were enrolled into this study. Healthy volunteers with NFG, who did not take any medications for diabetes, hypertension, and/or dyslipidemia, received annual medical health checkups for common disease screening at the Okayama Southern Institute of Health. NFG and T2D subjects agreed to measure serum vaspin levels. T2D subjects were treated with oral hypoglycemic agents (n = 98), insulin treatment (n = 79), or both (n = 17). All recruited T2D subjects agreed to undergo computed tomography and measure high-sensitivity C-reactive protein (hsCRP), oxidized low-density lipoprotein (LDL), leptin, adiponectin, and vaspin levels.

For single nucleotide polymorphism (SNP) genotyping of the *SERPINA12* (*vaspin*) gene and measurement of serum vaspin levels, we further collected 1138 Japanese subjects with various BMI levels (male:female ratio, 470:668; age, 53.4 ± 12.1 yr; BMI, 27.6 kg/m^2), who agreed to undergo computed tomography examinations to measure the visceral fat area (VFA) and subdermal fat area (SFA) (n = 697). We enrolled the subjects in three categories, *i.e.* normal weight (BMI $<$ 25.0 kg/m^2), overweight (25.0 $\leq$ BMI $<$ 30.0 kg/m^2), and obese (30.0 $\leq$ BMI kg/m^2). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the ethical committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences (Registration numbers 20, 51, and 66), Kyoto University, and also by all the appropriate institutional review boards.

Results

Serum vaspin levels well-correlated with insulin resistance

T2D subjects (n = 275; age, 61.6 ± 0.80 yr) revealed significantly higher levels of BMI, waist circumference, systolic blood pressure, plasma glucose, total and LDL-cholesterol, and lower high-density lipoprotein-cholesterol compared with NFG subjects (n = 259; age, 36.6 ± 0.73 yr) (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The measurement of vaspin levels in NFG subjects revealed that the majority displayed vaspin levels less than 3 ng/ml. A minor fraction (n = 18 or 6.9%) displayed much higher levels, ranging from 10 to 34 ng/ml (Fig. 1, A and B). The percentage of the population with such higher serum vaspin levels is also similar in NFG subjects, and the majority displayed vaspin levels less than 3 ng/ml; 7.2% of T2D subjects (n = 20) displayed much higher levels, ranging from 10 to 40 ng/ml (Fig. 1, C and D). By comparing the major population with vaspin levels less than 3 ng/ml (Vaspin$_{Low}$), vaspin levels in T2D subjects were significantly higher than NFG subjects (0.99 ± 0.04 *vs.* 0.86 ± 0.02 ng/ml; $P < 0.01$). Vaspin levels in T2D subjects were still significantly higher than NFG subjects (1.03 ± 0.06 *vs.* 0.83 ± 0.06 ng/ml; $P = 0.038$) by analysis of covariance, adjusting age as a covariant. In Vaspin$_{Low}$ T2D subjects, vaspin levels significantly correlated with BMI (R = 0.193; $P = 0.002$), SFA (R = 0.146; $P = 0.016$), homeostasis model of assessment for insulin resistance (HOMA-IR) (R = 0.142; $P = 0.022$), hsCRP (R = 0.128; $P = 0.042$), and leptin (R = 0.169; $P = 0.019$) levels, but not with VFA, oxidized LDL, and adiponectin levels. The linear regression analysis was followed by a stepwise multiple regression analysis using serum vaspin levels as the dependent variables to further analyze the significant predictors. Age, gender, BMI, SFA, VFA, Log$_{10}$HOMA-IR, Log$_{10}$hsCRP, and Log$_{10}$leptin levels were used as independent variables. By stepwise analysis in model 1, only Log$_{10}$HOMA-IR independently correlated with serum vaspin levels, and other variables demonstrating significant simple correlation with serum vaspin levels did not enter the equation at significant levels in model 2 (Supplemental Table 2).

