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ORIGINAL ARTICLE

Association of *SELP* Polymorphisms with Soluble P-Selectin Levels and Vascular Risk in Patients with Type 2 Diabetes Mellitus: A Case–Control Study

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Abstract

P-selectin, an adhesion molecule, is encoded by *SELP* and known as biomarker of endothelial as well as platelet dysfunction. *SELP* polymorphisms (rs6136, rs6127, and rs6125) and raised levels of soluble P-selectin (sP-selectin) have been associated with several disease conditions. The present study was aimed to determine the association of *SELP* variants and sP-selectin levels as well as vascular risk in Type 2 diabetes mellitus (T2DM) patients. The frequency of rs6136, rs6127, and rs6125 was assessed by restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR). sP-selectin levels were measured using commercially available kits. Haplotypes were constructed using PHASE software. The data obtained from the above-said analyses was subjected to suitable statistical analyses. sP-selectin levels (ng/ml) were significantly higher in patients as compared to controls ($p < 0.001$). Out of total, 22% of patients were found to have very high vascular risk, 43.2% with high vascular risk, while 34.4% with moderate vascular risk. For both rs6136 and rs6127, frequency of variant allele was found to be significantly higher in patients as compared to controls and accounted for 2.4- and 1.5-fold risk of disease development, respectively. CAG was found to be associated with 4.5-fold risk towards disease development. In contrast, AGG was conferring the protective effect. Significantly high sP-levels were observed in patients with homozygous wild genotype of rs6136, all genotypes of rs6127, and heterozygous genotype of rs6125 as compared to respective controls. Significant difference was observed in P-selectin levels within moderate-risk category for rs6136. When compared between the categories, significant difference was observed for rs6136 and rs6127. Furthermore, patients with haplotypes AAA, AGA, and AGG were found to have significantly high sP-selectin levels as compared to controls. Significant difference in sP-selectin levels was observed within very high-risk as well as high-risk category. When compared between the categories, significant difference was observed for AGA and AGG haplotypes. The

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studied polymorphisms of *SELP* have shown significant association with sP-selectin levels as well as vascular risk in T2DM patients.

Keywords Arterial stiffness · Endothelial dysfunction · Platelet hyperactivity · *SELP* · SNP

Abbreviations

ABI	Ankle brachial index
baPWV	Brachial-ankle pulse wave velocity
CAD	Coronary artery disease
CR	Consensus repeat
DM	Diabetes mellitus
EtBr	Ethidium bromide
ECTIM	Etude Cas-Témoin de l'Infarctus du Myocarde
ELISA	Enzyme-linked immunosorbent assay
ICMR	Indian Council of Medical Research Guidelines
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
MI	Myocardial infarction
PB	Punjab
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
sP-selectin	Soluble P-selectin
SPSS	Statistical package for Social science
T2DM	Type 2 diabetes mellitus
WHO	World Health Organization

Introduction

Diabetes mellitus (DM) is a chronic hyperglycemic and hypercoagulable state, affecting 422 million people worldwide (World Health Organization (WHO) 2016). The prevalence of diabetes is increasing very rapidly in India. According to International Diabetes Federation (IDF), 69.2 million cases of diabetes were reported from India with the prevalence of 8.7% (IDF 2015). T2DM is the most prevalent form of diabetes, accounting for approximately 90% of total diabetes cases worldwide (WHO 2016). Impairment of insulin-mediated glucose disposal (insulin resistance) and secretion of insulin by β-cells of pancreas are documented as the major cause of T2DM development (Stumvoll et al. 2005). It is associated with micro-vascular and macro-vascular complications. The macro-vascular complications are manifested as accelerated atherosclerosis that results into severe peripheral vascular disease, premature coronary artery disease (CAD), and increased risk of cerebrovascular diseases (Beckman et al. 2002; Creager et al. 2003). The metabolic milieu of T2DM patients, including insulin resistance, hyperglycemia, and release of excess free fatty acids, affecting vascular wall by

series of events including oxidative stress, increased coagulation, endothelial dysfunction, impaired fibrinolysis, and platelet hyperreactivity. These events in turn increase inflammation, enhanced vasoconstriction, and thrombus formation, resulting in development of atherosclerotic vascular complications (Beckman et al. 2002; Ferroni et al. 2004; Nesto 2004; Ferreiro et al. 2010).

P-selectin is one of the most important markers of endothelial dysfunction and platelet activation. It is a 140-kD adhesion molecule, stored in α-granules of platelets and Weibel–Palade bodies of endothelial cells (Ley 2003). During development of inflammatory response, it is translocated to the surface of endothelial cells and platelets (Blann et al. 2003; Raman et al. 2013). Furthermore, the ligands present on leukocytes bind to endothelium as well as platelets, increasing the expression of P-selectin on the surface of these cells (Marso and Stern 2004). Thus, increased sP-selectin levels may reveal its release from activated platelets and damaged endothelial cells (Pawelczyk et al. 2017). Along with other biomarkers, circulating levels of P-selectin were suggested as important indicator for platelet hyperactivity as well as endothelial dysfunction (Barac et al. 2007; Pawelczyk et al. 2017). Increased levels of sP-selectin were detected in various inflammatory disease conditions including T2DM, prediabetes, hypertension, coronary heart disease (CHD), myocardial ischemia (MI), CAD (Hillis et al. 2002; Lim et al. 2004; Aref et al. 2005; Gokulakrishnan et al. 2006; Bielinski et al. 2015; Huang et al. 2016).

P-selectin is encoded by *SELP* located on chromosome 1q21–q24 spanning > 50 kb and contains 17 exons, encoding structurally distinct domains (Barbaux et al. 2001). Variations in *SELP* may contribute to predisposition to endothelial as well as platelet dysfunction play and may regulate the physiologic response to vascular complications. *SELP* inactivation in atherosclerosis prone ApoE−/− mice showed decreased monocyte recruitment to neointima formation sites after carotid artery injury and markedly reduced atherosclerosis plaque formation (Manka et al. 2001). Various single-nucleotide polymorphisms (SNPs) of *SELP* have been linked with susceptibility towards different disease conditions including diabetic retinopathy, MI, thrombo-embolic stroke, asthma, ischemic stroke, and CAD (Kee et al. 2000; Tregouet et al. 2002; Barbaux et al. 2001; Bourgain et al. 2003; Zee et al. 2004; Volcik et al. 2006; Ay et al. 2007; Penman et al. 2015). Furthermore, genetic variations of *SELP* were also reported to be associated with the development of arterial stiffness (Zhu et al. 2008). Three non-synonymous (ns) SNPs were found to be associated with various disease conditions and sP-selectin levels in different populations. An nsSNP rs6136 (T715P) is the most extensively studied *SELP* variant till date. It was observed to have a protective effect on MI in both ECTIM (Etude Cas-Témoin de l'Infarctus du Myocarde) and ECTIM extension studies, whereas neutral effect was observed in other studies (Liu et al. 2005; Volcik et al. 2006). Another nsSNP rs6127 (D603N) was reported to be associated with increased risk of albuminuria in diabetic patients, recurrent spontaneous abortions, and MI in previous studies (Tregouet et al. 2002; Liu et al. 2005; Dendana et al. 2012). Third important nsSNP rs6125 (V209M) was found to be highly prevalent patients with ventricular fibrillation (Elmas et al. 2010). Out of these, rs6127 and rs6125 were reported to have putative deleterious effect on P-selectin protein structure as well as function (Kaur et al. 2017a, b).

