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Original article

The impact of *SELP* gene Thr715Pro polymorphism on sP-selectin level and association with cardiovascular disease in Saudi diabetic patients: A cross-sectional case-control study

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ABSTRACT

Background: Cardiovascular diseases (CVD) are leading cause of mortality in patients with type 2 diabetes mellitus (T2DM). Increased soluble sP-selectin and 715Thr > Pro polymorphism were studied in CVD and T2DM, but association between them hasn't been explored in Saudi Arabia. We aimed to assess sP-selectin levels in T2DM and T2DM-associated CVD patients in comparison to healthy control cohort. Also, we sought to investigate relationship between Thr715Pro polymorphism and sP-selectin levels and disease state.

Methods: This is a cross-sectional case-control study. sP-selectin level (measured by Enzyme-linked immunosorbent assay) and prevalence of Thr715Pro polymorphism (assessed by Sanger sequencing) were investigated in 136 Saudi participants. The study comprised 3 groups: group1 included 41 T2DM patients; group 2 (48 T2DM patients with CVD), and group 3 (47 healthy controls).

Results: sP-selectin levels were significantly higher in diabetics and diabetics + CVD groups as compared to the corresponding control. In addition, results showed that the prevalence of 715Thr > Pro polymorphism is 11.75 % in the study population amongst the three study groups (9.55 % *Thr/Pro*, and 2.2 % *Pro/Pro*). No statistical difference was found between sP-selectin levels in subject carrying the wildtype genotype of this polymorphism and these who carry the mutant gene. There could be an association between this polymorphism and T2DM, whilst the polymorphism may protect diabetic patients from having CVD. However, odds ratio is not statistically significant in both cases.

Conclusion: Our study supports the previous researches' results that Thr715Pro is neither influencing the sP-selectin level nor the risk of CVD in T2DM patients.

Abbreviations: ACE-I, Angiotensin-converting enzyme inhibitors; ARB, Angiotensin II receptor blockers; BMI, Body-mass index; CAM, Cell adhesion molecule; CCB, Calcium channel blockers; CVD, Cardiovascular disease; DM, Diabetes mellitus; ELISA, Enzyme-linked immunosorbent assay; Gp1bα, Platelet glycoprotein 1b-alpha; IDF, International Diabetes Federation; IR, Insulin resistance; PMN, Polymorphonuclear leukocytes; pP-selectin, Platelet P-selectin; PSGL-1, P-selectin glycoprotein ligand-1; SELP, P-selectin gene; sP-selectin, Soluble P-selectin; T2DM, Type 2 diabetes mellitus; vWF, Von-Willebrand factor; WPb, Weibel-Palade Bodies.

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1. Introduction:

