ACTIVATED PLATELETS AND PLATELET-DERIVED MICROPARTICLES AS POTENT STIMULATORS OF NEUTROPHIL EXTRACELLULAR TRAPs FORMATION

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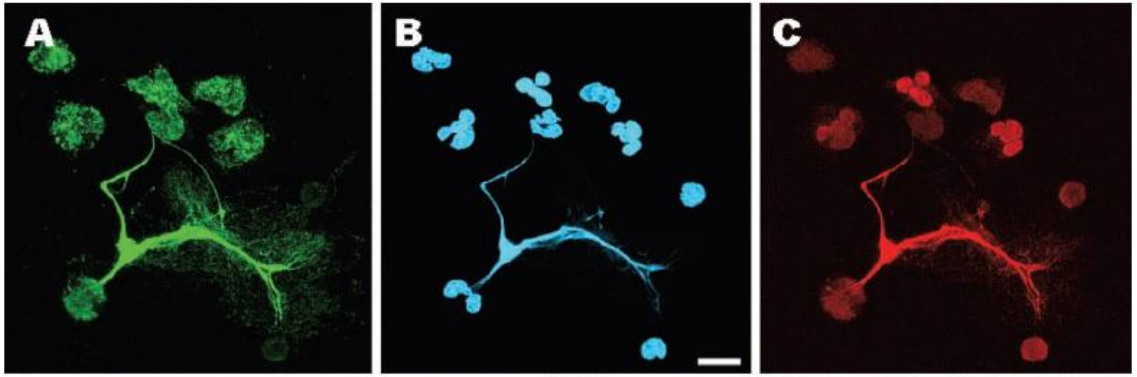
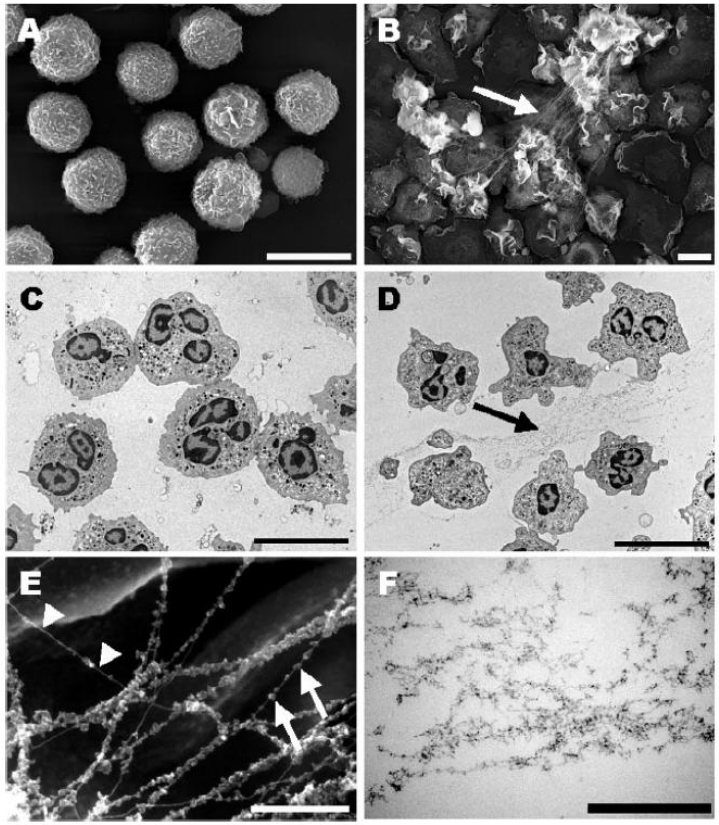
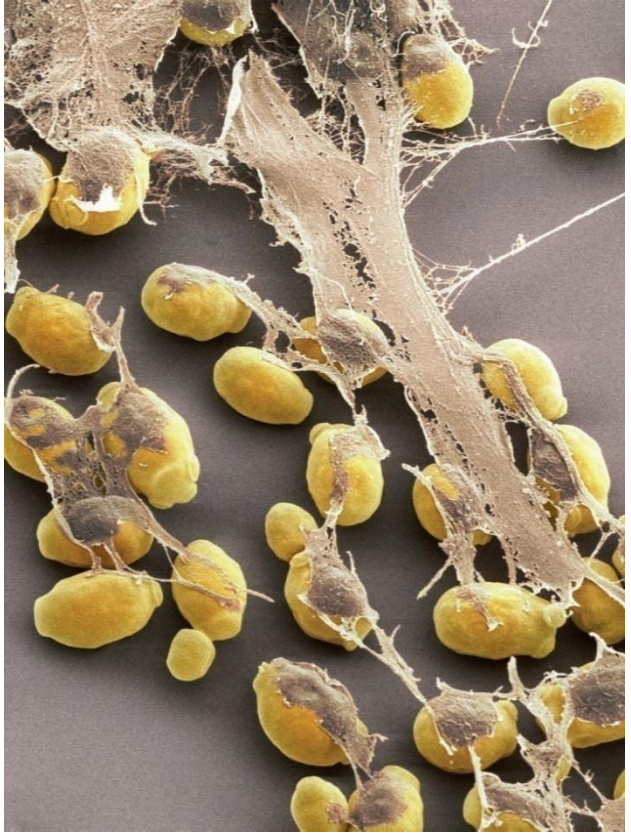
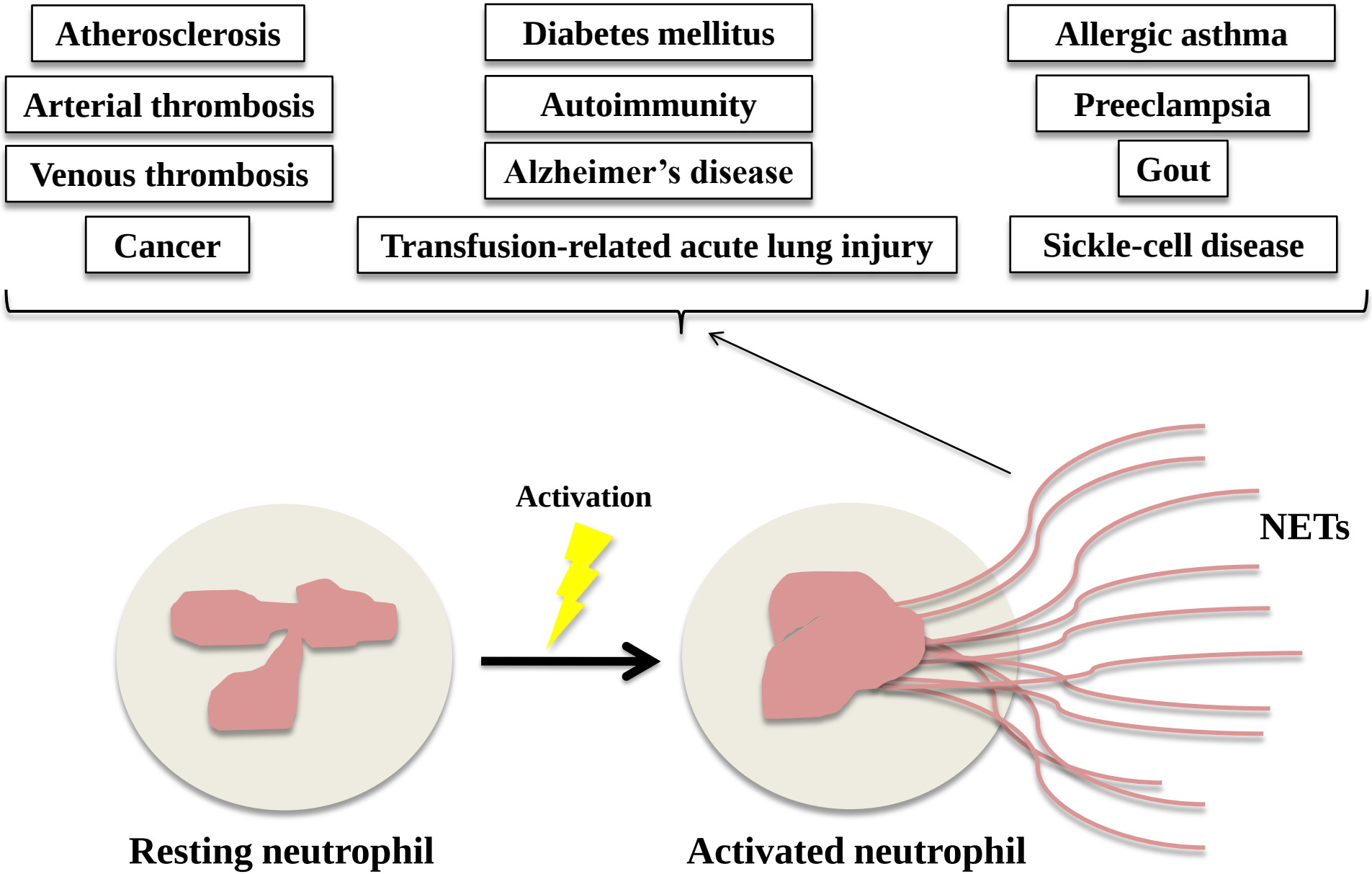


