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Pravastatin reduces levels of the glycoprotein IIa subunit from the fibrinogen receptor on platelet-derived microparticles in patients with type 2 diabetes

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Abstract

Objective: To evaluate the effect of pravastatin on the number, cellular origin and antigenic composition of microparticles (MP) in patients with type 2 diabetes mellitus.

Methods: 48 patients with type 2 diabetes were treated for 8 weeks in a cross-over study with pravastatin 40 mg. Before and after treatment, MP were stained with annexin V, with antibodies directed against tissue factor (TF), and with cell-specific antibodies including the platelet specific monoclonal antibody CD 61 (anti-glycoprotein IIIa) and analyzed by flow cytometry.

Results: Total number of MP was the same before (434 (327; 591) X10^6/L and after (446 (315; 595) X10^6/L; P=0.9) pravastatin treatment. Also the cellular origin of MP was similar, i.e. the MP were mainly platelet-derived. Platelet derived MP did, however, expose reduced levels of glycoprotein IIIa per MP after pravastatin treatment (113.2 (98.4; 133.5) vs 106.0 (87.3; 126.0) (mean fluorescence)) (P=0.004). The number of TF positive MP and the exposure of TF per MP remained unchanged during pravastatin treatment.

Conclusion: Although the total number of MP was unaffected by pravastatin, treatment resulted in a reduction of glycoprotein IIIa exposure on platelet derived MP from patients with type 2 diabetes. Reduced glycoprotein IIIa, and thereby reduced exposure of the fibrinogen receptor on MP may be responsible in part for the risk reduction of cardiovascular disease by statin treatment.
Introduction

Diabetes mellitus type 2 is characterized by the presence of multiple cardiovascular risk factors including hypercholesterolemia (1), increased inflammation (2) and coagulation activation (3). An increased level of circulating microparticles (MP) has been suggested to be one of the procoagulant determinants in patients with type 2 diabetes that may contribute to the high risk for atherothrombotic events, such as myocardial infarction (4). MP are small membrane vesicles, released from blood cells or endothelial cells upon activation or during apoptosis (5; 6). MP may promote coagulation activation and thereby atherothrombosis by providing a procoagulant surface via the presence of phosphatidylserine in the external membrane leaflet as well as exposure of tissue factor (TF), the initiator of the coagulation cascade (7; 8) and/or their exposure of various procoagulant receptors involved in platelet adhesion and aggregation. MP may additionally determine the process of arterial thrombosis by accumulation in complicated atherosclerotic plaques and exposure of TF locally (9).

The majority of MP is derived from platelets. MP levels increase upon platelet stimulation with potent stimuli such as thrombin, collagen and shear stress. The high level of MP observed in patients with type 2 diabetes and with coronary syndromes is thought to be related to platelet activation and oxidative stress (10) (11). Accordingly, several studies showed that the number of platelet derived MP is reduced during treatment with anti-oxidative agents such as vitamin C (11) and cholesterol-lowering treatment with eicosapentaenoic acid (12) and bezafibrate (13).

The composition of MP membranes has been shown to vary in various clinical situations and this may have functional implications (10). High levels of TF exposing MP are considered to be associated with procoagulant conditions, such as sepsis (8), and may contribute to an increased thrombotic tendency. One study reported that the number of TF exposing MP is increased in patients with type 2 diabetes, but this was not associated with increased ex-vivo thrombin formation (14). The density of procoagulant receptors such as the glycoprotein IIbIIIa complex, the main platelet fibrinogen receptor on platelet derived MP of which glycoprotein (GP) IIIa forms one subunit, varies with the stimulus that has induced the MP formation (15). Differences in exposure of GPIIbIIIa on MP were shown to have an effect on fibrinogen binding (15).
Treatment with HMG-CoA reductase inhibitors (cholesterol lowering drugs known as statins) can reduce the risk for cardiovascular complications by 25% in patients with or without type 2 diabetes (16; 17). There is emerging evidence that the beneficial effects of statins may also involve an effect on oxidative stress, inflammation, and coagulation besides their lipid lowering effect (18). For instance, it was shown that statins can reduce TF expression on monocytes and vascular cells (19; 20). In addition, several studies showed that statins have an effect on platelet activation and aggregation (21). However, it is not known whether statins influence the number and properties of MP, and in particular of platelet-derived MP, in patients with type 2 diabetes. The two objectives of the present study were (1) to assess the effect of pravastatin on the level and cellular origin of MP and (2) to evaluate its effect on the composition of MP.