Diabetes mellitus (DM) is a chronic syndrome composed of a group of metabolic noncommunicable diseases (NCD) sharing similar characteristics, remarkably hyperglycemia, with different pathology and etiologies. In 2015, International Diabetes Federation (IDF) reported that there were 415 million diabetics worldwide with high prevalence in the Middle East(Ogurtsova et al. 2017). In Kingdom of Saudi Arabia, diabetes level has been elevated to over 25 % with predominance of type 2 diabetes mellitus (T2DM) by 32.8 % (Meo 2016). T2DM mainly is a genetic disease and a combination of insulin resistance (IR) due to inappropriate response to the insulin by the insulin-sensitive tissues, and insulin insufficiency- a result of defective secretion of insulin by β-cells in pancreas. Insulin resistance is defined as impaired response to insulin by target cells (adipose tissues, liver and muscles) and subsequently the failure of intake and utilization of the circulating glucose (Semple et al. 2011; Galicia-Garcia et al. 2020). Due to chronic hyperglycemia, people with T2DM are at high risk of macrovascular complications such as cardiovascular diseases (CVD) (DeFronzo et al. 2015; Chawla et al. 2016). It is well known that risk factors influencing CVD are augmented in T2DM, these include hypertension, hyperinsulinemia, dyslipidemia, endothelial dysfunction and changes in hemostatic factors such as platelet hyper-reactivity (Zalewski et al. 2006). Additionally, people with diabetes are 2–4 times more likely to have coronary heart disease than people without diabetes (Li et al. 2017). Interestingly, insulin resistance and insulin insufficiency are independent factors for endothelial cell dysfunction and ultimately vascular stiffness, which serves as a predictive marker for CVD (Hill et al. 2021). Hemostatic and prothrombotic state alterations involved in overt diabetes include elevated levels of thrombin, and circulating tissue factor (Vazzana et al. 2012). Altered platelet glycosylation renders the membrane less fluidic and increases adhesion molecules like P-selectin and GpIIb/IIa (Vazzana et al. 2012; Li et al. 2017). P-selectin is a highly polymorphic cell adhesion molecule (CAM), single chain glycoprotein, and is the largest protein of selectin family; (E-, L-, and P-selectin). McEver and Martin (McEver and Martin 1984) were the first who discovered the presence of P-selectin in platelets after they had developed its monoclonal IgG antibody and they confirmed that it's a platelet membrane glycoprotein. Alternative splicing of mRNA causes deletion of a 40-residue transmembrane segment domain and generation of soluble P-selectin (sP-selectin) (Fox 1994). As P-selectin is expressed in activated platelets and vascular endothelium, it was believed that it influences CVD, peripheral vascular disease and atherosclerotic plaque progression (Woollard et al. 2014). There is strong evidence from previous animal models and clinical studies to support the idea that elevated P-selectin levels in T2DM patients are related to the development of atherothrombotic illness (Aref et al. 2005). Many gene variants have been reported in *SELP* gene, but most studied ones were three polymorphisms: Tyr715Pro (rs6136), Asn562Asp (rs6127), and Ser290Asn (rs6131). The most intensively studied variant of P-selectin is Tyr715Pro (Panzer et al. 2008). Thr715Pro polymorphism is an A:C substitution at exon 13 of amino acid 715 at the ninth tandem consensus repeat results in amino acid proline substituting threonine, and clinically it's reported as benign (Herrmann et al. 1998). This missense mutation is located 15 amino acids before the transmembrane domain. Subramanian and coworkers have studied the influence of Thr715Pro on the P-selectin protein synthesis, post-translational processing, and subsequently the function of P-selectin through a transfection study. It was found that 715Pro P-selectin is less terminally glycosylated. Moreover, it failed the post-translation modification by Golgi enzymes unlike the wild-type P-selectin (715Thr) leading to less mature P-selectin. The influence of this mutation on structure leads to a less P-selectin exposure on the cell surface. Moreover, this observation was confirmed by the less platelet-monocyte aggregates in 715Pro individuals compared to the wildtype ones (Subramanian et al. 2012). Moreover, Thr715Pro has been reported for association with numerous diseases; such as CVD and atherosclerosis (Kaur et al. 2019), rheumatoid arthritis (Burkhardt et al. 2014), Systemic lupus erythematous (SLE) (Morris et al. 2009), and cancer (Tan et al. 2012). The purpose of this study was to determine the possible use of sP-selectin as a surrogate marker for cardiovascular diseases in T2DM, and to assess the association between Thr715Pro polymorphism and development of CVD in T2DM patients.

2. Methods:

This is a cross-sectional case-control study that has been conducted between September 2019 and November 2020. Ethical approval was granted by the Institutional Review Board at Imam Abdulrahman Bin Faisal University (IRB-PGS-2018–03-139). Participants were recruited from King Fahd Hospital of University, Al-Khobar, Eastern Province, Saudi Arabia. The sample size was estimated using (n = z^2 × p(1-p) / e^2) formula and a total of 136 Saudi Arabian subjects both genders with age ranged from 20 to 93 years were recruited and divided into three groups; CVD + T2DM, T2DM and healthy controls (age and gender matched).

Case group: includes only T2DM patients with coronary vascular disease (CVD).
Case-index group: includes T2DM who have not been diagnosed with hypertension or CVD.
Control group: Healthy volunteers who have not been diagnosed with DMT2, CVD, or hypertension. Current smokers or smokers<3 years were excluded as well.

A written informed consent was voluntarily obtained from all participants after the study being explained in details. All the enrolled participants were Saudi with Saudi ancestors living in the Eastern province of Saudi Arabia. Major chronic diseases; such as cancer, severe liver failure and coagulation disorders were all considered as general exclusion criteria. Patients who had clinical evidence of infection, and pregnant women were also excluded.

2.1. Preparation of participants:

Participants have been instructed to fast for at least 12 h before blood collection; drinking water was allowed. Demographic data; such as age, gender, history of smoking and the family history of diabetes have been collected using data collection sheet specifically designed for this study.

Venous blood was collected from all participants into two Ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (4 mL), and Potassium Oxalate and red top plain tubes. One tube of EDTA samples was used to perform complete blood count (CBC) and to measure the glycated hemoglobin (Hb-A1c), whilst the other EDTA tube was stored in −80 °C freezer for genotyping studies. Potassium Oxalate tubes were settled to measure the fasting blood glucose (FBG), whereas the serum separated from the red-top tubes was divided into two parts; an aliquot that was stored at −80 °C for P-selectin measurement, and the rest was sent for lipid profiling (total cholesterol, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL)).

2.2. Measurement of P-selectin:

Frozen serum aliquots were thawed on ice for enzyme-linked immunoassay (ELISA) (Human P-Selectin/CD62P ELISA Kit, MOLEQULE-ON, Auckland city, New Zealand). The procedure was followed as described in the instruction manual of the above-mentioned kit. The absorbance of the end-point reaction was measured by ELx808TM microplate reader (BioTek, USA) at 450 nm. A standard curve was created and the concentration of unknown samples was calculated from the standard curve's equation.

