Beneficial Effects of Antioxidants in Regulating NET Generation Within the Course of Atherosclerosis

Keywords
NETs, Vitamin C, Flavonoids, Neutrophils

Abstract

Introduction

There are numerous publications confirming the involvement of neutrophils in the pathomechanism of atherosclerosis. The aim of this study was to determine the effects of flavonoids (quercetin and luteolin) and vitamin C on neutrophil extracellular trap (NET) formation and regulation of NET markers, including circulating free DNA (cfDNA) and myeloperoxidase (MPO). Additionally, we assessed the expression of NADPH oxidase complex subunits and superoxide anion generation in neutrophils.

Material and methods

Whole blood samples were collected from patients with atherosclerosis. Neutrophils were stimulated with lipopolysaccharide (LPS), and the effects of flavonoids and vitamin C on NET release, NADPH oxidase subunit expression (p47phox, p67phox, Rac1), and superoxide production were evaluated.

Results

Atherosclerotic patients showed increased NETosis, with elevated NET release, MPO, and cfDNA levels. Expression of NADPH oxidase subunits p47phox and p67phox, as well as superoxide anion generation, were significantly higher compared to controls. LPS further enhanced these effects. Treatment with quercetin, luteolin, and vitamin C reduced NET formation, NADPH oxidase expression, and superoxide production in stimulated neutrophils.

Conclusions

Flavonoids and vitamin C modulate NET generation by affecting NADPH oxidase activity and reactive oxygen species production, suggesting potential therapeutic use in atherosclerosis. Further and more detailed analysis could allow for the potential application of these compounds as adjunctive therapy in the treatment of atherosclerosis.

INTRODUCTION

According to the World Health Organization, cardiovascular diseases (CVD) constitute the most common cause of death worldwide. In 2019, 17.9 million deaths were recorded due to CVD, accounting for 32% of the total number of deaths. Regardless of the region, the high mortality rate from cardiovascular diseases is largely determined by disorders underlying atherosclerosis[1]. There is a constant search for new predictors of CVD[2,3].

An important role in the development of atherosclerosis is played by the immune process involving immune system cells and the mediators released by those cells. There is substantial evidence indicating the crucial, although not fully understood, role of neutrophils (PMNs, polymorphonuclear neutrophils) in the pathogenesis of atherosclerosis[4]. It has been proven that the presence of neutrophils within the atherosclerotic plaque is associated with both its formation and development as well as with destabilization. Neutrophils promote inflammatory responses and recruit immune-competent cells to atherosclerotic lesions, among other mechanisms, by releasing reactive oxygen species (ROS) and numerous enzymes[5].

The wave of experiments conducted on neutrophils was initiated by Brinkmann et al. who had discovered an interesting aspect of the biology of those cells, related to the formation and release of neutrophil extracellular traps (NETs) into the extracellular space, through a form of cell death known as NETosis[6]. Neutrophil extracellular traps are composed of numerous proteins and enzymes as well as strands of DNA and histones[7–11]. The results of many experiments have revealed that the NET generation requires the activity of the NADPH oxidase complex that initiates the production of reactive oxygen species by reducing molecular oxygen to superoxide anion[12,13].

Numerous scientific studies have shown that the NET generation plays a significant role in the body's immune response to infection. Neutrophil extracellular trap structures are attributed with the ability to bind, neutralize, and eliminate a variety of pathogens, including bacteria, fungi, protozoa,

and viruses[6,11,14–18]. By creating a physical barrier, NETs facilitate the degradation of 'trapped' pathogens and their virulence factors, thereby preventing the spread of microorganisms in the body[19]. Although the beneficial role of NETs in fighting pathogens has been proven, an excessive amount of traps may contribute to damage to surrounding tissues[20]. It has been observed that the increased NET generation, as well as a deficiency in DNase, which is responsible for the degradation of the traps, may be associated with the pathogenesis of autoimmune diseases as well as cardiovascular disorders, including atherosclerosis[21]. To date, studies analyzing the formation of neutrophil extracellular traps within the course of atherosclerosis are few and primarily involve experiments conducted on animal models[22]. Figure 1 presents a model of the involvement of neutrophils and NETs in the pathophysiology of atherosclerosis.

