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Association between Genetic Variants Linked to Premature Ovarian Insufficiency and Inflammatory Markers: A Cross-Sectional Study

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Abstract

Background: Premature menopause (PM) is the cessation of ovarian function before age 40. PM women are more likely to have cardiovascular diseases (CVDs), diabetes, and mental disorders. This is the first study that assessed the association of single nucleotide polymorphisms (SNPs) with anti-heat shock protein 27 (Hsp27), High-sensitivity C-reactive protein (hs-CRP), and PM and serum pro-oxidant-antioxidant balance (PAB), as putative risk factors for CVDs. We aimed to explore the association of oxidative stress markers with eight different SNPs shown to be related to premature menopause.

Materials and Methods: In this cross-sectional research, we included 183 healthy women and 117 premature menopausal women. We determined baseline characteristics for all participants and measured serum hs-CRP, anti-HSP-27 antibody titer, and PAB levels using the established methods. Genotyping for eight SNPs was done using the tetra amplification refractory mutation system polymerase chain reaction (Tetra-ARMS PCR) and allele-specific oligonucleotide PCR (ASO-PCR) methods.

Results: We found a significant difference between mean serum PAB levels and the genetic variant of rs16991615 (P=0.03). ANCOVA showed a significant effect of the genotypes rs4806660 and rs10183486 on hs-CRP serum levels in the case and control groups, respectively (P=0.04 and P=0.007). ANCOVA also showed an association between rs244715 genotypes and anti-hsp27 serum levels in the case group (P=0.02). There was a significant effect of the genotypes of rs451417 on the serum hs-CRP level in the control group (P=0.03).

Conclusion: There was a significant association of the genetic variants related to PM with oxidative stress and inflammatory markers (serum PAB, anti-hsp27 antibody, and hs-CRP). Accordingly, this seems to be an effective approach to predicting susceptible subjects for cardiovascular and mental disorders as well as various cancers.

Keywords: Genetic Variant, hs-CRP, Inflammatory Marker, Premature Ovarian Insufficiency

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Introduction

Premature menopause (PM), also known as primary ovarian insufficiency, is determined by amenorrhea before the age of 40 years (1). Fifteen to thirty percent of women with PM are familial, suggesting an essential role of genetic etiologies in the occurrence of PM (2). Multiple studies showed the effect of different genetic loci on age at natural menopause (3). Postmenopausal women are more susceptible to cardiovascular diseases (CVDs) and diabetes (4). Additionally, due to estrogen deficiency,

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PM women are prone to feel depression and anxiety (5). Early detection of PM women who are at higher risk of developing CVDs, diabetes, or depression via proper markers, will help us reduce the burden of these disorders.

Recent studies indicated that inflammation and oxidative stress predisposed individuals to CVDs and diabetes (6). The serum pro-oxidant-antioxidant balance (PAB) assay is a quick, cheap, and simple method for determining the pro and antioxidant activities (7). High serum PAB levels indicated enhanced production of reactive oxygen species (ROS) that may lead to oxidative stress (8). Recent studies also exhibited that serum PAB values were associated with the anti-Hsp27 antibody titers (9). Anti-Hsp27 antibody released in response to hsp-27 secretion could be taken into account for identifying oxidative events (10). Numerous investigations showed the association between PAB levels and risk factors for CVDs (11-13). Recently, anti-hsp27 levels have been suggested as a risk factor for CVDs (7). In addition, another study demonstrated the potential role of anti-hsp27 in predicting depression due to oxidative stress (14). However, high levels of hs-CRP as an inflammatory marker has shown a predictor nature for diabetes mellitus 2 occurrence (15). In the recent research, it was also shown that elevated hs-CRP levels were related to anxiety (13, 16).

As mentioned before, the role of genetic factors in PM has been well established. Extensive research has shown that mutations, such as SNPs, in a number of these genes were involved in PM (17, 18). Recently, a study showed significant association of eight single nucleotide polymorphisms (SNPs) (rs16991615 of *MCM8* gene, rs244715 of *ZNF346* gene, rs451417 of *MCM8* gene, rs1046089 of *PRRC2A* gene, rs7246479 of *TMEM150B* gene, rs4806660 of *TMEM150B* gene, rs10183486 of *TLK1* gene, rs2303369 of *FNDC4* gene) with PM in the northeastern population of Iran (19).

