Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells

Rosa Suades1,2; Teresa Padró1,2; Rodrigo Alonso3; Pedro Mata3; Lina Badimon1,2,4
1Cardiovascular Research Center (CSIC-ICCC), IIB-Sant Pau, Barcelona, Spain; 2CIBERobn – Pathophysiology of Obesity and Nutrition, Barcelona, Spain; 3Jimenez Diaz Foundation, Madrid, Spain; 4Cardiovascular Research Chair, UAB, Barcelona, Spain

Summary
Hyperlipidaemia is a causal factor in the etiopathogenesis of atherosclerosis. Statins are the cornerstone drug therapy for LDL-cholesterol (LDL-c) lowering, that exert beneficial effects beyond lipid lowering. Circulating microparticles (cMPs), microvesicles released by activated cells into the bloodstream, are markers of vascular and inflammatory cell activation with tentative role in disease progression. However, the role of statins on cMPs seems controversial. We aimed at the evaluation of the effects of lipid-lowering treatment (LLT) on cMP generation in patients in primary prevention of atherosclerosis. A case-control study was conducted in hypercholesterolaemic patients receiving LLT with statins and normocholesterolaemic controls (LLT+ and LLT-, respectively, n=37/group), matched by age, gender and LDL-c levels. cMPs were characterised by flow cytometry using annexin-V and cell-specific antibodies. In LLT+-patients overall numbers of cMPs (p<0.005) were lower than in controls. Levels of cMPs carrying parent-cell markers from vascular and circulating cell origin (platelet, endothelial cell, pan-leukocyte and specific-leukocyte subsets) were significantly lower in blood of LLT+ compared to LLT-patients. Moreover, MPs from LLT+-patients had reduced markers of activated platelets (αMβ2-integrin), activated inflammatory cells (αvβ3-integrin) and tissue factor. The effect of LLT on cMP shedding was found to be accumulative in years. cMP shedding associated to cardiovascular risk in LLT+-patients. In summary, at similar plasma cholesterol levels patients on statin treatment had a significant lower number of cMPs carrying markers of activated cells. These findings indicate that statins protect against vascular cell activation.

Keywords
Atherosclerosis, circulating microparticles, inflammation, statins, thrombosis

Introduction
Effective treatment of hyperlipidaemia (HL) is of great importance in the overall management of vascular risk and prevention of cardiovascular disease (CVD). Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitors, are the drug of choice for controlling lipid levels and reducing morbidity and mortality among patients with cardiovascular risk (1, 2). Beyond lipid-lowering effects, statins exert many pleiotropic effects, which involve anti-inflammatory, anti-atherogenic and anti-thrombotic effects (3-5). Pleiotropic effects of statins are mainly caused by inhibition of protein prenylation. Isoprenylation, a post-translation protein modification, adds intermediates of the mevalonate pathway (such as geranylgeranyl pyrophosphate –GGPP–) to small G proteins (Rho, Rac, Rab) and facilitates their cell membrane attachment and the subsequent activation of transmembrane signaling (6, 7). Thus, statins are involved in the modification of G protein functions, which mainly include cytoskeleton assembly, gene regulation, cell growth and motility, protein and lipid trafficking, and hence statins may affect membrane microparticle shedding. To evidence these tentative effects of statins in cell membranes in patients treated clinically we designed this study.

Circulating microparticles (cMPs) are submicron membrane vesicles shed from activated cells into the bloodstream (8). cMPs may originate from cells that are involved in the pathogenesis of atherothrombotic diseases and exposed to lipids in the circulation such as endothelial, leukocyte, and platelets (8-11). Indeed, it has been recently shown that circulating and platelet-derived microparticles enhance thrombosis on atherosclerotic plaques (12). Circulating MPs are also found in the plasma of healthy subjects...
Suades et al. Lipid-lowering treatment impact on microparticles

(13), but the number of cMPS increases in patients with existing cardiovascular disease (14-16) and with risk factors for CVD (17, 18). In fact, cMPS also seem to have substantial effects on early phases of atherogenesis (19, 20).

There are controversial results regarding the effects of lipid-lowering on cMP release (21). Diamant et al. reported that statins may stimulate endothelial-derived microparticle (eMP) release in vitro (22, 23). In contrast, other studies have shown that fluvastatin promotes inhibition of the release of eMPS in vitro (24). Atorvastatin has been reported to increase circulating eMPS in vivo (25) but also to reduce expression of activation markers on platelet-derived microparticles (pMPS) in patients with peripheral arterial occlusive disease (26, 27) and with type-1 diabetes and dyslipidaemia (28). Association of simvastatin with losartan decreased monocye-, endothelial- and platelet-MP numbers in patients with hypertension and type-2 diabetes (29, 30) and pravastatin reduced β3-integrin on pMPS in type-2 diabetes (31). Finally, pitavastatin could only decrease pMPS in association with eicosapentaenoic acid in the adiponectin-responder group of both hyperlipidaemic and diabetic patients (32). Recently, the increased plasma TF activity shown in hyperlipidaemic mice and monkeys was found to be reduced by simvastatin treatment (33).

