Atorvastatin reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived microparticles in patients with peripheral arterial occlusive disease

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Summary

We investigated the effects of statin treatment on platelet-derived microparticles (PMPs) and thrombin generation in atherothrombotic disease. Nineteen patients with peripheral arterial occlusive disease were randomised to eight weeks of treatment with atorvastatin or placebo in a cross-over fashion. Expression of GPIIIa (CD61), P-selectin (CD62P), tissue factor (TF, CD142) and phosphatidylserine (PS; annexin-V or lactadherin binding) was assessed on PMPs. Thrombin generation in vivo was assessed by measurement of prothrombin fragment 1+2 in plasma (F1+2) and ex vivo by using the calibrated automated thrombogram (CAT). During atorvastatin treatment, expression of TF, P-selectin and GPIIIa was significantly reduced vs. placebo (p<0.001 for all). No effect on annexin-V or lactadherin binding was seen. Thrombin generation was significantly reduced during atorvastatin as assessed by both the CAT assay (p<0.001) and by measurements of F1+2 (p<0.01). Subsequent in vitro experiments showed that when TF on microparticles (MPs) was blocked by antibodies, the initiation of thrombin generation was slightly but significantly delayed. Blocking PS on MPs using annexin-V or lactadherin resulted in almost complete inhibition of thrombin generation. In conclusion, atorvastatin reduces thrombin generation and expression of TF, GPIIIa and P-selectin on PMPs in patients with peripheral vascular disease. Microparticle-bound TF slightly enhances initiation of thrombin generation whereas negatively charged surfaces provided by MPs or lipoproteins could reinforce thrombin generation. Statins may inhibit initiation of thrombin generation partly through a microparticle dependent mechanism but the main effect is probably through reduction of lipoprotein levels.

Keywords

Atorvastatin, peripheral arterial occlusive disease, platelet-derived microparticles, thrombin generation, tissue factor

Introduction

Peripheral arterial occlusive disease (PAOD) is characterised by generalised atherosclerosis and an increased risk of atherothrombotic complications (1). Patients with PAOD have increased plasma levels of soluble platelet activation markers, increased platelet aggregability, enhanced thrombin generation and an altered fibrinolytic potential (2). These factors may contribute to the increased frequency of cardiovascular complications seen in this patient group. Statin therapy has widespread beneficial effects on the cardiovascular system through its lipid-lowering effect (3), and this beneficial effect has been documented in various patient populations, including patients with PAOD (4). However, it has also been postulated that statins have other, “non-cholesterol-related” effects relevant to cardiovascular disease, including improved endothelial function, reduced oxidative stress, reduced platelet adhesion, and plaque-stabilising effects (5).

During cellular activation small membrane particles are generated and released from the activated cells. These particles, so-called microparticles (MPs), are small vesicles 0.1–1.0 µm in diameter and are derived from various cell types such as platelets, endothelial cells, leukocytes and erythrocytes (6). Microparticles may be biologically active and this activity can be driven by way of their lipid and protein fractions. The bioactive lipid content in MPs contributes to transcellular activation of other cells, e.g. platelets and endothelial cells (7). MPs also harbor various cytokines, growth factors and proteases connecting them to inflammation, immune responses, angiogenesis, and cancer (8, 9). Platelet-derived microparticles (PMPs) are often described as having a negatively charged membrane (exposure of phosphatidylserine [PS]) and may ex-
press various surface antigens including glycoprotein (GP) IX (CD42a), GP Ib (CD42b), platelet-endothelium adhesion molecule-1 (PECAM-1, CD31) and GPIIb/IIIa (CD41/CD61) (10). In addition, PMPs can express other markers on their surface which reflect cellular activation, such as P-selectin (CD62P) and tissue factor (TF; CD142) (11). The exposure of TF and PS on PMPs is thought to contribute to the initiation and propagation of the coagulation cascade (12).

