



## Prevention of platelet-polymorphonuclear leukocyte interactions: new clues to the antithrombotic properties of parnaparin, a low molecular weight heparin

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**Background and Objectives.** Heparin might possess anti-thrombotic properties other than anticoagulation. The aim of the present study was to test the effects of a low-molecular weight heparin, parnaparin, on adhesive molecule-mediated platelet-polymorphonuclear (PMN) leukocyte interactions and on PMN function.

**Design and Methods.** Platelets and PMN were isolated from citrated blood from healthy subjects. Pre-activated platelets incubated with PMN under dynamic conditions formed mixed cell aggregates. In previous experiments PMN were stimulated *in vitro* by purified P-selectin or formyl-methionyl-leucyl-phenylalanine (fMLP). Dual color flow cytometry was used to detect the formation of platelet-PMN mixed cell aggregates, and PMN activation was tested for by measuring L-selectin shedding, tissue factor expression and PMN degranulation. The effect of parnaparin was compared to that of unfractionated heparin.

**Results.** Parnaparin, at a concentration of 0.3-0.8 IUaXa/mL, inhibited the formation of mixed cell aggregates (48.8±9.7% of total PMN population) by up to 60% in a concentration-dependent manner, while heparin inhibited aggregation up to 40%. Parnaparin, (0.3-0.8 IUaXa/mL), prevented L-selectin shedding from PMN, which was induced by purified P-selectin (5 µg/mL) or fMLP (0.5 µmol/L) by 65% and 67%, respectively. Inhibition was independent of incubation time (5-20 min). Parnaparin (0.8 IUaXa/mL) also inhibited tissue factor expression on PMN (% of positive cells), which was induced by P-selectin or fMLP (185±10 and 241±80% of basal value, respectively). Parnaparin protected PMN from degranulation after challenge with either stimulus (>95% inhibition). All the effects of parnaparin were observed with heparin at similar concentrations, although to a lesser extent and were often not significantly different from events in controls.

**Interpretations and Conclusions.** In conclusion, the process of depolymerization of heparin to obtain low molecular weight parnaparin resulted in an increased, anticoagulant-independent effect on PMN function. Thus, the overall anti-thrombotic properties of parnaparin may be partly due to a leukocyte-mediated anti-inflammatory effect.

Key words: low-molecular weight heparin, platelet-PMN interaction, parnaparin, inflammation, selectins.

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Recent studies on the pathogenesis of thrombosis point towards the crucial role of inflammation, particularly inflammatory cells such as leukocytes and their interaction with platelets and vascular cells during thrombogenesis.<sup>1,2</sup>

Epidemiological evidence suggests a positive correlation between the number of leukocytes, in particular polymorphonuclear cells (PMN), and the risk of ischemic vascular disease. Indeed, a higher PMN count has been associated with an increased risk of acute myocardial infarction and its recurrence or transient ischemic attacks. Other studies reported increased *ex vivo* functional responsiveness as well as *in vivo* PMN activation and platelet-leukocyte interactions in different clinical manifestations of ischemic heart disease and suggested an active role of these cells in the progression of vascular

occlusion.<sup>2-7</sup> Platelet-leukocyte aggregates have been observed in peripheral blood from patients with unstable angina, myocardial infarction and other acute coronary syndromes,<sup>8-9</sup> or mechanical heart valve replacement.<sup>10</sup> They have also been considered a predictive index of acute re-occlusion following percutaneous coronary surgery.<sup>7-9</sup> Platelet-leukocyte aggregates have also been found in patients with cancer and positively correlated with thrombotic events.<sup>11</sup> PMN leukocytes may be activated by adhesive molecules of the selectin family,<sup>12</sup> primarily by P-selectin expression on platelets.<sup>13,14</sup> The interaction of P-selectin with its counter-receptor on PMN, P-selectin glycoprotein ligand-1 (PSGL-1), and the consequent adhesion of platelets to PMN results in the activation of leukocytes and the formation of stable platelet-leukocyte

aggregates.<sup>14-17</sup> Several antithrombotic/ antiplatelet drugs have been studied to block the formation of mixed cell aggregates. It was found that blocking the glycoprotein IIb/IIIa receptor by abciximab and tirofiban in acute myocardial infarction only partially prevented the formation of platelet-leukocyte aggregates and the surface expression of Mac-1.<sup>18,19</sup>

