Microparticle shedding by erythrocytes, monocytes and vascular smooth muscular cells is reduced by aspirin in diabetic patients

La aspirina reduce la liberación de micropartículas eritrocitarias, monocitarias y de células del músculo liso vascular en pacientes diabéticos

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ABSTRACT

Introduction and objectives: Diabetes mellitus is associated to an enhanced risk for cardiovascular disease and its prevalence is increasing. Diabetes induces metabolic stress on blood and vascular cells, promoting platelet activation and vascular dysfunction. The level of vascular cell activation can be measured by the number and phenotype of microparticles found in the circulation. Here we had the objective of investigating the effect of a platelet-inhibitory dose of acetylsalicylic acid on the number and type of microparticles shed to the circulation.

Methods: Forty-three diabetic patients were enrolled in the study and received a daily dose of 100 mg of acetylsalicylic acid for 10 days to cover the average platelet life-span in the circulation. Before and after the intervention period, circulating microparticles were characterized and quantified by flow cytometry.

Results: Type 1 diabetic patients had about twice tissue factor-positive circulating microparticles (derived both from platelets and monocytes) and endothelial-derived E-selectin positive microparticles than type 2 diabetic subjects. Acetylsalicylic acid treatment significantly inhibited platelets since cyclooxygenase 1 derived thromboxane generation levels were reduced in a 99%. Microparticles derived from erythrocytes, activated monocytes and smooth muscle cells were significantly reduced after 10 days of acetylsalicylic acid.

Conclusions: These results indicate that a) vascular and blood cells in type 1 diabetic patients are exposed to a more sustained stress shown by its specific microparticle origin and levels; b) acetylsalicylic acid treatment inhibits vascular wall cell activation and microparticle shedding; and c) the effects of acetylsalicylic acid are similar in type 1 and 2 diabetes.

Keywords: Diabetes mellitus; Aspirin; Circulating Cell-Derived Microparticles; Tissue Factor; Activated Cells.

RESUMEN

Introducción y objetivos: La Diabetes mellitus está asociada a un mayor riesgo de enfermedad cardiovascular y su prevalencia está en aumento. La diabetes induce a un estrés metabólico en las células vasculares, promoviendo la activación plaquetaria y la disfunción vascular. El nivel de activación de las células vasculares se puede medir con el número y fenotipo de las micropartículas circulantes. Por ello, investigamos el efecto del ácido acetilsalicílico en el número y tipo de micropartículas liberadas a la circulación.

Métodos: Se reclutaron 43 pacientes diabéticos que recibieron una dosis diaria de 100 mg de ácido acetilsalicílico durante 10 días. Antes y después de este período se caracterizaron y cuantificaron las micropartículas circulantes por citometría de flujo.

Resultados: Respecto a los tipo 2, los diabéticos tipo 1 presentaron el doble de micropartículas circulantes plaquetarias y monocitarias factor tisular positivas y de micropartículas endoteliales E-selectina positivas. El ácido acetilsalicílico inhibió las plaquetas circulantes (reducción de la generación de tromboxano en un 99%). Cien mg de ácido acetilsalicílico redujeron los niveles de micropartículas derivadas de eritrocitos, monocitos activados y células del músculo liso.

Conclusiones: Estos resultados indican que: a) las células vasculares y sanguíneas en los diabéticos tipo 1 están expuestas a un mayor estrés sostenido reflejado en los niveles de micropartículas de ciertos orígenes celulares; b) el tratamiento con ácido acetilsalicílico inhibe la activación y liberación de micropartículas de las células de la pared vascular; y c) los efectos del ácido acetilsalicílico son similares en diabéticos tipo 1 y 2.

Palabras clave: Diabetes mellitus; Ácido Acetilsalicílico; Micropartículas Circulantes Derivadas de Células; Factor Tisular; Células Activadas.

Abbreviations

ABB, annexin binding buffer; ANCOVA, analysis of covariance; ARB, angiotensin II receptor blockers; ASA, acetylsalicylic acid; AV, annexin V; cMPs, circulating microparticles; COX-1, cyclooxygenase 1; CVD, cardiovascular disease, DM, diabetes mellitus; FITC, fluorescein isothiocyanate; FSC, forward scatter; PBS, phosphate buffered saline; PE, phycoerythrin; PFP, platelet free plasma; PPP, platelet poor plasma; PRP, platelet rich plasma; PS, phosphatidylserine; RT, room temperature; SMA, smooth muscle actin; SMC, smooth muscle cell; SSC, side scatter; TF, tissue factor; and TXB2; thromboxane B2.

Abreviaciones

ABB, anexina *binding buffer*; ANCOVA, análisis de la covariancia; ARAII, antagonistas del receptor de angiotensina II; AAS, ácido acetilsalicílico; AV, anexina V; MPc, micropartículas circulantes; COX-1, ciclooxigenasa 1; ECV, enfermedad cardiovascular, DM, diabetes mellitus; FITC, fluoresceína isothiocianato; FSC, *forward scatter*; PBS, *phosphate buffered saline*; PE, ficoeritrina; PLP, plasma libre de plaquetas; PPP, plasma pobre en plaquetas; PRP, plasma rico en plaquetas; FS, fosfatidilserina; TA, temperatura ambiente; SMA, *smooth muscle actin*; CML, célula muscular lisa; SSC, *side scatter*; FT, factor tisular; y TXB2; tromboxano B2.

INTRODUCTION

Diabetes mellitus (DM) is largely associated to microvascular and macrovascular complications and an enhanced risk for cardiovascular disease (CVD)[1]. The increasing prevalence of DM worldwide has led to estimate that in 2030, approximately 552 million people would have DM, of whom more than 95% would have type 2 DM[2]. A number of mechanisms for the increased CV risk in diabetes have been proposed, including increased tendency toward intracoronary thrombus formation, increased platelet reactivity and worsened endothelial dysfunction[3]. As up to 80% of individuals with diabetes mellitus will die of CV causes, evidence-based therapies to reduce CVD are of utmost importance.

