Introduction

P-selectin is a 140-kDa integral membrane glycoprotein located in the alpha granules of platelets and the Weibel-Palade bodies of endothelial cells (1). P-selectin is a member of the selectin family that binds to a dimeric mucin (P-selectin glycoprotein ligand-1 [PSGL-1]) expressed on the surface of leukocytes. P-selectin-PSGL-1 interaction is involved in leukocyte adhesion to endothelial cells and platelets. P-selectin is expressed on the platelet or endothelial surface only upon cell activation (3). A soluble form has been identified in plasma and its level was found to increase by the cleavage of the membrane bound form from the cell surface (4). Elevated level of the soluble P-selectin (sP-selectin) has been found in a number of different disorders like diabetes mellitus or ischemic cardiovascular disease (5–9). It is still a matter of debate whether sP-selectin is a causative factor in atherosclerotic or thrombotic processes or merely a marker of platelet activation (10).

The P-selectin gene is located on chromosome 1q21 to 1q24 and is highly polymorphic (11). Among the 13 gene missense polymorphisms, the Thr715Pro variant has been most intensively studied (11–17). Contradictory data have been published about the association of Thr715Pro polymorphism with cardiovascular disease (CVD) (18). The presence of Pro715 allele has been shown to have a “protective” effect on myocardial infarction (MI) in two extensive studies (11, 12), but some authors re-

Summary

Increased levels of soluble P-selectin (sP-selectin) have been shown in a number of different disorders, e.g. diabetes mellitus (DM) and cardiovascular disease (CVD). Several studies have attempted to demonstrate the association of the most intensively examined variant of P-selectin gene polymorphism (Thr715Pro) with sP-selectin levels in healthy subjects and in CVD, but contradictory data have been reported. To clarify the effect of Pro715 allele on the sP-selectin levels in type 2 DM, we analysed this polymorphism in diabetic patients and compared these data with sP-selectin levels. Type 2 DM patients \( n=119 \), 48 BMI-matched non diabetic individuals – consisting mostly of overweight subjects – and 57 healthy volunteers were included in the study. The Thr715Pro polymorphism was analysed by PCR-RFLP, while sP-selectin levels were measured by ELISA. Significantly elevated sP-selectin levels were found in both DM and in overweight subjects compared to healthy controls. We confirmed previous reports that in healthy Pro715 allele carriers lower sP-selectin levels could be measured; however, this difference was only significant in case of lean subjects. No significant difference was detected in sP-selectin level among DM and overweight individuals according to this genotype. However, significant difference was observed in sP-selectin levels in older DM patients compared to younger ones, but these levels were not accounted for by the Thr715Pro polymorphism. We suggest that in type 2 DM individuals, the significantly elevated sP-selectin levels are not due to the Thr715Pro P-selectin gene polymorphism.

Keywords

Platelet activation, soluble P-selectin, Thr715Pro P-selectin polymorphism, type 2 DM

Investigation of Thr715Pro P-selectin gene polymorphism and soluble P-selectin levels in type 2 diabetes mellitus

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Platelets and Blood Cells


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Financial support:
This study was supported by a „Mecenatura Grant“ 2002 of the Medical and Health Science Center at the University of Debrecen, OTKA grant T049392, and the Bolyai János fellowship.

Received November 6, 2006
Accepted after resubmission April 3, 2007
Prepublished online June 12, 2007
doi:10.1160/TH06–11–0628
ported no such effect (13, 14). Other polymorphisms (e.g. Ser290Asn, Asn562Asp) of this gene carried by the same haplotype were associated with an increased risk for MI in a study investigating French and Northern Irish populations (19). On the other hand, in a recent report (20), the Thr715Pro phenotype, together with Ser290Asn and Asn562Asp polymorphisms were excluded as major contributors to macrovascular complications in type 2 DM. Nevertheless, the exact association between the presence of mutated alleles or specific haplotypes of P-selectin gene and their impact related to sP-selectin levels in several diseases, is still unclear.