Materials and methods
Patients and study design
A group of 50 patients with type 2 diabetes was studied in a cross-over trial, described in detail elsewhere (22). Plasma samples for MP evaluation were available from 48 patients. Patients were recruited from the outpatient clinic of the Slotervaart Hospital, Amsterdam, The Netherlands. Men and women between 18 and 80 years of age with diabetes type 2 for at least one year and serum cholesterol levels of 5.0-10.0 mmol/l were eligible for the study. Excluded were patients with acute medical conditions: surgery during the previous 3 months, deep venous thrombosis or pulmonary embolism during the previous 3 months, significant renal, hepatic, metabolic or thyroid diseases, alcohol abuse, or known familial hypercholesterolemia. Patients were not concurrently receiving other lipid lowering, antithrombotic, or hormonal treatment, but the use of acetylsalicylic acid was allowed. Patients kept their regular diet during the study period. An open-label randomized cross-over design was used. One half of the subjects started out with pravastatin (Selektine, Bristol Myers Squibb, The Netherlands) 40 mg per day for 8 weeks, while the other half received no-treatment. Patients visited the out patient clinic at day one and after the first period of 8 weeks, whereupon pravastatin or no-treatment was crossed over for another 8 week period, and after 16 weeks at the end of the study. At these visits blood samples were taken. The active treatment and its possible effects on the measured variables
were presupposed to be washed out after 8 weeks. Laboratory outcomes at 8 and 16 weeks were compared, each patient being his/her own control. All patients gave informed consent and the study was approved by the institutional Ethical Review Board of the Slotervaart Hospital, Amsterdam.

**Control group**

Ten subjects without type 2 diabetes were recruited from the outpatient eye clinic from the Slotervaart hospital and from the personnel of the Slotervaart hospital and served as a control group.

**Reagents and assays for MP evaluation**

Anti-Glycophorin A (glyco-A)-PE (clone JC159, IgG1,κ) was obtained from Dako A/S (Glostrup, Denmark). IgG1-PE (clone X40), IgG1/2α-FITC (clone X40/X39), CD8-PE (clone SK1, IgG1), CD14-PE (clone MAEpG, IgG2b), CD20-PE (clone L27, IgG1) were obtained from Becton Dickinson (San Jose, CA, USA). CD62E-PE (clone HAE-1f, IgG1) was obtained from Ancell (Bayport, MN, USA). CD54-PE (clone 84H10, IgG1), CD62p-PE (clone CLB-Thromb/6, IgG1) and CD66b-FITC (clone 80H3, IgG1,κ) from Coulter/ImmunoTech (Marseille, France) and CD144-FITC (rabbit polyclonal) from MedSystems (Vienna, Austria). Annexin V-APC was obtained from Caltag Laboratories (Burlingame, CA, USA). CD61-PE (clone VI-PL2, IgG1,κ) and anti-TF-FITC (rabbit polyclonal) from American Diagnostics, Inc. (Greenwich, CT, USA). CD4-PE (clone CLB-T4/2, 6D10, IgG1) and CD66e-PE (clone CLB-gran/10, 1H4Fc, IgG1) were obtained from CLB (Amsterdam, The Netherlands). CD106-FITC (clone 1.11B1, IgG1) was obtained from Calbiochem (Darmstadt, Germany) and IgG2b (clone MCGb) was obtained from IQ Products (Groningen, The Netherlands).