In general, the effects of statins on cMPS were associated to reduction in low-density-lipoprotein cholesterol (LDL-c) levels. To further understand whether the beneficial effects of statins on cardiovascular disease are also related to effects on cell activation independent of their lipid lowering effect, we designed this study analysing cMP numbers, cell source and their transported activation cell markers in blood of subjects with the same LDL-c levels with or without statin treatment.

Study design, materials and methods

Clinical study population

The present nested case-control study was part of the ongoing SAFEHEART study (34). Briefly, SAFEHEART is an open, multicentre, long-term prospective cohort study in a well-molecularly defined familial hypercholesterolaemia (FH) population. Demographic and clinical characteristics data, cardiovascular history, classic cardiovascular risk factors and current treatment for hypercholesterolaemia were obtained from all subjects using a standardised report form at the inclusion in the cohort. Data related to lipid-lowering treatment (LLT) included statin, dose, time of treatment and compliance. Adherence to lipid lowering treatment was assessed by indirect method with a single question, as previously described (35). Maximum statin dose was defined as previously described: simvastatin 80 mg/day, pravastatin 40mg/day, lovastatin 80 mg/day, fluvastatin 80 mg/day, atorvastatin 80 mg/day, rosvastatin 20–40 mg/day (36). Cases were randomly selected from the group of hypercholesterolaemia and with negative genetic testing of FH that were receiving a stable LLT, at least one year before the inclusion, according to clinical guidelines (37-39). The control group included subjects from the same cohort with the same LDL-c levels that were not on LLT, matched by age, gender, and demographics. Neither patients nor control subjects present pregnancy, sepsis or infection or have history of cancer, since these conditions are known to independently impair MP number. The results of the study are presented in accordance with STROBE guidelines. The study was approved by the local ethics committee and was conducted according to the Declaration of Helsinki. A written informed consent was obtained from all participants prior to the study.

Blood sampling for analysis

Venous blood was withdrawn from the cubital vein without tourniquet using a 20-gauge needle after 10-14 hours (h) of fasting into serum, EDTA and 3.8% sodium citrate tubes, for biochemical, genotyping and microparticle analysis, respectively.

Serum (30 minutes [min] at 37°C, 30 min at 4°C, 1258 x g for 15 min at 4°C) and EDTA-plasma (1258 x g for 20 min) were prepared for lipid profile and DNA analysis. Samples were processed for assay within first 2 h. Total cholesterol, triglycerides, and high-density-lipoprotein (HDL)-cholesterol were measured by standardised enzymatic methods. Serum LDL-cholesterol concentration was calculated using the Friedewald formula (40). Lipoprotein (a) levels were measured using a turbidimetric method performed by suspension of latex particles coated with rabbit IgG anti-Lp (a) gene (Abbott Diagnostics, Abbott Park, IL, USA) in an Architect autoanalyzer c16000 (Abbot). For molecular genetic analysis, genomic DNA was isolated from whole blood using standard methods and the genetic diagnosis of FH made using a DNA microarray (LIPOCHIP) in a central laboratory (Progenika, Bilbao, Spain) (41).

For flow cytometric analysis of cMPS, blood cells were removed from citrated blood by low-speed centrifugation at 1258 x g for 20 min at room temperature (RT) in order to avoid in vitro platelet activation, as previously described (42, 43). Platelet-poor plasma (PPP) was carefully aspirated, leaving about 0.1 cm undisturbed layer on top of the cells. A second centrifugation step (1258 x g for 10 min, RT) was made to ensure the complete removal of cells and obtain the platelet-free plasma (PFP). All samples were processed identically and within 60 min after extraction. Samples were tested with a cell counter for the absence of residual cells after centrifugation. PFP aliquots were snap-frozen in liquid nitrogen and stored at -80°C until flow cytometric studies.

Circulating microparticle isolation

The cMP fraction was isolated from PFP by a two-step high speed centrifugation (42, 43). Briefly, frozen PFP aliquots were thawed on melting ice for 1 h and centrifuged for 30 min at 20,000 x g to pellet cMPS. Then, supernatant was discarded and the cMP-enriched pellet was washed once with citrate-phosphate buffered saline solution (citrate-PBS; 1.4 mmol/l phosphate, 154 mmol/l NaCl, 10.9 mM trisodium citrate, pH 7.4) before a second equal centrifugation step was made. Finally, the remaining cMP pellet was resuspended in citrate-PBS to a final volume of 100 μl.

Thrombosis and Haemostasis 110.2/2013 © Schattauer 2013
Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as described by Nieuwland et al. (43, 44) with slight modifications. Briefly, washed cMP suspensions (5 μl) diluted in 30 μl phosphate-buffered saline (PBS) containing 2.5 mM CaCl$_2$ were incubated (20 min, RT, dark) with combinations of BD-horizon V450-conjugated annexin V (5 μl) with two specific monoclonal antibodies.