Previously, in the healthy Pro715 allele carriers significantly lower levels of sP-selectin were measured (15), but contradictory results were published in CVD patients, and only two studies investigated the relation of this polymorphism with type 2 DM (16, 20). The goal of this study was to establish the effect of the Thr715Pro P-selectin polymorphism on sP-selectin levels in type 2 DM patients and to compare these data with healthy and overweight subjects.

Materials and methods

Subjects
Healthy volunteers (n=57) with body mass index (BMI) <25 kg/m², type 2 diabetic patients (diagnosed according to WHO criteria, n=119) and a body mass index (BMI)-matched non-diabetic study group consisting of overweight and obese subjects (n=48, BMI-matched non-DM group) were recruited in the study. Patients were enrolled from the Outpatients Clinic of the 1st Department of Internal Medicine, University of Debrecen, Hungary. Type 2 DM patients were treated as required by antihyperglycaemic agents or diet, and none of them had thromboembolism. Other exclusion criteria were severe symptomatic vascular diseases such as angina, intermittent claudication, transient ischemic attack, malignancy, pregnancy, impaired liver or renal function and infectious diseases. Healthy controls did not suffer from cardiovascular, neoplastic, metabolic or inflammatory disease, as observed by careful examination and routine laboratory tests. Overweight and obese subjects were without history or clinical evidence of diabetes; however, six individuals displayed impaired glucose tolerance and 15 subjects had mild hyperlipoproteinaemia. An additional classification of type 2 diabetic patients was also made, that allowed a pairwise comparison of a subgroup of diabetic patients (age-matched DM group, n=57) with healthy controls.

To further dissect the possible association of the Thr715Pro polymorphism with the DM group, all patients were enrolled into two subgroups according to age, gender, smoking habit and the median level of demographical parameters: subjects with values below median (lower half) and patients with values equal to or above the median (upper half). The levels of sP-selectin in these subgroups were analysed and the effect of the Thr715Pro polymorphism on sP-selectin levels was examined.

This study is a cross-sectional, analyst-blinded case-control study. All participants gave written informed consent. The study was approved by the Ethical Committee of the University of Debrecen.

Blood samples and flow cytometry
Venous blood was obtained into Vacutainer tubes containing 0.105 M sodium citrate (Becton Dickinson, San Jose, CA, USA) by atrumatic venipuncture when DM patients attended the Outpatients Clinic for follow-up appointments. Blood sampling conditions were designed to avoid artefactual activation of platelets during phlebotomy. Within 2 hours (h) of collection, 40 µl of all samples were fixed in 1 ml 1% paraformaldehyde and kept at room temperature (RT) for minimum 1 h. Platelet number was determined in each case by Advia 120 Hematology System (Bayer Diagnostics, Tarrytown, NJ, USA). Fixed whole blood samples were centrifuged at 1,300 x g for 15 minutes (min) at RT. The pellet was washed in 1 ml phosphate-buffered saline (PBS), then centrifuged as above and finally resuspended in PBS.

Platelets were identified by a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to GPIIX (CD42a). Platelet activation was detected by phycoerythrin (PE)-labeled anti-P-selectin (CD62-PE, Becton Dickinson). Fixed platelets were incubated with saturating concentrations of FITC- and PE-labeled antibodies for 20 min in the dark at RT. As a control for immunolabeling with anti-CD62, platelets were incubated with PE-coupled non-immune mouse IgG1 antibody. Ten thousand dual-color labeled platelet events were acquired on a FACSCalibur flow cytometer by using the CellQuest 3.2 software (Becton Dickinson). Results were expressed as percentage of double positive platelets.

Laboratory assays
Plasma soluble P-selectin was analysed by ELISA (R&D Systems, Minneapolis, MN, USA) commercial kits following the manufacturer’s instructions. All plasma samples were centrifuged immediately at 2,000 x g for 15 min at RT, aspirated and stored at −70°C until analysis. This procedure was completed within 30 min of blood drawing. Blood glucose, total cholesterol, and triglyceride values were measured on Hitachi analyser (Roche, Mannheim, Germany) and LDL-cholesterol levels were calculated by the Friedewald formula. HbA1c was measured by HPLC (BioRad, Hercules, CA, USA) and the fibrinogen levels were determined by the Clauss-method on Stago Compact (Stago, Asnières, France). CRP was measured by a turbidimetric assay on Integra 400 analyser (Roche).

