CHAPTER 6

A treatment strategy of pravastatin, vitamin E and homocysteine-lowering medication is associated with reduced blood platelet lysosomal degranulation in patients with renal impairment

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Results from the Anti-oxidant Therapy In Chronic renal insufficiency study

Abel Thijs MD[1], Prabath WB Nanayakkara MD, PhD[1], Dirk P van Asseldonk MD[1,2] Piet M ter Wee MD, PhD[3], Frans J van Ittersum MD, PhD[3] Peter C. Huijgens MD, PhD[4], Coen van Guldener MD PhD[5], Coen D.A. Stehouwer MD, PhD[6]

From the Departments of Internal Medicine[1], Gastroenterology[2], Nephrology[3], Haematology[4], Institute for Cardiovascular Research[1,5,6], VU University Medical Centre, Amsterdam, the Netherlands, the Department of Internal Medicine[5], Amphia Ziekenhuis, Breda, The Netherlands and the Department of Internal Medicine[6], Maastricht University Medical Centre, Maastricht, The Netherlands

Abstract

Background
Blood platelet activation in patients with mild to moderate renal impairment may contribute to the excess cardiovascular risk observed in these patients. We hypothesized that a treatment strategy primarily designed to reduce the level of oxidative stress may reduce platelet activation in patient with mild to moderate renal impairment.
Study design and intervention, setting and participants
We performed a side study in a randomized, double-blind, placebo-controlled clinical trial (www.clinicaltrials.gov: NCT00384618) to investigate the effect on blood platelet activation of a treatment regimen consisting of pravastatin to which vitamin E supplementation was added after 6 months and homocysteine-lowering therapy after another 6 months.
The study was carried out in 93 patients with mild-to-moderate renal impairment (Creatinine clearance 15 - 70 mL/min per 1.73 m2) from six out-patient departments of internal medicine/nephrology.
Outcomes and measurements
Platelet activation was measured at baseline and at 6, 12, 18 and 24 months after randomisation. Platelet activation was assessed by fluorescence cytometric analysis of the expression of markers indicating various aspects of blood platelet activation: CD42b, CD62P, CD63 and PAC-1 on blood platelets.
Results
Compared with placebo, active treatment resulted in a statistically significant reduction of blood platelet lysosomal degranulation (CD63 expression, point estimate and 95% confidence interval of generalized estimating equations coefficient -0.034 (-0.055 – -0.013)) . We did not detect a statistically significant effect on other parameters of platelet activation
Conclusion
In patients with mild to moderate renal impairment a treatment strategy consisting of cholesterol-lowering, antioxidant and homocysteine-lowering medication resulted in reduction of blood platelet lysosomal degranulation. The clinical significance of this finding requires further study.

Introduction

Patients with chronic kidney disease (CKD) are at greatly increased risk of cardiovascular disease [1-6]. Recent studies have shown that this increased risk is not confined to patients with end stage renal disease (CKD stage 5), as it is also apparent in patients with stage 3 and 4 CKD (estimated glomerular filtration rate (eGFR), 59-30 and 29-15 ml/min, 1.73m^2) [3;6], and cannot entirely be explained by a higher prevalence of traditional cardiovascular risk factors in CKD [7;8].

Platelet activation is a multistep key mechanism in atherothrombotic disease [9-11]. An attractive hypothesis to explain the increased risk of cardiovascular disease in CKD is that CKD is associated with increased platelet activation. Notably, oxidative stress is increased in CKD [12] and plays an important role in platelet activation [13]. Indeed, we and others [14;15] have shown that platelet activation is enhanced in CKD.

