Platelet function in obese children and adolescents

J. Lohse1; J. Schweigel1; A. Naeke1; MA. Lee-Kirsch1; G. Siegert2; S. Bergmann2; E. Kuhlisch3; M. Suttorp1; R. Knöfler1

1Department of Paediatrics, University Hospital Carl Gustav Carus, Technical University of Dresden, Germany; 2Department of Laboratory Medicine, University Hospital Carl Gustav Carus, Technical University of Dresden; 3Department of Medical Biometry and Bioinformatics, University Hospital Carl Gustav Carus, Technical University of Dresden

Keywords
Platelet aggregation, obesity, whole blood, children

Summary
Platelet hyperaggregability contributes to thromboembolic events of obesity in adulthood. In obese children hyperaggregability was described in platelet rich plasma. We investigated platelet aggregation in children with obesity and lipometabolic disorders in whole blood. Patients, material, methods: Specimens from patients with overweight (n = 35), hypercholesterolaemia and normal weight (n = 5), overweight plus combined lipometabolic disorder (n = 5) and healthy controls (n = 20) were investigated. Aggregation and ATP release were induced by ADP (20 μmol/l), collagen (1 μg/ml) and thrombin (0.5 U/ml) using a lumiaggregometer. Results: Overweight children and normal weight patients with hypercholesterolaemia exhibited no significant differences in platelet aggregation compared to controls. Contrastingly, in patients with obesity plus lipometabolic disorder the aggregation rate was significantly higher (p < 0.05) suggesting a hyperaggregable state. Conclusion: Obviously in obese children a hypercoagulable state exists and the slight hyperaggregability observed in whole blood in this cohort might contribute to that. Any effort should be undertaken to avoid obesity in children especially in those countries where the prevalence of obesity in childhood is continuously increasing.

Schlüsselwörter
Plättchenaggregation, Adipositas, Vollblut, Kinder

Zusammenfassung

Correspondence to:
Judith Lohse, M.D.
Department of Paediatric Haematology and Oncology University Hospital Carl Gustav Carus, Technical University of Dresden
Fetscherstr. 74, D-01307 Dresden, Germany
Tel. +49/(0)351/458 35 22, Fax +49/(0)351/458 58 64
E-mail: judith.lohse@uniklinikum-dresden.de

Obesity which is defined as body mass index (BMI) above the 97th age- and gender-related percentile is a well known risk factor for cardiovascular morbidity and mortality in adulthood and presently shows an alarming increased prevalence also in childhood and adolescence. In Germany the prevalence of overweight defined as BMI above the 90th age- and gender-related percentile in 3 to 17 years old children and adolescents is about 15% and of obesity about 6% (1).

The metabolic complications of obesity, such as elevated blood pressure, dyslipidaemia and insulin resistance may already become relevant in childhood and adolescence (2–4). For instance one third of all obese children have mild hypertension and elevated triglyceride (TG) and total cholesterol (TC) levels are present in about 25% of these children (5–7).

It is well known that in adults the combination of obesity and lipometabolic disorder leads to an increased risk of venous thromboembolism (8–10). In contrast, the incidence of thrombosis in obese children is not elevated compared to children with a
normal body weight. In a Canadian paediatric cohort study obesity was identified as a predisposing factor for thromboembolic events in only 2% of patients included (11). The low incidence of venous thromboembolism in obese children compared to adults might be due to a reduced thrombin generation capacity and an enhanced antithrombotic potential of the vessel wall (12). However, in obese children an imbalance in the haemostatic system seems to exist resulting in a hypercoagulable state caused by an increased fibrinogen level, coagulation factor VII activity, and plasminogen activator inhibitor of type 1 (PAI-1) level (13–16).

Platelets contribute to the development of atherosclerosis and its cardiovascular complications (17). Accordingly, platelet hyperaggregability has already been reported in obese adult patients (18, 19) but data on platelet function in obese children are limited to only one study investigating platelet aggregation in platelet rich plasma (6). Because data on platelet function in the more physiological milieu of whole blood are not available, the aim of this study was to investigate platelet function from obese children and adolescents using a whole blood method.

Patients

Sixty-five paediatric patients (mean age: 12.5 years, range 3 to 19 years) all of them cared for some years as outpatients were included in the study. As shown in Table 1, they were categorized into four groups (A-D) dependent on their BMI and lipometabolic profile.