Heparin has been shown to modulate the platelet-PMN interaction through several mechanisms: interference with P- and L-selectin-dependent cell adhesion,<sup>20-22</sup> or prevention of platelet activation induced by proteases released from PMN.<sup>23,24</sup> Moreover, heparin and other glycosaminoglycans may inhibit the release of lysosomal enzymes and production of superoxide by activated PMN.<sup>25</sup>

Parnaparin is the sodium salt of a low molecular weight heparin obtained by depolymerization of heparin from porcine intestinal mucosa. Like other low molecular weight heparins it has low hemorrhagic potential while maintaining the antithrombotic activity of unfractionated heparin.<sup>26-28</sup> It is currently used in post-operative prophylaxis of deep vein thrombosis and in the treatment of venous disorders with thrombotic risk. We compared the effects of parnaparin on the formation of platelet-PMN aggregates and PMN activation *in vitro*, with those of unfractionated heparin.

## Design and Methods

### Reagents

Trisodium citrate, NaHCO<sub>3</sub>, KH<sub>2</sub>PO<sub>4</sub>, glucose, CaCl<sub>2</sub>, NaCl, KCl and MgCl<sub>2</sub>·6H<sub>2</sub>O were supplied by Carlo Erba (Milan, Italy). Dextran was supplied by Amersham Biosciences (Milan, Italy). Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), HEPES, thrombin, formyl-methionyl-leucyl-phenylalanine (fMLP) and the fibrinogen-derived peptide Arg-Gly-Asp-Ser (RGDS) were purchased from Sigma (Milan, Italy). The Fix and Perm kit from Caltag was purchased from Valter Occhiena (Turin, Italy), ALEXA-FLUOR<sup>®</sup> 488 Protein Labeling Kit was from Space (Milan, Italy). The IO Test 3 Fixative Solution was provided by Immunotech (IL, Milan, Italy). Unfractionated heparin (sodium heparin 5,000 IU/mL) was supplied by Biologici Italia Laboratories (Novate Milanese, Milan, Italy) and parnaparin (Fluxum<sup>®</sup> 3.200 IUaXA/0.3mL) was supplied by Alfa-Wassermann (Bologna, Italy). Purified P-selectin from human platelets was kindly provided by Dr. Virgilio Evangelista, Consorzio Mario Negri Sud (Santa Maria Imbaro, Italy).

### Monoclonal antibodies

A specific monoclonal antibody (MoAb) against myeloperoxidase from Caltag was purchased from Valter Occhiena; MoAb against human tissue factor

CD142, clone HTF-1 from Pharmingen, was purchased from Becton Dickinson (Milan, Italy); MoAb against CD14, clone RMO52, MoAb against CD45, clone J33, MoAb against the platelet glycoprotein IIIa (CD61), clone SZ21, and against L-selectin (CD62L), clone Dreg 56, were all purchased from Immunotech (IL, Milan, Italy).

## Methods

### Preparation of platelets and PMN

Blood was collected from healthy volunteer blood donors who had not received any medication for at least 2 weeks. Subjects were informed that part of their blood would be collected for research purposes, that their privacy would be protected and were then asked to give their written consent.