Genome wide association studies (GWAS) have recognized a polymorphism, (i.e. rs16991615) of mini-chromosome preservation 8 homologous recombination repair factor of *MCM8* gene that was involved in the age of natural menopause (20). Correspondingly, rs1046089 and rs4806660 polymorphisms were located on Proline Rich Coiled-Coil 2A (*PRRC2A*) and transmembrane (*TMEM*) genes, respectively. These are associated with the age at menopause (21). GWAS also identified several other variants that are associated with primary ovarian insufficiency (POI) (22). However, few research studies have been done on the association between this variants and PM risk. We selected the most common variants, including eight SNPs while their role in PM was recently established in the northeastern population of Iran (19). We aimed to evaluate association of the SNPs variants with PAB, hs-CRP, and anti-Hsp27.

Material and Methods

Study subjects

In this cross-sectional research, we included 183 healthy women as the controls and 117 patients with PM. All of them had originally participated in the Mashhad study, a cohort study that was accomplished over ten years with the joining of 9704 individuals (35-65 years old). The cases had a menopause history before 40 years old, twelve non-stop months without menstruation, and serum follicle-stimulating hormone (FSH) levels up to 40 IU/L (repeated at four-week intervals). Exclusion criteria for cases were the history of genetically confirmed diseases surgeries affecting menstruation, and drugs affecting menstruation. On the other hand, the controls were up to 40 years old without a history of menopause, disorders related to menstruation, and medication for conditions affecting menstruation or infertility. Using procedures endorsed by the Mashhad University of Medical Science Ethics Committee (IR.MUMS.MEDICAL.REC.1398.658), informed consent was acquired from all individuals. We determined baseline characteristics such as age, body mass index (BMI), smoking habits, and physical-activity-level (PAL). Moreover, we measured serum hs-CRP, anti-HSP-27 antibody titer, and PAB in all participants.

Physical-activity-level and body mass index measurements

We assessed PAL in the all participants by using the specific questionnaire, a modified version of the SHHS/MONICA questionnaire. Based on the subject PAL score, they were categorized into: extremely inactive (<1.40), sedentary active (1.40-1.69), moderator active (1.70-1.99), vigorously active (2-2.4), and extremely active (>2.40). In addition, we determined BMI by dividing the person's weight (kg) by the square of height (m^2).

Oxidative stress markers measurement

After 12-14 hours of fasting, blood sample was collected from the all participants in Vacutainer tubes. Blood samples were centrifuged at 5000 g, 4°C, for 15 minute to separate serum. It was followed by analyzing hs-CRP, anti-HSP-27, and PAB serum levels. Serum hs-CRP was measured using the Alycon analyzer (ABBOTT, USA) with a detection limit of 0.06 mg/l. As previously described, anti-HSP-27 antibody titers were assessed using the in-home enzyme-linked immune sorbent (ELISA) method (23, 24). PAB was measured using the method described by Alamdari et al. (25).

Genotyping

Extracting DNA

Genomic DNA extraction was performed from 200 µl blood using Parstous blood DNA extraction kit (Parstous, Iran) based on the manufacturer's protocol. To check quality of the extracted DNA, electrophoresis on 1% agarose gel was applied (Parstous, Iran). Quantification of extracted DNAs was also assessed by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, USA) with 260 and 280 nm of wavelengths.

Tetra-ARMS PCR

Tetra amplification refractory mutation system polymerase chain reaction (Tetra-ARMS PCR) was done in an overall volume of 15 µl, including 7.5 µl master mix (Parstous, Iran), 2 µl genomic DNA, 1.5 µl water, and 1 µl of each primer (4 µM). Primer 1 software was used to design the primers. Tetra-ARMS PCR was conducted as follows: an initial denaturation step at 95ºC for 5 minutes, then 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 58ºC for 30 seconds and extension at 72ºC for 40 seconds, followed by one extension cycle at 72ºC for 5 minutes.