Vaspin serum levels in Vaspin$_{High}$ population are genetically defined

We next measured serum vaspin levels in samples derived from the parents of three independent Vaspin$_{High}$

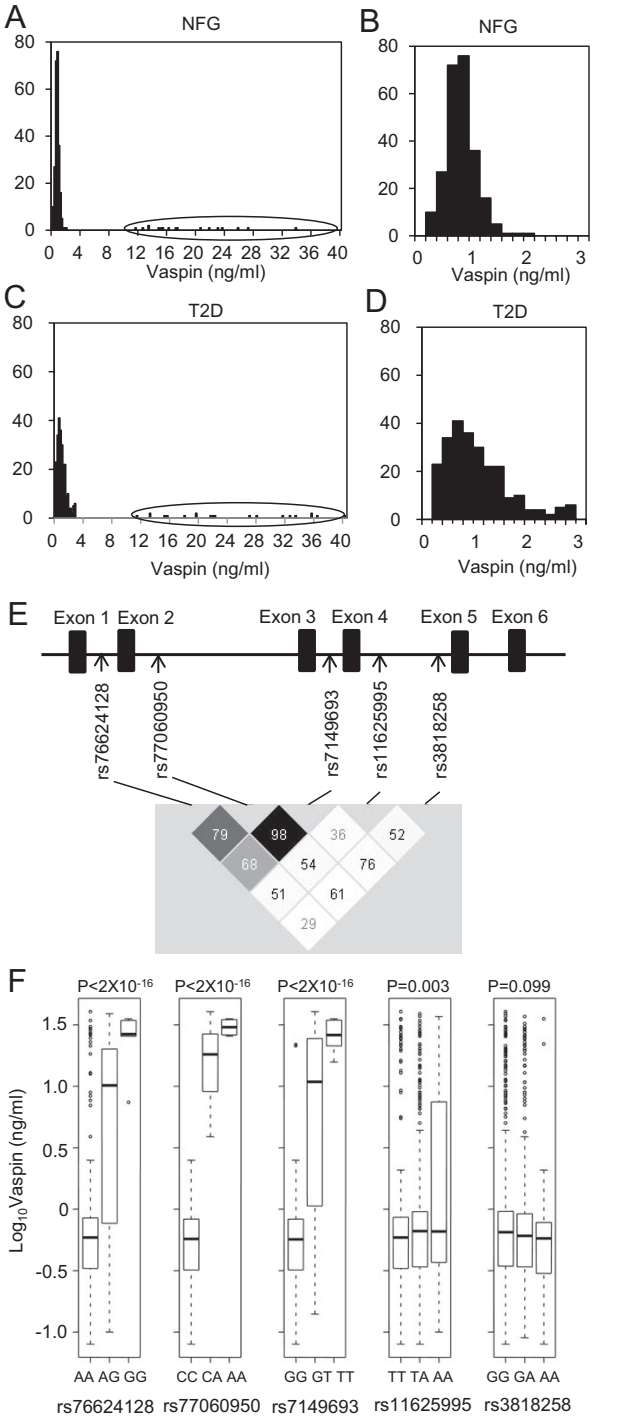

FIG. 1. Serum vaspin levels in the subjects with NFG and the patients with T2D. A, A minor fraction (6.9%) of NFG (n = 259) displays much higher levels (*circled*), ranging from 10 to 34 ng/ml. B, The majority of subjects displayed vaspin levels less than 2 ng/ml. C, Similarly, 7.2% of the patients with T2D (n = 275) displayed much higher levels (*circled*), ranging from 10 to 40 ng/ml. D, The majority of the patients with T2D displayed vaspin levels less than 3 ng/ml. E, The localization of five SNP identified at *SERPINA12* (vaspin) gene and linkage disequilibrium coefficients between five SNP in the *SERPINA12* gene. F, Serum vaspin levels in the subjects with various genotypes. Among five SNP, rs77060950 completely links the serum vaspin levels, *i.e.* CC (0.6 ± 0.4 ng/ml), CA (18.4 ± 9.6 ng/ml), and AA (30.5 ± 5.1 ng/ml).

