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Lack of influence of the COX inhibitors metamizol and diclofenac on platelet GPIIb/IIIa and P-selectin expression *in vitro*

Dirk Scheinichen*[1], Holger-Andreas Elsner[2], Rodin Osorio[1], Björn Jüttner[1], Werner Gröschel[1], Karsten Jaeger[1] and Siegfried Piepenbrock[1]

Address: [1]Department of Anesthesiology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany and [2]Department of Transfusion Medicine, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

Email: Dirk Scheinichen* - scheinichen.dirk@mh-hannover.de; Holger-Andreas Elsner - elsner.holger-andreas@mh-hannover.de; Rodin Osorio - scheinichen.dirk@mh-hannover.de; Björn Jüttner - juettner.bjoern@mh-hannover.de; Werner Gröschel - groeschel.werner@mh-hannover.de; Karsten Jaeger - KJaeger@sjs-bremen.de; Siegfried Piepenbrock - piepenbrock.siegfried@mh-hannover.de

* Corresponding author

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Abstract

Background: The effect of non-steroidal anti-inflammatory drugs (NSAIDs) for reduced platelet aggregation and thromboxane A_2 synthesis has been well documented. However, the influence on platelet function is not fully explained. Aim of this study was to examine the influence of the COX-1 inhibiting NSAIDs, diclofenac and metamizol on platelet activation and leukocyte-platelet complexes, *in vitro*. Surface expression of GPIIb/IIIa and P-selectin on platelets, and the percentage of platelet-leukocyte complexes were investigated.

Methods: Whole blood was incubated with three different concentrations of diclofenac and metamizol for 5 and 30 minutes, followed by activation with TRAP-6 and ADP. Rates of GPIIb/IIIa and P-selectin expression, and the percentage of platelet-leukocyte complexes were analyzed by a flow-cytometric assay.

Results: There were no significant differences in the expression of GPIIb/IIIa and P-selectin, and in the formation of platelet-leukocyte complexes after activation with ADP and TRAP-6, regarding both the time of incubation and the concentrations of diclofenac and metamizol.

Conclusions: Accordingly, the inhibitory effect of diclofenac and metamizol on platelet aggregation is not related to a reduced surface expression of P-selectin and GPIIb/IIIa on platelets.

Background

Hemostasis is characterized by a close interaction of vascular, plasmatic and cell components. Under physiological conditions, each of these biological systems is in a state of equilibrium. Interferences induced by medical drugs can lead to uncontrollable peri- and postoperative hemorrhage. Platelets act on the process of coagulation in various ways and therefore play a pivotal role in hemostasis. The activation of platelets is associated with a change in the expression of surface antigens, of which P-selectin and the fibrinogen receptor complex GIIb/IIIa are of special importance.

P-selectin is an adhesion molecule which is synthesized by megakaryocytes and incorporated in the platelet α-granules. After stimulation of platelets, P-selectin is rapidly transported to the cell surface by fusion of the α-granule membrane with the plasma membrane. P-selectin is involved in the adhesion of activated platelets to endothelium, monocytes and granulocytes. Findings from previous studies have shown that platelet activation is a potent stimulus for platelet-neutrophil complex formation and neutrophil function [1,2].

Like P-selectin, GPIIb/IIIa is an α-granule membrane protein [3]. Activation increases the number of GPIIb/IIIa complexes expressed on the platelet surface and transforms GPIIb/IIIa complexes to a state that is able to bind to fibrinogen or *von Willebrand* factor [4], which is the prerequisite for platelet aggregation. It becomes increasingly apparent that platelet-leukocyte complexes have an important function. These complexes are mediated by platelet CD62P expression and leukocyte β2 integrins or PSGL-1 and play a prominent role in hemostasis and inflammation [5,6]. Neutrophils with platelets attached have been described to display a more activated pattern of adhesion molecule expression, to produce more superoxide, and to ingest more bacteria than neutrophils not associated with platelets [2]. Accordingly, the percentage of platelet-leukocyte complexes reflects the state of platelet and leukocyte activation.

The pharmacological mechanism of non-steroidal anti-inflammatory drugs (NSAIDs) is the intracellular interaction with the cyclooxygenase (COX), one of the central enzymes in the eicosanoid metabolism. NSAIDs inhibit the synthesis of cyclooxygenase and subsequently lead to a decreased production of prostaglandines [7] and other substances, interfering with platelet activation, e.g. thromboxane A_2 (TXA_2) [8].

There are controversial results regarding the influence of diclofenac on hemostasis [9-11]. For metamizol, a dose-dependent effect on platelet aggregation through the inhibition of TXA_2 synthesis in platelets has been reported [12,13]. To further study the effect of the NSAIDs diclofenac and metamizol on platelet function, we have investigated the surface expression of GPIIb/IIIa and P-selectin as well as the formation of platelet-monocyte/granulocyte complexes, using a flow cytometric technique.