Genetic analysis
Genetic analysis of Thr715Pro polymorphism was performed as previously described (15) with some minor modifications. Genomic DNA was extracted from anticoagulated blood by QIaamp DNA blood kit (Qiagen, Hilden, Germany). Primers were designed by Primer3 and used to amplify exon 13 of P-selectin gene. The sequences of the oligonucleotide primers were 5’-TTTCTGAGCCTGTAATGC-3’ and 5’-ATTTACCTTGGCACAGTTGG-3’. Polymerase chain reaction (PCR) was performed in a total volume of 50 µl containing 100 ng of DNA, 10 pmol of each primer, 200 µM dNTPs, 1.5 mM MgCl2, 10% DMSO and 2 units Taq DNA polymerase (Roche). In restriction fragment length polymorphism (RFLP), after the initial denaturation at 94°C for 5 min, amplification was carried out for 40 cycles of 94°C for 30 seconds (sec), 60°C for 60 sec and 72°C for 60 sec, and the final extension at 72°C for 10 min. The PCR product (198 bp) was digested by EcoR11 (Fermentas, Vilnius,
Lithuania) and the digested products were run on a 3% agarose gel and visualized under UV light by ethidium bromide staining. In the presence of Thr715Pro mutation, a new (163 bp) DNA product could be detected during analysis.

**Statistical analysis**

Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Most outcome continuous parameters were non-normally distributed; therefore analyses were performed on log transformed data for Student’s independent t-test analysis. Differences in various parameters among study groups were tested using analysis of variance and chi²-test as appropriate. Deviations from the Hardy-Weinberg equilibrium was analysed using the chi²-test in each group. Multiple regression analysis was computed for checking the association of baseline characteristics with sP-selectin level. Univariate analysis of variance was used to adjust for significant variables and check for differences in sP-selectin levels within the different genotypes. P<0.05 was regarded as statistically significant. Statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

**Results**

There were marked differences in the baseline demographical and laboratory parameters and platelet activation markers between study groups (Table 1). Besides BMI, age also differed significantly between DM patients and healthy controls, since we could only enroll younger volunteers. Both the plasma level of sP-selectin and the percentage of platelet P-selectin were significantly increased in the BMI-matched non-DM subjects and in the type 2 DM group upon comparison with healthy individuals (Table 1).

As follows the frequency of Thr715Pro P-selectin genotype did not vary significantly between study groups. Healthy: 77.2% (AA, n=44); 22.8% (AC, n=13); BMI-matched non DM: 81.3% (AA, n=39), 18.7% (AC, n=9); Type 2 DM: 74.8% (AA, n=89), 23.5% (AC, n=28), 1.7% (CC, n=2). All groups were in Hardy-Weinberg equilibrium. There was no subject with CC genotype in the healthy and BMI-matched non-DM groups. The CC genotype was rare in DM group, thus these subjects were pooled into the subgroup of patients with the AC genotype.

In healthy Pro715 allele carriers, lower P-selectin levels could be measured, but the difference was not significant. In type 2 DM patients, sP-selectin levels were significantly increased compared to controls, but no difference (p=0.642) was observed in the sP-selectin levels between the two genotypes (Fig. 1).

The association of BMI and sP-selectin levels was analysed in all non-diabetic subjects. The levels of sP-selectin in healthy carriers of the C allele with BMI < 22.4 kg/m² were significantly (p=0.004) lower compared to controls with the AA genotype. However, this difference was not detectable in healthy subjects with higher BMI (≥ 22.4 kg/m²). In BMI-matched non-DM subjects, sP-selectin levels were elevated compared to healthy controls, with no difference (p=0.777) between AA and AC carriers (Fig. 2).

