Increased levels of platelet activation markers are positively associated with carotid wall thickness and other atherosclerotic risk factors in obese patients

Éva Csongrádi1,2; Béla Nagy Jr3; Tibor Fulop5; Zsuzsa Varga1; Zsolt Karányi1; Mária T. Magyar3; László Oláh5; Mária Papp6; Andrea Faccskó4; János Kappelmayer2; György Paraghi1; Miklós Káplár1
11st Department of Medicine, Medical and Health Science Center, University of Debrecen, Hungary; 12th Department of Medicine, Medical and Health Science Center, University of Debrecen, Hungary; 1Department of Cardiovascular-Renal Research, University of Mississippi Medical Center, Jackson, Mississippi, USA; 1Department of Clinical Biochemistry and Molecular Pathology, Medical and Health Science Center, University of Debrecen, Hungary; 2Department of Physiology & Biophysics, Center for Excellence in Cardiovascular-Renal Research, University of Mississippi Medical Center, Jackson, Mississippi, USA; 3Department of Neurology, Medical and Health Science Center, University of Debrecen, Hungary; 4Department of Medicine, Division of Nephrology, University of Mississippi Medical Center, Jackson, Mississippi, USA; 5Department of Neurology, Medical and Health Science Center, University of Debrecen, Hungary; 6Department of Ophthalmology, Medical and Health Science Center, University of Debrecen, Hungary.

Summary
The role of platelets in the development of atherosclerosis and obesity-related prothrombotic state is still under investigation. In this cross-sectional cohort study, we measured the levels of different platelet activation markers and evaluated their relationship with carotid intima-media thickness (IMT) along with other atherosclerotic risk factors in obese patients with or without atherosclerotic co-morbidities. We enrolled 154 obese patients, including 98 with either hypertension, type 2 diabetes mellitus or dyslipidaemia, 56 without these co-morbidities and 62 age- and sex-matched healthy controls. Platelet P-selectin expression and the number of platelet-derived microparticles (PMPs) were measured by flow cytometry; soluble P-selectin levels were analysed by ELISA and Thr715Pro P-selectin polymorphism was determined by PCR-RFLP. Carotid IMT was examined by ultrasonography. The levels of platelet activation parameters were significantly elevated in all obese subjects with increased carotid IMT compared to healthy controls. There was no effect of Thr715Pro genotype on soluble P-selectin levels in obese individuals contrary to normal subjects. Significant and positive association was revealed between carotid IMT and platelet P-selectin (p=0.0001), soluble P-selectin (p=0.039) and PMP (p=0.0001) levels. After adjusting for multiple variables, independent association was found between soluble P-selectin and fibrinogen (p=0.007), PMP levels and body mass index (p<0.0001) as well as platelet P-selectin and carotid IMT (p=0.012) plus plasminogen activator inhibitor-1 (p=0.009). In conclusion, P-selectin and PMP levels showed positive associations with abnormal carotid IMT and other risk factors in obesity suggesting a critical role of enhanced platelet reactivity in atherosclerotic wall alteration.

Keywords
Atherosclerosis, platelets, carotid intima-media thickness, P-selectin, microparticles, obesity, Thr715Pro polymorphism

Introduction
Enhanced platelet activation has been observed to occur in a number of diseases with atherosclerosis such as coronary artery disease (1–3), cerebrovascular disease (4, 5) and type 2 diabetes mellitus (DM) (6, 7); however, only limited data are available in terms of obesity. Previous studies demonstrated that activated platelets played a functional role in the initiation of atherosclerosis in mice (8, 9). Furthermore, co-morbidities like hypertension, hyperglycaemia or dyslipidaemia are additional major risk factors for atherosclerotic disease associated with platelet hyperactivity in humans (10–12). Activated platelets induce P-selectin (CD62) expression, an adhesive membrane glycoprotein from α-granules resulting in transient attachment of platelets to leukocytes and endothelial cells during atherothrombotic and inflammatory processes (13). In the meantime, secreted soluble P-selectin circulates at an increased level in the plasma and is involved in further cellular interactions (14). Microparticles are small membrane particles shed from activated blood cells and endothelial cells promoting coagulation and inflammation via their procoagulant surface properties (15). Elevated numbers of platelet-derived microparticles (PMPs) were found in vascular diseases and type 2 DM (16).

