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Platelets as a source of information on oxidative stress, inflammation and thrombotic readiness. Model and clinical research

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INTRODUCTION

Platelets are heterogeneous cells in number, size, but also in their response to external factors. High inter-individual variability of platelets depends on genetic and environmental factors that affect, e.g., platelet reactivity. We hypothesize that in older age we may observe higher generation of reactive oxygen species (ROS), increased platelet reactivity or elevated inflammation. Platelet phenotyping is an interesting approach that would provide insight into differences in platelet function.

Resolvin E1 (RvE1) is a bioactive **metabolite of omega-3 fatty acids**, precisely eicosapentaenoic acid (EPA) and also a lipid mediator with anti-inflammatory properties, synthesized with 5-lipoxygenase (5-LOX). The platelet glycoprotein VI inhibitor (GPVI) is specific to platelets, and it plays an important role in both aggregation and activation of platelets, which are induced by collagen. In addition, a novel approach was to use collagen (instead of more commonly used agonists, such as ADP) to **verify the effect of RvE1 on platelets via the platelet receptor for collagen.**

The main aim of the conducted research was to verify the research hypotheses:

1. Bioactive food components, such as resolvin E1, can reduce platelet aggregation and reactivity, and inhibit ROS generation.
2. People with increasing age have higher inflammation, higher platelet ROS generation, and increased platelet activation and reactivity.

MATERIALS AND METHODS

STUDY POPULATION:

Ninety-five people aged 20 to 70 years (50 women and 45 men with a mean age 40 ± 15 years) participated in this study. Healthy volunteers were recruited at the Department of Haemostasis and Haemostatic Disorders (Medical University of Lodz) between March 2021 and November 2022, according to the inclusion and exclusion criteria. The research was carried out due to the approval by the Bioethical Commission at the Medical University of Lodz on June 2020 (RNN/153/20/KE). The basic characteristics of the study groups are shown in Table 1.

RESEARCH METHODS:

- ROS generation in isolated platelets (fluorescence measurement using FACSCanto II flow cytometer),
- platelet activation and reactivity in platelet-rich plasma (measurement of P-selectin expression and fibrinogen binding capacity using FACSCanto II flow cytometer),
- platelet reactivity in platelet-rich plasma and isolated platelets (measurement of optical aggregation using Chrono-Log 490-4D aggregometer), and platelet reactivity in whole blood (measurement of impedance aggregation using Multiplate aggregometer),
- platelet membrane fluidity (measurement of fluorescence anisotropy using Perkin-Elmer spectrofluorometer).

STATYSTICAL ANALYSIS:

A statistical analysis was performed using Statistica 13.1 software (Statsoft, Poland) and GraphPad Prism v.9.1.1. (San Diego, CA, USA). The normality of the distribution of the analysed variables was assessed using the Shapiro-Wilk test. Data were presented as mean ± SD (variables with normal distribution) or as median and interquartile range (IQR) (variables with non-normal distribution). Statistical significance of differences between two groups was assessed using the paired Student's t-test or the unpaired Student's t-test (variables with normal distribution) and the Wilcoxon's singed-rank test or Mann-Whitney U-test (variables with non-normal distribution), while to compare differences between more than two groups, ANOVA for repeated measures with the post hoc Dunnett's test (variables with normal distribution) or Friedman test with post hoc analysis with Dunn's multiple comparisons test (variables with non-normal distribution) were applied. The differences in the analysed variables were considered to be statistically significant if the p value was <0.05.

RESULTS

Table 1. Characteristics of selected parameters among healthy donors (n = 95) depending on age

