Estrogen receptor 1 gene polymorphisms (PvuII and XbaI) are associated with type 2 diabetes in Palestinian women

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Background. Type 2 diabetes mellitus (T2DM) is a multifactorial disease where both genetic and environmental factors contribute to its pathogenesis. The PvuII and XbaI polymorphisms of the estrogen receptor 1(ESR1) gene have been variably associated with T2DM in several populations. This association has not been studied in the Palestinian population. Therefore, the aim of this study was to investigate the association between the PvuII and XbaI variants in the ESR1 and T2DM and its related metabolic traits among Palestinian women. Methods. This case–control study included 102 T2DM and 112 controls in which PvuII and XbaI variants of the ESR1 gene were genotyped using amplicon based next generation sequencing (NGS). Results. Allele frequencies of both PvuII and XbaI variants were not significantly different between patients and control subjects (P>0.05). In logistic regression analysis adjusted for age and BMI, the ESR1 PvuII variant was associated with risk of T2DM in three genotypic models (P< 0.025) but the strongest association was observed under over-dominant model (TT+CC versus TC) (OR=2.32, CI=1.18-4.55 adjusted P=0.013). A similar but nonsignificant trend was also observed for the ESR1 XbaI variant under the over-dominant model (AA+GG versus AG) (OR=2.03, CI=1.05-3.95; adjusted P=0.035). The frequencies of the four haplotypes (TA, CG, CA, TG) were not significantly different in the T2DM patients compared with control group (P>0.025). Among diabetic group, an inverse trend with risk of CVD was shown in carriers of CG haplotype compared to those with TA haplotype (OR=0.28, CI=0.09 - 0.90; adjusted P=0.035). Further, stratified analyses based on ESR1 PvuII and XbaI genotypes revealed no evidence for association with lipid levels (TC, TG, HDL, LDL). Conclusions. This is the first Palestinian study to conclude that ESR1 PvuII and XbaI variants may contribute to diabetes susceptibility in Palestinian women. Identification of genetic risk markers can be used in defining high risk subjects and in prevention trials.
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Abstract

**Background.** Type 2 diabetes mellitus (T2DM) is a multifactorial disease where both genetic and environmental factors contribute to its pathogenesis. The PvuII and XbaI polymorphisms of the estrogen receptor 1 (ESR1) gene have been variably associated with T2DM in several populations. This association has not been studied in the Palestinian population. Therefore, the aim of this study was to investigate the association between the PvuII and XbaI variants in the *ESR1* and T2DM and its related metabolic traits among Palestinian women.

**Methods.** This case–control study included 102 T2DM and 112 controls in which PvuII and XbaI variants of the *ESR1* gene were genotyped using amplicon based next generation sequencing (NGS). **Results.** Allele frequencies of both PvuII and XbaI variants were not significantly different between patients and control subjects (P>0.05). In logistic regression analysis adjusted for age and BMI, the *ESR1* PvuII variant was associated with risk of T2DM in three genotypic models (P<0.025) but the strongest association was observed under over-dominant model (TT+CC versus TC) (OR=2.32, CI=1.18-4.55 adjusted P=0.013). A similar but nonsignificant trend was also observed for the *ESR1* XbaI variant under the over-dominant model (AA+GG versus AG) (OR=2.03, CI=1.05-3.95; adjusted P=0.035). The frequencies of the four haplotypes (TA, CG, CA, TG) were not significantly different in the T2DM patients compared with control group (P>0.025). Among diabetic group, an inverse trend with risk of CVD was shown in carriers of CG haplotype compared to those with TA haplotype (OR=0.28, CI=0.09 - 0.90; adjusted P=0.035). Further, stratified analyses based on *ESR1* PvuII and XbaI genotypes revealed no evidence for association with lipid levels (TC, TG, HDL, LDL).
Conclusions. This is the first Palestinian study to conclude that ESR1 PuvII and XbaI variants may contribute to diabetes susceptibility in Palestinian women. Identification of genetic risk markers can be used in defining high risk subjects and in prevention trials.

Introduction

Type 2 Diabetes (T2DM) is a multifactorial disease that caused by a complex combination of genetic and environmental factors. Identification of genetic polymorphisms associated with diabetes may lead to prediction of disease development and prevention of its vascular complications (Slominski et al. 2018). Recently, several reports have revealed the role of estradiol in regulating energy metabolism (Hevener et al. 2018). It was evident that estrogens increase hepatic insulin sensitivity, stimulate insulin synthesis in islets of Langerhans, prevent β-cell apoptosis and improve insulin action in skeletal muscles (Meyer et al. 2011).

Estrogen exerts its physiological functions through the estrogen receptors (ESR1, ESR2, and a G-protein coupled cell surface receptor (GPER)) and might prevent menopause syndrome, CVD and diabetes (Gupte et al. 2015). Estrogen receptor 1 (ESR1) is broadly expressed in adipose tissue, skeletal muscle, liver, and immune cells. It is a ligand-activated transcription factor that regulates a large number of genes in diverse target tissues. (Hevener et al. 2018). Animal studies reported that male and female ESR1 gene knockout mice developed features of the metabolic syndrome including obesity caused by impaired fatty acid oxidation, glucose intolerance, and impaired insulin sensitivity, thus revealed the critical role of ESR1 in metabolic homeostasis (Heine et al. 2000; Ribas et al. 2010).