**Blood sampling and laboratory methods**

Blood samples were obtained by standard venipuncture between 9 and 11 am, after 12 hours of fasting. For MP isolation blood was collected into 3.2% trisodium citrate (Becton Dickinson). Blood cells were removed by centrifugation for 20 minutes at 2,000 x g and 20 °C. The plasma samples were snap frozen and then stored at -70 °C until use. Total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were determined by using standard laboratory procedures within one hour after sampling. HbA1κ was determined by high performance liquid chromatography as described elsewhere (23).
Isolation of microparticles

After removal of cells, 250 μL of plasma was centrifuged for 30 minutes at 17,570 x g and 20 °C. Subsequently, 225 μL of (MP-free) plasma was removed. The remaining (MP-enriched) plasma, 25 μL, was diluted with 225 μL of phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4), containing 10.9 mmol/L trisodium citrate to prevent coagulation activation. MP were resuspended and centrifuged for 30 minutes at 17,570 x g at 20 °C. Again, 225 μL of the supernatant was removed and MP were resuspended in the remaining 25 μL. For flow cytometry, 25 μL MP suspension was diluted with 85 μL PBS/citrate buffer, of which 5 μL was used per incubation with MoAb and annexin V.

Flow cytometric analysis

MP analysis was performed and MP were identified as described previously (8; 24). Briefly, MP (5 μL) were diluted in 35 μL PBS containing 2.5 mmol/L CaCl₂ (pH 7.4). After incubation for 15 minutes at room temperature, 5 μL TF-FITC, 5 μL annexin V-APC and 5 μL PE-labeled cell-specific MoAbs or isotype-matched control antibody was added. The mixtures were incubated in the dark for 15 minutes at room temperature. Subsequently, 200 μL PBS/calcium buffer was added and the suspensions centrifuged for 30 minutes at 17,570 x g and 20 °C. Finally, 200 μL of (MP-free) suspension was removed. The MP were resuspended with 300 μL PBS/calcium buffer before flow cytometry. All samples were run for 1 minute during which the flow cytometer analyzed approximately 70 μL of the suspension. To estimate the total number of MP X10⁶/L, the number of MP (N) identified by forward scatter (FSC), sideward scatter (SSC) and binding to Annexin V was used in the formula: Number X10⁶/L = N x [100/5] x [355/70] x [250]. To estimate the marker positive number of MP X10⁶/L, the number of MP (N) found in the upper right (marker positive and TF positive)- and lower right (marker positive and TF negative) quadrants of the flow cytometry analysis (FL1 versus FL2, corrected for isotype control and autofluorescence) was used in the same formula. The samples were analyzed in a FACSCalibur flow cytometer with CellQuest-PRO software (Beckton Dickinson, San Jose, CA, USA). FSC and SSC were set at logarithmic gain. To distinguish MP from events due to noise, MP were identified on FSC, SSC and binding of annexin V. To identify annexin V-positive events, a threshold was placed in a MP sample prepared without addition of calcium, which is necessary for annexin V binding. To identify MP that bound cell-specific MoAbs,
MP were incubated with identical concentrations of isotype-matched control antibodies to set the threshold. FITC-fluorescence was measured in the FL-1 channel, PE-fluorescence in the FL-2 channel and APC-fluorescence in the FL-4 channel.

Statistical analysis
Continuous variables are represented as median and 25-75% quartiles. Statistical differences between before and after pravastatin treatment were tested by Wilcoxon Rank test. Statistical differences between type 2 diabetes patients and controls were tested by Mann-Whitney U test. A P-value of <0.05 was taken to indicate statistical significant difference. All computations were performed by using SPSS 11.0.

Results
Subject Characteristics and reductions of lipid levels after pravastatin treatment
Baseline clinical characteristics from the 48 patients with type 2 diabetes are presented in table 1. The median age of the patients was 59 years. Patients were overweight, with a median body mass index (BMI) of 29 kg/m².