Methods

Following approval of the local Ethics Committee, 3 ml whole blood was collected in sodium citrate disposable blood sampling tubes (0.3 mL, 0.106 mol L^{-1}, Monovette®, Sarstedt, Nümbrecht, Germany) from 20 healthy blood donors. Blood samples were drawn from a peripheral vein, using 18-gauge needles. To keep *ex vivo* platelet activation as low as possible, blood samples were processed within 30 minutes after drawing.

Diclofenac, kindly supplied for experimental purposes by Merck dura (pure pulverized, Merck dura GmbH, Darmstadt, Germany) was added in final concentrations of 150 ng ml^{-1}, 1500 ng ml^{-1} and 3000 ng ml^{-1}. Metamizol (Ratiopharm GmbH, Ulm, Germany) was added in final concentrations of 6 μg ml^{-1}, 60 μg ml^{-1} and 120 μg ml^{-1}. Both drugs were dissolved with phosphate-buffered saline.

IgG isotype controls were applied to detect non-specific staining. Samples were incubated with diclofenac and metamizol at 37°C for 5 and 30 minutes, respectively, with gentle agitation. Controls did not contain drugs. For platelet identification and glycoprotein IIb/IIIa (GPIIb/IIIa) expression changes samples were stained with 6 μl anti-GPIIb/IIIa PE (phycoerythin)-conjugated monoclonal antibody (CD41-PE, clone P2, Beckman-Coulter, Krefeld, Germany). To detect platelet activation and leukocyte-platelet complex formation under basal conditions, samples were stained with 6 μl FITC (fluorescein isothiocyanate) – monoclonal antibody against P-selectin (CD62P-FITC, clone CLB/Thromb/6, Beckman-Coulter) or against leukocytes (CD45-FITC, clone KC56, Beckman-Coulter), respectively. Samples were incubated at 37°C for 10 minutes in the dark. To evaluate platelet reactivity, samples were activated with 6 μM TRAP-6 (Thrombin Receptor Activating Peptide-6, Bachem, Heidelberg, Germany) or 10 μM ADP (Adenosine Diphosphat, Sigma Chemicals, Deisenhofen, Germany) for 10 minutes at 37°C before staining with the monoclonal antibodies. After addition of 2 mL phosphate-buffered saline containing 1% bovine serum albumin, activation was stopped by transferring the sample onto ice.

Flow cytometry analysis

The flow cytometer was equipped with an argon laser with a wave length of 488 nm (Epics XL®, Beckman-Coulter). For each sample a minimum of 25,000 platelets or 15,000 leukocytes were analysed. The green fluorescence emission for CD62P and CD45 was measured by the corresponding photomultiplier (FL 1, 525 nm). The photomultiplier for FL-2 (575 nm) was used for recognition of platelets (CD41-PE). Forward scatter, sideward scatter, FL 1 and FL 2 were assessed using the logarithmic mode. Mean channel fluorescence emission was analyzed in histograms and platelet-leukocyte complexes in dot plots using a PC-software package (EXPO® 2.0, Beckman-Coulter).

Whole blood assays of platelets require correct identification and separation of platelets from erythrocytes or debris. For this reason platelets were identified based on

their high density expression of the specific platelet antigen CD41-PE in the FSC/FL2 dot plot. The platelets were gated by setting a polygonal gate and transferred to a FL-1 (CD62P-FITC) and FL-2 (CD41-PE) histogram. The mean channel expression of FL-1 and FL-2 was used to determine the activation-dependent expression density of GPIIb/IIIa and P-selectin according to TRAP-6 stimulation.

To analyze platelet-leukocyte complexes, leukocytes were marked in a FL1-histogram according to their high CD45 expression. The CD45-positive cells were displayed in a FSC/SSC dot plot. Dependent on their specific FSC and SSC characteristics monocytes and granulocytes were gated by setting a polygonal gate and were individually displayed in FL-2 versus FSC dot plots for analysis of platelet-monocyte and platelet-granulocyte complexes.

Statistical analysis

Histogram data were expressed as the mean channel intensity, aggregates of platelets and leukocytes as the percentages of the respective sub-population (mean ± SD). Differences between the groups were evaluated by the analysis of variance (ANOVA) for repeated measurements. $P < 0.05$ was considered significant.

