Journal of
Cardiology and Vascular Medicine

JScholar
Fostering Scholarly Communication

Research Article Open Access

Ponatinib and Risk of Thrombotic Events: *In Vitro* Study on Platelet Functions

Maria Ilaria Del Principe[1], Giovangiacinto Paterno[1], Lucrezia Gambardella[2], Elisa Buzzatti[1], Rosa Vona[2], Raffaele Palmieri[1], Pierantonio Menna[3], Camilla Cittadini[2], Domenico Del Principe[4], Adriano Venditti[1] and Elisabetta Straface[2*]

[1]Hematology Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Rome, Italy
[2]Biomarkers Unit, Center for Gender-Specific Medicine, Istituto Superiore di Sanità, Rome, Italy
[3]Clinical Pharmacology, University Campus Bio-Medico, Rome, Italy
[4]Former Professor, Pediatrics, Department of Health and Cell Biology, University of Rome "Tor Vergata", Rome, Italy

***Corresponding author:** Elisabetta Straface, Biomarkers Unit, Center for Gender-Specific Medicine, Istituto Superiore di Sanità, Rome, Italy, Tel: +39 06 4990 2443, E-mail: elisabetta.straface@iss.it

Received Date: June 10, 2021 **Accepted Date:** July 10, 2021 **Published Date:** July 12, 2021

Citation: Maria Ilaria Del Principe (2021) Ponatinib and Risk of Thrombotic Events: In Vitro Study on Platelet Functions. J Cardio Vasc Med 7: 1-8.

Abstract

Ponatinib is the only tyrosine kinase inhibitor effective in T315I mutated Philadelphia chromosome-positive leukemias, but it uses could contributed to increased thrombotic episodes. Therefore, the effect of ponatinib on platelet functions was evaluated. This work is aimed to: i) evaluate *ex vivo* the effects of different doses of ponatinib on platelet (PLT) functions and ii) investigate whether ponatinib affects PLT functions in a sex-dependent way.

PLTs from 25 males and 20 females healthy donors aged between 28 – 64 years were treated in rich plasma with different doses of ponatinib (0.03, 0.06 and 0.1 μM). By flow cytometry analyses PLT viability and activation were evaluated. We found that exposure to ponatinib 0.1 μM induces: i) a significant increase in the percentage of necrotic and apoptotic PLTs in both sexes and ii) a significant ($p < 0.05$) increase in the percentage of activated PLTs only in males. Moreover, we found that ponatinib 0.1 μM induces, in both sex, shedding of phosphatidylserine positive microvesicles (+ 84% in males and +57% in females), which have higher pro-coagulant activity than activated PLTs. An increase in necrotic PLTs may be linked to thrombocytopenia and inflammation, often occurring in ponatinib-treated patients. Further studies are needed to evaluate if lower dosages have less impact on PLT functions and whether the effect of ponatinib is gender dependent. This information can help optimizing ponatinib use minimizing occurrence of thromboembolic complications.

Keywords: Apoptosis; Necrosis; Platelets; Ponatinib; Tyrosine Kinase Inhibitor; Thrombosis

Introduction

Ponatinib is a third-generation tyrosine kinase inhibitor (TKI), approved for the treatment of Chronic Myeloid Leukemia (CML) and Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) patients, refractory or intolerant to standard therapy [1]. This drug is designed to overcome resistance-inducing mutations in the ABL kinase domain and is the only TKI with activity against the threonine-to-isoleucine mutation at position 315 (T315I). This mutation involves a residue set in front of hydrophobic pocket of the kinase causing the lack of a possible hydrogen bond that is the main characteristic for the selectivity and efficacy of first and second TKIs [2].

In open label multicenter phase II trial (PACE), ponatinib was tested in 449 patients affected by Ph+ ALL and CML resistant or intolerant to nilotinib or dasatinib. The initial reported of this trial showed major cytogenetic response rates equal to 56% by 12 months [1]. However, PACE trial observed that ponatinib was associated with a non-negligible number of cardiovascular complications, as arterial occlusive or venous thromboembolic events [3]. For this reason, ponatinib was withdrawn from the market for a short period in late 2013. In January 2014, considering that this drug was the only option for CML or Ph+ ALL patients with T315I mutation or resistant to other TKIs, Food and Drug Administration (FDA) readmitted ponatinib.