Nine parts of blood were mixed with one part 3.8% trisodium citrate as anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 180g for 20 minutes. Washed platelets were prepared from PRP and PMN isolated from the remaining blood by dextran sedimentation followed by Ficoll-Hypaque gradient separation, as previously described.<sup>14,15</sup>

Cells were resuspended in HEPES-Tyrode buffer (pH 7.4) containing 129 mmol/L NaCl, 9.9 mmol/L NaHCO<sub>3</sub>, 2.8 mmol/L KCl, 0.8 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1 mmol/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 5.6 mmol/L glucose, 10 mmol/L HEPES and 1 mmol/L CaCl<sub>2</sub>.

The absence of monocytes (0.02±0.02% of CD14-positive cells; n=25) and platelets (no CD61-positive cells were observed as contaminants in the CD45-positive PMN populations) in the suspensions of purified PMN was confirmed by flow cytometry. PMN viability was tested by trypan blue exclusion: viability was not affected by incubation for 30 minutes with parnaparin or heparin (up to 30 IU aXa/mL). Flow cytometry was also used to rule out the presence of leukocytes (less than 0.001% of CD45-positive cells in CD61-positive platelet populations) in the platelet suspensions.

### Preparation of pre-activated platelets for platelet-PMN mixed aggregate formation

Washed platelets were labeled with a specific MoAb against glycoprotein IIIa (CD61, fluorochrome FITC). The concentrations of MoAb were selected according to the manufacturers' instructions. After washing and labeling, platelets were stimulated with thrombin (0.5 U/mL, 2 min, room temperature), fixed with IO Test 3 Fixative Solution at room temperature for 1 hour and then washed twice. To minimize fibrinogen binding and platelet clumping, platelets were activated in the presence of 800 µmol/L RGDS.<sup>14,15</sup> In these conditions P-selectin was expressed on more than 90% of platelets, as previously reported.<sup>29</sup>

### Mixed platelet-PMN aggregate formation

To a 250  $\mu\text{L}$  suspension containing  $2 \times 10^8$  pre-activated platelets/mL (prepared as described above), vehicle (saline solution) and parnaparin or unfractionated heparin were added immediately before 250  $\mu\text{L}$  of a PMN suspension ( $10^7$  untreated PMN/mL), to obtain final cellular concentrations of  $1 \times 10^8$  platelets/mL and  $5 \times 10^6$  PMN/mL, corresponding to a platelet:PMN ratio of 20:1. The cells were incubated in siliconized glass tubes and the tubes were placed in an aggregometer (Chrono-Log, Mascia Brunelli, Milan, Italy) at  $37^\circ\text{C}$  with constant stirring (1,000 rpm) for 5 minutes. Reactions were stopped by adding equal volumes of ice-cold 2x fixing solution (IO Test 3 Fixative Solution). Samples were kept at  $4^\circ\text{C}$  in the dark and analyzed by flow cytometry within 18 hours.<sup>10</sup>

### Evaluation of PMN activation

All PMN were labeled with a MoAb against CD45 and with the specific MoAb for the markers of leukocyte activation (L-selectin or tissue factor). After 5 minutes' incubation at  $4^\circ\text{C}$  with vehicle (saline solution) and parnaparin or heparin, labeled PMN suspensions were placed in aggregometer cuvettes (as described above) for 1 minute and stimulated with 5  $\mu\text{g}/\text{mL}$  of purified P-selectin or 0.5  $\mu\text{mol}/\text{L}$  of fMLP. Reactions were stopped after 3 minutes by adding an equal volume of ice-cold 2x fixing solution. The experiment was repeated with parnaparin/heparin incubation periods of 0, 5, 10 and 20 minutes, to test the effect of incubation time.

### PMN degranulation

Intracellular myeloperoxidase content was evaluated as a marker of PMN activation/degranulation. After preincubation with parnaparin or heparin and stimulation with purified P-selectin or fMLP (as described for PMN activation), CD45-labeled PMN were treated with the Fix and Perm kit according to the manufacturer's instructions and labeled with a MoAb against myeloperoxidase. The myeloperoxidase-positive PMN were evaluated by flow cytometry.