2.3. Genotypic analysis

DNA was extracted by QlAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the instructions provided in the datasheet. The eluted DNA was measured for concentration and purity by DS-11 Series Spectrophotometer (Denovix, Wilmington, USA). The sequence amplified of Thr715Pro gene variant was 195 bp. The sequence of the forward primer used was 5′AGCTGTGAAATGCTCA GAAC3′ and the reverse primer was 5′ATTGTACCTTGGCAGGTTG G'3. The PCR reaction was performed in a total volume of 25 µl with 100 ng of DNA template and 1 µl of each primer (10 pmol/µ). The cycling program was as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 1 min, and extension at 72 °C for 1 min, final extension at 72 °C for 10 min, and then a final hold at 4 °C.

2.4. DNA gel purification & sequencing:

PCR product was purified from agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) kit. Purified samples were sent for fragment analysis PCR, SNP genotyping using DNA Sanger sequencing (applied Biosystem ABI 3730X, Hong Kong) (Sanger, Nicklen et al. 1977). This method consists of three main steps; chain termination PCR, size separation by capillary gel electrophoresis, and ultimately analysis and determination of DNA sequence. Results had been read and interpreted using sequence reading tools such as CodonCode Aligner V. 9.0.1 and FinchTV version 1.4.0.

2.5. Statistical analysis

All variables were tested for normality with Shapiro Wilk's test and p-value<0.05 suggested that data was significantly deviating from normal distribution. Normally distributed data were presented as mean ± standard deviation, while non-normally distributed data were presented as medians with interquartile range. Chi-square test was used for comparison between categorical variables. For comparison between continuous variables, student-test (Mann-Whitney *U* test (non-parametric)) and one-way ANOVA (Kruskal Wallis test (non-parametric)) were used. Odd ratio was calculated to measure the association between sP-selectin 715Thr > Pro and the risk of developing CVD. SPSS V.22 and Graph Pad Prism V.5 were used to conduct statistical comparisons and correlation studies. A confidence Interval (CI) of 95 % was calculated and p-value $\leq$ 0.05 was considered statistically significant.

3. Results:

3.1. Baseline characteristics of the study population

The baseline characteristics including the demographic and laboratory parameters among the study groups are summarized in Table 1. BMI and age variables varied significantly between the healthy and the other study groups (p = 0.001). The difference between groups in age could be explained by the young age of subjects in the control group as we could only enroll the younger volunteers. It was also found that P-selectin level was the highest in the disease index group (223.4 ± 88.9) as compared to the cardiovascular disease cases (202 ± 78.1) and the healthy group (174.4 ± 88.6). Moreover, P-selectin level differed significantly among the three groups (p = 0.028). Additionally, The ROC curve analysis indicated that the area under the curve of P-selectin to discriminate between control and disease index group (T2DM patients) was 74.67 (p= < 0.0001) and for the comparison between the control group and cases group (DM + CVD) was 66.65 (p = 0.0062) as indicated in curve depicted in Fig. 1, which means a moderate sensitivity of the test.

3.2. P-selectin levels in different subject categories in the study groups and correlation with laboratory parameters

Comparisons of the P-selectin levels between smokers and non-smokers and hypertensive vs non-hypertensive CVD patients and those on certain medications vs those who are not receiving medicines are presented in the supplementary file **Table S1** and **Table S2**, respectively. Statistical analysis revealed that neither smoking status nor hypertension affect the P-selectin level significantly in this group (p = 0.2 and 0.477, respectively). The results also showed that there was a significant difference in P-selectin levels between patients who are taking beta-blockers and these who do not take the drugs (p = 0.024); whilst the difference was not significant for all other medicine categories.

The results in Table 2 showed that P-selectin levels were positively correlated to the HBA1c and triglycerides levels in diabetic patients; although this correlation was weak but was statistically significant (r = 0.324 and p = 0.039) and (r = 0.393 and p = 0.011), respectively. Also, the mean platelet volume was significantly correlated with the P-selectin levels in diabetic patients (r = 0.499 and p = 0.006). Additionally, there was a positive moderate correlation between diastolic blood pressure and P-selectin levels in diabetic patients only (r = 0.416 and p = 0.002). Furthermore, the difference in P-selectin levels between males and females, and smokers and non-smokers was not statistically significant. On the other hand, the P-selectin level was significantly different between premenopausal and postmenopausal women in the T2DM group (p = 0.016) as shown in **Table S3** in the supplementary section.

