

SHBG Genetic Variability and Glucose Tolerance in T2DM Patients, Gestational Diabetics, and Women with PCOS in Comparison with the Control Czech Population Sample

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Abstract

Background: Sex hormone-binding globulin (SHBG) belongs to the factors contributing to the pathophysiology of type 2 diabetes mellitus (T2DM). We determined genotypic frequencies of the single nucleotide polymorphisms (SNPs) rs6259 and rs6257 in T2DM patients, offspring of T2DM patients, gestational diabetics, patients suffering from polycystic ovary syndrome (PCOS), and in healthy adult Czechs. 1687 volunteers entered the study. The aim was to compare genetic constellation between the groups and to study the possible association of the SNPs with biochemical and anthropometric markers of insulin sensitivity.

Methods: TaqMan (LC480, Roche) was used for genotyping, statistical evaluation was carried out using Statgraphics Centurion version XVI and NCSS 2007.

Results: The SNPs distribution was similar between the groups. We found lower SHBG concentrations in diabetics and PCOS patients. The rs6259 SNP was associated with SHBG levels: in the NN carriers, the concentration was significantly higher in comparison with DD and DN. Unexpected results were observed when association of the rs6259 SNP with insulin sensitivity was assessed. In spite of higher SHBG concentration, which is considered to be protective factor, the NN homozygotes exhibited systematically higher stimulated glucose levels during the 3-hour oral glucose tolerance test and lower Cederholm index of insulin sensitivity.

Conclusions: Genetic analysis confirmed the association between rs6259 NN genotype and higher SHBG levels. Furthermore, the NN genotype showed higher stimulated glycemia and lower insulin sensitivity. This observation seems intriguing considering established protective effect of higher SHBG levels in relation to T2DM and should be verified on a larger group of probands.

Keywords: Insulin sensitivity; Type 2 diabetes mellitus; Gestational diabetes mellitus; Sex hormone-binding globulin; Genetic polymorphism

Abbreviations: AUC: Area Under the Curve; BMI: Body Mass Index; HOMA: Homeostasis Models Assessment; OGTT: Oral Glucose Tolerance Test; OR: Odds Ratio; PCOS: Polycystic Ovary Syndrome; SHBG: Sex Hormone-Binding Globulin; SNP: Single Nucleotide Polymorphism; T2DM: Type 2 Diabetes Mellitus

Introduction

Sex hormone-binding globulin (SHBG) is the primary plasma transport protein for sex steroid hormones. The *SHBG* gene is located on the short arm of chromosome 17 and the major transcript is encoded by 8 exons, spanning approximately 3.2 Kb. The gene product is a 373 amino acid glycoprotein produced mainly by liver. It regulates the bioavailability of sex steroids to target tissues and its biological half-life is 7 days. Simplistically, as long as bound to SHBG, steroid hormones are inactive and serve as a reservoir for future use. In terms of molecular biology, the multiple interactions between SHBG and its putative receptors in various target tissues (breast, prostate, liver, epididymis) suggest involvement of SHBG in physiology that is more complex than the simple transport of sex steroids in serum [1,2]. SHBG has actually emerged as one of the manifold factors that contribute to the type 2 diabetes mellitus (T2DM) pathophysiology. In addition to epidemiological studies, which demonstrate a consistent relationship between lower levels of SHBG in serum and T2DM [3-5], also genetic studies reveal that specific *SHBG* gene polymorphisms

can causally affect insulin sensitivity and T2DM risk [3,4]. The exact mechanism underlying these findings is not known. Besides the contribution of genetic studies, there is also clinical evidence that the associations between insulin sensitivity and steroids may be causal. Hyper-androgenic disorders characterized by low SHBG levels such as polycystic ovary syndrome (PCOS) in women result in an increased risk of T2DM and are very strongly associated with insulin resistance [6-9]. Second, insulin lowering interventions lead to increased SHBG levels, suggesting that insulin can causally influence sex steroid dynamics [10,11]. Third, during pregnancy, in both first and second trimester SHBG appears to be lower among women who subsequently developed gestational diabetes mellitus. Furthermore, lower levels of SHBG were observed in those patients who developed severe gestational

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diabetes mellitus and required insulin therapy during the last months of pregnancy [12]. Noteworthy, multivariate analysis conducted on the American population suggested that SHBG measured from nonfasting first-trimester sera was the best predictor of gestational diabetes mellitus [13]. Also other studies confirm SHBG as the optimal first-trimester biomarker to predict subsequent gestational diabetes mellitus [14-16]. Finally, prospective studies show that the levels of sex hormones are altered in individuals who have been diagnosed with T2DM many years later [17]. Despite this evidence pointing to a causal role of sex steroids in T2DM or/and gestational diabetes mellitus, further research and convincing arguments are required. Prospective studies establish whether a risk factor is or is not present prior to disease diagnosis. However, disease processes can start many years before disease diagnosis, which means that altered hormone levels observed prospectively could be a consequence rather than cause of early disease processes. Overall, there is no doubt these observations provide support for an expanded role of SHBG in the pathophysiology of insulin resistance, gestational diabetes mellitus, and T2DM.