Table 1: Clinical characteristics of untreated (LIT$^-$) and treated (LIT$^+$) patients.

<table>
<thead>
<tr>
<th>Risk factor for CVD</th>
<th>Male /Female (n)</th>
<th>LIT$^-$-patients</th>
<th>LIT$^+$-patients</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes mellitus (n, %)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Hypertension (n, %)</td>
<td>1 (2.7%)</td>
<td>7 (18.9%)</td>
<td>p = 0.02</td>
<td></td>
</tr>
<tr>
<td>Tobacco consumption (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Current</td>
<td>10 (27%)</td>
<td>12 (32.4%)</td>
<td>p = 0.61</td>
<td></td>
</tr>
<tr>
<td>- Ex-smokers</td>
<td>6 (16.2%)</td>
<td>5 (15.5%)</td>
<td>p = 0.74</td>
<td></td>
</tr>
<tr>
<td>- Never</td>
<td>21 (56.8%)</td>
<td>20 (54.1%)</td>
<td>p = 0.82</td>
<td></td>
</tr>
<tr>
<td>Obesity (BMI &gt; 30)</td>
<td>0 (0%)</td>
<td>7 (18.9%)</td>
<td>p = 0.005</td>
<td></td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
<td></td>
</tr>
<tr>
<td>Waist diameter (cm)</td>
<td>101.0 (98.0–106.0)</td>
<td>87.0 (78.0–99.0)</td>
<td>p &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Corneal arcus</td>
<td>0 (0%)</td>
<td>12 (32.4%)</td>
<td>p = 0.0002</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>203.0 (186.0–228.0)</td>
<td>210.0 (192.0–240.0)</td>
<td>p = 0.56</td>
<td></td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>130.0 (110.0–147.4)</td>
<td>134.2 (109.0–159.6)</td>
<td>p = 0.35</td>
<td></td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>55.0 (47.0–64.0)</td>
<td>55.0 (48.0–63.0)</td>
<td>p = 0.74</td>
<td></td>
</tr>
<tr>
<td>TC / HDL-C ratio</td>
<td>4.0 (3.3–4.5)</td>
<td>3.9 (3.1–4.5)</td>
<td>p = 0.88</td>
<td></td>
</tr>
<tr>
<td>Lp(a) (mg/dl)</td>
<td>14.8 (6.0–32.7)</td>
<td>21.0 (10.9–48.5)</td>
<td>p = 0.13</td>
<td></td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>97.0 (67.0–124.0)</td>
<td>82.0 (64.0–120.0)</td>
<td>p = 0.50</td>
<td></td>
</tr>
<tr>
<td>High-sensitive CRP (mg/dl)</td>
<td>1.7 (1.0–2.7)</td>
<td>1.0 (0.7–2.4)</td>
<td>p = 0.04</td>
<td></td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>85.5 (78.5–91.5)</td>
<td>86.0 (78.0–91.0)</td>
<td>p = 0.91</td>
<td></td>
</tr>
</tbody>
</table>

Medication

<table>
<thead>
<tr>
<th>Medication</th>
<th>LIT$^-$-patients</th>
<th>LIT$^+$-patients</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEI</td>
<td>0 (0%)</td>
<td>3 (8.1%)</td>
<td>p = 0.08</td>
</tr>
<tr>
<td>ARB</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Anti-platelet drugs</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>CCB</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Statins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Simvastatin</td>
<td>0 (0%)</td>
<td>10 (27%)</td>
<td>p = 0.0007</td>
</tr>
<tr>
<td>- Atorvastatin</td>
<td>0 (0%)</td>
<td>18 (48.6%)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>- Rosuvastatin</td>
<td>0 (0%)</td>
<td>7 (18.9%)</td>
<td>p = 0.0054</td>
</tr>
<tr>
<td>- Fluvastatin</td>
<td>0 (0%)</td>
<td>2 (5.4%)</td>
<td>p = 0.15</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>0 (0%)</td>
<td>4 (10.8%)</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>HRT</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Tiazidic diuretics</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Diurasa</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Metformin</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
<tr>
<td>Glinidas</td>
<td>0 (0%)</td>
<td>1 (2.7%)</td>
<td>p = 0.31</td>
</tr>
</tbody>
</table>

For personal or educational use only. No other uses without permission. All rights reserved.
(mAbs, 5 μl each) labelled with fluorescein isothiocyanate and phycoerythrin, or with the isotype-matched control antibodies (see Suppl. Table 1, available online at www.thrombosis-online.com) and, then, diluted with 2.5 mM CaCl₂-PBS buffer before being analysed on a FACSCantoII™ flow cytometer (Becton Dickinson, San Diego, CA, USA). Acquisition was performed for 1 min per sample. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with gain settings in the logarithmic scale. cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to cell-specific mAb. Granulocyte's percentage was inferred by subtracting agranulocytes (lymphocytes plus monocytes) from total leukocytes instead of labelling with specific mAb. Gate limits were established following two criteria: 1) calibration using a Flow Check YG Size Range Calibration Kit (Polysciences, War- rington, PA, USA) (45) and 2) with an in vitro platelet-derived microparticle population as positive control (46) (see Suppl. Figure 1A-D, available online at www.thrombosis-online.com) since cali-