Group A comprised the normal weight controls and consisted of 20 children (mean age: 10.3 years, range: 3 to 18 years) in present healthy condition. Twelve of them showed up for follow-up examination after diagnosis of malignant or non-malignant tumours (acute lymphoblastic leukemia n = 3, solid tumours n = 9) and had successfully undergone antineoplastic and/or surgical treatment without long-term sequelae which had been terminated at least two years before entering this study. Eight children were presented with the suspicion of bleeding disorder which was excluded by the determination of appropriate coagulation parameters including platelet function testing.

Group B consisted of 35 patients (mean age: 12.8 years, range: 3 to 18 years) with overweight of whom 30 also suffered from obesity who presented for follow-up investigation of overweight children as outpatients.

Group C included 5 patients (mean: 15.9 years, range: 14 to 18 years) with normal weight and hypercholesterolaemia with lipid-lowering treatment (mean cholesterol level at time of determination of platelet function 5.7 mmol/l; range: 4.8–7.1 mmol/l; reference level: < 5.2 mmol/l).

Group D comprised 5 patients (mean age: 15.5 years, range: 9 to 19 years) with obesity and combined lipometabolic disorders such as hypertriglyceridaemia with hypercholesterolaemia and elevated low density lipoproteins (LDL; n = 2), hypertriglyceridaemia with elevated LDL and decreased high density lipoproteins (HDL; n = 1), hypercholesterolaemia and elevated LDL (n = 1), hypertriglyceridaemia and decreased HDL (n = 1).

Methods

Blood sampling

Blood was collected from peripheral veins into plastic tubes containing 3.2% trisodium citrate (Sarstedt, Germany). Nine volumes of blood were anticoagulated with one volume of the citrate solution. The samples were stored for at least 10 min at room temperature. Haematocrit, leukocyte and thrombocyte counts were determined by an electronic counter in an additional ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood sample. The study protocol and the informed consent form were approved by the local Ethics Committee. Written informed consent was obtained from all patients respectively the parents.

Test procedures

Lumi-aggregometry in whole blood

Samples were tested using the Whole Blood Lumi-Aggregometer type 560 VS (Chrono-log Corporation, Havertown, USA). Platelet aggregation was determined by the impedance technique (20), and the adenosine triphosphate (ATP) release reaction was measured using the luciferin-luciferase system (21).

The final concentrations of agonists were as follows: adenosine diphosphate (ADP) 20.0 μmol/l, collagen 1.0 μg/ml and thrombin 0.5 U/ml. In case of thrombin only the ATP release was measured. All reagents were purchased from Chronolog Corporation (Havertown, USA).

The calibration of the system and the measurement of aggregation and ATP release were performed according to the manufacturer’s instructions. For the test procedure 450 μl whole blood were mixed with 450 μl isotonic saline and 100 μl luciferin-luciferase reagent in plastic cuvettes.
and stirred at 1000 rpm. In the case of aggregation measurements without ATP release 500 μl of whole blood and 500 μl of saline were used.

The aggregation curves were recorded over a period of 6 min after addition of agonist. As demonstrated in Figure 1, the aggregation curve was characterized by:
- maximal aggregation (Ohms) after 6 min,
- lag phase (min) defined as time interval from the addition of agonist to the start of aggregation,
- aggregation rate (degree) which corresponds the aggregation velocity and
- area over the curve (cm²) within 6 min after agonist addition.

The ATP release curves were recorded until the peak was reached corresponding to the maximal ATP amount released from platelets. The free ATP content in the sample was subtracted from the peak height which was transformed into the ATP amount using an ATP standard. Furthermore, the lag time (min) defined as time interval from the addition of agonist to the start of aggregation, the maximal ATP release was not different between the two groups. No differences among these patient’s groups were found for the collagen-induced aggregation and the ADP- and collagen-induced aggregation curves without simultaneous determination of the ATP release.

Comparing both groups for aggregation with simultaneous measurement of ATP release reaction, the lag-time of ADP-induced aggregation curve was significantly shortened and the ADP-induced aggregation rate was significantly higher in overweight patients (Table 2). The thrombin-induced ATP release showed a shortened time to peak in obese children whereas the maximal ATP release was not different between the two groups. No differences among these patient’s groups were found for the collagen-induced platelet aggregation and the ADP- and collagen-induced ATP release.