There are only sporadic reports on *SELP* polymorphisms in relation to sP-selectin levels in T2DM. To the best of our knowledge, this is the first report on investigation of *SELP* polymorphisms in relation to sP-selectin levels as well as vascular risk in T2DM patients in Asia. The frequency and functional influence of *SELP* SNPs in particular population can help in better understanding of the role of P-selectin in genetic susceptibility to develop future vascular complications.

Materials and Methods

Study Participants

The present case–control study recruited 250 T2DM patients including 152 males and 99 females, with glycated hemoglobin (HbA1c) $\geq$ 6.5% and aged 30–80 years, from Carewell Heart & Superspeciality Hospital, Amritsar (PB). HbA1c levels of patients were measured by fully automated Alere AfinionTM analyzer using manufacturer's protocol (Afinion-AS100, Alera Technologies AS, Norway). Age- and gender-matched 264 healthy controls including 157 males and 107 females were also enrolled from the adjoining areas. The samples from both T2DM patients as well as controls have been collected in year 2013–2014. All the study participants belonged to different regions of North India. The information including disease history, disease duration, smoking/alcoholic status, dietary pattern, and total physical activity was gathered from all the participants on a pre-designed proforma. Individuals having HbA1c less than 6.5, pregnant women, nursing mothers, having other chronic diseases, and age < 30 and > 80 years were excluded from the study. Written voluntary informed consent was obtained from all the study participants and the study protocol was approved by ethics committee of Guru Nanak Dev University, Amritsar (PB), India, according to Indian Council of Medical Research guidelines (ICMR 2006) adapted from declaration of Helsinki (2004).

The vascular risk in the study participants was assessed by measurement of arterial stiffness (Yamashina et al. 2013) (Supplementary Table 1). Arterial stiffness was measured non-invasively by baPWV test using bidirectional automated Doppler ultrasound (VP-2000/1000-Colin Corporation, Hyayashi Komaki, Japan) (Suzuki et al. 2001; Munakata et al. 2012; Kaur et al. 2017a, b). The data regarding baPWV and ABI were collected from the clinical records of the patients.

Genotyping of *SELP* Variants

Five ml of peripheral blood sample was collected from all the study participants and subjected to deoxyribonucleic acid (DNA) and serum isolation. Genomic DNA was extracted from 2 ml of blood sample (with ethylenediaminetetraacetic acid (EDTA)) using inorganic method (Miller et al. 1988). The quantity and quality of DNA was assessed by yield gel (0.8%) electrophoresis using Lamda DNA (GeNei, Bangalore). The DNA samples were stored at − 80 °C till further use. Three nsSNPs, i.e., rs6136, rs6127, and rs6125 (V209M) of *SELP* gene, were genotyped in the present

Table 1 PCR primers and conditions

SNP	Primer sequence	T_m (°C)	Annealing temperature (°C)	Amplicon size (bp)
rs6136 (A/C)	F: 5′-TGACCTTTTACATTCTGCCTCA-3′	57.63	64	520
	R: 5′-ACACTGAAGAGGAGGGGTGAT-3′	60.20		
rs6127 (G/A)	F: 5′-AAGCTTACCATCCTCCCACTG-3′	59.44	50	514
	R: 5′-TCAGCTGTTTGCTAAACCCAAA-3′	58.97		
rs6125 (G/A)	F: 5′-CAAGTAGAGATGGATAGCTTGGGT-3′	45.83	60	403
	R: 5′-TGGAGAAAGCTCATTGTGTCC-3′	47.62		

Table 2 List of restriction enzymes and restriction digestion conditions

SNP	Enzyme	Restriction site	Incubation conditions
rs6136 (A/C)	*HincII*	GTYRAC CARYTG	At 37 °C for 16 h
rs6127 (G/A)	*Tsp45I*	GTSAC CASTG	At 65 °C for 2 h
rs6125 (G/A)	*BsaAI*	YACGTR RTGCAY	At 37 °C for 2 h

study. The nucleotide sequence containing the above-said variants was amplified using PCR in thermo-cycler (Eppendorf, Germany). The primers for all the three SNPs were designed using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). PCR was accomplished using the standard protocol in the reaction mixture of 20 µl containing 0.3 U Taq polymerase, 1X Taq buffer A with 15 mM $MgCl_2$, 200 µM of deoxynucleotide (dNTP) mix, 0.3 µM primer mix (forward and reverse primer each), and 1 µl (50 ng/µl) of genomic DNA. The PCR conditions involved 7-min denaturation at 94 °C, then 35 cycles each of 30 s at 94 °C for denaturation, at 50–65 °C (specific for each SNP) for annealing, at 72 °C for extension, and a final extension step of 7 min at 72 °C (Table 2). The PCR products were analyzed on 1.5% (w/v) agarose gel pre-stained with ethidium bromide (EtBr). Genotyping was carried out by RFLP using specific restriction enzymes (New England Biolabs, USA) under different conditions (Table 2). The digested products obtained were analyzed on 2.5% (w/v) agarose gel pre-stained with EtBr. Ten percent of representative samples of each variant having different genotypes were confirmed by Sanger sequencing. In case of rs6136, a PCR product of 520 bp was obtained and was further subjected to restriction digestion using *HincII*. The restriction digestion products obtained were 276, 244 bp in case of homozygous wild genotype and 520, 276, 244 bp for heterozygous genotype (Fig. 1a). The electropherograms of representative samples of rs6136 are shown in Fig. 1b and c. For rs6127, a PCR product of 514 bp was obtained and was subjected to restriction digestion using *Tsp45I*. The restriction digestion products obtained were 220, 169, 125 bp for homozygous wild genotype; 345, 169 bp for homozygous variant genotype; and 345, 220, 169, 125 bp for heterozygous genotype (Fig. 2a). The electropherograms of representative