### Binding of parnaparin and heparin

Parnaparin and heparin were treated with the ALEXA-FLUOR<sup>®</sup> 488 Protein Labeling Kit according to the manufacturer's instructions. The activity of fluoresceinated parnaparin and heparin was verified by standard coagulation tests. CD45-labeled PMN were then incubated with 0.5 IUaXa/mL of fluorescent heparins for 3 minutes and after fixation, the amount of fluorescence associated with cells (equivalent to parnaparin or heparin binding) was evaluated by flow cytometry.

### Analyses by flow cytometry

Flow cytometric analysis was performed with an EPICSXL-MCL flow cytometer (Beckman Coulter, IL, Milan, Italy). PMN were characterized by a combination of size evaluation and leukocyte specific marker CD45 (PC5) gating. Contamination with monocytes was excluded using the monocyte-specific marker CD14 (PE). Mixed cell aggregates were detected as the presence of platelet CD61-positive fluorescence in 10,000 CD45-positive PMN. Results were expressed as the percentage of CD45 cells with positive CD61 fluorescence, representing the percentage of PMN with at least one adhering platelet. PMN activation, determined by the presence of L-selectin, tissue factor and myeloperoxidase, as well as the binding of fluorescent parnaparin and heparin to PMN were evaluated in 5,000-10,000 CD45-positive events. Arbitrary units of FITC fluorescence were recorded, as was the percentage of FITC-positive PMN observed in the gated population.

### Statistical analysis

Results are reported as means and SEM derived from the number of replications stated in the results. Statistical analysis was performed by ANOVA and Dunnett or Tukey tests.

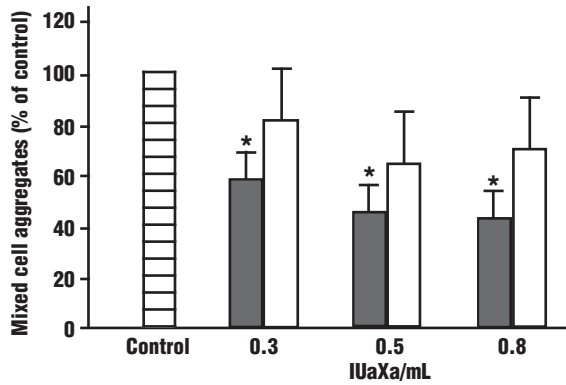
## Results

### Platelet-PMN mixed cell aggregates

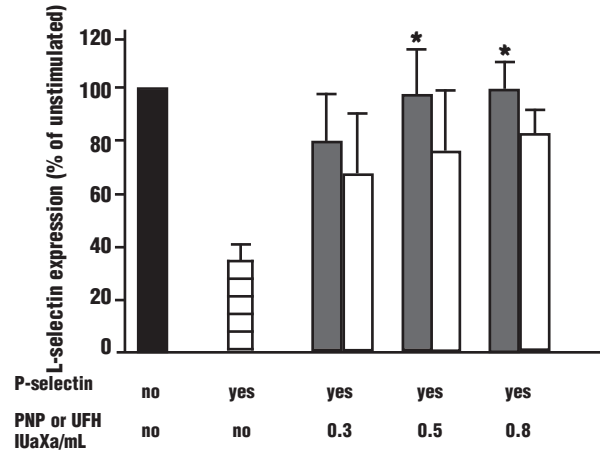
To determine the effect of parnaparin on the formation of mixed cell aggregates, pre-activated platelets were added to unstimulated PMN in the presence of vehicle and parnaparin or heparin. The results, reported in Figure 1, indicate that parnaparin (0.3-0.8 IUaXa/mL) statistically significantly reduced the formation of mixed cell aggregates in a concentration-dependent manner. Heparin only partially reduced aggregate formation and the difference was not statistically significant.