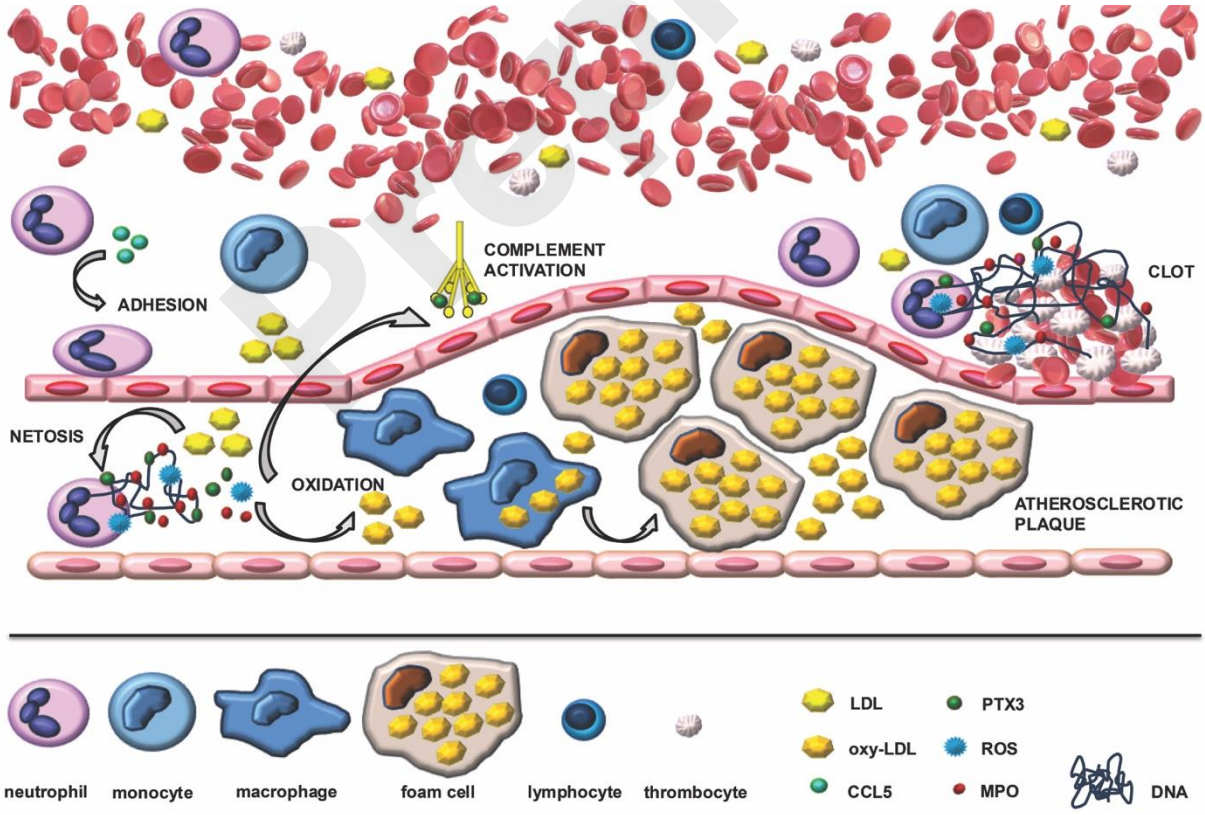


Figure 1. Model of the involvement of neutrophils and NETs in the pathophysiology of atherosclerosis.

Cholesterol crystals induce the formation of NETs, which in turn affect LDL oxidation, activation of monocytes/macrophages and the complement system. In addition, NETs participate in the formation of clots.

Given the consequences of excessive formation and release of NETs, it seems crucial to thoroughly understand the extent of that phenomenon and the potential for its control in patients with atherosclerosis, which has been set as the objective of our study. The strategic role of NADPH oxidase and ROS in generating neutrophil extracellular traps has led us to investigate the potential use of substances with antioxidant properties, such as flavonoids and vitamin C, which could provide numerous beneficial effects in patients with atherosclerosis by limiting the release of NETs.

