

## New *in vitro* effects of clopidogrel on platelets in hyperlipidemic and healthy subjects

*Hiperlipidemili ve sağlıklı olgularda trombositler üzerinde klopidogrelin yeni in vitro etkileri*

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### Abstract

**Objective:** We aimed to detect novel *in vitro* effects of clopidogrel on platelets by assessment of the following parameters: malondialdehyde, glutathione, nitrite, aggregation response, and expressions of P-selectin, fibrinogen, apolipoprotein A1, apolipoprotein B, and phosphatidylserine.

**Materials and Methods:** Platelets were obtained from healthy (n: 9) and hyperlipidemic (n: 9) volunteers. Expressions of P-selectin, fibrinogen, apolipoproteins A1/B and phosphatidylserine with and without clopidogrel were assayed by flow cytometry. Malondialdehyde, glutathione, aggregation and nitrite levels were also assayed.

**Results:** Without clopidogrel, the baseline values of platelet aggregation, malondialdehyde, and expressions of P-selectin, fibrinogen and phosphatidylserine were significantly higher, whereas nitrite and expression of apolipoproteins A1/B were significantly lower in hyperlipidemics than in the healthy group. In both groups, clopidogrel significantly reduced aggregation and expression of fibrinogen, but it elevated nitrite levels. Clopidogrel significantly decreased P-selectin and phosphatidylserine expression and malondialdehyde but increased expressions of apolipoproteins A1/B only in hyperlipidemics.

**Conclusion:** It seems that clopidogrel has some new *in vitro* antiplatelet effects. The present study is a basic *in vitro* study to suggest new insights into the effects of clopidogrel on platelet functions.

(*Turk J Hematol* 2010; 27: 99-108)

**Key words:** Platelets, hyperlipidemia, clopidogrel, apolipoprotein A1, apolipoprotein B, oxidative stress, nitrites, phosphatidylserine, P-selectin, glycoproteins IIb/IIIa

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### Özet

**Amaç:** Klopidogrelin trombositler üzerinde yeni *in vitro* etkilerini tayin etmek: Malondialdehit, glutatyon, nitrit, agregasyon cevabi, P-selektin, fibrinojen, apolipoprotein A1, apolipoprotein B ve fosfatidilserin ekspresyonları.

**Yöntem ve Gereçler:** Sağlıklı (n: 9) ve hiperlipidemik (n: 9) olgulardan trombositler elde edildi. Klopidogrelli ve klopidogrel-siz trombositlerde P-selektin, fibrinojen, apolipoprotein A1, apolipoprotein B ve fosfatidilserin ekspresyonları flow sitometre ile tayin edildi. Malondialdehit, glutatyon, agregasyon ve nitrit seviyeleri de tayin edildi.

**Bulgular:** Klopidogrel yokluğunda, hiperlipidemilerde kontrollere göre trombosit agregasyonu, malondialdehit, P-Selektin, fibrinojen ve fosfatidilserin ekspresyonunun başlangıç değerleri yüksek; bununla birlikte nitrit, apolipoprotein A1 ve apolipoprotein B ekspresyonlarının ise daha düşüktü. Her iki grupta, klopidogrel anlamlı düzeyde agregasyonu ve fibrinojen ekspresyonunu azalttı, fakat nitrit seviyelerini artırdı. Klopidogrel sadece hiperlipidemilerde P-selektin ve fosfatidilserin ekspresyonunu ve malondialdehiti azalttı ancak apolipoprotein A1 ve apolipoprotein B ekspresyonlarını artırdı.

**Sonuç:** Görüldüğü üzere klopidogrel bazı yeni *in vitro* antiplatelet etkilere sahiptir. Bu çalışma, trombosit fonksiyonları üzerinde klopidogrelin etkilerine yeni bir bakış açısı sağlayan temel *in vitro* bir çalışmadır. (Turk J Hematol 2010; 27: 99-108)

**Anahtar kelimeler:** Trombositler, hiperlipidemi, klopidogrel, Apolipoprotein A-I, Apolipoprotein B, oksidatif stres, nitrite, fosfatidilserin, P-selektin, Glikoprotein IIb/IIIa

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## Introduction

Atherothrombotic events due to vasoactivity, inflammation and also lipids may affect platelet function. Antiplatelet therapy is one of the most effective therapies for treatment of atherothrombotic and other associated diseases [1-4].

Platelet composition and function abnormalities in patients with hyperlipoproteinemia suggest that the circulating levels of lipids influence the platelet markers such as P-selectin [5]. In fact, it has been reported that increased low-density lipoprotein (LDL), especially oxidized-LDL, reduces platelet and macrophage nitric oxide (NO) synthase expression, and thus platelet activation increases [6]. There is some evidence of these lipoproteins interacting with platelets via specific receptors. It has been reported that there are high-density lipoprotein (HDL) and LDL binding sites on platelets, and those binding sites are reduced in familial hyperlipidemia [7,8]. In our previous study, we had detected that apolipoprotein-A1 (anti-apo-A1) was able to bind to the platelet surface indicating the presence of apo-A1 binding sites on platelets by a flow cytometric method [9].