### L-selectin expression on the surface of PMN

Figure 2 shows that PMN activation induced by purified P-selectin reduced L-selectin expression on PMN by more than 60%. This effect was inhibited by parnaparin in a concentration-dependent manner, up to almost complete inhibition at 0.8 IUaXa/mL. A similar, though not statistically significant effect was obtained with heparin (Figure 2). The effect of parnaparin was not time-dependent (0, 5, 10 and 20 min) at any concentration used (*data not shown*). The shedding of L-selectin from the surface of PMN was also tested by stimulating PMN with the bacterial-derived peptide fMLP, which induced a marked decrease in L-selectin on the cell surface (more than 50%). All concentrations of parnaparin tested significantly reduced



**Figure 1.** Formation of platelet-PMN mixed cell aggregates. Vehicle (horizontal-lines column), parnaparin (gray columns) or heparin (white columns) were added to the suspension of pre-activated platelets immediately before PMN. The reaction was stopped after 5 minutes' incubation under dynamic conditions at 37°C. The formation of platelet-PMN mixed aggregates was evaluated by flow cytometry. Data are reported as percent of control values (in the absence of drug), which corresponded to 48.8±9.7% of platelet-PMN aggregates; \**p*<0.05 significantly different from control, by one way ANOVA-Dunnett's test. Means and SEM, n=3-5.



**Figure 2.** L-selectin expression on PMN, activated by purified P-selectin. PMN were incubated with vehicle (control, horizontal-lines column), parnaparin (PNP, gray columns) or unfractionated heparin (UFH, white columns) for 5 minutes before stimulation by purified P-selectin (5 µg/mL). The expression of L-selectin on the PMN surface was evaluated by flow cytometry. Data are reported as the percent of L-selectin observed on unstimulated PMN (black column), corresponding to 33.1 ± 8.7 % (n=12). \**p*<0.05 significantly different from control, by one way ANOVA-Dunnett's test. Means and SEM, n=5-12.

the fMLP-induced decrease in L-selectin and complete inhibition was achieved at 0.8 IUaXa/mL. Heparin only partially inhibited this reduction, and the difference was not statistically significant (Figure 3).

### PMN degranulation induced by P-selectin

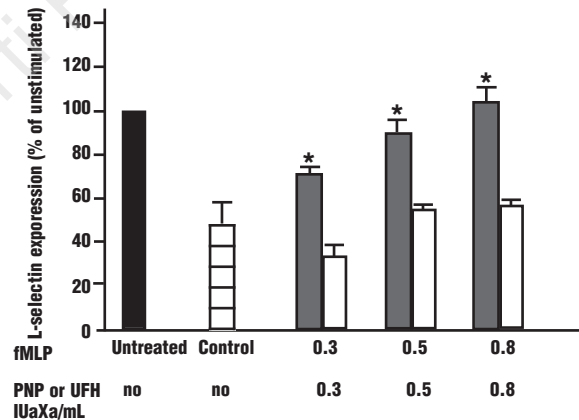
Activation was also evaluated by determining the myeloperoxidase content of the PMN, as an index of PMN degranulation. Purified P-selectin induced degranulation of PMN by more than 95%, an extent similar to that induced by 0.5 µmol/L fMLP (residual myeloperoxidase content: 4.5±3.2% of basal; n=3). Both parnaparin and heparin prevented purified P-selectin-induced PMN degranulation in a concentration-dependent manner, but parnaparin produced a greater effect than heparin at the two lower concentrations (Figure 4).

### Tissue factor expression

Parnaparin reduced the percentage of PMN expressing tissue factor after 3 minutes' incubation in dynamic conditions with no stimuli from 4.0 ± 0.9 to 1.6 ± 0.4%. The inhibitory effect of parnaparin was even more pronounced when PMN had undergone prior stimulation by either purified P-selectin or fMLP (Figure 5).

### Binding of parnaparin and heparin

Fluorochrome Alexa-Fluor® 488-labeled parnaparin and heparin (0.5 IUaXa/mL) rapidly bound to PMN in suspension. The amount of fluorescence associated with PMN after 3-minutes' incubation was 98.7±0.3% and 99.7±0.3% (n=3) of the total PMN, respectively. Fluorescence was detected by flow cytometry.