MATERIALS AND METHODS

Materials

The study was conducted in a group of 22 patients with newly diagnosed atherosclerosis of the lower extremity arteries (11 women and 11 men), patients of the Academic Family Medicine Practice Clinic in Bialystok, with range of age 49-79. The research procedures were carried out before the initiation of treatment. The patients exhibited symptoms of intermittent claudication, and the ankle-brachial index was <0.9. The diagnosis was confirmed by Doppler ultrasound of the lower extremity arteries, which revealed arterial stenosis. Exclusion criteria included: the presence of chronic diseases other than hypertension; the use of statins and dietary supplements containing vitamin C, other flavonoids, or antioxidants.

In the control group, 21 healthy individuals (10 women and 11 men) were enrolled, who were blood donors at the Regional Blood Donation and Blood Treatment Center in Bialystok, aged between 35 and 50 years, with a low likelihood of atherosclerotic disease. After obtaining written consents from the study participants, 9 ml of blood was drawn from the antecubital vein into a tube with the

anticoagulant EDTA-K3, which was used for neutrophil isolation, and 6 ml was drawn into a tube with a clot activator to obtain serum.

The study was approved by the Bioethics Committee of the Medical University of Bialystok – resolutions no. R-I-002/285/2017 and R-I-002/391/2018.

Methodology

WBC Count and Blood Smear Analysis

The total leukocyte count (WBC) was assessed using the chamber method, direct counting with a light microscope, and a Bürker hemocytometer (Superior Marienfeld).

The peripheral blood smear was assessed in preparations made on glass slides, stained by means of the May-Grünwald-Giemsa method (Aqua-Med), under a light microscope.

Biochemical Tests

In the group of patients with atherosclerosis of the lower extremity arteries (11 women and 11 men), a panel of biochemical tests was performed, which included the following parameters: total cholesterol, LDL, HDL, triglycerides, glucose, alkaline phosphatase, calcium, phosphorus, and homocysteine.

PMN Isolation and Incubation

Preliminary isolation of neutrophils was performed using the density gradient centrifugation method with the Polymorphprep™ reagent (Axis-Shield). The next step involved sorting the neutrophils using a MACS® Separator with CD16 MicroBeads (Miltenyi Biotec). Neutrophil sorting was carried out according to the standard procedure outlined in the company's research protocol attached to the device and reagents.

The isolated neutrophils were suspended in the RPMI-1640 medium (Gibco®, Life Technologies™) enriched with 4% fetal calf serum (PAN Biotech), and supplemented with the antibiotic Penicillin-Streptomycin (Sigma Life Science). The cell suspension was then applied to

96-well plates (Falcon). The neutrophils were pre-incubated for 30 minutes with flavonoids: quercetin – 40 µM (ROTH) and luteolin – 10 µM (ROTH) as well as vitamin C (Sigma-Aldrich) at a concentration of 2 mM. Subsequently, the cells were stimulated with LPS at a concentration of 10 µg/mL (Sigma-Aldrich) for one hour at 37°C with a 5% CO2 flow in an incubator (NUAIRE™).

Protein Isolation from PMNs

After the incubation, the cell supernatants were collected from each well and stored at –20°C for the purpose of the further analysis.

After incubation, the protein was isolated from the neutrophils using sonication (SonicVibra Cell) in the presence of protease inhibitors (Protease Inhibitors Cocktail, Sigma–Aldrich). Protein concentration was determined by means of a fluorescence method with the Qubit Protein Assay Kit (Invitrogen™), according to the procedure outlined in the official research protocol attached to the kit and the Qubit® 2.0 Fluorometer (Invitro-gen™).

Visualization of NETs

The incubation of neutrophils at 37°C with a 5% CO2 flow in the incubation chamber was recorded by the In Cell Analyzer 2200 Microscope System (GE Healthcare Life Sciences) microscope system. The main structures forming NETs were evaluated: DNA and myeloperoxidase. In order to stain the total DNA, Hoechst 33342 dye (InvitrogenTM) was used. Myeloperoxidase was stained by means of mouse monoclonal anti-MPO antibodies conjugated with fluorescein (Clone 8E6, MolecularProbes®).

Assessment of Neutrophil Myeloperoxidase Expression

After 1.5 hours of incubation, neutrophils were permeabilized by means of FACS Permeabilizing Solution 2 (BD Biosciences) and labeled with monoclonal mouse anti-MPO antibodies (Clone 8E6, MolecularProbes®). The data obtained on the FACSCalibur flow cytometer (BD Biosciences) were analyzed by means of FlowJo software (Tristar Inc.).