Several polymorphisms in the human *SHBG* gene have been found to be associated with circulating levels of SHBG [18-23], insulin sensitivity [24], and other sex steroid-dependent conditions such as breast cancer [25-27], prostate cancer [28], and reduced bone mineral density [19]. In particular, the rs6259 (G>A) SNP in exon 8, encoding a substitution of amino acid asparagine (according to the standard 1-letter amino acid nomenclature abbreviated as „N“) for aspartic acid (abbreviated as „D“) at the position 356 (D356N, referred to as D>N in our study and described elsewhere also as D327N [23,26,29]) has been associated with increased plasma levels of SHBG in N carriers [4,22,28]. It has been suggested that the modification affects a probable glycosylation site and thus increases the half-life of the protein [23]. The N variant is believed to be the anchor site for an additional carbohydrate group, which decreases the rate of clearance of SHBG from circulation [30]. Higher SHBG levels among the N allele carriers have been reported by several independent studies [4, 22,23,31], but no significant difference was reported by some other groups [21,26,32-34]. Also the rs6257 polymorphism (C>T) was associated with SHBG levels in the literature [19,35]. The mechanism of this effect is not clear, as this SNP is located in an intron 1, however, this intronic region harbors a potential binding site for the hepatocyte nuclear transcription factor-3/ forkhead box, which allows the possibility of functional implications [35].

The aim of our study was to examine relations between the SNP variants in the *SHBG* gene and biochemical, anthropometric and other clinical characteristics in order to detect the possible association of the two polymorphisms with the pathogenic markers of T2DM, gestational diabetes, and PCOS in the Czech population representatives.

Materials and Methods

Study subjects

Adult volunteers aged 18-70 years entered the study, see more details in the table 1. All the participants met the following criteria of the selection: patients suffering from T2DM were diagnosed by the criteria of the World Health Organization [36]; gestational diabetics met the 0.5-1 year interval after the childbirth and were without other pathologies (i.e. hormonal disturbances, infections, organ disorders, mental illness etc.); patients with PCOS were defined according to the European Society of Human Reproduction and Embryology consensus [37]; group of offspring of T2DM patients reflects that T2DM was present in one or in both parents of the examined subject; healthy

controls were without family history of T2DM, PCOS, or gestational diabetes. All the T2DM patients were well compensated either only by diet (41.6 %), or by diet and peroral antidiabetic drugs (39.8 %), or by insulin (18.6 %). The study protocol was in accordance with the institutional ethic guidelines and the national rules and all the subjects gave their written informed consent to participate in the study.

Clinical and biochemical characterization

Body weight, height, waist and hip circumferences were measured [38] in all participants in order to calculate body mass index (BMI) and to evaluate body fat distribution by means of waist circumference and waist to hip ratio. Furthermore, 5 skinfolds [38] and body composition according to bioimpedance method (*Tanita AB-140 Viscan, Tanita BC-480*) were determined.

Venous blood samples were obtained after an overnight fast. Glucose metabolism was characterized by blood glucose (Beckman Glucose Analyser 2), immunoreactive insulin (Immunotech IRMA, Czech Rep), C-peptide (Immunotech IRMA, Czech Rep), proinsulin (DRG Diagnostics, Germany), and also by glucagon (IBL-International, Germany).