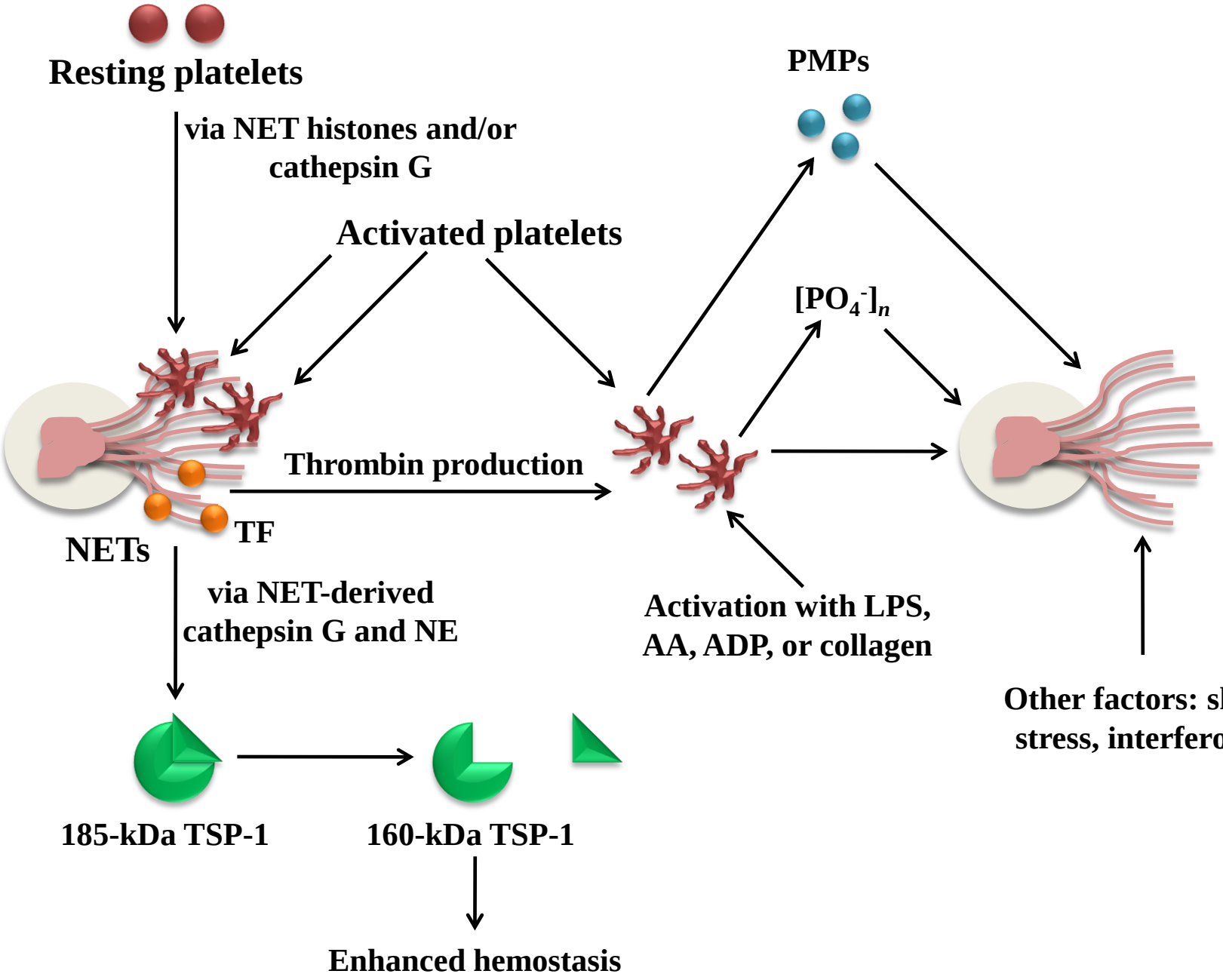




Brinkmann V *et al*. *Science* 2004; **303**: 1532-5 and Nahrendorf N *et al*. *Science* 2015; **349**: 237-9



Slightly modified from Moschonas IC *et al*. *Atherosclerosis* 2019; **submitted**



Moschonas IC *et al*. *Atherosclerosis* 2019; **submitted**

Materials and methods I

- Isolation of neutrophils with double-ficoll gradient, followed by resuspension with RPMI 1640 medium, supplemented with 2% HI human serum

- Preparation of platelet-rich plasma (PRP; 250,000 platelets/μL)

- Addition of neutrophils (200,000) to 24-well plates, in which poly-L-lysine coverslips were pre-placed

- Pre-activation of PRP with 20 μM ADP in an aggregometer for 5 min under stirring conditions (1,200 RPM)

- Addition of PRP to neutrophils at a physiological platelet/neutrophil number ratio, incubation at 37°C and 5% CO_2

- Alternatively, addition of PRP to neutrophils at a physiological platelet/neutrophil number ratio, then activation of PRP with 50 μM ADP *in situ*, incubation at 37°C and 5% CO_2

Materials and methods II

- Preparation of washed platelets (250,000/μL)

- Stimulation with 0.2 U/mL thrombin for 30 min, under stirring conditions (500-600 RPM)

- Centrifugation at 1,000 × *g* for 15 min at RT

- Overlay of PMPs-rich supernatant on 20% sucrose, followed by centrifugation at 2,000 × *g* for 10 min at RT