Quantification of Free Circulating DNA (cfDNA)

The concentration of free circulating DNA in blood serum and in the supernatants obtained after neutrophil incubation was determined by means of the Circulating DNA Quantification Kit (Abcam) according to the provided instructions.

Determination of MPO Concentration

The concentration of myeloperoxidase in the serum and in the supernatants obtained after neutrophil incubation was determined using the ELISA immunoassay (Human Myeloperoxidase Quantikine ELISA Kit, R&D Systems).

NADPH Oxidase Protein Expression Analysis

The expression of selected subunits of NADPH oxidase: p47phox, p67phox, Rac1, was measured by Western blot in the obtained cell lysates.

The cell lysate suspended in Laemmli buffer was subjected to SDS-PAGE electrophoresis. In the next step, the fractions of separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories) that, after blocking non-specific sites, was incubated with appropriate primary antibodies: anti-p47-phox, anti-p67-phox, and anti-panRac (Santa Cruz Biotechnology). After washing with 0.1% TBS-T [(10x Tris-Buffered Saline) (Bio-Rad Laboratories) and Tween 20 – Electrophoresis Reagent (Sigma-Aldrich)], the nitrocellulose membrane was incubated with secondary antibodies conjugated with alkaline phosphatase (Jackson Immuno Research). The addition of the BCIP/NBT Liquid Substrate System (Sigma-Aldrich) allowed for the detection of immunoreactive protein bands, the intensity of which was assessed using the ImageJ program and expressed in arbitrary units.

Superoxide Detection via Cytochrome c Reduction Assay

Using the method developed by McCord and modified by Bhuyan[23], the 'oxidative burst' was assessed based on the measurement of superoxide anion generation released by neutrophils.

A solution of cytochrome c in phosphate buffer (KH2PO4/K2HPO4) at pH 7.8 containing 0.1 mM EDTA was added to two parallel samples with isolated neutrophils. The concentration of cytochrome c was 15 mg/mL. Superoxide dismutase (SOD) with an activity of 5000 U/mL was added to the reference sample, and buffer to the test sample. Then, after the addition of quercetin – 40 µM (ROTH), luteolin – 10 µM (ROTH), vitamin C – 2 mM, and LPS – 10 µg/mL (Sigma-Aldrich) to both test samples, incubation was carried out at 37°C, and after 1.5 hours, the absorbance was read at λ = 550 nm. The generation of superoxide anion was expressed as nmol of reduced cytochrome c, using the absorption coefficient of 21.1 mmol/L-1cm-1 at 550 nm.

Statistical Analysis

The obtained research results were processed by means of statistical tests with the Statistica 13.1 software. The results are presented as mean values, summarized in tables or graphs, with the standard deviation (±SD) indicated. The statistical significance of differences was tested using the Student's t-test and one-way analysis of variance (ANOVA). The normality of data distribution was assessed using the Shapiro-Wilk test. The Spearman method was applied to analyze the correlations between the parameters. The minimum level of significance was set at $p < 0.05$.

RESULTS

WBC and Leukogram

The mean white blood cell count in the peripheral blood in the control group was $6.19x10^3$ cells/µL, while in patients with atherosclerosis it was $6.92x10^3$ cells/µL.

No significant differences were observed in the numbers of individual leukocyte populations in peripheral blood between patients with atherosclerosis and the control group. A summary of the mean values of leukocyte populations in the control group and in patients with atherosclerosis is presented in Table 1.

Table 1. Basic diagnostic parameters of patients and the control group.

Group	**Control** *n*=21				**Patients** *n*=22			
Sex	Women *n*=10		Men *n*=11		Women *n*=11		Men *n*=11	
Parameter	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*
WBC [x10^3/μL]	**6.20**	*1.13*	**6.19**	*1.07*	**6.44**	*1.65*	**7.40**	*1.61*
Blood smear [%]:								
neutrophils	**49**	*10*	**57**	*6*	**59**	*11*	**57**	*6*
lymphocytes	**44**	*10*	**34**	*6*	**31**	*10*	**35**	*5*
monocytes	**5**	*2*	**6**	*2*	**7**	*2*	**6**	*2*
eosinophils	**2**	*1*	**2**	*1*	**4**	*2*	**2**	*1*
basophils	**0**	-	**0**	-	**0**	-	**0**	-