The recommendation of low-dose acetylsalicylic acid (ASA) for the primary prevention of CV events in adults with DM has been the subject of controversy[2,3]. ASA is a nonsteroidal anti-inflammatory drug, and is the most commonly used antiplatelet agent due to its low cost and relative lack of adverse effects when administered in low doses. ASA inhibits platelet thromboxane A2 formation, a potent vasoconstrictor and platelet agonist, through the acetylation of cyclooxygenase 1 (COX-1) in the serine-530 position, thus preventing the arachidonic acid binding to the enzymatic active site[4,5]. Beyond the inhibitory effect on thromboxane formation, ASA may have pleiotropic effects, involving antioxidant and anti-inflammatory effects[6], but the effects of ASA on preventing cell activation from the vascular compartment and microparticle shedding still remain unclear.

Circulating microparticles (cMPs) are small phospholipid microvesicles of 0.1 to 1 μm diameter, shed by activated endothelial or blood cells and defined by both size and expression of cell-specific antigens on their surface[7]. Recent studies have shown a key role of these MP on thrombosis, inflammation and angiogenesis[8,9], which are essential in the development of diabetic complications. Although present in plasma of healthy individuals, elevated numbers of specific activated subsets of MPs have been reported in vascular disorders[10-13]. cMPs originate from cells and contain phosphatidylserine

(PS) and distinct surface proteins depending on their cells of origin or parental cells. cMPs can originate from platelets, endothelial cells, leukocytes, erythrocytes and smooth muscle cells (SMC)[14]. Some have strong procoagulant properties due to exposure of anionic phospholipids, as PS, in a similar fashion as activated platelets and provide a catalytic surface that may promote coagulation since PS facilitates the binding of the coagulation factors and the assembly of the coagulation complexes, accelerating the formation of thrombin[15]. As reviewed[16], patients with DM and diabetic complications have different cellular cMPs patterns, and blood levels of platelet-derived MPs, endothelial-derived MPs, and total Annexin V $(AV)^+$ MPs are significantly increased in type 1 DM. In type 2 DM patients, higher levels of total[17], platelet, leukocyte, monocyte and endothelial-derived cMPs have been observed compared to matched control subjects[18-21].

Thus, the aim of this study was to determine the effect of ASA administration on MP shedding and phenotype in diabetic patients.

MATERIALS AND METHODS

Diabetic patients

A total of 43 primary-care diabetic patients (men and women) aged between 41 and 73 years and treated according to international guidelines were recruited for the study in the outpatient clinic of the Hospital de la Santa Creu i Sant Pau in Barcelona. The patients included in the study had type 1 or 2 DM, and exclusion criteria were as follows: aspirin or ibuprofen ingestion within the last 10 days, contraindications to aspirin or beta-blocking agents, peptic ulcer and increased bleeding risk as well as past history of cancer, inflammatory disorders, sepsis, infection or pregnancy.

The Institutional Review Board of the hospital approved the study protocol, and the trial was conducted according to the Declaration of Helsinki. All participants gave written consent before participation in the study. After screening and inclusion in the study, a

medical record was administered to obtain lifestyle, medical and therapeutic data, and baseline measurements were performed. After that, patients were entered at the intervention period where they were administered 100 mg ASA daily for 10 days. Blood samples were taken before (baseline) and after the intervention period with ASA.

To ensure compliance and efficacy of ASA treatment, Cyclooxygenase-1 (COX-1) inhibition was proven by measuring the inhibition of thromboxane B2 (TXB2) formation with a commercial Enzyme Immunoassay Kit (Thromboxane B2 (TXB2) Express Eia Kit- Monoclonal, Cayman Chemical) following manufacturer's recommendations.

Control subjects

As control subjects we included 38 moderate-high CV risk subjects free of diabetes and CVD matched for sex, age, classical CV risk factors and statins use. Controls belong from the SAFEHEART cohort[22], an open, multicentre, long-term prospective study. Any of the control subjects were undergoing ASA therapy. Demographic and clinical characteristics data, CV history, classic CV risk factors and current treatment for hypercholesterolemia were obtained from all subjects using a standardized report form at inclusion. 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

Venous blood was withdrawn from the cubital vein without tourniquet using a 20-gauge needle after 10-14 hours of fasting into 3.8% sodium citrate tubes. Blood cells were removed by low-speed centrifugation (250×g, 15 min) at room temperature (RT) in order to avoid *in vitro* platelet activation. Platelet rich plasma (PRP) was carefully aspirated, leaving about 0.1 cm undisturbed layer on top of the cells. A second centrifugation step (11,000×g, 10 min, RT) was made to ensure the complete removal of cells and platelets in order to obtain the platelet poor plasma (PPP). A third

centrifugation step (11,000×g, 2 min, RT) was performed to ensure the complete removal of platelets and to obtain the platelet free plasma (PFP). All samples were processed identically and within 60 minutes after extraction. PFP aliquots of 250 µL were immediately frozen in liquid nitrogen and stored at -80ºC until processing for isolation and quantification of cMPs.

Circulating microparticles isolation and quantification

The cMP fraction was isolated from PFP by a two-step high-speed centrifugation. Briefly, 250 µL of frozen PFP aliquots were thawed on melting ice for 1 hour and centrifuged at 20,000×g for 30 min at 30ºC to pellet cMPs. The supernatants were 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 pellets were resuspended in 100 µL citrate-PBS.

Triple-label flow cytometric analysis was performed as described by Suades et al.[23]. Briefly, 5 µL of washed cMP suspensions were diluted in 30 µL PBS buffer containing 2.5 mM $CaCl_2$ (Annexin Binding Buffer, ABB). Thereafter, combinations of 5 µL of V450-conjugated AV (BD-horizon) with two specific monoclonal antibodies (mAb, 5 µL each, **Table 1**) labeled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), or the isotype-matched control antibodies were added. Samples were incubated 20 min at RT in the dark and diluted with ABB before being immediately analyzed on a FACSCantoII™ flow cytometer (except for MPs from SMC). SMC-derived MPs were quantified as previously described[14]. Summarizing, 5 µL of the cMPs suspension were incubated 20 min at RT in the dark with 5 µL AV-V450 and 5 µL CD142-FITC (tissue factor, TF) in a final volume of 50 µL ABB. cMPs were fixed with 450 µL ABB/paraformaldehyde 2% during 30 min and centrifuged at 20,000×g for 30 min to pellet cMPs. After eliminating the supernatant, cMPs were permeabilized with 20 µL of ABB/saponin 0.1% 20 min at RT in the dark. After permeabilizing, 5 µL of smooth

muscle actin (SMA)-α-PE were added to the cMPs suspension and incubated 20 min at RT in the dark and finally diluted with ABB prior to flow cytometer analyses.