Results

Influence of metamizol and diclofenac on GPIIb/IIIa receptor expression

Compared to the control (22.9 ± 8.1) the mean fluorescence intensity of GPIIb/IIIa antigen expression on resting platelets after 5 minutes incubation not statistically significant influenced in either concentration (Metamizol: 6 μg ml^{-1}: 23.5 ± 11.5; 60 μg ml^{-1}: 22.5 ± 10.9; 120 μg ml^{-1}: 24.1 ± 12.1; Diclofenac: 150 ng ml^{-1}: 23.1 ± 12.4, 1500 ng ml^{-1}: 22.9 ± 11.9; 3000 ng ml^{-1}: 24.3 ± 12.3). After TRAP-6 stimulation, we noted a non significant increased GPIIb/IIIa expression (49.9 ± 20.5; 48.7 ± 17.7; 49.0 ± 22.3) with metamizol compared to the control (45.3 ± 16.8). ADP stimulation had no effect on GPIIb/IIIa receptor expression (control: 30.7 ± 5.8 vs. 29.9 ± 3.0; 28.5 ± 3.4; 30.1 ± 3.7, respectively). Incubation with diclofenac did not affect GPIIb/IIIa expression after TRAP-6 (41.7 ± 10.7; 44.3 ± 12.0; 42.9 ± 5.2) and ADP (29.0 ± 3.4; 28.3 ± 4.3; 28.6 ± 4.8) activation. These results were independent of the incubation time (Fig. 1, 2).

Influence of metamizol and diclofenac on P-selectin receptor expression

Similar to the results of GPIIb/IIIa, P-selectin expression on resting platelets was not influenced by metamizol (2.0 ± 0.2; 1.8 ± 0.2; 2,1 ± 0.3) and diclofenac (1.9 ± 0.2; 2.1 ± 0.2; 1.7 ± 0.2) compared to control (1.9 ± 0.1). Agonist stimulation with ADP (control: 13.7 ± 0.3 vs. 13.6 ± 0.4; 13.6 ± 0.5; 14.4 ± 0.4) or TRAP-6 (control: 18.4 ± 2.5 vs. 17.7 ± 4.2; 16.2 ± 2.9; 18.7 ± 6.4) had no significant effect on P-selectin following supplementation with metamizol. Comparable results were obtained for diclofenac incubation. Incubation time had no effect on P-selectin expression, either (Fig. 1, 2).

Influence of metamizol and diclofenac on the percentage of platelet-monocyte and -granulocyte complex formation

Metamizol as well as diclofenac did not influence the formation of platelet-monocytes and platelet-granulocyte complexes after activation with both agonists (Fig. 3, 4).

Discussion

In this study whole blood samples were incubated with the NSAIDs diclofenac and metamizol at various concentrations incubation times. The activation of platelets was induced by TRAP-6 and of ADP, respectively. The influence of both NSAIDs on platelet behaviour was examined with regard to the expression of GPIIb/IIIa and P-selectin. Additionally, the formation of complexes between platelets and leukocytes – differentiated in granulocytes and monocytes – was studied. Neither substance showed a substantial influence on receptor expression and formation of complexes.

It is commonly accepted that the main mechanism for the alteration of platelet function caused by NSAIDs is the interference with the eicosanoid metabolism, particularly the inhibition of the enzyme cyclooxygenase (COX). Both substances tested in this study are non-selective COX inhibitors with great analgesic capacity. Despite their frequent clinical usage, few data exist about diclofenac and metamizol regarding their effect on platelet receptor expression and complex formation.

Various *in vitro* and *ex vivo* studies demonstrated a reduction of TXA_2 synthesis and platelet aggregation caused by the interference of diclofenac [15-17] and metamizol [14-19] with COX-1. With this respect we focused on platelet receptor expression rather than platelet aggregation.

ADP initiates a positive feedback reaction by inducing other agonists of platelet aggregation, e.g. TXA_2, which enable a complete activation of platelets. This activation can be observed as a biphasic process using an aggregometer. In this process TXA_2 acts as the substantial mediator of irreversible aggregation. Although such a process of platelet aggregation is unlikely to occur *in vivo*, it can be assumed that the COX-dependent platelet activation by ADP has a greater effect than the stimulation by thrombin or TRAP-6 [20].

Previous studies suggested that ADP-mediated platelet aggregation can be suppressed at least partially by diclofenac [21] and metamizol [22]. By contrast, in our