subjects. All individuals from the $Vaspin_{High}$ group had either parent with high serum vaspin levels, suggesting an autosomal dominant or additive inheritance model (Supplemental Table 3). Genomic DNA derived from three independent $Vaspin_{High}$ subjects and three independent $Vaspin_{Low}$ subjects was used for *de novo* sequencing by Illumina Genome Analyzer (Supplemental Methods). Five novel SNP unique to the $Vaspin_{high}$ group were identified (Fig. 1E), and we further investigated the allele frequency of these SNP and serum vaspin levels in the Japanese subjects with various BMI (n = 1138) (Supplemental Table 4). Because we enrolled the normal, overweight, and obese Japanese subjects, the average BMI (27.6 ± 4.2 kg/m^2) was higher, and the sample subjects may not represent the general population of Japan. This population consisted of 1028 subjects in the $Vaspin_{Low}$ group and 110 subjects in the $Vaspin_{High}$ group. In the $Vaspin_{Low}$ group, unlike T2D, BMI and Log_{10}hsCRP were negatively correlated with serum vaspin levels. In contrast, Log_{10}HOMA-IR was positively correlated with serum vaspin levels like T2D. In the $Vaspin_{High}$ group, BMI was the independent variable to predict the serum vaspin levels. Serum vaspin levels were higher in women than men; however, the above-mentioned correlations were similar in both men and women (Supplemental Table 5).

Among five SNP, rs77060950 most accurately predicted the serum vaspin levels, *i.e.* CC (0.6 ± 0.4 ng/ml), CA (18.7 ± 9.5 ng/ml), and AA (30.5 ± 5.1 ng/ml) (Supplemental Table 6 and Fig. 1F). The CC genotype completely matched to the $Vaspin_{Low}$ group (n = 1027), and CA/AA genotype corresponded to the $Vaspin_{High}$ group (n = 110). Thus, rs77060950 was a candidate for the functional SNP for the transcriptional regulation of vaspin gene expression, and SNP rs76624128 and rs7149693 were in the strong linkage disequilibrium with rs77060950 (Supplemental Table 6). Such strong linkage was also true in NFG and impaired fasting glucose/diabetes mellitus subgroups, defined by the fasting plasma glucose levels (Supplemental Table 7). Although serum vaspin levels were tightly associated with rs77060950, various clinical parameters obtained in the cross-sectional study were not significantly different in CC, CA, and AA genotypes of rs77060950 (Supplemental Table 8).

We found GATA transcription factor consensus binding site around rs77060950 by TFSEARCH and MOTIF search programs (Fig. 2A). We compared the luciferase activities and gel shift by using the minor (A) and major (C) alleles of rs77060950 (Supplemental Methods). Luciferase activities were significantly higher in the minor allele construct, pGL4–175A vector, compared with the major allele construct, pGL4-175C vector (Fig. 2B). Luciferase activities were higher in pGL4-295A, pGL4-