Acquisition was performed at 1 minute per sample. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with the settings in the logarithmic scale. cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to AV and reactivity to cell-specific mAb (**Figure 1**).

The contribution of granulocytes was inferred by subtracting agranulocytes (lymphocytes plus monocytes) from total leukocytes instead of labeling with specific mAb. Gate limits were established following the criteria previously described[23]. The lower detection limit was placed as a threshold above the electronic background noise of the flow cytometer. To identify positive stained events, thresholds were also set based on samples incubated with the same final concentration of isotype-matched control antibodies after titration experiments. AV binding level was corrected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer (PBS).

Data were analyzed with the FACSDiva™ software (version 6.1.3, Becton Dickinson). The cMP concentration (number of cMPs per µL of PFP) was determined according to Nieuwland's formula[15], based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events (N), as follows: $cMPs/\mu L = N \times (V_f/V_a) \times ({}_{Vt/}FR) \times (1/V_i)$ [where V_f(µL) = final volume of washed cMP suspension, V_a(µL) = volume of washed cMP suspension used for each labeling analysis, V_t(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 analyzed in one minute), 1 is the µL unit of volume, and V_i(µL) = original volume of plasma used for microparticle isolation]. Flow rate was measured before each experiment. To reduce background noise, buffers were prepared on the same day and filtered through 0.2 µm pore size filters under vacuum.

Statistical analysis

Statistical analyses were performed using the SPSS Statistical Analysis System (version 22.0). Descriptive statistics [mean ± sd or n (%)] were used to describe the baseline characteristics of the patients and the outcome variables. To analyze the changes after the ASA treatment a 2-tailed Student's *t* test for paired samples was performed with the data obtained before and after the intervention. One-way analysis of covariance (ANCOVA) and the Bonferroni post-hoc test were used to compare the differences of changes in outcome variables according to the type of diabetes. Changes in cMPs after the ASA therapy were considered the primary outcome. *P* was considered significant when <0.05.

RESULTS

Baseline characteristics

Baseline characteristics of the 43 diabetic patients included in the study (13 with type I DM and 30 with type II DM, with an average of 17 years of disease evolution) are shown in **Table 2**, and baseline characteristics of control subjects can be found in Supplemental Table 1. The mean age of the diabetic population was 55 years old, ~56% were men, ~21% were current smokers, ~93% had dyslipidemia and ~91% were hypertensive. A ~53% of the patients were receiving angiotensin-converting enzyme inhibitors, ~65% angiotensin II receptor blockers (ARB), ~7% β-blockers, ~19% doxazosin, ~37% diuretics, ~77% statins and ~33% were receiving ezetimibe.

Patients with type I DM showed a mean of 25 ± 10 years of disease evolution while type II diabetics showed a mean of 13 ± 9 years of diabetes evolution (*P*= 0.001, one-way ANOVA). At baseline, type I diabetic patients had about twice TF^{+} cMPs from platelet and monocyte origin and endothelial-derived $CD62E^{+/-}$ cMPs than type II diabetic subjects (**Figure 2**), possibly reflecting life-long exposure to metabolic stress. In fact, baseline levels of these cMPs correlated positively with the time of disease

progression (Spearman's rho = 0.371, 0.402, 0.330 and 0.335, *P*= 0.014, 0.008 0.029 and 0.025, for platelet- and monocyte-derived TF^+ and endothelial-derived $CD62E^{+/-}$ cMPs, respectively).

Compared to controls, patients before the ASA intervention presented higher levels of all cMPs quantified except for erythrocyte-derived ($CD235ab^+$), $CD11b^+/AV^+$, and overall TF ($CD142^+$)-loaded cMPs, which were similar to control values (Supplemental Table 2).

Inhibition of platelet activation

TXB2 levels in serum (obtained in the same blood extraction than citrate tubes) were measured before and ten days after 100 mg ASA intervention as a measure of efficacy of ASA. After ASA treatment, COX-1 inhibition was above 99%, as TXB2 concentration at baseline was about 18.8+2.8 ng/ml and 0.26+0.19 ng/ml after the intervention, indicating the absence of ASA pseudo-resistance and that compliance was excellent.

ASA-induced changes in circulating microparticles

The overall effects of ASA were similar in both types of diabetes (**Table 3**). Therefore, the changes of microparticle shedding after ASA intervention were expressed considering together both types of diabetes.

As depicted in **Figure 3**, after 10 days of ASA, cMPs from erythrocyte origin ($CD235a^+/AV^+$) decreased by ~17%. Monocyte-derived cMPs decreased by ~22% ($CD14^+/AV^+$) and cMPs from activated monocytes also decreased (~22 and 29% for $CD11b^+/CD14^+/AV^+$ and $CD142^+/CD14^+/AV^+$ cMPs, respectively). Furthermore, SMC-derived cMPs decreased by ~52% ($SMA\text{-}\alpha^+/AV^+$) and TF-expressing SMC-derived cMPs ($CD142^+/SMA\text{-}\alpha^+/AV^+$) also decreased by ~54%.

Moreover, levels of $CD11b^+/CD14^+/AV^+$, $SMA\text{-}\alpha^+/AV^+$ and $CD142^+/SMA\text{-}\alpha^+/AV^+$ after the ASA intervention achieved similar concentrations of those of non-diabetic high-CV risk controls.

Plasma concentration of total cMPs (AV^+) and cMPs originated from platelets ($CD61^+/AV^+$), endothelial cells ($CD146^+/AV^+$), lymphocytes ($CD3^+/AV^+$) and granulocites ($CD45^+/CD3^-/CD14^-/AV^+$) were not affected by the ASA treatment. ASA intervention did not influence cMPs from activated platelets, endothelial cells, lymphocytes or granulocytes.

DISCUSSION

cMPs are able to bind to circulating cells or endothelium and participate in intercellular communication through a broad spectrum of transmission of inflammatory information and cell activation, cell survival and apoptosis, endothelial function, vascular remodeling and angiogenesis, thus accelerating CVD progression. Therefore, strategies to decrease cMPs may delay CV complications.

ASA is given for the primary and secondary prevention of CVD in diabetic patients. While secondary prevention by low doses of ASA seems far demonstrated[24,25], the balance benefit/risk of ASA administration for the primary prevention of CVD in these patients is still a controversial matter[4-6,26-28], taking into account its collateral effects, specially upper gastrointestinal bleeding.