ESR1 gene, which encompasses 140 kb of DNA, is found on chromosomes 6q25.1. It is a highly polymorphic gene containing more than 1,600 single nucleotide polymorphisms (SNPs). Two
SNPs in \textit{ESR1}, both located in the first intron, PvuII (rs2234693) and XbaI (rs9340799), are the most extensively investigated variants and reported to be associated with T2DM (Huang et al. 2006; Mohammadi et al. 2013), metabolic syndrome (MetS) (Zhao et al. 2018) and other diseases (Onland-Moret et al. 2005; Silva et al. 2010; Weng et al. 2015). These polymorphisms might interfere with the estrogen effect by altering \textit{ESR1} gene expression via altering the binding of its own transcription factors (Gomes-Rochette et al. 2017). Diabetes and obesity had reached an alarming rate among Palestinians especially among refugees (El Kishawi et al. 2014; Shahin et al. 2015). In 2015, the Palestinian Ministry of Health (PMOH) reported that cardiovascular diseases were the leading cause of death and diabetes mellitus was the fourth cause of death among Palestinians (Health Annual Report Palestine 2015). A recent study conducted on three refugee camps in the West Bank, revealed that the overall prevalence of obesity and overweight was 63.1% (for obesity: 42% in women and 29.2% in men; overweight 25.8 and 28.9% in women and men; respectively). The prevalence of MetS among obese and overweight adults was 69.4% with no gender-based differences (Damiri et al. 2018). Obese and overweight individuals are at high risk of both cardiovascular diseases and T2DM (Alberti & Zimmet 1998). Estrogens deficiency is an important obesity-triggering factor which enhances metabolic dysfunction and thus predisposing to T2DM and cardiovascular diseases among menopausal women (Lizcano & Guzman 2014). A meta-analysis study investigating the new-onset of T2DM in postmenopausal women following estrogen replacement therapy, revealed a 30% lower relative risk [RR 0.7 (CI, 0.6–0.9)] of diabetes compared with placebo (Salpeter et al. 2006). However, the use of estrogen to prevent chronic diseases is still challenging and controversial (Bolton 2016).
As *ESR1* gene is a potential candidate gene for susceptibility to T2DM, we hypothesized that the *ESR1* variants (PvuII and XbaI) might be associated with T2DM, diabetic complications and related metabolic traits in Palestinian diabetic women.

**Materials & Methods**

**Study design and participants**

This case control study includes 214 Palestinian women (102 with T2DM and 112 non diabetic controls). All participants were unrelated, aged >40 years and selected within the period of 2016-2017 from the United Nations Relief and Works Agency for Palestine Refugees (UNRWA) clinics (Hebron and Ramallah, Palestine). Diagnosis of T2DM was based on World Health Organization (WHO) criteria (fasting plasma glucose ≥126 mg/dl and/or currently on treatment for diabetes)(Kumar et al. 2016). The diagnosis of T2DM was confirmed based on patients’ medical records reporting initial diagnosis of diabetes mellitus after age of 40, receiving oral hypoglycemic agents without insulin for at least one year after initial diagnosis, and currently on treatment for diabetes. Patients with probable type 1 diabetes who received continuous insulin therapy since diagnosis were excluded. The anthropometric measurements were collected from their medical records using a standard questionnaire that included age, age at diagnosis, sex, family history, diabetic complications and medication for diabetes. BMI was calculated as kilograms divided by the square of height in meters. Blood pressure was measured in sitting position, on the left arm, after a 5-min rest by a health clinic worker, with a mercury sphygmomanometer. Blood samples (5 ml) after a 12-h minimum fast was collected in EDTA tubes for biochemical tests as described in our previous work (Sabarneh et al. 2018). Plasma glucose, cholesterol, HDL cholesterol, and triglyceride were determined using standard methods of commercial kits (Human, Wiesbaden, Germany). LDL cholesterol was calculated based on the Friedewald formula. The control group
was selected from individuals who came to the same clinic for an annual health check-up, they were eligible to be included if they had no prior diagnosis for T2DM, no family history in first-degree relatives, their fasting glucose<126 mg/dl and BMI>25. All the participants provided written informed consent to participate in the study, the study protocol was approved by Al-Quds University Research Ethics Committee (Rf no. 2/SRC/4).

Amplicon based next generation genotyping and bioinformatics

Genomic DNA was extracted from whole blood (300 μl) using genomic QIAamp DNA purification kit according to the manufacturer instructions (Qiagen, Hilden, Germany). All DNA samples were genotyped for the T/C, rs2234693, and A/G, rs9340799, also known PvulII and XbaI polymorphisms, respectively using amplicon based next generation sequencing (NGS). Briefly, two primers (forward and reverse) were used to target the 2 SNPs as previously described (Motawi et al. 2015). Both primers were modified with over hanged Illumina adaptor sequences at the 5' ends (italic bolded, Table S1) to target a partial sequence of 119 bp in length with a final product of 186 bp using conventional thermocycler.

The PCR product was visualized and captured on a 1.5% agarose gel, cleaned by Agencourt AMPure XP system (X1, Beckman Coulter Genomics, A63881) and eluted in 25 μl elution buffer.

All purified products were subjected to a second round of amplification to assign unique index sequences (barcode) for each sample using Nextera XT Index Kit (Illumina). Five microliter from each barcoded sample were pooled together, mixed and spin down. Then, 100 μl of the pooled product was cleaned by Agencourt AMPure XP system (X1)(Beckman Coulter Genomics, A63881), and eluted in 50 μl elution buffer. Library purity and quantity were evaluated by 4200 TapeStation System (Agilent Technologies, Inc., Santa Clara, CA) using D1000 ScreenTape kit (Agilent Technologies, Inc., Santa Clara, CA) and by Qubit® Fluorometer (Invitrogen, Carlsbad,
CA) using Qubit dsDNA high-sensitivity (HS) assay (Invitrogen). Concentration of 4 nM was prepared. 20K reads for each sample was targeted. Samples were deep sequenced on NextSeq 500/550 machine using the 150-cycle Mid Output Kit (Illumina).

The obtained DNA sequences were uploaded on galaxy program (https://usegalaxy.org/). Workflow of filtration included Illumina adaptor trim, quality selection of Q>20 with minimal read length of 100 bp. Four virtual probe sequences were used to identify the PvuII and XbaI variants (Table S1, supplementary materials). The genotypes were determined based on the calculated ratio between the read counts for wild type and mutant alleles, for both SNPs (PvuII and XbaI) in each individual sample.