ASO-PCR

We carried out allele-specific oligonucleotide PCR (ASO-PCR) in a total volume of 15 µl, containing 7.5 µl master mix (Parstous company), 2 µl genomic DNA, 1.5 µl water, and 1 µl of each primer (4 µM). Using the Primer 3 software, the primers were designed. ASO-PCR was performed as follows: an initial denaturation step at 95ºC for 7 minutes in one cycle, afterward 35 cycles of denaturation at 95ºC for 30 seconds, annealing for 30 seconds, extension at 72ºC for 30 seconds, followed by a final extension at 72°C for 7 minutes in one cycle.

Statistical analysis

SPSS software version 24 (SPSS Inc., USA) and SAS JMP Pro (SAS Institute Inc., USA) version 13 were used to analyze the data. All measurements were performed in triplicate. Kruskal-Wallis and Bonferroni correction tests were used to compare mean differences between different groups. Additionally, we used ANCOVA test to analyze SNP genotypes by removing the potential confounding variables. These factors (including PAL and age factors) influence oxidative stress marker levels. A P value below 0.05 was considered statistically significant ($P<0.05$). Multiple testing correction was applied using the Bonferroni correction. To describe the qualitative and quantitative variables, mean ± SD and frequency were reported, respectively. Chi-square and Fisher's exact tests were applied to measure association of qualitative variables. Moreover, mean of quantitative variables between the two groups was compared by an independent t test. Data mining techniques, including logistic regression (LR) and decision tree (DT) algorithms, were used to analyze data.

Logistic regression model

LR model is a prevalent statistical model to evaluate association between various predictor variables and binary outcomes in medicine, public health, etc (26).

Let Y_i denotes the response variable and takes the values of 0 or 1 depending on whether a response occurs or not. $\boldsymbol{X}$ is the vector of covariates associated with the response variable, $\boldsymbol{\beta}$ is the corresponding vector of regression coefficients. Subsequently, association between the covariates and binary response variable can be investigated as follows:

$$logit\{E(Y_i)\}=logit\{Pr(Y_i=1|\ \boldsymbol{X}, \boldsymbol{\beta})\} = \boldsymbol{\beta}^T \boldsymbol{X}.$$

The statistical model known as logistic regression, or LR, is used to model dichotomous targets and investigate how explanatory variables affect the dichotomous target variable. The likelihood of including each record in the target groups is also shown in LR (27). The LR ability to demonstrate a strong direct or inverse relationship between the target and inputs or explanatory variables is its primary advantage. Additionally, it is adaptable (28).

Decision tree model

One of the analyses of artificial intelligence that emerged toward the end of the 20th century is data mining (29). In other words, data mining is the process of finding hidden information within large data sets. Data classification is one of the important issues that researchers face during this process. Classification issues can be addressed using a variety of approaches (30). In the medical field, DT model can be used in a variety of ways (31). It is widely used and studied in these fields, due to its simplicity, clarity, and ability to extract straightforward rules (32). Components, nodes, and branches make up DT. Therefore, there are three kinds of nodes: i. A root node is the result of splitting all records into two or more distinct subsets, ii. The internal nodes are a possible connection point in the tree structure between the leaf nodes at the bottom and the root nodes at the top, iii. Leaf nodes that display the final results of the records obtained from division of tree target groups. Likelihood of placing records in target groups is indicated by the tree branches, originated from the root node and internal nodes (33). The Gini impurity index is used by the DT algorithm to choose the best variable.

$$Gini(D) = 1 - \sum_{i=1}^{m} P_i^2$$

In this calculation, P_i is probability of the record in D belonging to the class C_i and it is estimated by $|C_i, D|/|D$.