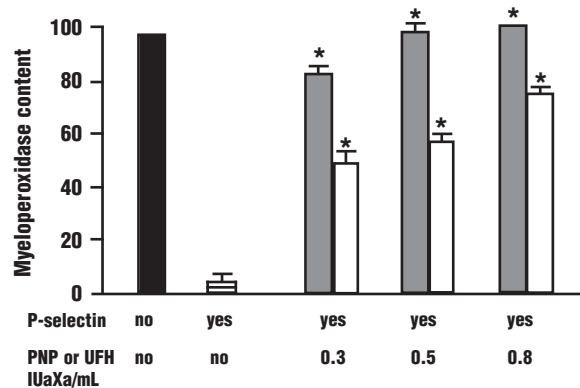


**Figure 3.** L-selectin expression on PMN, activated by fMLP. PMN were incubated with vehicle (control, horizontal-lines column), parnaparin (PNP, gray columns) or unfractionated heparin (UFH, white columns) for 5 minutes before stimulation by fMLP (0.5 µmol/L). The expression of L-selectin on PMN surface was evaluated by flow cytometry. Data are reported as the percent of L-selectin observed on unstimulated PMN (black column), corresponding to 48.3 ± 11 % (n=6). \**p*<0.05 significantly different from control, by one way ANOVA-Dunnett's test. Means and SEM; n=3-6.

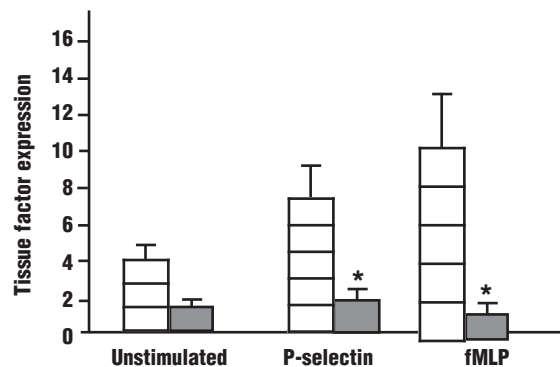
## Discussion

We report here that parnaparin, a low molecular weight heparin, is able to modify PMN leukocyte activation in a similar and even more effective way than unfractionated heparin. Parnaparin was used *in*





**Figure 4.** Intragranular myeloperoxidase content of PMN stimulated with purified P-selectin. PMN were incubated for 5 minutes in the absence (horizontal-lines column) or presence of parnaparin (PNP, gray columns) or unfractionated heparin (UFH, white columns) and stimulated for 3 min by purified P-selectin (5 µg/mL), then fixed and permeabilized. Data are expressed as percent of myeloperoxidase-positive PMN (means and SEM, n=3). \*p<0.05 significantly different from controls by the ANOVA-Tukey test; the effects of parnaparin (at 0.3 and 0.5 IUaXa/mL) there also significantly different from those of the same concentrations of unfractionated heparin.



**Figure 5.** Tissue factor expression on PMN. PMN suspensions were incubated for 3 minutes with 0.8 IUaXa/mL parnaparin (gray columns) or without parnaparin (horizontal-lines columns) and unstimulated or stimulated by purified P-selectin (5 µg/mL) or fMLP (0.5 µmol/L) for 3 min. The percent of tissue factor-positive PMN was evaluated by flow cytometry and reported as a mean and SEM, n=4. \*p<0.05 significantly different from control group by paired t test.