- Ultracentrifugation at 100,000 × *g* for 2 h at 4°C

- Resuspension with 10 mM PBS

- Quantitative determination of the PMPs protein content using the BCA assay

- Qualitative determination using annexin V-FITC, anti-CD62P-PE and anti-CD61-PerCP

- Addition of 1-60 μg/mL PMPs to neutrophils (200,000) attached to poly-L-lysine coverslips, incubation for 3.5 h at 37°C and CO_2

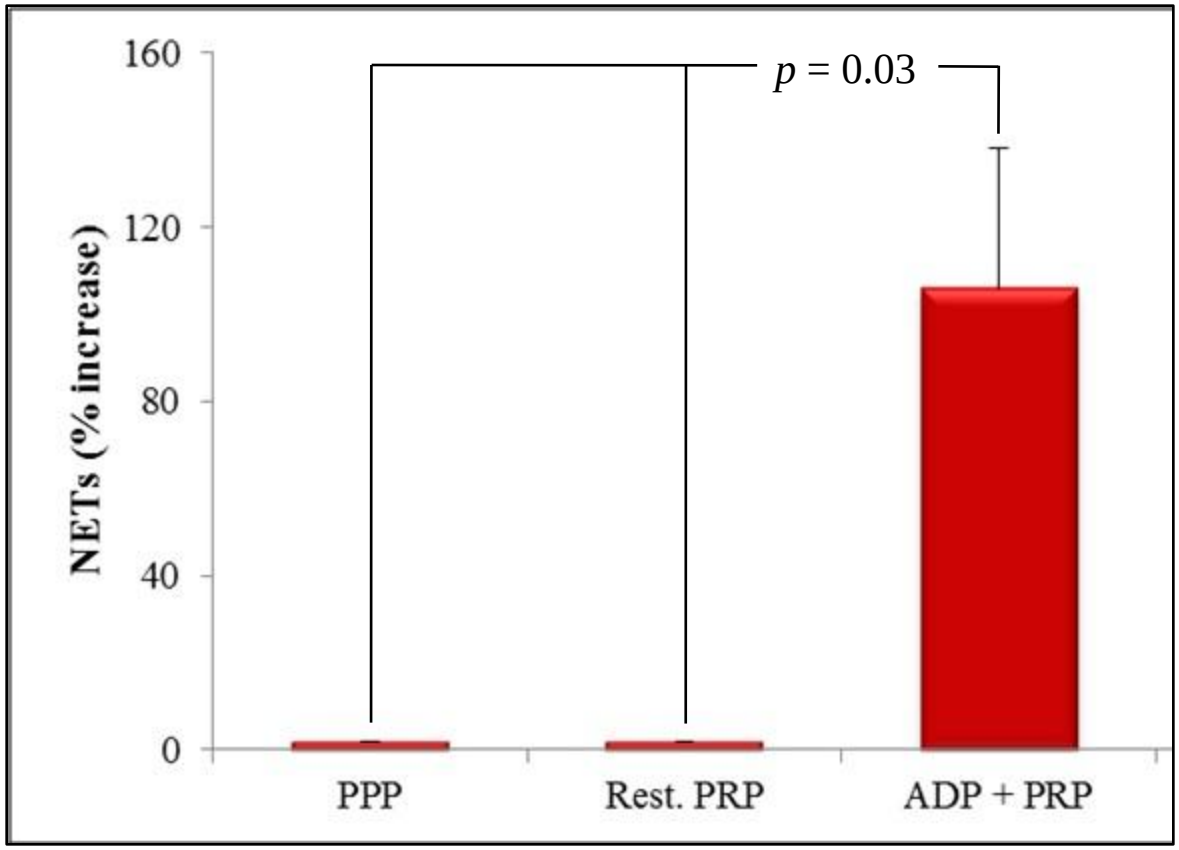
Materials and methods III

- Fixation of neutrophils using 4% paraformaldehyde, permeabilization with 0.5% Triton X-100