The main findings of this study are that 100 mg/day of ASA decrease microparticle shedding and activation from erythrocyte, monocyte and SMC origin in diabetic patients without a previous vascular event. To our knowledge, this is the first time that MP shedding from several cells of the vascular compartment after ASA treatment has been investigated in diabetic patients.

ASA did not reduce circulating platelet MP number nor activated platelet shedding in diabetic individuals, in agreement with previously reported data showing that ASA intake has no effect on platelet-derived cMPs in type 2 DM[29]. This lack of effect on platelet MP shedding has been also observed in healthy volunteers[30], in which 100 mg ASA were administered for 7 days and no differences in the number of platelet-derived

cMPs were detected. There were no differences either in endothelial-derived cMPs or in TF^+ cMPs or AV^+ cMPs between ASA or placebo treated healthy volunteers. It has also been shown in a case-control study on diabetic patients[17], in which diabetics who undergone ASA treatment did not show significant differences in the number of platelet-derived cMPs compared to patients who were not undergoing this treatment. A study on stable coronary disease also showed that ASA did not modify platelet-derived cMP levels in these patients[31].

Here, we observed that TF^+ cMPs from monocyte and SMC origin decreased after ASA treatment. Cell-derived microparticles promote thrombus formation *in vivo* in a TF-dependent manner[32,9], and high levels of TF^+ cMPs in diabetic patients are reported to be involved in transcellular signaling or angiogenic processes other than the classical procoagulant function of TF^+ MPs shown before[12]. Moreover, TF^+ monocyte-derived MPs constitute the second largest pool of thrombogenic MPs after MPs originated from platelets[33]. Additionally, we observed a decreased activated monocyte-derived ($CD11b^+/CD14^+/AV^+$) MP shedding after the 10 days of ASA treatment. Interestingly, high concentrations of cMPs derived from activated monocytes were detected in type-2 diabetic patients compared to control subjects[21]. A relationship between $CD11b^+$ cMPs and degree of CV risk and atherosclerotic plaque burden has been previously observed in subjects with different grades of CVD burden[34].

Additionally, we observed that at entry type I diabetic patients had about twice the TF^+ cMPs from platelet and monocyte origin and endothelial-derived $CD62E^{+/-}$ cMPs than type II diabetic subjects, attributable to the long life exposure to metabolic stress in type I diabetic patients. These results are in accordance with Sabatier et al., who found that type 1 diabetic patients presented significantly higher numbers of platelet and endothelial microparticles and demonstrated that type 1 and type 2 DM patients depicted distinct MP profile[17]. Nevertheless, 90% of type 2 diabetic patients were under statins therapy which may potentially contribute to the lower MP levels observed.

Diabetic patients are usually polymedicated, as shown in **Table 1**. It is provable that concomitant medication may affect MP shedding, but this issue is partially solved by the study design, as we have quantified cMPs before and after the 10 days intervention for each patient, in which concomitant medication was not modified. This study is not exempt of limitations. It was not possible to obtain data on the effects of ASA in our study controls (patients at high CV risk but without diabetes and CVD), because 10 days ASA intervention were not justified according to the Ethics Committee of our Institution. Current guidelines do not specify a CV risk threshold from which aspirin should be used systematically in diabetic patients for the primary prevention of CVD. Although the investigated population corresponds to real life diabetic patients treated as per guidelines without clinical evidence of atherosclerotic disease attending a metabolism clinic, it may not be representative for the total diabetic population at high CVD risk.

CONCLUSIONS

A ten days intervention with ASA in primary prevention of diabetic patients (treated as per guidelines) does not affect platelet MP shedding. Interestingly cells that show reduction of MP shedding, and hence passivation of activation, are smooth muscle cells and immune cells. These cell types have shown a significantly reduced shedding of membrane vesicles carrying epitopes of cell activation.

In summary, our results indicate that a) type I and II diabetic patients have a different profile of microparticle shedding, reflecting a more sustained stress in type I diabetics; b) ASA-treatment inhibits vascular wall cell activation and microparticle shedding; and, c) the effects of ASA are similar in type I and II diabetes.

CONFLICTS OF INTEREST

None declared.

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REFERENCES

1. Fox CS, Coady S, Sorlie PD, D'Agostino RB Sr, Pencina MJ, Vasan RS, et al. Increasing Cardiovascular Disease Burden Due to Diabetes Mellitus: The Framingham Heart Study. Circulation. 2007;115:1544–1550.

2. Authors/Task Force Members, Rydén L, Grant PJ, Anker SD, Berne C, Cosentino F, et al. ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD: the Task Force on diabetes, pre-diabetes, and cardiovascular diseases of the European Society of Cardiology (ESC) and developed in collaboration with the European Association for the Study of Diabetes (EASD). Eur Heart J. 2013;34:3035-87.

3. Pignone M, Alberts MJ, Colwell JA, Cushman M, Inzucchi SE, Mukherjee D, et al. Aspirin for primary prevention of cardiovascular events in people with diabetes: a position statement of the American Diabetes Association, a scientific statement of the American Heart Association and an expert consensus document of the American College of Cardiology Foundation. Circulation. 2010;121:2694-2701.

4. Patrono C. Aspirin as an antiplatelet drug. N Engl J Med. 1994;330:1287–94.

5. Eikelboom JW, Hankey GJ, Thom J, Bhatt DL, Steg PG, Montalescot G, et al. Incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: determinants and effect on cardiovascular risk. Circulation. 2008;118:1705–1712.

6. Egger G, Burda A, Obernosterer A, Mitterhammer H, Kager G, Jürgens G, et al. Blood polymorphonuclear leukocyte activation in atherosclerosis: effects of aspirin. Inflammation. 2001;25:129-135.

7. Jy W, Horstman LL, Jimenez JJ, Ahn YS, Biró E, Nieuwland R, et al. Measuring circulating cell-derived microparticles. J Thromb Haemost. 2004;2:1842-1851.

8. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation. 2000;101:841-843.

9. Suades R, Padró T, Vilahur G, Badimon L. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. Thromb Haemost. 2012;108:1208-19.

10. Namba M, Tanaka A, Shimada K, Ozeki Y, Uehata S, Sakamoto T, et al. Circulating platelet-derived microparticles are associated with atherothrombotic events: a marker for vulnerable blood. Arterioscler Thromb Vasc Biol. 2007;27:255-256.