3.3. Detection of Thr715Pro missense polymorphism by Sanger sequencing and its impact on P-selectin level in study groups:

Forward and reverse Sanger sequencing of the purified PCR products were read by CodonCode Aligner V. 9.0.1 and FinchTV version 1.4.0 as shown in Fig. 2.

The diagram below (Fig. 3**)** demonstrates the distribution and prevalence of Thr715Pro polymorphism among the three study groups. It revealed that 88.23 % of the study population have carried the wildtype gene (*Thr/Thr*), while 9.55 % carried the heterozygous gene (*Thr/Pro*). On the other hand, only 2.2 % have been shown to carry the homozygous mutant gene (*Pro/Pro*). There was no difference in the P-selectin level between subjects carrying the wild-type alleles (AA) and these carrying the hetero/homozygous mutant (AC/CC) genotypes in all studied groups as showed in Table 3.

3.4. Association between Thr715Pro polymorphism and CVD in T2DM patients

The results showed that the odds for having type 2 diabetes when carrying the polymorphism is higher than that in the control

Table 1
Demographic and basic laboratory characteristics among the study groups.

Groups	Healthy (n = 47)	Disease index (Type 2 DM) (n = 41)	Cases (CVD + T2DM) (n = 48)	*p*-value
Demographic parameters				
Male: Female %(n)	49:51 (23:24)	39:61 (16:25)	73:27 (35:13)	0.004*
Age (years) ‡	35 (20–46)	51 (32–74)	60 (59–93)	0.001*
BMI (kg/m^2)	27.25 ± 5.50	31.78 ± 6.33	32.58 ± 7.07	0.001*
Duration of DM (<10 years) (n)	0	3.8 ± 2.48 (15)	2.27 ± 1.75 (15)	0.67
Duration of DM (≥10 years) (n)	0	19.88 ± 7.32 (26)	19.39 ± 5.84 (33)	0.286
Smokers				
Never smokers (%)	98	78	58	0.001*
Current smokers (%)	0	10	21	
Former smokers (%)	2	12	21	
Hypertension (%)	0	0	83	–
History of diabetes (%)	64	88	77	0.032*
Laboratory parameters				
Fasting glucose (mg/dl)	91 ± 9.2	157.6 ± 58.2	168.8 ± 76.6	0.001*
HbA1c (%)	5.3 ± 0.5	8.2 ± 1.6	8.2 ± 1.6	0.001*
Total Cholesterol (mg/dl)	178.6 ± 30.2	171.5 ± 37.5	176.7 ± 46.3	0.678
LDL- Cholesterol (mg/dl)	111.7 ± 25.9	102.1 ± 31.2	109.2 ± 42.8	0.404
HDL- Cholesterol (mg/dl)	55.4 ± 13.2	49.6 ± 12.5	40.2 ± 12	0.001*
Triglyceride (mg/dl)	78.6 ± 39.6	134.1 ± 115.2	151.5 ± 92.7	0.001*
Platelets ($\times 10^3/\mu l$)	272.6 ± 91.2	268.2 ± 73.7	238.3 ± 58.9	0.061
MPV (fl)	9.1 ± 1.1	8.8 ± 0.8	8.9 ± 0.9	0.499
P-selectin level (ng/ml)	174.4 ± 88.6	223.4 ± 88.9	202 ± 78.1	0.028*
Systolic blood pressure (mmHg)	119.1 ± 14.1	127.4 ± 10.2	136.5 ± 21.3	0.001*
Diastolic blood pressure (mmHg)	78 ± 9.1	77.7 ± 7.1	78.3 ± 14.2	0.965

BMI: body-mass index, MPV: mean platelet volume. * Statistically significant. † Data are reported as (mean ± SD) or percentage. ‡ Age was represented by median (range). * = p value ≤ 0.05.

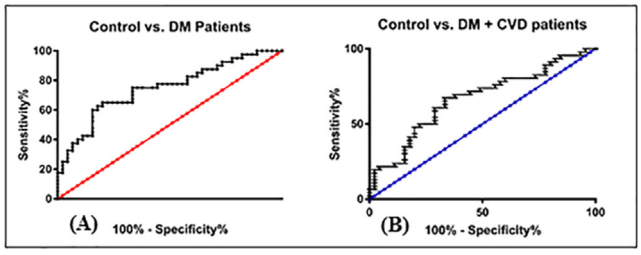

Fig. 1. ROC curve analysis indicating the sensitivity of P-selectin test for discrimination between **(a)** control and disease index group, **(b)** control and case group. DM: Diabetes Mellitus, CVD: Cardiovascular disease.

group (odd ratio = 1.73 and CI = 0.524–5.346), indicating that there is an association between the polymorphism and the risk of type 2 diabetes; however, this association is not statistically significant (p = 0.53). On the other hand, the odds of having type 2 diabetes and CVD when carrying the polymorphism was lower than the odds for healthy subjects indicating that carrying the polymorphism is not associated with having CVD in T2DM patients and could be protective (odd ratio = 0.715 and CI = 0.2227 to 2.819). This also indicates that this conclusion is not statistically significant (p = 0.74) as revealed in Fig. 4.