Biochemical Test Results in Atherosclerosis Patients

No significant differences were observed in the levels of the measured biochemical parameters between women and men. The mean levels of total cholesterol, LDL cholesterol, glucose, and homocysteine were found to have exceeded the reference values in women, and in the case of men the level of glucose was found to have exceeded the reference values. The case-by-case analysis has revealed that 55% of women and 12% of men had elevated total cholesterol levels. LDL cholesterol levels were above the reference values in 45% of women and 34% of men. High triglyceride levels were observed in 23% of women, while in men, the levels were within the reference range. Furthermore, 55% of both women and men had elevated glucose levels. The level of alkaline phosphatase in women was within the normal range. Alkaline phosphatase levels were reduced in 12% of men. The levels of total calcium and phosphorus were normal in the study group of women and men. Elevated homocysteine levels above the reference values were found in 67% of women and 50% of men. The results are summarized in Table 2.

Table 2. Patients' biochemical diagnostic tests.

Group	**Patients** *n*=22				
Sex	Women *n*=11		Men *n*=11		**References**
Parameter	Mean	*SD*	Mean	*SD*	
TC [mg/dL]	**213.67**↑	*42.84*	**173**	*35.87*	<190
LDL-C [mg/dL]	**121.33**↑	*42.22*	**101.11**	*25.37*	< 115 in the absence of risk factors; < 100 severe risk factors, diabetes without organ complications; < 70 with risk: disease
HDL-C [mg/dL]	**64.33**	*13.83*	**56.56**	*15.34*	>40
TG [mg/dL]	**116.33**	*65.78*	**88.67**	*21.01*	<150
Glc [mg/dL]	**105**↑	*14.35*	**105**↑	*9.03*	70-100
ALP [IU/L]	**58.89**	*14.95*	**53.63**	*17.38*	37-123
tCa [mmol/L]	**2.45**	*0.11*	**2.48**	*0.08*	2.25-2.75
PHOS [mmol/L]	**1.15**	*0.07*	**1.11**	*0.11*	0.74-1.52
HCY [µmol/L]	**16.49**↑	*6.42*	**13.24**	*4.37*	4.44-13.56

↑ - exceeding reference values. Abbreviations: TC, total cholesterol; LDL-C: low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglyceride; Glc, glucose; ALP, alkaline phenyl phosphatase; tCa, total calcium; PHOS, phosphorus; HCY, homocysteine.

NET Formation Analysis

The microscopic analysis has revealed that neutrophils in patients with atherosclerosis exhibit a significantly higher ability to spontaneously release extracellular traps as compared to neutrophils in healthy individuals (Table 3).

In the presence of LPS, neutrophils from both atherosclerosis patients and the control group produced a significantly higher amount of NETs, with values being higher in atherosclerosis patients as compared to the control group.

It has also been found that in both groups, preincubation of neutrophils in the presence of quercetin and luteolin significantly reduced NET release following LPS stimulation. The amount of NETs released from neutrophils after preincubation with luteolin was higher in patients as compared to the control group. Preincubation of neutrophils with vitamin C or with vitamin C combined with luteolin resulted in a reduction in NET release after LPS stimulation in the healthy group whereas no significant changes were observed in the patient group.

Neutrophils from healthy individuals preincubated with luteolin generated a significantly lower amount of NETs as compared to cells preincubated with quercetin or vitamin C.

The analysis of the results with regard to sex in the control group has revealed significantly higher amounts of NETs after LPS stimulation of neutrophils in women as compared to men.

Table 3. Percentage of neutrophils forming NETs.

Control	**Mean [%]**	**5.03**	**9.07[a]**	**5.53[b]**	**3.51[cf]**	**5.55[dg]**	**4.93[e]**
	SD	***1.95***	***2.09***	***1.18***	***0.57***	***1.32***	***2.26***
Patients	**Mean [%]**	**8.66***	**14.85*[a]**	**9.78[b]**	**9.69*[c]**	**10.24**	**11.57**
	SD	*1.47*	*1.58*	*5.14*	*4.82*	*5.9*	*8.69*
Incubation procedure	PMNs	+	+	+	+	+	+
	LPS	−	+	+	+	+	+
	QCT	−	−	+	−	−	−
	LT	−	−	−	+	−	+
	Vit C	−	−	−	−	+	+