We hypothesised that a treatment strategy to decrease oxidative stress may decrease platelet activation in CKD stage 3 – 4. To screen for this hypothesis, we measured platelet activation in a side study of the Anti-oxidant Therapy in Chronic renal insufficiency (ATIC) trial, a trial that compared a treatment strategy consisting of pravastatin, vitamin E and homocysteine lowering medication to placebo in patients with CKD stage 3 and 4 against a background of well-controlled blood pressure (www.clinicaltrials.gov identifier NCT00384618). The primary results of this trial have been reported elsewhere [16]

Results

Baseline characteristics (Table 1)
Out of 93 included patients, six withdrew after the baseline measurement and 87 underwent at least one of the following platelet activity measurements and were included in the final analysis (Figure 1). After two years, 77% in the treatment group and 83% in the placebo group were still taking the drugs. Compliance at each follow up visit was defined as consumption of at least 80% of the scheduled tablets since the previous visit. Four patients in the treatment group and two patients in the placebo group were found to consume more than 60% but less than 80% of the allocated tablets during the study period; all others took at least 80% of their scheduled tablets. None of the patients used non-steroidal antiinflammatory drugs or other platelet inhibitors.

LDL-cholesterol, homocysteine and oxidized LDL levels during the study (Table 2)
After 24 months there was a strong and statistically significant reduction of LDL-cholesterol, oxidized LDL and homocysteine level with the treatment strategy.

Renal function
After 24 months, the mean eGFR (MDRD formula) had decreased from 35 to 33 mL/min per 1.73m^2 in the placebo group and increased from 32 to 35 mL/min per 1.73m^2 in the treatment group ($p = 0.89$ for between-group difference).

Platelet activation (figure 2)
There were no statistically significant differences at baseline between the two groups. After 24 months, there was no difference in expression between the treatment group

and the placebo group of CD42b, CD62P and PAC-1 over time (point estimate and 95% confidence intervals of GEE coefficients respectively -0.004 (-0.036 – 0.028), -0.011 (-0.025 – 0.003) and -0.020 (-0.048 – 0.009)).There was, however, an overall statistically significant difference in CD 63 expression between the treatment group and the placebo group (point estimate and 95% confidence interval of GEE coefficient -0.034 (-0.055 – -0.013), p=0.001). In the first 12 months, the rate of decline of the CD63 expression was higher in the treatment than in the placebo group. At 24 months, this difference was no longer statistically significant. Finally, the point estimates of all platelet activation parameters decreased in both groups during the study. This was, however, only statistically significant for CD42b ($p<0.01$). These results were not materially altered when analyses were adjusted for smoking, baseline eGFR, urinary albumin excretion or blood pressures, changes in these variables during the study, or duration of use of renin-angiotensin system inhibitors prior to the study (data not shown).

Discussion

The main finding of this study is that, in patients with mild to moderate non-diabetic renal impairment who had no manifest arterial occlusive disease (thus were not using platelet inhibitors) and had well-controlled blood pressure, 24 months of treatment with a strategy consisting of initially pravastatin with the addition after 6 months of vitamin E and homocysteïne-lowering therapy, we could not detect a change in platelet activation different from placebo, with the exception of a decrease in the expression of CD63 in the first 12 months of the study. The difference in the expression of CD63 at baseline does not corrobate this result, since the statistical method we used to analyse the data corrects for differences at baseline.

In our study population, an effect of the treatment strategy on platelet activation measured by CD42b, CD62P and PAC-1 expression should have been detectable for several reasons. Firstly, the level of platelet activation as compared to healthy, normal individuals was substantial increased (data not shown) [14]). Others also have found platelet activation in CKD patients that was similar to that observed in our study group, although the intensity of activation is not easily comparable with our study due to lack of standardisation of platelet activation parameters [15]. Secondly, flow cytometry is a very sensitive and reproducible technique to measure characteristics of cells or cell fragments [17]. It thus is more sensitive to minimal alterations in platelet function than is ex-vivo testing of spontaneous platelet aggregation. It requires minimal blood sample handling, diminishing the risk or extent of ex vivo platelet activation. All platelet activity measurements were done on the same fluorescence cytometer by the same investigator, and the fluorescent reagents used were titrated weekly against beads with standard fluorescence intensity. Other ways of automated platelet activation (e.g. analysis with the help of the Platelet Function Analyser 100 (PFA 100)) measurement have in this setting not been compared to flow cytometry. In addition, since the effect of a therapeutic strategy on platelet function alteration is to be expected to occur within several weeks given the half life of blood platelets of about one week, this trial should have been long enough to detect such an effect. The power to detect even small differences in these parameters was high, as is evident from the 95% confidence intervals of the GEE coefficients. Indeed, the therapeutic strategy had a clear effect on plasma levels of cholesterol, homocysteine and oxLDL, all consistent with a diminishing level of oxidative stress in the treatment group, compared to the placebo group.