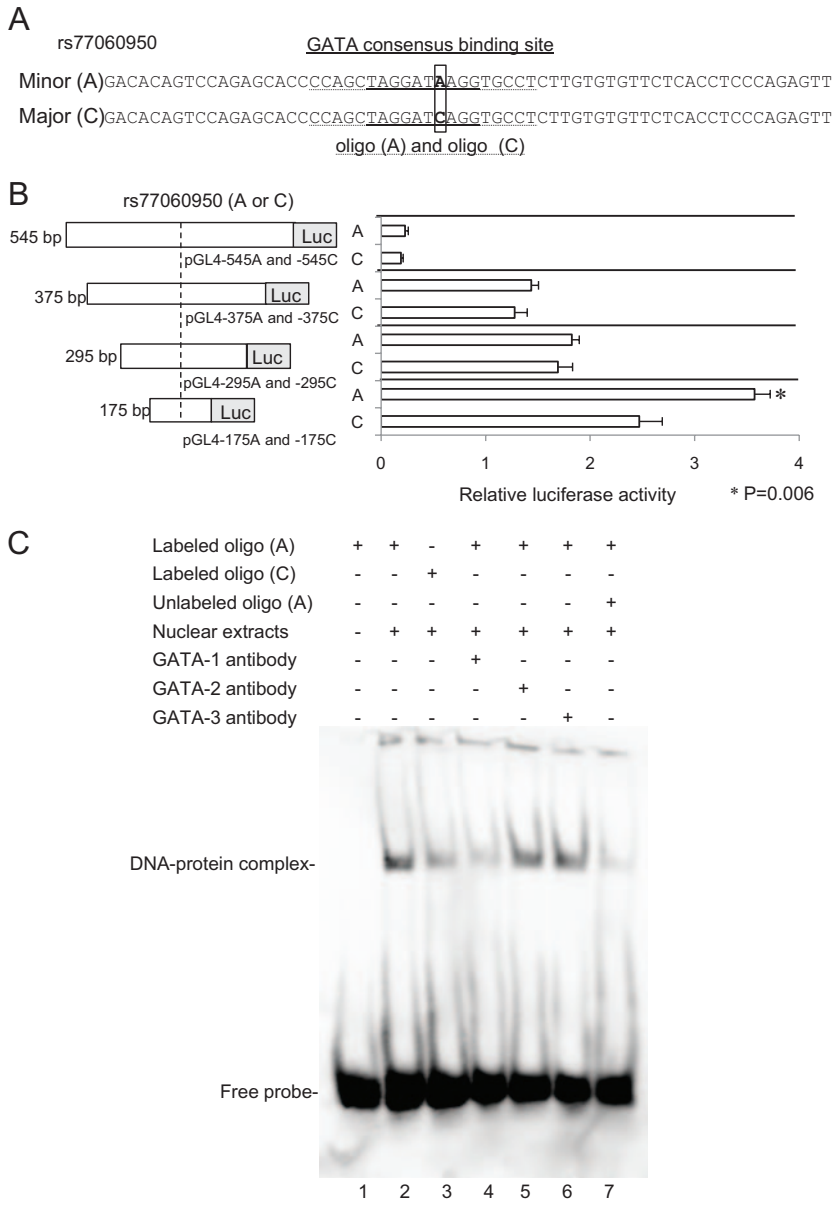

FIG. 2. Luciferase assay and EMSA. A, In the flanking region of rs77060950, GATA transcription factor binding site (*underlined*) is found, and oligos (A) and (C) were prepared (*dotted underline*). B, Luciferase assay using various pGL4 constructs. Higher luciferase activity was observed in pGL4–175A compared with pGL4–175C vector ($P = 0.006$). C, EMSA. The intense DNA-protein complex using oligo (A), minor allele sequence, was observed compared with oligo (C), major allele sequence (lanes 2 and 3). The DNA-protein complex was reduced by the addition of 100-fold excess of unlabeled oligo (A) (lane 7). The addition of anti-GATA-1, -GATA-2, and -GATA-3 antibodies did not demonstrate supershift (lanes 4–6).

375A, and pGL4-545A vectors compared with pGL4-295C, pGL4-375C, and pGL4-545C vectors, respectively, although they were not statistically significant. We next tested the binding of the rs77060950 minor and major allele sequences by GATA with EMSA and demonstrated DNA-protein complexes using oligo (A), minor allele sequence, and the intensity of the complexes was much weaker in oligo (C), major allele sequence (Fig. 2C). We also observed no supershifting and competition for binding by the treatment of anti-GATA-1, -GATA-2, and -GATA-3-specific antibodies in the major and minor allele sequences (Fig. 2C).

Discussion

A major finding of the current study is that approximately 7% of the Japanese population revealed much higher serum vaspin concentrations in both NFG and T2D subjects. Furthermore, $Vaspin_{High}$ subjects are genetically defined by minor allele sequence (A) of rs77060950. Another significant finding is that serum vaspin levels closely correlated with HOMA-IR rather than anthropometric parameters both in T2D subjects and the genotyped Japanese population.