In this work we carried out a randomised double-blind placebo-controlled study in order to investigate the effect of atorvastatin on PMPs and thrombin generation in patients with PAOD. As we found that atorvastatin reduced the expression of various molecules on PMPs including TF, we also investigated the influence of MPs on thrombin generation in additional *in vitro* experiments.

### Methods

#### Patients and study design

Nineteen patients (14 women and 5 men; 65 ± 6 years old) with PAOD and hypercholesterolaemia were recruited from the Department of Surgery, Karolinska University Hospital, Huddinge, Sweden. All patients had a clinical diagnosis of intermittent claudication verified by either ultrasound duplex or angiography. Patients with angina pectoris, previous acute myocardial infarction, ischaemic stroke and those who had undergone coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI) were excluded from the study in order to avoid influences of antithrombotic and/or antiplatelet drugs on the investigated biochemical variables. Patients already on lipid-lowering and/or aspirin therapy were also excluded. The patients arrived at the laboratory in the morning after a 12-hour fast. They were asked to abstain from alcohol, tobacco smoking and caffeine-containing beverages on the experimental day. After 15 minutes (min) of acclimatisation in a supine position, blood samples were taken from an antecubital vein.

The design of the study is presented in Figure 1. All patients performed a baseline investigation when they were free from aspirin. Thereafter the patients were randomised in a double-blind, cross-over fashion to receive either aspirin (320 mg; one tablet of Alka Seltzer® per day) and atorvastatin (80 mg) once daily, or aspirin (320 mg daily) and matching placebo. The treatments were given for eight weeks each separated by a four-week wash-out period when only aspirin was administered. All other pharmacological treatment was kept constant during the study.

#### Laboratory investigations

Total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were assayed enzymatically using reagents from Synchron LX System(s) (Beckman Coulter, Dublin, Ireland). The low-density lipoprotein cholesterol (LDL-C) fraction was estimated by using the Friedewald formula (LDL-C = total cholesterol – (0.45 × TG + HDL-C) expressed in mM). Overnight excretion of 11-dehydro-thromboxane B2 (11-dehydro TxB2) in urine, reflecting platelet function *in vivo*, was determined by using a commercially available enzyme immunoasay (Cayman Chemical Co., Ann Arbor, MI, USA), and a sample work-up procedure developed by Perneby et al. (13).

### Haemostatic measurements

Antecubital venous blood was sampled, without a tourniquet, into test tubes containing 1/10 0.129 M sodium citrate. Plasma was collected after centrifugation at 2,000 x g for 10 min and then frozen at −70°C until analysis.

Thrombin generation *in vivo* was assessed by determination of prothrombin fragment 1+2 (F1+2; Enzygnost F1+2,Behringwerke AG, Marburg, Germany). Thrombin generation in plasma was determined by using the calibrated automated thrombogram (CAT) as originally described by Hemker et al. (14) and according to the instructions of the manufacturer (Thrombinscope BV, Maastricht, the Netherlands). Thrombin generation in this assay was initiated by the addition of 20 μl of both platelet-poor plasma (PPP) reagent and FluCa reagent to 80 μl of plasma, giving final concentrations of 5 pM tissue factor and 4 μM phospholipid. The calculated area under the curve (AUC) represents the total amount of thrombin generated over time and is below called the endogenous thrombin potential (ETP). Time to the start of thrombin generation (lag time), maximal concentration of thrombin generation (peak thrombin) and time to maximal thrombin generation (time to peak thrombin) are also assessed in CAT analysis (14).

