Clopidogrel has no effect on D-dimer and thrombin-antithrombin III levels in patients with peripheral arterial disease undergoing peripheral percutaneous transluminal angioplasty

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Objective: **Coagulation activation markers are significantly elevated in patients with peripheral arterial disease compared with healthy controls. The more severe the disease, the higher the markers. Increased coagulation activation may contribute to the disease process and the risk of complications in patients with peripheral arterial disease, particularly after endovascular intervention. Animal studies have shown that clopidogrel significantly inhibits coagulation activation. The aim of this study was to determine whether combination of aspirin and clopidogrel affects thrombin-antithrombin III and D-dimer in patients with intermittent claudication undergoing angioplasty, compared with aspirin alone.**

Methods: **This was a double blind, randomized placebo-controlled trial conducted in a vascular unit in a tertiary referral center. One hundred thirty-two patients with intermittent claudication were randomized to clopidogrel and aspirin or placebo and aspirin, with a loading dose 12 hours before endovascular intervention. D-dimer and thrombin-antithrombin III (TAT) levels were measured using enzyme-linked immunosorbent assay at baseline, 1 hour before, and 1 hour, 24 hours, and 30 days after intervention in 103 patients who underwent endovascular intervention.**

Results: **There was a significant rise in D-dimer levels at 1 hour and 24 hours after angioplasty in both groups (placebo group: 63.69, 141.45, 122.18 ng/mL; clopidogrel group: 103.79, 159.95, 134.69 ng/mL), but no difference between the two groups ($P = .514$). Similarly there was a significant rise in TAT levels at 1 hour after angioplasty in both groups (placebo group: 2.93, 6.16 μg/L; clopidogrel group: 3.39, 5.27 μg/L), with no significant difference between the two groups ($P = .746$).**

Conclusion: **Endovascular intervention results in a significant increase in TAT and D-dimer. The addition of clopidogrel to aspirin has no effect on TAT and D-dimer before or after endovascular intervention. (J Vasc Surg 2005;42:252-8.)**

Coagulation activation has been shown to be significantly increased in patients with peripheral arterial occlusive disease.[1-6] This has been implicated in the increased risk of this group of patients for cardiovascular, cerebrovascular, and peripheral vascular events, and hence the significantly increased risk of mortality compared with age-matched controls.[7-9] These risks are even higher at around the time of endovascular or surgical intervention. Coagulation activation markers increase further after coronary[10-12] and peripheral endovascular intervention.[13] It is unclear, however, whether this increase in response to intervention contributes to the increased risk of periprocedural events or whether medical intervention to reduce coagulation activation will translate into a reduced risk for such events.

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Support for this work was provided by funding from Sanofi-Synthelabo for consumables and reagents.

Competition of interest: none.

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0741-5214/$30.00

doi:10.1016/j.jvs.2005.04.027

Patients with peripheral arterial disease are routinely administered antiplatelet treatment in the form of aspirin. Clopidogrel is a prodrug metabolized in the liver to an active compound that is an adenosine 5′-diphosphate (ADP)-receptor antagonist, which mediates its antiplatelet effect. Clopidogrel is increasingly used, particularly in combination with aspirin, in high-risk patients. The benefits of combination aspirin-clopidogrel in non-ST elevation myocardial infarction[14] and in patients undergoing coronary stenting have been identified in randomized controlled trials.[15] These benefits are probably mediated through the improved antiplatelet effect of the combination of drugs.

However, platelet ADP receptors have also recently been shown to contribute to the initiation of intravascular coagulation through rapid exposure of tissue factor,[16] which is the major initiator of coagulation. Furthermore, ex vivo inhibition of the $P2Y_{12}$-ADP receptor by clopidogrel administration diminished rapid exposure of tissue factor in mice.[16] As early as 1994, Savi and colleagues[17] had shown that clopidogrel was able to inhibit platelet-induced tissue factor expression in rats, whereas aspirin was ineffective.[17] In separate studies, it was shown that clopidogrel significantly inhibited shear-induced coagulation in rats whereas aspirin did not,[18] and that clopidogrel inhibited thrombus formation in a stasis-induced venous thrombosis rat mod-

el.[19] These findings suggested that the clinical benefits observed with use of clopidogrel may be partly due to inhibition of coagulation activation.