Results

Study population characteristics

We enrolled 117 women as the case and 183 women as the control group who were matched for BMI and smoking status. Participants in the case (PM) and control groups were 55.21 ± 5.56 and 54.62 ± 2.89 years old, respectively. Additionally, physical activity level of the case group was significantly higher than that of the control group [1.77 (0.27) vs. 1.68 (0.27); P=0.003]. Serum level PAB, hs-CRP, and anti-Hsp27 were not significantly different between the case and control groups (Table 1). As shown in Table 2, in this research, eight SNPs were examined, including rs16991615, rs244715, rs451417, rs1046089, rs7246479, rs4806660, rs10183486, and rs2303369.

Table 1: Demographic features and characteristics of the study population

Characteristics	PM cases (n=117)	Control (n=183)	P value
Age (Y)	55.21 ± 5.56*	54.62 ± 2.89*	0.4
Anti-hsp27	0.22 (0.34)**	0.23 (0.32)**	0.846
hs-CRP	1.78 (2.81)**	2.39 (4.52)**	0.443
PAB	81.90 (86.66)**	108.64 (94.24)**	0.111
PAL	1.77 (0.27)**	1.68 (0.27)**	0.003#
Smoking			0.392
Non-smoker	86 (37.1)***	146 (62.9)***	
Ex-smoker	10 (52.6)***	9 (47.4)***	
Current-smoker	19 (40.4)***	28 (59.6)***	
TC (mg/dl)	207.8 ± 35.8*	188.8 ± 33.1*	<0.001#
TG (mg/dl)	111.0 (82.0-162.5)	117.0 (80.0-159.0)	0.684
LDL-C (mg/dl)	128.39 ± 33.96*	110.47 ± 33.42*	<0.001#
HDL-C (mg/dl)	47.45 ± 9.33*	45.06 ± 11.34*	0.040#
FBG (mg/dl)	88.74 ± 20.06*	88.96 ± 31.43*	0.948
BMI (kg/m^2)	28.78 ± 5.06*	29.34 ± 4.22*	0.323

PM; Premature menopause, Anti-hSP; Anti-heat shock protein, hs-CRP; High sensitivity C reactive protein, PAB; Pro-oxidant-antioxidant balance, PAL; Physical activity level, TC; Total cholesterol, TG; Triglycerides, LDL-C; Low-density lipoproteins-cholesterol, HDL-C; High-density lipoproteins-cholesterol, FBG; Fasting blood glucose, BMI; Body mass index, SD; Standard deviation, IQR; Interquartile range, *; Mean ± SD, **; Median (IQR), *; n (%), and #; Significant at a level of 0.05.**

Association of single nucleotide polymorphisms and serum pro-oxidant-antioxidant balance levels

Serum PAB levels in the control group were significantly different (P=0.031) between the three genotypes of rs16991615: AA, GA, and GG. After adjusting confounders, including age and level of physical activity, there was no significant association between serum PAB levels in either group and the other selected SNP genotypes (Table 2).

Association of single nucleotide polymorphisms and serum hs-CRP levels

After adjusting for physical activity level and age in the both group, one-way ANCOVA was conducted to determine statistically significant difference between genotypes of the selected SNPs and hs-CRP levels s. Based on the obtained results, it was shown that the genotypes of rs4806660 significantly affected serum hs-CRP levels in the case group after adjusting for confounding variables (P=0.042). Additionally, the genotypes of rs10183486 significantly affected the hs-CRP level in the control group (P=0.007). On the other hand, in the control group, Kruskal-Wallis Test exhibited significant difference in serum hs-CRP levels among the each genotype of rs10183486 (TT, CT, and CC, P=0.014). The other results were not significant (Table 2).

Association of single nucleotide polymorphisms and anti-hsp27

The results showed significant effect of rs244715 genotypes on anti-hsp27 antibody titer in the case group after adjusting for confounding variables (P=0.023). Moreover, significant effect of the genotypes of rs451417 on the hs-CRP level was observed in the control group (P=0.031). The other results did not show any association between the genotypes of SNPs and anti-hsp27 antibody titer (Table 2).