On the other hand, the expression of P-selectin, fibrinogen, glycoprotein (Gp) IIb/IIIa, and circulating platelet-derived microparticles (PDMPs) on platelets are used as clinical markers for platelet activation status in several disorders including hyperlipidemia [10-13]. Additionally, occurrence of apoptosis-like events in platelets has been confirmed by *in vivo* and *in vitro* studies. Phosphatidylserine (PS) appears on the outer cell membranes during the early stages of apoptosis. PS exposure serves as a procoagulant stimulus and a signal for phagocytic clearance of apoptotic cells including platelets as well as being an activation marker [14-16]. Reactive oxygen species have a number of important pro-atherogenic effects such as oxidation of LDL and activation of platelets [17]. It has been reported that *in vivo* platelet "release reaction" and malondialdehyde (MDA) formation are increased in hyperlipidemic patients [18].

The target of most of the antiplatelet treatments is either platelet agonist receptors or platelet fibrinogen receptor GpIIb/IIIa [19]. Several antiplatelet drugs have been developed to inhibit platelet activity in acute thrombotic events [20]. Clopidogrel, which is a thienopyridine derivative, blocks platelet aggregation irreversibly by binding to P2Y<sub>12</sub>, one of the adenosine diphosphate (ADP) receptors on platelets. Clopidogrel affects GpIIb/IIIa complex activation. Several studies have reported that the essential action of thienopyri-

dine derivatives occurs upon their derivatization in the liver [21-23]. However, various other studies have reported that clopidogrel had different *in vitro* antiplatelet effects on platelets [24-26]. Although those studies have been carried out in different mediums such as whole blood, isolated platelets or platelet-rich plasma (PRP), the consensus of those studies is that hepatic biotransformation is not required for platelet inhibition by clopidogrel, which seems contrary to most of the *in vivo* studies. Considering those studies, our aim was to investigate some new effects of clopidogrel on platelets, which were obtained from healthy and hyperlipidemic volunteers under *in vitro* conditions. The novel *in vitro* effects of clopidogrel on the following parameters were investigated: apo A and apolipoprotein B (apo B) binding sites, expression of P-selectin and fibrinogen, PS exposure on the surface of platelets, aggregation response, MDA, glutathione (GSH) and nitrite levels in platelets. We think that our findings obtained under *in vitro* conditions will be useful in future studies to determine new targets for the drug.

## Materials and Methods

### Subjects and Blood Sampling

The study group consisted of 9 hyperlipidemic (7 males and 2 females) patients and 9 normolipidemic, age- and sex-matched control subjects (6 males and 3 females). This study was performed in accordance with the Declaration of Helsinki. Written evidence of informed consent was obtained from each participant. Table 1 shows some characteristics of the

**Table 1. Some characteristics of the healthy and hyperlipidemic groups**

	Healthy Group	Hyperlipidemic Group
n	9	9
Age, years	46 ±4.65	49±6.96 (NS)
Male/Female	6/3	7/2 (NS)
BMI, kg/m <sup>2</sup>	24.11±3.21	28.55±2.92*
T-CHO, mmol/L	4.66±0.20	7.70±0.36***
LDL-C, mmol/L	2.98±0.24	5.50±0.31***
VLDL-C, mmol/L	0.57±0.22	1.13±0.13***
TG, mmol/L	1.32±0.18	2.44±0.23***
HDL-C, mmol/L	1.51±0.25	0.94±0.14**

BMI: Body mass index; T-CHO: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; VLDL-C: Very low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; TG: Triglyceride; \* p<0.05, \*\* p<0.01, \*\*\* p<0.001, NS: not significant

dyslipidemic group and healthy volunteers. Apo A and apo B levels were within the normal ranges. Exclusion criteria were: secondary hyperlipidemia (renal, liver, thyroid and cardiovascular disease), fasting glucose > 6.1 mmol/L, HbA<sub>1c</sub> (%) > 6, alcohol consumption, smoking, and use of anti-platelet, anti-coagulant or lipid-lowering drugs within the previous eight weeks. All subjects were normotensive (<140/90 mmHg) and fasting glucose levels were in the normal range. The levels of total cholesterol (T-CHO) and triglyceride (TG) were determined by enzymatic methods using test kits with a Hitachi 917 analyzer. HDL-cholesterol (C) was measured by the dextran sulfate-Mg<sup>2+</sup> precipitation method. LDL-C and very low-density lipoprotein (VLDL)-C were calculated by the formula of Friedewald.

Fasting venous blood samples were obtained with a 21-gauge butterfly needle from healthy and hyperlipidemic volunteers. Whole blood was collected into tubes containing 3.2% sodium citrate and used for flow cytometric analysis. PRP was used for aggregation and other analysis.

#### Drug

Clopidogrel was obtained from Sanofi Synthelabo, France. In the reactions, 10 µM clopidogrel was used at a final concentration, which is close to the therapeutic concentration in human plasma reported by Cruz et al. [24].