The aim of this study was to determine whether combination aspirin-clopidogrel treatment has any affect on coagulation activation in patients with intermittent claudication undergoing endovascular intervention. The anticoagulation markers (thrombin-antithrombin III [TAT] and D-dimer) measured were the secondary outcome measures in a randomized controlled trial investigating the effect of combination aspirin-clopidogrel treatment on platelet activation (primary outcome measure) in patients undergoing endovascular intervention.[20] The results of this study showed that combination aspirin-clopidogrel reduces platelet activation more effectively than aspirin alone in this group of patients.

Fibrin D-dimer is a degradation product of cross-linked fibrin that is brought about by plasmin. The latter is converted from its precursor plasminogen by the action of tissue plasminogen activator. TAT is a single, polypeptide serum protein synthesized by the liver that forms the complex with activated thrombin, destroying its enzymatic activity. D-dimer and TAT complexes therefore serve as markers of coagulation activation. Because platelet ADP receptors contribute to intravascular coagulation through rapid exposure of tissue factor, clopidogrel, which is an ADP-receptor antagonist, may reduce coagulation activation.

METHODS

All patients between the ages of 18 and 80 years referred to the Vascular Unit of the Aberdeen Royal Infirmary with lifestyle-limiting claudication of the lower limbs and evidence on duplex scanning of arterial stenosis or occlusion in either the aortoiliac or femoropopliteal segments suitable for angioplasty were considered for participation in the study. Eligible participants were included if they were able to provide informed consent and satisfied inclusion and exclusion criteria (Table I). Patients were recruited between March 2002 and January 2003.

The main outcome measure for the randomized controlled trial was platelet activation on which the power calculation was based and which has been published.[20,21] Coagulation markers were the secondary outcome measures of the study.

Interventions. Patients were randomized to receive either 75 mg clopidogrel and 75 mg aspirin daily for 30 days, with a loading dose of 300 mg clopidogrel administered 12 hours before planned intervention, or 75 mg aspirin and placebo daily, with a loading dose of placebo administered 12 hours before intervention. A control group receiving no antiplatelet medication was not included in this study, as this was considered unethical. The drugs were supplied in identical packs by the Trial Drugs Pharmacy Department at Aberdeen Royal Infirmary.

Patients underwent digital subtraction angiography followed by endovascular intervention. Endovascular procedures were performed by one of two experienced consultant interventional radiologists. Intravenous heparin (3,000 to 5,000 units) was used in all procedures performed. The dose given depended on the patient's weight.

Table I. Patient inclusion and exclusion criteria

Inclusion criteria:
- Hemoglobin >10 g/dL
- Platelet count $>150 \times 10^9$/L; normal APTT/INR/fibrinogen
- Aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase <3 times upper normal limit
- Serum creatinine <2 times upper normal limit
- Body mass index <33
- Age, 18 to 80 years
- No contraindication to aspirin or clopidogrel

Exclusion criteria
- History of hematologic malignancy
- Acute illness ≤14 days before randomization
- Transfusion of whole blood or red blood cells ≤14 days before randomization
- Known or suspected drug or alcohol abuse
- Taking steroids, other drugs with antiplatelet effects (eg, NSAIDs), warfarin or heparin
- History of bleeding diathesis or coagulopathy
- History of severe neutropenia (neutrophil count $<1.8 \times 10^9$/L)
- History of thrombocytopenia (platelet count $<150 \times 10^9$/L)

APTT, Activated partial thromboplastin time; *INR,* international normalized ratio; *NSAIDs,* nonsteroidal anti-inflammatory drugs.