Abnormalities in the coagulation processes with higher levels of prothrombotic factors (17) and enhanced levels of platelet acti-
Platelet activation markers and IMT in obesity

Csongrádi et al. Platelet activation markers and IMT in obesity

Materials and methods

Subjects

This was a cross-sectional, analyst-blinded, case-control study involving 154 obese subjects compared with 62 age- and sex-matched healthy controls. All participants gave written informed consents. The study was approved by the Ethics Committee of the University of Debrecen. Obese subjects were enrolled from the 1st and 2nd Departments of Internal Medicine Outpatients Clinic at the University of Debrecen. Exclusion criteria for enrollment included active angiopathy, intermittent claudication, transient ischemic attack, malignancy, pregnancy, impaired liver or renal function and infectious diseases.

Hypertension was defined in accordance to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7), issued by the U.S. National Heart, Lung and Blood Institute (NHLBI), as an office-based measured blood pressure of ≥140/90 mmHg (after 5 minutes [min] of rest, with the arm supported and properly sized and placed cuff) and/or the presence of antihypertensive drug therapy. In accordance with the American Diabetes Association 2004 criteria, type 2 DM was defined by a measured fasting glucose of ≥7.0 mM, or use of insulin and/or oral hypoglycaemic agents. Presence of dyslipidaemia was defined as an elevation in measured fasting total cholesterol (≥25.2 mM), LDL-cholesterol (≥23.4 mM), and/or use of lipid-lowering medications. The oral glucose tolerance test (OGTT) was performed by collecting venous blood samples after an overnight fasting and after 2 hours (h) following an oral load of 75 g glucose. Insulin resistance index was assessed by using the homeostasis model assessment of insulin resistance (HOMA-IR) as previously described (29). Plasma atherogenic index was calculated as log(triglyceride/HDL-cholesterol) (30). Normal controls were recruited among healthy volunteers from the Departments of Medicine and Ophthalmology who underwent a detailed medical history, physical examination and routine laboratory tests and were free of any cardiovascular, cancer, metabolic or inflammatory disease.

Blood sampling and flow cytometric analysis

Venous blood samples for platelet analysis were obtained between 8:00–10:00 a.m. by atraumatic venepuncture into Vacutainer® tubes containing 0.105 M sodium citrate (Becton Dickinson, San Jose, CA, USA) when patients attended the Outpatients Clinic for follow-up appointments. Blood sampling conditions were designed as previously described (7). Platelet number was determined in each case by Advia 120 Hematology System (Bayer Diagnostics, Tarrytown, NJ, USA). Briefly, 40 μl of all samples were fixed in 1 ml 1% paraformaldehyde and kept at room temperature (RT) for minimum 1 h. Platelets were identified by a fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody to GPIIX (CD42a). Platelet activation was detected by phycoerythrin (PE)-labelled anti-P-selectin (CD62-PE, Becton Dickinson). Fixed platelets were incubated with saturating concentrations of antibodies for 20 min in the dark at RT. As a control for immunolabelling with anti-CD62 antibody, platelets were incubated with PE-coupled non-immune mouse IgG. A total of 10,000 dual-colour labelled platelet events were acquired on a FACSCalibur flow cytometer by using the CellQuest 3.2 software (Becton Dickinson). Results were expressed as the percentage of double positive platelets. The number of PMPs was analysed by the standardised methods we used before (31). Briefly, platelet-poor plasma (PPP) was obtained from whole blood by centrifugation at 1,550 x g for 20 min at 25°C. Five hundred μl of PPP was spun down at 13,000 x g for 2 min to get rid of platelet debris, and then centrifuged at 16,100 x g for 30 min at 25°C to isolate PMPs. We used fluorescent beads Tru-COUNT (Becton Dickinson) with standard size and amount for enumeration of PMPs below 1 μm. Beads were first processed, and then clinical samples were measured within a standard collection time (30 seconds [sec]). The number of PMPs was calculated based on the event count from the bead tube collected for the same time period. PMPs were gated into a restricted area by forward scatter (FSC) and side scatter (SSC) parameters and then identified by their CD42a positivity.
Table 1: Overall study groups demographics and vascular risk factors.