Statistical analysis
The t-test was used for the comparison of the analyzed platelet function parameters with equal variances between the different groups. In case of inhomogeneous variances the Welch-test, a modified t-test, was applied. Because of the significant difference of the haematocrit and the leucocyte count in groups A and B those parameters were considered as covariates. Consequently, the comparison of two groups concerning thrombocyte parameters was performed by an analysis of covariance.

The influence of the lipometabolic parameters, such as total-cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides, on the parameters of aggregation and ATP release was investigated by linear regression analyses and by the determination of the correlation coefficients according to Pearson. Due to the multicollinearity of the metabolic parameters the major factors were extracted via principal component analysis. Besides leucocyte count, haematocrit and age the main component “lipometabolic parameter” (LDL-Cholesterol, HDL-Cholesterol, lipoprotein (a)) was used to calculate the partial correlation coefficients as well as the corresponding p-values.

The significance level for all tests was taken as p less than 0.05. All tests were performed using software SPSS, version 16.0 (SPSS, Inc., Chicago Illinois).

Results
Patients
The characteristics of patients included in the study are shown in Table 1. Patients in groups B, C and D were significantly older compared to the control group (group A). The overweight patients (group B) showed significantly higher values for the haematocrit and the leucocyte count compared to the control group.

Comparisons
Overweight patients to controls
No significant differences were detected between normal weight controls (group A) and overweight children (group B) for all parameters of ADP- and collagen-induced aggregation curves without simultaneous determination of the ATP release.

Comparing both groups for aggregation with simultaneous measurement of ATP release reaction, the lag-time of ADP-induced aggregation curve was significantly shortened and the ADP-induced aggregation rate was significantly higher in overweight patients (Table 2). The thrombin-induced ATP release showed a shortened time to peak in obese children whereas the maximal ATP release was not different between the two groups. No differences among these patient’s groups were found for the collagen-induced platelet aggregation and the ADP- and collagen-induced ATP release.

<table>
<thead>
<tr>
<th>group</th>
<th>A (n = 20)</th>
<th>B (n = 35)</th>
<th>C (n = 5)</th>
<th>D (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (years)</td>
<td>10.3 ± 4.8</td>
<td>12.8 ± 4.0 *1</td>
<td>15.9 ± 1.9 **1</td>
<td>15.5 ± 4.0 *1</td>
</tr>
<tr>
<td>gender (M/F)</td>
<td>12/8</td>
<td>21/14</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>16.8 ± 2.8</td>
<td>30.0 ± 5.8 ***1</td>
<td>20.3 ± 2.3 *1 **2</td>
<td>28.7 ± 4.5 ***1</td>
</tr>
<tr>
<td>body weight (kg)</td>
<td>37.3 ± 20.1</td>
<td>77.1 ± 25.7 ***1</td>
<td>56.5 ± 7.4 **1</td>
<td>79.5 ± 15.1 ***1</td>
</tr>
<tr>
<td>haemoglobin (mmol/l)</td>
<td>8.1 ± 0.7</td>
<td>8.5 ± 0.7</td>
<td>8.8 ± 0.2</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td>haematocrit</td>
<td>0.39 ± 0.04</td>
<td>0.41 ± 0.03 *1</td>
<td>0.42 ± 0.01</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>leucocyte count (G/l)</td>
<td>6.6 ± 1.5</td>
<td>8.3 ± 2.1 **1</td>
<td>7.4 ± 2.28</td>
<td>7.8 ± 0.9</td>
</tr>
<tr>
<td>platelet count (G/l)</td>
<td>267 ± 66</td>
<td>303 ± 64</td>
<td>279 ± 67</td>
<td>229 ± 62</td>
</tr>
</tbody>
</table>

data given as means ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, versus group 1A, 2B

<table>
<thead>
<tr>
<th>Tab. 1</th>
<th>Patients’ characteristics</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>age (years)</td>
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<tr>
<td>A (n = 20)</td>
<td>10.3 ± 4.8</td>
</tr>
<tr>
<td>B (n = 35)</td>
<td>12.8 ± 4.0 *1</td>
</tr>
<tr>
<td>C (n = 5)</td>
<td>15.9 ± 1.9 **1</td>
</tr>
<tr>
<td>D (n = 5)</td>
<td>15.5 ± 4.0 *1</td>
</tr>
</tbody>
</table>

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Normal weight patients with hypercholesterolaemia to controls

No significant differences for all measured platelet function parameters were found between the normal weight controls (group A) and the normal weight patients with hypercholesterolaemia (group C).