Blood samples were taken at baseline before clopidogrel or placebo was administered, 1 hour before the endovascular intervention, and then at 1 hour, 24 hours, and 30 days after the endovascular intervention. Blood samples were collected using a 21-gauge needle inserted into an antecubital vein with the cuff applied to the upper arm. The cuff was removed once the first trickle of blood appeared into the first of two 1:10 3.2% sodium citrate Vacutainers (Beckton Dickinson, Franklin Lakes, NJ). Ten milliliters of blood collected in sodium citrate Vacutainers was centrifuged at 3,000 rpm at 21°C for 10 minutes. The serum was stored at −80°C until the enzyme-linked immunosorbent assay (ELISA) was performed. An Enzygnost TAT micro ELISA kit (Dade Behring, Marburg, Germany) was used to measure TAT and a Dimertest Gold EIA kit (AGEN, Brisbane, Australia) was used to measure D-dimer.

Randomization. Pharmacists dispensed placebo or active drug packs according to a computer-generated randomization process that balanced the control and treatment groups with respect to sex, diabetes mellitus, and smoking status using a minimization method. The decision to accept or reject participants was made and informed consent obtained from participants before randomization, ensuring allocation concealment. The code was held by the Trial Drugs Pharmacy Department and was only revealed to the researchers once recruitment, data collection, and laboratory analyses were complete. Participants were enrolled by the first author (KC), and participants assigned and drug packs provided by the Trial Drugs Pharmacy