4. Discussion:

Cardiovascular diseases and strokes are serious outcomes for the vascular complications of diabetes. Hence, early diagnosis of CVD in diabetic patients is pivotal to ensure early intervention and combating the disease. In this respect, P-selectin is proven to be one of the signature biomarkers for detecting the prothrombotic

Table 2
Correlation between plasma P-selectin levels and laboratory parameters in the three groups.

Groups	**Healthy**		**Disease index (Type 2 DM)**		**Cases (CVD + T2DM)**	
Age	*r*	*p*-value	*r*	*p*-value	*r*	*P*-value
< 45	0.143	0.344	−0.159	0.587	0.308	0.614
≥ 45	0	0	−0.440	0.587	−0.061	0.699
BMI						
<25	−0.178	0.542	0.800	0.200	–	–
25–30	−0.330	0.211	0.510	0.090	0.143	0.626
>30	−0.271	0.293	0.528	0.007*	0.018	0.925
Laboratory parameters						
Fasting glucose	−0.129	0.387	0.151	0.345	0.169	0.252
HbA1c	−0.031	0.834	0.324	0.039*	−0.038	0.798
Total Cholesterol	0.128	0.391	0.103	0.521	0.085	0.564
LDL- Cholesterol	−0.058	0.698	0.186	0.244	0.147	0.317
HDL- Cholesterol	0.245	0.085	−0.198	0.215	−0.145	0.317
Triglyceride	−0.138	0.345	0.393	0.011*	−0.022	0.884
Platelets	0.080	0.592	0.156	0.329	0.090	0.054
MPV	0.003	0.985	0.499	0.006*	−0.069	0.614
Systolic blood pressure	−0.068	0.651	−0.130	0.418	−0.076	0.610
Diastolic blood pressure	−0.187	0.209	0.416	0.002*	0.063	0.670

(r): correlation coefficient. * Statistically significant at p ≤ 0.05.

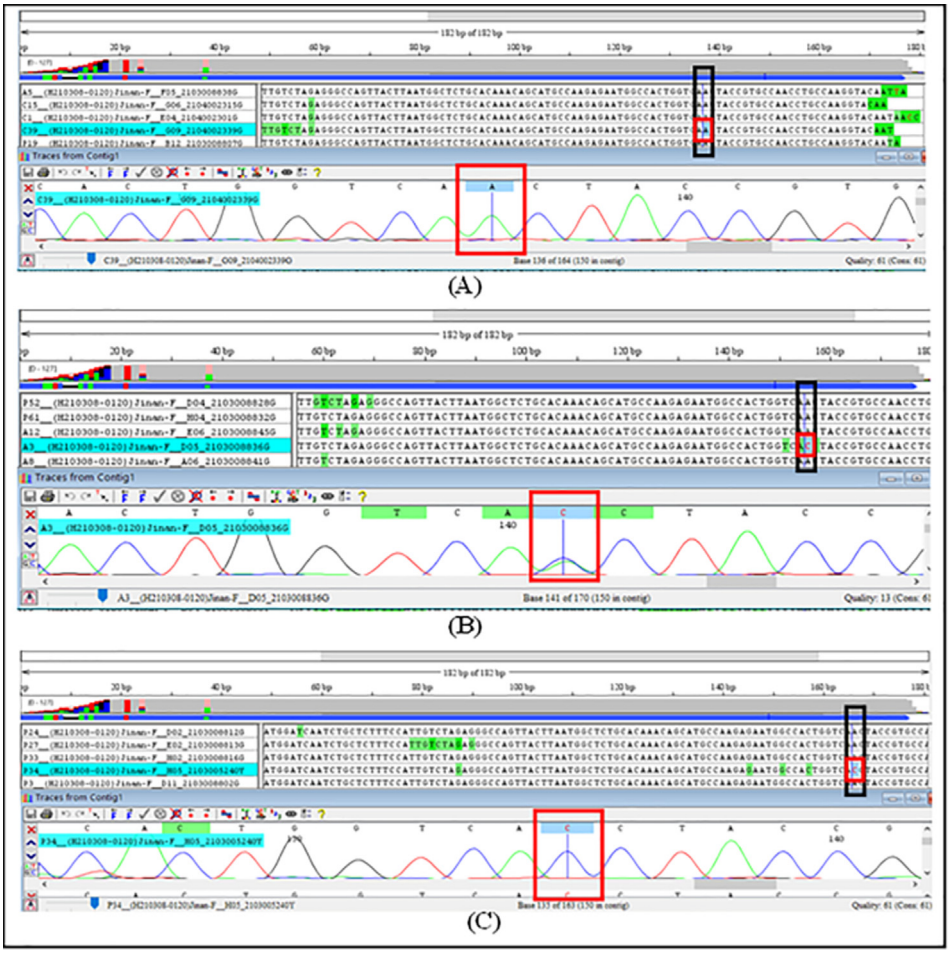

Fig. 2. Illustration of DNA fragment sequencing readings by CodonCode Aligner program **(A)** An example of a wildtype genotype sample from control group (A/A), **(B)** An example of a heterozygous genotype sample from control index group (A/C), and **(C)** An example of a mutant genotype sample from cases group (C/C).