Statistical analysis
Statistical analysis was performed with the SPSS package, version 19.0 (SPSS, Inc., Chicago, IL, USA). All tests were two-tailed and P<0.05 was considered significant. The mean values and standard errors were reported in tables. Genotype and allele frequencies in T2DM and control subjects were tested by multivariable logistic regression analysis using five genetic models: codominant, dominant, over-dominant, recessive and additive with adjustment for age and BMI using R statistics (V 3.5.1; SNPassoc package)(Gonzalez et al. 2007). The genotype frequencies were tested for Hardy–Weinberg equilibrium by calculating a \( \chi^2 \) statistic and corresponding P-value as previously described by (Mayo 2008). The risk to T2DM was estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs). Linkage disequilibrium (LD), coefficient (D') for haplotypes and their frequencies were performed using R statistics (V 3.5.1; SNPassoc package)(Gonzalez et al. 2007). Multivariate linear regression models taking into account age and BMI were performed to assess associations between SNPs and T2DM complications.
All quantitative parameters were normalized before analysis using the “bestNormalize” R package (https://github.com/petersonR/bestNormalize)(Peterson 2017). To minimize the chance of obtaining type I error, Bonferroni’s adjustment was applied. P<0.025 was adopted as the significant threshold unless otherwise specified. Quanto 1.2.4 software was used to estimate the statistical power (http://biostats.usc.edu/Quanto.html). The power of any test of statistical significance was <50%.

Results

Biochemical and Genetic analysis

Anthropometric and biochemical characteristics of study individuals are presented in Table 1. In comparison with the control group, all clinical parameters showed statistically significant increase in T2DM patients (P<0.05). However, no significant difference in diastolic blood pressure was observed among the two group (P=0.4). The T2DM subjects were significantly older (P=0.0001) but the control group were at the same mean age at T2DM diagnosis in cases (P>0.05) (Table 1). The cases had higher mean BMI than control subjects but was at borderline significance (P=0.054). Among T2DM patients, 10.8, 8.8, 7.8 and 5.9% had cardiovascular diseases (CVD), nephropathy, diabetic foot and retinopathy, respectively. The genotyping distribution for both variants was consistent with Hardy Weinberg equilibrium in cases and control groups and in all subjects (p>0.05). Our results showed that the heterozygous genotypes TC and AG of the ESR1 PvuII and XbaI polymorphisms were significantly elevated in T2DM patients compared with control individuals (P =0.04, P=0.01 respectively), while, the frequencies of C and G alleles of the ESR1 PvuII and XbaI polymorphisms, respectively, were comparable among cases and controls (P>0.05) (Table 2). However, upon Bonferroni’s adjustment, only the heterozygous genotype AG of the ESR1 XbaI remained significantly higher in diabetic group (P=0.01).
PvuII and XbaI Polymorphisms in *ESR1* gene and risk to type 2 diabetes

To estimate the effect of the genotypes on the disease, logistic regression analysis was performed using five genetic models adjusted to age and BMI: additive, dominant, codominant, recessive and over-dominant. In the case of the PvuII T/C rs2234693 polymorphism, T2DM patients showed significantly different genotypes distribution compared to control group in three models, carriers of TC genotype had higher risk to T2DM compared to those of TT genotype (OR=2.84, CI=1.32-6.11; P=0.024)(Table 3). In the dominant model, the TC+CC genotype carriers was significantly at higher risk to T2DM than TT genotypes (OR = 2.50, CI= 1.21-5.14; P = 0·011). In the over-dominant model (TT+CC versus TC), the association was also significant (OR=2.32, CI=1.18-4.55;P=0.013)(Table3). The analysis of the Xba rs9340799 A/G polymorphism showed a trend for association with T2DM in the over dominant model (AA+GG versus AG)(OR= 2.03 , CI=1.05-3.95;P=0.035)(Table3).

Biochemical characteristics of T2DM individuals according to *ESR1* PvuII and XbaI genotypes are shown in Table 4. It demonstrated insignificant association of the investigated parameters across genotypes of both polymorphisms (P>0.05). A stratified analysis of combined genotypes with respect to different genetic models also revealed no significant association with any biochemical or clinical parameter (data not shown).

**LD estimation between *ESR1* SNPs and haplotype analysis**

The polymorphisms tested were in LD (normalized Lewontin’s D’ = 0.91). Among all subjects, the most common haplotype, TA, had a frequency of 55%, and the CG haplotype had a frequency of 40% while two haplotypes CA and TG had frequencies of 2.9% and 2.2%, respectively. The frequencies of the four haplotypes were not significantly different in the T2DM patients compared with control group as shown in Table 5. In logistic regression analyses, adjusting for the same
potential confounders (age and BMI) used in the genotype models, none of the four possible haplotypes were associated with increased risk of diabetes (global P=0.14) (Table 5).

**Association of ESR1 PvuII and XbaI Polymorphisms with CVD in diabetic group**

The association of PvuII and XbaI SNPs within the ESR1 gene and CVD risk was evaluated among diabetic group (n=102) using logistic regression after adjustment of age and BMI. Under the dominant model, the combined PvuII TC+CC genotypes have a trend to lower risk of CVD compared with TT genotype (OR=0.25, CI =0.07-0.93;P=0.039). A similar trend was observed in the combined genotypes (AG+GG) of the ESR1 XbaI compared to AA (OR=0.26, CI=0.07-0.99;P=0.049) as shown in Table 6. The frequency of CG haplotype was statistically higher in diabetic patients without CVD (46%) compared to those with CVD (23%) (OR=0.28, CI=0.09-0.90; adjusted P=0.035). On the other hand, we did not find any association of ESR1 PvuII and XbaI polymorphisms with the other T2DM complications including nephropathy, retinopathy and diabetic foot (data not shown).