<table>
<thead>
<tr>
<th>Demographic parameters</th>
<th>Healthy controls (n=62)</th>
<th>Obese patients Total (n=154)</th>
<th>Without atherosclerotic co-morbidity (n=56)</th>
<th>With atherosclerotic co-morbidity (hypertension and/or type-2 DM and/or dyslipidaemia) (n=98)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39.7 ± 10.0</td>
<td>40.6 ± 11.1</td>
<td>40.4 ± 11.8</td>
<td>39.5 (30–51)</td>
</tr>
<tr>
<td>Gender, female/male (%)</td>
<td>66.1/33.9</td>
<td>61.7/38.3</td>
<td>64.3/35.7</td>
<td>60.2/39.8</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.1 ± 1.96</td>
<td>22.4 (20.6–23.9)</td>
<td>38.2 ± 7.7</td>
<td>37.0 (31.9–43.0)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>79.5 ± 8.15</td>
<td>79 (73–86)</td>
<td>118.7 ± 18.3</td>
<td>116 (105–128)</td>
</tr>
<tr>
<td>Never smokers (%)</td>
<td>71.0</td>
<td>52.6</td>
<td>53.6</td>
<td>52.1</td>
</tr>
<tr>
<td>Previously smokers (%)</td>
<td>8.0</td>
<td>27.0</td>
<td>35.7</td>
<td>21.9</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>21.0</td>
<td>20.4</td>
<td>10.7</td>
<td>26.0</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>0</td>
<td>49.4</td>
<td>0</td>
<td>77.6</td>
</tr>
<tr>
<td>Type-2 diabetes mellitus (%)</td>
<td>0</td>
<td>20.1</td>
<td>0</td>
<td>31.6</td>
</tr>
<tr>
<td>Dyslipidaemia (%)</td>
<td>0</td>
<td>55.8</td>
<td>0</td>
<td>87.8</td>
</tr>
<tr>
<td>Vascular risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>115.2 ± 9.75</td>
<td>120 (110–120)</td>
<td>128.1 ± 15.8</td>
<td>130 (120–138)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>72.7 ± 7.17</td>
<td>70 (70–80)</td>
<td>80.9 ± 7.3</td>
<td>80 (80–80)</td>
</tr>
<tr>
<td>Carotid intima-media thickness (mm)</td>
<td>0.50 ± 0.08</td>
<td>0.49 (0.44–0.56)</td>
<td>0.58 ± 0.12</td>
<td>0.58 (0.49–0.67)</td>
</tr>
</tbody>
</table>

Genetic analysis of the Thr715Pro polymorphism of P-selectin gene was performed as previously reported (7). Briefly, DNA was extracted from anticoagulated blood by QIAamp DNA blood kit (Qiagen, Hilden, Germany). Primers with the 5′-TTTCTGACGCTGTGAAAATGC-3′ and 5′-ATTGTACCTTGGCAGGTTGG-3′ sequences were used. 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 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 EcoR111 (Fermentas, Vilnius, Lithuania) and the digested products were run on a 3% agarose gel and visualised under ultraviolet (UV) light by ethidium bromide staining. In the presence of Thr715Pro mutation, a new (163 bp) DNA product could be detected during analysis.