The more rapid decline of the expression of CD63 in the treatment group compared to the placebo group is a remarkable finding. CD63 expression indicates lysosomal degranulation. Therefore, diminished CD63 expression is suggestive of diminished lysosomal degranulation. Since lysosomal contents could play a role in the inflammatory phase of atherosclerotic lesions, this attenuation of lysosomal degranulation is of considerable mechanistic interest. In contrast, the expression of CD42b, CD62P and PAC-1 reflects changes in alpha granules, the conformation of the fibrinogen receptor, the level of expression of the fibrinogen and Von Willebrand Factor receptors, i.e. variables more likely to contribute to the later stages of atherothrombosis (e.g. platelet aggregation and adhesion), since these factors are known to play a role in these processes. Not much is known about the function of blood platelet lysosomes nor on the regulation of their exocytosis. [18] It is assumed that exocytosis of lysosomes is the final step of the degranulation of blood platelets (after the exocytosis of dense granules and α-granules).[19] Whether this inhibition of lysosomal degranulation plays a *clinically* significant role in the inhibition of the inflammatory process in the long run is not known, and requires further study.

Our study had several limitations. Firstly, the effects of the different treatments could not be analysed separately. Indeed, recent evidence shows that anti-oxidant therapy may – apart from a hypothesised positive effect on platelet activation – also have a negative effect on platelet activation by diminishing the levels of oxidised high density lipoprotein (HDL). Oxidised HDL particles seem – at least in vitro – to have a platelet inhibitory effect. It is not known whether this effect on oxHDL is irrespective of the way a anti-oxidant state is reached, and we cannot exclude that in this trial the individual anti-oxidant treatments had opposite effects on blood platelet activation [20;21]. The literature on this subject is, however, scarce. Secondly, there is a tendency of the point estimates of the platelet activity parameters to decrease with time, making it more difficult to discern an effect on the treatment strategy group. The reason for this time-effect is not clearly understood. It could be a trial effect (all patients being carefully followed and treated for e.g. hypertension). Several studies indicate a platelet inhibitory effect of antihypertensives including RAS inhibitors [22-24].

In conclusion, we demonstrated that a treatment strategy designed primarily to reduce oxidative stress consisting of pravastatin, α-tocopherol acetate and homocysteine lowering, had an inhibitory effect on platelet lysosome degranulation in a population of patients with in stage 3 and 4 CKD. However, this treatment strategy had no significant effect on other parameters of platelet activation..Whether this is clinically relevant (i.e. associated with less progression of atherothrombosis) remains to be investigated.

Concise methods

Patients

Between May 2001 and December 2002, patients with an eGFR of 15-70 mL / min per 1.73 m^2 (according to the Cockcroft-Gault equation) without manifestations of occlusive vascular disease or diabetes mellitus from out-patient clinics of seven hospitals near or in Amsterdam, the Netherlands were screened for eligibility for participation in the ATIC study.

Design

Participants were randomised, after stratification for prior use of angiotensin-converting enzyme inhibitors (ACE inhibitors) or angiotensin receptor blockers (ARBs), creatinine clearance (between 15-39 and 40-70 ml / min per 1.73 m^2) and age (between 20-49 and 50-80 years). Randomisation was carried out centrally by means of a computer-generated sequence involving randomised blocks of four and concealed envelops were kept by one hospital pharmacist. After randomisation, participants in the treatment group were treated with pravastatin 40 mg/day; six months later α-tocopherol acetate 300 mg/day was added, and six months thereafter folic acid 5 mg/day, pyridoxine hydrochloride100 mg/day and cyanocobalamin 1 mg/day in one tablet was added. Patients continued this therapy for another 12 months (Figure 1). Patients in the placebo group received matching placebos at the onset of the study, and 6 and 12 months thereafter. Group assignment was blinded for patients as well as investigators. Adherence to therapy was assessed by counting left-over pills. Subjects not using ACE inhibitors or ARBs at inclusion received an ACE inhibitor (fosinopril 10 mg/day) for at least two weeks before the baseline measurements and randomisation. Those who were on ARBs continued their ARBs. During the following visits, blood pressure was controlled according to a standard protocol in which hydrochlorothiazide (a loop diuretic if eGFR<30 mL/min), metoprolol, amlodipine or doxazosine were added in that order to achieve a blood pressure of <140/90 mmHg. We excluded individuals with diabetes mellitus (ADA criteria), active vasculitis, nephrotic syndrome, renal transplantation, fasting total cholesterol > 7 mmol/L, cholesterol-lowering therapy within three months prior to inclusion or ischemic coronary, cerebrovascular or peripheral arterial disease. Ninety-three patients (out of 118 eligible patients) took part in the study (Figure 1). Written informed consent was obtained from all participants and the study was approved by the ethical committees at each centre.