Figure 1: Comparison of cMP numbers and cellular origins between LLT- and LLT+ patients. A) Box and whisker plots showing number of total, AV+, and AV–cMPs per microliter of platelet-free plasma (cMPs/μl PFP), both in controls without LLT (n=37) and subjects under LLT (n=37). Lines within boxes represent median values, the upper and lower boxes lines represent the 25th and 75th percentiles, respectively, and the upper and lower bars outside the boxes represent the 10th and 90th percentiles, respectively. B) Pie charts showing distribution of cMPs from (I) controls and (II) subjects on LLT by cell origin, represented by the percentage of total cMPs from each cell type. Used markers were CD41+/CD61+ for platelet, CD146+/CD31+ for endothelial cell, CD45+ for total leukocyte, CD3+ for lymphocyte and CD14+ for monocyte origins accounting for agranulocytes and, finally, granulocytes were inferred subtracting agranulocytes subpopulation from leukocytes fraction. Numbers indicate percentages of each marker relative to all cMP population and, percentages of leukocyte subpopulations relative to total leukocytes are expressed in brackets. *p<0.0001, **p<0.02 vs control group (U-Mann Whitney test).
Microparticles have different properties of FSC/SSC compared with biologic MPs (47, 48). The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer. To identify positive marker events, thresholds were set based on samples incubated with the same final concentration of isotype-matched control antibodies after titration experiments. Phosphatidylserine-positive cMPs were labelled using annexin V in the presence of 2.5 mM CaCl$_2$, since calcium is essential for annexin V conjugation. Annexin V binding level was corrected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer.

Data were analysed with FACS Diva™ software (version 6.1.3, Becton Dickinson). The concentration (number of cMPs per µl of plasma) was determined according to Nieuwland's procedure (44), based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events (N), as follows: cMPs/µl = N x (Vi/Va) x (Vi/FR) x (1/Vi) [Vi(µl) = final volume of washed cMP suspension, Va(µl) = volume of washed cMP suspension used for each labelling analysis, Vt(µl) = total volume of cMP suspension before fluorescence-activated cell sorting analysis, FR(µl/min) = flow rate of the cytometer at low mode (the average volume of microparticle suspension analysed in 1 min), 1 is the µl unit of volume, and Vi(µl) = original volume of plasma used for microparticle isolation].

Flow rate was measured before each experiment. Intra-assay CV of cMP counts was 3.1%, while inter-assay CV was 5.4%. To reduce background noise, buffers were prepared on the same day and filtered through 0.2 µm pore size filters.

**Statistical analysis**

Sample size was selected based on the average of studies focusing on cMP analysis by FACS. All data are presented as medians (interquartile range), except when indicated. An initial descriptive analysis was carried out using number of cases and percentages for qualitative variables and median and interquartile range for quantitative variables. Frequencies of qualitative variables (risk factors and medications) were compared between two groups by using the Chi-square analysis. Median values of quantitative variables were compared with non-parametric tests. The statistical significances between patient and control groups were determined with U-Mann Whitney and multiple comparisons by Kruskal-Wallis, and when significant, Bonferroni post-hoc analysis was used to assess intergroup differences. StatView was used for all statistical tests and a p<0.05 was considered statistically significant.
Results

Clinical characteristics of the study population

A total of 37 untreated (LLT) and 37 statin-treated (LLT*) non-FH subjects from the Spanish SAFEHEART cohort were included in the analysis. Demographic, biochemical, and clinical data of both groups as well as classical coronary risk factors are shown in Figure 1. The LLT* - patient group was composed by 14 men and 23 women with a median age at inclusion of 53 years (range from 48 to 62 years) and the untreated group consisted of 18 men and 19 women with a median age of 43 years (range from 41 to 51 years). Total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides were similar in both groups. There were no differences either in gender, mean age, BMI, type 2 diabetes mellitus, current tobacco consumption, and Lp(a) levels, between the two groups. However, hypertension and obesity were more frequent in LLT* group. In LLT* -patients, the dose of simvastatin use was 20 mg (n=10), atorvastatin 20 mg (n=18) and rosuvastatin 10 mg (n=7). Fluvastatin was not included in the statistical analysis of cMP reduction depending on LLT* duration since only 5.4% of LLT*-patients (2 out of 37) were taking this drug. Only one case (2.7% of LLT*-patients) was receiving maximum statin dose. The mean duration of LLT was 7.5 ± 1.0 years (range 2-22 years).