Table 1. Patients’ characteristics

<table>
<thead>
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<th>Type 2 diabetes patients(n=48)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>59 (53-64)</td>
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<tr>
<td>Sex ratio (M/F)</td>
<td>24/24</td>
</tr>
<tr>
<td>Body mass index: BMI (kg/m²)</td>
<td>29.1 (26.8-33.1)</td>
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<tr>
<td>Diabetes duration (years)</td>
<td>6.0 (3.0-10.8)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.9 (6.3-7.8)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.3 (5.6-6.9)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.1 (3.5-4.4)</td>
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<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 (1.0-1.4)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 (1.3-2.9)</td>
</tr>
</tbody>
</table>

Data are presented as medians with 25th-75th percentiles.

Patients were well controlled for their diabetes, median HbA1c was 6.9%. Control characteristics are presented in table 2. The median age was similar to that of the patients with type 2 diabetes, 60 years. Total cholesterol, LDL-cholesterol and HDL-cholesterol were all significantly lower in the controls compared to the patients with type 2 diabetes.

After treatment by pravastatin statistically significant reductions of total cholesterol (-1.4 mmol/l (-1.9, -1.0)), LDL cholesterol (-1.3 mmol/l (-1.74, -0.95))
Table 2. Control characteristics

<table>
<thead>
<tr>
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<th>Controls (n=10)</th>
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<tr>
<td>Age (years)</td>
<td>60 (52.5-63.5)</td>
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<tr>
<td>Sex ratio (M/F)</td>
<td>6/4</td>
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<tr>
<td>Body mass index: BMI (kg/m²)</td>
<td>26.3 (24.8-28.2)*</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.8 (4.0-5.4)*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.7 (2.0-2.8)*</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.8 (0.7-1.4)*</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.7 (1.9-3.6)</td>
</tr>
</tbody>
</table>

Data are presented as medians with 25th-75th percentiles. Patients and controls differed significantly (\(^*\)) in BMI (P=0.02), total cholesterol (P=0.002), LDL-cholesterol (P<0.001) and HDL-cholesterol (P=0.008).

and triglycerides (-0.19 mmol/l (-0.55, 0.08)) were achieved in the patients with type 2 diabetes. HDL levels did not change during treatment.

Numbers and Cellular Origin of circulating MP

In patients with type 2 diabetes (n=48) and in controls (n=10) the total number of annexin positive MP and annexin-TF positive MP were measured (Figure 1A and 1B). No difference was observed in the total number of MP between controls and patients (523 (377-614) X10⁶/L vs. 434 (327-591) X10⁶/L; P=0.3). However, the number of TF positive MP was significantly lower in controls compared to patients (6 (5-16) X10⁶/L vs. 17 (9-24) X10⁶/L; P=0.03). MP number between before and after pravastatin treatment remained unchanged (434 (327-591) X10⁶/L vs. 446 (315-595) X10⁶/L; P=0.9). Also the number of TF positive MP did not change after pravastatin treatment (17 (8-24) X10⁶/L vs. 16 (10-28) X10⁶/L; P=0.6).

![Figure 1](image)

**Figure 1.** A) Total mean number of annexin positive MP was similar before and after pravastatin treatment (n=48) and in controls (n=10). B) Total mean number of TF positive (and annexin positive) MP did not change after pravastatin treatment. The mean number of TF positive MP was significantly lower in controls compared to patients with type 2 diabetes (**; P=0.03).
The effect from pravastatin on the number of MP derived from different cell types was assessed in a subgroup of 20 patients (Figure 2). MP from platelets (CD61) constituted the largest proportion of total MP, 68 (60; 77)%. The numbers of MP derived from platelets, T-helper (CD4) and T-suppressor (CD8) lymphocytes, monocytes (CD14), B-lymphocytes (CD20), granulocytes (CD66b), erythrocytes (glyco-A) and endothelial cells (CD62e) remained unchanged after pravastatin treatment in accordance to total MP number. Also the number of CD54, CD62p, CD106, CD144 (endothelial cells) and CD66e (epithelial cells and granulocytes) positive MP was similar before and after pravastatin treatment (data not shown).