Procedures

Clinical data

All patients were examined in the fasting state in a supine position in a temperature-controlled room. Firstly, data were collected with regard to age, medication and smoking status (having smoked in the past year) and a detailed history was obtained to exclude clinically relevant peripheral, cerebral and coronary vascular disease. Thereafter, height and weight were measured with the individuals wearing light clothing. After 30 minutes of rest, blood pressure was measured with an oscillometric device (Colin Press-Mate, model BP-8800, Komaki-City, Japan) and expressed as the mean value of six measurements over a period of 30 minutes. Mean arterial pressure was calculated as (2 * diastolic pressure + systolic pressure) / 3. Blood samples to perform flow cytometry (see below) were drawn from the antecubital vein with a 19-gauge needle, without stasis or vacuum**.** The first 5 ml of blood were discarded.

Flow cytometry on blood platelets

Flow cytometry on whole blood was performed as previously described [25;26] with minor modifications. In brief, blood was anticoagulated with 0.38% sodium citrate and

immediately fixated with 1% formaldehyde (methanol-free, 1 hour). After fixation, 5 μl blood was labelled with fluorescein isothiocyanate (FITC)- or R-phycoerythrin (PE)-conjugated monoclonal antibodies for 20 minutes in 50 μl of phosphate-buffered saline with 0.1% human serum albumin (PBS-HSA) at room temperature. Monoclonal antibodies used were directed against glycoprotein IIb/IIIa (CD41, fibrinogen receptor, PE-labelled, Dako, Glostrup, Denmark), the activated form of glycoprotein IIb/IIIa (PAC-1, FITC-labelled, Becton Dickinson), Von Willebrand factor receptor (CD42b, glycoprotein Ib, FITC-labelled, Dako), P-selectin (CD62P, FITC-labelled, CLB, Amsterdam, The Netherlands) and glycoprotein 53 (CD63, FITC-labelled, Coulter, Marseille, France). Staining with FITC-labelled IgG1 (ITK, Uithoorn, The Netherlands) was performed as appropriate isotype control. After labelling, the cell suspension was diluted further with 2 ml PBS-HSA. Flow cytometry was performed with a FACScan cytometer (Becton Dickinson Benelux NV, Belgium). Only glycoprotein IIb/IIIa-positive particles in whole blood were considered to be platelets [27] and included in the analysis of a blood sample. FITC-conjugated antibody labelling intensity was expressed as mean fluorescence intensity (set against isotype control). The investigators who performed and read the flow cytometry (AT, DPA) were blinded tot treatment and control group.

Renal function and other laboratory analyses
Plasma creatinine concentration was assessed by a kinetic Jaffé method.
Renal function was estimated by the Modification of diet in renal disease (MDRD) study equation (eGFR in mL/min, per Levey equation 7) [28]; and by the Cockcroft-Gault and Dubois formulas (creatinine clearance in mL/min, 1.73m^2) because at the time of study design it was unclear which method was best. [29;30] .
Total cholesterol, HDL cholesterol, and triglycerides were measured by routine laboratory methods. We calculated LDL cholesterol by use of the Friedewald formula [31] (two participants had triglyceride levels of >4.5mmol/L and their LDL values were not used in the evaluation).
Plasma total (free plus protein-bound) homocysteine level was measured with an automated fluorescence polarization immunoassay analyzer (IMx; Abbott Laboratories, Abbott Park, Illinois, USA).
Urinary albumin was measured in a 24-hour urine collection and analyzed using a microalbumin antiserum analyzer (Beckman Array 360 Analyzer; Global instrumentation Inc., Clearwater, Minnesota USA)
The plasma concentration of oxLDL was measured by a competitive enzyme linked immunosorbent assay (Mercodia, Uppsala, Sweden).