Currently, it is ongoing the evaluation whether lower dosages of ponatinib (30 *vs* 15 mg) reduce the incidence of cardiovascular events maintaining the same clinical efficacy as 45 mg. Although, there is experimental evidence that ponatinib inhibits critical platelet (PLT) functions such as activation and aggregation [4] and induces thrombocytopenia [3] several mechanisms underlying the thrombotic effects of ponatinib remain still unclear.

PLTs play a pivotal role in hemostasis. When injury occurs, they have to undertake a range of processes to form the hemostatic plug or thrombus. Different PLT subpopulations are involved in this process: pro-coagulant PLTs and aggregating PLTs [5].

Pro-coagulant PLTs are characterized by balloon shape and phosphatydilserine (PS) exposure, necessary for coagulation assembly and thrombin generation. PLTs exposing PS on their surface are able to bind coagulation factors (prothrombin, Va, IXa and Xa) and to control thrombin and fibrin generation, forming fibrin coat. The pro-coagulant subpopulation includes both apoptotic and necrotic PLTs, both showing PS on the surface [6]. Apoptotic PLTs are characterized by depolarization of mitochondrial membrane and activation of caspase 3. Necrotic PLTs, also defined COAT-PLTs (collagen and thrombin activated platelets), express on their surface high levels of *a* -granule proteins, including fibrinogen, fibronectin, vWF, factor V, and thrombospondin. Their formation occurs secondary to PS exposure, most likely as a consequence of the assembly of PLT secretion products and plasma factors at the outer membrane. Both apoptotic and necrotic PLTs can release microparticles (MPs) that expose PS and have a thrombin-generating potential [7-9].

The absence or excess of pro-coagulant PLTs have been linked to bleeding or thrombotic disorders, respectively [10]. Aggregating PLTs are characterized by activated integrin *a IIb-β3* (fibrinogen receptor) that, interacting with fibrin, causes clot retraction. They contain numerous pseudopods and strongly secrete bioactive molecules such as adenosine diphosphate (ADP), adenosine triphosphate (ATP) and serotonin.

This study is aimed to investigate: i) the *ex vivo* effects of different doses of ponatinib on PLT functions; ii) whether the influence exerted by ponatinib on PLT functions might differ in feminine versus masculine gender, considering the sex-based potential differences in drug efficacy and toxicity [11].

Materials and Methods

Selection of platelet donors

Forthy-five non-smoking volunteers (25 males and 20 females aged between 28 and 64 years) were recruited for this study at the Institute of Hematology University "La Sapienza" of Rome (Italy). Following the rules of good medical practice, the nature and purpose of the study were explained to all participants who then gave their informed consent. The investigation conformed to the principles outlined in the Declaration of Helsinki. All participants had stopped taking aspirin or NSAIDs at least one week before the beginning of the study. Premenopausal women using contraceptives and postmenopausal women using substitution therapy were excluded from the study.

To obtain more comparable results, for each donor the blood was collected in 2 tubes and the first was discarded.

In this study informed consent by healthy donors was obtained. The study was approved the ethics committee of the Istituto Superiore di Sanità (approval number: 10751).

Platelet isolation and treatment

PLTs were isolated by fresh whole blood samples from 45 volunteers. The blood samples were collected in acid-ci-

trate-dextrose tubes (ACD; NIH formula A), and immediately centrifuged at 200 g for 12 min at room temperature to separate platelet-rich plasma (PRP). After isolation, PLTs were treated in PRP with different doses of ponatinib (0.03, 0.06 and 0.1 μM) and incubated for 30 minutes a 37°C. After incubation with ponatinib, all samples were centrifuged at 700 g for 5 min and washed three times with phosphate buffer saline. After resuspending the centrifuged PLT pellet there were no aggregates.

Platelet viability

Quantitative evaluation of necrosis was performed by flow cytometry analysis by using 5μM calcein-AM (Molecular Probes).

Apoptosis was performed by flow cytometry using Annexin V Apoptosis Detection Kit FITC (eBioscience). All samples were analyzed by FACSCalibur cytometer (Becton Dickinson, Mountain View, CA, USA) in the FL1 and FL3 channels to determine the percentage of dead cells.