#### Flow Cytometric Analysis

Expressions of P-selectin and fibrinogen on the surface of platelets were assayed according to the modified flow cytometric analysis method of Shattil et al. [27] as previously described. Briefly, citrated whole blood was diluted (1:10) in phosphate-buffered saline (PBS) (8 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM KCl, 125 mM NaCl, 5 mM glucose and 0.5 g/L albumin) and mixed gently. Whole blood (50 µL) was incubated with clopidogrel (10 µM) for 5 min at 37°C. The following steps were not used so that platelet activation during the assay could be minimized: washing, centrifugation, gel filtration, and mixing vigorously. All blood samples were stimulated with ADP (10 µM) for 5 min. Then, they were added to polypropylene tubes containing the appropriate amounts of fluorescein isothiocyanate (FITC)-labeled antibodies such as anti-CD62P-FITC (to detect platelet activation marker, P-selectin) (Immunotech, Coulter) or anti-fibrinogen-FITC (to detect platelet activation marker, platelet bound fibrinogen) (polyclonal rabbit antihuman fibrinogen-FITC, Dako Cytomation). The samples were incubated for 15 min at room temperature without stirring, and then an equal volume of 0.02 g/ml para-formaldehyde was added to stop the reaction. In parallel experiments, the same procedure was performed without addition of the drug. The samples were further diluted with PBS and kept in the dark at 4°C until flow cytometric measurement.

Diluted whole blood samples (with or without the drug) were stimulated with ADP (10 µM) for 10 min to detect PS exposure (platelet early apoptosis) on the surface of the platelets. It has been reported that PS exposure on the surface of cells is commonly measured by flow cytometry of fluorescently labeled annexin V binding to these procoagulant phospholipids. Annexin V-FITC (25 µg/ml) (Immunotech Coulter) was added to the samples and the tubes were kept on ice for 10 min. Then, 400 µL of ice-cold binding buffer (10 mM HEPES/NaOH (pH 7.4) 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) was added to the blood samples, and flow cytometric analysis was performed.

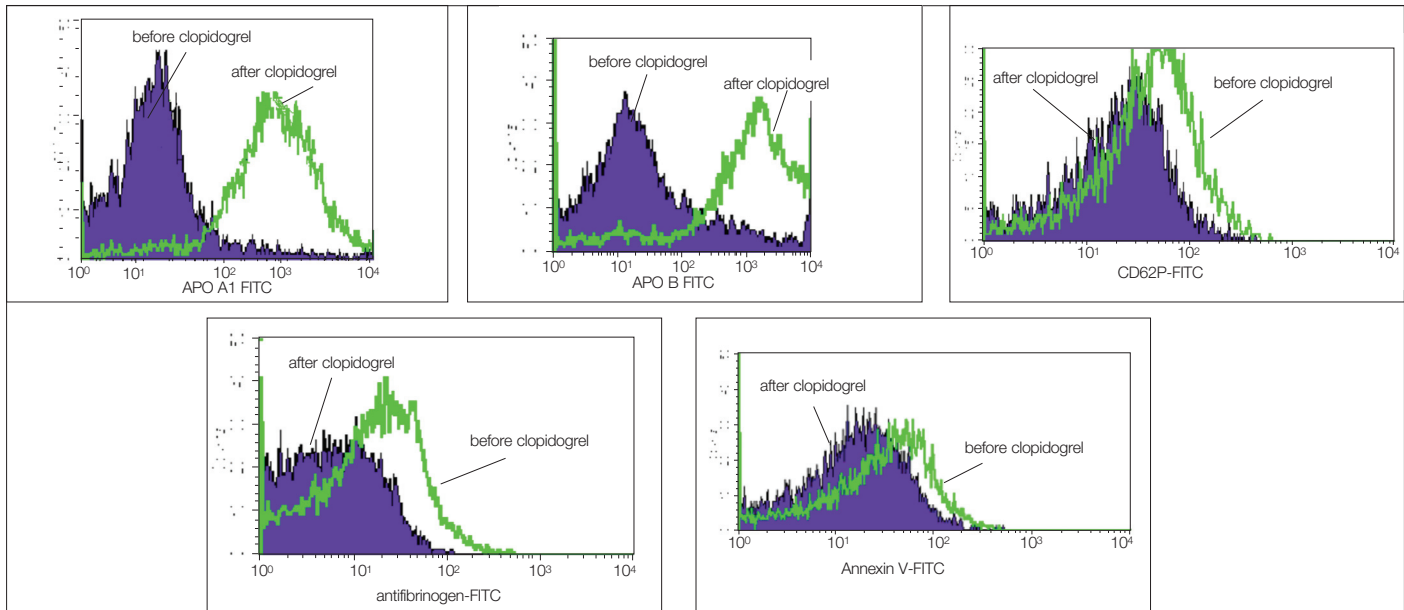
We used the same method in our previous study with a small modification for flow cytometric assay for apo-A1 and apo B, which were bound to the surface of platelets [9]. Diluted whole blood samples (with or without clopidogrel) were stimulated with ADP (10 µM) for 5 min. Monoclonal antibodies (MoAbs) against apo-A1 or apo B were added (1/100 diluted as optimal Moab concentration). After 15 min incubation at room temperature in the dark, 5 µL of FITC-labeled secondary antibody was added to the tubes. Then, the tubes were mixed and incubated for 15 min at room temperature in the dark. The tubes were centrifuged and the cells were washed with PBS two or three times by centrifugation to remove unbound FITC-labeled secondary antibody. Progression of the reaction was stopped by adding an equal volume of 0.02 g/ml para-formaldehyde. The samples were diluted with PBS and kept at 4°C prior to flow cytometric analysis.