**Discussion**

The prevalence of diabetes has increased worldwide, most markedly in the world’s middle-income countries which reflects an increase in associated risk factors such as being overweight or obese (World Health Organization 2016). At menopause, women experience health challenges due to estrogen deficiency. The risk of T2DM is increasing with postmenopause status interacting with other risk factors i.e hypertension, dyslipidemia and obesity (Ren et al. 2018). Estrogen may regulate insulin action directly via actions on insulin-sensitive tissues. In skeletal muscle, ESR1 is thought to have a positive effect on insulin signaling and GLUT4 expression. It has been shown that stimulation of estrogen receptor with its agonist propylpyrazoletriyl (PPT) increased insulin-stimulated glucose uptake in skeletal muscles (Gorres et al. 2011). Although not all studies are in...
agreement, PvuII and XbaI and other polymorphisms across the ESR1 gene have been associated with risk of T2DM as reported in the Chinese (Huang et al. 2006), African–Americans, European–Americans (Sale et al. 2004), Hungarians (Speer et al. 2001) and Egyptian women (Motawi et al. 2015). In this study, the frequency for ESR1 PvuII and XbaI alleles and genotypes in Palestinian women (n=214) was evaluated. Our results reported that the ESR1 PvuII C and XbaI G allele frequencies were similar to other studies among Arab population (El-Beshbishy et al. 2015; Motawi et al. 2015). However, several studies showed distinct prevalence for these two polymorphisms, particularly the XbaI alleles (Ganasyam et al. 2012). Discrepancies in allelic frequencies between reports may be attributed to the ethnic variability of the studied populations, heterogeneity of the analyzed diseases and sample size.

Most often, deviation from the HWE is caused by a small sample size and poor genotyping quality. In this study, minimization of genotyping errors was achieved by amplicon based NGS. It provides a lower cost per sample alternative to restriction fragment length polymorphism (RFLP), probe based Taq-man PCR and Sanger sequencing. RFLP, a traditional genotyping method, requires a high quality and quantity of DNA sample and numerous steps including amplification, digestion with restriction enzymes and gel electrophoresis which can be a laborious and time-consuming process. Moreover, confirmatory sequencing must be performed when a sample shows confused banding pattern particularly the bands of low molecular size. In our analysis, DNA sequences with a quality score of more than 20 ( >Q20), Q20 represents an error rate of 1 in 100 with a corresponding call accuracy of 99%, were selected. Amplicon based NGS requires advanced instrumentation, however and despite this limitation, we believe that it is the method of choice for genotyping studies and for rapid genetic screening of several hundred to thousands of samples.
To our knowledge, this is the first report to investigate whether *ESR1* PvuII and XbaI alleles and their genotypes and haplotypes are associated with T2DM among Palestinian women. In this study, the additive, dominant, co-dominant and recessive genotypic models of association were tested, all were adjusted for age and BMI. The *ESR1* PvuII variant, supported evidence for association with risk of T2DM in three genotypic models (P< 0.025) but the strongest association was observed in the over-dominant model, in which heterozygous carries confer a higher risk compared to homozygotes (Table 3). Although such association toward the heterozygotes could result from genotyping error, this is unlikely to be the case in our study as we used variant specific probes as described above.

One possible explanation is that the variant allele may have a very strong dominant effect so that there is little difference between the effects of the variant homozygotes and heterozygotes. Given that the majority of participants in both cases and controls were heterozygotes, the real effect among the variant homozygotes can only be assessed by much larger study in the future. On the other hand, the over-dominant model (upon Bonferroni correction) showed a trend for association of XbaI SNP with T2DM (OR=2.03, CI=1.05-3.95 P=0.035) which was unexpected given the tight linkage disequilibrium between these two polymorphisms. These results could also be attributed to the poor statistical power due to small sample size. Considering the associations of both SNPs found in other studies, we believe that further study involving a larger number of participants is needed to elucidate the exact role of these polymorphisms in susceptibility to T2DM. However, in 2018, a meta-analysis including eight studies indicated that PvuII, rather than XbaI polymorphism, was associated with T2DM (OR=0.673, 95% CI=0.550 - 0.823). In that study, the C allele of PvuII polymorphism showed a protective role in T2DM in Chinese people while the G allele of XbaI polymorphism is related to a reduced risk for T2DM in Caucasian...
population (Yang et al. 2018). Thus, we believe that physiological pathway to diabetes may vary among different population and those differences are reflected in part by genetic differences. Therefore, the results of our study should be considered exploratory and confirmed by additional studies including other polymorphisms/genes as one single gene polymorphisms are not enough to answer the pathogenesis of a complex disease such as T2DM. Further, it is well known that estrogens regulate the cardiovascular system via their direct effects on the vessel wall and indirect effects on total cholesterol and triglycerides metabolism (Liping et al. 2013). Among diabetic group, stratified analyses based on ESR1 PvuII and XbaI genotypes revealed no evidence for association with lipid levels (TC, TG, HDL, LDL). Moreover, we did not find any association between the two polymorphisms (PvuII and XbaI) and TC in control group (n=114). One limitation of this study is the lack of information about subjects who received hormone replacement therapy and/or lipid lowering agent which may affect their serum lipid profile, therefore, we were unable to study the interaction between these variants and hormone replacement therapy on lipid profile which was, however, beyond the scope of this study. It is reported that the effect of PvuII and XbaI polymorphisms on serum lipids depend on several other genes not on ESR1 gene alone. Similarly, Matsubara et al. (1997) and Almeida et al. (2006) did not find any association between the two polymorphisms and serum lipid profile in post-menopausal women (Almeida et al. 2006; Matsubara et al. 1997). A recent study conducted on a cohort of post-menopausal Brazilian women reported no effects of PvuII SNP of ESR1 gene on patient’s serum TC, LDL, HDL, and TG, while XbaI SNP was associated to changes in TG and total lipids mainly in obese and overweight woman (Gomes-Rochette et al. 2017). In contrast, Egyptian study showed that both PvuII and XbaI SNPs have been associated with increased levels of triglycerides, total cholesterol and LDL (Motawi et al. 2015).
The mechanism by which intronic polymorphisms of *ESR1* gene might confer increased risk of T2DM is not fully understood. However, it is reported that the PvuII and XbaI polymorphisms change the *ESR1* gene expression by altering the binding of its own transcription factors. Moreover, linkage disequilibrium between these two polymorphisms with other polymorphisms in the *ESR1* gene, such as TA tandem polymorphism in the promoter region could affect gene expression or function (Herrington et al. 2002). Polymorphisms in *ESR1* were first thought to be potential risk factors for the development of CVD; but a meta-analysis including 10 case-control studies demonstrated that the *ESR1* SNPs PvuII and XbaI are not associated with risk of CVD (Morselli et al. 2017). In this study we have noted an inverse trend between these polymorphisms and risk of CVD in diabetic group. Our findings cannot be a definitive conclusion to identify *ESR1* genotype/haplotype that could be useful in identification of diabetic women that are more prone to develop CVD, due to small sample size with limited statistical power to detect interactions or perform subgroups analyses which was the most obvious limitation of this study.