Table 2: Association of genotypes related to PM with lipid profile in the studied patients (n=300)

SNPs	Markers	PM (n=117)					Control (n=183)				
rs16991615		AA	GA	GG	P1	P2	AA	GA	GG	P1	P2
	Anti- hsp27	0.28 (0.34)	0.21 (0.28)	0.23 (0.39)	0.51	0.48	0.28 (0.47)	0.19 (0.25)	0.25 (0.33)	0.15	0.27
	hs-CRP	2.44 (4.60)	1.30 (1.37)	1.88 (2.90)	0.23	0.34	1.62 (3.30)	2.87 (4.43)	1.89 (4.34)	0.28	0.88
	PAB	77.16 (65.34)	93.34 (66.98)	72.92 (103.01)	0.53	0.62	34.77 (-)[a]	117.01 (79.70)[b]	94.47 (124.92)[b]	**0.03***	0.08
rs244715		GG	AG	AA	P1	P2	GG	AG	AA	P1	P2
	Anti-hsp27	0.18 (0.35)[a]	0.28 (0.44)[b]	0.16 (0.26)	0.25	0.02	0.44 (-)	0.24 (0.34)	0.22 (0.27)	0.67	0.75
	hs-CRP	1.50 (4.08)	2.22 (3.43)	1.60 (2.62)	0.52	0.41	2.21 (5.54)	2.52 (4.91)	2.38 (4.35)	0.82	0.75
	PAB	81.91 (57.02)	71.53 (95.59)	89.74 (88.78)	0.56	0.56	-	112.52 (138.48)	108.64 (78.41)	0.40	0.39
rs451417		AA	CA	CC	P1	P2	AA	CA	CC	P1	P2
	Anti-hsp27	0.22 (0.38)	0.28 (0.24)	0.17 (0.39)	0.92	0.57	0.23 (0.16)	0.18 (0.25)[a]	0.24 (0.38)[b]	0.10	**0.03***
	hs-CRP	1.76 (2.90)	2.14 (3.86)	1.57 (2.30)	0.72	0.54	2.51 (5.45)	2.34 (4.32)	2.57 (4.63)	0.69	0.63
	PAB	77.16 (52.12)	15.02 (115.79)	91.54 (56.14)	0.38	0.46	137.26 (-)	88.29 (101.15)	108.64 (98.35)	0.83	0.99
rs1046089		AA	GA	GG	P1	P2	AA	GA	GG	P1	P2

Table 2: Continued.

SNPs	Markers	PM (n=117)					Control (n=183)				
rs7246479		AA	TA	TT	P1	P2	AA	TA	TT	P1	P2
	Anti-hsp27	0.16 (0.25)	0.19 (0.27)	0.30 (0.40)	0.19	0.16	0.31 (0.36)	0.23 (0.25)	0.23 (0.33)	0.47	0.58
	hs-CRP	1.49 (1.48)	1.68 (2.76)	2.08 (3.28)	0.34	0.54	6.58 (12.10)[a]	2.31 (3.75)[b]	2.32 (4.79)[b]	**0.03***	0.11
	PAB	88.19 (-)	64.86 (87.74)	90.13 (68.0)	0.56	0.73	-	94.47 (77.76)	121.27 (140.21)	0.18	0.15
rs4806660		CC	TC	TT	P1	P2	CC	TC	TT	P1	P2
	Anti-hsp27	0.25 (0.54)	0.22 (0.31)	0.21 (0.31)	0.37	0.54	0.38 (0.41)	0.24 (0.35)	0.22 (0.26)	0.24	0.11
	hs-CRP	2.20 (7.46)[a]	2.14 (3.16)	1.36 (2.30)[b]	0.23	0.04	2.52 (23.73)	2.33 (4.38)	2.42 (4.64)	0.49	0.27
	PAB	92.86 (-)	80.30 (86.66)	76.81 (84.60)	0.37	0.42	63.58 (-)	105.45 (121.63)	108.64 (103.96)	0.51	0.46
rs10183486		TT	CT	CC	P1	P2	TT	CT	CC	P1	P2
	Anti-hsp27	0.21 (0.17)	0.21 (0.34)	0.22 (0.42)	0.87	0.66	0.35 (-)	0.17 (0.32)	0.25 (0.32)	0.19	0.56
	hs-CRP	1.23 (6.05)	1.56 (2.67)	2.08 (2.85)	0.50	0.33	1.23 (19.81)	2.76 (4.97)[a]	1.82 (3.06)[b]	**0.01***	**0.007***
	PAB	147.12 (-)	85.82 (64.65)	76.81 (81.96)	0.15	0.12		64.07 (110.91)	114.48 (89.91)	0.72	0.49
rs2303369		TT	CT	CC	P1	P2	TT	CT	CC	P1	P2
	Anti-hsp27	0.21 (0.49)	0.22 (0.36)	0.22 (0.29)	0.65	0.33	0.31 (0.26)	0.23(0.34)	0.23 (0.32)	0.37	0.24
	hs-CRP	2.71 (6.00)	1.76 (2.84)	1.40 (2.47)	0.05	0.12	4.07 (7.15)	2.33 (4.54)	2.27 (3.95)	0.18	0.44
	PAB	91.30 (63.19)	74.66 (79.07)	81.90 (90.88)	0.88	0.91		73.55 (105.36)	112.82 (116.35)	0.78	0.95