Levels and cellular origin of circulating microparticle population

The total number of blood microparticles (based on FSC/SSC characteristics) was significantly lower in LLT*-patients, as shown in Figure 1A. Both phosphatidylserine (PS)-positive or annexin V-binding (AV+∗) and PS negative (AV−) microparticles were detected (Figure 1A); however, the majority of all circulating microparticles were AV+ in both studied groups. Levels of AV+ and AV- cMPs and AV- cMPs were significantly lower in blood of LLT+ than in LLT- patients (p<0.0001 in all cases).

Circulating microparticles showed a different parental cell origin in LLT+ and LLT- patients. Figure 1B illustrates the pattern of circulating microparticles according to their cell source marker. Whilst percentages of total circulating platelet-derived (CD41+/CD61−; pMP) (84.8% LLT−/81.8% LLT+) microparticles did not significantly change, there were significant changes in the white cell type and vessel wall-derived cMPs between both groups (Figure 1B). Endothelial cell-derived cMPs (CD146+/CD31−; eMPs) were significantly lower in LLT+ patients (Figure 1B). The pan-leukocyte-derived cMPs (CD45+; LMPs) were higher in LLT+ patients but there were significant differences between the LLT+ patients. Pro-inflammatory cell-derived cMPs (lymphocyte- [CD45+/CD3+, tMPs] plus monocyte- [CD14+, mMPs]) were significantly lower in LLT+ patients (Figure 1B).

Annexin V-positive circulating microparticles

Total number of microparticles shed from the different parental cells is depicted in Figure 2. Since most of all cMPs (> 86% in average) contained phosphatidylserine in their surface (annexin V-binding (AV+)) microparticles were AV+ and AV- microparticles were significantly lower in blood of LLT patients (p<0.0001 in all cases).

Figure 3: Activation status and tissue factor-bearing cMPs in LLT and LLT* patients. Box and whisker plots of total AV+ cMPs per µl of PFP bearing (A) activated αβ-integrin marker (PAC1+) and both PAC1 and P-selectin markers (PAC1+/CD62P+) representing AV+ cMPs from activated platelets, B) M-integrin marker (CD11b+) and both αβ-integrin and monocyte markers (CD11b+/CD14+) corresponding to AV+ cMPs from activated leukocytes (a-LMPs and monocytes, and C) tissue factor (CD142+; TF+cMPs) and both monocyte marker and TF (CD142+; TF+cMPs) in non-LLT (n=37) and LLT (n=37) patients. Data are given as a box and whisker plot as described in Figure 1. *p<0.0001 vs control group (U-Mann Whitney test).

For personal or educational use only. No other uses without permission. All rights reserved.
V+), we specifically studied this subpopulation. LLT+-patients were found to have significantly lower platelet-, endothelial cell-, leucocyte-, lymphocyte-, and monocyte-derived AV+-cMP subsets than LLT-group (p<0.0001, p<0.0001, p<0.001, p<0.01 and p<0.0001, respectively) (Figure 2). There was a general lowering of activation of vascular and inflammatory cells.

**Activation cell markers and tissue factor in circulating microparticles**

AV+-cMPs shed by activated platelets and activated leucocytes were significantly reduced in LLT+-patients (Figure 3). Specifically, levels of AV+-pMPs carrying the activated α\textsubscript{IIb}\textbeta\textsubscript{3} integrin marker PAC1+ and both PAC1+ and P-selectin markers together (PAC1+/CD62P+) were significantly lower in the LLT+-patients (p<0.0001 and p<0.0001, respectively) (Figure 3A). cMPs derived from activated leucocytes (CD11b+) and monocytes (CD11b+/CD14+) were also significantly lower in AV+-cMPs of LLT+-patients (Figure 3B).

Total levels of circulating MP derived from activated tissue factor-bearing cells (TF; CD142+-cMPs) were significantly lower in LLT+-patients (Figure 3C). Thus, higher number of total annexin V-positive circulating (AV+-cMPs) and monocyte-derived (AV+-mMPs) microparticles had TF in LLT than in LLT+-patients (p<0.0001 in both cases) (Figure 3C). Therefore, platelet-derived MP carrying α\textsubscript{IIb}\textbeta\textsubscript{3}-integrin and P-selectin (PAC1+/CD62P+), leucocyte-derived MP carrying MAC-1 (CD11b+) and tissue factor (CD142+) bearing cMPs of LLT group showed three-fold, three-fold and two-fold lower median values, respectively, than the LLT+ group.

When focusing on relative amounts of cMPs bearing activation markers, AV+-MPs carrying markers of platelet activation (PAC1+ and PAC1+/CD62P+) and tissue factor from platelet origin (CD142+/TSP1+) given as percentage of the total AV+-cMPs were significantly lower in LLT+ compared to LLT- (Figure 4A). cMPs carrying TF were also significantly lower in LLT+-patients (n=37) and LLT- (n=35), in the latter classified according to the type of statin they were taken (simvastatin, atorvastatin or rosuvastatin). Data are given as median (interquartile range). *p<0.05 vs LLT+ (Mann-Whitney). Differences in LLT+ due to statin drugs were not significant (Kruskal-Wallis).