**Conclusions**

The present study suggests that the PvuII polymorphism in the *ESR1* gene is associated with increased risk of type 2 diabetes but not with lipid profile. The interaction between XbaI genotype and T2DM still need to be clarified. Identification of genetic risk markers can be used in defining high risk subjects and in prevention trials. The mechanism by which the *ESR1* PvuII and XbaI polymorphisms might be related to CVD-among diabetic group- needs further investigation with larger sample size. Also, future studies are warranted to replicate our results and to explain the influence of these intronic polymorphisms on diabetes susceptibility in Palestine.
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References

Alberti KG, and Zimmet PZ. 1998. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 15:539-553. 10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S

Almeida S, Fiegenbaum M, de Andrade FM, Osorio-Wender MC, and Hutz MH. 2006. ESR1 and APOE gene polymorphisms, serum lipids, and hormonal replacement therapy. *Maturitas* 54:119-126. 10.1016/j.maturitas.2005.09.009

Bolton JL. 2016. Menopausal Hormone Therapy, Age, and Chronic Diseases: Perspectives on Statistical Trends. *Chem Res Toxicol* 29:1583-1590. 10.1021/acs.chemrestox.6b00272

Damiri B, Abualsoud MS, Samara AM, and Salameh SK. 2018. Metabolic syndrome among overweight and obese adults in Palestinian refugee camps. *Diabetol Metab Syndr* 10:34. 10.1186/s13098-018-0337-2

El-Beshbishy HA, Tawfeek MA, Al-Azhary NM, Mariah RA, Habib FA, Aljayar L, and Alahmadi AF. 2015. Estrogen Receptor Alpha (ESR1) Gene Polymorphisms in Pre-eclamptic Saudi Patients. *Pak J Med Sci* 31:880-885. 10.12669/pjms.314.7541

El Kishawi RR, Soo KL, Abed YA, and Muda WA. 2014. Obesity and overweight: prevalence and associated socio demographic factors among mothers in three different areas in the Gaza Strip-Palestine: a cross-sectional study. *BMC Obes* 1:7. 10.1186/2052-9538-1-7

Ganasyam SR, Rao TB, Murthy YS, Jyothy A, and Sujatha M. 2012. Association of Estrogen Receptor-alpha Gene & Metallothionein-1 Gene Polymorphisms in Type 2 Diabetic Women of Andhra Pradesh. *Indian J Clin Biochem* 27:69-73. 10.1007/s12291-011-0179-2

Gonzalez JR, Armengol L, Sole X, Guino E, Mercader JM, Estivill X, and Moreno V. 2007. SNPassoc: an R package to perform whole genome association studies. *Bioinformatics* 23:644-645. 10.1093/bioinformatics/btm025

Gorres BK, Bomhoff GL, Morris JK, and Geiger PC. 2011. In vivo stimulation of oestrogen receptor alpha increases insulin-stimulated skeletal muscle glucose uptake. *J Physiol* 589:2041-2054. 10.1113/jphysiol.2010.199018

Gupte AA, Pownall HJ, and Hamilton DJ. 2015. Estrogen: an emerging regulator of insulin action and mitochondrial function. *J Diabetes Res* 2015:916585. 10.1155/2015/916585

Health Annual Report Palestine. 2015. Palestinian Ministry of Health. 2015, https://www.site.moh.ps/Content/Books/NWNJX7RJ2Bn4FSEGYiH43a2tiAAzKBnseGnUECaqWqYZndsbCePy_JQWguvkHTR4Xk4zUpdT450oWxH11BhIbVxwpgWVy2wiwHdGcM5K7aZ.pdf, accessed 05 January 2019.

Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, and Cooke PS. 2000. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A* 97:12729-12734. 10.1073/pnas.97.23.12729
Herrington DM, Howard TD, Brosnihan KB, McDonnell DP, Li X, Hawkins GA, Reboussin DM, Xu J, Zheng SL, Meyers DA, and Bleecker ER. 2002. Common estrogen receptor polymorphism augments effects of hormone replacement therapy on E-selectin but not C-reactive protein. Circulation 105:1879-1882.

Hevener AL, Zhou Z, Moore TM, Drew BG, and Ribas V. 2018. The impact of ERalpha action on muscle metabolism and insulin sensitivity - Strong enough for a man, made for a woman. Mol Metab 15:20-34. 10.1016/j.molmet.2018.06.013

Huang Q, Wang TH, Lu WS, Mu PW, Yang YF, Liang WW, Li CX, and Lin GP. 2006. Estrogen receptor alpha gene polymorphism associated with type 2 diabetes mellitus and the serum lipid concentration in Chinese women in Guangzhou. Chin Med J (Engl) 119:1794-1801.