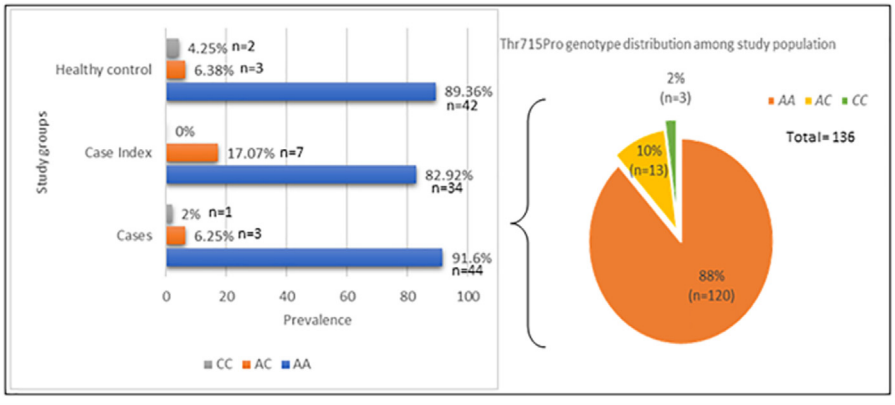

Fig. 3. Thr715Pro variant distribution among the three study groups.

Table 3
P-selectin levels in wildtype alleles vs hetero/homozygous mutant alleles.

Genotype	*AA*	*AC + CC*	*p*-value*
Cases (CVD + T2DM) (n)	179.33 (44)	132.83 (4)	0.110
Disease Index (T2DM) (n)	228.32 (34)	199.41 (7)	0.486
Healthy (n)	204.84 (42)	170.41 (5)	0.437

AA = wildtype, AC = heterozygous mutant, and CC = homozygous mutant. *p-value was calculated by Mann-Whitney test.

state and the hypercoagulability of the platelets in T2DM and its associated CVD (Karmakar et al. 2015). Therefore, the main objective of the current study was to assess P-selectin levels in diabetic patients and diabetic patients having CVD in comparison to a healthy control cohort. Also, we aimed at investigating the distribution of Thr715Pro polymorphism in the study groups and its possible association with P-selectin levels and the disease state.

The results of the current study indicated that P-selectin levels are significantly higher in the diabetic and diabetic + CVD groups as

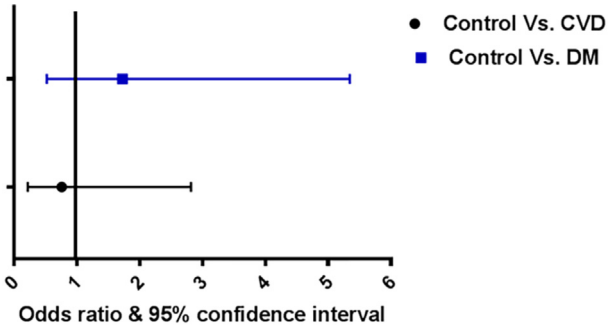

Fig. 4. Odd-ratio calculated for control vs CVD groups and control vs T2DM groups. Blue = 1.73 odd ratio and CI = 0.524–5.346, black = 0.715 odd ratio and CI = 0.2227–2.819 indicating that both odds are not statistically significant ($p > 0.5$ in both cases). DM: Diabetes Mellitus, CVD: Cardiovascular disease.

compared to the corresponding control. It was also shown that P-selectin test has a good sensitivity to discriminate healthy subjects from those having diabetes or diabetes + CVD. P-selectin levels were positively correlated to diastolic blood pressure, HBA1c, triglycerides, and mean platelet volume in diabetic patients.

In addition, the results showed that the prevalence of Thr715Pro polymorphism is 11.75 % in the study population amongst the three study groups; of these 9.55 % showed the heterozygous genotype and 2.2 % have the homozygous allele. In this regard, results showed that there was no statistical difference between P-selectin levels in subjects carrying the wildtype genotype of this polymorphism and those who are carrying the mutant gene. There could be an association between this polymorphism in the P-selectin gene and contract of type 2 diabetes as indicated by the odd ratio. Additionally, the polymorphism could protect TDM patients from developing CVD, although the odd ratio was not statistically significant.