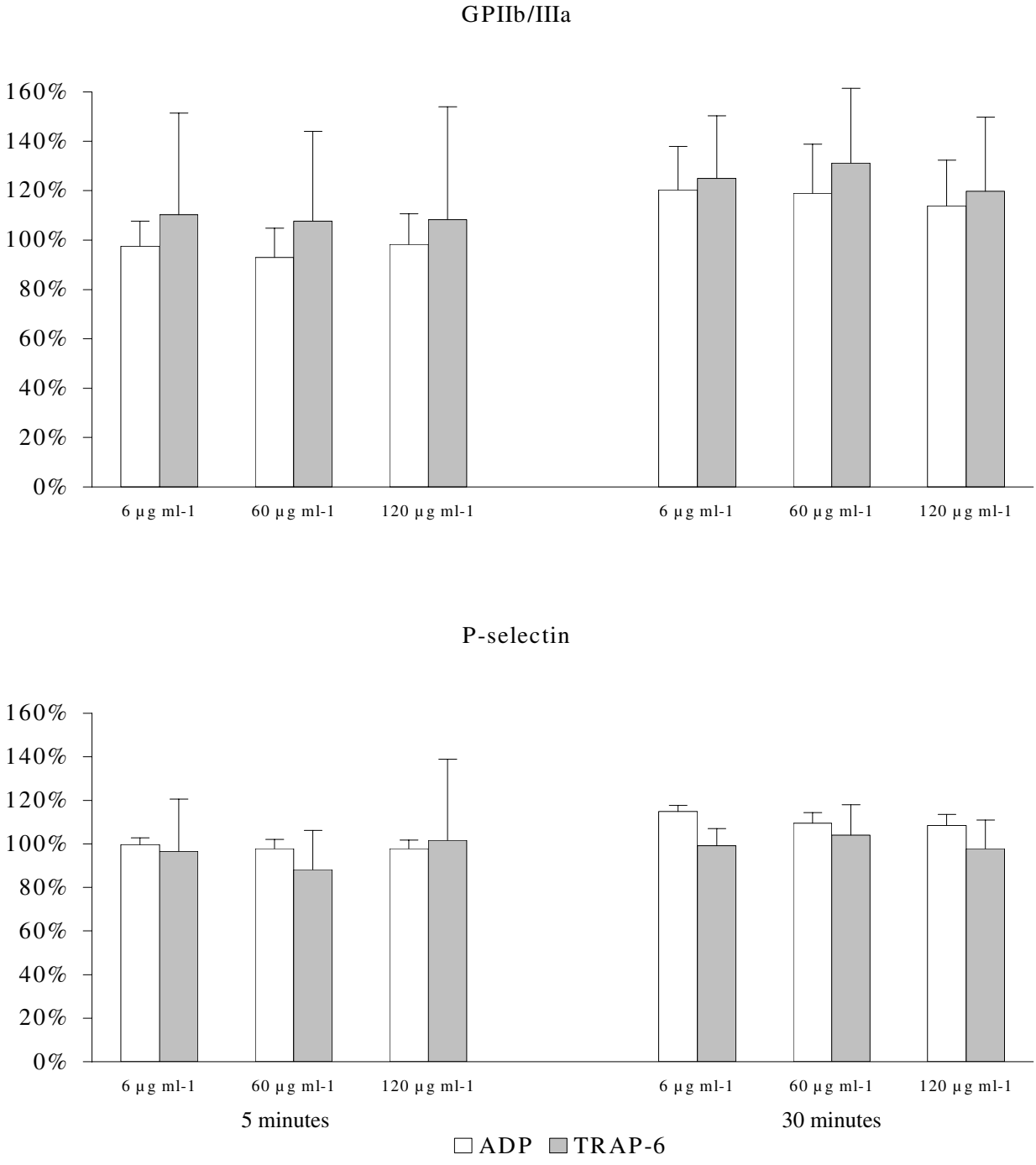

Figure 1
Effects of metamizol after 5 and 30 minutes incubation on the expression of P-selectin and GPIIb/IIIa on the surface of ADP and TRAP-6 activated platelets. Results are given as percentage of change from the control (100%) of the the mean fluorescence intensity (n = 20, mean ± SD).

