SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of the associations between platelet activation markers and adipokines, and atherosclerosis in obesity

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University of Debrecen
Doctoral School of Health Sciences
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Members of the Examination Committee: Dániel Bereczki, MD, PhD, DSc
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The Examination takes place at the Conference Room in the Building of the Faculty of Public Health, University of Debrecen, on July 4, 2017 at 11 a.m.

Head of the Defense Committee: Róza Ádány, MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Building A of the Department of Medicine, Faculty of Medicine, University of Debrecen, on July 4, 2017 at 1 p.m.
1. INTRODUCTION

Atherosclerotic cardiovascular diseases are identified as leading causes of mortality worldwide. Atherothrombosis as a critical component of cardiovascular events is the acute crisis of atherosclerosis. Obesity is considered one of the major manageable risk factors of atherosclerosis besides hypertension, type 2 diabetes mellitus (T2DM), dyslipidemia and smoking. Obesity, due to its global epidemic, the severity of the consequences and the lack of efficient treatment has become one of the major public health problems worldwide. Obesity is often accompanied by comorbidities predisposing to atherosclerosis, namely, hypertension, T2DM and dyslipidemia. It should be noted that nearly one-third of obese individuals do not suffer from any atherosclerotic concomitant diseases.

The increased platelet activation in atherosclerotic cardio- and cerebrovascular diseases and in conditions predisposing atherosclerosis: hypertension, type 2 diabetes mellitus and dyslipidaemia is adequately demonstrated by increased levels of surface and soluble P-selectin and platelet-derived microparticles (PMP). Nevertheless, in obesity, prior to the study described in the thesis, platelet activation marker levels were assessed only in small-scale studies with obese patients yielding partially ambiguous results. Due to the Thr715Pro P-selectin polymorphism, the soluble P-selectin level varies in healthy individuals; and based on literature evidence the homo- and heterozygous forms associated with lower levels are likely to have a protective effect against atherosclerotic processes. The effect of Thr715Pro P-selectin polymorphism on P-selectin levels was not evaluated in obese populations previously. Moreover, in obese individuals, essential aspects of the correlations between platelet activation markers and vascular risk factors, prothrombotic parameters and common carotid artery intima-media thickness (carotid IMT) as a marker of subclinical atherosclerosis remained unexplored.

In abdominal obesity, visceral fat accumulation causes dysregulation of adipose tissue functions, leading to the overexpression of most adipokines including leptin, resistin, TNF-α, IL-6, and hyposecretion of adiponectin. There is increasing evidence that obesity-associated altered adipokine levels are closely involved in the pathogenesis of atherosclerotic vascular diseases. Although the association of adipokines with traditional vascular risk factors such as low-level chronic inflammation characteristic of visceral obesity and hypertension, metabolic abnormalities (i.e. hyperglycaemia, dyslipidaemia) frequently associated with obesity are well known and acknowledged; their relationship with the platelet activation markers playing a crucial role in atherosclerotic and atherothrombotic processes has not yet been evaluated in
Obese individuals, as well as the associations with hypercoagulation and hypofibrinolysis markers are insufficiently clarified. Despite its importance, essential aspects of the relationship between classical adipokines and the carotid artery thickness have not yet been clarified in obese patients.

Bearing in mind the above aspects, in our study conducted in obese individuals we aimed to discover the factors and associations which may have importance with regards to the accelerated atherosclerosis and increased cardiovascular risk characteristic of obesity, by analysing platelet activation and the altered adipokines profile and evaluating their unknown or insufficiently characterised relationship with the major factors of atherosclerosis.

2. OBJECTIVES

Describing the associations between platelet hyperactivation and atherosclerosis in obesity

In the first phase of our work we aimed to evaluate the platelet hyperactivation markers and analyse their associations with atherosclerosis in a relatively large population of obese patients and in their subcohorts by comorbidities predisposing for atherosclerosis. The following aims were pre-specified for these studies:

- Evaluating platelet activation by assessment of platelet surface P-selectin expression, soluble P-selectin levels and platelet-derived microparticles levels in the whole obese cohort as well as in the subcohorts of obese patients with and without atherosclerotic comorbidity and in healthy controls, respectively.
- Studying the effect of Thr715Pro P-selectin polymorphism on soluble P-selectin plasma level in obese patient and control populations.
- Analysing correlations of platelet activation parameters with vascular risk factors and markers of prothrombotic coagulation abnormalities.
- Determining the vascular risk factors independently linked to platelet activation markers.
- Evaluating the relationship between platelet hyperactivity markers and carotid IMT.
analysing the associations between adipokines and atherosclerosis in obesity

In the second phase of our work we aimed to characterise the associations not yet studied or insufficiently explored between altered adipokines plasma levels typical in obesity, and the factors playing a key role in the atherosclerosis pathomechanism. We aimed to answer the following questions during these studies:

- Whether the presence of atherosclerosis predisposing comorbidities have an impact on the plasma level of the classic adipokines i.e. leptin, adiponectin, resistin, TNF-α and IL-6 in obese individuals.
- Evaluating the correlations of adipokines with haemostatic parameters, platelet activation markers in particular.
- Determining the haemostatic parameters which are independently linked to adipokine levels.
- Analysing the relationship between adipokine plasma levels and carotid artery wall thickness.
- Exploring which adipokines and haemostatic parameters can be considered independent predictors of carotid IMT.

