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Estrogen receptor alpha single nucleotide polymorphism as predictor of diabetes type 2 risk in hypogonadal men.

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Short title: ESR1 and diabetes

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Abstract

Estradiol (E2) is apart from its role as a reproductive hormone also important for cardiac function and bone maturation in both genders. It has also been shown to play a role in insulin production, energy expenditure and in inducing lipolysis. The aim of the study was to investigate if low circulating testosterone or E2 levels in combination with variants in the estrogen receptor alpha (*ESR1*) and estrogen receptor beta (*ESR2*) genes were of importance for the risk of type-2 diabetes.

The single nucleotide polymorphisms *rs2207396* and *rs1256049*, in *ESR1* and *ESR2*, respectively, were analysed by allele specific PCR in 172 elderly men from the population based Tromsø study. The results were adjusted for age.

In individuals with low total (≤ 11 nmol/L) or free testosterone (≤ 0.18 nmol/L) being carriers of the variant A-allele in *ESR1* was associated with 7.3 and 15.9 times, respectively increased odds ratio of being diagnosed with diabetes mellitus type 2 ($p = 0.025$ and $p = 0.018$, respectively).

Lower concentrations of E2 did not seem to increase the risk of being diagnosed with diabetes.

In conclusion, in hypogonadal men, the *rs2207396* variant in *ESR1* predicts the risk of type 2 diabetes.

Introduction

Estrogens are considered as “female sex hormones” but, in both sexes, they have a crucial role in regulating not only the reproductive system but also many other of the functions in the human body, e.g. the cardiovascular function, bone mineral density and the metabolism [1-3].

In males estradiol (E2) is primarily produced at extragonadal sites by CYP19 controlled aromatization of testosterone [4, 5]. In an alternative pathway, androstenedione is converted to estrone (E1), which subsequently is metabolized to E2. Previously, E2 has been shown to inhibit insulin gene receptor transcription, promote insulin resistance and also to up-regulate CYP19 expression in mature adipocytes [6-8]. Estradiol has also been shown to increase insulin biosynthesis [9].

Low testosterone levels in men has in numerous studies and also in a meta-analysis comprising more than 6000 men been associated with increased risk of type 2 diabetes (referred to as diabetes hereafter) [6]. It has also been shown that testosterone replacement therapy may improve insulin sensitivity [10], whereas androgen deprivation increases insulin resistance in men [11]. However, whether this is attributed to testosterone *per se* or to the parallel changes in E2 has been discussed. Estrogen action is mediated through estrogen receptor α (ESR1), estrogen receptor β (ESR2) and a G-protein coupled cell surface receptor [12, 13].

Total estrogen resistance due to a germ line mutation in the *ESR1* gene was in 1994 reported in a 28-years old man with continued linear growth into adulthood. He also presented with osteoporosis and early atherosclerosis as well as glucose intolerance and hyperinsulinemia [11]. However, testosterone concentration was within normal range and the man had gone through normal puberty, suggesting that lack of estrogen action rather than testosterone deficiency was the cause of his diabetes. Estrogen resistance due to homozygous germ line mutations in the

ESR1 gene is however extremely rare, only one kindred described to date. Nevertheless, one cannot exclude inherited differences in estrogen secretion and response, which may have more subtle effects on the metabolic system and over time contribute to the etiology of diabetes. Animal studies are supporting this theory, since *ESR1* knockout mice suffer from increased fasting plasma glucose levels and increased insulin insensitivity [14]. In males suffering from mutations in the gene coding for aromatase increased insulin resistance, increased concentrations of insulin and diabetes have been found [15-18]. However, Carani et. al. found no changes in metabolic parameters in a patient with a mutation in the gene coding for aromatase [19].

Both the *ESR1* and *ESR2* genes contain several single nucleotide polymorphisms (SNPs). We have previously reported that *G* to *A* substitution in SNP *rs2207396*, situated in intron 6, of the *ESR1* was associated with higher risk of developing azoospermia in childhood cancer survivors [20], pointing to a functional role of this polymorphism. With respect to *rs1256049* SNP in *ESR2*, a synonymous substitution of *GTG* to *GTA* in, *exon 5* was first shown to be associated with ovulatory dysfunction in women of Chinese origin [21] and later also with male infertility in Caucasian patients [22].