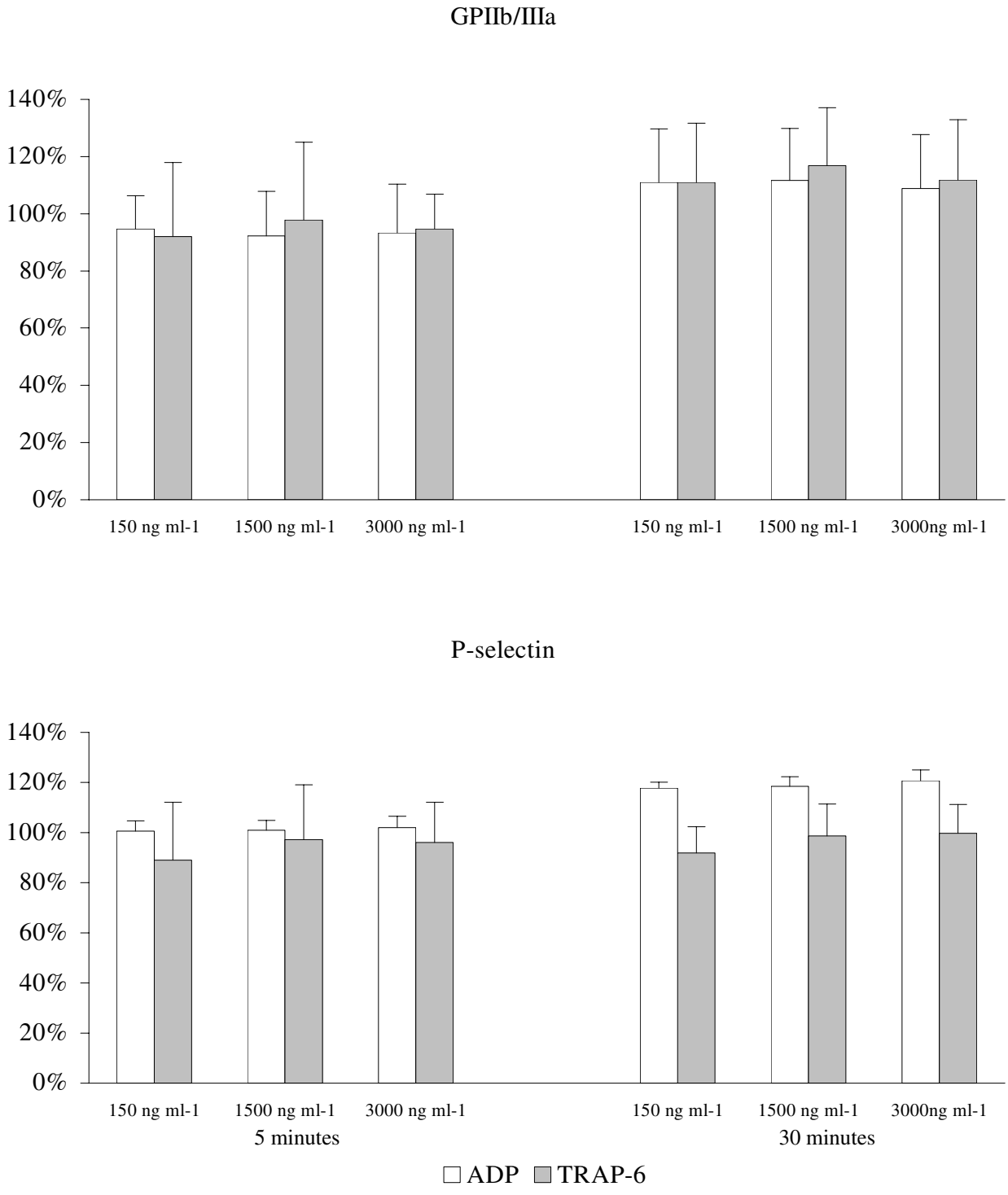

Figure 2
Effects of diclofenac after 5 and 30 minutes incubation on the expression of P-selectin and GPIIb/IIIa on the surface of ADP and TRAP-6 activated platelets. Results are given as percentage of change from the control (100%) of the the mean fluorescence intensity (n = 20, mean ± SD).