Platelet-derived microparticles were assayed using a BD FACScan (measurements of CD42a/CD142) or a Beckman Coulter Gallios flow cytometer (measurements of CD42a/CD61 and CD42a/CD62p) as described previously (15). Briefly, a microparticle-enriched pellet was obtained by high-speed centrifugation from plasma samples and incubated for 20 min at room temperature (RT) with annexin-V-FITC (AbDSerotec, Clone VAA-33, Oxford, UK), lactadherin-FITC (MFG-E8, Haematologic Technologies, Essex Junction, VT, USA), CD42a-PE (Glycoprotein IX, BD, Clone Alma-16, Franklin Lakes, NJ, USA), CD142-FITC (TF, AbDSerotec, Clone CLB/TF-F5), CD61-FITC (GPIIIa, BD, NJ, USA) or CD62P-FITC (P-selectin, BD). To measure the amount of platelet fragments, samples were incubated with CD42a-PE and phallolidin-FITC (Sigma-Aldrich, St. Louis, MO, USA) (15). Forward scatter (FSC) was set against FL1 to determine the upper and lower gate limits (0.5 μm, 0.9 μm and 3.0 μm beads, Megamix beads, American Diagnostica, Stamford, CT, USA). The acquired settings and gates were later controlled with fresh platelet-rich plasma (PRP), where platelets and PMPs could be clearly distinguished. Fresh PRP, which has a low phallolidin level, were also used to determine the cut-off point for phallolidin positive events in the plasma.
MP-gate. In order to ensure minimal disturbance from non-specific binding of the antibodies, we investigated samples treated with non-specific FITC-conjugated mouse IgG. The PMP population was gated and visualised on a dot plot using labelled antibodies directed against CD42a/CD142, CD42a/CD62P or CD42a/CD61 (Gallios). The mean fluorescence intensity (MFI) of investigated samples was translated into Molecules of Equivalent Soluble Fluorochrome (MESF) using calibration standard type IIIB (Schwartz 98), Quantum™ 26–FITC (Bang Laboratories, Fishers, IN, USA). The intra- and interassay CVs for CD42a/CD62P with regard to MESF values are 6.2% and 7.2%, respectively, and the intra- and interassay CVs for CD42a/CD142 are 7.1% and 6.4% (15).

**In vitro experiments**

*In vitro* experiments were performed to investigate the influence of MPs on thrombin generation, and the relative contribution of TF and the negatively charged surface provided by MPs. An MP-enriched pellet was obtained from five patient samples collected during placebo treatment, as described earlier. The pellet was then added to commercial plasma (Haemochrom, Diagnostica, Essen, Germany) with no addition of TF or phospholipids. To further investigate the possible mechanisms of MPs on thrombin generation, experiments were also performed after addition of either a polyclonal antibody (American Diagnostica) or a monoclonal antibody against TF (AbDSerotec, Clone CLB/TF-5). A negative/isotype control mouse antibody (IgG1, AbDSerotec) was used to investigate possible unspecific effects of addition of an antibody in the CAT-assay. The effects of the TF antibodies were determined by adding them to recombinant TF in normal (pool) plasma; in the presence of antibodies lag time was increased in a concentration-dependent manner (see results section for further information). For comparison, blocking of the negatively charged PS was also investigated by testing the effects of addition of recombinant annexin-V (final concentration 22 μM) or lactadherin (final concentration 40 μM) in normal plasma.

**Statistics**

Based on data on MP expression of CD62P in patients with claudication it was estimated that 20 patients were needed to detect a 30% difference between treatments with a power of 80% at a p<0.05 significance level (two sided test). We considered normality to be present if skewness was above –1 and below 1. Data that are normally distributed are presented as means ± SEM (± SD in Table 1), whereas skewed data are presented as medians and 25th and 75th percentiles (F1+2 data only). Normally distributed data were analysed by Student’s paired t-test. Skewed data (F1+2) were logarithmically transformed and then analysed by Student’s paired t-test. Statistical calculations were performed using SPSS.
(16.0, SPSS Inc., Chicago, IL, USA) and Graphpad Prism (5.0b) software. A p-value of <0.05 was considered to be statistically significant. The study was approved by the Ethics Committee of the Karolinska Institute.