### Table 2: cMP profiling according to statin drugs in LLT+ compared to LLT- patients

<table>
<thead>
<tr>
<th>Annexin V+</th>
<th>Marker</th>
<th>LLT+</th>
<th>LTT-</th>
<th>Simvastatin</th>
<th>Atorvastatin</th>
<th>Rosuvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>cMPs (AV+)</td>
<td>Total cMPs</td>
<td>5238 (1773–10925)</td>
<td>1762 (1162–1877) *</td>
<td>1684 (1098–2082) *</td>
<td>1858 (1432–2062) *</td>
<td></td>
</tr>
<tr>
<td>eMPs (CD146+/CD31+/AV+)</td>
<td>8 (1–34)</td>
<td>0.34 (0.2–2) *</td>
<td>2 (0.25–4) *</td>
<td>2 (0.75–3) *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMPs (CD45+/AV+)</td>
<td>338 (164–660)</td>
<td>104 (78–187) *</td>
<td>202 (136–275) *</td>
<td>208 (124–240) *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cMPs (CD45+/CD3+/AV+)</td>
<td>16 (6–43)</td>
<td>5 (4–13)</td>
<td>6 (3–12) *</td>
<td>14 (14–17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mMPs (CD14+/AV+)</td>
<td>68 (23–147)</td>
<td>13 (8–31) *</td>
<td>15 (7–28) *</td>
<td>42 (15–62)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMPs (PAC1+/AV+)</td>
<td>MPs carrying activation markers</td>
<td>34 (24–74)</td>
<td>14 (4–21) *</td>
<td>10 (6–14) *</td>
<td>10 (9–20.5) *</td>
<td></td>
</tr>
<tr>
<td>LMPs (CD11b+/AV+)</td>
<td>90 (56–252)</td>
<td>22 (11–48) *</td>
<td>24 (20–48) *</td>
<td>53 (20–150)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF+cMPs (CD142+/AV+)</td>
<td>82 (54–174)</td>
<td>39 (24–68) *</td>
<td>35 (16–51) *</td>
<td>43 (17–94) *</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

© Schattauer 2013

*Figure 4: Annexin V-positive levels of cMPs according to time of LLT. Box and whisker plots showing number of AV+-cMPs per µl of PFP corresponding to cMPs (CD3+/CD45+), activated-LMPs (CD11b+) and TF+-cMPs (CD142+) related to exposure years to LLT (n=37). Data are given as a box and whisker plot as described in Figure 1. *p<0.05 (U-Mann Whitney test).
significantly lower in LLT+ compared to LLT-patients (p<0.05; see Suppl. Figure 3, available online at www.thrombosis-online.com). Similarly, AV+-MPs bearing markers of inflammatory cell activation (CD11b+) and tissue factor from monocyte origin (CD142+/CD14+) showed a reduction trend (see Suppl. Figure 3, available online at www.thrombosis-online.com). The percentage of AV+-cMPs from activated inflammatory cells (CD11b+) relative to all LMPs (CD45+) was significantly lower in statin-treated patients than non-treated subjects (16.8 [8.7-36.9] vs 26.7 [13.5-51.3], p<0.05, respectively). Contrarily, percentage of total pMPs (CD41+/CD61+) and LMPs (CD45+) did not differ between the statin and non-statin groups (Figure 1B). No correlation was found between AV+-cMPs numbers (TF+-cMPs or activated cell-cMPs) and haematologic counts (platelet, leukocyte, lymphocyte and monocyte counts), showing that the effect of statins was on cell activation and not in cell numbers.

LLT-specific decrease in cMP levels is time-dependent

The decrease of cMP shedding driven by lipid-lowering treatment was directly associated with the duration of LLT treatment. Specifically, those patients who had received LLT during 10 or more years had significantly lower levels of AV+-cMPs (CD45+/CD3+), activated AV+-LMPs (CD11b+) and tissue factor-bearing AV+-cMPs (CD142+) when compared to subjects under LLT for less than ten years (p<0.05 in all cases, Figure 4). The effects of statins on cMPs were not found different between statins (Table 2). Therefore, differences due to different statin drugs were not significant (Kruskal-Wallis). The slight use of ezetimibe in LLT+ patients (11%) did not exert any difference either (data not shown).