Overweight patients with combined lipometabolic disorders to controls

The ADP-induced platelet aggregation without simultaneous determination of ATP release reaction showed a significant increase of aggregation rates in group D compared to controls (Fig. 2). The aggregation rate in response to collagen was also significantly higher in group D.

In samples using the luciferin-luciferase reagent the determination of ADP-induced aggregation showed a significant higher aggregation rate in group D with 77° compared to the controls with a mean aggregation rate of 67° (p = 0.044). Concerning the collagen-induced platelet aggregation a significant increase was seen for the area over the curve with 84.2 cm² in group D vs. 65.5 cm² in controls (p = 0.041). No significant differences were found for the ATP release reaction induced by all of the three agonists.

Correlation of platelet function with lipometabolic parameters in overweight patients

As shown in Table 3, in overweight patients (group B) the triglyceride and the total-cholesterol serum levels were significantly correlated to the collagen-induced aggregation rate and area over the curve in samples without luciferin-luciferase reagent. The other lipometabolic parameters (LDL-cholesterol, lipoprotein (a), leptin) were not related to any of the platelet function parameters.

Discussion

In paediatric overweight patients a slight hyperaggregability of platelets was detected in whole blood. However, when analyzing the platelet response to three agonists differences were detected only for the adenosine diphosphate (ADP)-induced platelet aggregation. Samples contained the luciferin-luciferase reagent (LLR) which is needed for the luminescence-based determination of adenosine triphosphate (ATP) release from platelets. It must be kept in mind that LLR itself is able to increase the agonist-induced platelet aggregation in whole blood and in platelet rich plasma (22). The underlying mechanism is unclear but most likely this phenomenon is caused by one or more reagent components.

Comparable data in the literature can be depicted only from studies perfomed in platelet rich plasma (PRP) but not in whole blood. All authors describe a marked hyperaggregability in PRP samples from obese children and adults concerning ADP-

Table 2 Comparison of the ADP-induced platelet aggregation with simultaneous determination of ATP release reaction and thrombin-induced platelet ATP release between normal weight controls (group A) and overweight children (group B)

<table>
<thead>
<tr>
<th>parameters measured by the lumiaaggregateometer</th>
<th>controls (group A; n = 20)</th>
<th>overweights (group B; n = 35)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP-induced platelet aggregation and ATP release reaction</td>
<td>lag-time (min)</td>
<td>0.49 ± 0.20</td>
<td>0.39 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>aggregation rate (°)</td>
<td>67 ± 13</td>
<td>73 ± 7</td>
</tr>
<tr>
<td></td>
<td>maximal aggregation (°)</td>
<td>13.2 ± 4.1</td>
<td>14.9 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>area over the curve (cm²)</td>
<td>47.8 ± 18.9</td>
<td>54.9 ± 20.9</td>
</tr>
<tr>
<td></td>
<td>maximal ATP release (nmol)</td>
<td>1.1 ± 0.8</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>time to peak (min)</td>
<td>2.99 ± 0.61</td>
<td>3.10 ± 0.65</td>
</tr>
<tr>
<td>thrombin-induced ATP release</td>
<td>maximal ATP release (nmol)</td>
<td>1.7 ± 0.8</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>time to peak (min)</td>
<td>1.0 ± 0.19</td>
<td>0.88 ± 0.16</td>
</tr>
</tbody>
</table>

data given as means ± SD, *p < 0.05

Fig. 2 Comparison of the ADP- (a) and collagen-induced (b) aggregation rate (α in °) in samples without luciferin-luciferase reagent between controls (group A; n = 20) and overweight children with combined lipometabolic disorder (group D; n = 5)

Tab. 3 Correlations between selected parameters of aggregation and ATP release curves and lipometabolic parameters in overweight patients (group B, n = 35)

<table>
<thead>
<tr>
<th>collagen-induced triglyceride</th>
<th>cholesterol</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>aggregation rate</td>
<td>0.481</td>
<td>0.027*</td>
<td>0.469</td>
</tr>
<tr>
<td>area over the curve</td>
<td>0.548</td>
<td>0.010*</td>
<td>0.515</td>
</tr>
</tbody>
</table>

p: p-value; r: correlation coefficient; n.s.: not significant; *p < 0.05
and collagen-induced aggregation (6, 18, 19). Additionally, a higher frequency of spontaneous platelet aggregation in PRP from obese paediatric patients was reported by Cacciari et al. (23). Platelets in obese paediatric patients are in an activated status, a finding which is also supported by Widhalm et al. (24) showing an enhanced plasma concentration of substances released from activated platelets like thromboglobulin and platelet factor 4. Obviously the different results obtained from investigations in PRP and whole blood are due to the presence of erythrocytes and leukocytes in the whole blood samples. It is well known that these cells influence the platelet activation process and it must be stressed that platelet aggregation studies in whole blood might reflect more precisely the physiological conditions in the blood than studies performed on isolated cells such as in PRP (25–28).