Parameter	Donors <40 y.o. (n = 51)	Donors ≥40 y.o. (n = 44)	P value
PLT [*10^9/L]	239 (207-273)	262 (217-295)	ns
MPV [fL]	10.5 (9.9-11.2)	10.3 (9.7-10.6)	ns
RBC [*10^{12}/L]	4.83 ± 0.49	4.72 ± 0.47	ns
WBC [*10^9/L]	6.02 ± 1.49	6.22 ± 1.30	ns
NEU [*10^9/L]	2.98 (2.48-3.89)	3.16 (2.69-3.93)	ns
LYM [*10^9/L]	2.02 (1.70-2.51)	2.03 (1.68-2.25)	ns
CRP [mg/dL]	1.1 (0.6-2.3)	1.3 (0.7-1.7)	ns
PLR	117.0 (98.8-131.6)	128.1 (105.1-164.1)	**0.036**
NLR	1.3 (1.1-1.9)	1.6 (1.3-2.1)	**0.020**
MPVLR [fL/10^9/L]	5.0 (4.0-6.5)	5.2 (4.5-6.2)	ns
SII	338.5 (252.7-462.6)	420.4 (323.1-531.2)	**0.013**
Total cholesterol [mmol/L]	4.95 ± 0.81	5.63 ± 1.17	**0.001**
LDL-cholesterol [mmol/L]	2.95 ± 0.71	3.52 ± 1.02	**0.002**
HDL-cholesterol [mmol/L]	1.51 (1.30-1.75)	1.52 (1.25-1.72)	ns
Non-HDL-cholesterol [mmol/L]	3.41 ± 0.86	4.10 ± 1.13	**0.001**
Triglycerides [mmol/L]	1.03 (0.74-1.31)	1.18 (0.97-1.51)	**0.015**
Glucose [mmol/L]	5.14 (4.91-5.36)	5.40 (5.14-5.73)	**0.002**
ROS generation [%]			
collagen	5.5 ± 2.5^A	5.9 ± 2.5^B	ns
thrombin	9.5 (7.9-12.3)^C	9.5 (7.4-12.2)^D	ns
Platelet aggregation [A_{max}, %]			
collagen	84.0 (79.0-88.0)^E	82.0 (78.5-86.0)^F	ns
ADP	73.0 (54.0-83.0)^G	79.0 (74.3-83.5)^H	**0.030**
AA	83.1 ± 7.7^I	84.3 ± 7.8^J	ns
CD62-positive platelets [%]	1.7 (1.1-2.2)^K	2.0 (1.5-2.6)^L	ns
fibrinogen-positive platelets [%]	3.0 (2.6-4.1)^M	3.8 (2.4-6.6)^N	ns

Data were presented as mean ± SD (normal distribution) or as median (IQR) (non-normal distribution). Student's t-test (normal distribution) or Mann-Whitney U-test (non-normal distribution) was used to assess the significance of differences. AA – arachidonic acid; ADP - adenosine diphosphate; CRP – C-reactive protein; HDL – high-density lipoproteins; LDL – low-density lipoproteins; LYM – lymphocytes; MON – monocytes; MPV – mean platelet volume; MPVLR – MPV/lymphocyte; NEU – neutrophils; NLR – neutrophil/lymphocyte; PLR – platelet/lymphocyte, PLT – platelets; RBC – red blood cells; SII – platelet x neutrophil/lymphocyte; WBC – white blood cells.
A: n=42; B: n=33; C: n=45; D: n=39; E: n=49; F n=33; G: n=23; H: n=20; I: n=18; J: n=20; K: n=41; L: n=29; M: n=41; N: n=27

Effect of RvE1 on platelet activation and reactivity in platelet-rich plasma

Table 2. Effect of RvE1 on platelet activation and reactivity based on expression of P-selectin (CD62) on platelets without activation and with collagen activation (n = 14)

	Control	RvE1 10 nM	P value	RvE1 100 nM	P value
PLATELETS AT REST					
no activation	1.5 (1.0-2.1)	1.4 (0.9-2.3)	ns	1.3 (1.1-2.3)	ns
collagen activation	11.9 (8.0-18.7)	10.5 (5.6-18.2)	**0.029**	6.7 (5.1-17.1)	**0.001**
PLATELETS AFTER 1 HOUR AT RT					
no activation	3.6 (1.5-5.1)	3.5 (1.8-4.6)	ns	3.0 (2.1-5.4)	ns
collagen activation	16.1 (7.6-26.1)	12.3 (7.0-24.9)	ns	9.5 (7.7-20.0)	ns

Results were presented as a percentage of CD62-positive platelets as median (IQR). Significance of differences was assessed by the Friedman test with Dunn's multiple comparisons test.

Table 3. Effect of RvE1 on platelet activation and reactivity based on exogenous fibrinogen binding to platelets without activation and with collagen activation (n = 14)

	Control	RvE1 10 nM	P value	RvE1 100 nM	P value
PLATELETS AT REST					
no activation	3.5 ± 1.9	3.4 ± 1.8	ns	3.3 ± 1.6	ns
collagen activation	45.8 ± 18.5	44.1 ± 21.9	ns	43.9 ± 22.9	ns
PLATELETS AFTER 1 HOUR AT RT					
no activation	4.0 ± 3.0	3.7 ± 2.4	ns	3.3 ± 2.0	**0.040**
collagen activation	39.0 ± 18.7	33.4 ± 20.2	ns	32.7 ± 20.7	ns

Results were presented as a percentage of fibrinogen-positive platelets as mean ± SD. Significance of differences was assessed by the ANOVA with Dunnett's multiple comparisons test.