Laboratory assays

We analysed the plasma levels of soluble P-selectin by using commercially available ELISA (R&D Systems, Minneapolis, MN, USA) kit according 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. Blood glucose, total cholesterol, LDL- and HDL-cholesterol and triglyceride levels were measured on Hitachi analyser (Roche, Mannheim, Germany). HbA₁c was measured by HPLC (BioRad, Hercules, CA, USA), fibrinogen by Clauss-method, while plasminogen activator inhibitor (PAI)-1 levels and factor VIII activity were determined on Stago Compact (Stago, Asniéres, France). CRP was measured by a turbidimetric assay on Integra 800 analyser (Roche). Serum insulin concentration was measured by commercially available radioimmunoassay kit (MP Biomedicas, Orangeburg, NY, USA).
Table 2: Laboratory parameters associated with atherosclerotic risk and platelet activation markers in the study groups. n: case-number. \(x \pm SD: mean \pm standard \ deviation. m (q1–q3): median (lower-upper quartiles). 1Significant differences between healthy control and total obese groups. 2Significant differences between healthy controls and obese patients without atherosclerotic co-morbidity. 3Significant differences between healthy controls and obese patients with atherosclerotic co-morbidity. 4Significant differences between the two obese subgroups related to atherosclerotic co-morbidity. HbA1C=Haemoglobin A1C; HOMA-IR=insulin resistance homeostasis model assessment; LDL-cholesterol=low-density lipoprotein cholesterol; HDL=high-density lipid cholesterol; hs-CRP=high-sensitivity C-reactive protein; PAI-1=plasminogen activator inhibitor-1; PMP=platelet-derived (CD42a positive) microparticles.

<table>
<thead>
<tr>
<th>Laboratory parameters</th>
<th>Healthy controls (n=62)</th>
<th>Obese patients (n=154)</th>
<th>Without atherosclerotic co-morbidity (n=56)</th>
<th>With atherosclerotic co-morbidity (hypertension and/or type-2 DM and/or dyslipidaemia) (n=98)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (mM)</td>
<td>4.59 ± 0.54</td>
<td>4.6 (4.3–5.0)</td>
<td>6.23 ± 2.67</td>
<td>5.3 (4.9–6.6)</td>
</tr>
<tr>
<td>HbA1C(%)</td>
<td>5.43 ± 0.43</td>
<td>5.4 (5.2–5.7)</td>
<td>6.25 ± 1.44</td>
<td>5.8 (5.4–6.4)</td>
</tr>
<tr>
<td>Fasting plasma insulin (μU/l)</td>
<td>16.0 ± 7.94</td>
<td>14.9 (10.9–21.1)</td>
<td>42.2 ± 26.3</td>
<td>35.2 (25.1–47.7)</td>
</tr>
<tr>
<td>Triglyceride (mM)</td>
<td>0.93 ± 0.42</td>
<td>0.9 (0.6–1.1)</td>
<td>1.99 ± 2.16</td>
<td>1.5 (1.1–2.2)</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>4.47 ± 0.67</td>
<td>4.3 (4.0–5.0)</td>
<td>5.22 ± 1.26</td>
<td>5.1 (4.55–5.8)</td>
</tr>
<tr>
<td>LDL-cholesterol (mM)</td>
<td>2.47 ± 0.77</td>
<td>2.4 (2.0–3.1)</td>
<td>3.0 ± 0.87</td>
<td>2.9 (2.5–3.6)</td>
</tr>
<tr>
<td>HDL-cholesterol (mM)</td>
<td>1.75 ± 0.51</td>
<td>1.7 (1.4–2.0)</td>
<td>1.39 ± 0.39</td>
<td>1.3 (1.1–1.6)</td>
</tr>
<tr>
<td>Atherogen index</td>
<td>2.78 ± 0.72</td>
<td>2.75 (2.25–3.25)</td>
<td>4.08 ± 1.31</td>
<td>4.0 (3.13–4.78)</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>1.51 ± 1.59</td>
<td>0.93 (0.50–2.10)</td>
<td>7.76 ± 7.29</td>
<td>5.09 (2.70–10.34)</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>2.94 ± 0.53</td>
<td>2.95 (2.59–3.28)</td>
<td>4.14 ± 0.90</td>
<td>4.12 (3.38–4.79)</td>
</tr>
<tr>
<td>Factor VIII activity (%)</td>
<td>165.9 ± 55.7</td>
<td>159 (125–200)</td>
<td>206.1 ± 78.1</td>
<td>190 (146–254)</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>47.2 ± 14.2</td>
<td>47.5 (36.6–58.7)</td>
<td>83.1 ± 15.7</td>
<td>86.6 (73.5–93.8)</td>
</tr>
</tbody>
</table>