11. Sinning JM, Losch J, Walenta K, Böhm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. Eur Heart J. 2010;32:2034-2041.

12. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW, Radder JK. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Circulation. 2002;106:2442-2447.

13. Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, et al. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. J Am Coll Cardiol. 2005;45:1622-1630.

14. Leroyer AS, Isobe H, Lesèche G, Castier Y, Wassef M, Mallat Z, et al. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. J Am Coll Cardiol. 2007;49:772-7.

15. Nieuwland R, Berckmans RJ, McGregor S, Böing AN, Romijn FP, Westendorp RG, et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. Blood. 2000;95:930-935.

16. Wang Y, Chen LM, Liu ML. Microvesicles and diabetic complications-novel mediators, potential biomarkers and therapeutic targets. Acta Pharmacol Sin. 2014;35:433–443.

17. Sabatier F, Darmon P, Hugel B, Combes V, Sanmarco M, Velut JG, et al. Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. Diabetes. 2002;51:2840–2845.

18. Nomura S, Inami N, Shouzu A, Urase F, Maeda Y. Correlation and association between plasma platelet-, monocyte- and endothelial cell-derived microparticles in hypertensive patients with type 2 diabetes mellitus. Platelets. 2009;20:406-14.

19. Feng B, Chen Y, Luo Y, Chen M, Li X, Ni Y. Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus. Atherosclerosis. 2010;208:264-9.

20. Zhang X, McGeoch SC, Johnstone AM, Holtrop G, Sneddon AA, MacRury SM, et al. Platelet-derived microparticle count and surface molecule expression differ between subjects with and without type 2 diabetes, independently of obesity status. J Thromb Thrombolysis. 2014;37:455-63.

21. Omoto S, Nomura S, Shouzu A, Nishikawa M, Fukuhara S, Iwasaka T. Detection of monocyte-derived microparticles in patients with Type II diabetes mellitus. Diabetologia. 2002;45:550-555.

22. Alonso R, Andres E, Mata N, Fuentes-Jimenez F, Badimon L, Lopez-Miranda J, et al; SAFEHEART Investigators. Lipoprotein(a) levels in familial hypercholesterolemia: an important predictor of cardiovascular disease independent of the type of LDL receptor mutation. Journal of the American College of Cardiology. 2014;63:1982-9.

23. Suades R, Padró T, Alonso R, López-Miranda J, Mata P, Badimon L. Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolaemia patients. Thromb Haemost. 2014;111:111-121.

24. Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ. 2002;324:71-86.

25. Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy. I. Prevention of death, myocardial infarction and stroke by prolonged antiplatelet therapy in various categories of patients. BMJ. 1994;308:81–106.

26. Belch J, MacCuish A, Campbell I, Cobbe S, Taylor R, Prescott R, et al. The prevention of progression of arterial disease and diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease. BMJ. 2008;337:a1840.

27. Sanmuganathan PS, Ghahramani P, Jackson PR, Wallis EJ, Ramsay LE. Aspirin for primary prevention of coronary heart disease: safety and absolute benefit related to coronary risk derived from meta-analysis of randomised trials. Heart. 2001;85:265-271.

28. Ogawa H, Nakayama M, Morimoto T, Uemura S, Kanauchi M, Doi N, et al. Low-dose aspirin for primary prevention of atherosclerotic events in patients with type 2 diabetes: a randomized controlled trial. JAMA. 2008;300:2134-2141.

29. Duarte RC, Gonçalves LH, Campos FM, Filho OA, Alves MT, Fernandes AP, et al. Effect of acetylsalicylic acid on platelet activation and oxidative profile in a set of Brazilian patients with type 2 diabetes mellitus. Blood Coagul Fibrinolysis. 2015;26:123-30.

30. Lubsczyk B, Kollars M, Hron G, Kyrle PA, Weltermann A, Gartner V. Low dose acetylsalicylic acid and shedding of microparticles in vivo in humans. Eur J Clin Invest. 2010;40:477-482.

31. Camargo LM, França CN, Izar MC, Bianco HT, Lins LS, Barbosa SP, et al. Effects of simvastatin/ezetimibe on microparticles, endothelial progenitor cells and platelet aggregation in subjects with coronary heart disease under antiplatelet therapy. Braz J Med Biol Res. 2014;47:432-437.

32. Biró É, Sturk-Maquelin KN, Vogel GMT, Meuleman DG, Smit MJ, Hack CE, et al. Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. J Thromb Haemost. 2003;1:2561–2568.

33. Angelillo-Scherrer A. Leukocyte-derived microparticles in vascular homeostasis. Circ Res. 2012;110:356-69.

34. Chironi G, Simon A, Hugel B, Del Pino M, Gariepy J, Freyssinet JM, Tedgui A. Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. Arterioscler Thromb Vasc Biol. 2006;26:2775–80.

Table 1. Cell surface molecules for circulating microparticle identification and characterization.

mAb	Alternative name	Expression	Conjugation	Clone	Company
Annexin V	PS-binding protein	Widely expressed	V450	--	BD Biosciences
IgG1γ	--	--	FITC/PE	X40	BD Biosciences
IgG1k	--	--	FITC/PE	MPOC21	BD Pharmingen
CD142	Tissue Factor	Widely expressed	FITC	VD8	Sekisui diagnostics
CD61	β_3-integrin	Platelets	PE	VI-PL2	BD Pharmingen
PAC-1	$\alpha_{IIb}\beta_3$-integrin	Activated Platelets	FITC	PAC1	BD Biosciences
CD62P	P-Selectin	Activated Platelets	PE	AK-4	BD Pharmingen
CD146	Melanoma Cell Adhesion Molecule	Endothelial Cells	FITC	P1H12	BD Pharmingen
CD62E	E-Selectin	Endothelial Cells	PE	68-5H11	BD Pharmingen
CD235a	Glycoporin A	Erythrocytes	FITC	11E4B-7-6	Beckman Coulter
CD3	T-cell co-receptor	T-Lymphocytes	FITC	HIT3a	BD Pharmingen
CD45	Leukocyte Common Antigen (LCA)	Leukocytes	PE	Immu-19.2	Beckman Coulter
CD11b	macrophage-1 antigen (Mac-1)	Neutrophils, leukocytes	FITC	VIM12	Molecular Probes
CD14	LPS-receptor	Macrophages, monocytes	PE	M5E2	BD Pharmingen

SMA-α	Smooth Muscle Actin α	Smooth muscle cells	PE	1A4	R&D Systems

FITC denotes fluorescein isothiocyanate; LPS, lipopolysaccharide; mAb, monoclonal antibody; PE, phycoerythrin; PS indicates phosphatidylserine.