Effect of RvE1 on collagen-induced platelet aggregation in whole blood

Table 4. Effect of RvE1 on collagen-induced platelet aggregation in whole blood (impedance aggregation) (n = 10)

	Control	RvE1 10 nM	P value	RvE1 100 nM	P value
platelets at rest	59.0 (54.0-68.0)	60.0 (54.5-69.0)	ns	54.0 (51.0-67.5)	ns
platelets after 1 h at RT	51.5 (43.0-57.8)	52.0 (41.0-55.8)	ns	50.5 (44.3-63.0)	ns

Results were presented in arbitrary units (U) as median (IQR). Significance of differences was assessed by the Friedman test with Dunn's multiple comparisons test.

Effect of RvE1 on collagen-induced platelet aggregation in platelet-rich plasma

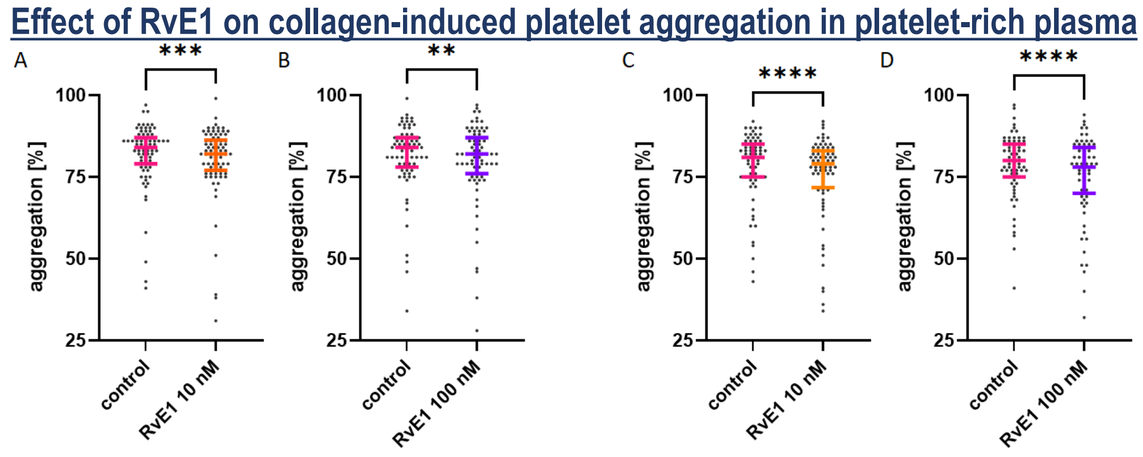

Figure 1. Effect of RvE1 on collagen-induced platelet aggregation in platelet-rich plasma (optical aggregation) (n = 80; A, B – platelets at rest, and n = 74; C, D – platelets after 1 h at RT)

*The graphs present the dot-plots with median (horizontal bar) and IQR (whiskers). Significance of differences was assessed by the Wilcoxon's singed-rank test. ** p<0.01; *** p<0.001; **** p<0.0001*

Effect of RvE1 on collagen-induced platelet aggregation in isolated platelets

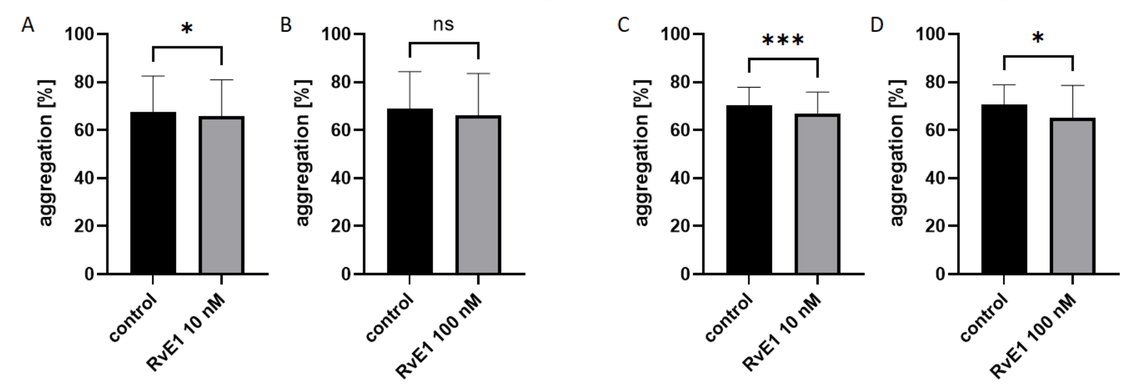

Figure 2. Effect of RvE1 on collagen-induced platelet aggregation in isolated platelets (optical aggregation) (n = 10)

*Data were presented as mean ± SD. Significance of differences was assessed by the paired Student's t-test. A, B – platelets at rest; C, D – platelets after 1 h at RT. * p<0.05; *** p<0.001*