Platelet activation and adhesion

PLT activation was evaluated by using monoclonal anti-CD-62 IgG-Phycoerythrin-conjugated (1:20; Becton Dickinson). Homotypical adhesion (PLT-PLT) was evaluated in term of PAC-1 positivity by using monoclonal anti PAC-1 IgG-FITC-conjugated (1: 20; Becton Dickinson).

All samples were stained at 4 °C for 30 min and analyzed on a FACSCalibur cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser. At least 20,000 events have been acquired.

Detection of MPs

PLTs resuspended in PRP were centrifuged at 5.000*g* for 15 minutes at 4°C to obtain platelet-poor plasma (PPP). The PPP was further centrifuged at 20.000*g* for 20 minutes to sediment the MPs. The pellet containing MPs was incubated with FITC-conjugated annexin V or a phycoerythrin (PE) mouse anti-Human CD-62P (BD Biosciences) for 30 minutes at room temperature and analyzed by flow cytometry.

Statistical analyses

Cytofluorimetric results were analyzed by using the Kolmogorov–Smirnov test using Cell Quest Software. At least 20,000 events were acquired. The percentage of PLT positives to annexin V, CD62 and PAC-1 were used to provide a semi-quantitative analysis. Results are displayed as average value ± standard deviation, unless otherwise specified. Statistical analysis was performed by one-way analysis of variance (ANOVA). $p < 0.05$ was considered as a threshold for a significant difference.

Results

As shown in figure 1, we found that *in vitro* exposure to ponatinib boosts, in a dose dependent manner, the formation of different PLT subpopulations. In particular, especially at the concentration of 0.1 μM, ponatinib induces a significant increase in the percentage of necrotic and apoptotic PLTs (PS^+-PLTs) in both sexes (Figure 1 A and B).

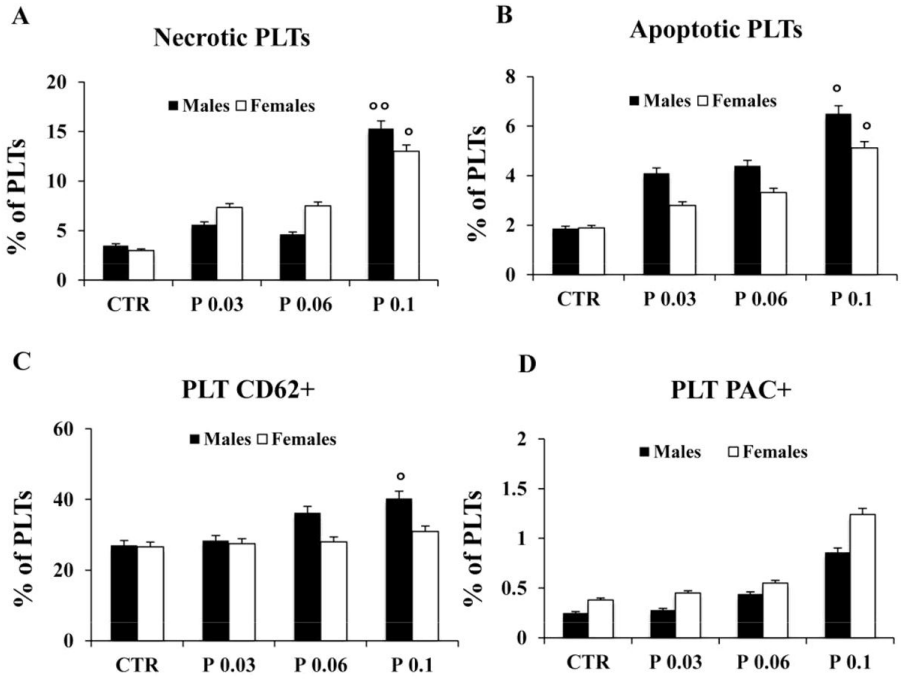

Figure 1: Percentage of different platelet populations after ponatinib treatment

Moreover, a significant ($p < 0.05$) increase in the percentage of activated PLTs (evaluated in terms of positivity to CD62,) was found only in males after treatment with ponatinib 0.1 μM (Figure 1 C). No significant increase in the percentage of PAC positive PLTs was found in either males or females and for all ponatinib concentrations used (Figure 1 D).

Moreover, considering that PLT size is a marker of PLT functions and that large PLTs have a higher thrombotic potential, the morphology (size and granularity) of the different PLT populations was evaluated by flow cytometry.