Since subnormal testosterone levels, either due to the male sex hormone action *per se* or due to parallel decrease in E2, were found to be associated with increased risk of metabolic derangements [23] we wished to investigate whether this association is modified by polymorphisms in the above mentioned SNPs in the *ESR1* and *ESR2* genes.

Materials and methods

Subjects

The study population and the methods have been presented previously [23]. Shortly, the Tromsø Study is an on-going populations based health survey of the inhabitants from the municipality of Tromsø, mainly focusing on lifestyle related diseases. A subgroup consisting of 68 men with consistent total testosterone ≤ 11.0 nmol/L and 104 men with consistent total testosterone > 11.0 nmol/L were recruited from the fifth Tromsø study. When they were invited to participate in this study in 2005 they were between 60 and 80 years old.

Blood samples for analysis of levels of testosterone and E2, glucose, insulin and for genetic analyses were drawn 08.00 am. An oral glucose tolerance test (OGTT) with ingestion of 75 g glucose was performed. Diabetes was defined as having either non-fasting glucose > 11.1 mmol/L, fasting glucose > 7.0 mmol/L or 2-hour glucose load > 11.1 mmol/l Height and weight were measured in subjects wearing light clothing without shoes. Body mass index (BMI) was calculated (kg/m^2). All participants were genotyped for *rs2207396* in *ESR1* and *rs1256049* in *ESR2*. Free testosterone was calculated according to Vermeulen's formula [24].

All participants signed an informed consent form and the study was approved by the Regional Research Ethics Committee, North-Norway.

Sex hormone analyses and biochemistry

Serum total testosterone was analysed by electrochemical luminescence immunoassay using an automated clinical chemistry analyser (Modular E, Roche Diagnostics GmbH, Mannheim, Germany). The total analytical precision expressed as the sum of intra and interassay coefficients

of variation (CV_a) were 5.6 and 4.7 and the reference values were 10-28 nmol/L and < 0.20 nmol/L, respectively. Serum E2 levels were measured using DELFIA® Estradiol-kit (Perkin Elmer, Turku, Finland). The total analytical precision expressed as the sum of interassay coefficients of variations (CV) 20% at 30 pmol/L and 10% at 280 pmol/L. Measuring range was 8-1500 pmol/L. For the E2 analysis, serum samples from 5 participants were lacking thus limiting the study population to 167 males for all calculations involving E2.

Serum SHBG and insulin were analysed by immunometry based on chemiluminescence using an automated clinical chemistry analyser (Immunulite 2000, Diagnostic Product Corp., Los Angeles, USA). The CV_{as} were 5.2 and 8.8, respectively.

Allele specific PCR

To identify the different SNPs, allele specific PCR was used and PCR conditions established to generate both a short allele-specific and a longer control band in the presence of the variant, and only the longer control band in its absence. For the *ESR1* variant *rs2207396*, the PCR amplification volume was 25 μ l containing 25 ng genomic DNA, 45 mM KCL, 10 mM Tris-HCL, 0.2 mM dNTP (Fermentas GmbH, St. Leon-Rot, Germany), 0.1% Tween 20 (Scharlau Chemie S.A, Barcelona, Spain), 1.5 mM $MgCl_2$, 0.5 μ M forward primer (Invitrogen, Edinburgh, Scotland) (Table I), 0.5 μ M reverse primer, 0.45 μ M of either one of the allele specific primers, 0.5 U DNA Polymerase (DyNAzyme II, Finnzymes, Espoo, Finland). Amplifications were carried out for 42 cycles (Eppendorf, Hamburg, Germany); each cycle including denaturation for 1min at 96°C, primer annealing at 53.5°C for 30 sec, and primer extension at 72°C for 3 min, with an initial denaturation step for 3 min at 96°C and a final extension at 72°C for 5 min.

For *rs1256049* in *ESR2* gene, the PCR amplification volume was 25 μ l containing 25 ng genomic DNA, 45 mM KCL, 10 mM Tris-HCL, 0.2 mM dNTP 0.25 μ M forward primer, 0.25 μ M reverse primer and 0.3 μ M of either one of the allele specific primers [22] and 0.5 U DNA Polymerase. Amplifications were carried out for 40 cycles; and primer annealing at 57°C for 30 sec, whereas all other procedures were the same as for *ESR1*.