ROS generation in isolated platelets induced by collagen and thrombin

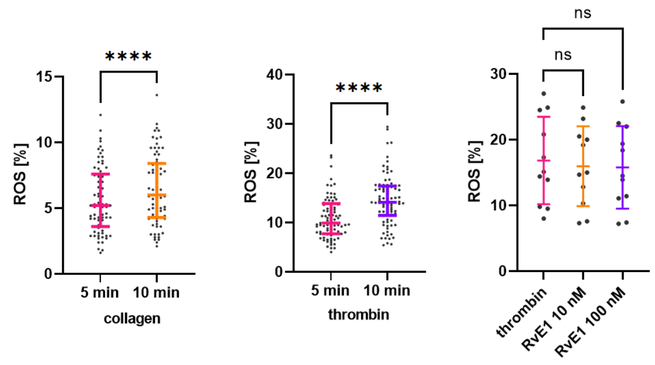

Figure 3. ROS generation in platelets stimulated with collagen (n = 75), thrombin (n = 84), and effect of RvE1 on thrombin-induced ROS generation (n = 11)

*The graphs present the dot-plots with median (horizontal bar) and IQR (whiskers), and mean (horizontal bar) and SD (whiskers) for effect of RvE1. Significance of differences was assessed by the Wilcoxon's singed-rank test (collagen, thrombin) and by the ANOVA with Dunnett's multiple comparisons test (effect of RvE1). **** p<0.0001*

Effect of RvE1 on platelet membrane fluidity – fluorescence anisotropy

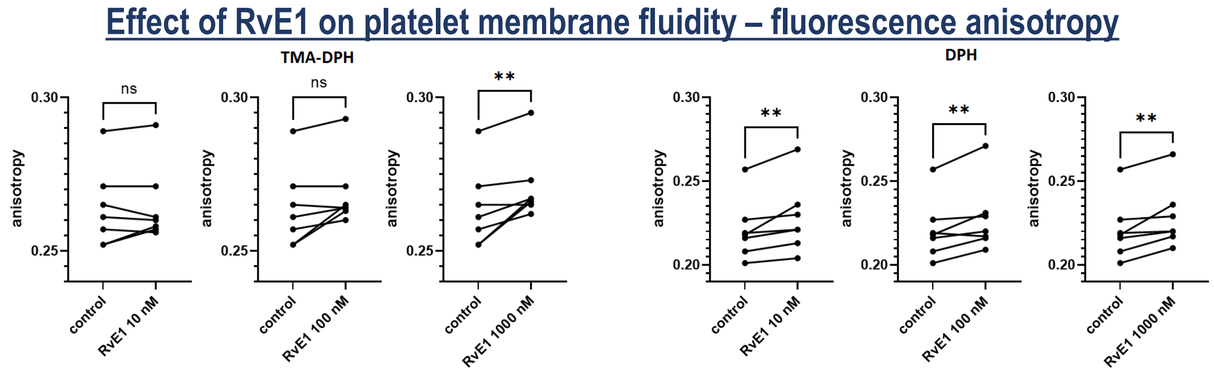

Figure 4. Effect of RvE1 on platelet membrane fluidity with fluorescent probes: TMA-DPH (n=7) and DPH (n=7)

*Significance of differences was assessed by the Friedman test with Dunn's multiple comparisons test (TMA-DPH) and by the ANOVA with Dunnett's multiple comparisons test (DPH). ** p<0.01*

CONCLUSIONS

1. No age-related differences were observed in ROS generation (collagen- and thrombin-induced), collagen- and AA-induced platelet aggregation, and inflammatory markers such as CRP and MPVLR. Statistically significant **age-dependent differences** were noted only for ADP-induced platelet aggregation and inflammatory markers - **PLR, NLR, SII.**
2. RvE1 **does not affect platelet activation *in vitro* and collagen-induced platelet activation** (reactivity) based on measurements of CD62 expression and fibrynogen binding.
3. RvE1 **can reduce platelet reactivity by inhibiting collagen-induced platelet aggregation, in platelet-rich plasma and isolated platelets**, but not in whole blood.
4. **No reduced ROS generation was noted in platelets in the presence of RvE1**.
5. A slight increase in fluorescence anisotropy (both TMA-DPH and DPH) was observed in the presence of RvE1 (concentration dependent), which may indicate **maintenance of platelet membrane fluidity in the presence of RvE1 with a minor tendency to increase membrane stiffness.**
6. In conclusion, this was the **first attempt to explore and clarify the molecular mechanism of RvE1's effect on platelets**, aimed at connecting platelet membrane stiffening with the anti-aggregation effect of this compound. The association between platelet reactivity and platelet membrane fluidity is a hypothesis, requiring further research.