Data are presented as level of Anti-hsp27, hs-CRP and PAB in serum of studied. P1; P value for the Kruskal-Wallis test, P2; P value for the analysis of covariance (ANCOVA) with age and physical activity level (PAL), as model covariates, *; Significant P<0.05. Anomalous letters indicate a significant difference. PM; Premature menopause, SNP; Single nucleotide polymorphism, Anti-HSP; Anti-heat shock protein, hs-CRP; High sensitivity C reactive protein, and PAB, Pro-oxidant-antioxidant balance.

Relationship of biochemical and SNPs predictors with PM, using LR and DT models

All variables were found to be significantly associated with PM (P<0.050) in the multiple LR model (Table 3). PAB, PAL, TG, rs10183486, rs451417, BMI, and rs244715 were found to be significantly associated with PM (P<0.05), after adjusting for the effect of other variables in the model. Additionally, rs10183486, PAB, PAL, and TG were the most significant variables (they have more LogWorth in Table 3).

Table 3: Parameters estimating of the LR model

Source	LogWorth	Importance	P value
PAB	145.178		<0.001
PAL	85.894		<0.001
TG	67.938		<0.001
rs10183486	39.144		<0.001
rs451417	15.160		<0.001
BMI	11.486		<0.001
rs244715	3.436		<0.001

LR; Logistic regression, PAB; Pro-oxidant-antioxidant balance, PAL; Physical activity level, TG; Triglycerides, and BMI; Body mass index

Figure 1 illustrated the outcomes of DT training for biochemical factors. DT algorithm determined various PM risk factors and categorized them into the four layers. According to the DT model, the first variable (root) is of the most significant classifying data, whereas the subsequent variables have different levels of significance (12). Figure 1 illustrated that age, followed by hs-CRP and anti-hsp27, had the most significant impact on the PM development risk.

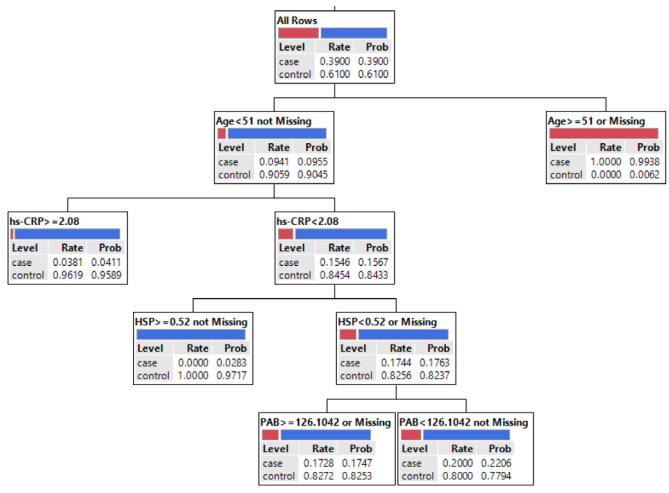

Fig.1: Decision tree for inflammatory risk factor of premature ovarian insufficiency diagnosis. hs-CRP; High sensitivity C reactive protein, HSP; Heat shock protein, and PAB; Pro-oxidant-antioxidant balance.