In our DM group, age did affect the sP-selectin levels. There was a significant difference (p<0.05) in sP-selectin levels among older (median age ≥ 54 years, 112.6 ± 61.7 ng/ml) and younger (median age < 54 years, 69 ± 47.1 ng/ml) patients when all DM

<table>
<thead>
<tr>
<th>Healthy (n=57)</th>
<th>Type 2 DM (n =119)</th>
<th>BMI-matched non-DM (n =48)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic parameters (median, range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>35.1</td>
<td>65.5</td>
<td>37.5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>38 (22–61)</td>
<td>54 (18–76)</td>
<td>45.5 (20–63)</td>
</tr>
<tr>
<td>BMI (kg/m2, mean ± SD)</td>
<td>22.1 ± 1.9</td>
<td>31 ± 6.4</td>
<td>29.8 ± 2.8</td>
</tr>
<tr>
<td>Duration of DM (years)</td>
<td>0</td>
<td>8 (1–32)</td>
<td>0</td>
</tr>
<tr>
<td>Never smokers (%)</td>
<td>95</td>
<td>76</td>
<td>69</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>5</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>Hypertensive (%)</td>
<td>0</td>
<td>82</td>
<td>54</td>
</tr>
<tr>
<td>Laboratory parameters (mean, 1st and 3rd quartile)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>4.6 (4.25–4.95)</td>
<td>9.4 (6.7–12)</td>
<td>5.1 (4.8–5.4)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.1 (5–5.7)</td>
<td>7.8 (6.7–8.9)</td>
<td>5.6 (5.2–5.8)</td>
</tr>
<tr>
<td>Total Cholesterol (mM)</td>
<td>4.7 (4.1–5.4)</td>
<td>5.4 (4.4–6.6)</td>
<td>5.4 (4.6–6.2)</td>
</tr>
<tr>
<td>LDL-Cholesterol (mM)</td>
<td>1.9 (1.4–2.3)</td>
<td>2.8 (2.2–3.4)</td>
<td>2.4 (1.4–3.5)</td>
</tr>
<tr>
<td>Triglyceride (mM)</td>
<td>0.9 (0.6–1.1)</td>
<td>3.3 (1.3–3.2)</td>
<td>1.7 (0.9–2)</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.0 (2.5–3.3)</td>
<td>4.1 (3.2–4.8)</td>
<td>3.6 (2.9–4.1)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.8 (0.6–2.1)</td>
<td>5.8 (1.7–7)</td>
<td>4.9 (1.6–4.2)</td>
</tr>
<tr>
<td>Flow cytometric parameters (mean, 1st and 3rd quartile)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface P selectin (%)</td>
<td>1.0 (0.4–1.2)</td>
<td>1.8 (0.7–2.5)</td>
<td>1.9 (0.6–2.3)</td>
</tr>
<tr>
<td>Soluble P selectin (ng/ml)</td>
<td>39.4 (25.6–49.1)</td>
<td>91.3 (43.8–126.9)</td>
<td>54.4 (42–61)</td>
</tr>
</tbody>
</table>

Table 1: Characteristics of all study groups.
subjects were pooled, but genotype did not influence P-selectin level in the subgroups studied (Table 2). Since age significantly affected sP-selectin levels, we compiled two age-matched groups and created 57 pairs; one consisting of DM patients and the other of healthy controls. P-selectin levels were significantly ($p < 0.001$) higher in the DM group (mean: 79.4 ng/ml, quartiles: 39.7–121 ng/ml) as compared to the age matched healthy control group (mean: 46.6 ng/ml, quartiles: 33.2–57.6 ng/ml).

The sP-selectin levels did not vary with gender in the DM group. There was no statistically significant difference between male (94 ± 65.5 ng/ml) and female (86.2 ± 44.6 ng/ml) patients, and no variation could be demonstrated when the different sexes were analysed according to genotype. The sP-selectin levels did not differ with the smoking status either, since no statistically significantly difference was found between 'never smoker' DM patients (86.2 ± 49.7 ng/ml) and 'current' DM smokers (105 ± 80 ng/ml). Here, too, no difference was found in the sP-selectin levels according to genotypes (Table 2).