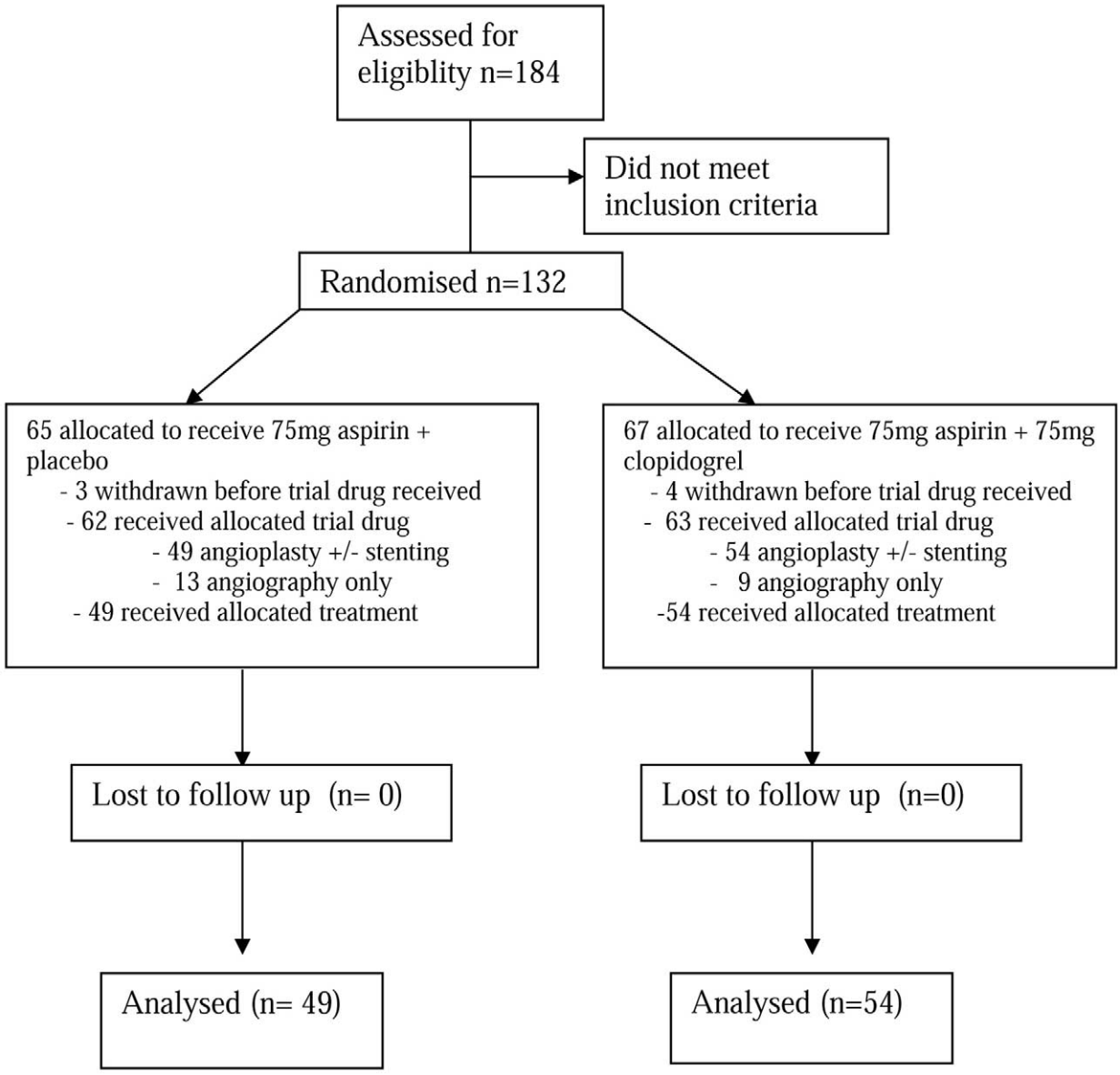


Fig 1. Flow of participants through the study.

Department. All study personnel and participants were blinded to treatment assignment for the duration of the study. Full ethical approval for the study was granted by the local ethics committee. Permission for Sanofi-Synthelabo to supply clopidogrel for the purposes of the study was obtained from the Medicines Control Agency.

Statistical analysis. All data analysis was carried out according to pre-established analysis plans. Results are presented as mean values and 95% confidence intervals. Between subjects, analysis of variance (ANOVA) was used to compare placebo and clopidogrel groups. $P < 0.05$ was accepted as statistically significant. Within subjects, ANOVA was used to estimate treatment effect.

RESULTS

Fig 1 shows the flow of participants through each stage. Between March 2002 and January 2003, 132 patients were recruited and randomized, 65 to placebo and 67 to the clopidogrel group. Seven patients were excluded after randomization because of failure to attend for angiography or diagnosis of malignancy after randomization but before administration of treatment. One hundred twenty-five proceeded to angiography, and 103 underwent vascular intervention.

In 22 patients, endovascular intervention was deemed unfeasible or too risky, mainly because of a single patent calf

Table II. Baseline demographics and clinical characteristics

	Claudicants (n = 132)		
	Total (n = 132)	*Placebo group (n = 65)*	*Clopidogrel group (n = 67)*
Males:females	102:30	50:15	52:15
Mean age (yrs) (range)	65.7 (43-80)	65.4 (46-80)	66.1 (43-80)
Smoking (%)			
never smoked	8 (6)	3 (4.6)	5 (7.5)
ex-smoker >1 yr	55 (42)	27 (41.5)	28 (41.8)
ex-smoker <1 yr	24 (18)	13 (20.0)	11 (16.4)
smoker	45 (34)	22 (33.8)	23 (34.3)
Diabetes mellitus (%)	23 (17)	11 (16.9)	12 (17.9)
Mean serum cholesterol (mmol/L)*	3.9 ± 2.13	3.68 ± 2.23	4.15 ± 2.02
Mean body mass index*	25.8 ± 4.0	26.1 ± 3.9	25.5 ± 4.1
Median ankle-brachial pressure index (range)	0.64 (0.36-1.14)	0.63 (0.36-1.14)	0.65 (0.36-0.91)
Intervention			
None	7	3	4
Angiography only	22	13	9
Transluminal PTA	62	29	33
Subintimal PTA	16	10	6
Stenting	25	10	15
Site of disease treated			
None	29	16	13
Aortoiliac segment	31	15	16
Femoropopliteal segment	71	34	37
Aortoiliac + fempop segments	1	0	1

PTA, Percutaneous transluminal angioplasty.
*Data are ± standard deviation.

vessel. A duplex examination failed to detect significant proximal calf vessel disease, which substantially increased the risk of intervention. After discussion and agreement, angioplasty was not performed.