Results

Plasma lipids, thromboxane generation and haematological variables

As expected, atorvastatin treatment significantly reduced cholesterol levels, i.e. plasma levels of total cholesterol decreased from 7.0 ± 1.1 to 4.1 ± 0.8 mM, and the levels of LDL cholesterol from 4.8 ± 0.8 to 2.2 ± 0.6 mM. Atorvastatin also reduced triglyceride levels from 2.0 ± 1.3 to 1.4 ± 0.6 mM (p<0.001 for all) whereas HDL cholesterol levels were not significantly changed (1.27 ± 0.4 and 1.35 ± 0.4 mM during placebo and atorvastatin treatment, respectively).

Aspirin treatment reduced the excretion of 11-dehydro TxB₂ into urine from 76 ± 34 ng/mmol creatinine (pretreatment) to 16 ± 7 and to 15 ± 5 ng/mmol creatinine during placebo and atorvastatin treatment, respectively (p<0.001). The excretion of 11-dehydro TxB₂ remained stable during the randomised treatments (p=0.29 between treatments). Aspirin had no significant influence on the other haemostatic variables measured. Atorvastatin had no significant effects on platelet, leukocyte or red blood cell counts, or haemoglobin values (data not shown).

Thrombin generation

Concentrations of F1+2 in plasma decreased during atorvastatin therapy (from 1.25 [1.07, 1.39] nM with placebo to 1.06 [0.90,1.31] nM during atorvastatin treatment; p=0.004, Fig. 2A). Furthermore, the amount of thrombin formed over time (endogenous thrombin generation; ETP) was significantly lower during atorvastatin treatment compared with placebo (1,684 ± 286 vs. 1,883 ± 282 nM x min; p<0.001, Fig. 2B). Peak thrombin levels were also significantly reduced during atorvastatin treatment compared with placebo (328 ± 42 vs. 347 ± 54 mM, p<0.02), as was time to peak (5.0 ± 0.6 vs. 5.2 ± 0.7 min, p<0.02). No change was seen in lag time (2.51 ± 0.40 vs. 2.35 ± 0.42 min, p=0.7).

Platelet-derived microparticles

The percentage of particles in the microparticle gate positive for phalloidin was 9.7 ± 1.9% indicating acceptable sample handling with low numbers of platelet fragments in plasma samples (11, 15). Expression of CD61, CD62P and CD142 was significantly reduced during atorvastatin treatment compared with placebo (Fig. 3 C–E). On the other hand atorvastatin had no effect on PS expression on platelet-derived microparticles as measured by either annexin-V or lactadherin binding (Fig. 3 A, B). No statistically significant correlations could be seen between reductions in lipid levels and reductions in expression of CD61, CD62P or CD142 on microparticles following atorvastatin treatment.

Influence of PMPs on thrombin generation

In order to investigate the possible influence of MPs on thrombin generation, additional in vitro experiments were performed using CAT analysis. As can be seen from Fig. 4 and Table 1, thrombin generation was significantly increased when MPs sampled from patients at baseline (n=5) were added to a commercial normal plasma pool (p<0.001 for all parameters). Total thrombin generation (ETP) increased from 929 ± 72 nM*min in the absence of MPs to 1,438 ± 24 nM*min in plasma pool samples to which MPs had been added. Furthermore, lag time was reduced from 24.7 ± 3.2 to 8.4 ± 1.2 min, peak thrombin levels were increased from 60 ± 12 to 135 ± 5.4 nM and time to peak was reduced from 36.7 ± 2 to 15.3 ± 2 min in the presence of MPs.

Figure 2: The effect of atorvastatin on thrombin generation.
Figure 3: The effect of atorvastatin on platelet-derived microparticles.

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In order to investigate the effects of TF and PS provided by MPs on thrombin generation, some dose-response experiments were performed. The effects of two different TF antibodies (see Methods) on thrombin generation in normal plasma were investigated using the original CAT assay (i.e. where TF at 5 pM is used to initiate thrombin generation). The effects of increasing concentrations of the antibodies were measured against a positive control (i.e. antibodies added to plasma in the absence of TF). An antibody concentration of 40 ng/ml prolonged lag time by 93% in relation to the positive control and was chosen for the experiments. The addition of a mouse IgG1 had no effect in the CAT-assay (results not shown).