Statistically significant differences ($p<0.5$): * – with control group, a – with unstimulated PMNs, b – between PMNs+LPS and PMNs+30'QCT+LPS, c – between PMNs+LPS and PMNs+30'LT+LPS, d – between PMNs+LPS and PMNs+30'VitC+LPS, e – between PMNs+LPS and PMNs+30'(VitC+LT)+LPS, f – between PMNs+30'LT+LPS and PMNs+30'QCT+LPS, g – between PMNs+30'LT+LPS and PMNs+30'VitC+LPS. Abbreviation: PMNs, neutrophils; LPS, lipopolysaccharide; QCT, quercetin; LT, luteolin; Vit C, vitamin C.

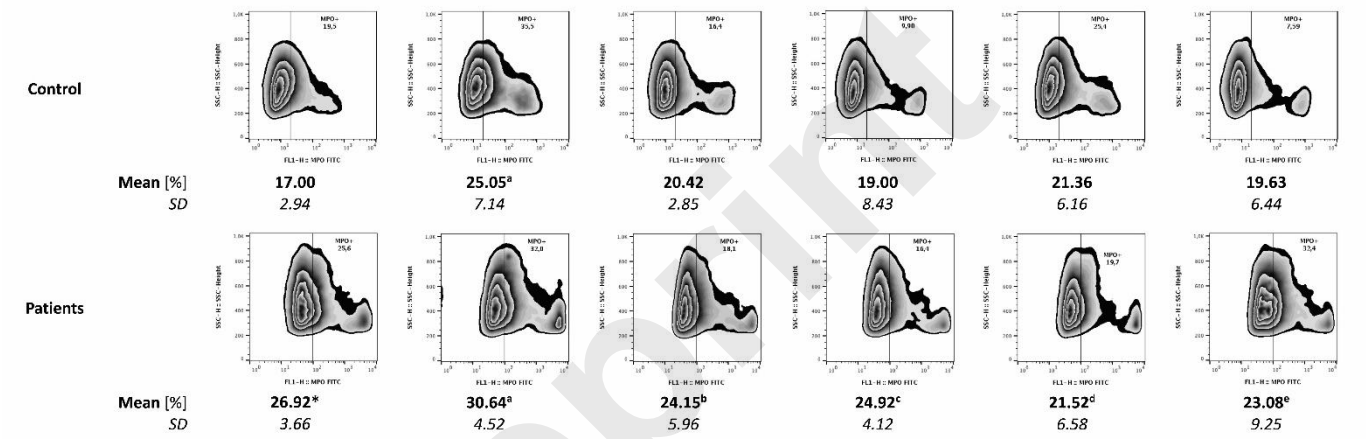
Quantification of MPO-Expressing Neutrophils

The Flow cytometry analysis (Figure 2) has revealed a higher percentage of MPO-positive (MPO+) neutrophils among unstimulated PMNs in patients with atherosclerosis as compared to healthy individuals.

The study results have revealed that in both the patient group and healthy individuals, LPS stimulation of cells significantly increased the percentage of MPO+ neutrophils as compared to unstimulated PMNs.

It has been observed that in patients, preincubation of neutrophils with flavonoids such as quercetin and luteolin, as well as vitamin C or vitamin C combined with luteolin, reduced the percentage of MPO+ neutrophils after LPS stimulation.

No differences have been observed in the results between the female and male groups.



Incubation procedure							
	PMNs	+	+	+	+	+	+
	LPS	–	+	+	+	+	+
	QCT	–	–	+	–	–	–
	LT	–	–	–	+	–	+
	Vit C	–	–	–	–	+	+

Figure 2. Percentage of MPO+ neutrophils in patient and control group.

Statistically significant differences ($p<0.5$): * – with control group, a – with unstimulated PMNs, b – between PMNs+LPS and PMNs+30'QCT+LPS, c – between PMNs+LPS and PMNs+30'LT+LPS, d – between PMNs+LPS and PMNs+30'VitC+LPS, e – between PMNs+LPS and PMNs+30'(VitC+LT)+LPS. Abbreviation: PMNs, neutrophils; LPS, lipopolysaccharide; QCT, quercetin; LT, luteolin; Vit C, vitamin C.