3. SUBJECTS AND METHODS

3.1. Obes and control study populations

We enrolled 154 obese patients (age: 40.6 ± 11.1 years; 95 female and 59 male; BMI: 38.2 ± 7.72 kg/m²) from the Obesity Outpatient Clinic at the Department of Medicine, University of Debrecen. Sixty-two age- and sex-matched healthy volunteers (age: 39.7 ± 10.0 years; 41 female and 21 male, BMI: 22.1 ± 1.96 kg/m²) were recruited from the staff of the Departments of Medicine and Ophthalmology to serve as a control group. Obese group included two subgroups: 98 obese patients suffering from comorbid conditions predisposing them for atherosclerosis (hypertension and/or T2DM and/or dyslipidemia) and 56 age-, sex- and BMI-matched obese subjects free of atherosclerotic concomitant diseases. BMI was calculated as body weight (kg) divided by the square of body height (m). Obesity was defined as a body mass index (BMI) ≥30 kg/m². Subjects with malignancy, impaired liver or renal function, pregnancy, alcohol or drug dependence, infectious diseases, as well as severe symptomatic cardiovascular diseases such as active angina, intermittent claudication and
transient ischemic attack were excluded. All participants gave written informed consent. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and approved by the Ethics Committee of the Faculty of Medicine, University of Debrecen.

3.2. Laboratory measurements

Laboratory measurements were performed at the Department of Laboratory Medicine and the Research Laboratory of the Department of Medicine, University of Debrecen by internationally validated and standardized methods.

3.2.1. Blood drawing and sample preparation

Venous blood samples were taken after an overnight fast by vacutainer technique. Plasma samples were kept frozen at -70°C for ELISA measurements.

For flow cytometric analysis of platelet surface P-selectin expression, within 2 hours of sample collection, 40 μl whole blood samples were fixed in 1 ml 1% paraformaldehyde and kept at room temperature (RT) for a minimum of 1 hour. Fixed whole blood samples were centrifuged at 1300 x g for 15 minutes at RT. The pellet was washed in 1 ml phosphate-buffered saline (PBS) buffer, then centrifuged as above and finally resuspended in 0.5 ml PBS.

To study the amount of PMPs, platelet-poor plasma (PPP) was obtained from whole blood anticoagulated with sodium citrate by centrifugation at 1550 x g for 20 minutes at RT. Five hundred μl of PPP was spun down at 13000 x g for 2 minutes to get rid of platelet debris, and then centrifuged at 16100 x g for 30 minutes at RT to isolate MPs.

3.2.2. Chemical / immunochemical and hematological laboratory assays

Serum glucose, total cholesterol, LDL-C, HDL-C, triglyceride values were measured on a Hitachi analyser, applying hexokinase method for serum glucose; enzymatic, colorimetric tests for total cholesterol, LDL-C, HDL-C and triglyceride. To confirm non-diabetic status in obese and lean study participants, we applied an oral glucose tolerance test. HbA1c was determined by high-performance liquid chromatography. Serum insulin concentration was analysed by a commercially available radio-immunoassay kit. Insulin resistance index was estimated by using the homeostasis model assessment of insulin resistance (HOMA-IR). The value of hs-CRP was assessed by turbidimetric assay on an Integra 800 analyser. Fibrinogen plasma level was determined by the Clauss method on an STA Compact instrument.
activity was measured on an STA Compact instrument. Platelet count and MPV were determined by Advia 120 Hematology System. Plasma levels of leptin, adiponectin, resistin, TNF-α, IL-6, soluble P-selectin and PAI-1 antigen were analysed by commercially available ELISA kits, according to the manufacturer’s instructions.

3.2.3. Flow cytometric analysis

Platelets were identified by a monoclonal antibody to GPIX (CD42a). Platelet surface P-selectin expression was detected by using anti-CD62 monoclonal antibody. Fixed platelets were incubated with saturating concentrations of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to GPIX (CD42a) and phycoerythrin (PE)-labelled anti-P-selectin (CD62-PE) for 20 minutes in the dark at RT. As a control for immunolabeling with anti-CD62 antibody, platelets were incubated with PE-coupled non-immune mouse IgG1 antibody. 10,000 dual-colour labelled platelet events were acquired on a FACSCalibur flow cytometer by using the CellQuest 3.2 software. Results were expressed as the percentage of double positive platelets.