All the samples, which were prepared as described above, were analyzed on a Beckman Coulter, EPICS XL-MCL flow cytometer. The flow cytometer was equipped with a 488 nm argon ion laser. The platelet population was identified by forward scatter for cell size and by side scatter for cell granularity. Alignment of the instrument was checked by calibration beads daily. An electronic bitmap was placed around the platelet population and CD41a-FITC was used to form a gate. The results were expressed as the percentage of antibody-positive platelets. Nonspecific and background fluorescence was determined by the use of FITC conjugated immunoglobulin G (IgG). Fifty thousand platelets were counted in each tube.

#### Platelet Aggregation

Platelet-rich plasma and platelet-poor plasma (PPP) were prepared by differential centrifugation. PRP was obtained by centrifugation of citrated whole blood for 8 min at 1500 rpm. PPP was obtained by centrifugation of PRP for 10 min at 3500 rpm.

Clopidogrel was incubated with PRP (500 µL) for 10 min. ADP (Chronolog, final concentration 5 µM) was used to stimulate platelet aggregation. The aggregation responses were assayed on Chronolog-Lumi aggregometer and recorded as the mean percentage of maximum platelet aggregation both with and without clopidogrel.



**Figure 1.** Flow cytometric histograms of antibodies (ApoA, Apo B, CD62P-, antifibrinogen, annexinV) in a hyperlipidemic patient with or without clopidogrel. Mean channel values were used for the evaluation of negative and positive results. An unstained sample and another sample combined with related monoclonal antibodies (without clopidogrel) were used as negative controls. More than 20% shift in channel numbers is regarded as a cut off point for the negative/positive results

#### GSH and MDA Analyses

Platelet-rich plasma (1.5 ml) was incubated with clopidogrel (10  $\mu$ M) at 37°C for 15 min. After centrifugation at 10,000 rpm for 15 min, the platelet pellet was obtained. After washing twice with Tris-NaCl EDTA buffer, the platelet pellet, which was treated either with or without clopidogrel, was suspended in distilled water. It was frozen and thawed four times and then centrifuged at 10,000 rpm for 15 min. The supernatant was used for GSH assay and 15% metaphosphoric acid was added according to the Mergel and Anderman method [28]. The results were expressed as microgram per 10<sup>9</sup> platelets.

The precipitate was solubilized with Tris-NaCl buffer containing 1% Triton X-100 for 8 h and then centrifuged. After solubilization, platelet crude membrane MDA levels were assayed as a product of lipid peroxidation in supernatants with thiobarbituric acid reactive substances (TBARS) method [29]. The results were expressed as nmol/mg crude protein. The protein concentration was determined according to the Bradford method and bovine serum albumin was used as the standard [30]. The sensitivities of GSH and MDA are as follows: GSH: 0.4-100  $\mu$ M, and MDA: 0.1  $\mu$ M.

#### Nitrite Assay

After centrifugation of PRP at 10,000 rpm for 15 min, the platelet pellet was obtained and washed twice with PBS buffer. The washed platelets were incubated with clopidogrel (10  $\mu$ M) and 1.44 mmol/L NADPH for 1 h at 37°C. Then, each sample, treated with or without clopidogrel, was incubated for 1 h at

37°C after the addition of 20 mU nitrate reductase, which reduced nitrate to nitrite. The platelets were frozen and thawed four times. After centrifugation, the supernatant was allowed to react with Griess reagent to form a chromophore; its absorption was measured subsequently at 546 nm. Sodium nitrite (0.2 to 4  $\mu$ M) was used as the standard [6]. The sensitivity of nitrite was 2  $\mu$ M.

#### Statistical Analysis

The results were presented as mean  $\pm$  SD. Mean differences between the healthy and patient groups were calculated with the nonparametric Mann-Whitney U test. Analyses before and after the drug addition were compared using the Wilcoxon signed-rank test. Statistical analyses were performed with SPSS software. P values <0.05 were considered significant.