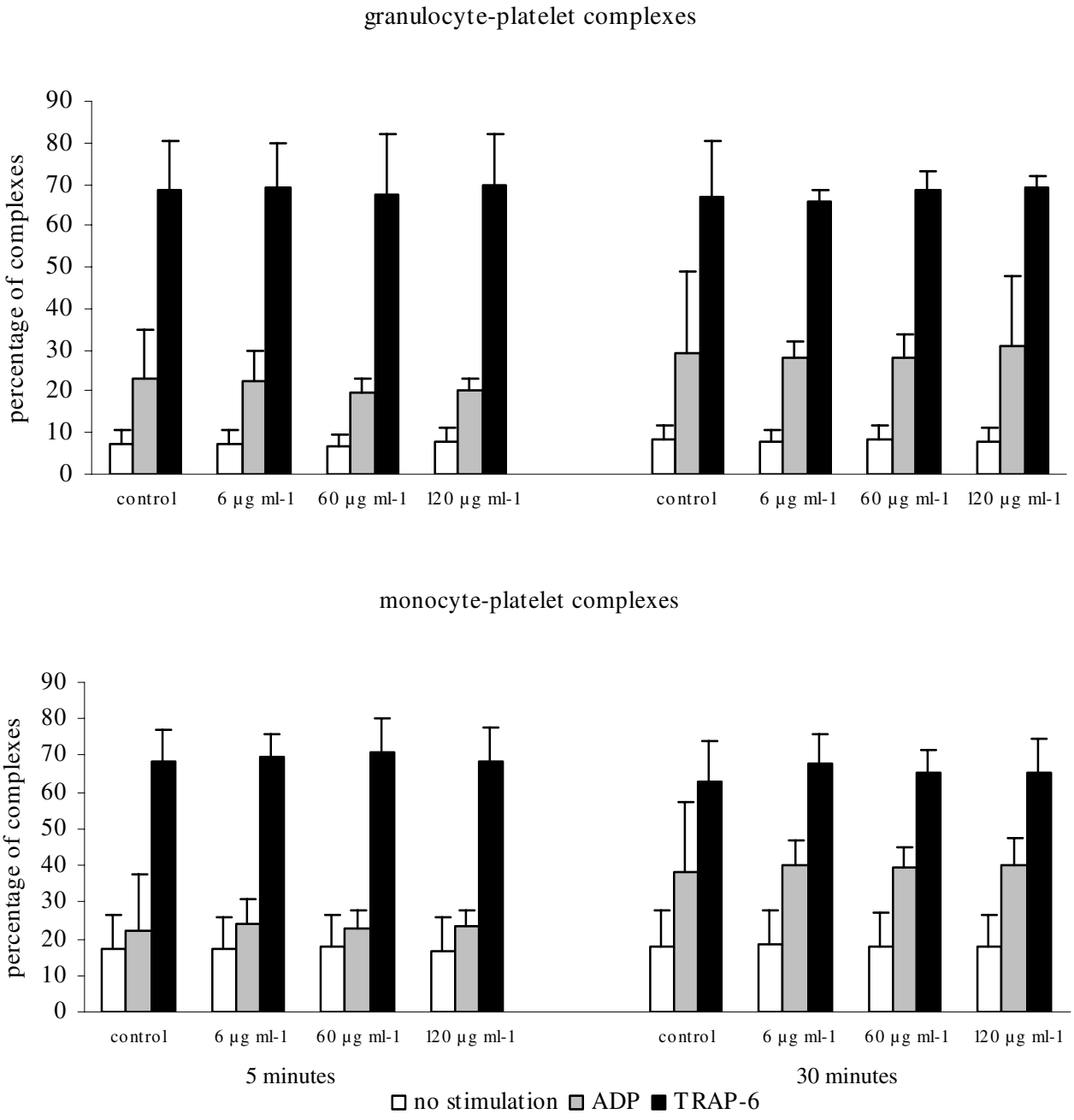

Figure 3
Metamizol effect after 5 and 30 minutes incubation on the percentage of granulocyte- and monocyte-platelet complexes after stimulation with ADP and TRAP-6. Values are expressed as the mean ± SD (n = 20).

study no substantial changes in receptor expression or complex formation, neither by diclofenac or Metamizol, were observed for this type of platelet aggregation. These findings are in accordance with the study of Domínguez-Jiménez et al. [23], who observed no reduction of GPIIb/IIIa expression using diclofenac following ADP stimulation. However, in contrast to our study they used antibodies directed against neoepitopes generated by activation-