*vitro* at concentrations comparable to those obtained in plasma during anticoagulant treatment.<sup>27</sup>

Heparin has been used as an anticoagulant and antithrombotic agent for more than 60 years. Its anti-inflammatory effect has been investigated and primarily attributed to the blockade of P- and L-selectins.<sup>20-22</sup> In this context, we tested the effect of both heparin and parnaparin on platelet-PMN leukocyte interactions mediated by P-selectin. Thrombin-preactivated, fixed platelets were used as a source of P-selectin; resting PMN were able to bind to these platelets. In these conditions parnaparin significantly reduced the formation of mixed cell aggregates and did so more efficiently than the corresponding concentrations of unfractionated heparin.

Parnaparin also prevented PMN activation induced by purified P-selectin, measured as L-selectin shedding, degranulation and tissue factor expression. In view of the reported effects of heparin on selectin-mediated adhesion of leukocytes,<sup>22</sup> it is reasonable to speculate that parnaparin may also down-regulate the interaction of P-selectin and PMN. However, other mechanisms cannot be ruled out as both drugs also exerted an inhibitory effect on PMN activation triggered by the chemotactic bacterial peptide fMLP. This is in agreement with previous reports showing that heparin and related molecules are able to prevent elastase release induced by fMLP,<sup>30</sup> a strong inducer of L-selectin shedding from PMN<sup>31-33</sup> via mechanisms involving the protein kinase C pathway.<sup>33</sup> The inhibitory effect of parnaparin and heparin on fMLP-induced L-selectin shedding may be explained by the

reported ability of heparin and its structural analogs to interact with CXC-like receptors, such as chemokine receptors, and to negatively modulate the signaling and intracellular cascade of events mediated by inositol triphosphate.<sup>34-37</sup> L-selectin shedding could also be due to ADAM-17 proteolytic cleavage.<sup>31</sup> In this case, the anti-protease activity of heparin<sup>23,24</sup> could play a role in the protective effect of the drug.

The relatively greater effects of parnaparin with respect to heparin could be due to chemico-physical differences between the two molecules. Indeed, parnaparin has a higher negative charge, corresponding to a greater content of negatively charged sulphate groups per unit of specific anti-Xa activity, than that of unfractionated heparin.<sup>26,27</sup> In fact, the ratio of SO<sub>3</sub>/COO<sup>-</sup> is 2.27 for parnaparin and 2 for unfractionated heparin (with specific biological activity of 89,66 and 202 IUaXa/mg for parnaparin and unfractionated heparin, respectively).<sup>38</sup> This is consistent with previous observations that the efficiency of different preparations of heparin in preventing selectin adhesive interactions was found to depend on the replacement of carboxylic groups with sulphate groups.<sup>21</sup> In contrast, when the carboxyl group was reduced to an alcohol group, no blocking activity was observed.<sup>21</sup> It can be hypothesized that the different steric hindrance due to the smaller mass of the molecule may play a role in the greater anti-adhesive properties of parnaparin than unfractionated heparin. It is accepted that PMN participate in different steps of inflammation-related thrombogenesis,<sup>39</sup> inducing platelet and endothelium activation,<sup>40,41</sup> as well as in plasma hemostasis, promoting

prothrombinase and fibrin formation.<sup>42</sup>

Upon activation PMN can express biologically active tissue factor on their surface.<sup>43,44</sup> We report here as a preliminary finding that parnaparin was effective in preventing tissue factor expression induced by either purified P-selectin or fMLP, thus reducing the potential prothrombotic role of these inflammatory cells.

In conclusion, parnaparin significantly reduced the formation of P-selectin-dependent platelet-PMN aggregates and prevented purified P-selectin- or fMLP-dependent PMN activation. The effects of parnaparin were concentration-dependent and stronger than those of unfractionated heparin.

Further studies are now required to assess the efficacy of parnaparin on platelet-PMN interactions *in vivo* and the possible clinical benefit of this heparin on the inflammatory component of thrombotic disease.

*NM and CC conceived, designed and executed the experiments, analyzed the data and drafted the manuscript. MB, MBD and GdG contributed to the discussion of data and revised the manuscript. GdG gave final approval for its submission for publication.*

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