To verify the results of the allele-specific PCR, 3 selected PCR samples representing each genotype, were purified using a JETquick-kit according to the manufacturer's recommendation (Genomed, Löhne, Germany) and directly sequenced. The sequencing reaction was performed using ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's recommendation (Applied Biosystems, Austin, USA) and the results were obtained using a 3130x/Genetic Analyzer (Applied Biosystems, Hitachi, Austin, USA).

Statistics

The subjects were categorised into groups with subnormal and normal testosterone level. For total testosterone the cut off level was set to 11 nmol/L, **as has been used for previous studies on these subjects**, whereas it was 0.18 nmol/L for the calculated level of free testosterone [25, 26].

Using binary regression analysis, the odds ratio (OR) for diabetes in men with low testosterone levels, with the group having normal concentration of this sex hormone as reference, was assessed.

In order to explore whether fluctuations in testosterone levels mirror corresponding changes in E2 concentration, we tested correlation between these two parameters using Spearman's test.

The association between E2 and the OR for diabetes in the entire study population was assessed using binary regression analysis with median E2 (74.0 pmol/L) as a cut-off point for categorising E2.

For the combination of total/free testosterone and the genotype four different groups were defined: a) Normal (total/free) testosterone + *GG*; b) Normal (total/free) testosterone + *AA/AG*; c) Subnormal (total/free) testosterone + *GG*; d) Subnormal (total/free) testosterone + *AA/AG*. For each genotype we primary performed an interaction analysis (testosterone subnormal/normal * genotype) in order to explore whether there was a statistically significant interaction between sex hormone levels and genotype in relation to risk of developing diabetes.

The interaction analysis was also done for E2*genotype with median E2 levels as cut off for dichotomising the hormone concentrations. Since there are no well-defined subnormal E2 levels for men, we even performed the interaction analysis with E2 as continuous variable

Similar strategy was applied for the *ESR2* genotypes with the exception that the analysis of differences in *ESR2* genotype distribution among males with normal levels of testosterone Fisher's exact test was used since one of the subgroups contained 0 subjects.

Calculations were adjusted for age.

Statistical significance was defined as $p < 0.05$.

For statistical analysis, IBM SPSS Statistics 20 (version 20, IBM Corp., New York, USA) was used.

Results

Total and free testosterone

The measured levels of total and free testosterone are shown in figure 1. Subnormal levels of total testosterone, irrespective of allele, did not increase the OR of having been diagnosed with diabetes (95% CI: 0.7-6.0; $p = 0.196$). Subnormal levels of free testosterone, irrespectively of allele, increased the OR of having been diagnosed with diabetes (OR= 4.3, 95% CI: 1.2-11.0; $p = 0.019$.) compared to individuals with normal levels of free testosterone.

Estradiol

The distribution of E2 in the study population is shown in figure 2. There was a statistically significant although weak positive correlation between testosterone and E2 ($p < 0.0005$, β : 0.327).

The OR of having diabetes in those with E2 below median did not differ from those with E2 above this value (OR=1.4; 95% CI: 0.460-4.184; $p = 0.571$).

ESR1

The frequency of the variants of *rs2207396* SNP in *ESR1* was *GG* $n=98$ (57.0%), *AG* $n=61$ (35.5%) and *AA* $n=13$ (7.6%), which is in Hardy Weinberg equilibrium.

In the cohort, in total 15% of the men were diagnosed with diabetes. Of these, 53% had the *AA* or the *AG*-variant. No statistical significant difference in the OR between the different allele groups was observed (OR = 1.6; 95% CI: 0.569-4.901; $p = 0.350$).

Interaction between ESR1 and testosterone

There was a statistically significant interaction between both total and free testosterone groups (subnormal and normal) and allele groups in relation to the OR of having been diagnosed with diabetes ($p = 0.026$ and $p = 0.013$, respectively).

In carriers of an *A*-allele and subnormal levels of total testosterone, the OR of having diabetes was 7.3 (95% CI: 1.3-41.7; $p = 0.025$) as compared to men homozygous for the *G*-allele (Table II). For those with low free testosterone, men with the *A*-variant had 15.9 times increased OR (95% CI: 1.6-166.7; $p = 0.018$) for diabetes compared to counterparts with the *G*-variant (Table III). In men with normal levels of total or free testosterone, carriers of an *A*-allele, as compared to men homozygous for the *G*-allele did not differ significantly with respect to the diabetes risk ($p = 0.354$ and $p = 0.323$, respectively) (Table II).