**Platelet activation parameters**

| Platelet P-selectin (%) | 0.94 ± 0.73 | 0.72 (0.39–1.18) | 1.80 ± 1.68 | 1.30 (0.69–2.26) | 1.82 ± 1.80 | 1.18 (0.68–2.02) | 1.78 ± 1.61 | 1.43 (0.70–2.30) |
| Soluble P-selectin (ng/ml) | 37.3 ± 15.0 | 35.7 (25.1–45.9) | 55.1 ± 34.0 | 45.2 (38.7–58.0) | 54.9 ± 38.0 | 44.3 (38.7–54.9) | 55.3 ± 31.8 | 48.0 (38.7–61.1) |
| PMP (n/jl plasma) | 174.9 ± 118.7 | 165 (68–241) | 543.2 ± 431.8 | 392 (234–715) | 494.5 ± 368.8 | 362.5 (236–614.5) | 571.1 ± 463.7 | 407 (227–777) |

**Carotid duplex ultrasound examination**

Carotid vessel wall examination was performed at the Neurosonography Laboratory of the Department of Neurology, University of Debrecen. All subjects were examined immediately after blood sampling using a colour-coded HP SONOS 4500 (Hewlett Packard, Andover, MA, USA) carotid duplex equipment with a 7.5 MHz linear transducer. Both diameter and area reductions were measured according to the European Carotid Surgery Trial (ECST) method (32). For screening, the internal carotid artery (ICA) stenosis was classified in categories of 10% taking into account the peak systolic velocity in the jet of the stenosis, the broadening of the stenotic and poststenotic spectrum, the peak systolic velocity in the poststenotic ICA, and the direction of ophthalmic flow. Occlusion was diagnosed in the complete absence of detectable flow in and above the stenosis and the presence of corresponding indirect haemodynamic criteria (33). On-line measurements of IMT were performed in both common carotid arteries (CCA) at about 10 mm proximal to the carotid bulb or 20 mm proximal to the flow divider. IMT was measured between the leading edge of the first echogenic line (lumen-intima interface) and the second echogenic line (upper layer of the adventitia) in the far (deeper) artery wall. All measurements were performed on
frozen enlarged images at the end of a heart cycle (end-diastole), and the transducer was in the medio-lateral directions (34). In each plaque free common carotid artery segment, three measurements of IMT were performed at 1-mm increment. The mean IMT of the six values in each patient was calculated.

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 (ANOVA) with Bonferroni’s multiple comparison or chi-square test as appropriate. Deviations from the Hardy-Weinberg equilibrium were also analysed using the chi-square test in each group. Spearman’s linear regression analysis was computed for checking the association of baseline characteristics with platelet activation markers. Multivariate regression analysis was performed to adjust for independent effect of each significant variable. The p≤0.05 probability level was regarded as statistically significant. Analysis was performed by using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Baseline demographic characteristics of all study groups are shown in Table 1. The obese and non-obese cohorts did not show any significant differences in terms of age (40.6 ± 11.1 vs. 39.7 ± 10.0 years), gender distribution (female/male; 62/38% vs. 66/34%) and current smoking habit (20.4% vs. 21.0%). Obese subjects had excessive body mass index (BMI) (38.2 ± 7.72 vs. 22.1 ± 1.96 kg/m²) and waist circumference values (118.7 ± 18.3 vs. 79.5 ± 8.2 cm) compared to healthy controls. In addition, mean carotid IMT was also significantly enlarged in all obese patients compared to healthy controls (0.58 ± 0.12 mm vs. 0.50 ± 0.08 mm). In order to evaluate at what extent obesity in the presence and absence of other risk factors for early atherosclerosis had an influence on platelet activation, these patients were further divided into two subgroups according to their additional co-morbidities: i) in those with hypertension, or type 2 DM, or dyslipidaemia (n=98), and ii) in patients without such complications (n=56). Among obese subjects there was no significant difference in BMI and waist circumference as well as systolic and diastolic blood pressure values; however, a significant (p<0.05) intra-group difference in IMT was shown compared to those without any diseases (0.60 ± 0.12 mm vs. 0.54 ± 0.11 mm) (Table 1). Laboratory parameters and platelet activation markers measured in the study groups are demonstrated in Table 2. Between the obese sub-cohorts significant (p<0.05) difference was found in the level of fasting plasma glucose, HbA1C, triglyceride, and total cholesterol. Both forms of P-selectin showed a significant elevation in obese patients when compared to healthy...
individuals (platelet P-selectin: 1.80 ± 1.68% vs. 0.94 ± 0.73%; soluble P-selectin: 55.1 ± 34.0 ng/ml vs. 37.3 ± 15.0 ng/ml). Additionally, PMP levels (543.2 ± 431.8/μl vs. 174.9 ± 118.7/μl) were significantly (p<0.05) higher in all obese than normal subjects. Of note, these platelet activation parameters – especially PMPs – demonstrated increased levels in obese subjects even in the absence of co-morbidities and still higher levels in those with co-morbidities compared to healthy controls. However, these differences were not significant between the two obese subgroups according to the statistical analysis (Table 2).