#### Results

Figure 1 shows Flow cytometric histograms of antibodies (ApoA, Apo B, CD62P-, antifibrinogen, annexinV) in a hyperlipidemic patient with or without clopidogrel. Figure 2A shows the expression of fibrinogen and Figure 2B shows the expression of P-selectin on the ADP-stimulated platelet surface. Before clopidogrel was added, there were significant differences in the expression of both P-selectin (%) and fibrinogen (%) between the hyperlipidemic and the healthy groups. In both groups, the expression of fibrinogen (%) was significantly decreased after

clopidogrel; however, clopidogrel significantly decreased P-selectin expression on platelets only in hyperlipidemics.

Phosphatidylserine exposure with ADP-stimulated platelets is shown in Figure 3. Before clopidogrel, there were significant differences in PS exposure (annexin V) between the hyperlipidemic and the healthy groups. After clopidogrel, platelet PS exposure (annexin V) was significantly decreased in the hyperlipidemic group, but not in the healthy group.

We measured the levels of MDA and GSH in platelets. Figure 4A shows the levels of MDA and Figure 4B shows the levels of GSH before and after clopidogrel in the healthy and hyperlipidemic groups. Before clopidogrel, there were significant differences in the levels of MDA between the hyperlipidemic group and the healthy group, but not in GSH levels. MDA levels significantly decreased after clopidogrel in the hyperlipidemic group. On the other hand, GSH levels remained unchanged after clopidogrel in both groups

Platelet aggregation responses before and after clopidogrel are shown in Figure 5. Before clopidogrel, there were significant differences in the aggregation (%) status between the hyperlipidemic patients and the healthy volunteers. After clopidogrel, platelet aggregation response significantly decreased in both healthy and hyperlipidemic groups (Figure 5A). Figure 5B shows the aggregation responses before and after clopidogrel in the hyperlipidemic group.

Nitrite levels in platelets are shown in Figure 6. Before clopidogrel, there were significant differences in the levels of nitrite between the hyperlipidemic and the healthy groups. In both groups, platelet nitrite levels significantly increased after clopidogrel.

Figure 7A shows the expression of platelet apo-A1 before and after clopidogrel in the hyperlipidemic group and healthy volunteers. Before clopidogrel, there were significant differences in the expressions of apo-A1 and apo B on the platelet surface between the hyperlipidemic patients and the healthy group. The expression of platelet apo-A1 significantly increased after clopidogrel in the hyperlipidemic group, but did not change in the healthy group.

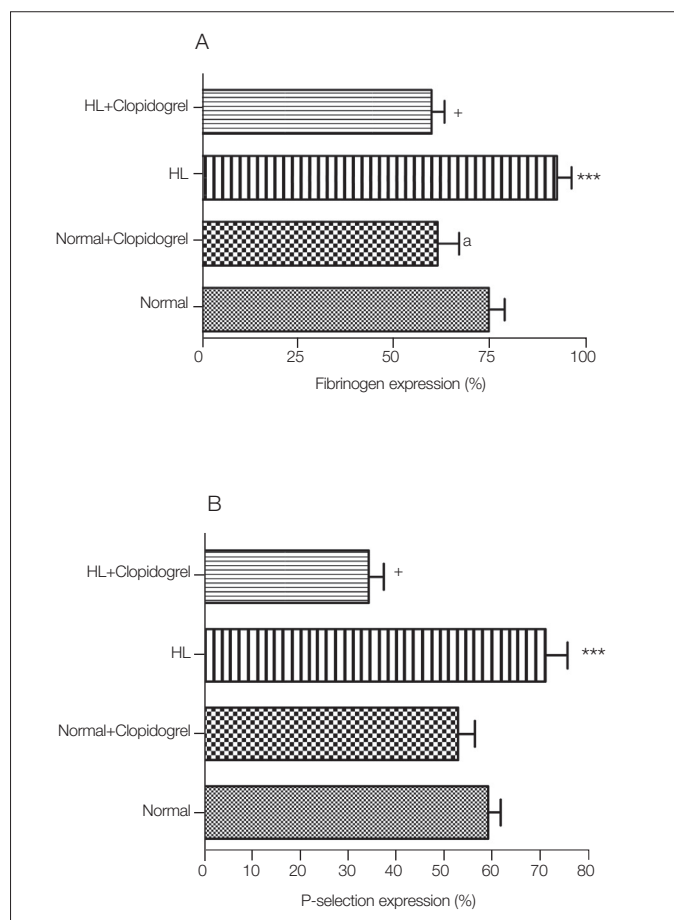
Figure 7B shows the expression of platelet apo B before and after clopidogrel in the hyperlipidemic and healthy groups. The apo B expression on the platelet surface significantly increased after clopidogrel in the hyperlipidemic group, but did not change in the healthy group.

## Discussion

It has been reported that circulating activated platelets and increased thrombotic risk are related to many cardiovascular events such as angioplasty, stroke, diabetes mellitus, and hyperlipidemia [31-34]. In the present study, using a flow cyto-

metric method, we found that both P-selectin and fibrinogen expressions on the surface of platelets were significantly higher in the hyperlipidemic group compared to the healthy group. In our previous study, we had detected that the receptor numbers of GpIIb/IIIa, GpIIIa and P-selectin were significantly higher in hyperlipidemic patients [12]. In our present study, we confirmed our previous findings indicating that platelets are activated in the circulation in hyperlipidemic patients.