### Table 2: sP-selectin levels in type 2 DM patients according to the Thr715Pro genotype.

<table>
<thead>
<tr>
<th>Groups</th>
<th>AA genotype</th>
<th>AC+CC genotype</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DM group (n=119)</td>
<td>89.9 (43.8–130)</td>
<td>95.6 (42.6–122.6)</td>
<td>0.642</td>
</tr>
<tr>
<td>Males (n=78)</td>
<td>91.5 (42.2–132.5)</td>
<td>102.3 (40.6–163.6)</td>
<td>0.726</td>
</tr>
<tr>
<td>Females (n=41)</td>
<td>86.4 (44.2–128.9)</td>
<td>85.5 (64.1–120.3)</td>
<td>0.746</td>
</tr>
<tr>
<td>Never smokers (n=86)</td>
<td>87.8 (43.9–127.7)</td>
<td>80 (42.6–115.4)</td>
<td>0.901</td>
</tr>
<tr>
<td>Current smokers (n=27)</td>
<td>90.9 (41.2–159.4)</td>
<td>126 (63.3–171.2)</td>
<td>0.293</td>
</tr>
<tr>
<td>Older (n=61)*</td>
<td>111.4 (63–162.7)</td>
<td>117 (81.7–122.7)</td>
<td>0.607</td>
</tr>
<tr>
<td>Younger (n=58)*</td>
<td>64.7 (36.4–86.1)</td>
<td>79.2 (39.5–125.4)</td>
<td>0.412</td>
</tr>
<tr>
<td>Higher BMI (n=60)**</td>
<td>82.5 (40–130.2)</td>
<td>91.8 (39.9–122.9)</td>
<td>0.523</td>
</tr>
<tr>
<td>Lower BMI (n=56)**</td>
<td>94.9 (47.2–129.5)</td>
<td>99.9 (60.2–98.7)</td>
<td>0.860</td>
</tr>
<tr>
<td>Longer DM duration (n=59)***</td>
<td>92.5 (44.2–132.6)</td>
<td>88.7 (53.5–113.9)</td>
<td>0.902</td>
</tr>
<tr>
<td>Shorter DM duration (n=58)***</td>
<td>88.5 (41.2–130.3)</td>
<td>107.4 (39.5–163.6)</td>
<td>0.499</td>
</tr>
</tbody>
</table>

Figure 1: Comparison of soluble P-selectin levels in healthy and type 2 DM individuals according to the Thr715Pro genotype. Significant difference in sP-selectin level between AA and AC+CC genotypes was found in case of healthy controls by Student’s independent t-test.

Figure 2: Soluble P-selectin levels in all non-diabetic subjects with different BMI values. In the group on the right the BMI matches with that of the type 2 DM patients. Significant difference was found only in the lower half of healthy controls according to BMI.

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In the DM group, multiple regression analysis was used to test for significant association between sP-selectin and all the continuous variables. Only age was found to be significant in this response. After adjustment for this parameter along with the categorical variables (gender, blood pressure, smoking habit), no significant (p=0.204) difference was determined in sP-selectin level by univariate analysis of variance among patients with AA and AC+CC genotypes (data not shown).

Discussion

This study revealed that in type 2 DM individuals, the significantly elevated sP-selectin values did not vary according to the Thr715Pro P-selectin gene polymorphism. No significant difference was found in the effect of this polymorphism in the various subgroups of DM patients. We could confirm previous reports that in healthy Pro715 allele carriers a lower sP-selectin levels could be measured, but this was only true in lean subjects. No difference was detected among DM and overweight individuals according to genotype. Although significant difference was observed in sP-selectin level among older and younger DM patients, sP-selectin levels were not accounted for by this polymorphism.