Data on inhibition of TF and PS are displayed in Table 1. As can be seen, addition of a monoclonal TF antibody to MP-enriched plasma resulted in a slight but significant increase in lag time and a slight decrease in thrombin generation. Addition of a polyclonal antibody against TF yielded similar results as the monoclonal antibody (results not shown).

The effect of blocking PS by incubation of normal plasma containing added MPs with increasing concentrations of annexin-V or lactadherin were also investigated. As can be seen from Figure 4, both annexin-V (Fig. 4A) and lactadherin (Fig. 4B) inhibited thrombin generation in a concentration-dependent manner, and resulted in a much stronger inhibition of thrombin generation than addition of TF antibodies did.

Drug compliance and adverse events

All randomised patients completed the trial. Compliance with medication was, according to pill count, 95% for placebo and 98% for atorvastatin. Two mild adverse events were reported; one patient had diarrhea during placebo treatment whereas another patient reported nausea and cramps in the calf during atorvastatin treatment. Two patients on active treatment had ALAT concentrations three times above the upper normal limit and one patient showed slightly elevated creatinine kinase (3.9 mm/l) during atorvastatin treatment.

Discussion

In the present study, we investigated the effects of atorvastatin on platelet-derived microparticles and thrombin generation in patients with PAOD treated with aspirin. We found significant reductions in expression of TF, P-selectin and GPIIIa on PMPs, whereas the amount of PS on PMPs, as measured by binding of annexin-V or lactadherin, was not influenced by atorvastatin. Thrombin generation in vivo, as estimated by prothrombin fragment 1+2 measurements, as well as the ex vivo-measured ETP, were significantly reduced during atorvastatin treatment compared with placebo. Thus, atorvastatin treatment has effects on haemostatic variables, which are expected to be beneficial in terms of atherothrombotic complications.

To assess whether atorvastatin influenced PMP PS exposure, we measured the binding of both annexin-V and lactadherin. Annexin-V binding is commonly used to assess PS exposure. Lactadherin is a glycoprotein of ~50 kDa, which binds to phosphatidylserine in a calcium-independent manner and has recently been reported to reflect PS exposure in a more sensitive manner than annexin-V binding (16). In the present study both annexin-V binding and lactadherin binding remained unchanged during treatment with atorvastatin indicating that statin treatment does not affect PS exposure on PMPs. In contrast, expression of TF, P-selectin and GPIIIa were all significantly reduced during atorvastatin treatment. The observed reduction in expression of platelet-specific markers may be interpreted as being indicative of reduced platelet activation. However, as we did not determine the concentration of PMPs, but determined the antigen expression of TF, P-selectin and GPIIIa on PMPs, we cannot say for sure whether our findings reflect reduced PMP counts, or reduced antigen expression without affecting PMP counts. Viewed in view of the findings by Sommeijer et al (24), who found reduced expression of GPIIIa (CD61) but no effect on PMP counts in patients with type 2 diabetes treated with pravastatin, it is likely that it is the antigen expression that is affected by atorvastatin in our study. Anyhow, it would not be controversial to conclude that our data which show a highly sig-
significant reduction in expression of TF, P-selectin and GPIIIa, reflect platelet inhibiting effects of atorvastatin treatment.

Several (17, 18) but not all investigators (19) have found platelet-inhibiting effects of statins. One postulated mechanism by which statin treatment may attenuate platelet function is through changes in the cholesterol content of platelet membranes, leading to alterations in membrane protein expression and function (20), but if this affects microparticle formation is unknown. Another proposed mechanism is reduced platelet production of thromboxane A2 (21). However, in the present study all patients were on aspirin, so it is unlikely that statin-induced inhibition of thromboxane formation was mechanistically involved. Statin treatment has, however, been reported to suppress TF gene expression and decryption of TF (22), and it has been speculated that negatively charged surfaces could activate TF. It is of note that some lipoproteins are negatively charged, and a reduction in lipoproteins such as very low-density lipoprotein (VLDL) and LDL may thus lead to reduced TF/FVIIa downstream activation of the coagulation cascade and thereby reducing thrombin generation and platelet activation. In addition, reduction in plasma levels of negatively charged lipoproteins may reduce coagulation propagation by reducing surfaces for assembly of the tenase complex and prothrombinase complex thus reducing thrombin formation. This could be mechanisms through which atorvastatin reduces exposure of platelet activation markers on MPs.