Kumar R, Nandhini LP, Kamalanathan S, Sahoo J, and Vivekanadan M. 2016. Evidence for current diagnostic criteria of diabetes mellitus. World J Diabetes 7:396-405. 10.4239/wjd.v7.i17.396

Liping D, Lihua H, Zhitao J, Taohong H, Huili M, Lina Z, Xincheng Q, and Caiyi L. 2013. A meta-analysis of correlation of ER gene polymorphisms and risk in Chinese population with coronary heart disease Life Science 110:595-598.

Lizcano F, and Guzman G. 2014. Estrogen Deficiency and the Origin of Obesity during Menopause. Biomed Res Int 2014:757461. 10.1155/2014/757461

Matsubara Y, Murata M, Kawano K, Zama T, Aoki N, Yoshino H, Watanabe G, Ishikawa K, and Ikeda Y. 1997. Genotype distribution of estrogen receptor polymorphisms in men and postmenopausal women from healthy and coronary populations and its relation to serum lipid levels. Arterioscler Thromb Vasc Biol 17:3006-3012.

Mayo O. 2008. A century of Hardy-Weinberg equilibrium. Twin Res Hum Genet 11:249-256. 10.1375/twin.11.3.249

Meyer MR, Clegg DJ, Prossnitz ER, and Barton M. 2011. Obesity, insulin resistance and diabetes: sex differences and role of oestrogen receptors. Acta Physiol (Oxf) 203:259-269. 10.1111/j.1748-1716.2010.02237.x

Mohammadi F, Pourahmadi M, Mosalanejad M, Jamali H, Ghabadifar MA, and Erfanian S. 2013. Association of Estrogen Receptor alpha Genes PvuII and XbaI Polymorphisms with Type 2 Diabetes Mellitus in the Inpatient Population of a Hospital in Southern Iran. Diabetes Metab J 37:270-277. 10.4093/dmj.2013.37.4.270

Morselli E, Santos RS, Criollo A, Nelson MD, Palmer BF, and Clegg DJ. 2017. The effects of oestrogens and their receptors on cardiometabolic health. Nat Rev Endocrinol 13:352-364. 10.1038/nrendo.2017.12

Motawi TM, El-Rehany MA, Rizk SM, Ramzy MM, and El-Roby DM. 2015. Genetic polymorphism of estrogen receptor alpha gene in Egyptian women with type II diabetes mellitus. Meta Gene 6:36-41. 10.1016/j.mgene.2015.08.001

Onland-Moret NC, van Gils CH, Roest M, Grobbe DE, and Peeters PH. 2005. The estrogen receptor alpha gene and breast cancer risk (The Netherlands). Cancer Causes Control 16:1195-1202. 10.1007/s10552-005-0307-5

Peterson RA. 2017. Estimating normalization transformations with bestNormalize.

Ren Y, Zhang M, Liu Y, Sun X, Wang B, Zhao Y, Liu D, Liu X, Zhang D, Liu F, Cheng C, Liu L, Chen X, Zhou Q, and Hu D. 2018. Association of menopause and type 2 diabetes mellitus. Menopause. 10.1097/GME.0000000000001200

Ribas V, Nguyen MT, Henstripe DC, Nguyen AK, Beaven SW, Watt MJ, and Hevener AL. 2010. Impaired oxidative metabolism and inflammation are associated with insulin resistance in ERalpha-deficient mice. Am J Physiol Endocrinol Metab 298:E304-319. 10.1152/ajpendo.00504.2009

Sabarneh A, Ereqat S, Cauchi S, AbuShamma O, Abdulhafez M, Ibrahim M, and Nasreddin A. 2018. Common FTO rs9939609 variant and risk of type 2 diabetes in Palestine. BMC Med Genet 19:156. 10.1186/s12881-018-0668-8
Sale MM, Freedman BI, Langefeld CD, Williams AH, Hicks PJ, Colicigno CJ, Beck SR, Brown WM, Rich SS, and Bowden DW. 2004. A genome-wide scan for type 2 diabetes in African-American families reveals evidence for a locus on chromosome 6q. *Diabetes* 53:830-837.

Salpeter SR, Walsh JM, Ormiston TM, Greyber E, Buckley NS, and Salpeter EE. 2006. Meta-analysis: effect of hormone-replacement therapy on components of the metabolic syndrome in postmenopausal women. *Diabetes Obes Metab* 8:538-554. 10.1111/j.1463-1326.2005.00545.x

Shahin Y, Kapur A, and Seita A. 2015. Diabetes care in refugee camps: the experience of UNRWA. *Diabetes Res Clin Pract* 108:1-6. 10.1016/j.diabres.2015.01.035

Silva IV, Rezende LC, Lanes SP, Souza LS, Madeira KP, Cerri MF, Paes MF, Daltoe RD, Chambo-Filho A, Guimaraes MC, Graceli JB, and Rangel LB. 2010. Evaluation of PvuII and XbaI polymorphisms in the estrogen receptor alpha gene (ESR1) in relation to menstrual cycle timing and reproductive parameters in post-menopausal women. *Maturitas* 67:363-367. 10.1016/j.maturitas.2010.08.006

Slominski B, Myśliwska J, Ryba-Stanisławowska M, Skrzypkowska M, and Myśliwiec M. 2018. Estrogen receptor alpha gene polymorphism and vascular complications in girls with type 1 diabetes mellitus. *Mol Cell Biochem* 437:153-161. 10.1007/s11010-017-3103-0

Speer G, Cseh K, Winkler G, Vargha P, Braun E, Takaes I, and Lakatos P. 2001. Vitamin D and estrogen receptor gene polymorphisms in type 2 diabetes mellitus and in android type obesity. *Eur J Endocrinol* 144:385-389.

Weng H, Zhang C, Hu YY, Yuan RX, Zuo HX, Yan JZ, and Niu YM. 2015. Association between Estrogen Receptor-alpha Gene XbaI and PvuII Polymorphisms and Periodontitis Susceptibility: A Meta-Analysis. *Dis Markers* 2015:741972. 10.1155/2015/741972

World Health Organization. 2016. [http://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf?sequence=1](http://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf?sequence=1), accessed 05 January 2019.