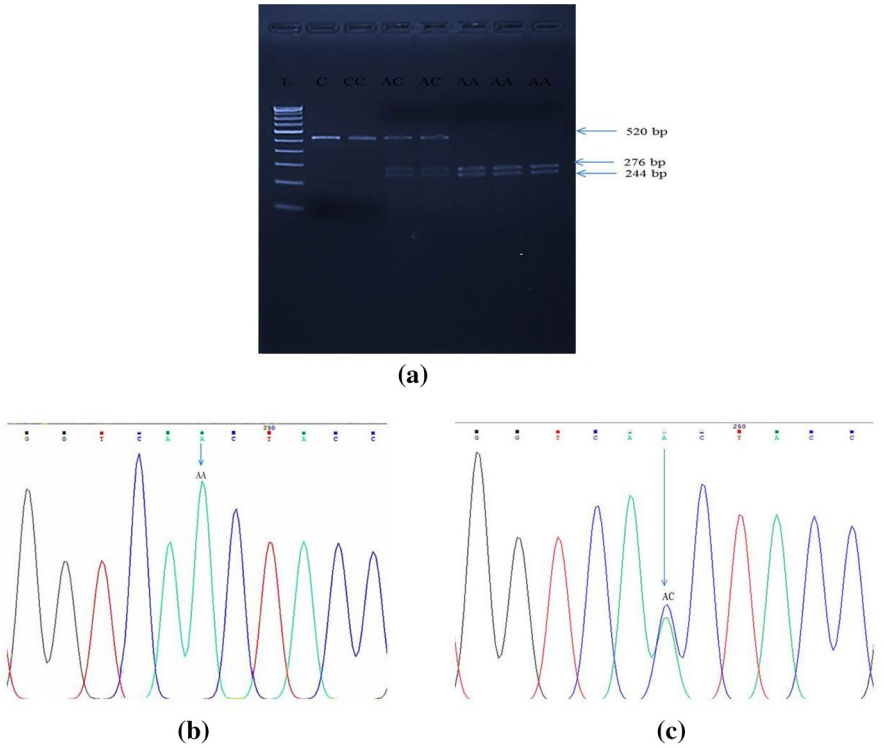

Fig. 1 **a** Representative gel showing PCR product of size 520 bp and restriction digestion products obtained for rs6136. L represents 100-bp ladder, C represents negative control. Products obtained were 276, 244 bp for AA genotype and 520, 276, 244 bp for AC genotype; **b** electropherograms of representative samples of rs6136 confirming homozygous wild genotype and **c** heterozygous genotype

samples of rs6127 are shown in Fig. 2b–d. Furthermore, in rs6125 a PCR product of 403 bp was obtained and was further subjected to restriction digestion using *BsaAI*. The restriction digestion products obtained for rs6125 were 295, 108 bp for homozygous wild genotype; 403 bp for homozygous variant genotype; and 403, 295, and 108 bp for heterozygous genotype (Fig. 3a). The electropherograms of representative samples of rs6125 are shown in Fig. 3b–d.

Assessment of sP-Selectin Levels

For serum separation, 3 ml of blood sample was incubated at 37 °C for 30 min and centrifuged at 400×*g* for 15 min. Serum sP-selectin levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's protocol (RayBiotech, USA). In brief, 100 µl of pre-diluted sample was added to anti-Human P-selectin-coated microtitre plate and incubated for 2.5 h at 25 ± 2 °C. The solution was discarded and plate was washed four times with 1× wash buffer. Then, 100 µl of 1× biotinylated antibody was added to each well and incubated at

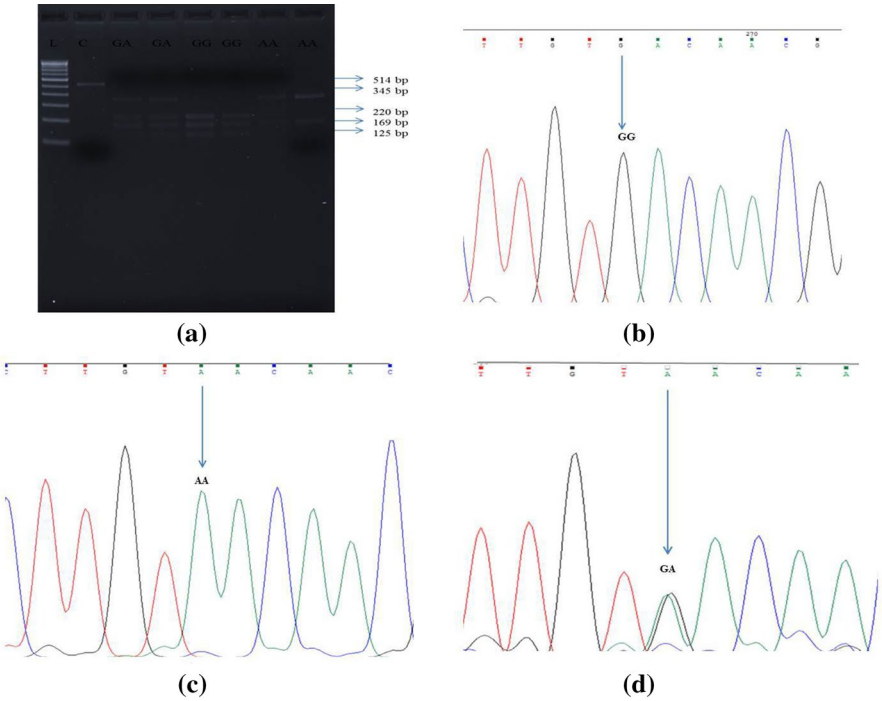

Fig. 2 **a** Representative gel showing PCR product of size 514 bp and restriction digestion products obtained for rs6127. L represents 100-bp ladder, C represents negative control Products obtained were 220, 169, 125 bp for GG genotype; 345, 169 bp for AA genotype; and 345, 220, 169, 125 bp for GA genotype; **b** electropherograms of representative samples of rs6127 confirming homozygous wild genotype, **c** homozygous variant genotype, and **d** heterozygous genotype

25 ± 2 °C with gentle shaking for 1 h. After incubation, well contents were discarded and the washing was done four times to remove unbounded biotinylated antibody. Afterwards, 100 µl of Streptavidin-HRP solution was added to each well and the plate was incubated for 45 min at 25 ± 2 °C. After final washing, 100 µl of TMB substrate was added to each well and incubated for 15 min at 25 ± 2 °C. At last, 50 µl of stop solution was added to each well, which changed the color from blue to yellow. The intensity of the color was measured at 450 nm (BIO-RAD, iMark™ Microplate Reader, USA).