MPO Concentrations Assessment

No significant differences have been observed between the myeloperoxidase (MPO) concentration in the supernatants of unstimulated neutrophils from patients with atherosclerosis and the concentration in the cellular supernatants of healthy individuals (Table 4).

It has been observed that LPS stimulation of neutrophils resulted in a significant increase in MPO levels in the cellular supernatants, both in patients with atherosclerosis and in healthy individuals, as compared to the supernatants of unstimulated neutrophils.

It has been found that in both studied groups, preincubation of PMNs with quercetin, luteolin, vitamin C, and vitamin C combined with luteolin led to a significant reduction in MPO concentrations in the cellular supernatants as compared to the supernatants of PMNs after LPS stimulation.

The analysis of myeloperoxidase levels in blood serum has revealed significantly lower concentrations of that protein in the serum of patients with atherosclerosis as compared to the values obtained in the serum of healthy individuals.

No differences have been observed between the MPO concentrations obtained in women and those in men.

Table 4. Concentration of MPO, cfDNA, and superoxide anion in patient and control samples.

Mean *SD* Group	PMNs	PMNs+LPS	PMNs+30'QCT+LPS	PMNs+30'LT+LPS	PMNs+30'Vit C+LPS	PMNs+30'(Vit C+LT)+LPS	Serum
MPO [ng/mL]							
Control	**28.08** *4.25*	**38.57**[a] *6.23*	**27.89**[b] *3.68*	**28.19**[c] *4.11*	**28.09**[d] *4.20*	**27.73**[e] *4.36*	**556.4** *339.2*
Patients	**30.28** *3.65*	**35.97**[a] *8.01*	**27.69**[b] *4.62*	**29.11**[c] *4.17*	**28.76**[d] *4.07*	**27.71**[e] *4.04*	**273.3*** *108.2*
cfDNA [ng/mL]							
Control	**24.36** *5.93*	**39.20**[a] *17.01*	**28.52** *8.50*	**27.90**[c] *11.91*	**28.87**[d] *7.40*	**28.10** *16.51*	**27.91** *6.29*
Patients	**30.42*** *7.82*	**36.39**[a] *10.19*	**27.33**[b] *10.19*	**29.57** *12.72*	**27.27**[d] *7.06*	**31.25** *7.70*	**24.30*** *2.94*
Superoxide anion radical [mmol/$L^{-1}cm^{-1}$]							
Control	**2.62** *1.14*	**4.52**[a] *1.42*	**2.87** *1.86*	**3.68** *0.19*	**1.27**[dg] *0.48*	**3.13** *2.35*	-
Patients	**2.89** *0.60*	**7.63***[a] *2.38*	**4.34**[b] *1.73*	**4.48**[c] *1.54*	**4.06***[d] *1.51*	**1.82**[eijk] *0.61*	-

Statistically significant differences ($p<0.5$): * – with control group, a – with unstimulated PMNs, b – between PMNs+LPS and PMNs+30'QCT+LPS, c – between PMNs+LPS and PMNs+30'LT+LPS, d – between PMNs+LPS and PMNs+30'Vit C+LPS, e – between PMNs+LPS and PMNs+30'(LT+Vit C)+LPS, g – between PMNs+30'LT+LPS and PMNs+30'Vit C+LPS, i – between PMNs+30'QCT+LPS and PMNs+30'(Vit C+LT)+LPS, j – between PMNs+30'LT+LPS and PMNs+30'(Vit C+LT)+LPS, k – between PMNs+30'Vit C+LPS and PMNs+30'(Vit C+LT)+LPS. Abbreviation: PMNs, neutrophils; LPS, lipopolysaccharide; QCT, quercetin; LT, luteolin; Vit C, vitamin C; MPO, myeloperoxidase; cfDNA, cell free DNA.

cfDNA Concentrations Assessment

The assessment of free circulating DNA levels has revealed significantly higher concentrations in the supernatants of unstimulated neutrophils from patients with atherosclerosis compared to the values obtained in the control group (Table 4).

It has been observed that both in patients with atherosclerosis and in healthy individuals, the cfDNA level in the supernatants of PMNs after LPS stimulation was significantly higher as compared to the concentration in the supernatants of unstimulated neutrophils.