In this study, we also observed that PS exposure, lipid peroxidation and aggregation response of platelets were all higher, whereas platelet nitrite levels were lower in hyperlipidemics than in the healthy group. It has been shown that increased PS exposure is an important signal of early apoptosis, platelet activation and procoagulant stimulus [14-16,35,36]. The increase of PS exposure, which we observed in this study, may be related to increased oxidative stress (high MDA levels), because oxidative stress is one of the mediators of apoptosis [37]. Due to the increasing platelet aggregation response and PS exposure, we can speculate that platelets from hyperlipid-



**Figure 2.** Mean values for expressions of fibrinogen (A) and P-selectin (B) in platelets of different study groups.

Normal: Platelets from the healthy group; HL: Platelets from the hyperlipidemic group

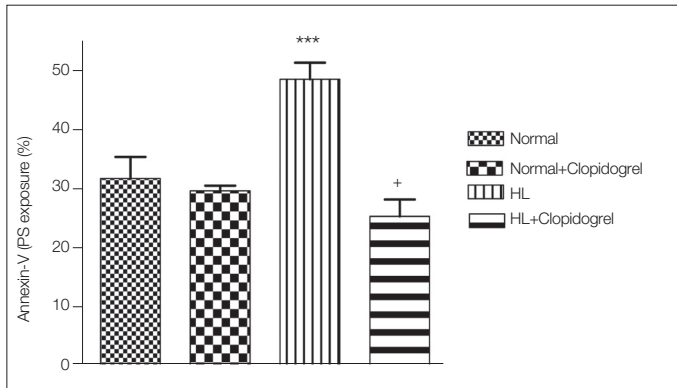
\*\*\* P <0.001 vs normal

a, P <0.01 vs normal

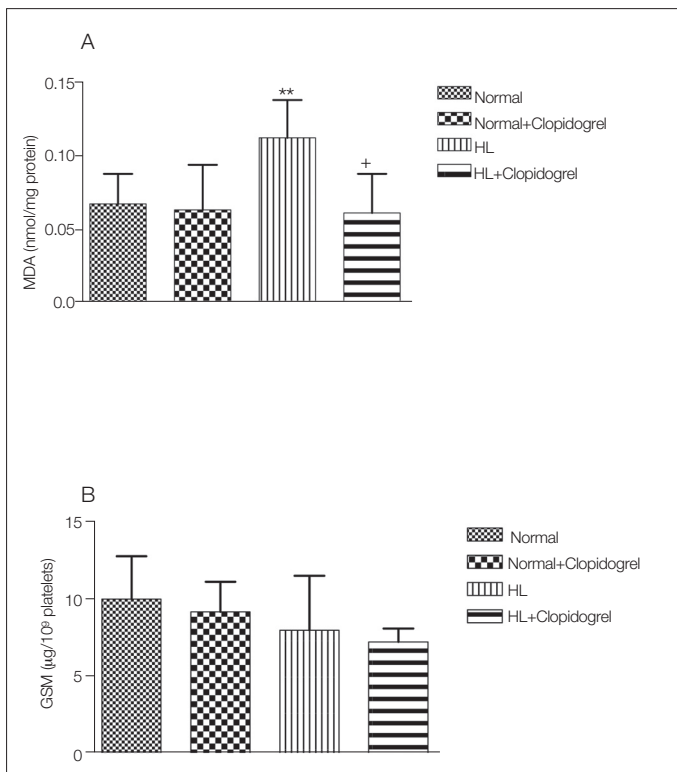
+, P <0.01 vs HL

emic subjects are more susceptible to ADP and the apoptosis-activation process.

In fact, it has been reported that elevated LDL, especially oxidized-LDL, may reduce NO synthase expression in platelets and macrophages. Due to the decrease in NO, platelet activation and cellular production of oxygen radicals

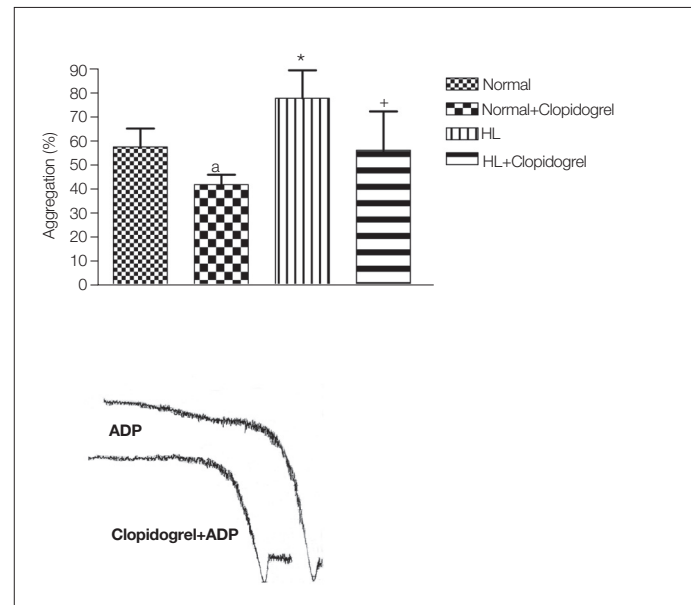


**Figure 3.** Mean values of annexin-V (PS exposure) in platelets of different study groups. Normal: Platelets from the healthy group; HL: Platelets from the hyperlipidemic group  
\*\*\*, P <0.001 vs normal  
+, P <0.01 vs HL