Statistical Analyses

Sample size for genetic association was calculated by CaTS Power calculator (http://csg.sph.umich.edu/abecasis/CaTS/) to achieve a power of 80 percent, by taking assumptions of 8.7% prevalence of diabetes in India (IDF 2015) and an odds ratio of 1.5 ($\alpha = 0.05$, 95% CI). Genotypic and allelic frequencies in patient and control groups were compared by Odds ratio using MedCalc software (https://www.medcalc.org/). Genetic models were determined using Web-Asso test program (http://

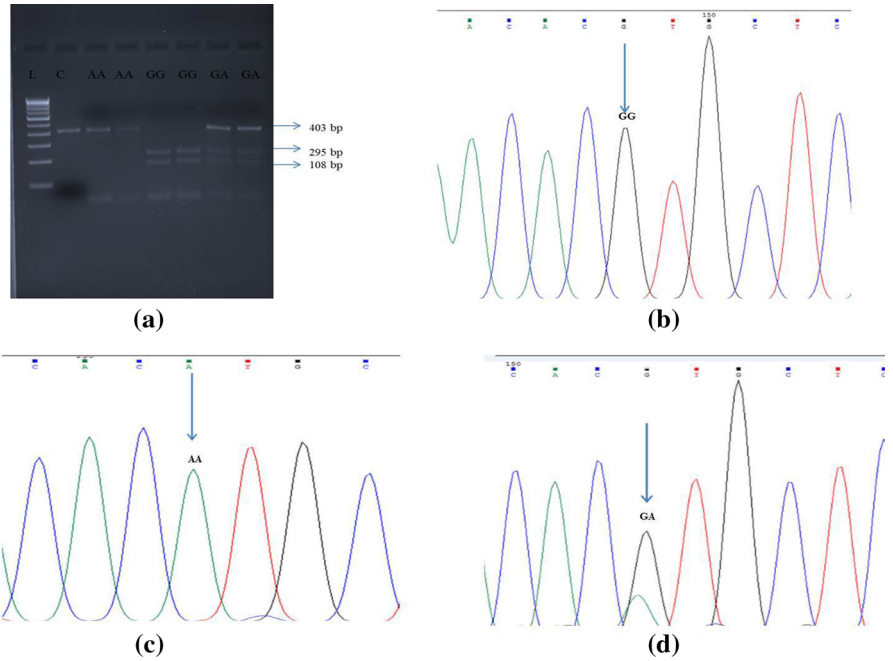

Fig. 3 **a** Representative gel showing PCR product of size 403 bp and restriction digestion products obtained for rs6125. L represents 100-bp ladder, C represents negative control. Products obtained were 295, 108 bp for GG genotype; 403 bp for AA genotype; and 403, 295, and 108 bp for GA genotype; **b** electropherograms of representative samples of rs6125 confirming homozygous wild genotype, **c** homozygous variant genotype, and **d** heterozygous genotype

www.asso-web.com/). Haplotypes were constructed by PHASE software version 2.1.1 (Stephens et al. 2001). Linkage disequilibrium was determined using Haploview version 4.2 (Barrett et al. 2005). sP-selectin levels (mean ± SD) were compared in different genotype/haplotype combinations within the groups by one-way ANOVA, followed by Tukey's multiple comparison post hoc test, if applicable. sP-selectin levels were compared in different genotypic/haplotype combinations between the groups by Student's *t* test. Box whisker plot was used to find out all the outliers from the whole data. All the statistical analysis was performed using statistical package for Social science (SPSS) version 18.0 (IL, USA and Chicago). The p value < 0.05 were considered to be statistically significant for all the analyses.

Results

The mean age of patients was 54.23 ± 9.23 years, with 53.73 ± 10.03 years in males and 55.02 ± 7.86 years in female subjects. The mean age of healthy controls was 54.04 ± 9.40 years, with 57.78 ± 7.51 years in males and 48.56 ± 9.22 years in females. Vascular risk stratification was carried out in patients by combination of

baPWV and ABI values. Out of total, 22% ($n=56$) of patients were found to be with very high risk (Cat. I), 43.2% ($n=108$) with high risk (Cat. II), 34.4% ($n=86$) with moderate risk (Cat. III). One case was found to have low risk, and thus excluded from the further analysis (Fig. 4).

In the present study, three nsSNPs (rs6136, rs6127, rs6125) were subjected to genotypic and haplotypic analyses. In case of rs6136, a significant difference was observed in genotypic and allelic distribution between patients and controls (Table 3). Frequency of homozygous wild genotype was significantly low in patients (85.20%) as compared to controls (93.6%), while heterozygosity was found to be significantly high (14.8%) in patients than controls (6.44%). The frequency of C allele was significantly high in patients (7.4%) as compared to controls (3.22%), conferring 2.4-fold risk of disease development (OR 2.4, 95% CI 1.33–4.32, $p=0.0035$). No significant difference was observed in genotypic as well as allelic distribution between the vascular risk categories ($p>0.05$) (Table 4).

For rs6127, a significant difference was observed in genotypic and allelic distribution between patients and controls (Table 3). The frequency of homozygous variant genotype was found to be significantly high in patients (34.8%) as compared to controls, while frequency of homozygous wild genotype was low in patients (27.27%) as compared to controls (20.40%). The heterozygosity was found to be less prevalent (44.80%) in patients as compared to controls (51.14%), but the difference was not found to be significant ($p>0.05$). The frequency of A allele was also significantly high in patients (57.2%) as compared to controls (47.15%) and it was found to be associated with 1.5-fold risk of disease development (OR 1.5, 95% CI 1.17–1.91, $p=0.0013$). There were suggestive evidences of association of both dominant (GA/AA vs.GG; OR 0.15, 95% CI 0.04–0.51, $p<0.001$) and recessive models (AA vs. GG/GA; OR 10.19, 95% CI 2.33–44.27, $p<0.001$) with disease development. Furthermore, when genotypic/allelic frequencies were compared between vascular risk

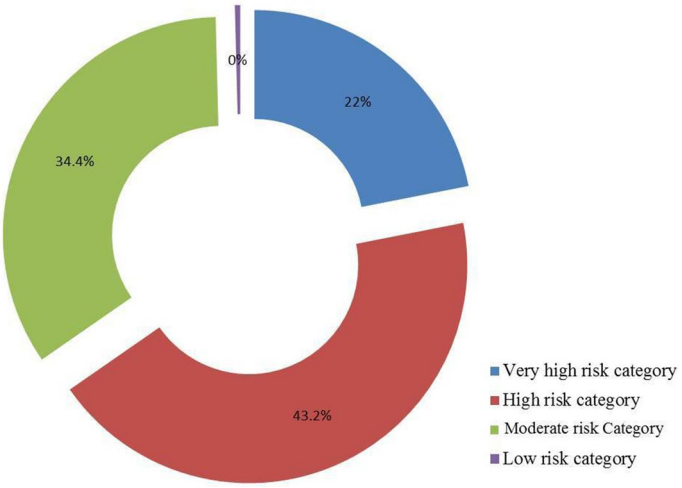

Fig. 4 Vascular risk stratification on the basis of combination of baPWV and ABI

Table 3 Genetic distribution of *SELP* polymorphism in patients and control subjects