The results of some previous studies also suggest that MP production can be influenced by statin treatment. In vitro, fluvastatin has been reported to inhibit endothelial cell-derived microparticle shedding, partly through Rho small GTPases, key regulators in cytoskeleton remodelling (23). In vivo, GPIIIa expression was reduced in MPs from patients with type 2 diabetes mellitus and treated with pravastatin (24). Furthermore, circulating monocyte-derived MPs were reduced in hypertensive patients treated with a combination of losartan and simvastatin (25). Our present findings that atorvastatin reduces platelet-specific markers on MPs, and TF expression, are in line with the results of a recent study by our group in patients with type 1 diabetes showing significant reductions of TF, GPIIIa and P-selectin expression during atorvastatin treatment (26). Thus, the present data extend information on the effect of atorvastatin on MPs to a non-diabetic group of patients with manifest atherosclerotic disease, i.e. patients with PAOD.

As both thrombin generation and TF expression on MPs were reduced during treatment, we investigated the effect of MPs on thrombin formation in vitro. Adding a microparticle-enriched pellet obtained from plasma of PAOD patients to commercial normal plasma enhanced all parameters of the thrombogram compared to negative control (Table 1). Thus, ETP and peak thrombin concentration increased, and lag time and time to thrombin peak were shortened in the presence of microparticles from the patients. These results were obtained without any added TF or phospholipids, which is a slight modification of the original method described by Hemker et al. (14).

The issue of TF expression on MPs has been lively debated (27), and it has been questioned if the TF molecule expressed on MPs is intact, and whether it is in an active or inactive state in the circulation. Our experimental data indicate that TF measured on MPs in the present study has a small but statistically significant enhancing effect on thrombin generation. When TF was blocked by the addition of antibodies to samples containing MPs the initiation of thrombin generation (lag time) and time to peak of thrombin generation were delayed and peak thrombin generation slightly but significantly decreased, findings which are in agreement with the hypothesis that TF present on cells can initiate formation of small amounts of thrombin (12).

Our findings are however different from those reported in a study by Diamant et al. (28). In that study TF-positive microparticles from type 2 diabetes patients initiated thrombin generation, but the mean thrombin generating capacity induced by MPs from patients was significantly lower than what could be induced by MPs from healthy control subjects. Pre-incubation of MPs from both controls and patients with a polyclonal anti-TF antibody did not significantly affect thrombin generation. The reasons for the discrepancy between our findings and those reported by Diamant et al. are unknown, but may be explained by the use of different antibodies and different patient populations from which the MPs were obtained. Diamant et al. did not investigate if phosphatidylserine exposed on the MPs had any effect on thrombin generation. Our data suggest that the main effect of MPs on thrombin generation is exerted by the negatively charged surface provided by MPs. When blocking the surface of MPs with annexin-V or lactadherin we observed strong and concentration-dependent inhibition of thrombin generation (see Fig. 4). However, as dis-

**What is known about this topic?**

- Peripheral arterial occlusive disease (PAOD) is associated with an increased risk of atherothrombotic complications.
- Statins have been shown to exhibit vascular protective effects, including some potentially anti-thrombotic properties.
- Platelet-derived microparticles (MPs) are released from activated platelets and considered to be procoagulant due to the exposure of negatively charged surfaces and tissue factor (TF).