We used fluorescent beads Tru-COUNT with standard size and amount for enumeration of PMPs. 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.

3.2.4. Thr715Pro P-selectin genotype analysis

For genetic analysis of the Thr715Pro polymorphism of P-selectin gene, DNA was extracted from whole blood anticoagulated with sodium citrate. Primers with the 5′-TTTCTGCAGCTGTGAAATGC-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. 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 Eco91I and the digested products were run on a 3% agarose gel and visualised under ultraviolet light by ethidium bromide staining. In the presence of Thr715Pro mutation, a new (163 bp) DNA product could be detected during analysis.
3.3. Common carotid duplex ultrasound examination

Common carotid vessel wall examination was performed at the Ultrasound Laboratory of the Department of Neurology, University of Debrecen, using a colour-coded HP SONOS 4500 carotid duplex machine with a 7.5 MHz linear transducer. Online measurements of IMT were performed in both common carotid arteries 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 mediolateral direction. In each plaque-free common carotid artery segment, three measurements of IMT were performed at 1 mm increments. The mean IMT of the six values in each patient was calculated.

3.4. Statistical analysis

Statistical analysis was performed by using SAS™ for Windows™ 9.3 and SPSS (version 13) computer software. The Kolmogorov-Smirnov test was used for evaluating the normality of data distribution. Non-normally distributed parameters were transformed logarithmically. Differences in various parameters among study groups were tested using one-way ANOVA with post-hoc tests (Bonferroni, Tukey-Kramer Newman-Keuls). Correlations between continuous variables were assessed by using Spearman’s and Pearson’s test. A chi-square test was performed to determine the associations of discrete variables. Associations of multivariate parameters were evaluated by multiple regression analysis with the backward stepwise method. With probability value of \( p \leq 0.05 \) the result was considered statistically significant.

4. RESULTS

4.1. Plasma levels of the markers of platelet hyperactivation, hypercoagulation and hypofibrinolysis

The plasma levels of platelet activation markers: platelet surface P-selectin expression, soluble P-selectin, and the number of PMP were significantly higher in obese subjects compared to healthy control (surface P-selectin: 1.30 (0.69-2.26) % vs. 0.72 (0.39-1.18) %, \( p \leq 0.0001 \); soluble P-selectin: 45.2 (38.7-58.0) ng/ml vs. 35.7 (25.1-45.9) ng/ml, \( p \leq 0.0001 \);
PMP: 392 (234-715) n/μl vs. 165 (68-241) n/μl, p=0.0001. There were no significant difference in platelet activation marker levels between two obese subgroups related to atherosclerotic comorbidities (surface P-selectin: 1.18 (0.68-2.02) % vs. 1.43 (0.70-2.30) %, p=0.671; soluble P-selectin: 44.3 (38.7-54.9) ng/ml vs. 48.0 (38.7-61.1) ng/ml, p=0.551; PMP: 362.5 (236-614.5) n/μl vs. 407 (227-777) n/μl, p=0.467). Platelet number, MPV, fibrinogen level, factor VIII activity and PAI-1 antigen plasma level were also significantly elevated in obese individuals, than healthy subjects, and these haemostatic parameters did not significantly differ in obese subgroups.

4.2. Effect of P-selectin Thr715Pro polymorphism on soluble P-selectin level

The frequency of Thr715Pro P-selectin polymorphism (AA: Thr715Thr, AC: Thr715Pro, CC: Pro715Pro) was similar in obese and control study groups, in obese subjects: 76.0% AA (n=117), 22.7% AC (n=35), 1.3% CC (n=2), in healthy controls: 72.6% AA (n=45), 27.4% AC (n=17),(χ² test p>0.05). 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. Among healthy subjects the soluble P-selectin levels were significantly lower in heterozygous individuals for P-selectin Thr715Pro polymorphism compared to those with wild-type (AA) genotype (25.1 (17.9-41.9) ng/ml vs. 36.6 (27.8-50.0) ng/ml, p=0.023). There was no significant difference between hetero/homozygous and wild-type genotypes of P-selectin Thr715Pro polymorphism in obese group (49.7 (40.7-62.3) ng/ml vs. 44.3 (38.5-55.1) ng/ml, p=0.19). Similarly, no significant difference was observed between AC+CC versus AA genotypes of P-selectin Thr715Pro polymorphism among obese patients when they were recruited into the two subgroups according to the presence or absence of co-morbidities.