To give an account on the role of P-selectin as a biomarker for predicting vascular complications in T2DM and its consequence CVD, it was recently reported that P-selectin along with vWF can be used as biomarkers for the hypercoagulable state and risk of CVD in T2DM, which seems to be in line with our findings (Karmakar et al. 2015). Likewise, it was reported that enhanced P-selectin expression and over activation of platelets is associated with T2DM poor glycemic control, which increases the risk to develop vascular complications in these patients (Neubauer et al. 2010). In a similar context, Devaraj and colleagues (Devaraj et al. 2002)reported that P-selectin level was significantly elevated in T2DM patients suffering from macrovascular complications in comparison to the control subjects, which is in concordance with our finding that measuring P-selectin has a good sensitivity to discriminate healthy individuals from diabetics or diabetics having CVD. However, the use of soluble P-selectin as a biomarker of CVD risk events remains uncertain, in particular when comparing its advantages to other biomarkers that are already in use, and due to lack of evidence from human-based trials (Woollard and Chin-Dusting 2007; Antoniades et al. 2010).

Moreover, P-selectin expression was reportedly found in atherosclerotic plaques and indicates platelets activation. In line with this reported information, it was also documented that P-selectin levels and other platelet activation markers are higher in obese patients suffering from hypertension, diabetes or dyslipidemia than in non-obese subjects; and is associated with thickness of carotid wall resulting from atherosclerosis events (Csongrádi et al. 2011). This is in harmony with our finding that P-selectin levels are correlated significantly to BMI ranges (30 kg/m^2 or more) indicating pre-obese or obese patients and triglycerides levels in diabetic group. It was also reported that sP-selectin level could be serving as a predicting marker for myocardial infarction in hypertensive patients (Varughese et al. 2007). This sounds to be in line with our study results that showed a significant positive moderate correlation between sP-selectin levels and high diastolic blood pressure in diabetic patients, which is a well-established risk factor for CVD. In another study that had been held in United Kingdom, P-selectin showed to be significantly higher in hypertensives compared to the normotensive control group and diastolic blood pressure demonstrated to be a predictor marker for P-selectin level (Lip et al. 1995). It was also reported that P-selectin levels increase during impaired glucose tolerance (i.e., prediabetes state) as well as in diabetes when compared to the levels in subjects with normal glucose tolerance indicating that early disruption of glycemic control is associated with prompt changes in P-selectin levels (Gokulakrishnan et al. 2006; Neubauer et al. 2010; Genc et al. 2012). This is in agreement with our results that showed a positive correlation between P-selectin levels and HBA1c values in the study groups. These changes can be explained by the altered glycosylation, which is implicated in platelets abnormal activity in cases of T2D and coronary heart disease (Li et al. 2017). In addition, a similar finding was reported by Nagy and colleagues (Nagy et al. 2007)stating that higher levels of sP-selectin were measured in diabetic patients when compared to healthy subjects, which is also in agreement with our results. However, the study also indicated that the levels were not affected by the presence or absence of the well–studied Thr715Pro polymorphism that is also discussed in the current study.

Regarding the impact of Thr715Pro polymorphism on the levels of P-selectin in the study groups, we found no effect of the polymorphism on the P-selectin levels. However, we have to point out that this conclusion was based on a small sample size in which the polymorphism was not significantly prevalent amongst the participants. Having stated this, another study reported that Thr715Pro polymorphism did not have an effect on P-selectin levels in obese patients with comorbidities like hypertension, dyslipidemia and diabetes as compared to the corresponding non-obese controls (Csongrádi et al. 2011). In contrast, another study (Ay et al. 2007) reported that sP-selectin levels are lower in individuals with Thr715Pro polymorphism than in these who carry the wildtype genotype. This can be justified too by the impaired terminal glycosylation of P-selectin as an impact of 715Pro, which might be masked in this study by the small sample size as mentioned previously. In this regard, we showed that presence of this polymorphism might be protective against development of CVD in diabetic patients. In the same context, Julia Ferrari *et al.* (Ferrari et al. 2007) reported no difference between patients and controls regarding the presence of Thr715Pro polymorphism and that its presence is not associated with risk factors or severity of stroke, which is in line with our findings in this current study.

Recently, a Mexican study by Herrera and colleagues (Herrera-Maya et al. 2020) showed that Thr715Pro polymorphism was not associated with acute coronary syndrome risk and was associated with lower plasma levels of sP-selectin, which is in agreement of our results that indicated that the polymorphism was protective in the DM + CVD group as indicated by the odd ratio calculation. Perhaps, results were not statistically significant due to the low prevalence of the polymorphism in our study population. Also, it was reported that the presence of C allele, which indicates Thr715Pro variant is associated with lower levels of sP-selectin in white people and South Asians, whilst the association with sP-selectin level in blacks could not be documented as the polymorphism was rare in this ethnic group (Miller et al. 2004). These findings are correlated with our results where the polymorphism was rare in our study group and there was no significant difference in sP-selectin levels between participants who carry the wildtype variant and these who carry the polymorphism.