In the controls of all PLT populations examined (PS+, CD6+ and PAC+) no significant sex differences in the size and granularity have been found (Table 1). Moreover, we found that, in both males and females, PAC^+-control PLTs had a higher size and granularity than the other PLT populations. After ponatinib treatment, especially at concentration of 0.1 μM, in both sex an increase of size has been detected only in PS^+-PLTs (Table 1). Interestingly, this increase was significantly higher in males than in females ($p < 0.05$). Moreover, after ponatinib treatment, especially at concentration of 0.1 μM, in PLT positives to PS and PLT positives to PAC the granularity increased significantly especially ($p < 0.05$) in males.

Table 1: Morphology (size and granularity) of platelet subpopulations

Size in males				
PLT	**Controls**	**Ponatinib 0.1 μM**	%Δ	**p value**
subpopulations				
PS+	**112±16**	**148±15**	**+32%**	**p≤0.05**
CD62+	**110±19**	**103±26**	**-6.4%**	
PAC+	**283±92.4**	**158±34**	**-44.2%**	
Size in females				
PLT	**Controls**	**Ponatinib 0.1 μM**	%Δ	**p value**
subpopulations				
PS+	**120±13**	**124±11**	**+3.3%**	
CD62+	**117±12.7**	**108±8.4**	**-7.8%**	
PAC+	**265±53.7**	**145±52**	**-45.3%**	
Granularity in males				
PLT	**Controls**	**Ponatinib 0.1 μM**	%Δ	**p value**
subpopulations				
PS+	**68±22**	**87.5±35**	**+28.7%**	**p≤0.05**
CD62+	**65.5±30.5**	**61.5±27**	**-6.1%**	
PAC+	**253.3±25**	**344.5±33**	**+36%**	**p≤0.05**
Granularity in females				
PLT	**Controls**	**Ponatinib 0.1 μM**	%Δ	**p value**
subpopulations				
PS+	**94.8±16.7**	**103±27.8**	**+8.6%**	
CD62+	**78.25±15**	**76±20**	**-2.9%**	
PAC+	**278.5±22.5**	**350±41.3**	**+25.7%**	**p≤0.05**

*Platelet size was expressed as the media of median value of the FSC parameter.
Platelet granularity was expressed as the media of median value of the SSC parameter

Furthermore, we also measured the percentage of MPs released into the plasma by the PLTs. Interestingly, with respect to controls we found that ponatinib 0.1 μM, although in a non-significant way, increases the percentage of MPs positive to PS in both sexes (+84 % in males and +57% in females). In both males and females no differences in the percentage of MPs CD62 positives were detected after treatment with ponatinib 0.1 μM.

Discussion

Considering that thrombosis is a well-known side effect of ponatinib [12], we tested, by flow cytometry, the impact of different concentration of ponatinib on PLT viability and functions. In this experimental study, we found that the effects of ponatinib on PLTs are dose dependent and that PLTs from males appear to respond more significantly than those of females at ponatinib 0.1 μM. The ponatinib 0.1 μM is a clinically relevant ponatinib plasma concentration able to suppress the outgrowth of BCR-ABL1 mutants [13]. Interestingly, Marchesi and colleagues [14] have previously shown that 0.1 μM was a ponatinib steady-state plasma concentrations measured in a patient with Ph-positive ALL treated with 45 mg/day as starting dose of ponatinib and then reduced to 30 mg/day to resolve neuronal toxicity occurred during therapy [14].

Based on our preliminary results, we suggested that *in vitro* exposure to ponatinib boosts, in a dose dependent manner: i) the formation of different PLT subpopulations: necrotic, apoptotic and activated platelets (Table 2); and ii) PS positive MPs shedding. PS is a phospholipid that, in resting PLTs, is located almost exclusively at the inner monolayer. When PLTs activate and become apoptotic and/or necrotic, PS translocates to the outer leaflet of the plasma membrane, leading to the shedding of MPs that are pro-coagulants and pathogenic markers of thrombotic disorders and vascular damage [15]. Literature data report that MPs originating from PLTs represent approximately 25% of the pro-coagulant activity in the blood [16] and that their surface exhibits 50- to 100-fold higher pro-coagulant activity than the surface of activated PLTs [16]. The strongest pro-coagulant activity of MPs is mainly related to negatively charged PS, which electrostatically attracts the positively charged segment of clotting proteins such as factors VII, IX, X and prothrombin inducing thrombogenesis [17-18].