Table 2. Baseline characteristics of the 43 diabetic patients studied.

	ALL	Type I Diabetics (n=13)	Type II Diabetics (n=30)	*P*
Age (years)	55±10	47±5	59±10	<0.0001
Males [n (%)]	24 (55.8)	7 (53.8)	17 (56.7)	0.798
Current smokers [n (%)]	9 (20.9)	3 (23.1)	6 (20)	0.317
Dyslipidemia [n (%)]	40 (93.0)	8 (61.5)	29 (96.7)	0.001
Hypertension [n (%)]	40 (90.7)	10 (76.9)	30 (100)	0.02
Years of evolution	17±10	25±10	13±9	0.001
Retinopathy [n (%)]	5 (11.6)	3 (23.1)	2 (6.7)	0.655
Nephropathy [n (%)]	1 (2.3)	1 (7.7)	0 (0)	0.06
Polyneuropathy [n (%)]	3 (7)	1 (7.7)	2 (6.7)	0.564
Cardiovascular event [n (%)]	0 (0)	0 (0)	0 (0)	1.000
Body Mass Index (kg/m^2)	25.70±3.04	24.38±2.21	26.12±3.25	0.086
Body Mass Index >25 kg/m^2 [n (%)]	21 (48.8)	5 (38.5)	17 (56.7)	0.011
Systolic Blood Pressure (mmHg)	138±14	132±15	141±12	0.054
Diastolic Blood Pressure (mmHg)	81±8	80±8	81±9	0.840
Glucose (mmol/L)	8.5±2.8	8.9±3.9	8.3±2.1	0.529
HbA1c (%)	7.8±0.7	7.9±0.5	7.7±0.8	0.447
Triglycerides (mmol/L)	1.4±1.2	1.5±2.0	1.3±0.7	0.753
Total cholesterol (mmol/L)	4.8±0.9	5.3±1.0	4.6±0.8	0.027
LDL cholesterol (mmol/L)	2.6±0.7	2.6±0.6	2.6±0.7	0.185
HDL cholesterol (mmol/L)	1.4±0.4	1.5±0.5	1.3±0.4	0.805
LDLc/HDLc ratio	2.1±0.8	1.8±0.7	2.2±0.8	0.142
Non-HDL cholesterol (mmol/L)	3.5±0.8	3.8±0.9	3.3±0.8	0.150
Potassium (mEq/L)	4.8±0.3	4.6±0.3	4.8±0.2	0.014
Urea (mmol/L)	7.5±1.8	6.8±1.5	7.8±1.8	0.079
Creatinine, plasma (mmol/L)	82.1±16.6	76.8±18.5	85.0±15.7	0.142
Creatinine, urine (pmol/L)	8.1±3.6	8.7±4.1	7.8±3.4	0.505
Glomerular filtration rate (mL/min)	50.3±19.5	57.1±3.6	49.4±20.6	0.625
Antidiabetic agents [n (%)]				
Metformin	22 (51.2)	0 (0)	22 (73.3)	<0.0001
Sulfonylurea	5 (11.6)	0 (0)	5 (16.7)	<0.0001
Glinides	8 (18.6)	0 (0)	8 (26.7)	<0.0001
Sitagliptin	6 (13.9)	0 (0)	6 (20.0)	<0.0001
Insulin	35 (81.4)	11 (84.6)	24 (80)	0.428
Antiplatelet agents [n (%)]	15 (34.9)	3 (23.1)	12 (40)	0.012

Antihypertensive agents [n (%)]				
ACE Inhibitor	23 (53.5)	8 (61.5)	15 (50)	0.523
ARB	28 (65.1)	3 (23.1)	25 (83.3)	<0.0001
β- Blockers	3 (7.0)	1 (7.7)	2 (6.7)	0.317
α- Blockers	8 (18.6)	4 (30.8)	4 (13.3)	0.851
Diuretics	16 (37.2)	2 (15.4)	14 (46.7)	0.002
Lipid-lowering agents [n (%)]				
Statins	33 (76.7)	6 (46.2)	27 (90)	<0.0001
Ezetimibe	14 (32.6)	1 (7.7)	13 (43.3)	0.001

P value from one-way ANOVA for quantitative variables and from Chi-square analysis for qualitative variables. ACE Inhibitor indicates angiotensin-converting-enzyme inhibitor; ARB, angiotensin-II receptor blocker; HbA1c, glycated hemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein.

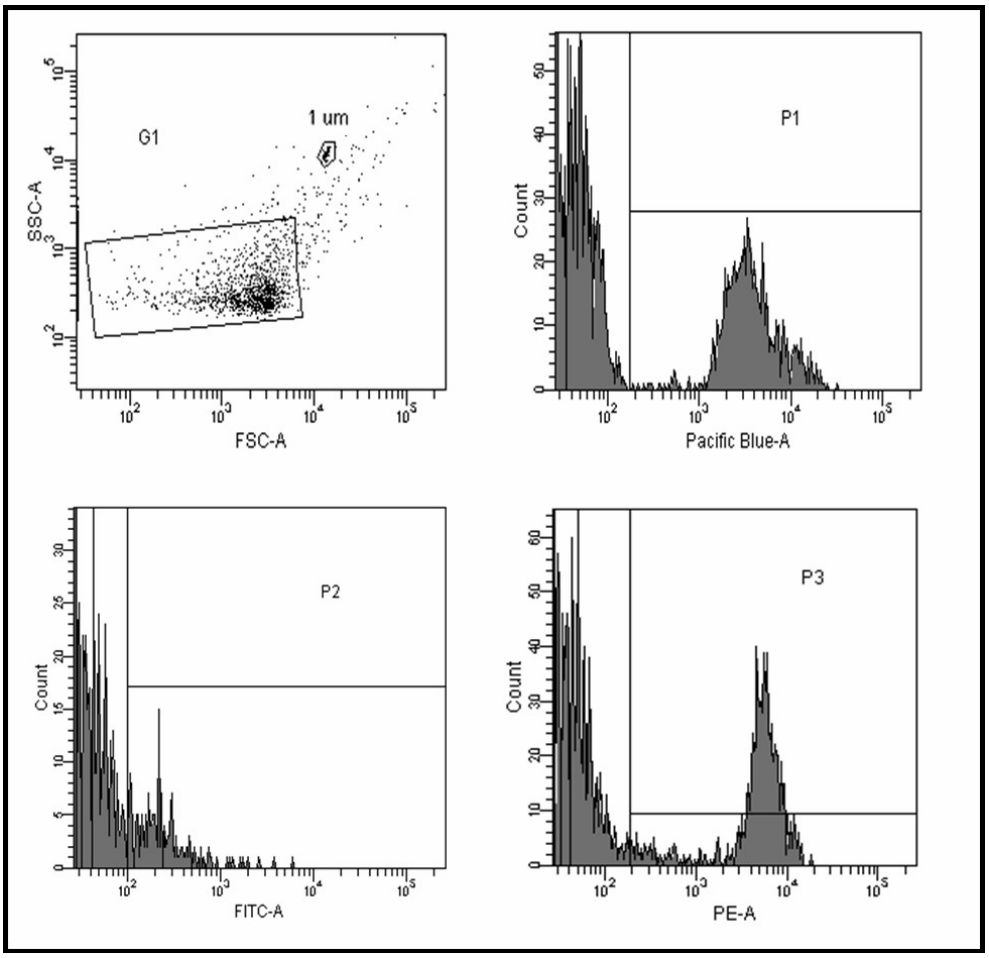
Table 3. Changes in circulating microparticles after the acetylsalicylic acid intervention according to the type of diabetes.