Forty-nine patients in the placebo group and 54 patients in the clopidogrel group underwent endovascular intervention. The median ankle-brachial pressure index in the affected limb in the placebo group was 0.63 and in the clopidogrel group 0.65. Fifteen patients in the placebo group and 16 in the clopidogrel group had treatment of the aortoiliac segment disease, and 34 patients in the placebo group and 37 in the clopidogrel group had femoropopliteal segment disease treated. One patient in the clopidogrel group had both iliac and femoropopliteal segment disease treated. Stenting of the iliac segment was required in 13 patients in the placebo group and 15 in the clopidogrel group. No stents were inserted in the femoropopliteal segment.

None of these patients was lost to follow-up. Data from 103 patients who underwent endovascular intervention were analyzed. All follow-up visits were completed by March 2003.

Four patients in the placebo group stopped the trial medication early because of rash (n = 2), loss of medication (n = 1), or voluntarily (n = 1). Seven patients in the clopidogrel group stopped taking the trial medication early because of the development of a rash (n = 2), upper gastrointestinal bleeding (n = 1), epigastric pain (n = 1), ischemic stroke (n = 1), loss of medication (n = 1), and no apparent reason (n = 1). Two patients failed to take their loading dose on time and one patient was seen for the 30-day postintervention visit on day 38.

Analysis was on a modified intention-to-treat basis, and results from all patients who underwent endovascular intervention irrespective of compliance with trial medication were analyzed. Patients who did not undergo endovascular intervention were not included in the analysis. Data from all 103 patients who underwent endovascular intervention were analyzed at all time intervals. Table II shows the baseline demographics and clinical characteristics of the patients in both groups, the type of intervention performed, and the site of disease treated.

D-dimer. D-dimer levels at baseline were similar between the placebo group (89.33 ng/mL) and the clopidogrel group (73.01 ng/mL) ($P = .89$). Fig 2 shows the results of D-dimer levels in the placebo and clopidogrel groups at different time-points in the study in the form of box plots. The administration of the clopidogrel loading dose did not have an affect on D-dimer levels, as shown by the lack of any significant change in D-dimer levels in the clopidogrel group at 1 hour before the intervention.

Percutaneous transluminal angioplasty had a significant effect on D-dimer levels, however. At 1 hour after angioplasty, D-dimer levels were significantly elevated compared with baseline in both the clopidogrel and placebo groups. Similarly, D-dimer levels at 24 hours after angioplasty remained significantly elevated compared with baseline in both groups. The highest D-dimer levels were reached at 1 hour after angioplasty in both the clopidogrel and placebo groups. By 30 days after angioplasty, D-dimer levels had