Interestingly; it's been found in a cross-sectional study that's been held in India, that MPV was significantly higher in diabetic group compared to the control group (non-diabetic non-coronary artery disease (Kodiatte et al. 2012). Moreover, the study exhibited that diabetic group who had HbA1c < 6.5 % showed a significant lower MPV compared to the diabetic group whose HbA1c was $\geq$ 6.5 %; indicating that chronic elevation of blood glucose affects platelets reactivity. This is consistent with our finding that MPV was significantly correlated to P-selectin levels in diabetic patients indicating platelets hyperactivity. Nevertheless, on the other side of the coin, another study which had been held in Italy; Gieseppe D. L. and coworkers proved that there was no association between MPV with platelet reactivity through measuring MPV and the pP-selectin, besides the lack of association between MPV and coronary artery disease (De Luca et al. 2013). The fold of increase in pP-selectin was considered as a marker for platelet reactivity, and platelets were stimulated via collagen and then confirmed by another stimulant; U46119. On the other hand, P-selectin levels were significantly different between the premenstrual and post-menstrual women in DM group and that could rise another question about whether sex hormones influence platelet reactivity and subsequently P-selectin levels in our population. In 1996, Bernard Jilma and co-workers demonstrated that estradiol can affect P-selectin levels after measuring it at different stages of the menstrual cycle in healthy women. Likewise, the effect of estradiol on P-selectin level was confirmed in men who received 10 mg of 17-estradiol (E2) (Jilma et al. 1996). Their study revealed that the increase of serum E2 was inversely proportional to the P-selectin levels. In this study, it might be suggested that sex hormones have an impact on P-selectin, and therefore, on platelet reactivity.

In the present study, we found that DM + CVD patients who administer beta-blockers have significantly lower levels of sP-selectin compared to other drugs, with the exception of angiotensin receptor blockers (ARBs), in which patients who take the latter class of drugs had lower levels of sP-Selectin than those who are taking beta-blockers. This can be justified by the relatively less patients on ARB compared to patients who were on beta-blockers, besides the huge mean difference between users and no-users in both groups. In this context, it was reported that beta-blockers increase the count of platelets in patients with chronic heart failure, which means that beta-blockers decrease the MPV (Gibbs et al. 2001). In this line, Turgay Celik *et al* has reported in their clinical study that sP-selectin and MPV were significantly lower in hypertensive patients post-treatment with nebivolol (a medication belongs to beta-blocker family) (Celik et al. 2007). This is in agreement with our study, which indicated that beta-blockers treatment was significantly associated with lower sP-selectin levels. This suggests that beta-blockers could be a mean to decrease the hypercoagulable state in CVD patients. In contrast, we reported that there was almost no relationship between P-selectin and MPV in CVD group, which might suggest that beta-blockers could have no influence on MPV in this population or a minimum correction effect of platelet reactivity by the intervening medications.

5. Conclusions

To our knowledge, it's the first study reporting the prevalence of Thr715Pro in the Middle East and its influence on P-selectin level besides its correlation to CVD. In summary, to assess the potential use of role of sP-selectin as a biomarker in diabetes and/or diabetes-related CVD patients, the present study should be reproduced in a larger representative population to give a decisive account on the actual changes in this marker levels in this category of patients. Similarly, a larger sample size is needed to explore the representativeness of the Thr715Pro polymorphism amongst the Saudi population so that its correlation with disease status in T2DM and T2DM + CVD patients could be properly investigated. For this reason, we recommend the establishment of research clinics to minimize patient noncompliance and ensure effective patient contribution to research activities under the supervision of their treating physicians. This will help to underscore factors contributing to disease pathophysiology and validate the results of scientific research. In turn, the network of a clinics and research laboratories will be more successful. As P-selectin level was the highest in T2DM group and beta blockers showed its efficient effect to reduce the level of P-selectin in DM + CVD group. Hence, it is recommended to assess the clinical outcome of the prophylaxis use of this medication in T2DM patients to reduce the risk of CVD. Furthermore, 17β-estradiol hormone has shown its effect as vascular protective, and hence; it would be recommended to study the hormonal impact on platelet reactivity and P-selectin level in our population.

Ethical approval/Research involving human participants and/or animals:

The study was reviewed and approved by the institutional review board at Imam Abdulrahman Bin Faisal University (IRB#PGS-2018–03-139). Participation was voluntary and authors declare that all ethical considerations were properly followed.

Informed consent:

A written informed consent was voluntary obtained from all participants after the study being explained in details.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103579.

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