In the last decade, genetic analyses of P-selectin polymorphisms in healthy individuals, patients with CVD (11–14, 19) and even stroke patients (17) were highly in focus. These studies provided additional evidence that the P-selectin gene is highly polymorphic (11) and some of the 13 missense mutations may have an association with sP-selectin levels (13–15). Among these polymorphisms, mostly the Thr715Pro seemed to have a substantial effect on the sP-selectin levels (14, 15, 17). It is located in the last consensus repeat region of the P-selectin molecule, and it is possible that the substitution of threonine for proline may induce a conformational change in the precursor protein which may influence its intracellular transportation and secretion (14). In addition, it is still unknown whether the Pro715 allele is associated with the reduction in the membrane-bound form of P-selectin (14) or shedding of distal fragments of the receptor (20). Although Miller et al. (15) found that the Pro715 allele is associated with reduced sP-selectin levels in white and south asian healthy individuals, it is still controversial if this effect may be “protective” on survival following acute thrombotic events by decreasing the risk for myocardial infarction (11, 12, 19) or sP-selectin levels (13). Furthermore, Carter et al. (14) could not confirm this beneficial effect of the Pro715 allele in relation to MI or severe stenosis; however, lower levels of plasma sP-selectin were measured in those with Pro715 allele compared with those who were homozygous for Thr715. These findings confirmed the results of Barbaux et al. (13), though in this study sP-selectin levels were determined from serum samples. On the other hand, Pro715 allele was not found to be associated with higher prevalence and greater degree of albuminuria as a hallmark of endothelial dysfunction in type 2 DM (16). No association was observed in the haplotype frequency of Thr715Pro, Asn562Asp and Ser290Asn polymorphisms with CVD complications in type 2 DM patients (20). Nevertheless, the exact association between the presence of Pro715 allele or specific haplotypes of this P-selectin gene and their impact related to sP-selectin levels in several diseases is still unclear.

Although several polymorphisms have also been described in the promoter region of the P-selectin gene, no significant associations of –1817 T/C, –1969 G/A, and –2123 C/G polymorphisms with the sP-selectin levels were seen (14). We investigated only the Thr715Pro polymorphism, since this proved to be the most relevant to influence sP-selectin levels due to its localization and our goal was to establish its effect on sP-selectin level in type 2 DM patients and two different control groups.

The levels of sP-selectin did not vary according to the Thr715Pro P-selectin polymorphism in type 2 DM group. Similar results were observed between sP-selectin level and the presence of this genotype in the BMI-matched non-diabetic control group. No significant difference was seen in several subgroups of DM patients according to different baseline characteristics.

Previous studies have shown age- and sex-dependence of sP-selectin levels (13, 14). However, another report found no correlation between sP-selectin levels and age in type 2 DM (5). In our DM group, males displayed elevated values compared to females, but the difference was not significant. We found that sP-selectin values were significantly increased in diabetic patients, but this difference was not modulated by the presence of the Pro allele.

In the DM group, no significant association of sP-selectin level was found with BMI. There was no such association even after subgrouping them according to their genotype. On the contrary, in the healthy group, carriers of Pro715 allele with BMI < 22.4 kg/m² had significantly lower sP-selectin levels compared with subjects possessing the wild genotype; however, this phenomenon was not detectable in healthy individuals with BMI ≥ 22.4 kg/m². In overweight individuals, this effect on sP-selectin level was undetectable. Barbaux et al. (13) also revealed a weak correlation between sP-selectin levels and BMI only in their healthy controls, and no association was found in CVD patients.

We determined no significant difference in sP-selectin levels in 'never smoker' DM patients compared with 'current' DM smokers. Some studies observed an association between Pro715 allele and lower sP-selectin levels, but this was true only in nonsmokers with CVD (13, 14). However, others failed to find this dependence in a healthy cohort (15).

In our DM group, only the age was found to be significantly associated with sP-selectin. After adjustment for age and the categorical variables, no significant difference was found in sP-selectin. In our study, the number of patients with homozygous genotype was very low, as such these cases were pooled with the heterozygous cases.

We conclude that in type 2 DM patients, the significantly elevated P-selectin values are not affected by the Thr715Pro P-selectin gene polymorphism. Furthermore, even in non-diabetic subjects with BMI comparable to the DM group, P-selectin values are elevated, and this elevation is not modulated by the presence of Thr715Pro. Further studies are required to investigate the significance of this polymorphism and the haplotypes of additional P-selectin polymorphisms in type 2 DM and in obesity.

Acknowledgements

We thank Dr. Giovanni de Gaetano (Campobasso, Italy) for useful comments. The assistance of Anikó Győrfi Veszprémi, Erika Dzsudzsák, Zsuzsa Simon, Zsolt Karányi, Ildikó Beke Debreceni and Ildikó Trefán are greatly acknowledged.
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