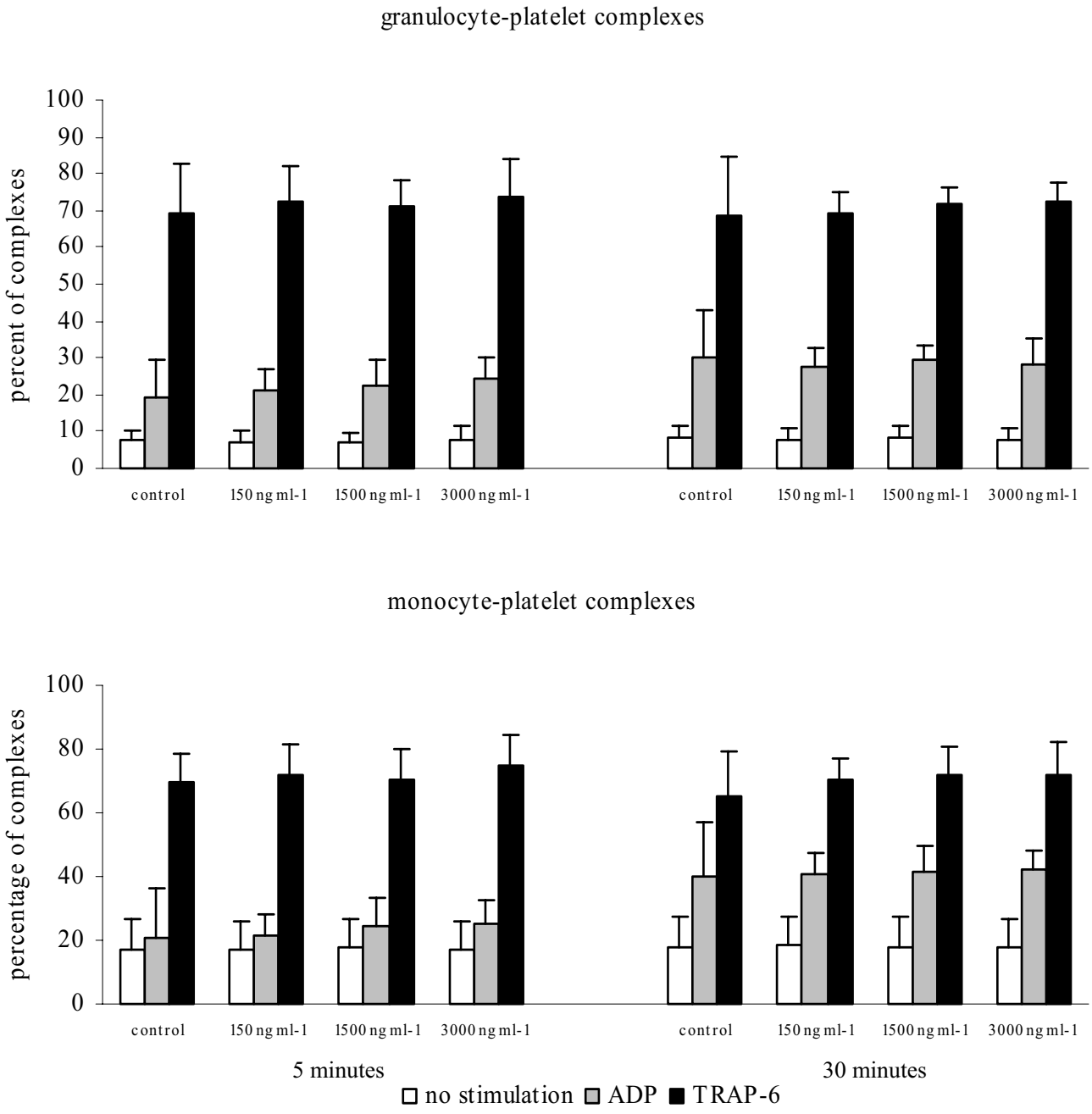

Figure 4
Diclofenac effect after 5 and 30 minutes incubation on the percentage of granulocyte- and monocyte-platelet complexes after stimulation with ADP and TRAP-6. Values are expressed as the mean ± SD (n = 20).

dependent conformational changes in the GPIIb/IIIa complex.

Although statistically not significant, it is apparent that metamizol tends to inhibit P-selectin expression following stimulation by ADP. This effect is basically weak but increases with higher concentrations of metamizol and

with incubation time. However, since higher metamizol concentrations are usually not achievable *in vivo*, this effect might be disregarded in the clinical situation.

The idea of a COX-independent effect on platelets is supported by the study of Andrioli and colleagues [24], who found that diclofenac increases platelet activation probably through the inhibition of the COX pathway, leading to a shift of the arachidonic acid metabolism to the 12-lipoxygenase pathway. Its metabolites 5-, 12-, and 15-hydroxyeicosatetraenoic acid seem to support platelet aggregation [25]. However, the latter effect may weakened by inhibitory effects of 12-hydroxyeicosatetraenoic acid on TXA_2-induced platelet aggregation, as proposed by Fonlupt et al. [26], whereby the dose may be critical for either activating or inhibiting effects [27,28].

In conclusion, our study demonstrates that at low and even at very high doses, diclofenac and metamizol do not lead to impaired platelet aggregation, as measured by platelet-leukocyte adhesion, or expression of GPIIb/IIIa and P-selectin.

Competing interests
None declared.

Authors' contributions
DS and RO initially designed the study. BJ carried out the statistical analysis and interpretation. WG, KJ and RO collecting, processing and measured blood samples. DS, HAE and SP primarily drafted the manuscript. All authors read and approved the final manuscript