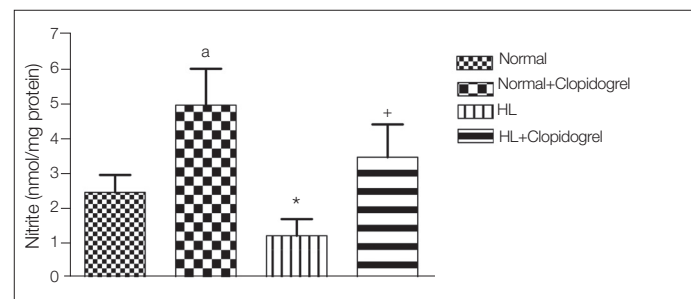


**Figure 4.** Mean MDA (A) and GSH levels (B) in platelets of different study groups. Normal: Platelets from the healthy group; HL: Platelets from the hyperlipidemic group  
\*\* P <0.01 vs normal  
+, P <0.01 vs HL

may increase [38]. The measurement of nitrite levels instead of NO has been used in several studies since it is an indirect indication of NO production. As it is known, when LDL is at high concentrations and when it is together with an agonist such as ADP (as in our study), it triggers platelet hyperactivity and vascular damage as an independent stimulant [39,40]. Therefore, in this study, both elevated plasma LDL levels and platelet low nitrite levels observed in the hyperlipidemic subjects may be some of the factors responsible for the increase in platelet activation, platelet lipid peroxidation and apoptosis. One of the platelet abnormalities, observed in the hyperlipidemic group, is the change in membrane receptor responses. It has been shown that patients with



**Figure 5.** (A) Platelet aggregation responses (%) of the study groups. Normal: Platelets from the healthy group; HL: Platelets from the hyperlipidemic group  
\* P <0.05 vs normal  
a, P <0.01 vs normal  
+, P <0.001 vs HL  
(B) Platelet aggregation plots from the hyperlipidemic group



**Figure 6.** Mean values of nitrite levels in platelets of different study groups. Normal: Platelets from the healthy group; HL: Platelets from the hyperlipidemic group  
\* P <0.05 vs normal  
a, P <0.01 vs normal  
+, P <0.001 vs HL

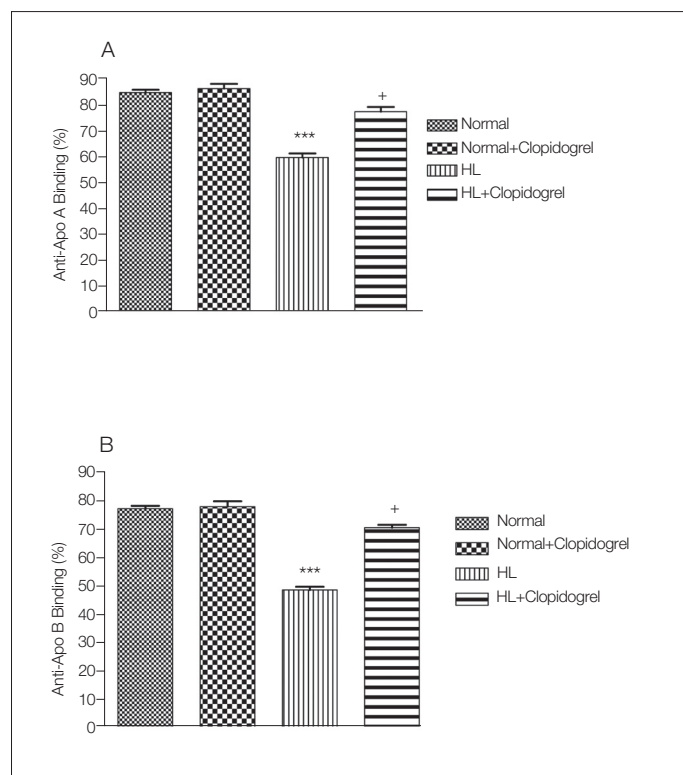
atherosclerosis, diabetes and hyperlipidemia have significantly lower platelet LDL and HDL binding sites [7,8,41]. In our study, expressions of both apo B (also LDL) and apo A (also HDL) on platelets were lower in the hyperlipidemic patients. This is possibly related to the reactivity potential of platelets in the hyperlipidemic group. Based on our findings, the increase in platelet activation, apoptosis, oxidative status (MDA), aggregation, altered platelet membrane binding features, and decreased platelet nitrite levels in hyperlipidemia might elevate thrombotic risk causing atherogenesis to progress.