To observe the effect of the most investigated polymorphism of P-selectin gene on elevated soluble P-selectin levels in obesity, we determined this genotype in all study patients. The frequency of Thr715Pro P-selectin polymorphism (A=715Thr; C=715Pro) was similar between the two study groups: 72.6% AA (n=45), 27.4% AC (n=17) in healthy controls, and 76.0% AA (n=117), 22.7% AC (n=35), 1.3% CC (n=2) in obese subjects (with chi-square test p>0.05). All analysed groups were in Hardy-Weinberg equilibrium. As there was no subject with CC genotype among healthy controls and it was rare in the obese group, for analytic purposes these subjects were pooled into the group with subjects of AC genotype.

Thr715Pro P-selectin polymorphism (A=715Thr; C=715Pro) was demonstrated increased levels in obese subjects even in the absence of co-morbidities and still higher levels in those with co-morbidities compared to healthy controls. However, these differences were not significant between the two obese subgroups according to the statistical analysis (Table 2).

To test whether each of the studied platelet activation markers as a dependent variable was associated with obesity after correction for baseline demographic and laboratory factors, multivariate regression analysis was also performed for multiple comparisons. Our key findings are summarised in Table 4. Significant independent associations were maintained between PMPs and BMI (p<0.0001), soluble P-selectin and fibrinogen (p=0.007), and plasma fibrinogen (p=0.001), and platelet P-selectin (p=0.01), and soluble P-selectin (p=0.001), and platelet P-selectin (p=0.007), and plasma fibrinogen (p=0.001), and platelet P-selectin (p=0.01), and soluble P-selectin (p=0.001), and platelet P-selectin (p=0.007), and plasma fibrinogen (p=0.001), and platelet P-selectin (p=0.01), and soluble P-selectin (p=0.001), and platelet P-selectin (p=0.007), and plasma fibrinogen (p=0.001), and platelet P-selectin (p=0.01), and soluble P-selectin (p=0.001), and platelet P-selectin (p=0.007), and plasma fibrinogen (p=0.001), and platelet P-selectin (p=0.01), and soluble P-selectin (p=0.001), and platelet P-selectin (p=0.007), and plasma fibrinogen (p=0.001), and platelet P-selectin (p=0.01), and soluble P-selectin (p=0.001), and platelet P-selectin (p=0.007), and plasma fibrinogen (p=0.001), and platelet P-selectin (p=0.01), and soluble P-selectin (p=0.001), and platelet P-selectin (p=0.007), and plasma fibrinogen (p=0.001).
telet P-selectin and PAI-1 (p=0.009). Moreover, platelet P-selectin was also significantly (p=0.012) and independently associated with carotid IMT.

Discussion

According to previous studies, in obesity platelets become activated because of dyslipidaemia (12, 35) and subsequent inflammatory processes (36). Hence, enhanced platelet activation may accelerate atherogenesis by stimulating endothelial cells and the development of related prothrombotic events via secreting several chemokines (e.g. IL-1β, PDGF, CD40L) (37). Thus, platelets demonstrate a link between metabolic and haemodynamic abnormalities in atherosclerotic diseases. Indeed, while previous clinical reports published a few data about the relationship between increased platelet activation and atherosclerosis by studying different number of obese patients lacking marked statistical power (19–22), so further aspects remained to be explored on this field. In this present study, we investigated the relationship between platelet activation parameters and several prothrombotic and inflammatory vascular disease markers in a large obese cohort and compared these data with healthy controls.