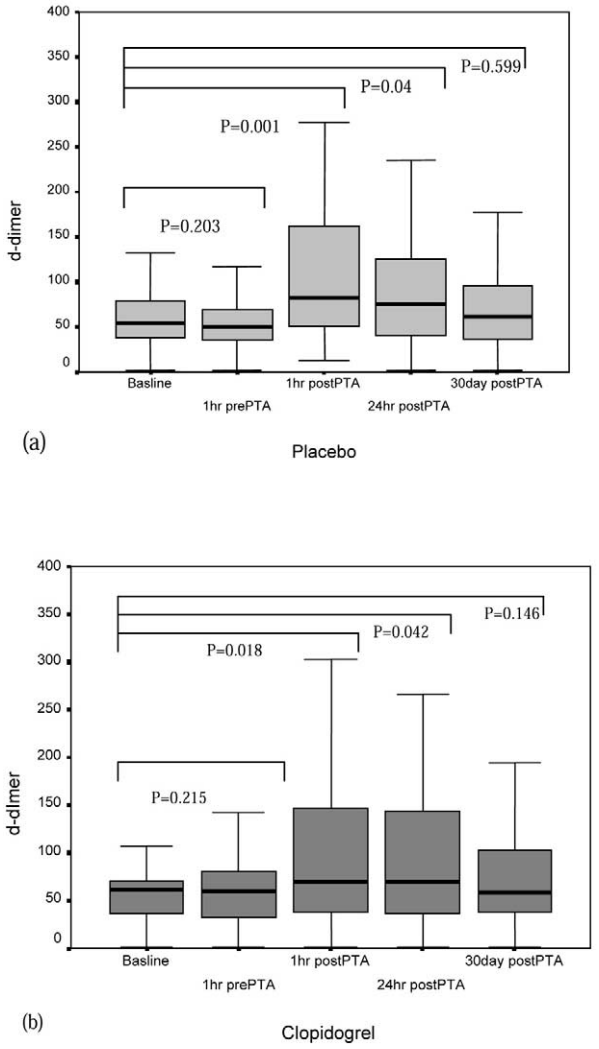


Fig 2. Box plots of D-dimer levels (ng/mL) in **(a)** the placebo and aspirin group and **(b)** clopidogrel and aspirin group. The bars represent median values, and the whiskers represent smallest and largest values. The top of box is the 75th percentile, and the bottom of box is the 25th percentile. *PTA*, Percutaneous transluminal angioplasty.

returned to baseline levels in both the clopidogrel and placebo groups.

ANOVA showed no difference between the clopidogrel and placebo groups ($P = .514$). Table III gives results and 95% confidence intervals for D-dimer levels at different time points in both groups.

Thrombin-antithrombin III. TAT levels at baseline were similar between the placebo group (3.09 μg/L) and the clopidogrel group (3.33 μg/L) ($P = .92$).

Fig 3 shows the results for TAT levels in the two groups at the different time points in the form of box plots.

TAT levels at 12 hours after the administration of a loading dose did not differ from baseline levels in both the placebo and clopidogrel groups; however, at 1 hour after angioplasty, TAT levels were significantly elevated compared with baseline in both groups. In the placebo group, TAT levels remained significantly elevated even at 24 hours after angioplasty. In the clopidogrel groups, although the TAT levels were higher than baseline at 24 hours after angioplasty, this was not statistically significant. At 30 days after angioplasty, TAT levels were down to baseline levels in both groups.

Table III. Between subjects analysis of variance for D-dimer levels*

Group	*Time of measurement*	*Mean*	*95% CI lower bound*	*95% CI upper bound*
Placebo	Baseline	89.33	45.58	133.07
	1 hr pre-PTA	63.69	48.39	79.00
	1 hr post-PTA	141.45	98.59	184.29
	24 hr post-PTA	122.18	81.61	162.75
	30 day post-PTA	78.64	59.88	97.40
Clopidogrel	Baseline	73.01	53.17	92.84
	1 hr pre-PTA	103.79	50.48	157.10
	1 hr post-PTA	159.95	84.72	235.19
	24 hr post-PTA	134.69	74.88	194.51
	30 day post-PTA	102.39	62.65	142.14

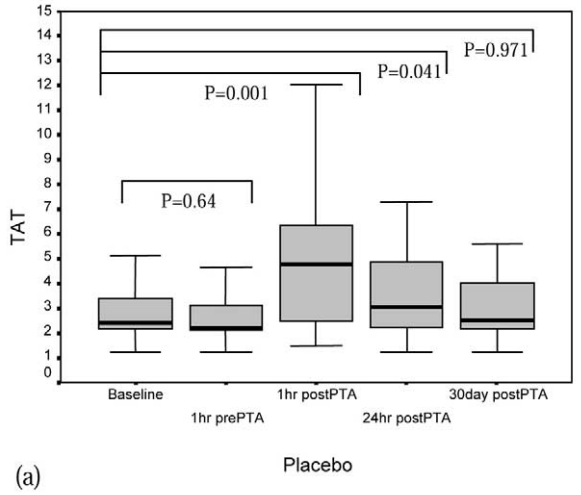
CI, confidence interval; *PTA*, percutaneous transluminal angioplasty.
Between subjects analysis of variance: $P = 0.514$.
*Mean value for healthy controls: 26.1 ng/mL[6].