New risk assessment criteria for thrombosis and cardiovascular diseases [42,43] and new drugs [44,45] for inhibition of coagulation are very important research areas. Clopidogrel is a drug that has been used for the secondary prevention of atherothrombotic events related to ischemia [46]. Although there are many *in vivo* reports related to the effects of clopidogrel on platelet functions, there are also various reports about its *in vitro* effects on platelets [24-26]. In those *in vitro* studies, it was shown that hepatic biotransformation is not required for platelet inhibition by clopidogrel. According to the findings of the study of De La Cruz et

al. [24], clopidogrel *in vitro* reduced ADP-induced platelet aggregation and increased endothelial NO production depending on dose. Arrebola et al. [25] showed that thromboxane B<sub>2</sub> production and collagen-induced platelet aggregation were inhibited, but prostacyclin synthesis did not change following *in vitro* incubation with clopidogrel. Weber et al. [26] reported that the *in vitro* inhibition of platelet aggregation by clopidogrel is selective for ADP and does not require hepatic bioactivation. Regarding their findings, the inhibitory effects of clopidogrel were selective for ADP because no inhibition of platelet aggregation was seen with collagen, thrombin or thromboxane A<sub>2</sub>. Additionally, they have shown that the antiaggregant activity of clopidogrel was associated with the platelets and was not dependent on the presence of the compound in the test buffer. They also discussed that platelets are capable of generating the putative active metabolite of clopidogrel. This possibility is supported by the time-dependence of platelet inhibitory actions of clopidogrel in their study. In rats, Savi et al. [47] reported that clopidogrel (40 mg kg<sup>-1</sup>) was less effective in hepatectomized rats as compared to normal control rats. In addition, clopidogrel did inhibit platelet aggregation in isolated, blood-perfused rat livers.

It seems that our results support some of their findings, but we have also observed some novel *in vitro* effects of clopidogrel. We found that *in vitro* clopidogrel significantly increased platelet nitrite levels in both hyperlipidemic and healthy groups. *In vitro* and *in vivo* studies show that clopidogrel may stimulate NO production via a Ca-dependent way in different cells. Thienopyridines enhance endothelial NO production, which influences vascular wall endothelial function, platelet function and inflammation [48]. In our study, the same mechanism may be responsible for the effects of clopidogrel on nitrite production.

It was reported in a study that there is a significant decrease in fibrinogen binding to GpIIb/IIIa by an aspirin and clopidogrel combination and a reduction in platelet P-selectin expression by clopidogrel alone [49]. Similarly, in our study, in the presence of ADP, clopidogrel reduced the fibrinogen expression on the platelet surface, and thereby, presumably decreased the platelet activation in both groups. On the other hand, *in vitro* addition of clopidogrel to platelets reduced increased P-selectin expression only in hyperlipidemic patients. Storey et al. [50] showed that clopidogrel and P2Y<sub>12</sub> receptor antagonist suppressed platelet aggregation, P-selectin expression and platelet-leukocyte conjugate formation, whereas aspirin had no such inhibitory effect. In our study, we also observed that clopidogrel has reducing effects on PS exposure, MDA levels and platelet aggregation



**Figure 7.** Mean values for expressions of apoA1 (A) and apoB (B) in platelets of different study groups.

Normal: Platelets from the healthy group; HL: Platelets from the hyperlipidemic group

\*\*\* P < 0.001 vs normal

+, P < 0.001 vs HL

response in hyperlipidemic patients. It has been reported that the antiaggregant effect of thienopyridines is more potent when aggregation is induced with ADP than with collagen. Although in this study we have not enlightened the exact mechanism of clopidogrel, we have speculated on the possible mechanisms inspired from *in vitro* and *in vivo* studies. Clopidogrel may block ADP-induced platelet aggregation via G protein activation and inhibition of adenyl cyclase or it may be totally independent from these factors. At the same time, clopidogrel may impair the transmembrane migration of PS, and thus PS exposure reduces.

In conclusion, the present study is a basic *in vitro* study to suggest new insights into the effects of clopidogrel on platelet functions. We observed that platelets from hyperlipidemic subjects may be more susceptible to agonists, activation and apoptosis. Disturbed lipid metabolism in those subjects may lead to physiochemical changes in platelet response and consequently may result in altered expression of surface membrane proteins, apoA and apoB. Those events may contribute to the atherosclerotic process by elevating thrombotic risk in hyperlipidemics. We have shown some novel effects (for example apo A and apo B binding sites on platelets) of clopidogrel under *in vitro* conditions. To our knowledge, there is no *in vivo* study related especially with the effects of clopidogrel on those platelet binding sites. It seems that clopidogrel, which is not subjected to biotransformation, especially affects hyperlipidemic platelets by reducing platelet thrombotic response and by altering membrane specifications. We are hopeful that our findings obtained under *in vitro* conditions can be used in further studies to determine new benefits or side effects of clopidogrel.

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### Conflict of interest

No author of this paper has a conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included in this manuscript.

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