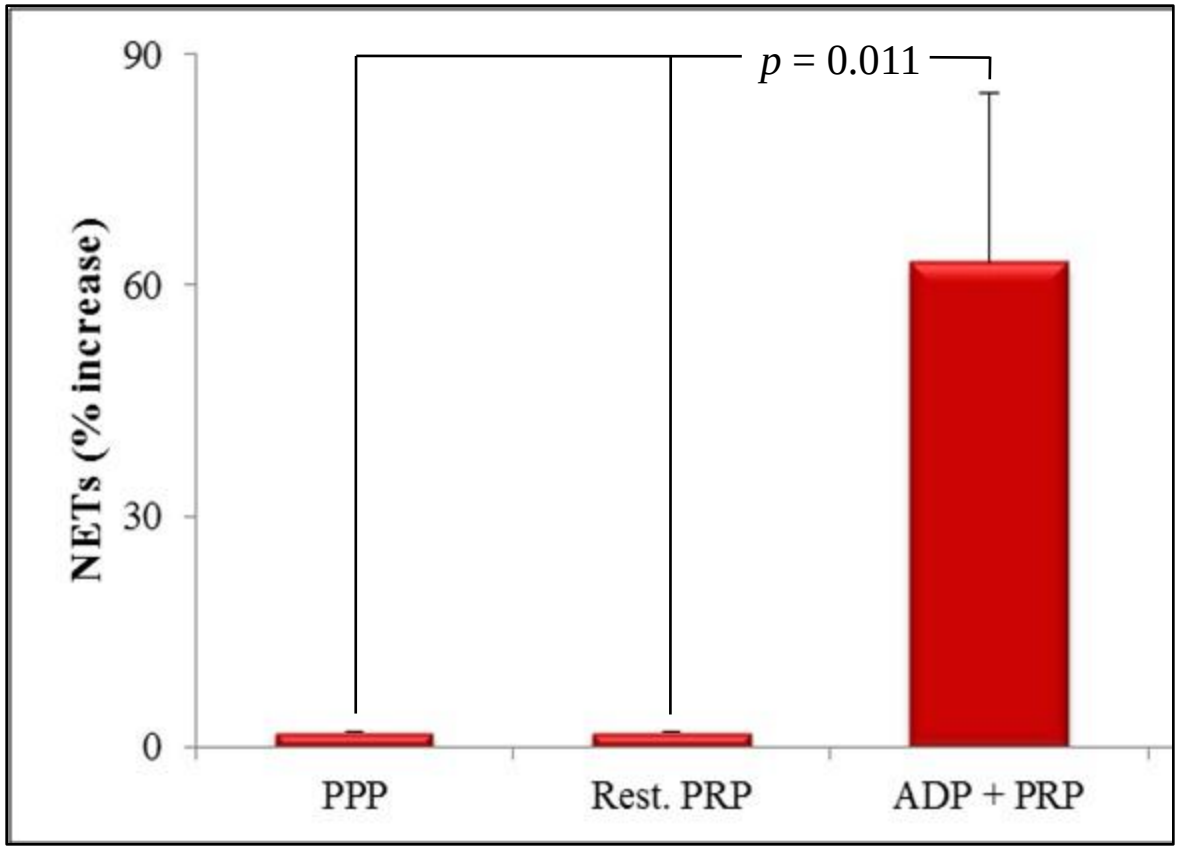
- Immunofluorescence protocol, using anti-myeloperoxidase (MPO) antibody and DAPI for DNA staining

- Visualization of neutrophils and NETs using confocal microscopy (magnification 63×), calculation of percentage of NETosis in 5 representative optical fields

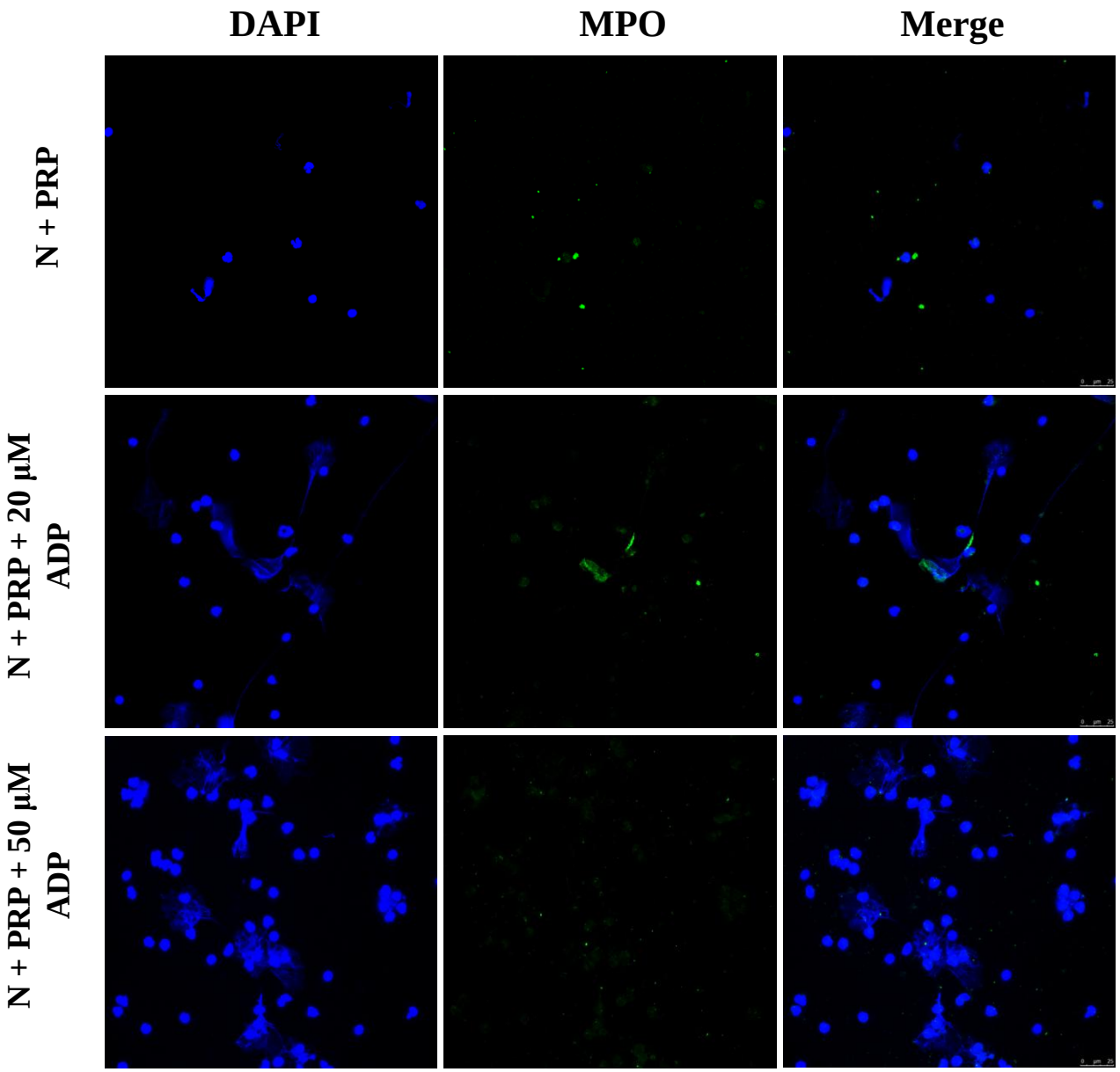
Results I: platelets pre-activated with ADP induce NETosis