Between-subjects ANOVA showed no difference between the clopidogrel and placebo groups with regards to TAT levels. Table IV shows the mean values as well as 95% confidence intervals for these values.

Adverse events. Two patients in each group developed a skin rash and two in each group developed a hematoma at the site of radiologic access that did not require surgical intervention. The number of patients who developed bruising at and around the site of access was slightly higher in the clopidogrel group (25 vs 16), but the difference between the two groups was not statistically significant. Two patients in the clopidogrel group had an ischemic stroke at day 7 and day 12 after intervention. One of these patients had stopped taking all medication immediately after the intervention, against medical advice. Melena secondary to bleeding from multiple small gastric ulcers developed in one patient. One patient in the clopidogrel group became hypotensive immediately after the intervention and was found to have a retroperitoneal hematoma. This did not require surgical intervention but resulted in a 7-day delay in discharge from the hospital.

DISCUSSION

This study shows that endovascular intervention in patients with intermittent claudication results in a significant increase in D-dimer and TAT despite the administration of heparin during the procedure. The addition of clopidogrel to aspirin does not affect coagulation activation in these patients, neither does it prevent or reduce the increase in coagulation activation observed after endovascular intervention.



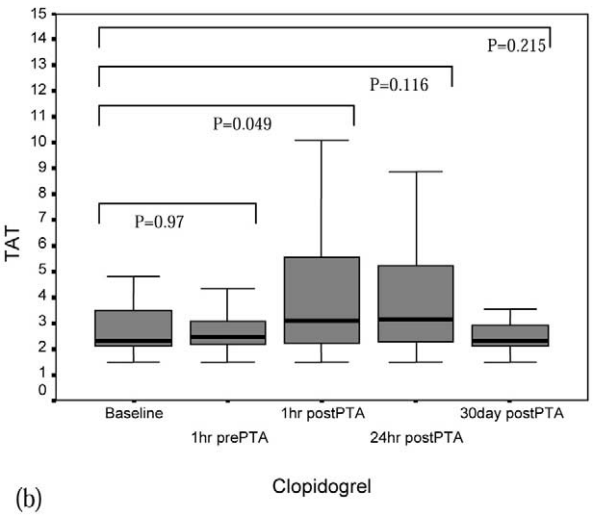


Fig 3. Box plots of thrombin-antithrombin III *(TAT)* levels (μg/L) in the **(a)** aspirin and placebo and **(b)** aspirin and clopidogrel groups. The bars represent median values, and the whiskers represent smallest and largest values. The top of box is the 75th percentile, and the bottom of box is the 25th percentile. *PTA*, Percutaneous transluminal angioplasty.

Endothelial damage results in exposure of subendothelial matrix molecules, leading to platelet adhesion and aggregation and the formation of a primary hemostatic plug. However, in addition to this function in hemostasis, platelets also contribute to thrombin formation and fibrin generation, not least through provision of anionic phospholipid, which serves as a template for the construction of the tenase and prothrombinase complexes.

Savi and colleagues[17] first showed that clopidogrel was able to inhibit platelet-induced tissue factor expression in endothelial cells in rats, whereas aspirin was ineffective. Later on, clopidogrel was found to inhibit shear-induced coagulation in rat blood, but ticlopidine and aspirin did not.[18] Clopidogrel was also found to significantly inhibit ex vivo thrombin generation triggered by low concentrations of tissue factor in rat platelet-rich plasma,[19] confirming the close relationship between platelet activation and thrombin generation leading to coagulation.