The 3-hour oral glucose tolerance test (oGTT) with 75 g of glucose load was performed in all subjects except of T2DM patients. Areas under the oGTT glycemic, C-peptide and insulin curves (AUC) were calculated. Lipid profile was assessed by total cholesterol, high density lipoprotein, low density lipoprotein, and triglyceride concentrations (analyser Integra 400+, Roche Diagnostics GmbH, Germany). To assess insulin sensitivity and beta-cell function, the following homeostasis models of insulin sensitivity were calculated: $1/HOMAR=1/(0 \text{ min insulin } [\mu\text{U/ml}] \times 0 \text{ min glucose } [\text{mmol/l}]/22.5)$ and $HOMAF=20 \times 0 \text{ min insulin } [\mu\text{U/ml}]/(0 \text{ min glucose } [\text{mmol/l}]-3.5)$, Matsuda index $= 104/\sqrt{(\text{mean } 0 \text{ min insulin } [\mu\text{U/ml}] \times \text{mean } 0 \text{ min glucose } [\text{mmol/l}] \times 0 \text{ min insulin } [\mu\text{U/ml}])}$, Cederholm index $= [75.000 + (0 \text{ min glucose } [\text{mmol/l}] - 120 \text{ min glucose } [\text{mmol/l}] \times 1.15 \times 180 \times 0.19 \times \text{body weight } [\text{kg}])/[120 \times \log(\text{mean insulin}) \times \text{mean glucose } [\text{mmol/l}]]$, and also insulinogenic index $=(30 \text{ min insulin } [\mu\text{U/ml}] - 0 \text{ min insulin } [\mu\text{U/ml}])/(30 \text{ min glucose } [\text{mmol/l}] - 0 \text{ min glucose } [\text{mmol/l}])$ evaluating beta-cell function.

Hormonal spectra (testosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstendione, estradiol, luteinizing hormone, follicles-stimulating hormone, SHBG) were assessed due to GC-MS, RIA or ELISA methods. Moreover, thyroid hormones and liver enzymes were evaluated (Cobas 6000).

Study subjects	n	Age (years)	BMI (kg/m ²)
T2DM patients	383	60.0 ± 8.1	30.7 ± 5.2
females	243	59.5 ± 8.4	31.4 ± 5.5
males	140	60.9 ± 7.6	29.7 ± 4.4
Offspring of T2DM	184	38.4 ± 11.9	25.7 ± 4.3
females	118	37.7 ± 12.8	24.8 ± 3.9
males	66	39.7 ± 10.2	27.1 ± 4.4
PCOS	397	28.1 ± 7.7	27.4 ± 6.8
Gestational diabetics	307	33.3 ± 5.1	24.3 ± 4.7
Controls	416	30.1 ± 10.1	23.7 ± 4.2
females	285	30.4 ± 10.9	23.5 ± 4.6
males	131	29.4 ± 7.9	24.2 ± 3.1
Total	1687		

Values are given as mean ± SD

Table 1: Study subjects.

In addition, standardized questionnaires monitoring demographic and anamnestic data regarding family T2DM or gestational diabetes incidence, quality of life, physical activity, eating behaviour, etc. [39-41] were collected from all participants.

SHBG genotyping

DNA extracted from peripheral blood leukocytes (QIAamp DNA Blood Kit, QIAGEN, Germany) was used to genotype for rs6259 (D>N) and rs6257(T>C) variants by ABI TaqMan SNP Genotyping Assays (LightCycler 480 System, Roche).

Statistical analysis

Data are given as means \pm SDs or percentage. The Chi-square test was used to assess deviation from Hardy-Weinberg equilibrium of the genotype frequencies. Allele/genotype/haplotype frequencies were compared by Chi-square test or Fisher's exact tests. Odds ratios (OR) and 95% Confidence Intervals were calculated according to MedCalc Software. Differences in biochemical and anthropometric data between the compared groups were tested by non-parametric Mann-Whitney test owing to the non-normal data distribution. To evaluate the relationships between dependent and factors, we have used the repeated measures ANOVA model consisting of subject factor, genotype as the between subject factor, time of oGTT as the within-subject factor, and interaction of genotype \times time of oGTT. ANOVA testing was followed by least significant difference (LSD) multiple comparisons with Bonferroni correction. The original dependent variable was transformed by a power transformation to attain a constant variance and symmetric distribution of the data and residuals [42]. Statistical software Stat graphic Centurion version XVI (Herndon, VA, USA) was used for calculations. The homogeneity of the data and residual was checked as described elsewhere [43,44]. Statistical software Statgraphics Centurion version XVI and NCSS 2007 from Number Cruncher Statistical Systems (Herndon, Utah, USA) was used for finding optimum transformation parameter and ANOVA testing, respectively. The p-values <0.05 (two tailed) were considered to be significant.