Yang J, Han R, Chen M, Yuan Y, Hu X, Ma Y, Wu M, Zhang X, Wang M, Jiang S, and Pan F. 2018. Associations of Estrogen Receptor Alpha Gene Polymorphisms with Type 2 Diabetes Mellitus and Metabolic Syndrome: A Systematic Review and Meta-Analysis. *Horm Metab Res* 50:469-477. 10.1055/a-0620-8553

Zhao L, Fan X, Zuo L, Guo Q, Su X, Xi G, Zhang Z, Zhang J, and Zheng G. 2018. Estrogen receptor 1 gene polymorphisms are associated with metabolic syndrome in postmenopausal women in China. *BMC Endocr Disord* 18:65. 10.1186/s12902-018-0289-4
Table 1 (on next page)

Demographic and biochemical characteristics of study subjects. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBS: Fasting blood sugar

Data are presented as mean (standard error); P<0.05 was considered significant. NA: Not applicable
Table 1 Demographic and biochemical characteristics of study subjects.
BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBS: Fasting blood sugar.

|                                | Control (n=112) | T2DM (n=102) | P value |
|--------------------------------|----------------|--------------|---------|
| Age at sampling (years)        | 48.9 (0.68)    | 59.9 (0.95)  | 0.0001  |
| Age at diagnosis (years)       | NA             | 49.6 (0.83)  |         |
| BMI (Kg/m²)                    | 32.5 (0.52)    | 34 (0.6)     | 0.054   |
| SBP (mmHg)                     | 122 (0.93)     | 136.2 (1.6)  | 0.0001  |
| DBP (mmHg)                     | 77.9 (0.85)    | 78.9 (1.02)  | 0.4     |
| FBS (mg/dl)                    | 90.1 (0.97)    | 161.8 (4.9)  | 0.0001  |
| TC (mg/dl)                     | 182 (3.4)      | 192 (4.02)   | 0.03    |

Data are presented as mean (standard error); P<0.05 was considered significant. NA: Not applicable
Table 2 (on next page)

Allele frequencies and genotypes distribution of ESR1 gene PvuII and Xbal polymorphisms in control and T2DM patients.

Bold numbers showed the significant correlation.
Table 2. Allele frequencies and genotypes distribution of ESR1 gene PvuII and XbaI polymorphisms in control and T2DM patients.

| Allele/genotype | All subjects | Control n(%) | T2DM n (%) | P value |
|-----------------|--------------|--------------|------------|---------|
| **PvuII**       |              |              |            |         |
| T               | 244 (57%)    | 133 (59%)    | 111 (54%)  | 0.46    |
| C               | 184 (43%)    | 91 (41%)     | 93 (46%)   | 0.46    |
| TT              | 70 (33%)     | 43 (38%)     | 27 (26%)   | 0.06    |
| TC              | 104 (49%)    | 47 (42%)     | 57 (56%)   | 0.04    |
| CC              | 40 (19%)     | 22 (20%)     | 18 (18%)   | 0.7     |
| **XbaI**        |              |              |            |         |
| A               | 247 (58%)    | 132 (59%)    | 115 (56%)  | 0.6     |
| G               | 118 (42%)    | 92 (41%)     | 89 (44%)   | 0.7     |
| AA              | 73 (34%)     | 44 (39%)     | 29 (28%)   | 0.09    |
| AG              | 101 (47%)    | 44 (39%)     | 57 (56%)   | **0.01**|
| GG              | 40 (19%)     | 24 (21%)     | 16 (16%)   | 0.35    |

P values lower than the Bonferroni threshold (P = 0.025) were considered statistically significant.
**Table 3** (on next page)

Association of *ESR1* PvuI and XbaI variants with T2DM

*P*-values were from logistic regression models adjusted for age and BMI, P<0.05 was considered significant, bold numbers showed the significant correlation.
### Table 3. Association of *ESR1* Pvu and Xba variants with T2DM

| PvuII SNP       | Model         | Genotype | OR(95% CI)         | *P-value* |
|-----------------|---------------|----------|--------------------|-----------|
|                 | Codominant    | T/T      | 1                  |           |
|                 |               | T/C      | 2.84 (1.32-6.11)   | **0.024** |
|                 |               | C/C      | 1.77 (0.66-4.73)   |           |
|                 | Dominant      | T/T      | 1                  |           |
|                 |               | T/C-C/C  | 2.50 (1.21-5.14)   | **0.011** |
|                 | Recessive     | T/T-T/C  | 1                  |           |
|                 |               | C/C      | 0.96 (0.41-2.25)   | 0.92      |
|                 | Overdominant  | T/T-C/C  | 1                  |           |
|                 |               | T/C      | 2.32 (1.18-4.55)   | **0.013** |
|                 | Log-additive  | ---      | 1.48 (0.92-2.37)   | 0.1       |

| Xba SNP         | Model         | Genotype | OR(95% CI)         | *P-value* |
|-----------------|---------------|----------|--------------------|-----------|
|                 | Codominant    | A/A      | 1                  |           |
|                 |               | A/G      | 2.15 (1.02-4.51)   | 0.1       |
|                 |               | G/G      | 1.18 (0.45-3.10)   |           |
|                 | Dominant      | A/A      | 1                  |           |
|                 |               | A/G-G/G  | 1.82 (0.91-3.66)   | 0.089     |
|                 | Recessive     | A/A-A/G  | 1                  |           |
|                 |               | G/G      | 0.76 (0.32-1.80)   | 0.53      |
|                 | Overdominant  | A/A-G/G  | 1                  |           |
|                 |               | A/G      | 2.03 (1.05-3.95)   | **0.035** |
|                 | Log-additive  | ---      | 1.21 (0.76-1.92)   | 0.42      |

*P values were from logistic regression models adjusted for age and BMI, P<0.025 was considered significant, bold numbers showed the significant correlation.*
Table 4 (on next page)

Demographic characteristics and biochemical measurements based on ESR1 Pvull and XbaI genotypes in diabetic women. TC: Total cholesterol; TG: Triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

Values are presented as mean (standard error); P-value was obtained by ANOVA, P<0.05 was considered significant.
Table 4. Demographic characteristics and biochemical measurements based on ESR1 PvuII and XbaI genotypes in diabetic women.