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References
1. Peters MJ, Heyderman RS, Hatch DJ, Klein NJ: **Investigation of platelet-neutrophil interactions in whole blood by flow cytometry.** *J Immunol Meth* 1997, **209:**125-135.
2. Peters MJ, Dixon G, Kotowicz KT, Hatch DJ, Heyderman RS, Klein NJ: **Circulating platelet-neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing.** *Br J Haematol* 1999, **106:**391-399.
3. Cramer EM, Savidge GF, Vainchenker W, Berndt MC, Pidard D, Caen JP, Masse JM, Breton-Gorius J: **Alpha-granule pool of glycoprotein IIb-IIIa in normal and pathologic platelets and megakaryocytes.** *Blood* 1990, **75:**1220-1227.
4. Liu YK, Nemoto A, Fang Y, Uemura T: **The binding ability to matrix proteins and the inhibitory effects on cell adhesion of synthetic peptides derived from a conserved sequence of integrins.** *J Biochem* 1997, **121:**961-968.
5. Gawaz M, Dickfeld T, Bogner C, Fateh-Moghadam S, Neumann FJ: **Platelet function in septic multiple organ dysfunction syndrome.** *Intensive Care Med* 1997, **23:**379-385.
6. Evangelista V, Manarini S, Rotondo S, Martelli N, Polischuk R, McGregor JL, de Gaetano G, Cerletti C: **Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: Evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18.** *Blood* 1996, **88:**4183-4194.
7. Vane JR: **The mechanism of action of anti-inflammatory drugs.** In *Advances in eicosanoid research* Edited by: Serhan CN, Perez HD. Berlin Heidelberg: Springer-Verlag; 2000:1-23.
8. Schafer AI: **Effects of nonsteroidal anti-inflammatory therapy on platelets.** *Am J Med* 1999, **106(Supl 5B):**25S-35S.
9. Schmidt A, Bjorkman S, Akeson J: **Preoperative rectal diclofenac versus paracetamol for tonsillectomy: effects on pain and blood loss.** *Acta Anaesthesiol Scand* 2001, **45:**48-52.
10. Perttunen K, Kalso E, Heinonen J, Salo J: **IV diclofenac in post-thoracotomy pain.** *Br J Anaesth* 1992, **68:**474-480.
11. Laitinen J, Nuutinen LS, Puranen J, Ranta P, Salomaki T: **Effect of a non-steroidal anti-inflammatory drug, diclofenac, on haemostasis in patients undergoing total hip replacement.** *Acta Anaesthesiol Scand* 1992, **36:**486-489.
12. Geisslinger G, Peskar BA, Pallapies D, Sittl R, Levy M, Brune K: **The effects on platelet aggregation and prostanoid biosynthesis of two parenteral analgesics: ketorolac tromethamine and dipyrone.** *Thromb Haemost* 1996, **76:**592-597.
13. Eldor A, Zylber-Katz E, Levy M: **The effect of oral administration of dipyrone on the capacity of blood platelets to synthesize thromboxane A2 in man.** *Eur J Clin Pharmacol* 1984, **26:**171-176.
14. Golanski J, Pietrucha T, Baj Z, Greger J, Watala C: **Molecular insight into anticoagulant-induced spontaneous activation of platelets in whole blood: Various anticoagulants are not equal.** *Thromb Res* 1996, **83:**199-216.
15. Failli P, Cecchi E, Tosti-Guerra C, Mugelli A, Laffi G, Zilletti L, Giotti A: **Effect of some cyclooxygenase inhibitors on the increase in guanosine 3':5'-cyclic monophosphate induced by NO-donors in human whole platelets.** *Br J Pharmacol* 1998, **123:**1457-1463.
16. Van Hecken A, Schwartz JI, Depre M, De Lepeleire I, Dallob A, Tanaka W, Wynants K, Buntinx A, Arnout J, Wong PH, Ebel DL, Gertz BJ, De Schepper PJ: **Comparative inhibitory activity of rofecoxib, meloxicam, diclofenac, ibuprofen, and naproxen on COX-2 versus COX-1 in healthy volunteers.** *J Clin Pharmacol* 2000, **40:**1109-1120.
17. Patrignani P, Panara MR, Sciulli MG, Santini G, Renda G, Patrono C: **Differential inhibition of human prostaglandin endoperoxide synthase-1 and -2 by nonsteroidal anti-inflammatory drugs.** *J Physiol Pharmacol* 1997, **48:**623-631.
18. Bozzo J, Escolar G, Hernandez MR, Galan AM, Ordinas A: **Prohemorrhagic potential of dipyrone, ibuprofen, ketorolac, and aspirin: mechanisms associated with blood flow and erythrocyte deformability.** *J Cardiovasc Pharmacol* 2001, **38:**183-190.
19. Abbate R, Pinto S, Gori AM, Paniccia R, Coppo M, Neri Serneri GG: **Activity of dipyrone on intraplatelet arachidonic acid metabolism: an in vitro study.** *Pharmacological Research* 1989, **21:**43-50.
20. Holmsen H: **Significance of testing platelet functions in vitro.** *Eur J Clin Invest* 1994, **24(Suppl 1):**3-8.
21. Cronberg S, Wallmark E, Soderberg I: **Effect on platelet aggregation of oral administration of 10 non-steroidal analgesics to humans.** *Scand J Haematol* 1984, **33:**155-159.
22. Eldor A, Polliack G, Vlodavsky I, Levy M: **Effects of dipyrone on prostaglandin production by human platelets and cultured bovine aortic endothelial cells.** *Thromb Haemostas* 1983, **49:**132-137.
23. Domínguez-Jiménez C, Díaz-González F, González-Álvaro I, Cesar JM, Sánchez-Madrid F: **Prevention of αIIbβ3 activation by non-steroidal anti-inflammatory drugs.** *FEBS Lett* 1999, **446:**318-322.
24. Andrioli G, Lussignoli S, Gaino S, Benoni G, Bellavite P: **Study on paradoxical effects of NSAIDs on platelet activation.** *Inflammation* 1997, **21:**519-530.
25. Nyby MD, Sasaki M, Ideguchi Y, Wynne HE, Hori MT, Berger ME, Golub MS, Brickman AS, Tuck ML: **Platelet lipoxygenase inhibitors attenuate thrombin- and thromboxane mimetic-induced intracellular calcium mobilization and platelet aggregation.** *J Pharmacol Exp Ther* 1996, **278:**503-509.
26. Fonlupt P, Croset M, Lagarde M: **12-HETE inhibits the binding of PGH2/TXA2 receptor ligands in human platelets.** *Thromb Res* 1991, **63:**239-248.
27. Cortelazzo S, Marchetti M, Orlando E, Falanga A, Barbui T, Buchanan MR: **Aspirin increases the bleeding side effects in essential thrombocythemia independent of the cyclooxygenase pathway: role of the lipoxygenase pathway.** *Am J Hematol* 1998, **57:**277-282.

28. Saeed SA, Afzal MN, Shah BH: **Dual effects of nimesulide, a COX-2 inhibitor, in human platelets.** *Life Sciences* 1998, **63:**1835-1841.

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