4.3. Associations between platelet activation markers and vascular risk factors

We analysed the relationships between the sensitive markers of platelet activation and vascular risk factors by correlation analysis. Strong significant (p≤0.01), positive correlations were detected between surface P-selectin and BMI (r=0.25), systolic (r=0.26) and diastolic (r=0.26) blood pressure values, HOMA-IR (r=0.32), HbA1c (r=0.20), fasting glucose (r=0.21), fasting insulin (r=0.28), triglyceride (r=0.26), total cholesterol (r=0.26), and PAI-1 antigen (r=0.30). Furthermore, significant (p≤0.05) and positive associations were revealed between platelet P-selectin expression and waist circumference (r=0.18), LDL-C (r=0.18), and
hsCRP (r=0.18). Surface P-selectin showed significant (p≤0.05), negative correlation with HDL-C (r=-0.16). There were no significant relationships of platelet P-selectin expression with fibrinogen plasma level and factor VIII activation.

Our study demonstrated close significant (p≤0.01) relationships of soluble P-selectin with the following vascular risk parameters: BMI (r=0.25), fasting insulin (r=0.27), HOMA-IR (r=0.32), triglyceride level (r=0.23), hs-CRP (r=0.23), and PAI-1 antigen level (r=0.26). Strong significant (p≤0.01) but negative correlation was observed between soluble P-selectin and HDL-C (r=-0.23). Additionally, soluble P-selectin associated significantly (≤0.05) and positively with waist circumference (r=0.16), LDL-C (r=0.17), and fibrinogen level (r=0.15). Soluble P-selectin did not correlate significantly with blood values, fasting glucose, HbA1c, total cholesterol, and factor VIII activity.

PMPs associated significantly with all investigated vascular risk factors, except total cholesterol. We verified highly significant (p≤0.01) positive correlation between PMPs and BMI (r=0.37), waist circumference (r=0.39), diastolic blood pressure value (r=0.23), fasting glucose (r=0.28), HbA1c (r=0.22), fasting insulin (r=0.32), HOMA-IR (r=0.38), triglyceride (r=0.30), hs-CRP (r=0.37), fibrinogen (r=0.39), and PAI-1 antigen plasma level (r=0.48). Furthermore, PMPs associated significantly (p≤0.05) with systolic blood pressure value (r=0.17), LDL-C level (r=0.15) and FVIII activity (r=0.18). Inverse significant (p≤0.01) correlation was showed between PMPs and HDL-C level (r=-0.20).

**Independent associations between platelet activation parameters and vascular risk factors**

We investigated the independent associations between platelet activation markers and vascular risk factor using multiple regression analysis. In our model platelet activation markers were the dependent variables, and BMI, systolic blood pressure, HOMA-IR, triglyceride, LDL-C, hs-CRP, fibrinogen, FVIII activity and PAI ag were the independent variable. Significant independent associations were maintained between platelet surface P-selectin and PAI-1 antigen (p=0.009), soluble P-selectin and fibrinogen (p=0.007), and PMPs and BMI (p<0.0001).

**4.4. Common carotid artery intima media thickness**

Carotid IMT was significantly enlarged in obese patients compared to healthy controls (0.58 ± 0.12 mm vs. 0.46 ± 0.04 mm, p<0.0001). Significant difference in IMT was shown between obese subjects with atherosclerotic comorbidity and obese individuals without these diseases (0.60 ± 0.12 mm vs. 0.54 ± 0.11, p=0.044).
4.5. Relationship between platelet activation markers and carotid intima-media thickness

We analysed the relationships of carotid IMT with platelet activation markers using correlation analysis. Notably, significant and positive univariate associations of carotid IMT with platelet P-selectin (r=0.37; p<0.0001), soluble P-selectin (r=0.17; p=0.039) and the level of PMPs (r=0.32; p=0.0001) were observed.

4.6. Effect of the atherosclerotic comorbidity of obesity on adipokin levels

No significant difference was observed in the plasma levels of leptin, adiponectin, resistin, TNF-α and IL-6 between the obese sub-cohorts related to atherosclerotic comorbidities. Leptin, resistin, TNF-α and IL-6 levels were significantly increased, while adiponectin was significantly decreased in both obese subgroups compared to lean healthy controls.