SNP	Patients $n=250$ (%)	Controls $n=264$ (%)	Odds ratio (95% CI)	p value	Dominant model Odds ratio (95% CI) p value	Co-dominant model Odds ratio (95% CI) p value	Recessive model Odds ratio (95% CI) p value
rs6136							
Genotype							
AA	213 (85.20)	247 (93.56)	1	1	–	–	–
AC	37 (14.80)	17 (6.44)	2.5239 (1.3812–4.6118)	0.0026**			
CC	0	0					
Allele							
A	463 (92.6)	511 (96.7)	1	1			
C	37 (7.4)	17 (3.22)	2.4021 (1.3344–4.3243)	0.0035**			
rs6127							
Genotype							
GG	51 (20.40)	72 (27.27)	1	1	0.15 (0.04-0.51) $p<0.001$	1.05 (0.57-1.94) $p=0.879$	10.16 (2.33-44.27) $p<0.001$
GA	112 (44.80)	135 (51.14)	1.1712 (0.7562–1.814)	0.4789			
AA	87 (34.80)	57 (21.60)	2.1548 (1.3199–3.5178)	0.0021**			
Allele							
G	214 (42.8)	279 (52.84)	1	1			
A	286 (57.2)	249 (47.15)	1.4975 (1.1707–1.9154)	0.0013**			
rs6125							
Genotype							
GG	18 (7)	3 (1.14)	1	1	1.46 (0.97-2.20) $p=0.067$	1.48 (1.16-1.89) $p=0.002$**	1.94 (1.31-2.87) $p=0.001$**
GA	214(86)	259 (98.10)	0.1377 (0.0400–0.4738)	0.0032**			
AA	18 (7)	2 (0.76)	1.5000 (0.2233–10.0769)	0.6765			

Table 3 (continued)

SNP	Patients $n=250$ (%)	Controls $n=264$ (%)	Odds ratio (95% CI)	p value	Dominant model Odds ratio (95% CI) p value	Co-dominant model Odds ratio (95% CI) p value	Recessive model Odds ratio (95% CI) p value
Allele							
G	250 (50)	265 (50.18)	1	1			
A	250 (50)	263 (49.82)	1.0076 (0.7888–1.2872)	0.9515			

OR odds ratio, *CI* confidence interval

**Represents p value significant at 0.01 level

Table 4 Comparison of genotypic/allelic distribution of *SELP* variants between the vascular risk categories

SELP variants	Cat. I $n=54$ (%)	Cat. II $n=109$ (%)	Cat. III $n=86$ (%)	Odds ratio (95% CI)			*p* value		
				Cat. I vs. Cat. II	Cat. II vs. Cat. III	Cat. I vs. Cat. III	p^a	p^b	p^c
rs6136									
Genotype									
AA	45 (83.33)	90 (82.56)	78 (90.69)	1	1	1			
AC	9 (16.66)	19 (17.59)	8 (9.30)	0.95 (0.40–2.26)	2.06 (0.85–4.96)	1.95 (0.70–5.41)	0.900	0.110	0.200
CC	0	0	0						
Alleles									
A	99 (91.66)	199 (91.28)	164 (97.67)	1	1	1			
C	9 (8.33)	19 (8.71)	8 (4.65)	0.95 (0.42–2.18)	1.96 (0.83–4.59	1.86 (0.70–4.99)	0.900	0.122	0.210
rs6127									
Genotype									
GG	7 (12.96)	25 (22.93)	18 (9.30)	1	1	1			
GA	23 (42.59)	58 (53.21)	31 (36.04)	1.41 (0.53–3.72)	1.34 (0.63–2.84)	1.90 (0.68–5.32)	0.48	0.434	0.217
AA	24 (44.44)	26 (23.85)	37 (43.02)	3.29 (1.20–9.0)	0.50 (0.23–1.11)	0.87 (0.41–1.84)	0.02*	0.08	0.723
Alleles									
G	37 (34.25)	108 (49.54)	67 (38.95)	1	1				
A	71 (65.74)	110 (50.45)	105 (61.04)	1.88 (1.16–3.04)	0.64 (0.43–00.97)	1.22 (0.74–2.022)	0.009**	0.037*	0.429
rs6125									
Genotype									
GG	1 (1.85)	10 (9.17)	7 (8.13)	1	1	1			
GA	47 (87.03)	94 (86.23)	72 (83.72)	0.5 (0.62–40.23)	0.91 (0.33–2.15)	0.218 (0.26–1.83)	0.130	0.86	0.16
AA	6 (11.11)	5 (4.48)	7 (8.13)	12 (1.11–128.84)	0.50 (0.111–2.24)	1.32 (0.41–4.14)	0.04*	0.36	0.64
Alleles									
G	49 (45.37)	114 (52.29)	86 (50)						

Table 4 (continued)

SELP variants	Cat. I $n=54$ (%)	Cat. II $n=109$ (%)	Cat. III $n=86$ (%)	Odds ratio (95% CI)			p value		
				Cat. I vs. Cat. II	Cat. II vs. Cat. III	Cat. I vs. Cat. III	p^a	p^b	p^c
A	59 (54.62)	104 (47.70)	86 (50)	1.31 (0.83–2.09)	0.91 (0.61–1.36)	0.83 (0.51–1.34)	2.09	0.65	0.45

*Represents p value significant at 0.0 level. **Represents p value significant at 0.01 level; Cat. I represents very high-risk category; Cat. II represents high-risk category; Cat. III represents moderate-risk categories; p^a denotes for p value of comparison between Cat. I and Cat. II; p^b denotes for p value of comparison between Cat. II and Cat. III; p^c denotes for p value of comparison between Cat. I and Cat. III

categories, heterozygosity was found to be more prevalent in high-risk category (53.21%) followed by very high-risk category (42.59%) and moderate-risk category (36.04%), but difference was not statistically significant (Table 4). Frequency of homozygous variant genotype was significantly low in high-risk category (23.85%) as compared to very high-risk category (44.44%) and moderate-risk category (43.44%). Furthermore, frequency of variant allele was found to be significantly high in very high-risk category (60.74%) in comparison with high-risk category (50.45%) and was found to be associated with 1.88-fold very high vascular risk (OR 1.88, 95% CI 1.16–3.04, p = 0.009). Moreover, when frequency of variant allele was compared between high-risk and moderate-risk categories (61.04%), it was found to be protective against disease development (OR 0.64, 95% CI 0.43–0.97, $p = 0.037$).

For rs6125, a significant difference was observed in genotypic distribution between patients and controls (Table 3). The prevalence of heterozygosity was significantly low (86%) in patients as compared to controls (98.10%). The frequency of homozygous variant as well as homozygous wild genotype was high in patients (7% each) than controls (0.76 and 1.14% respectively), although the differences were not statistically significant (Table 3). There were suggestive evidences of association of both co-dominant (AA/GA = GA/GG; OR 1.48, 95% CI 1.16–1.89, $p = 0.002$) and recessive models (AA vs. GG/GA; OR 1.94, 95% CI 1.31–2.87, $p = 0.001$) with disease development. Furthermore, frequency of homozygous variant genotype was found to be significantly high in very high-risk than high-risk category (Table 4). But, no significant difference was observed in other genotypic/allelic distribution between all the three vascular risk categories ($p > 0.05$).