Interaction between ESR1 and E2

No statistically significant interaction between E2, as dichotomized or continuous variable and ESR1 genotype in relation to the OR of having been diagnosed with diabetes ($p = 0.425$ and $p = 0.477$, respectively) was observed.

ESR2

In *ESR2*, the frequency of the variants of *rs1256049* was *GG* $n=159$ (92.1%), *AG* $n=12$ (7.3%) and *AA* $n=1$ (0.6%) which is in Hardy Weinberg equilibrium.

No statistical significant difference in the OR between the different allele groups was observed (OR = 1.1; 95% CI: 0.124-8.929; $p = 0.962$).

Interaction between ESR2 and testosterone

There was no interaction between total, nor free, testosterone (subnormal or normal) and genotype in relation to the OR of having been diagnosed with diabetes ($p = 0.999$ and $p = 0.678$, respectively).

Neither for subnormal levels of total nor free testosterone was there any statistically significant change in the OR of having been diagnosed with diabetes when carriers of an *A*-allele were compared with those homozygous for the *G*-allele (Table IV and table V).

Interaction between ESR2 and E2

There was no interaction between E2, as dichotomized or continuous variable, and genotype in relation to the OR of having been diagnosed with diabetes ($p = 0.668$ and $p=0.163$ respectively).

Discussion

The main finding of current study was significant interaction between the *ESR1* genotype of the SNP *rs2207396* and testosterone levels in relation to the risk of having been diagnosed with diabetes. In men with subnormal testosterone levels, carriers of the less frequent *A* variant presented with increased OR for diabetes as compared to those homozygous for *G*. However, in men with normal testosterone levels, the genotype did not matter. This effect does not seem to be mediated through the conversion of testosterone to E2, since there was only a weak correlation between the serum levels of this two hormones and E2 concentration did not affect the OR for having diabetes, regardless of the *ESR1* or *ESR2* genotype.

The finding that men with low androgen concentrations were at higher risk for diabetes has been reported before and is consistent with a recent meta- analysis comprising more than 6000 men [6]. Earlier studies have also found an association between variants in *ESR1* (*rs3020314* and *rs2234693*, which are not in linkage disequilibrium with *rs2207396*), and increased risk of developing diabetes [27, 28]. However, the interaction between low androgen levels and variants of the *ESR1* gene in relation to diabetes risk is a novel finding. Moreover, in current study, E2 was not associated with diabetes risk, neither as an independent factor nor in combination with any genotype tested. However, care should be taken when interpreting this result due to the 20% CV for the method used for analysis of E2.

The *G* to *A* change in *rs2207396* does not lead to a change of amino acid in the protein but might be in linkage disequilibrium with other genetic variations that could affect gene expression or function. However, another option is that polymorphisms in the SNP *rs2207396*, changes the secondary structure of the *ESR1* mRNA, possibly leading to changes in mRNA synthesis, splicing, maturation, transport, translation, or degradation [29]. The importance of this SNP is

stressed by the fact that same variation in the *ESRI* was previously reported to determine the risk of azoospermia in men who during childhood underwent cancer treatment [20].

The most obvious limitation to this study is the relatively small population size. However, since there were no obvious reasons to believe any selection bias related to the SNPs, we believe that our findings mirror a true biological association. The strength of the study is the genetic homogeneity of the subjects, which minimized noise due to ethnic origin.

In this study we have not corrected for body mass index (BMI). The reason for this was that as testosterone is inversely correlated to BMI [30]. Thus, inclusion of BMI in the analysis would lead to an over-adjustment of the effect of low testosterone levels. Furthermore, the genetic variant had most impact in men with low free testosterone, which is regarded as a parameter that is rather robust to the variation in BMI.

Our finding has biological and clinical implications. Further investigation of the role of *ESRI* in the pathogenesis of diabetes may help in understanding the biology of this frequent disease and in this context genetic and endocrine risk markers can be used in defining high risk subjects and in prevention trials.

In summary, this study shows that older men with low free testosterone levels have markedly increased risk of diabetes if they in addition are carriers of the *rs2207396 A* allele in the *ESRI* gene. A larger study on this topic is warranted.

Declaration of interests: None of the authors have financial and/or personal relationships with people or organizations that could inappropriately influence (bias) their work. The corresponding author had full access to all data in the study and had the final responsibility for the decision to submit the manuscript for publication.

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