In addition, the thrombogenic activity of MPs may also be due to the presence of membrane proteins such as: i) tissue factor (TF), that acts as receptor of the FVII/VIIa complex, which activates both factors IX and X to initiate thrombin formation, and ii) plasminogen activator inhibitor-1 and protein S activator that may augment thrombogenesis amplification by suppression of fibrinolysis [19].

Microparticle CD-62 positives are considered as derivate from activated PLTs and participate in reactions as platelets. In our preliminary study we found that ponatinib induced, although not significantly, an increase in the release of MP-PS positive in both males and females, highlighting a pro-coagulant effect of ponatinib 0.1 μM that, although not significant, appears to be higher in males.

On the basis of our preliminary data, we can hypothesize that an increase in necrotic and apoptotic PLTs may be linked to thrombocytopenia and inflammation, which often occur in patients treated with ponatinib. Since it has been shown the critical role of PLT CD-62 in arterial thrombogenesis by the formation stable PLT-leukocyte aggregates, their role and function in ponatinib-treated male may be crucial. CD-62 or P-selectin is constitutively expressed in the *a* -granules of resting PLTs and translocates to the surface of activated PLTs incorporated into the forming thrombus. Once incorporated in the thrombus, they promote recruitment of leukocytes [20] Based on this, PLT shedding is likely to be the main "reservoir" of the soluble form of CD-62 found in the plasma following thrombotic events. On PLT surface, CD-62 serves as a cell adhesion receptor to interact with other cell receptors, including P-selectin glycoprotein ligand-1 (PSGL-1) and GPIb-V-IX, forming heterotypic aggregates (PLTs- lymphocytes). Conversely, the presence of active glycoprotein αIIbβ3 at PLT surface (determined with PAC-1 an-

PLTs	MALES (n = 25)	p value	FEMALES (n = 20)	p value
Necrotic	+338 Δ%	p ≤0.01	+320 Δ%	p ≤ 0.01
Apoptotic	+261 Δ%	p ≤ 0.05	+168 Δ%	p ≤ 0.05
CD62+	+48 Δ%	p ≤ 0.05	+16 Δ%	
+PAC	+79 Δ%		+226 Δ%	

Table 2: Δ% of different platelet subpopulations after treatment with ponatinib 0.1 μM

tibody) is required for platelet-platelet interactions and plays a critical role in platelet thrombus formation [20].

A greater pro-coagulant effect of ponatinib in males was confirmed by the size measurement. Based on literature data, reporting that large PLTs are more reactive, produce more prothrombotic factors, and aggregate more easily [21], the size of all PLT populations analyzed was measured by flow cytometry.

According to Itterman et al., we found that in the controls, PLT size was similar between males and females [22]. Conversely, after ponatinib treatment (especially at concentration of 0.1 μM) in PS^+-PLTs, an increase of PLT size in both sex was detected. Interestingly, this increase was significantly ($p < 0.05$) higher in males [22].

The observation that, after treatment with ponatinib, the percentage of reactive PLTs was higher in males than females is the first demonstration of a gender-dependent effect of ponatinib on PLTs.

Further studies in patients with CML and ALL are needed to evaluate if ponatinib given at dosages of 15 and 30 mg has less effect on PLT functions and to confirm if this effect is indeed gender dependent. This will be critical to optimize the use of ponatinib also according to gender categorization.

Authorship Statement

All persons who meet authorship criteria are listed as authors and all authors certify that they have approved the version of the manuscript to be published and take responsibility for the content of the manuscript.

Author Contributions

MIDP, DDP and ES designed the study, interpreted data, and drafted the manuscript; LG, CC and RV: acquired data and performed analysis; PM, GP, EB and RP contributed to study design; AV critical reviewed manuscript.

Acknowledgments

The research study carried out with the free supply of pure ponatinib substance by Incyte.

References

1. Cortes JE, Kim DW, Pinilla-Ibarz J, le Coutre PD, Paquette R, et al. (2018) Ponatinib efficacy and safety in Philadelphia chromosome-positive leukemia: final 5-year results of the phase 2 PACE trial. Blood 132: 393-404.