Such unique logarithmic normal distribution of serum vaspin concentrations has not been reported in any other serum proteins. One exception may be the case with lipoprotein(a), which also demonstrates the logarithmic normal distribution of serum concentration. However, lipoprotein(a) is disulfide-linked to apolipoprotein B-100, and it contains two types of plasminogen-like kringle domains revealed the variation of the copy number, which resulted in the six phenotypes with varied serum concentration (14, 15). In contrast to the case with lipoprotein(a), we did not find any differences in the coding sequence of the *SERPINA12* (vaspin) gene in six families of $Vaspin_{High}$ and $Vaspin_{Low}$ groups revealed by *de novo* sequencing of PCR-amplified genomic DNAs (Supplemental Methods and Supplemental Table 9) using Illumina Genome Analyzer, and thus the variation of serum concentrations of vaspin could not be attributed to the difference of the protein structure and half-life of vaspin protein. Based upon these considerations, we speculated that serum vaspin levels are closely linked to transcriptional activities of the *SERPINA12* (vaspin) gene, which are determined by the minor allele sequence (A) of rs77060950. Actually, minor allele sequence (A) of rs77060950 alters a GATA transcription factor binding site associated with increased luciferase activity using pGL4 construct containing 175 bp flanking

rs77060950 and enhanced the formation of DNA-protein complex by EMSA compared with minor allele sequence (C) of rs77060950. Among the GATA transcription factors, GATA-2 and GATA-3 are specifically expressed in white adipocyte precursors, and their down-regulation sets the stage for terminal differentiation. GATA-3-deficient embryonic stem cells exhibit an enhanced capacity to differentiate into adipocytes, and defective GATA-2 and GATA-3 expression is associated with obesity (16). Although TFSEARCH and MOTIF search programs predicted that GATA-2 and GATA-3 are good candidates for the regulators for transcriptional activities of the *SERPINA12* gene, the lack of supershift in EMSA by anti-GATA-2 and -GATA-3 antibodies may suggest the presence of uncharacterized transcriptional factors that bind to the genomic DNA region surrounding the rs77060950, and more intensive characterization of molecular genetics may be required.

In a previous publication, Kempf *et al.* (17) reported the significant association of vaspin SNP rs2236242 with T2D in the KORA F3 study, with the AA genotype bearing an increased risk [adjusted odds ratio, 2.35 (1.59, 3.46) *vs.* AT/TT]. However, the comparison of various clinical parameters between $Vaspin_{High}$ and $Vaspin_{Low}$ groups revealed that only insulin resistance index (microunits per milliliter) was higher in the AA genotype compared with CC and CA genotypes, although it did not reach statistically significant differences. The frequency of population with higher vaspin levels of more than 10 ng/ml seems to be different in various ethnic groups, *i.e.* approximately 1% in a European population in previous reports (18, 19); thus, the clinical characterization of the $Vaspin_{High}$ group may be more difficult in a Caucasian population. Future cohort studies enrolling the Japanese population with higher serum vaspin levels with AA and CA genotypes of rs77060950 are required to demonstrate susceptibilities for the development of obesity, insulin resistance, diabetes, nonalcoholic fatty liver disease, and various vascular disorders.