**What does this paper add?**

- Aspirin treatment alone has no effect on thrombin generation or the PMP expression of TF, P-selectin or GPIIIa in patients with PAOD.
- Treatment with atorvastatin attenuates thrombin generation and reduces PMP expression of TF, P-selectin and GPIIIa without affecting the exposure of negatively charged surfaces in PAOD patients treated with aspirin.
- Microparticles enhance thrombin generation mainly through expression of negatively charged surfaces and to lesser degree through expression of TF.
- The major mechanism behind atorvastatin induced inhibition in thrombin generation does not seem to involve microparticles, but attenuation of a TF dependent effect on the initiation of thrombin generation cannot be excluded.
cussed above the lipoproteins VLDL and LDL (especially oxidised LDL) may be procoagulant, as they could support thrombin generation through expression of PS (29). It is therefore possible that the reduction in thrombin generation seen in the present study is also partly a result of VLDL and LDL reduction brought about by atorvastatin. This mechanism would thus be dependent on the lipid lowering effect of statins. Indeed, lipid-lowering treatment with the fibrate gemfibrozil also reduces thrombin generation (30).

In the present study all patients were put on treatment with 320 mg aspirin daily, but a baseline sample, taken before aspirin treatment was started (i.e. before the patients entered the double blind randomised part of the investigation) made it possible to assess the influence on aspirin on thrombin generation and PMPs. When we compared baseline and placebo data on GPIIIa, CD62P and TF on PMPs, and thrombin generation, no effect of aspirin could be seen. Thus, it is unlikely that 320 mg aspirin daily has any effect on PMPs or thrombin generation in patients with PAOD. Urinary excretion of the thromboxane metabolite 11-dehydro TxB2 was stable during randomised treatment, indicating that atorvastatin did not affect thromboxane generation in patients on aspirin treatment, which may be the case when hyperlipidemic patients free from aspirin are treated with statins (31).

There are limitations in the present study, which should be taken into account. As discussed briefly above, the flow cytometry method used is based on measurements of MFI, i.e. the amount of a specific antigen on the surface of a particle is measured through assessment of fluorescence intensity from specific antibodies bound to the antigen. The MFI is then translated into MESF (Molecules of Equivalent Soluble Fluorochrome) by using a standard curve. Lower MESF values thus indicate that the sample contains fewer MPs expressing the antigen, or that less antigen is bound to the MPs. Another possibility would be to measure the absolute numbers of PMPs. However, it could be argued that it is the total amount of TF and/or PS that determines the degree of thrombin generation and thus is pathophysiologically relevant. Another limitation is that we studied only platelet-derived microparticles. Thus it is possible that some of the TF activity that we blocked in vitro using TF antibodies was expressed on microparticles derived from other cell types, e.g. monocytes. We therefore cannot prove a mechanistic link between atorvastatin-induced reduction in TF expression on PMPs and the reduced thrombin generation we observed by assessing ETP and F1+2 concentrations in plasma. One limitation of the study may also be that we performed no ultracentrifugation of plasma samples to avoid contamination of platelets and/or platelet/other cell membrane fragments that may be present in frozen/thawed samples. However, using FITC-labelled phalloidin, a fungal toxin which binds with high affinity to intracellular actin, we have previously found that high-speed centrifugation (20,000 x g) yields low amounts of cells/ cell membrane fragments that may cause artefacts when measuring microparticles. In the present study around 10% of platelet microparticles were positive for phalloidin indicating acceptable sample handling and sample quality (15). A further limitation is the small number of patients in our study. However, the study was randomised, double-blind and placebo-controlled and yielded highly significant differences in PMP expression and thrombin generation.

In conclusion, atorvastatin reduces thrombin generation and expression of TF, P-selectin and GPIIIa on PMPs in patients with PAOD treated with aspirin. MPs may have a role in thrombin generation, where the mechanism mainly involves the presence of negatively charged surfaces provided by MPs and to a lesser extent involve TF expression. The reduction in thrombin generation seen in the present study of patients with PAOD is probably a result of reduced lipoprotein levels brought about by atorvastatin treatment, but according to our data it cannot be excluded that reduced TF expression on microparticles also may contribute.

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Conflict of interest
None declared.

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