4.7. Associations between adipokines and haemostatic parameters

We analysed the relationships between adipokine levels and haemostatic markers by correlation analysis. Each of the investigated adipokines showed a significant (p≤0.05) association with platelet count, MPV, PMP, fibrinogen and PAI-1 antigen levels. Leptin, resistin, TNF-α and IL-6 correlated positively, while adiponectin was negatively correlated with these haemostatic parameters. Notably, strong significant (p≤0.001) and positive univariate correlations of PMPs with leptin (r=0.44), resistin (r=0.30), TNF-α (r=0.35) and IL-6 (r=0.33) were detected, but a negative association with adiponectin (r=-0.36) was found. Resistin had significant and positive correlations with platelet surface and soluble P-selectin. Leptin was related significantly and positively with soluble P-selectin. In contrast, a significant inverse correlation was found between adiponectin and soluble P-selectin levels. TNF-α and IL-6 did not show significant associations with P-selectin values. Leptin, resistin and IL-6 demonstrated significant and positive relationships with FVIII activity. Finally, adiponectin and TNF-α had no associations with FVIII activity.

Independent associations between adipokines and haemostatic parameters

To test whether the investigated adipokines are independently associated with haemostatic parameters, multiple regression analysis was performed. In our study the markers of platelet activation, hypercoagulation and hypofibrinolysis were the dependent variables, and classic adipokines, age and gender were the independent variables. Significant independent
associations were retained between leptin and platelet count (p<0.0001), MPV (p=0.019), PMPs (p<0.0001), fibrinogen (p=0.001), FVIII activity (p=0.035); adiponectin and PAI-1 antigen (p=0.035); resistin and soluble P-selectin (p=0.002); TNF-α and PAI-1 antigen (p<0.0001); and IL-6 and fibrinogen (p=0.011).

4.8. Relationships of adipokines with carotid intima-media thickness

Based on correlation analysis between adipokines and carotid IMT, highly significant (p≤0.0001) and positive correlations of IMT with leptin (r=0.48), resistin (r=0.39), TNF-α (r=0.43) and IL-6 (r=0.45) were observed. A significant, but negative association was also found between IMT and adiponectin (r=−0.26, p=0.016).

4.9. Independent predictors of carotid intima-media thickness

When IMT was considered as a dependent variable, and adipokines, haemostatic markers, age, and gender were entered as independent variables in the model of multiple regression analysis, IMT still maintained independent associations with leptin (p=0.0005), adiponectin (p=0.019), IL-6 (p=0.001), MPV (p=0.0003), PMPs (p=0.008), FVIII activity (p=0.043) and age (p<0.0001).

5. DISCUSSION

5.1. Associations between platelet hyperactivation and atherosclerosis in obesity

As it is well known from previous studies, platelet activation plays an important role in atherosclerotic, atherothrombotic processes. The P-selectin expressed in the platelet surface during platelet activation facilitates those interactions between platelets, leukocytes and endothelial cells which are important for the completion of the atherothrombotic processes. Nevertheless, the soluble form of P-selectin is also abundant in the plasma during platelet activation and facilitates platelet-derived microparticles production and coagulation cascade activation. The platelet-derived microparticles are structures cleaved off from platelets which due to their procoagulant surface properties facilitate inflammatory and coagulation processes. Prior to our studies only few literature data in obese patients was available about the levels of these sensitive platelet activation markers and their role played in atherosclerosis. Increased platelet activation parameter levels were reported based on a few, small-scale studies conducted in obese cohorts. However, Samocha-Bonet et al. failed to detect a significant
increase in platelet activation in obese patients compared to healthy individuals. Considering the major role of platelet activation in atherosclerotic processes and the scarce and ambiguous literature evidence collected from obese individuals in this regard, assessment of platelet activation marker levels in a relatively large patient cohort was deemed important. In our studies the platelet surface P-selectin expression as well as the soluble P-selectin and platelet-derived microparticles levels were found significantly higher in the obese cohort compared to the controls. In an attempt to answer the question whether the increased platelet activation detected in obese individuals is a characteristic feature of obesity or a factor attributable to the presence of atherosclerotic comorbidities associated with obesity, platelet activation marker levels were analysed in the obese subcohorts with and without atherosclerotic comorbidities. Our results indicated that all three platelet activation marker levels examined were already significantly elevated in the obese patients free from atherosclerosis predisposing comorbidities compared to the control group and the presence of atherosclerotic comorbid conditions did not result in a further significant increase in platelet activation levels.

The Thr715Pro polymorphism is the most studied polymorphism of the P-selectin gene. Literature data indicate that the Thr715Pro polymorphism affects soluble P-selectin level in healthy individuals; in the presence of the Pro715 allele lower soluble P-selectin levels and a protective effect against the vascular processes were reported. The publication results on Thr715Pro polymorphism and cardiovascular diseases are ambiguous. Nagy et al. did not find any difference in the P-selectin levels as per the Thr715Pro polymorphism in patients with type 2 diabetes mellitus and in overweight individuals. To the best of our knowledge our study is the first to assess the effect of Thr715Pro polymorphism of P-selectin gene on soluble P-selectin levels in obese population. Our results provided evidence that in obese individuals, when compared to healthy controls, the Pro715 allele did not have a significant influence on the soluble P-selectin levels. When assessing the soluble P-selectin levels of heterozygote and wild type individuals in the two sub-cohorts of obese patients by the presence or absence of atherosclerotic comorbidity no significant difference in soluble P-selectin levels were found in either subgroup depending on the two genotypes.