LD is commonly measured by D′ and LOD values. The D′ value ranged from 0 to 1, where 0 indicates complete equilibrium and 1 indicates complete LD. LOD stands for log of the odds of there being LD between two loci and LOD score ≥ 3.0 commonly considered as a significant evidence of linkage. In present study, SNPs rs6127 and rs6125 were observed to be in strong linkage disequilibrium D′/LOD values 0.897/26.63 (Fig. 5). The genotype distributions of rs6127 polymorphism of *SELP* were according to Hardy–Weinberg equilibrium. These variants were further analyzed and eight haplotypes were constructed by phase program. Out of these, seven haplotypes with frequency ≥ 0.01 in patients or controls were subjected to further analyses (Table 5). AAA was the most prevalent (frequency > 0.1) haplotype in both the studied groups and was considered as reference. Frequency of AGG was significantly low in patients (30.8%) than controls (39%), where AGG conferred

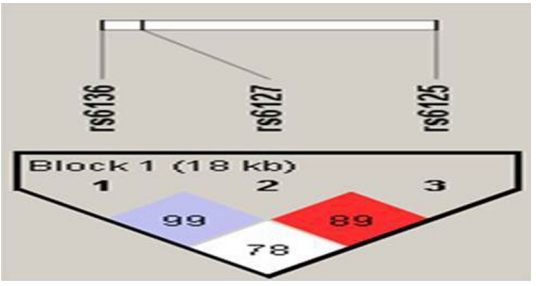

Fig. 5 Pairwise linkage disequilibrium between three studied polymorphisms of *SELP*. The red square represents strong LD ($D = 0.897$; LOD = 26.63); blue and white squares indicate non-significant LD (LOD < 3.00) (Color figure online)

Table 5 Comparison of haplotype distribution between patients and controls

Haplotype	Patients (*N*)	Freq.	Controls (*N*)	Freq.	OR	95% CI	*p* value
AAA	185	0.370	189	0.358		Ref.	
AGG	154	0.308	206	0.390	0.77	0.57–1.29	0.049*
AAG	73	0.146	54	0.102	1.38	0.92–2.08	0.110
AGA	51	0.102	62	0.117	0.84	0.55–1.29	0.420
CAG	18	0.036	4	0.007	4.59	1.52–13.84	0.006**
CAA	14	0.028	12	0.022	1.19	0.53–2.64	0.660
CGG	5	0.01	1	0.001	5.108	0.59–44.11	0.140

N number, *Freq*. frequency

*Represents statistical significance at 0.05 level

**Represents statistical significance at 0.01 level

the protective effect (OR 0.77, 95% CI 0.57–1.29, $p=0.049$). In contrast, CAG was found to be more prevalent in patients (3.6%) as compared to controls (0.7%), conferring 4.5-fold risk towards disease development (OR 4.69, 95% CI 1.52–13.84, $p=0.006$). Furthermore, frequency of AAG was high in patients (24.6%) than controls (10.2%), while contrary results were observed in case of AGA, but the results were not statistically significant ($p>0.05$). These haplotypes were also segregated according to vascular categories. Six haplotypes were observed with frequencies ≥ 0.01 in any of the risk category (Table 6). AAA was found to be the most prevalent (frequency >0.1) haplotype in two of the three categories and was used as the reference haplotype. No significant difference was observed in frequencies of any of the haplotypes between these vascular categories ($p>0.05$). AGG was the second most prevalent haplotype in very high-risk (28.7%) and moderate-risk (27.9%) categories, while it was the most prevalent haplotype in high-risk category (36.2%).

sP-selectin levels (ng/ml) were significantly high ($p<0.001$) in patients (169.02 ± 101.84) as compared to controls (132.54 ± 76.63). No significant differences in sP-selectin levels were observed between the vascular risk categories ($p>0.05$). However, patients with high vascular risk were found to have high sP-selectin levels (172.33 ± 85.98) as compared to patients with moderate risk (158.57 ± 95.47) as well as very high risk (152.39 ± 90.13). Furthermore, sP-selectin levels were also stratified according to various genotypes and haplotypes in patients, controls, and vascular risk categories. A criterion of subjects >2 for genotypes and ≥ 5 for haplotypes was used for further genotypic-phenotypic correlation analyses. sP-selectin levels were compared within as well as between patients and controls (Table 7). No significant difference was observed within patients as well as controls for all the studied variants. When compared between patients and controls, significantly high sP-levels were observed in patients with homozygous wild genotype of rs6136 ($p<0.001$), all genotypes of rs6127 ($p=0.022$; $p=0.011$; $p=0.028$, respectively), and heterozygous genotype of rs6125 ($p<0.001$) as compared to respective controls. Furthermore, sP-selectin levels were also compared in vascular risk categories (Table 8). Significant difference was observed in P-selectin levels within moderate-risk category for rs6136 ($p<0.001$). Patients with heterozygous genotype

Table 6 Comparison of haplotype distribution between vascular risk categories

Haplotypes	Cat. I (*N*)	Freq. $2n = 108$	Cat. II (*N*)	Freq. $2n = 218$	Cat. III (*N*)	Freq. $2n = 172$	OR (95% CI)	p^a	OR (95% CI)	p^b	OR (95% CI)	p^c
AAA	46	0.426	71	0.325	66	0.383	Ref.		Ref.		Ref.	
AAG	16	0.148	25	0.114	33	0.191	1.01 (0.49–2.09)	0.973	0.70 (0.38–1.31)	0.266	1.43 (0.71–2.91)	0.313
AGA	8	0.074	24	0.110	17	0.098	1.94 (0.80–4.69)	0.139	1.31 (0.65–2.66)	0.450	1.48 (0.58–3.72)	0.403
AGG	31	0.287	79	0.362	48	0.279	1.65 (0.94–2.88)	0.077	1.52 (0.93–2.49)	0.089	1.08 (0.59–1.94)	0.799
CAA	2	0.018	9	0.041	2	0.011	2.91 (0.60–14.10)	0.183	4.18 (0.87–20.07)	0.073	0.69 (0.09–5.12)	0.722
CAG	5	0.046	8	0.036	4	0.023	1.04 (0.32–3.36)	0.952	1.86 (0.53–6.46)	0.329	0.55 (0.14–2.19)	0.402

N number, *Freq*. frequency

Cat. I represents very high-risk category; Cat. II represents high-risk category; Cat. III represents moderate-risk categories; p^a denotes for p value of comparison between Cat. I and Cat. II; p^b denotes for p value of comparison between Cat. II and Cat. III; p^c denotes for p value of comparison between Cat. I and Cat. III

Table 7 Comparison of sP-selectin levels with different genotypes of *SELP* variants between T2DM patients and controls

SNP	Patients			Controls			$p^{\#}$
	Count ($n=201$)	Mean ± SD	p^{Ψ}	Count ($n=200$)	Mean ± SD	p^{Ψ}	
rs6136							
AA	173	169.16 ± 103.84	0.103#	184	132.11 ± 73.57	0.067#	< 0.001
AC	28	141.80 ± 76.25		16	115.52 ± 27.70		0.108
rs6127							
GG	40	192.01 ± 111.10	0.397	56	144.69 ± 74.35	0.393	0.022*
GA	90	157.72 ± 94.10		104	125.64 ± 79.07		0.011*
AA	71	173.61 ± 103.81		40	136.72 ± 70.23		0.028*
rs6125							
GG	12	141.41 ± 77.14	0.570	2	84.55 ± 81.10	–	–
GA	172	170.17 ± 101.48		198	133.03 ± 76.64		< 0.001
AA	17	158.66 ± 59.80		–	–		–

p^{Ψ}represents one-way ANOVA; $p^{\#}$represents independent *t* test; *represents significance at 0.05 level

were observed to have significantly high sP-selectin levels as compared to homozygous wild genotype. When compared between the categories, significant difference was observed for rs6136 and rs6127 ($p<0.05$).