2. Buffa P, Romano C, Pandini A, Massimino M, Tirrò E, et al. (2014) BCR-ABL residues interacting with ponatinib are critical to preserve the tumorigenic potential of the oncoprotein. FASEB J 28: 1221-36.

3. Casavecchia G, Galderisi M, Novo G, Gravina M, Santoro C, et al. (2020) Early diagnosis, clinical management, and follow-up of cardiovascular events with ponatinib. Heart Fail Rev 25: 447-56.

4. Loren CP, Aslan JE, Rigg RA, Nowak MS, Healy LD, et al. (2015) The BCR-ABL inhibitor ponatinib inhibits platelet immunoreceptor tyrosine-based activation motif (ITAM) signaling, platelet activation and aggregate formation under shear. Thromb Res 135: 155-60.

5. Agbani EO, Poole AW (2017) Procoagulant platelets: generation, function, and therapeutic targeting in thrombosis. Blood 130: 2171-9.

6. Jackson SP, Schoenwaelder SM (2010) Procoagulant platelets: are they necrotic? Blood 116: 2011-8.

7. Heemskerk JW, Mattheij NJ, Cosemans JM (2013) Platelet-based coagulation: different populations, different functions. J Thromb Haemost 11: 2-16.

8. Pietraforte D, Vona R, Marchesi A, Tarrisi de Jacobis I, Villani A, et al. (2014) Redox control of platelet functions in physiology and pathophysiology. Antioxid Redox Signal 21: 177-93.

9. Baaten CCFMJ, Ten Cate H, van der Meijden PEJ, Heemskerk JWM (2017) Platelet populations and priming in hematological diseases. Blood Rev 31: 389-99.

10. Tohidi-Esfahani I, Lee CSM, Liang HPH, Chen VMY (2020) Procoagulant platelets: Laboratory detection and clinical significance. Int J Lab Hematol Suppl 1: 59-67.

11. Soldin OP, Chung SH, Mattison DR (2011) Sex differences in drug disposition. J Biomed Biotechnol 2011: 187103.

12. Cortes JE, Kim DW, Pinilla-Ibarz J, le Coutre P, Paquette R, et al. (2013) A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. N Engl J Med 369: 1783-96.

13. O'Hare T, Shakespeare WC, Zhu X, Eide CA, Rivera VM, et al. (2009) AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. T Cancer Cell 16: 401-12.

14. Marchesi F, Salvatorelli E, Renzi D, Mengarelli A, Minotti G, et al. (2019) Efficacy and safety of low dose ponatinib in a case of Ph-positive acute lymphoblastic leukaemia. Br J Haematol 187: e15-e17.

15. Morel O, Morel N, Freyssinet JM, Toti F (2008) Platelet microparticles and vascular cells interactions: a checkpoint between the haemostatic and thrombotic responses. Platelets 19: 9-23.

16. Tans G, Rosing J, Thomassen MC, Heeb MJ, Zwaal RF, et al. (1991) Comparison of anticoagulant and pro-coagulant activities of stimulated platelets and platelet-derived microparticles. Blood 77: 2641–48.

17. Nomura S, Ozaki Y, Ikeda Y (2008) Function and role of microparticles in various clinical settings. Thromb Res 123: 8–23.

18. Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, et al. (1991) Platelet derived microparticles express high affinity receptors for factor VIII. J Biol Chem 266:17261–68.

19. Owens AP, Mackman N (2011) Microparticles in hemostasis and thrombosis. Circ Res 108: 1284-97.

20. Payrastre B, Missy K, Trumel C, Bodin S, Plantavid M, et al. (2000) The integrin alpha IIb/beta 3 in human platelet signal transduction. Biochem Pharmacol 60: 1069-74.

21. Korniluk A, Koper-Lenkiewicz OM, Kamińska J, Kemona H, Dymicka-Piekarska V (2019) Mean Platelet Volume (MPV): New Perspectives for an Old Marker in the Course and Prognosis of Inflammatory Conditions. Mediators Inflamm 2019: 9213074.

22. Ittermann T, Feig MA, Petersmann A, Radke D, Greinacher A, et al. (2019) Mean platelet volume is more important than age for defining reference intervals of platelet counts. PLOS ONE 14: e0213658.