Statistical analyses

Statistical analysis was performed with Intercooled Stata 7 for Windows with blinding to treatment group kept intact. All analyses were performed according to the intention-to-treat principle. Outcome variables were analyzed with generalized estimating equations (GEE), an established technique for the analysis of longitudinal, continuous outcome variables [32]. In the primary GEE model, the outcome variable studied (e.g. expression of CD42b on the platelet membrane) was analyzed as dependent variable using treatment strategy (1= intervention group, 0=placebo group) as key independent variable adjusted for time and, if appropriate, for previous observations, using extra independent variables. For example, a GEE coefficient of -0.004 for the outcome variable *X* means that for every time interval (1 month) the

expression of *X* decreased 0.004 percent point more in the treatment group than in the placebo group. GEE corrects for differences at baseline and regression to the mean. Since the platelet activation parameters had a skewed distribution we used log transformed data in the statistical analysis Data are presented in graphs indicating means with standard deviations. A P-value of <0.05 was considered to be statistically significant.

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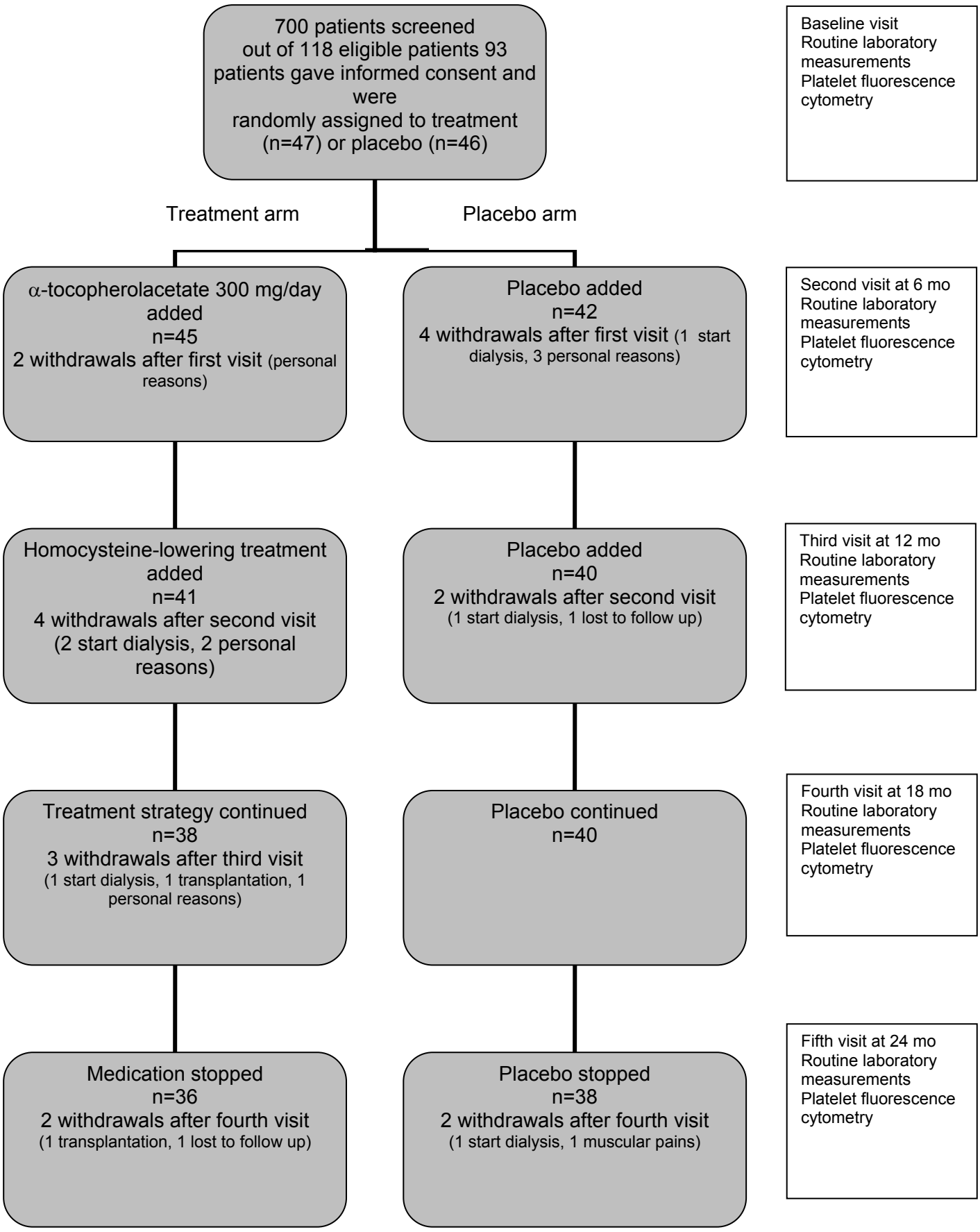
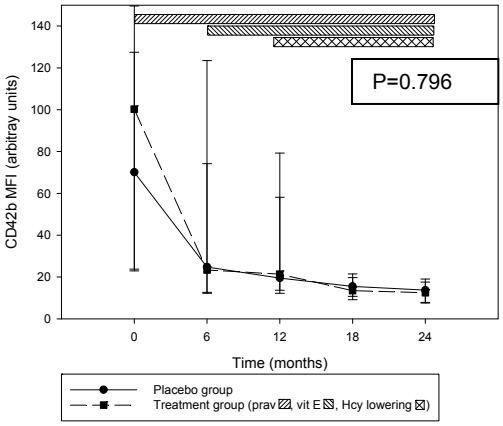
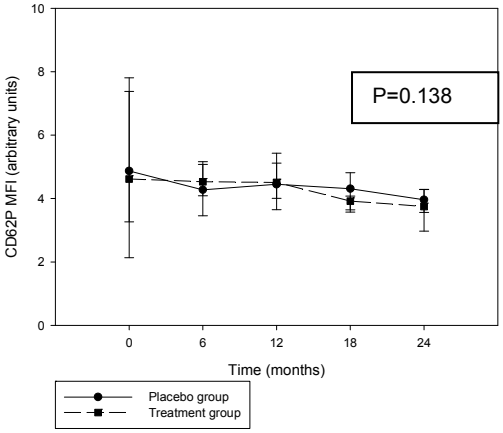
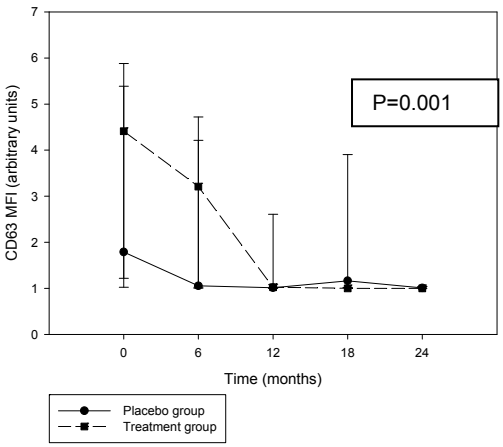