Exposure of annexin positivity, TF and GPIIIa on platelet derived MP

To assess whether pravastatin has an effect on the membrane composition of platelet derived MP the intensity of annexin V, TF and GPIIIa (determined by

Figure 3. Antigen level on platelet derived MP was measured in patients with type 2 diabetes (n=48) before (control) and after (treatment) pravastatin treatment. Mean fluorescence of CD61 antigen (IIla) reduced significantly after treatment (* P=0.004). Annexin V and TF exposure did not differ between before and after treatment (P=0.7 and P=0.4 respectively)
exposure of CD61 antigen or GPIIIa, one part of the GPIIbIIIa complex) staining per MP was measured (Figure 3). GPIIIa expression significantly decreased after pravastatin (113.2 (98.4-133.5) vs. 106.0 (87.3-126.0) mean fluorescence; P=0.004). Annexin V and TF exposure per platelet derived MP remained unchanged during treatment with pravastatin (P=0.7 and P=0.4 respectively).

Association of MP with cholesterol levels
We investigated the correlation of total as well as cell-specific MP with cholesterol levels. The total number of annexin V positive MP did not correlate with baseline levels of total cholesterol (P=0.2), LDL-cholesterol (P=0.3), HDL-cholesterol (P=0.5) or triglycerides (P=0.4). From the subgroups of cell specific MP, only the number of P-selectin positive MP (CD62p) correlated with total cholesterol levels (r=0.57, P=0.009). The number of MP from B-lymphocytes (CD20) and endothelial cells (CD66p) correlated with LDL-cholesterol levels (r=0.45; P=0.05 and r=0.70; P=0.001 respectively). MP derived from T suppressor cells (CD8) correlated with triglyceride levels (r=0.50; P=0.03).

In addition, to identify possible relationships between exposure of TF and GPIIIa on MP and lipid levels correlation studies were assessed. From the TF positive MP only the number of MP from granulocyte origin (CD66b) correlated with LDL-cholesterol (r=0.47, P=0.04). No correlation was observed between the other subtypes or total number of TF-positive MP with total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides levels. Exposure of GPIIIa and TF on platelet derived MP did not correlate with baseline cholesterol levels either (P=0.8 and P=0.4 respectively). Since both GPIIIa exposure and lipid levels decreased after pravastatin treatment, we also assessed the correlation between the changes (level after treatment minus level before treatment) of these parameters. However, again no correlation between delta GPIIIa exposure and delta total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides could be observed.

Discussion
Statin treatment significantly reduces the risk for cardiovascular events in patients with type 2 diabetes (17). Increasing evidence suggests that this is in part the result of other than the lipid lowering effects of these drugs. In the present study we investigated the effect of pravastatin on the number and composition of MP. The
major findings are as follows: while pravastin clearly reduced cholesterol levels in the diabetic patients, the total number of MP was not different after 8 weeks of treatment. Also the number of TF positive MP and the level of TF antigen per platelet derived MP did not change after pravastatin. Pravastatin treatment, however, significantly reduced GPIIIa antigen on MP from platelet origin. Since GPIIIa associates with GPIIb to form the platelet fibrinogen receptor GPIIbIIIa, reduction of GPIIIa exposure on MP may be a new aspect of the non-cholesterol-lowering effects of pravastin, speculated to be related to an effect on platelet activation.