Changes in AV^+ cMPs (cMP/µL PFP)	Type 1 diabetic patients [mean, (95% CI)]	Type 2 diabetic patients [mean, (95% CI)]	*P*
Total	-16.05 (-90.856, 58.75)	-23.78 (-62.3, 14.73)	0.835
Platelet-derived cMPs			
$CD61^+$	-23.89 (-88.486, 40.7)	-3.26 (-31.003, 24.48)	0.468
$CD61^+/CD142^+$	-4.27 (-28.047, 19.51)	1.31 (-5.142, 7.76)	0.524
$PAC\text{-}1^+$	3.76 (-2.185, 9.7)	2.5 (-3.519, 8.52)	0.797
$CD62P^+$	8.24 (-9.364, 25.84)	-2.21 (-11.494, 7.08)	0.239
$PAC\text{-}1^+/CD62P^+$	1.17 (-3.956, 6.29)	1.21 (-3.273, 5.69)	0.991
Endothelial-derived cMPs			
$CD146^+$	2.71 (-1.267, 6.69)	0.58 (-3.533, 4.69)	0.533
$CD62E^+$	1.17 (-3.956, 6.29)	1.21 (-3.273, 5.69)	0.991
$CD146^+/CD62E^+$	2.32 (-1.072, 5.71)	-0.34 (-4.237, 3.56)	0.416
Erythr*ocyte-derived cMPs*			
$CD235ab^+$	-18.55 (-44.949, -7.84)*	-11.76 (-26.327, -2.8)*	0.619
Leukocyte-derived cMPs			
$CD45^+$	-31.67 (-96.685, 33.35)	-3.18 (-21.002, 14.64)	0.232
$CD3^+/CD45^+$	0.58 (-12.509, 13.67)	-6.11 (-18.22, 6)	0.513
$CD14^+$	-11.42 (-22.922, -0.08)*	-7.73 (-15.673, -0.21)*	0.597
$CD14^+/CD11b^+$	-4.8 (-14.237, -4.63)*	-6.06 (-13.09, -0.97)*	0.836
$CD14^+/CD142^+$	-11.41 (-24.775, -1.95)*	-5.09 (-10.752, -0.57)*	0.284
$CD45^+/CD3^-/CD14^-$	-42.27 (-123.805, 39.26)	6.36 (-12.304, 25.03)	0.081
$CD11b^+$	-2.31 (-24.248, 19.64)	-6.45 (-21.813, 8.91)	0.758
$CD142^+$	-2.32 (-39.311, 34.67)	2.37 (-9.276, 14.02)	0.739
Smooth muscle cell-derived cMPs			

SMA-α⁺	-1.79 (-5.353, -1.77)*	-4.16 (-8.333, -0.81)*	0.463
CD142⁺/SMA-α⁺	-0.35 (-1.698, -0.1)*	-1.96 (-3.64, -0.27)*	0.225

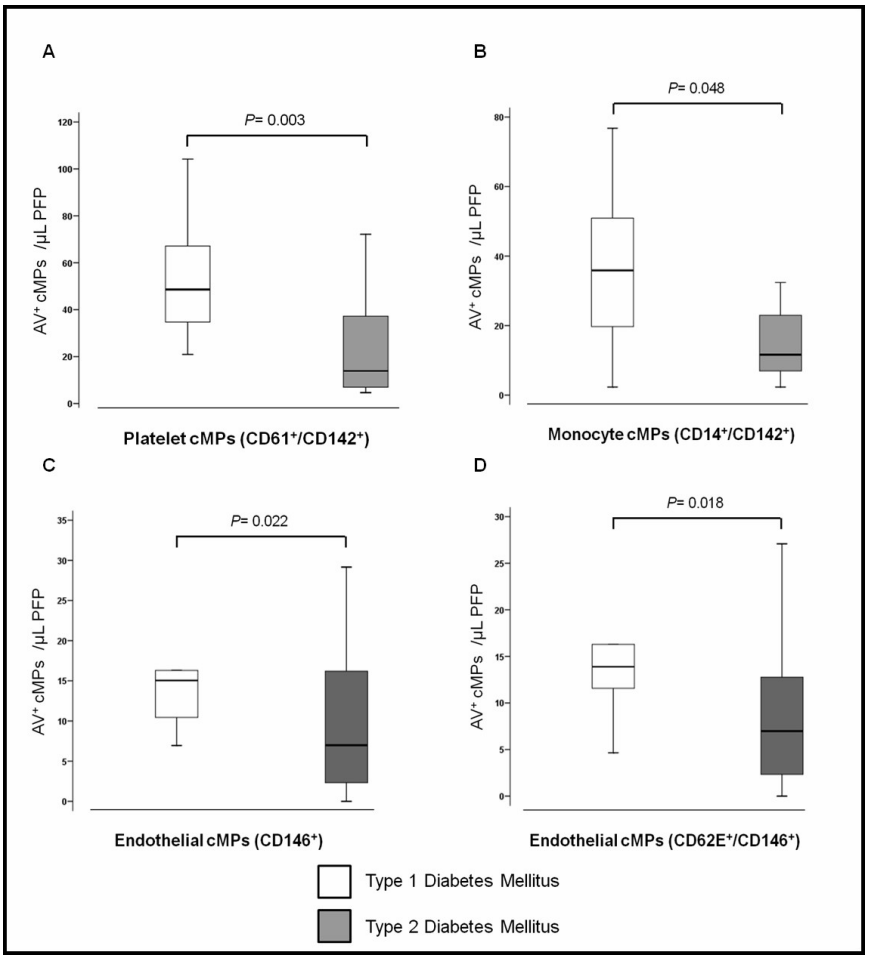
P value from the one-way ANOVA of the changes in cMPs after ASA intervention according to the type of diabetes. **P* <0.05 from the comparison between before and after the intervention (Student's *t* test for paired samples). ASA indicates acetylsalicylic acid; CI confidence interval and cMPs, circulating microparticles. Used markers were CD61 for platelets, CD146 for endothelial cells, CD235ab for erythrocytes, CD45 for total leukocytes, and CD3 for lymphocytes and CD14 for monocytes origins accounting for agranulocytes. Granulocytes were inferred subtracting agranulocytes subpopulation from leukocytes fraction and SMA-α was used for smooth muscle cells. The other CDs were used as biomarkers of cell activation (see **Table 1**). Type 1 diabetes, n=13; type 2 diabetes, n=30.