More participants with age<51 and hs-CRP≥2.08 were in the control group, according to the DT model, than those with age<51 and hs-CRP<2.08. Ninety-nine percent of the case group patients were ≥51 years old. Table 4 indicated specific PM developed by the DT model. Thus, age, hs-CRP, anti-hsp27, and PAB were determined as the most crucial variables in the DT model and diagnosis of PM. The receiver operating characteristics (ROC) curve for the DT model was performed to evaluate presentation of the model and comparisons (Fig.2).

Table 4: Detailed rules based on the DT model

Rules	Case (%)	Control (%)
R1: Age<51 and hs-CRP>2.08	4.11	95.89
R2: Age<51 and hs-CRP<2.08 and Anti-hsp27>0.52	2.83	97.17
R3: Age<51 and hs-CRP<2.08 and Anti-hsp27<0.52 and PAB>126.1042	17.47	82.53
R4: Age<51 and hs-CRP<2.08 and Anti-hsp27<0.52 and PAB<126.1042	22.06	77.94
R5: Age>51	99.38	0.62

DT; Decision tree, hs-CRP; High sensitivity C reactive protein, Anti-hSP; Anti-heat shock protein, and PAB; Pro-oxidant-antioxidant balance.

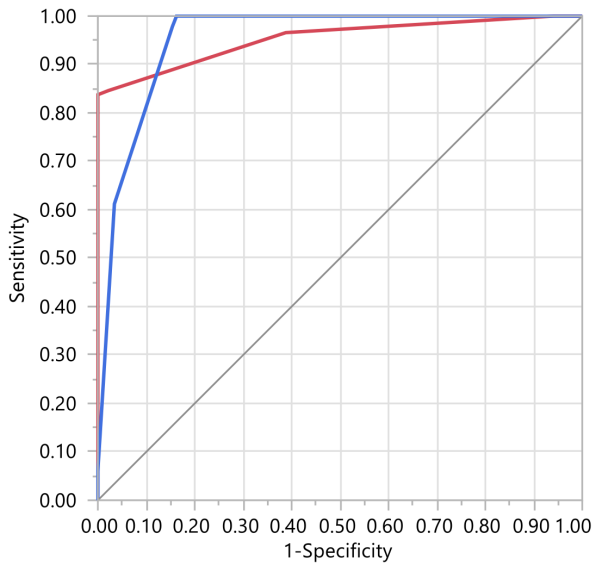

Fig.2: ROC curve of the DT model. ROC; Receiver operating characteristic and DT; Decision tree.

Discussion

Some recent studies investigated genetic determinants of PM (19). Women with PM were shown to develop disorders that are more prevalent or severe (33). CVDs, as an example, were found to be associated with oxidative stress markers (9). Interestingly, in the present study, for the first time, we aimed to investigate association of oxidative stress markers (including serum PAB, anti-hsp27 antibody, and hs-CRP) with eight different SNPs that were shown to be related to PM.

We found that rs244715 of the *ZNF346* gene and rs451417 of the *MCM8* gene were associated with anti-Hsp27 antibody titers. Thus, in the cases carrying rs244715 AG and rs451417 CC genotypes, anti-Hsp27 antibody levels were significantly greater than the controls. Both significant results were obtained after correcting for confounders, including PAL and age. As these SNPs were associated with PM (19), they may lead to CVDs, due to menopause and estrogen deficiency (34). An excess amount of Hsp27 to perform its antioxidant role may trigger the immune system to increase the corresponding antibody level (anti-hsp27 antibody), but this immune molecule may have pathological aspects (9, 27). PM was found to be significantly associated with an increased incidence of CVDs (33). Atherosclerosis progression was also shown to be related to the balance of serum Hsp27 and its antibody titers. Despite the protective role of Hsp27, anti-Hsp27 has a pathological role in atherosclerosis (35). Another study also showed that anti-Hsp27 antibody titers independently predicted depression, due to oxidative stress. Anti-Hsp27 antibody titers may exacerbate atherosclerosis. So Hsp27 and its antibody immune complex may be contributory, because of its pro-inflammatory property (9, 36). Recognizing PM women with AG genotypes of rs244715 and healthy women with CC genotype of rs451417 may screen women who are at risk of CVDs and mental disorders. Predicting and preventing these disorders may reduce the related socio-economic burdens.