TC: Total cholesterol; TG: Triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

| Parameter         | PvuII genotypes | XbaI genotypes | P value | P value |
|-------------------|-----------------|----------------|---------|---------|
|                   | CC              | TC             | TT      | AA      | AG    | GG    |         |         |
| BMI(Kg/m²)        | 34.5 (1.8)      | 33.7 (0.69)    | 34.1 (1.3) | 0.91     | 34.5 (1.24) | 33.5 (0.69) | 34.8 (1.9) | 0.67     |
| SBP(mmHg)         | 143.5 (3.50)    | 135.5 (2.2)    | 133 (2.9) | 0.09     | 134.6 (2.75) | 135.3 (2.27) | 142.4 (3.8) | 0.25     |
| DBP(mmHg)         | 79 (3.0)        | 78.8 (1.4)     | 78.7 (1.6) | 0.93     | (1.65) (79.9) | 78.5 (1.4) | 78.1 (3.1) | 0.79     |
| FBS(mg/dl)        | 169.85 (13.2)   | 162.82 (6.84)  | 154.44 (8.1) | 0.59     | 159.24 (9.39) | 159.14 (6.36) | 176.21 (13.98) | 0.46     |
| HbA1C             | 8.28 (0.47)     | 7.86 (0.26)    | 7.72 (0.38) | 0.62     | 7.83 (0.4)   | 7.78 (0.24)   | 8.46 (0.52)   | 0.46     |
| TC (mg/dl)        | 202.42 (11.68)  | 188.29 (5.3)   | 192.33 (7.39) | 0.45     | 191.52 (6.84) | 189.57 (5.59) | 200.6 (11.78) | 0.65     |
| TG (mg/dl)        | 250.07 (62.75)  | 192.87 (19.25) | 164.45 (6.84) | 0.2      | 164.9 (6.37)  | 212.01 (27.22) | 191.75 (15.81) | 0.42     |
| LDL(mg/dl)        | 118.3 (7.69)    | 105.95 (3.29)  | 105.59 (4.78) | 0.19     | 106.1 (4.47)  | 106.57 (3.53)  | 116.78 (7.48)  | 0.36     |
| HDL(mg/dl)        | 38.56 (2.8)     | 41.58 (1.19)   | 44.55 (1.62) | 0.11     | 44.38 (1.59)  | 41.14 (1.2)   | 39.69 (3.04)   | 0.2      |

Values are presented as mean (standard error); P -value was obtained by ANOVA, P<0.05 was considered significant.
Table 5 (on next page)

Estimated haplotype distributions in cases (T2DM) and control groups
Table 5. Estimated haplotype distributions in cases (T2DM) and control groups

| PvuII | XbaI | Frequency(case) | Frequency(control) | Frequency(total) | OR (95% CI)      | P-value |
|-------|------|-----------------|--------------------|------------------|------------------|---------|
| T     | A    | 0.5391          | 0.557              | 0.5485           | 1                | ---     |
| C     | G    | 0.4312          | 0.374              | 0.4013           | 1.36 (0.84 - 2.21)| 0.21    |
| C     | A    | 0.0247          | 0.0323             | 0.0286           | 2.28 (0.62 - 8.37)| 0.22    |
| T     | G    | 0.005           | 0.0367             | 0.0216           | 0.21 (0.02 - 2.66)| 0.23    |
Table 6 (on next page)

Association of *ESR1* PvuII and Xbal variants with CVD among T2DM cases

NA: not applicable.
Table 6. Association of *ESR1* PvuII and XbaI variants with CVD among T2DM cases

| PvuII SNP       | Model      | Genotype | Yes(%) | No(%) | OR (95% CI) | P-value |
|-----------------|------------|----------|--------|-------|-------------|---------|
|                 | Dominant   | T/T      | 54.5%  | 23.1% | 1           |         |
|                 |            | T/C-C/C  | 45.5%  | 76.9% | 0.25 (0.07-0.93) | 0.039  |
|                 | Recessive  | T/T-T/C  | 90.9%  | 81.3% | 1           |         |
|                 |            | C/C      | 9.1%   | 18.7% | 0.41 (0.05-3.57) | 0.36 |
|                 | Overdominant| T/T-C/C  | 63.6%  | 41.8% | 1           |         |
|                 |            | T/C      | 36.4%  | 58.2% | 0.42 (0.11-1.57) | 0.19 |

| Xba SNP        | Model      | Genotype | Yes(%) | No(%) | OR (95% CI) | P-value |
|----------------|------------|----------|--------|-------|-------------|---------|
|                 | Dominant   | A/A      | 54.5%  | 25.3% | 1           |         |
|                 |            | A/G-G/G  | 45.5%  | 74.7% | 0.26 (0.07-0.99) | 0.049  |
|                 | Recessive  | A/A-A/G  | 100%   | 82.4% | 1           |         |
|                 |            | G/G      | 0%     | 17.6% | 0.00 (0.00-NA) | 0.036  |
|                 | Overdominant| A/A-G/G  | 54.5%  | 42.9% | 1           |         |
|                 |            | A/G      | 45.5%  | 57.1% | 0.64 (0.18-2.31) | 0.49 |

| PvuII/XbaI     | Haplotype  | Yes(%) | No(%) | OR (95% CI) | P-value |
|----------------|------------|--------|-------|-------------|---------|
|                 | T/A        | 73%    | 52%   | 1           |         |
|                 | C/G        | 23%    | 46%   | 0.28 (0.09 - 0.90) | 0.035  |
|                 | C/A        | 4%     | 2%    | 2.75 (0.22 - 33.96) | 0.43 |

NA: not applicable.