When stratified according to the haplotypes, no significant difference ($p>0.05$) was observed in sP-selectin levels when compared within the studied groups (Table 9). Patients with AAA, AGA, and AGG haplotypes were found to have significantly high sP-selectin levels ($p<0.001$; $p=0.044$; $p=0.004$, respectively) as compared to controls. When stratified according to vascular risk, patients with AGG haplotype were observed with significantly low levels of sP-selectin as compared to patients with AAA haplotype ($p=0.013$) in very high-risk categories as well as AGG haplotype in both high-risk and moderate-risk categories ($p<0.001$; $p=0.001$, respectively). On the other hand, patients with AGA haplotype in moderate-risk category were observed with significantly elevated levels of sP-selectin as compared to very high-risk and high-risk categories ($p=0.010$; $p=0.021$, respectively) (Table 10).

Discussion

In the present analysis, significantly high levels of sP-selectin were observed in T2DM patients as compared to respective controls. These results are in concordance with the previous studies showing association of high levels of sP-selectin with diabetes along with adverse cardiovascular outcome (Ridker et al. 2001; Lim et al. 2004; Aref et al. 2005). In another study, increased levels of sP-selectin were reported in subjects with prediabetes and T2DM (Gokulakrishnan et al. 2006). Furthermore, elevated sP-selectin levels were also found to be correlated with glucose dysregulation in T2DM patients (Yngen et al. 2001). No significant difference in

Table 8 Comparison of sP-selectin levels in different vascular risk categories with different genotypes of *SELP* variants

SNP	Very high-risk category (Cat. I)			High-risk category (Cat. II)			Moderate-risk category (Cat. III)			$p^{\#}$		
	Count ($n=49$)	Mean ± SD	p^{Ψ}	Count ($n=84$)	Mean ± SD	p^{Ψ}	Count ($n=67$)	Mean ± SD	p^{Ψ}	p^{a}	p^{b}	p^{c}
rs6136												
AA	42	167.85 ± 108.52	0.665#	69	175.92 ± 99.87	0.493#	61	136.68 ± 67.57	< 0.001#	0.690	0.009**	0.103
AC	7	188.76 ± 165.40		15	194.98 ± 81.74		6	377.14 ± 65.22		0.906	< 0.001	0.024
rs6127												
GG	7	144.54 ± 87.60	0.213	19	216.80 ± 120.95	0.055	13	134.02 ± 85.05	0.080	0.163	0.042*	0.079
GA	21	140.72 ± 108.99		45	152.28 ± 80.91		24	192.08 ± 118.24		0.546	0.181	0.139
AA	21	202.14 ± 131.89		20	191.60 ± 101.27		30	137.21 ± 78.24		0.777	0.038*	0.052
rs6125												
GG	1	133.45 ± 0.00	0.289	6	187.32 ± 98.92	0.957	6	119.36 ± 71.50	0.069	–	0.203	–
GA	43	161.99 ± 118.86		73	176.25 ± 99.75		55	150.54 ± 82.47		0.490	0.123	0.592
AA	5	222.57 ± 126.96		5	183.53 ± 93.79		6	231.88 ± 178.50		0.595	0.557	0.923

p^{Ψ}represents one-way ANOVA; $p^{\#}$represents independent *t* test; *represents significance at 0.05 level; **represents significance at 0.01 level; p^{a} denotes for *p* value of comparison between Cat. I and Cat. II; p^{b} denotes for *p* value of comparison between Cat. II and Cat. III; p^{c} denotes for *p* value of comparison between Cat. I and Cat. III

Table 9 Comparison of sP-selectin levels with different haplotypes between T2DM patients and controls

SNP	Patients			Controls			$p^{\#}$
	Count	Mean ± SD	p^{Ψ}	Count	Mean ± SD	p^{Ψ}	
AAA	150	169.96 ± 101.85	0.491	140	130.95 ± 78.11	0.210	< 0.001
AAG	58	159.31 ± 82.67		37	154.40 ± 57.50		0.734
AGA	42	193.23 ± 121.37		47	148.76 ± 74.40		0.044*
AGG	126	162.41 ± 99.93		160	130.58 ± 78.86		0.004**
CAA	10	145.00 ± 54.16		12	114.04 ± 78.86		0.109
CAG	14	182.09 ± 75.00		3	90.31 ± 50.02		–

p^{Ψ}represents one-way ANOVA; $p^{\#}$represents independent *t* test; *represents significance at 0.05 level; **represents significance at 0.01 level

sP-selectin levels was observed between the vascular risk categories. However, the high-risk category was found to have highest sP-selectin levels followed by moderate-risk and very high-risk category. The reason behind non-significant difference could be the lesser number of individual in these categories. Furthermore, genetic variants as well as haplotypes of *SELP* may influence the sP-selectin levels. To ascertain this association, a genotypic-phenotypic correlation analyses was also performed by comparing sP-selectin levels among the *SELP* variants and haplotypes in both the study groups as well as vascular risk categories.

rs6136 (Thr715Pro) is a missense variant located in exon 13, which encodes for the last consensus repeat in P-selectin. This exon is located in close proximity to the exon encoding transmembrane domain and this domain is absent in sP-selectin produced as the result of alternative splicing (Marteau et al. 2008). It is postulated that variation at position 715 from threonine to proline can influence the stability of mRNA and induce conformational changes in precursor protein, affecting its intracellular secretion (Carter et al. 2003).Therefore, this may explicate the lower concentrations of sP-selectin in the individuals carrying this variant (Kee et al. 2000; Tregouet et al. 2002; Zee et al. 2004; Marteau et al. 2008). Furthermore, an in vivo study suggested proteolytic cleavage may also account for the presence of shorter P-selectin molecules in plasma (Berger et al. 1998). Proteolytic cleavage was also suggested as the mechanism for the production of soluble forms of E- and L-selectin (Marteau et al. 2008). Indeed, if sP-selectin is produced by proteolytic cleavage, then variation at position 715 may affect the sequence of cleavage site of membrane bounded P-selectin and result in decrease in P-selectin release. Remarkably, various studies have suggested association of high P-selectin levels with increased risk of cardiovascular complications but P715 allele was reported to be associated with decreased P-selectin levels, and thus suggested to be protective in nature (Kee et al. 2000; Tregouet et al. 2002; Zee et al. 2004). In the present study, no homozygous variant genotype was observed. This may be attributed to the low global minor allele frequency of rs6136, i.e., 0.0359. In concordance with the previous studies, patients with homozygous wild genotypes were found to have significantly elevated levels of sP-selectin as compared to controls. Furthermore, heterozygosity was also found