Results

In accordance with the literature data, we confirmed lower SHBG concentrations in T2DM patients (34.3 ± 20.9 nmol/l) and in PCOS women (38.6 ± 26.1 nmol/l) compared to the controls (67.8 ± 60.4 nmol/l); $p < 0.001$ in both comparisons. Our results also confirm the statistically significant association of rs6259 SNP with SHBG levels: in rs6259 NN genotype carriers, the concentration was higher (83.9 ± 69.0 nmol/l) in comparison with DD (50.5 ± 45.7 nmol/l; $p = 0.02$) and DN

(48.8 ± 38.9 nmol/l; $p = 0.04$) genotype. On the other hand, association of rs6257 SNP with SHBG levels did not reach statistical significance. Concerning genotypic frequencies, both the analyzed SNPs were consistent with the Hardy-Weinberg equilibrium. Comparison of the genotype distribution between the groups revealed that 1.6 % of the rare rs6259 NN genotype were in the group of gestational diabetics compared with 0.3-0.7 % in other groups; n.s. For details see the table 2. The analysis of the SNP rs6257 genotype distribution showed that rare CC homozygotes were represented by 1.6 % in the diabetic group compared with 0.7-1.1 % in the other groups; n.s. This corresponds with lower SHBG levels found in the group of diabetics. Though, the OR_{T2DM} for the minor allele was not significant ($OR_{T2DM} = 1.25$ with 95% CI [0.89; 1.74]; $p = 0.22$).

Unexpected results were observed when association of the SNPs with biochemical markers of insulin sensitivity was assessed. High SHBG concentration is considered to have protective effect in relation to insulin resistance and T2DM. In spite of this fact, homozygotes of the N variant in the rs6259 SNP exhibited systematically higher stimulated glucose levels during the 3-hour oGTT (p -level for AUC NN vs. DD <0.01; NN vs. DN=0.02), and lower Cederholm index of insulin sensitivity (DD: 71.7 ± 21.3 , DN: 72.1 ± 24.3 , NN: 57.7 ± 12.2 ; p -level NN vs. DD=0.03; NN vs. DN=0.04); see the differences in the particular minutes of the glycemic curve between the rs6259 genotypes in the figure 1. This finding is not the consequence of the above mentioned apparent proportion of gestational diabetics among the NN carriers. The observation of higher stimulated glucose concentration as well as lower Cederholm index of insulin sensitivity remained significant even after exclusion of all the gestational diabetics from the analysis. For completeness we add that stimulated insulin and C-peptide levels did not differ between the rs6259 genotypes.

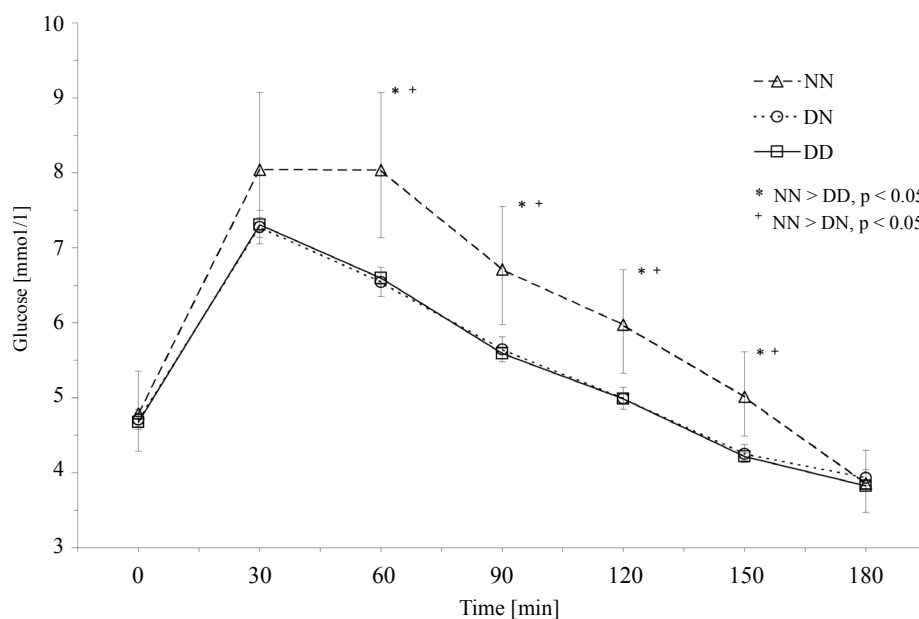
Evaluation of the relations between the two SNPs genotypes and steroid hormones (cortisol, testosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstendione, luteinizing hormone, follicles-stimulating hormone) as well as thyroid hormones (thyreostimulating hormone, free thyroxine, free triiodothyronine) did not show any association in men and women, also lipid profile (cholesterol, high density lipoprotein, low density lipoprotein, triglycerides) and hepatic enzymes (alanine aminotransferase, aspartate aminotransferase, gama-glutamyltransferase) were not different between the tested genotypes.