LLT-specific cMP profile is not determined by LDL-cholesterol levels

There was no correlation between lipid levels and activated cell-cMPs or TF+-cMPs. Further, to identify whether LDL-c plasma levels were promoting MP shedding, the studied groups were classified in two groups using the cut-off in LDL-c levels of 130 mg/dl according to guideline target in primary prevention. A 51% of LLT+ and 43% of LLT-patients were on-target LDL-c levels (Figure 5). Significant lower values in cMP levels were detected in LLT+ patients regardless of cholesterol levels. Therefore there is a

---

Figure 5: cMP profiling and LDL-cholesterol levels. Box and whisker plots of numbers of total cMPs, total AV+-cMPs and AV+-cMPs from platelets (pMPs, CD41+/CD61+), endothelial cells (eMPs; CD146+/CD31+), lymphocytes (EMPs, CD45+/CD3+) and monocytes (mMPs, CD14+) per µl of PFP in LLT (n=37) and LLT+(n=37)-patients classified in two groups: on- and off-target LDL-c levels (with clinical guidelines target LDL-cholesterol levels, <130 mg/dl for primary prevention). Data are given as a box and whisker plot as described in Figure 1A. *p<0.05 (Mann-Whitney test). Non-significant differences due to LDL-c levels (between on-target and off-target) were found.
clear effect of statins on vascular and inflammatory cells activation independent of plasma cholesterol levels.

**cMPs and cardiovascular risk**

Since LLT-subjects are characterised by having almost no CV risk factors, only TF⁺-cMPs significantly correlated with hs-CRP levels (p<0.05, R²=0.256; see Suppl. Figure 2, available online at www.thrombosis-online.com).

On the other hand, levels of cMPs in LLT⁺-subjects significantly correlated with CV risk scores (▶Figure 6). Specifically, Framingham risk scores (FRSs) for general cardiovascular disease at 10 years calculated using the National Cholesterol Education Program ranged from <5 to 48%. The mean FRSs did not differ among groups based on LLT treatment. However, within the range of FRSs in LLT⁺-patients, significant differences were found in pMPs expressing activated α₅β₃-integrin marker (PAC1⁺) and tissue factor-bearing cMPs (CD142⁺) levels between subjects with moderate to high Framingham risk and those with low Framingham risk (▶Figure 6A). The REGICOR (Registre Gironí del Cor) risk was also investigated. There was a significant increased activated pMPs, eMPs, LMPs, tLMPs, and activated LMPs in LLT⁺-pa-
patients with Regicor score risk of ≥5% compared to those who had lower score (<5%) (▶ Figure 6B).

Discussion

High cholesterol plasma levels are a causal factor in the ethiopathogenesis of atherosclerosis (49, 50). Hypercholesterolaemia induces entry of LDL-c in the intima, formation of modified LDL-c species and the triggering of an innate immunity response followed by acquired immunity complications (50). Induction of TF complicates the plaques with triggering of thrombotic events (50, 51). Statins are first-line drug therapy for LDL-c lowering in most patients (1, 2). Statins exert their beneficial effect by reducing cholesterol plasma levels, but other effects beyond lipid reduction have also been considered (3–5). We have recently found that cMPs and platelet-derived MPs enhance thrombosis on atherosclerotic plaques (12). Therefore, the inhibition of cMP release may have significant implications both in plaque identification and inhibition of plaque progression.

Here, we demonstrate that patients treated with LLT with statins have lower MP numbers, especially of platelet, leukocyte and endothelial cell-derived cMPs, than blood from untreated patients with the same plasma lipid levels. This study was designed to evidence effects of statin use in a population of primary prevention patients with a median of LDL-cholesterol in target levels (<130 mg/dl). Interestingly, blood from statin-treated patients had cMPs with reduced markers of cell activation. Markers from activated-platelets, inflammatory cells and endothelial cells were lower than in untreated patient’s blood. These results indicate a direct effect of statin in cell activation and membrane homeostasis.

Circulating MPs and, specifically platelet-derived MPs, play an important role in mural thrombosis and also in coagulation (12). Therefore the effects detected may have implications in the protection against atherosclerosis exerted by statins. It has been described that some statins could improve plasma adiponectin levels, a circulating adipokine that suppresses the attachment of monocytes to endothelial cells (EC) and stimulates nitric oxide (NO) production in vascular ECs ameliorating endothelial function (32, 52, 53). These data indicate that the NO-dependent anti-platelet properties by adiponectin could contribute to the decrease of pMPs and to the anti-atherosclerotic effects of statins (32). Also cMPs from T cells and monocytes have been found to induce endothelial dysfunction, activation and subsequent amplification of inflammation (54, 55), therefore contributing to atherosclerosis (50, 56).

Interestingly, low numbers of cell-activation markers, such as αIβ3-integrin (PAC1•), P-selectin (CD62P•), αM-integrin (Mac-1, CD11b•) and tissue factor (TF•, CD142•) were detected in cMPs of the statin-treated patients, suggesting that statins acting on various multiple cellular targets may exhibit anti-inflammatory and anti-thrombotic actions. This view is further supported by the statin-induced decrease in relative amounts of cMPs carrying markers of cell activation, especially evident in those of platelet origin.

Patients treated with statins had a better cMP profile depending on the time on-treatment, suggesting that chronic use of statins helps to reduce the vascular dysfunction burden in HL. Because patients were treated with four commonly used statins at low dose, we have been able to show that all statins are effective in the modulation of cMP shedding in these asymptomatic hypercholesterolaemic patients. Therefore, the duration of the treatment more than the type of statin seems to influence these effects.