When analysing the associations between platelet activation markers and the traditional risk factors, a significant positive correlation similar to the one reported by De Pergola et al. was found between the soluble P-selectin level and BMI, HOMA-IR, fasting insulin and triglyceride levels. In the current study further significant positive correlation of soluble P-selectin level with waist circumference and LDL-C level, moreover a significant inverse correlation with HDL-C level have been confirmed, respectively. In line with the
previous study results, strong correlation was demonstrated between platelet surface P-selectin expression and BMI. In our study the surface P-selectin correlated significantly not only with the anthropometric measurements but also with blood pressure readings and carbohydrate and lipid metabolism parameters. Murakami et al. reported a positive correlation of platelet-derived microparticles and BMI in obese individuals. Our analysis confirmed that BMI is an independent predictor of PMP level. Furthermore, our data revealed significant positive correlation of PMP level with waist circumference, systolic and diastolic blood pressure values and carbohydrate metabolism parameters. Although platelet microparticles level failed to show significant correlation with total cholesterol level in obese individuals, significant positive correlation with LDL-C and triglyceride and significant negative correlation with HDL-C levels were demonstrated, respectively.

The low-degree chronic systemic inflammatory marker, CRP, which is independently linked to central fat accumulation showed significant positive correlation with both platelet surface and soluble P-selectin levels as well as with PMP levels in our study.

In line with literature data, our study results showed an elevated level of parameters indicating a prothrombotic shift in the coagulation i.e. fibrinogen, factor VIII activity and PAI-1 antigen in the group of obese patients as compared to the healthy individuals with normal body weight. Our results indicated that the plasma levels of these prothrombotic factors in obese individuals are significantly increased compared to the control group even in lack of atherosclerotic diseases. In their previous study De Pergola et al. confirmed a significant positive correlation between soluble P-selectin and PAI-1 antigen levels, however they did not detect a significant link between soluble P-selectin and plasma fibrinogen levels.

In our study the soluble P-selectin level showed significant positive correlation with both PAI-1 antigen and fibrinogen levels. Fibrinogen proved to be an independent predictor of soluble P-selectin level. Our study data confirmed an independent, significant positive correlation between platelet surface P-selectin and PAI-1 antigen levels. Our study proved a significant positive correlation of the platelet microparticles level with all three prothrombotic parameters examined.

Our results demonstrated a strong relationship of platelet activation markers with vascular risk factors and parameters of prothrombotic coagulation. It should be highlighted that the PMP level showed significant correlation with the anthropometric measurements, blood pressure readings, carbohydrate and lipid metabolism parameters and the markers of hypercoagulation and hypofibrinolysis as well.
Carotid IMT is a reliable marker of subclinical atherosclerosis and an independent predictor of cardio- and cerebrovascular events. The significantly higher carotid artery wall thickness detected in the subcohort of obese patients without atherosclerotic comorbidity compared to healthy controls indicates that obese individuals, irrespective of the presence of atherothrombotic comorbidities, have subclinical atherosclerosis and consequently an increased atherothrombotic risk. Presence of atherosclerotic comorbidities in obese individuals resulted in a further significant increase of the carotid wall thickness compared to obese patients without comorbidities, thereby demonstrating that through different pathways the accompanying atherosclerotic diseases further increase the risk of developing atherosclerotic vascular diseases.

Previous studies reported a significant positive correlation between platelet P-selectin and carotid IMT in patients with type 2 diabetes mellitus, hypertension, dyslipidaemia and atherosclerotic cerebrovascular diseases. Our study conducted in obese patients provides evidence for the significant positive correlation of platelet surface and soluble P-selectin levels with carotid artery thickness. To the best of our knowledge this is the first study published in the literature that evaluates the relationship of platelet-derived microparticles level and carotid IMT confirming a strong correlation in obese individuals.

5.2. Relationships of adipokines with atherosclerosis in obesity

The literature data concerning the crucial role of the obesity associated altered adipokine secretion profile in the pathogenesis of the atherosclerotic vascular diseases are mainly based on experimental studies. The data collected in obese patients in this regard is significantly less and more debated compared to those from experimental models. In the second phase of our study, by exploring the associations of the adipokine profile distinctive of obese patients with the haemostatic parameters having a decisive role in atherosclerosis and carotid wall thickness, we aimed to contribute to the clarification of the debated role of adipokines in the atherosclerosis of obese patients.