Table 4: Multiple regression analysis for the association between platelet activation parameters and distinct vascular risk factors. BMI=body mass index; hs-CRP=high-sensitivity C-reactive protein; PAI-1=plasminogen activator inhibitor-1, IMT=intima-media thickness, PMP=platelet-derived (CD42a positive) microparticles.

<table>
<thead>
<tr>
<th></th>
<th>Platelet P-selectin (%)</th>
<th>Soluble P-selectin (ng/ml)</th>
<th>PMPs (n/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>p&lt;0.0001</td>
<td></td>
<td>p=0.0001</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>p=0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>p=0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid IMT (mm)</td>
<td>p=0.012</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
First, we measured platelet and soluble P-selectin levels along with the number of PMPs to evaluate platelet reactivity in all study subjects. Significantly elevated levels of platelet and soluble P-selectin as well as PMP levels were found in obese patients versus healthy subjects. Moreover, when obese patients were analysed regarding co-morbidities and compared to each other in the two patient subgroups, even higher levels of these markers especially PMP number were studied in the presence of further metabolic and vascular disorders. However, the increase was not statistically significant. All these data also imply that early lipid disorders may initiate the development of platelet hyperactivity. Nagy et al. proved in an animal model that enhanced P2Y12 receptor mediated signalling was also involved in the early generation of platelet hyperreactivity in mild and severe hypercholesterolaemia (38). In addition, complications like hypertension or diabetes accompanied with atherosclerosis may generate further elevation in platelet activation. In turn, platelets may have a functional role in the progress of atherosclerotic plaque formation in patients (39, 40). Chronic platelet activation with increased PMP number has been recently revealed also in the convalescent phase of stroke (41). Furthermore, Kappelmayr et al. published earlier that in the acute and chronic phase of cerebrovascular disease monocytes expressed tissue factor promoting enhanced blood coagulation, which was an indirect marker of increased platelet activation as well (42).

The cross-sectional association of platelet and soluble P-selectin levels with various cardiovascular risk factors in this study was consistent with previous reports (18, 39). We found the strongest association of platelet activation markers with parameters of visceral obesity and elevated insulin resistance rather than poor glucose metabolic control (HbA1c). Similarly, De Pergola et al. reported that soluble P-selectin concentrations were positively correlated with BMI, HOMA-IR, blood pressure, fasting insulin, triglyceride and PAI-1 levels. However, body fat content and insulin resistance were not independent determinants of soluble P-selectin levels according to multiple regression analysis (20). Recently, in the Bypass Angioplasty Revascularization Investigation 2 Diabetes study, BMI was positively, while HbA1c was negatively associated with platelet reactivity, and the levels of triglyceride and CRP were related to platelet P-selectin (43).

The association between platelet activation markers and low-degree inflammation status (C-reactive protein [CRP]) was evident in our study. CRP has been shown to be an independent mediator of central fat accumulation and insulin resistance in overweight adults (44), suggesting a component of obesity related disorders. Significantly increased levels of prothrombotic factors such as fibrinogen and factor VIII activity in obese individuals were consistent with previous findings (17, 45). There was a significant independent association between fibrinogen and soluble P-selectin in this study. Elevated PAI-1 antigen level was measured in patients with impaired glucose tolerance (46) and considered as a biochemical feature of insulin resistance syndrome (47). Furthermore, weight reduction resulted in a decrease of PAI-1 activity (22). Here we found similar association of significant univariate correlation of PAI−1 level with all platelet-associated parameters (p≤0.01), and surface P-selectin was significantly (p=0.009) independent after multivariate adjustment. Atherogenic index of plasma atherogenicity demonstrated a significant elevation in subjects with obesity especially in the presence of co-morbidities compared to healthy individuals, but did not correlate significantly with any platelet activation markers (30). Interestingly, no significant difference was formerly detected in the expression of platelet activation markers between small groups of non-obese and obese individuals by others (24); thus, there may be some additional risk factors like diet, lifestyle and genetic background which resulted in different conclusions in this aspect.