MP and type 2 diabetes
Previous observations that patients at risk for atherothrombotic complications, with acute coronary syndromes (25), hypercholesterolemia (11) and type 2 diabetes (4; 14) have enhanced levels of circulating procoagulant MP, suggest that MP play a role in cardiovascular disease. In the present study, the number of TF positive MP was indeed significantly higher in the diabetic patients compared to the controls (confirming the results of Diamant et al (14) in a group of 16 type 2 diabetic patients), although the total number of MP was similar in patients and controls. TF is the main initiator of the coagulation cascade and a higher number of TF positive circulating MP may promote coagulation activation and thereby increase the risk for thrombotic events like a myocardial infarction. Paradoxically, Diamant et al. (14) observed no effect of the higher percentage of TF exposing MP in patients with type 2 diabetes on in vitro thrombin formation and it was suggested that TF on MP may be more involved in angiogenesis or cell signaling rather than in atherothrombosis.

Effect of pravastatin on MP
MP composition depends on the cellular origin and the cellular processes triggering their formation (10). The observed reduction of GPIIIa exposure on platelet derived MP in the present study is therefore possibly related to the activation state of platelets. The GPIIbIIIa receptor of which GPIIIa is a subunit, is the main platelet receptor for fibrinogen and thereby crucial for thrombus formation. Release of this receptor from a storage pool to the cell membrane is upregulated in activated platelets (26). The density of GPIIbIIIa on the MP membrane can vary depending on the stimulus that induces the formation of MP, which has an effect on fibrinogen binding (15). We speculate that pravastatin
treatment inhibited platelet activation and thereby reduced the exposure of GPIIbIIIa on platelet derived MP. Indeed, there are various studies showing that statin treatment has an effect on platelet activation (27; 28) and platelet membrane composition (29; 30). The exact mechanism is not known but probably involves a change in cholesterol content of platelet intra- and extracellular membranes, which alters membrane traffic and fluidity, or a reduction in cytosolic calcium (29; 31). In particular, pravastatin has been found to reduce the expression of granule membrane protein 140 (32) and P-selectin (31). There is, as far as we know, no previous evidence that statins have an effect on GPIIbIIIa expression on platelets or platelet derived MP. Since the reduction of GPIIbIIIa is not correlated with reduction of lipid levels the effect seems to be a non-cholesterol-lowering effect of pravastatin.

We did not observe an effect of pravastatin on the total number of MP or on the number of cell specific MP. It has previously been suggested that the increased number of MP in patients with coronary syndromes and diabetes is associated with higher cholesterol levels and increased oxidative stress (11). An effect of pravastatin on MP levels was therefore expected since pravastatin not only reduces cholesterol levels in patients with type 2 diabetes (22) but is also associated with oxidative stress reduction in the vessel wall (33). Two previous studies indeed have shown that other cholesterol-lowering agents, eicosapentaenoic acid (fish oil) (12) and bezafibrate (13) reduced the number of circulating platelet derived MP. A study on the effect of oxidative stress reduction by vitamin C also showed that MP levels were reduced after treatment (11). We speculate that the lack of effect of pravastatin on MP numbers, in contrast to the other cholesterol-lowering agents, may be explained by differences in working mechanisms of the various drugs. The observation in our study that cholesterol levels did not correlate with the total number of MP suggests that processes other than lipid metabolism and oxidative stress are involved in MP formation in type 2 diabetes. This is further supported by the fact that the total number of MP number did not differ between the patients with type 2 diabetes and controls, although lipid levels were significantly higher in the patients with type 2 diabetes.

The exposure of TF on MP and the number of TF positive MP were not influenced by pravastatin treatment in the present study. TF exposure on MP is associated with clinical conditions that are associated with hypercoagulability, such as sepsis.
Pravastatin and microparticles

(8) and type 2 diabetes (14). Although statins have been described to reduce the expression of TF on vascular cells (19) and circulating monocytes (20) probably via an effect on isoprenoids (34), we could not confirm these findings on MP.

In conclusion, reduction of IIIa and therefore GPIIbIIIa exposure on MP from platelet origin may be a novel aspect of the non-cholesterol-lowering effects of pravastin and is probably related to an effect on platelet activation. Since GPIIbIIIa is the main platelet receptor for fibrinogen and crucial for thrombus formation, a reduced exposure on MP may contribute to the lower risk for arterial thrombosis during treatment with statins.

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References