When comparing the plasma adipokines levels in obese individuals with and without atherosclerotic comorbidity we aimed to clarify how the presence of atherosclerotic comorbidity influences the adipokines levels in obese patients. Our results provided evidence that the presence of comorbidities predisposing to atherosclerosis in obese patients do not result in any further significant changes in the levels of adipokines.
Platelet count and MPV are widely available platelet parameters in clinical practice. Previous data are contradictory regarding the association between platelet count and cardiovascular outcomes. Thaulow et al. published earlier that coronary heart disease mortality is associated with increased platelet concentrations. Moreover, higher platelet counts correlated with adverse clinical outcomes in patients with acute coronary syndrome. In contrast, other population-based studies reported that platelet count was not associated with the occurrence of cardiovascular events. MPV is a predictive biomarker of cardiovascular risk and prognosis. Larger platelets are more reactive, and have greater haemostatic potential affecting platelet function. Platelet number and size are mainly determined during megakaryocytopoiesis in the bone marrow. IL-6 plays a crucial role in stimulating megakaryocytopoiesis resulting in increased circulating platelet count and volume. TNF-α may also influence thrombopoiesis via inducing de novo synthesis of IL-6. Similarly, we found a significant positive association of IL-6 and TNF-α with the platelet count and MPV in obesity. Furthermore, in our present study, leptin and resistin also showed significant positive relationships with platelet number and size, whereas a significant negative correlation was detected between adiponectin and the above-mentioned platelet parameters. Leptin associated independently with these platelet indices.

Although sensitive markers of platelet activation, surface and soluble P-selectin, and PMPs, and altered adipokine profil are both deeply involved in atherogenesis, their associations are still unknown segments of the pathogenesis of atherosclerosis in subjects with obesity. In the present study, for the first time in the literature, we have demonstrated significant positive associations of resistin with platelet surface and soluble P-selectin. Furthermore, we have detected significant positive correlation of leptin with soluble P-selectin, while we have showed a significant negative association between adiponectin and soluble P-selectin. This study highlights the close relationship of all studied classic adipokines to PMPs with high thrombogenic activity. Additionally, based on multiple regression analysis, independent significant associations have been revealed between resistin and soluble P-selectin, and leptin and PMPs. These findings suggest, that the associations of adipokines with platelet activation parameters partially account for their atherogenic effects in obesity.

Platelet activation and activation steps of the coagulation cascade occur closely interrelated during atherothrombosis. Fibrinogen, as an important component of the coagulation pathway, was positively associated with leptin, resistin and IL-6 and negatively with adiponectin, similar to others’ observations. Moreover, we demonstrated a significant positive association between fibrinogen and TNF-α in obese individuals. The cross-sectional
significant positive associations of FVIII activity, a further key coagulation parameter, with leptin and IL-6 in this study was consistent with previous studies. Interestingly, Wannamethee et al. reported that the adiponectin level was positively correlated with FVIII; in our investigation, adiponectin was not found to be associated with FVIII activity. In addition, we established a significant positive correlation between FVIII activity and resistin in obese subjects. We confirmed previous report that leptin is independently associated with both of these hypercoagulation markers. Moreover, we demonstrated an independent relationship between IL-6 and fibrinogen. In agreement with earlier findings, we detected a significant positive correlation of hypofibrinolysis displayed by elevated plasma PAI-1 antigen level with leptin, TNF-α and IL-6, and a negative association between PAI-1 antigen and adiponectin. Furthermore, the current study revealed significant positive association between PAI-1 antigen and resistin plasma level. TNF-α is a potent stimulator of the obesity-linked elevation of PAI-1 expression in adipose tissue and may substantially contribute to the increased plasma concentrations of PAI-1 observed in obesity. In the present investigation, TNF-α had an independent relationship with PAI-1 antigen in obese individuals. In addition, our study showed, that adiponectin is also an independent predictor of PAI-1 antigen. Thus, our data provide further evidence that adipokines are closely associated with markers of hypercoagulability and impaired fibrinolysis.

According to previous studies, in obesity we found a significant positive association of IMT with leptin, and IL-6, and an inverse correlation with adiponectin. The present study demonstrates that obese adults, similar to obese children, have a significant positive relationship between resistin and IMT. Moreover, we showed here a possible influence of the plasma level of TNF-α on the initial phases of atherosclerosis in obese patients. In previous investigations of our research team, platelet surface and soluble P-selectin, and PMPs were significantly and positively correlated to IMT. Corroborating previous data, fibrinogen was related significantly and positively to IMT. In the study by Arslan et al. a significant positive association between MPV and IMT was detected in obese adolescents. We have verified this association in obese adults. Additionally, in our study patients, we demonstrated significant positive relationships of platelet count, FVIII activity and PAI-1 antigen with IMT. The independent relationships of leptin, adiponectin, IL-6 and age with IMT were evident in our study similarly to previous reports. Furthermore, our multiple regression analysis also retained MPV, PMPs and FVIII activity as independent predictors of IMT.