Results of large, well-controlled clinical trials have demonstrated that statins are effective in primary and secondary prevention of cardiovascular disease (1). In primary prevention results of clinical trials have shown less clear beneficial effects but yet reductions in coronary artery disease are evident (WOSCOP [57], AFCAP/TexCAP [58], ASCOT_LLA [59]) and even mortality was reduced in the JUPITER trial (60). However, the benefit on all-cause mortality has not been proved in a recent meta-analysis (61). For most hypercholesterolaemic patients unable to achieve recommended lipid level goals with therapeutic lifestyle changes, statins are considered first option for treatment.

The cardiovascular risk of each individual was calculated using the Framingham Heart Study equations for primary at 10 years as well as REGICOR risk charts (39, 62). Interestingly, cMP levels significantly correlated with Framingham CV risk score. In addition the REGICOR score (used to indentify high CV risk in the Spanish population) correlated significantly with the majority of specific cell-derived microparticles.

What is known about this topic?
- Blood cells and platelets release circulating microparticles (cMPs) with procoagulant activity.
- cMPs are increased in patients with coronary artery disease and with clustering of cardiovascular risk factors.
- Hypercholesterolaemia is a major risk factor for atherosclerosis and is associated with a pro-thrombotic state.
- Statins are hypolipidaemic drugs, which not only decrease plasma cholesterol levels but also exert beneficial effects in cardiovascular disease prevention due to their pleiotropic effects. However, there is very limited information about the effect of lipid-lowering therapy (LLT) with statins on cMPs.

What does this paper add?
- At equal LDL-cholesterol levels, patients treated with statins have less cMP levels and less cMPs carrying markers of parental cell activation (of platelet, lymphocyte, monocyte and endothelial cell origin).
- Beyond cholesterol lowering, statins have direct effects on reducing activated cell membrane shedding of cMPs.
- The effects of statins on cMP shedding are increased with years of treatment.
- Beyond cholesterol lowering the beneficial effects of statins are also due to vascular endothelial, platelet and inflammatory cell protection against activation.
This study has some characteristics and limitations that need to be discussed. First, this is a cross-sectional study from an observational (non-randomised) prospective clinical study and some bias related with the indication of treatment may have affected the results. However, all cases receiving LLT have been treated at least one year before the inclusion in the study with the same LLT. Indeed, LLT- patients had reduced their total cholesterol levels from 270 mg/dl (range 231-300 mg/dl) (maximum level that patients achieved before recruitment into SAFEHEART) to 210 mg/dl (range 192-240 mg/dl) by statin treatment. Our control group was matched to patients with similar cholesterol plasma levels but never-treated with statins. Interestingly, LLT- patients with higher exposure to lipids have fewer MPs, giving information of the protective role of statins in hypercholesterolaemic patients.

In conclusion, statins significantly reduce the shedding of blood cells and vascular cell MPs. The specific reduction of cMPS derived from activated parental cells demonstrates how statins can affect evolution of disease. The lower cMP shedding may ameliorate the vascular and inflammatory effects associated to the progression of atherothrombotic disease in asymptomatic patients contributing to statin protective effects (21). Benefits of statins operating at this level may explain their proved beneficial effects seen in patients with low cholesterol levels. In summary, effective lipid-lowering treatment with statins may prevent the development of premature cardiovascular disease by reducing vascular and inflammatory cell activation as detected by a reduced cMP shedding of the cell membranes. Further studies measuring changes in microparticle number and composition before and after LLT will help to complete our understanding of statin effects on the vessel wall and the role of microparticles as biomarkers of vascular disease.

Acknowledgements
The authors thank Dr. R Nieuwland and Chi M. Hau for the exceptional advice and expertise, Dr. J. Crespo for his help in flow cytometry, Marta Guindo for the technical support, and the Spanish Familial Hypercholesterolemia Foundation for the recruitment of participants. Authors are indebted to the FH patients and relatives for their valuable contribution and willingness to participate.

Conflicts of interest
None declared.

References

Abbreviations
AV, annexin V; BMI, body mass index; cMPS, circulating microparticles; CV, coefficient of variation; CVD, cardiovascular disease; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; eMPS, endothelial-derived microparticles; FACS, fluorescence-activated cell sorting; FSC, forward scatter characteristics; GGPP, geranylgeranyl pyrophosphate; HDL, high-density lipoprotein; HL, hyperlipidaemia; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HS-CRP, high sensitive C-reactive protein; LDL-c, low-density-lipoprotein cholesterol; LLT, lipid-lowering therapy; lMPS, lymphocyte-derived microparticles; LMPs, leukocyte-derived microparticles; LP(a), lipoprotein (a); mAb, monoclonal antibody; MPs, microparticles; mMPS, monocyte-derived microparticles; PS, phosphatidylserine; PFP, platelet-free plasma; pMPS, platelet-derived microparticles; SCC, side scatter characteristics; TC, total cholesterol; TF, tissue factor.