Results II: platelets activated with ADP in situ induce NETosis



Results III: representative confocal microscopy images of NETosis induced by pre-activated platelets or platelets activated in situ, with ADP



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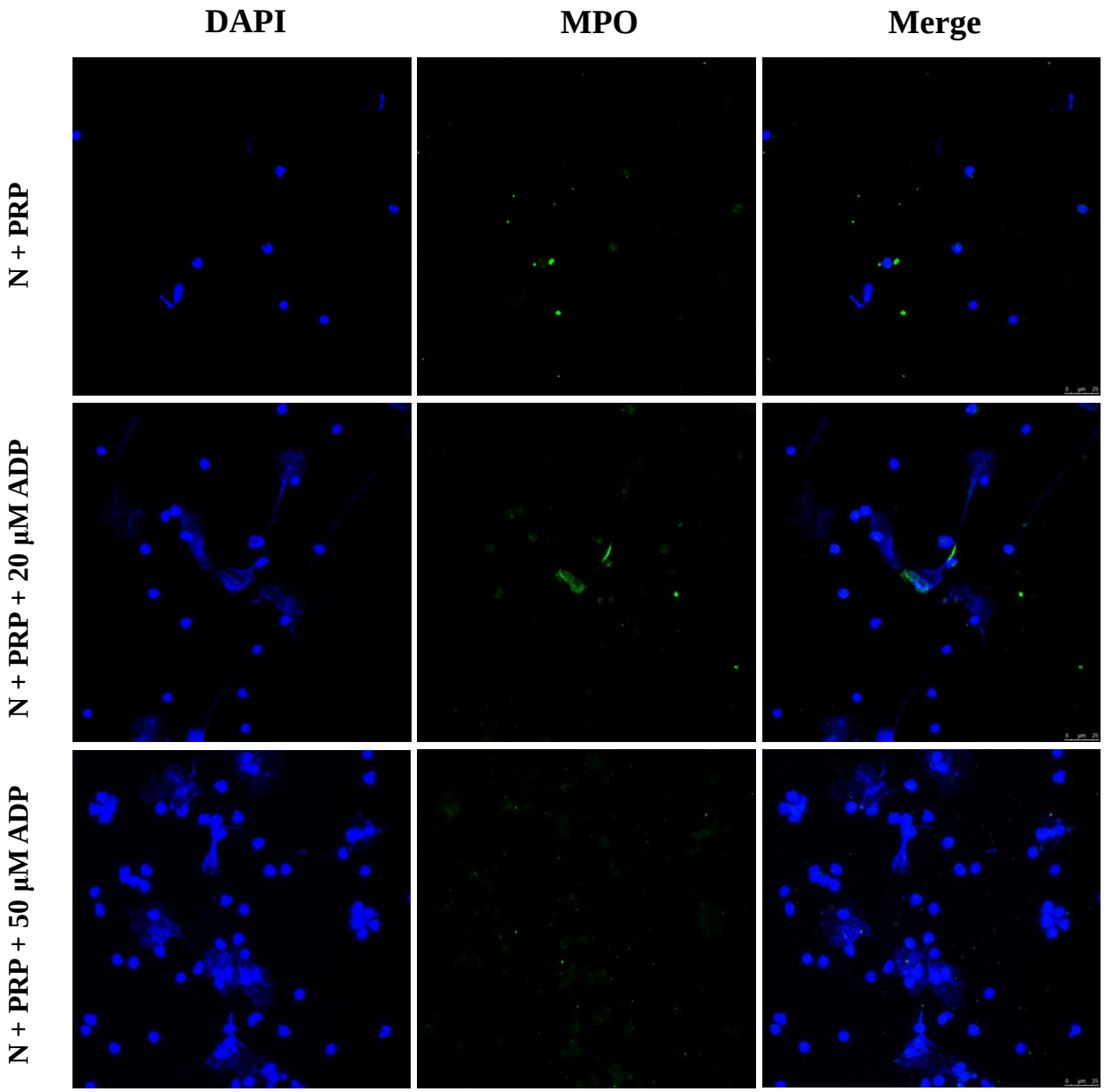
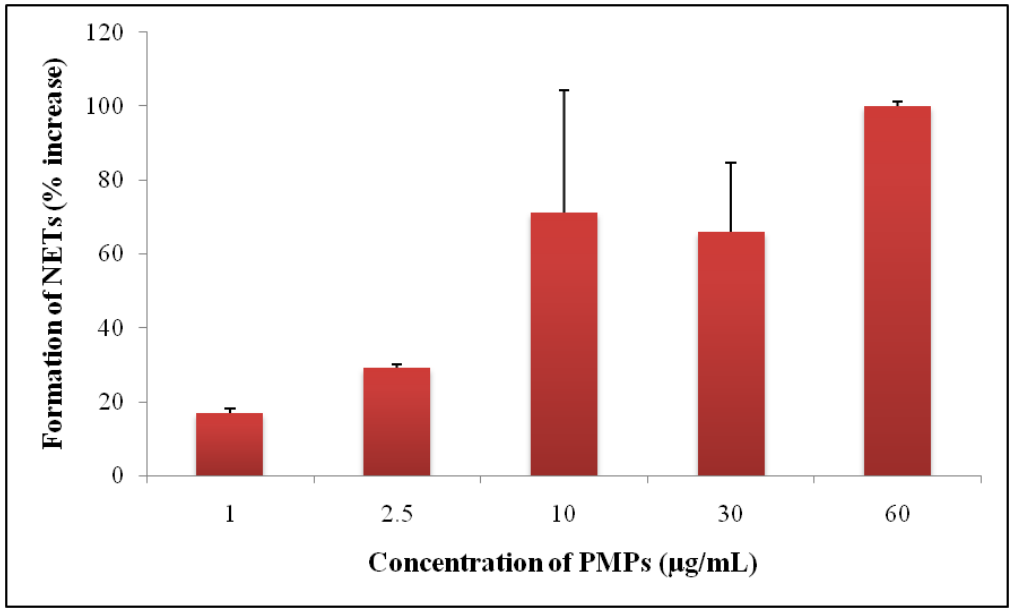
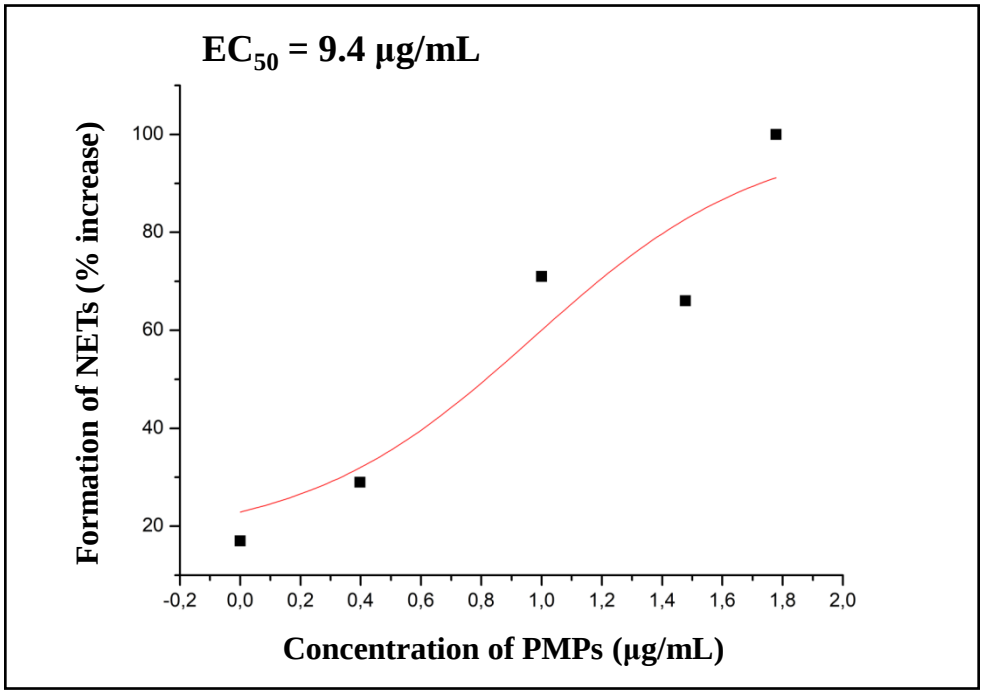
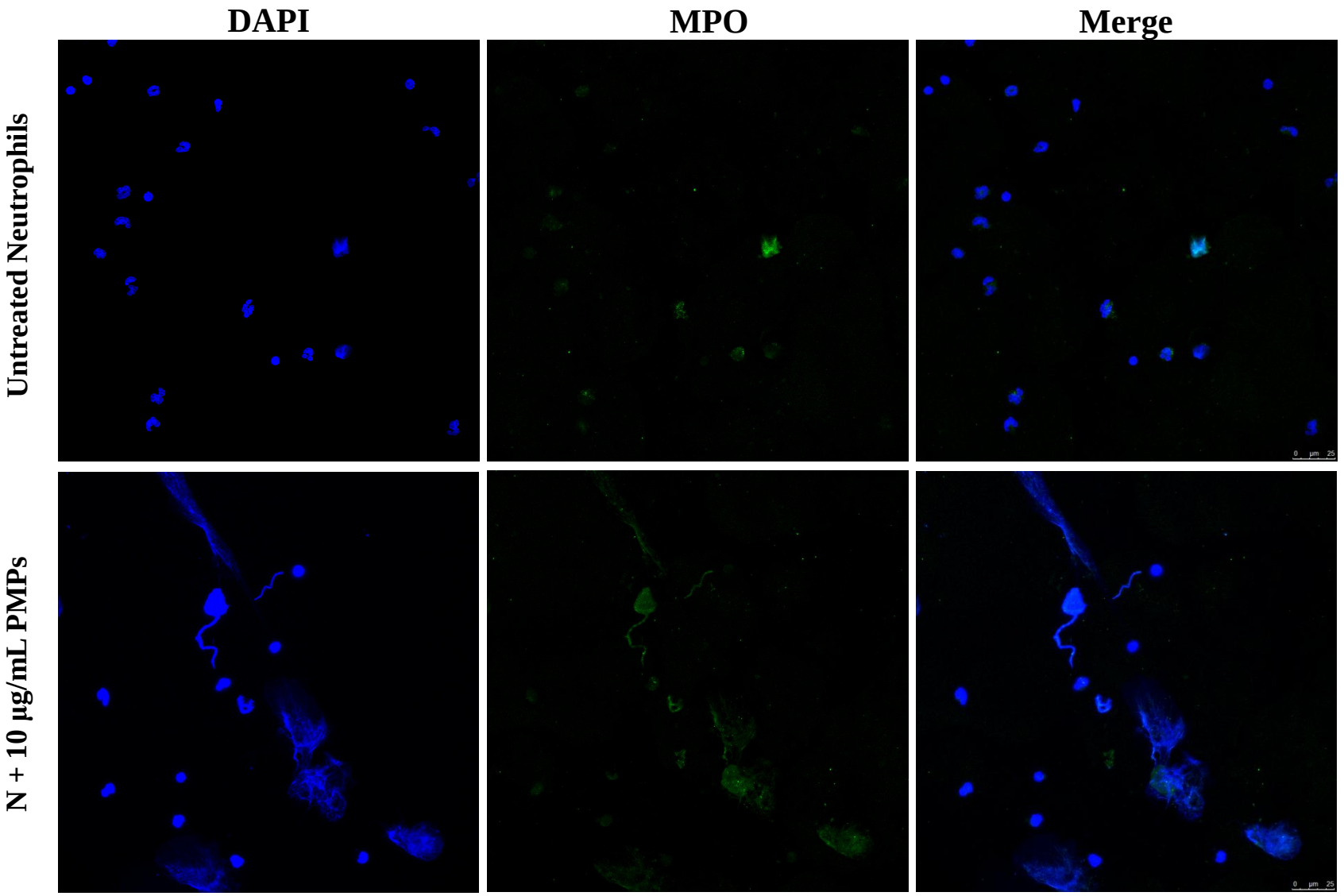


Results IV: PMPs induce NETosis in a concentration-dependent manner





Results V: representative confocal microscopy images of NETosis induced by PMPs, at the threshold concentration of 10 µg/mL



Conclusions

• ADP-stimulated platelets, either pre-activated, or activated *in situ*, induce the formation of NETs.

• PMPs, derived from thrombin-activated platelets, induce the formation of NETs in a concentration-dependent manner.

• Considering the implication of NETs in a vast number of pathophysiological conditions, including atherothrombosis, the aforementioned data provide evidence that platelets contribute to these diseases not only directly, but also indirectly, via the formation of NETs


Thank you very much for your attention!

ACKNOWLEDGMENTS

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