Figure 1. Gating and acquisition strategy for the detection of circulating microparticles in the FACS analysis.



Gate limits were established before analyses using a Flow Check YG Size Range Calibration Kit (Polysciences, Warrington, PA, USA). G1 was set according to cMPs size and granularity (defined as <1µm). AV^+ cMPs quantified in the Pacific Blue channel were selected (P1) from G1. cMPs binding $FITC^+$ (P2) and/or PE^+ (P3) labeled antibodies (see Table 1) were selected from P1 and quantified. cMPs denotes circulating microparticles; FITC, fluorescein isothiocyanate; and PE, phycoerythrin.

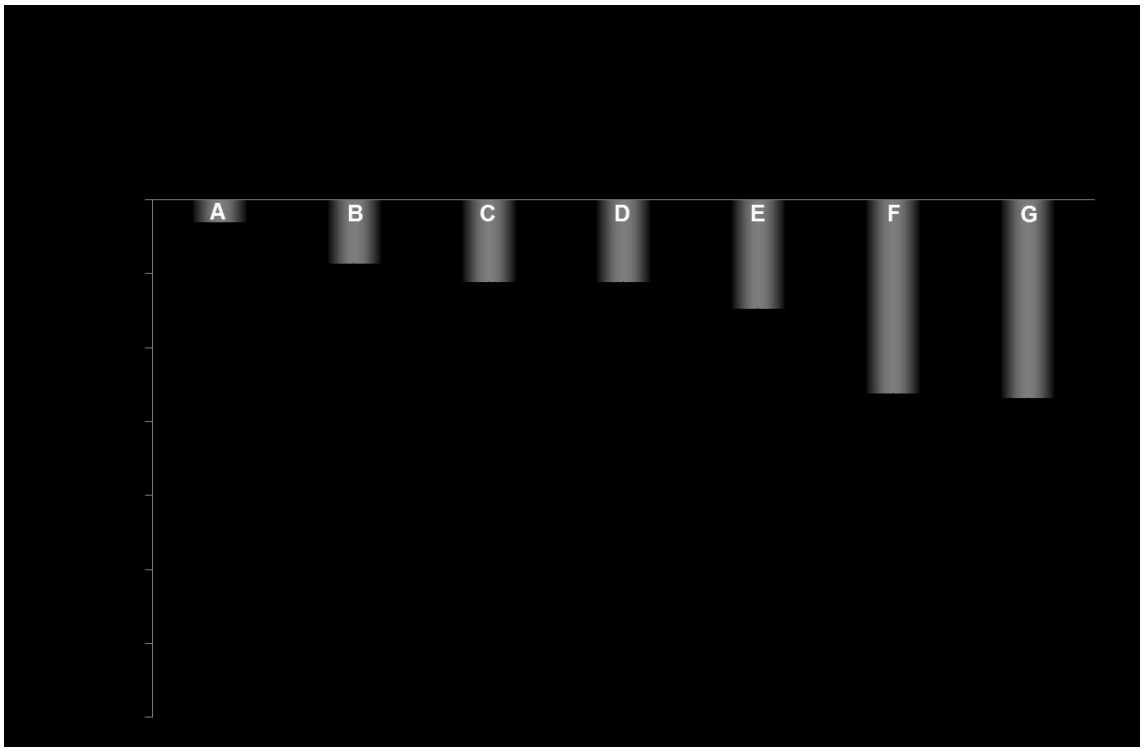
Figure 2. Differences in baseline Annexin V⁺ circulating microparticles according to the type of diabetes.



Box and whisker plots showing number of A) tissue factor positive (CD142⁺) cMPs from platelet (CD61⁺) and B) monocyte origin (CD14⁺) and C) endothelial-derived (CD146⁺) cMPs and D) activated endothelial cells (CD146⁺/CD62E⁺) cMPs before the acetylsalicylic acid intervention according to the type of diabetes. 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. White boxes represent Type 1 DM (n=13) and grey boxes Type 2 DM (n=30). cMPs denotes circulating microparticles; and DM, diabetes mellitus.

P value from the ANCOVA of the number of cMPs with type of diabetes as covariate.

Figure 3. Differences expressed in percentage of decrease in Annexin V^+ circulating microparticles between before and after aspirin intervention.



A: $CD61^+$; B: $CD235a^+$; C: $CD14^+$; D: $CD14^+/CD11b^+$; E: $CD14^+/CD142^+$; F: SMA-α^+ and G: SMA-$\alpha^+/CD142^+$. CD61 was used as a biomarker of platelets, CD235a for erythrocytes, CD14 for monocytes and smooth muscle actin (SMA)-α for smooth muscle cell (SMC) origins. CD142 (tissue factor, TF) and CD11b (α_M-integrin) were used as biomarkers of cell activation. *P<0.05, comparing before and after the intervention (Student's t test for paired samples).