Limited data are reported concerning the platelet ATP release in obese children. Interestingly, Raumaraa et al. (29) describe an exercise-related lowering of platelet ATP release in overweight men suggesting a positive effect of physical exercise on platelet activation. Tangorra et al. (30) detected a decreased thrombin-induced platelet response in PRP from obese children which was associated with a diminished release reaction compared to controls. This was not confirmed in our study where only the time to the peak of thrombin-induced ATP release was significantly shorter in overweight patients but not the amount of ATP released from platelets. Takaya et al. (31) reported an increased intracellular calcium content in platelets from obese children. As the speed of platelet release reaction is calcium dependent, these results could explain at least in part the faster ATP release found in our study.

Neither the extent of platelet aggregation nor of ATP release differed between normal weight controls and normal weight children with hypercholesterolaemia. For the interpretation of these findings, however, the low number of 5 patients with hypercholesterolaemia must be taken into consideration. Our results are in accordance with findings reported by Lowe et al. (32) and Corash et al. (33) but contrast the data from Carvalho et al. (34) who detected an increased platelet response to ADP and collagen in PRP and an increased nucleotide release in hyperlipoproteinaemia type II. These findings were also confirmed by DiMinno et al. (35) and Aviram et al. (36) investigating adults with hypercholesterolaemia.

Considering the low number of 5 patients with overweight and a combined lipometabolic disorder it is remarkable that in these patients statistical analysis revealed a significantly increased ADP- and collagen-induced platelet aggregation rate and area over the curve of the collagen-induced aggregation which was independent on the presence of LLR in the samples. Because comparable data are not available from the literature we hypothesize that the combination of overweight with a lipometabolic disorder causes a more pronounced hyperaggregability than the isolated presence of either overweight or a lipometabolic disorder.

In overweight children and adolescents a positive correlation of serum concentration of triglyceride and total cholesterol with the collagen-induced aggregation rate and area over the curve was detected. Several groups already investigated the influence of HDL- and LDL-cholesterol, triglyceride and remnant lipoproteins serum concentrations on platelet function (36–42). Gallistl et al. (38) demonstrated a positive correlation of serum cholesterol with the concentration of soluble P-selectin in overweight children and adolescents. The positive correlation of cholesterol, triglyceride and lipoproteins serum concentrations with the platelet ATP release reaction has not been reported so far.

Obesity in adults and children is associated with an elevated plasma leptin level (15, 18, 43). Corsonello et al. (18) observed an increase of platelet aggregation by in vitro modified leptin concentrations in PRP samples from healthy men and suspected an increased platelet aggregation in patients with obesity. Because this was not confirmed in overweight and obese men the same authors postulate a leptin resistance in obesity and negate a prothrombotic effect of leptin in obese patients. Assuming the existence of leptin resistance also in obese children the missing correlation of leptin plasma concentration with platelet aggregation parameters in the analysis presented may be explained.

By investigating platelet function in citrate anticoagulated whole blood from obese children we have shown that obesity is associated with a slight hyperaggregability of platelets. In case of combination of overweight with a lipometabolic disorder the differences to the control group became more pronounced. With regard to studies in whole blood no comparable data exist in the literature but investigations in platelet rich plasma have revealed a marked hyperaggregability in obese children and adults. The discrepancy can be explained by the presence of erythrocytes and leukocytes in the more physiological milieu of whole blood with their known influence on the platelet activation process.

Conclusion

Taking the data from studies of the haemostatic system in obese children together it is obvious that a hypercoagulable state exists in these patients and the slight hyperaggregability observed in our patients might contribute to that. Therefore, a temporary prophylactic anticoagulation of obese paediatric patients should be considered on individual basis in prothrombotic risk situations especially in case of postoperative immobilisation lasting several days.

Our results underline the necessity for national programmes to avoid obesity in children especially in the industrial nations where the prevalence of obesity in children is continuously increasing.

Acknowledgment

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Conflict of interest

No financial conflict.
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