We found that the rs4806660 and rs2303369 SNPs were associated with serum hs-CRP levels in PM women. Serum hs-CRP levels were significantly higher in the carriers of CC genotype of rs4806660 and TT genotype of rs2303369. In addition, we came to the conclusion that hs-CRP levels were significantly higher in the carrier individuals of AA genotype of rs7246479 and CT genotype of rs10183486, in our controls. Association of rs10183486 with hs-CRP levels was so strong, because it was significant either with or without removing confounders. Scientifics found a strong relationship between hs-CRP and serum PAB, due to the dysregulation of hs-CRP effects on the balance of pro-oxidants and antioxidants in the body. Menopause contributed to insulin resistance and the other cardiovascular risk factors (4, 37). hs-CRP has reportedly been associated with development of type 2 diabetes, because of endothelial adhesion molecule overproduction and insulin resistance of hs-CRP (38). In another study, it was shown that hs-CRP might predict diabetes mellitus type 2 (15). As a cardiovascular complication, in-stent restenosis (ISR) was demonstrated to be associated with higher hs-CRP levels (39). Attention to the CC genotype of rs4806660 and TT genotype of rs2303369 may help us identify PM women prone to cardiovascular and metabolic syndromes. Furthermore, in healthy women, the AA genotype of rs7246479 and CT genotype of rs10183486 may be helpful markers in predisposing to metabolic syndrome and mental disorders.

PAB levels were significantly higher in the women carrying GA genotype of rs16991615. Interestingly, there was a considerable difference between serum PAB levels in GA and AA genotypes of rs16991615. These significant associations were obtained without controlling confounders, including PAL and age. So, further investigations are needed to clarify it. Serum PAB was associated with the pathogenesis of CVDs. Risk factors of CVDs may elevate serum PAB, as a prognostic role in CVDs (9). Our findings indicated that many diseases, such as metabolic syndrome and various cancers, can be predicted. In other words, screening healthy women with GA and AA genotypes of rs16991615 may help us recognize people who are at high risk. It was found that oxidative stress might substantially increase the severity of Covid-19, as a global disaster (40). So, carriers of the

AA and GA genotypes of rs16991615 who have active menstrual cycles may develop more severe Covid-19.

Our study included some limitations. In this research, there was a small sample size. By using biochemical assays, we were unable to confirm premature menopause. Finally, according to our novel findings, further investigations are needed to validate our results. So, designing more studies is necessary for various regions with larger sample sizes.

Conclusion

Premature menopause SNPs may potentially influence oxidative stress/inflammatory markers. More precisely, there were significant effects of rs16991615 on serum PAB; rs244715 and rs451417 on the anti-hsp27 antibody titers, and four SNPs (rs7246479, rs4806660, rs10183486, and rs2303369) may affect hs-CRP levels. Accordingly, this seems to be helpful in predicting the susceptible subjects for several diseases, such as CVDs and mental disorders, as well as various cancers.

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Authors' Contributions

M.R.M., M.A., H.Gh.; Conception, Designed the experiments, and Drafting the manuscript. M.R.F.M., S.M., M.A.M., E.H.; Drafting the manuscript and Data analyzing. M.M.B., Sh.Y., A.R.E.; Designed the experiments and Revised the manuscript. M.M.Gh., A.E.D., E.A.; Performed the experiments and Drafting the manuscript. H.E., T.H.; Data analyzing and revising the manuscript. G.A.F.; Revising the manuscript and Conception. A.P., M.Gh.-M.; Conception and Corresponding author. All authors reviewed, considered, and approved the final manuscript.

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