Table 10 Comparison of sP-selectin levels between different vascular risk categories with haplotypes of *SELP*

Haplotype	Very high-risk category (Cat. I)			High-risk category (Cat. II)			Moderate-risk category (Cat. III)			$p^{\#}$		
	Count	Mean ± SD	p^{Ψ}	Count	Mean ± SD	p^{Ψ}	Count	Mean ± SD	p^{Ψ}	p^{a}	p^{b}	p^{c}
AAA	36	160.13 ± 77.91	0.006**	44	175.42 ± 80.57	0.011*	48	149.36 ± 89.29	0.116	0.394	0.159	0.575
AAG	16	162.94 ± 104.27[a]		23	128.99 ± 44.82		14	133.93 ± 61.07		0.172	0.744	0.317
AGA	6	101.25 ± 38.86		18	118.98 ± 47.30		17	204.47 ± 101.69		0.418	0.021*	0.010*
AGG	31	105.11 ± 43.42[a]		78	167.38 ± 91.03		46	162.09 ± 93.00		< 0.001	0.755	0.001**
CAG	3	284.07 ± 99.61		5	101.47 ± 40.79		4	268.20 ± 39.25		–	–	–

p^{Ψ}represents one-way ANOVA; $p^{\#}$represents independent *t* test; *represents significance at 0.05 level; **represents significance at 0.01 level; p^{a} denotes for *p* value of comparison between Cat. I and Cat. II; p^{b} denotes for *p* value of comparison between Cat. II and Cat. III; p^{c} denotes for *p* value of comparison between Cat. I and Cat. III; [a]$p = 0.013$

to be associated with high sP-selectin levels in patients than controls, but the results were not statistically significant. Furthermore, C allele was observed as a risk allele but its frequency was very low (0.074) as compared to A allele (0.926).

The nsSNP rs6127 is located within the seventh CR domain (exon 11) of the P-selectin protein. This domain is shown to be important for binding of P-selectin with its ligand, but particular functional significance of this variation is still incompletely understood (Patel et al. 1995; Ruchaud-Sparagano et al. 1998). Unlike position of rs6136, the rs6127 is located slightly away from transmembrane domain, and thus we could speculate that substitution of aspartate at asparagine at position 562 may not influence the sP-selectin release much by conformational changes at the cleavage site of P-selectin protein. In the previous studies, rs6127 was reported to be associated with susceptibility to ischemic vascular complications and high prevalence of albuminuria in T2DM (Liu et al. 2005; Zalewski et al. 2006). Furthermore, it was also suggested to be linked with increased risk of incident CAD in whites and MI in French and Northern Ireland population (Tregouet et al. 2002; Volcik et al. 2006). In the present investigation, all genotypes of rs6127 were accounted for significantly elevated levels of sP-selectin. Moreover, variant allele was found to a risk allele for T2DM as well as very high vascular risk and protective for high vascular risk.

The other variant, rs6125, is located within the first consensus repeat of P-selectin, near to the epidermal growth factor-like domain of *SELP* and is important for specificity and ligand binding (Revelle et al. 1996). In a previous study, rs6125 polymorphism was found to be with Primary Ventricular fibrillation in MI but not with sP-selectin plasma levels (Elmas et al. 2010). There is no other report showing association of this polymorphism with development of any other disease condition. In the present analyses, heterozygous genotype in rs6125 was found to be associated with significantly high levels of sP-selectin but in contrast associated with protective effect. Moreover, variant genotype was conferring 12-fold very high vascular risk in T2DM patients.

Furthermore, AGG haplotype including wild alleles of all the variants was shown to have protective effect against disease development. Moreover, patients with AGG haplotype were observed to have significantly high sP-selectin levels as compared to patients with AAG in very high-risk category and patients with AGG in high- and moderate-risk category. On the other hand, CAG haplotype with two variant alleles (rs6136 and rs6127) was found to be associated with 4.56-fold risk of disease development. It is postulated that rs6136 and rs6127 variants, located within CR domain region of P-selectin protein, may affect binding of P-selectin to its ligand, resulting in a protein with high efficiency of leukocyte recruitment to endothelium (Volcik et al. 2007). Additionally, patients with haplotypes AAA and AGA were also found to have significantly high sP-selectin levels as compared to controls. Moreover, in moderate-risk category, AGA haplotype accounted for significantly elevated sP-selectin levels than other two risk categories. There was only a single report showing haplotype distribution of *SELP* variants (rs6136, rs6127, and rs6133) in T2DM patients (Zalewski et al. 2006). In contrast to the previous study, significant difference in three marker haplotype distribution was observed between patients and control. However,

haplotypes including both AA and AG genotypes were most frequent in both the studied groups in the present study as well as in the previous study. This is the first report on determination of the association of genotypes/haplotypes of *SELP* with sP-selectin levels in vascular risk categories. Thus, this makes the present study more effective for explaining genetic association of *SELP* polymorphism to sP-selectin levels and with vascular complication in T2DM.

However, there were some limitations in the present study. This study can be extended to larger sample size to screen significant number of patients with respect to vascular risk. In addition, being an expensive clinical parameter, it was not possible to perform baPWV measurements in the control group.

Conclusion

In the present study, we made an attempt to establish a useful link between *SELP* polymorphism, sP-selectin levels, and vascular risk in T2DM patients. The studied SNPs of *SELP* have shown significant association with sP-selectin levels as well as vascular risk in T2DM patients. The variant allele of rs6136 was conferring 2.4-fold risk of disease development but no association with vascular risk in T2DM patients. Moreover, the variant allele of rs6127 was found to be associated with 1.5-fold risk of disease development as well as 1.88-fold very high vascular risk in T2DM. Furthermore, the polymorphism that occurred in consensus repeat region near the transmembrane region could induce a conformational change in the P-selectin and may affect the sP-selectin levels. The variation at position 562 from asparagine to aspartate and at 168 from valine to methionine may lead to higher levels of sP-selectin. Thus, the present study shows that P-selectin polymorphisms and levels are probably a promising diagnostic tool in depicting new aspects in understanding molecular T2DM susceptibility and risk of vascular complications. However, the exact pathophysiological mechanisms and further clinical implications of these findings require further investigations.

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Author Contributions RK carried out all the biochemical as well as statistical analysis and also drafted the manuscript, the other authors, JS and MK, critically reviewed the manuscript, and RK has provided the patient samples as well as information regarding their disease history.

Compliance with Ethical Standards

Conflict of interest All the authors state no conflict of interest in the manuscript.

Ethics Approval Written voluntary informed consent was obtained from all the study participants and the study protocol was approved by ethics committee of Guru Nanak Dev University, Amritsar (PB), India, according to Indian Council of Medical Research guidelines (ICMR 2006) adapted from declaration of Helsinki (2004).

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