Whether adipokines play a causal role in the development of atherosclerosis in obese patients needs to be evaluated in upcoming follow-up studies. Longitudinal studies are also
needed to clarify the role of adipokines in the long-term atherosclerotic cardiovascular outcome in obese individuals.

6. SUMMARY

Obesity is associated with accelerated atherosclerosis, resulting in increased cardiovascular morbidity and mortality. By investigating the relationship between platelet activation markers, adipokines and atherosclerosis in obesity, a disease with important public health implications, we intended to contribute additional data to the endeavours that serve a better understanding and thereby more efficient prevention and treatment of the pathogenesis of obesity-related accelerated atherosclerosis.

Based on our investigation we concluded that in obesity the activation of platelets is increased, which is manifested in elevated levels of surface P-selectin, soluble P-selectin, and the number of platelet-derived microparticles, regardless of the absence or presence of atherosclerotic comorbidities. While in the control group soluble P-selectin levels of individuals with hetero- and homozygous forms of Thr715Pro polymorphism of the P-selectin gene were significantly lower compared to those with the wild genotype of the gene, this difference could not be demonstrated in obese individuals. In our study we demonstrated significant correlations of platelet activation markers with blood pressure values, anthropometric, metabolic, and inflammation parameters as well as the prothrombotic markers of the coagulation system in obese individuals. Our results revealed a close relationship between platelet activation markers and common carotid intima-media thickness as a marker of pre-clinical atherosclerosis.

We verified in our investigation that plasma levels of the classic adipokines including leptin, adiponectin, resistin, TNF-α and IL-6 did not significantly differ in obese individuals with and without atherosclerotic comorbidities. We demonstrated significant correlations of adipokines with haemostatic parameters such as platelet hyperactivation, hypercoagulation, and hypofibrinolyis markers in obese subjects. Our study showed a close correlation between obesity-associated altered adipokine levels and common carotid wall thickness. In analysing the independent associations between carotid IMT and adipokine levels, and haemostatic parameters, we determined that leptin, adiponectin, IL-6, MPV, the number of platelet microparticles and factor VIII activity are predictive markers of common carotid wall thickness. Thus our results indicate a crucial role of altered adipokine profile, platelet
hyperactivation, and the close relationship between them in common carotid atherosclerosis in obese individuals.

7. NEW RESULTS, STATEMENTS AND CLINICAL RELEVANCE OF THE THESIS

- By detecting platelet hyperactivation in obese individuals without atherosclerotic comorbidity we provided evidence for obesity being a prothrombotic condition regardless of the presence of atherosclerosis predisposing comorbidities.
- As to the Thr715Pro P-selectin polymorphism we confirmed that with regards to soluble P-selectin the supposed protective effect of Pro715 allele against atherosclerotic processes is lost in obese individuals compared to healthy controls. Future discovery of the mechanism responsible for the different effect of Pro715 allele on soluble P-selectin levels in obese and control populations may contribute to the better understanding and influence of accelerated atherosclerosis in obesity.
- Strong correlations of platelet microparticles, platelet surface and soluble P-selectin levels with vascular risk factors, prothrombotic parameters and carotid IMT were demonstrated. Based on these correlations it can be assumed that the platelet activation markers have a key role in linking vascular risk factors, prothrombotic coagulation abnormality to atherosclerosis development in obesity.
- We concluded that the presence of comorbidities predisposing to atherosclerosis in obese individuals does not result in a further significant change of adipokine plasma levels.
- We revealed and reported for the first time in the literature the close correlation between adipokines and platelet activation markers in obesity.
- We pointed out the so far unrecognized, significant correlation between altered adipokine levels and prothrombotic coagulation abnormalities in obese patients.
- In our study we revealed novel links supporting the strong association of adipokine levels with carotid atherosclerosis in obese individuals. Exploring the exact mechanism of action by which the adipokines act in atherosclerosis through direct and haemostatic factors may provide promising specific vasculoprotective and therapeutic targets in the future.
- Our results therefore indicate the decisive role of the altered adipokine profile and platelet hyperactivation in the accelerated atherosclerosis and increased cardiovascular risk of obese individuals, irrespective of the presence of atherosclerotic comorbidities.
Hence, adipokines and platelet activation markers may serve as early biomarkers of an increased atherothrombotic risk in obesity.

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9. LIST OF PUBLICATIONS

List of publications related to the dissertation

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**Total IF of journals (all publications): 65.79**
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