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References

1. **Hida K, Wada J, Eguchi J, Zhang H, Baba M, Seida A, Hashimoto I, Okada T, Yasuhara A, Nakatsuka A, Shikata K, Hourai S, Futami J, Watanabe E, Matsuki Y, Hiramatsu R, Akagi S, Makino H, Kanwar YS** 2005 Visceral adipose tissue-derived serine protease inhibitor: a unique insulin-sensitizing adipocytokine in obesity. Proc Natl Acad Sci USA 102:10610–10615
2. **Wada J** 2008 Vaspin: a novel serpin with insulin-sensitizing effects. Expert Opin Investig Drugs 17:327–333
3. **Choi SH, Kwak SH, Lee Y, Moon MK, Lim S, Park YJ, Jang HC, Kim MS** 2011 Plasma vaspin concentrations are elevated in metabolic syndrome in men and are correlated with coronary atherosclerosis in women. Clin Endocrinol (Oxf) 75:628–635
4. **Cho JK, Han TK, Kang HS** 2010 Combined effects of body mass index and cardio/respiratory fitness on serum vaspin concentrations in Korean young men. Eur J Appl Physiol 108:347–353
5. **Youn BS, Klöting N, Kratzsch J, Lee N, Park JW, Song ES, Ruschke K, Oberbach A, Fasshauer M, Stumvoll M, Blüher M** 2008 Serum vaspin concentrations in human obesity and type 2 diabetes. Diabetes 57:372–377
6. **Lee MK, Jekal Y, Im JA, Kim E, Lee SH, Park JH, Chu SH, Chung KM, Lee HC, Oh EG, Kim SH, Jeon JY** 2010 Reduced serum vaspin concentrations in obese children following short-term intensive lifestyle modification. Clin Chim Acta 411:381–385
7. **Suleymanoglu S, Tascilar E, Pirgon O, Tapan S, Meral C, Abaci A** 2009 Vaspin and its correlation with insulin sensitivity indices in obese children. Diabetes Res Clin Pract 84:325–328
8. **Koiou E, Tziomalos K, Dinas K, Katsikis I, Kalaitzakis E, Delkos D, Kandaraki EA, Panidis D** 2011 The effect of weight loss and treatment with metformin on serum vaspin levels in women with polycystic ovary syndrome. Endocr J 58:237–246
9. **Cakal E, Ustun Y, Engin-Ustun Y, Ozkaya M, Kilinç M** 2011 Serum vaspin and C-reactive protein levels in women with polycystic ovaries and polycystic ovary syndrome. Gynecol Endocrinol 27:491–495
10. **Tan BK, Heutling D, Chen J, Farhatullah S, Adya R, Keay SD, Kennedy CR, Lehnert H, Randeva HS** 2008 Metformin decreases the adipokine vaspin in overweight women with polycystic ovary syndrome concomitant with improvement in insulin sensitivity and a decrease in insulin resistance. Diabetes 57:1501–1507
11. **Handisurya A, Riedl M, Vila G, Maier C, Clodi M, Prikoszovich T, Ludvik B, Prager G, Luger A, Kautzky-Willer A** 2010 Serum vaspin concentrations in relation to insulin sensitivity following RYGB-induced weight loss. Obes Surg 20:198–203
12. **Chang HM, Lee HJ, Park HS, Kang JH, Kim KS, Song YS, Jang YJ** 2010 Effects of weight reduction on serum vaspin concentrations in obese subjects: modification by insulin resistance. Obesity (Silver Spring) 18:2105–2110
13. **Oberbach A, Kirsch K, Lehmann S, Schlichting N, Fasshauer M,**

Zarse K, Stumvoll M, Ristow M, Blüher M, Kovacs P 2010 Serum vaspin concentrations are decreased after exercise-induced oxidative stress. Obes Facts 3:328–331
14. **Utermann G, Menzel HJ, Kraft HG, Duba HC, Kemmler HG, Seitz C** 1987 Lp(a) glycoprotein phenotypes. Inheritance and relation to Lp(a)-lipoprotein concentrations in plasma. J Clin Invest 80:458–465
15. **McLean JW, Tomlinson JE, Kuang WJ, Eaton DL, Chen EY, Fless GM, Scanu AM, Lawn RM** 1987 cDNA sequence of human apolipoprotein (a) is homologous to plasminogen. Nature 330:132–137
16. **Tong Q, Dalgin G, Xu H, Ting CN, Leiden JM, Hotamisligil GS** 2000 Function of GATA transcription factors in preadipocyte-adipocyte transition. Science 290:134–138
17. **Kempf K, Rose B, Illig T, Rathmann W, Strassburger K, Thorand B, Meisinger C, Wichmann HE, Herder C, Vollmert C** 2010 Vaspin (SERPINA12) genotypes and risk of type 2 diabetes: results from the MONICA/KORA studies. Exp Clin Endocrinol Diabetes 118:184–189
18. **Seeger J, Ziegelmeier M, Bachmann A, Lössner U, Kratzsch J, Blüher M, Stumvoll M, Fasshauer M** 2008 Serum levels of the adipokine vaspin in relation to metabolic and renal parameters. J Clin Endocrinol Metab 93:247–251
19. **Hida K, Poulsen P, Teshigawara S, Nilsson E, Friedrichsen M, Ribel-Madsen R, Grunnet L, Lund SS, Wada J, Vaag A** 2012 Impact of circulating vaspin levels on metabolic variables in elderly twins. Diabetologia 55:530–532