Table IV. Between subjects analysis of variance for thrombin-antithrombin III levels*

Group	*Time of measurement*	*Mean*	*95% CI lower bound*	*95% CI upper bound*
Placebo	Baseline	3.0864	2.5516	3.6211
	1 hr pre-PTA	2.9280	2.4433	3.4127
	1 hr post-PTA	6.1559	4.4435	7.7684
	24 hr post-PTA	3.8088	3.1831	4.4345
	30 day post-PTA	3.0984	2.6834	3.5134
Clopidogrel	Baseline	3.3304	2.6192	4.0416
	1 hr pre-PTA	3.3855	2.4993	4.2716
	1 hr post-PTA	5.2695	3.7117	6.8273
	24 hr post-PTA	4.7862	3.3511	6.2212
	30 day post-PTA	2.9885	2.4945	3.4826

CI, confidence interval; *PTA*, percutaneous transluminal angioplasty.
Between subjects analysis of variance: $P = .746$.
*Mean value for healthy controls, 2.36 μg/L[6].

Only recently has it been recognized that platelet ADP receptors contribute to the initiation of intravascular coagulation.[16] Under in vitro conditions, the $P2Y_{12}$ and the $P2Y_1$ ADP receptors were both found to be implicated in the exposure of tissue factor when whole blood was activated with collagen injection. Leon and colleagues[16] also showed that ex vivo inhibition of the ADP receptor by clopidogrel administration diminished this rapid exposure of tissue factor. These findings suggested that clopidogrel administration could reduce the level of coagulation activation in humans.

The combination of aspirin and clopidogrel, however, did not appear to have any effect on coagulation parameters (platelet-dependent thrombin generation, antithrombin III, TAT III complex, prothrombin fragment 1+2) in patients with nonvalvular atrial fibrillation.[21] Similarly, no effect was noted on coagulation activation markers when clopidogrel was administered in addition to aspirin in patients with non-ST elevation acute coronary syndromes.[22] Neither did clopidogrel have any effect on coagulation markers (prothrombin fragment 1+2) in patients undergoing elective coronary stenting.[23]

The results of our study support the findings of these studies that clopidogrel has no effect on coagulation activation markers. This is contrary to what would be expected given the demonstrated role of the ADP receptor in the initiation of coagulation.[16]

There are various possible explanations for the results of our study. Failure to show any difference with clopidogrel may be because of the limited statistical power of the study, although this is unlikely considering that similar findings have been published in the context of atrial fibrillation, coronary stenting, and non-ST-elevation myocardial infarction. Furthermore, our sample size was sufficient to show differences in

platelet activation[20] and to pick up differences in coagulation markers during and after endovascular intervention. The combination of clopidogrel and aspirin was significantly more effective at inhibiting platelet function than aspirin alone in this group of patients. The antiplatelet combination caused a significant reduction in flow cytometrically measured platelet P-selectin expression, fibrinogen binding, and ADP-stimulated fibrinogen binding compared with aspirin alone.[20]

The handling of the blood after collection may lead to artificially elevated coagulation markers, thus limiting the identification of any difference between the two groups. Great care was taken to standardize blood withdrawal and to process blood immediately after sampling to diminish artifactual coagulation activation, and the subjects themselves acted as their own controls.

Another possible explanation for failure to show a difference is that clopidogrel is a $P2Y_{12}$ ADP receptor antagonist and therefore fails to block the $P2Y_1$ ADP receptor. The latter has been shown to be involved in rapid tissue factor exposure, and it is possible that this alternative pathway, which remains unblocked, allows tissue factor exposure and initiation of coagulation to continue despite adequate inhibition of the $P2Y_{12}$ ADP receptor.

CONCLUSION

Our results show that combining clopidogrel and aspirin does not reduce the increased levels of TAT and D-dimer in patients with intermittent claudication and does not prevent the further increase in these coagulation markers seen after endovascular intervention in this group of patients. Any clinical benefit observed with combination treatment in this group of patients is therefore unlikely to be mediated through inhibition of coagulation activation.

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Submitted Jan 20, 2005; accepted Apr 19, 2005.