Figure 1
Flow of participants through each stage for both arms of the study







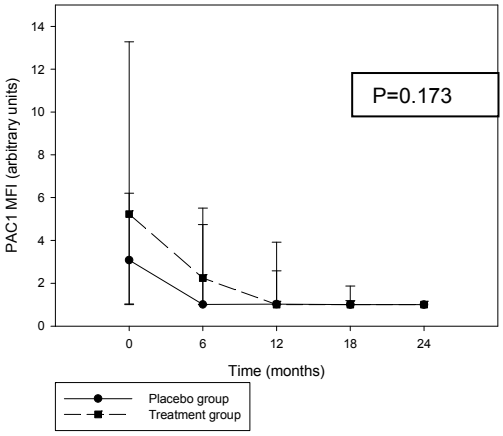


Figure 2
Changes in mean (SD) CD42b, CD62P, CD63 and PAC-1 expression on blood platelets, with the P values for between group differences.
Error bars indicate SD.
Abbreviations: prav, pravastatin, 40 mg/day; vit E, vitamin E, 300 mg/day; Hcy lowering, homocysteine lowering therapy (folic acid 5 mg/day, pyridoxine hydrochloride 100 mg/day, cyanocobalamin 1 mg/day)

Table 1. Baseline Characteristics of the participants *

Variable	Placebo group (n = 46)	Treatment group (n = 47)
Male gender *No. (%)*	29 (63)	24 (51)
Age *years*	52 ± 13	54 ± 11
BMI *kg/m2*	26 ± 4	27 ± 5
Smokers *No. (%)*	17 (37)	16 (34)
Blood pressure *mmHg*		
systolic	134 ± 22	136 ± 20
diastolic	78 ± 13	79 ± 11
mean	97 ± 15	98 ± 13
pulse	56 ± 13	57 ± 13
Lipids *mmol/L*		
total cholesterol	5.4 ± 1.0	5.8 ± 1.5
LDL cholsterol	3.3 ± 0.9	3.8 ± 0.9
HDL cholesterol	1.3 ± 0.4	1.2 ± 0.3
triglycerides		1.8 ± 1.0
Plasma homocysteine, *µmol/L*	22.5 ± 11.3	20.0 ± 6.8
OxLDL *U/L*	61 ± 16	68 ± 12
Renal function		
Plasma creatinine *µmol/L*	199 ± 70	211 ± 96
MDRD formula *mL/min, 1.73 m2*	35 ± 14	32 ± 13
Cockcroft-Gault formula *mL/min, 1.73m2*	39 ± 15	38 ± 16
Urinary albumin excretion (range) *mg/24h*	71 (3 - 2601)	45 (3-3420)
Antihypertensive medication *No.*		
ACE-inhibitors	28	33
Angiotensin receptor blockers	10	9
Diuretics	18	27
β-blockers	19	15
α-blockers	3	2
Calcium channel blockers	8	13
Underlying renal diseases *No. (%)*		
Hypertension	17 (37)	12 (26)
polycystic kidney disease	9 (20)	4 (8)

Abbreviations: ACE, angiotensin-converting enzyme; BMI body mass index (weight in kilograms divided by height in meters squared); HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDRD, Modification of Diet in Renal Disease; oxLDL, oxidized LDL

* Values are expressed as mean ± SD unless indicated otherwise

Table 2. Changes in Oxidized Low-Density Lipoprotein (LDL), LDL Cholesterol and Homocysteine levels during the study*

Variable		at baseline	at 6 months	at 12 months	at 18 months	at 24 months	P Value for between group differences during entire study period
Mean oxidized LDL U/L							< 0.001
	treatment group	67.48 ± 12.35	56.67 ± 13.49	55.97 ± 12.25	56.33 ± 14.19	57.31 ± 13.87	
	placebo group	60.57 ± 15.59	64.61 ± 15.07	63.19 ± 15.67	62 .11 ± 15.19	62 .13 ± 14.57	
Mean plasma LDL-cholesterol mmol/L							< 0.001
	treatment group	3.79 ± 0.93	2.65 ± 0.85	2.67 ± 0.76	2.74 ± 0.81	2.80 ± 0.77	
	placebo group	3.27 ± 0.88	3.38 ± 0.95	3.36 ± 0.92	3.39 ± 0.93	3.41 ± 1.07	
Mean plasma homocysteine µmol/L							0.001
	treatment group	20.16 ± 6.80	19.76 ± 6.46	17.31 ± 6.02	11.31 ± 4.66	10.45 ± 4.02	
	placebo group	22.45 ± 11.27	21.87 ± 9.91	18.71 ± 8.63	19.68 ± 10.44	20.22 ± 12.06	

* Values other than P values are expressed as mean ± SD