Carotid IMT is a strong predictor of future vascular events (28). We demonstrated an independent association between carotid IMT and surface P-selectin in obesity similarly to being observed in manifest atherosclerosis (39) and in cerebrovascular disease (5). In addition, there are other pieces of evidence about the contribution of increased IMT to atherosclerosis and intra-ventricular arterial thrombosis (48, 49). However, it has not been yet clarified whether activated platelets or vessel wall alteration may primarily cause or affect subsequent events.

Smoking also seems to contribute to atherothrombotic complications in this study, while previously and currently smoker subjects with co-morbidities showed significantly (p=0.004) increased IMT values, especially with significantly (p=0.012) higher levels of platelet CD62 versus the non-smoker individuals within the obese subgroup. There was even substantial difference (p<0.0001) in case of each platelet activation marker when smokers among obese persons having co-morbidities and the healthy cohort were compared to each other.

Another degranulation marker of activated platelets, CD63, seemed to be an additional risk factor for progression of carotid artery disease (50). Surprisingly, IMT did not correlate with soluble CD40L and CD40 plasma levels in occlusive carotid artery disease (51).

### What is known about this topic?
- Enhanced platelet activation has been shown in a number of diseases such as coronary artery and cerebrovascular disease and type 2 diabetes mellitus.
- However, only limited data are available about the correlations between platelet activation markers and the metabolic, inflammatory and vessel wall disorders in obesity.

### What does this paper add?
- Platelet P-selectin and platelet-derived microparticles (PMP) levels showed a positive and significant association with abnormal carotid IMT in obesity, which is a predictive marker of preclinical vascular events.
- The levels of platelet activation markers especially PMP number were also found significantly higher in those obese patients even without additional atherosclerotic co-morbidities compared to healthy individuals.
- A critical role of enhanced platelet reactivity was proved in atherosclerotic wall alteration in obese patients.
Thr715Pro P-selectin polymorphism was tested to study whether the increased soluble P-selectin levels were affected in those having Pro715 allele in obesity. We found no significant influence of this genotype on this platelet marker among obese subjects, even in the presence or absence of co-morbidities unlike in healthy persons. No effect of this polymorphism on increased soluble P-selectin levels was earlier shown by our group in type 2 DM and in a small number of non-DM patients versus normal subjects (7). Others claimed its “protective” effect by reducing soluble P-selectin levels in cardio- and cerebrovascular disease (25, 52). Further investigations may be needed to explore the significance of Pro715 allele in patients with different metabolic failures.

Our current study has some limitations. This paper reports a cross-sectional analysis of selected patient cohorts without longitudinal follow-up. Recruitment of obese subjects was performed at the Obesity Outpatient Clinic, and we did not screen a large primary (unselected) population, which might have contributed to potential selection bias. On the other hand, our subjects were inserted into either group according to their comprehensive laboratory and physical investigations and the measurement of platelet activation markers, and the statistical analyses were performed only after the study groups were completed up to the desired patient number. A longitudinal follow-up study is necessary to re-evaluate these significant associations at a later time point and then compare the development of clinical condition by demographical parameters to the change in the levels of platelet activation markers that would potentially indicate the trend of their relationship from another aspect.

In conclusion, we here report for the first time that the level of PMPs showed a positive and significant correlation with several key metabolic, inflammatory and prothrombotic parameters in obesity. Moreover, as an independent parameter it had a strong positive association with BMI. In addition, there was a positive association between platelet P-selectin with PAI-1 plus IMT, and the statistical analyses were performed only after the study groups were completed up to the desired patient number. A longitudinal follow-up study is necessary to re-evaluate these significant associations at a later time point and then compare the development of clinical condition by demographical parameters to the change in the levels of platelet activation markers that would potentially indicate the trend of their relationship from another aspect.

Acknowledgements
The authors thank Ms. Éva Lénárt, Erika Dzsudzsák and Ms. Anikó Györfi Veszprémi for their technical assistance.

Conflict of interest
None declared.

References

© Schattauer 2011

Thrombosis and Haemostasis 106.4/2011

Csongrádi et al. Platelet activation markers and IMT in obesity