As expected, anthropometric parameters (BMI, waist to hip ratio, waist circumference, percentage of fat) correlated negatively with the SHBG levels, however, they were not associated with the analyzed SNPs in the SHBG gene.

rs6257 (T>C)	T2DM patients	T2DM offspring	Gest. Diabetics	PCOS	Controls
TT (n=1357)	80.1	77.0	80.3	80.1	83.2
TC (n=311)	18.3	21.9	19.0	19.2	16.1
CC (n=16)	1.6	1.1	0.7	0.7	0.7
T carriership	89.3	88.0	89.8	89.7	91.2
C carriership	10.7	12.0	10.2	10.3	8.8
rs6259 (D>N)					
DD (n=1412)	82.5	79.3	85.9	83.4	86.0
DN (n=260)	17.2	20.1	12.5	16.1	13.3
NN (n=12)	0.3	0.5	1.6	0.5	0.7
D carriership	91.1	89.4	92.1	91.4	92.6
N carriership	8.9	10.6	7.9	8.6	7.4

Values are given as percentage

Table 2: SHBG gene polymorphisms rs6257, rs6259 - genotype and allele frequencies in the Czech cohorts.



Evaluated by repeated measures ANOVA model with Bonferroni correction; error bars represent transformed means for NN, DN, and DD genotypes with their 95% confidence intervals

Figure 1: SHBG polymorphism rs6259 and stimulated glucose levels during the 3h-oGTT.

We have also categorized all the participants according to the particular haplotype combinations of the two SNPs to assess the possible interference of the variants and its possible relation with the phenotypic parameters. We observed an additive effect of the SNPs concerning SHBG levels (36.5 ± 26.3 nmol/l in DDCC haplotype vs. 84.0 ± 69.0 nmol/l in NNTT haplotype; $p < 0.01$). Other significant associations with anthropometric or biochemical markers of glucose intolerance were not detected.

Discussion

The study should be interpreted within the context of its limitations. As regards the association found between the SNP rs6259 and SHBG levels, it is necessary to take into account several factors. Contraceptive use, thyroid hormones, BMI, gender and age are well known to alter SHBG concentration. Being aware of these facts, women who reported to take hormonal contraceptives were excluded from the analysis. Concerning thyroid hormones, no correlation was found between the thyreostimulating hormone, free thyroxine or free triiodothyronine and SHBG levels in our cohort. Age was not significantly different between the compared rs6259 NN, DN, and DD genotypes. The impact of BMI was compensated by use of Cederholm index of insulin sensitivity as body weight is integrated to the calculation of this index. In respect of known lower SHBG levels in men, the whole cohort of probands was subjected to the gender stratification. The statistics provided results supporting strong effect of the rs6259 NN genotype in women: the significance of higher stimulated glycemia levels and lower Cederholm index of insulin sensitivity remained unchanged after the exclusion of all males from the analysis. Notably, due to low proportion of the NN homozygotes among men, the statistics performed in males was not significant.

It is important to emphasize that glycemic curve during the 3h-oGTT remained within the normal range of glucose tolerance even in the NN genotype. However, the value of insulin sensitivity expressed

by mean of Cederholm index in the NN group corresponds with the values of obese people [45], which is clinically quite important. Our group of NN homozygotes is still relatively young (mean age 37.8 ± 13 years) and slim (mean BMI 24.4 ± 4.2 kg/m²). Slight differences in glycemic curve and insulin sensitivity in young age and slim figure can make a clinically significant difference in older age, especially when BMI rises, if there is a genetic predisposition present.

Low frequency of the minor alleles in both the evaluated SHBG gene polymorphisms places high demands on the abundance of the analyzed cohort. Especially in case of the noteworthy observation which was made on a group of rare homozygotes. Nevertheless, the finding of strong association between the rs6259 SNP NN genotype and biochemical markers shifted towards impaired glucose tolerance in spite of significantly higher SHBG levels could represent important insight to the pathophysiology of insulin resistance and T2DM aetiology. If confirmed on the representative group of rare homozygotes, the polymorphism would be another causal factor involved in the glycoregulation, acting independently of the SHBG concentration. Further investigation will follow to verify this challenging observation on the larger group of probands.

Conclusion

Our findings indicate lower SHBG concentrations in diabetics and PCOS patients in comparison with controls. Furthermore, genetic analysis confirms the association of rs6259 SNP with SHBG levels: homozygotic constellation NN in this SNP was associated with higher SHBG concentration. Interestingly, the NN genotype also exhibited higher stimulated glucose concentrations during the 3-hour oGTT and lower insulin sensitivity expressed by means of Cederholm index. This observation seems paradoxical considering established protective effect of higher SHBG levels in relation to T2DM risk and may help uncover new genetic factor affecting glucose homeostasis. The finding will be subjected to further investigation.

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