It has been found that in both studied groups, preincubation of neutrophils with vitamin C contributed to a decrease in cfDNA concentration in the supernatants of those cells after LPS stimulation. In patients with atherosclerosis, lower cfDNA concentrations have also been observed in the supernatants of neutrophils preincubated with quercetin. However, in the healthy group, preincubation of PMNs with luteolin resulted in a re-duction of cfDNA levels in the cellular supernatants.

The analysis of cfDNA in the whole blood serum has revealed lower concentrations of the NETs marker in the serum of patients with atherosclerosis as compared to the values obtained in the serum of healthy individuals.

No significant differences have been observed in cfDNA concentrations between the values in women and men.

Assessment of Superoxide Anion ($O_2^{\bullet-}$) Generation

The results of the studies have not shown significant changes in the amount of superoxide anion in the supernatants of unstimulated neutrophils from patients as compared to the control group (Table 4).

It has been observed that both in patients with atherosclerosis and in healthy individuals, PMNs released higher amounts of $O_2^{\bullet-}$ after LPS stimulation as compared to unstimulated cells, with significantly higher values in patients.

It has been shown that preincubation of PMNs from patients with antioxidant compounds such as quercetin, luteolin, vitamin C, or vitamin C combined with luteolin led to a reduction in the release of $O_2^{\bullet-}$ by those cells as compared to PMNs after LPS stimulation.

In the control group, the amount of $O_2^{\bullet-}$ in the supernatants of neutrophils preincubated with vitamin C was lower as compared to the amount obtained in the supernatants of PMNs preincubated with luteolin.

In the patient group, neutrophils preincubated concurrently with vitamin C and luteolin generated a lower amount of superoxide anion as compared to cells preincubated with quercetin, luteolin alone, or vitamin C alone.

No differences have been observed in the amount of generated $O_2^{\bullet-}$ between the female and male groups.

NADPH Oxidase Subunit Expression

The study results have shown that unstimulated neutrophils from patients with atherosclerosis exhibited a higher expression of the p47phox and p67phox subunits of NADPH oxidase as compared to unstimulated PMNs from healthy individuals (Figure 3).

No significant changes in the Rac1 protein expression have been observed in un-stimulated neutrophils from patients with atherosclerosis as compared to the expression obtained in unstimulated cells from healthy individuals.

It has been observed that LPS stimulation of neutrophils from healthy individuals resulted in a significant increase in the p47phox and p67phox expression as compared to unstimulated cells. LPS stimulation of neutrophils from patients with atherosclerosis enhanced the expression of those proteins; however, the difference was not statistically significant. After LPS stimulation, the p47phox and p67phox expression was significantly higher in patients as compared to the expression in cells from healthy individuals. In the case of Rac1, LPS stimulation led to a significant increase in the expression of that protein only in healthy individuals.

It has been found that in both study groups, preincubation of neutrophils with luteolin or vitamin C contributed to a reduction in the expression of p47phox and Rac1 in PMNs incubated with LPS as compared to cells incubated only with LPS. Additionally, in patients with atherosclerosis, a significantly higher expression of p47phox has been observed in neutrophils preincubated with luteolin and vitamin C as compared to cells from healthy individuals. Neutrophils from patients preincubated with quercetin showed a lower p47phox expression as compared to cells stimulated with LPS. In the control group, preincubation of neutrophils with vitamin C and luteolin reduced the expression of the studied protein as compared to LPS stimulation. The p47phox expression was significantly higher in neutrophils preincubated with vitamin C and luteolin in patients with atherosclerosis as compared to the expression in healthy individuals.

In the control group, a significantly lower expression of the p47phox protein has also been observed in neutrophils preincubated with luteolin as compared to cells preincubated with quercetin.

The studies have shown that in both groups, preincubation of neutrophils with quercetin or luteolin resulted in a reduction of the p67phox expression as compared to neutrophils stimulated with LPS.

In patients with atherosclerosis, a reduction in the Rac1 expression has also been observed in neutrophils preincubated with vitamin C and vitamin C combined with luteolin as compared to cells stimulated with LPS.

In the patient group, a lower expression of the Rac1 protein has been observed in neutrophils preincubated with vitamin C as compared to cells preincubated with quercetin.

No differences have been found in the expression of the p47phox, p67phox, and Rac1 proteins between the female and male groups.

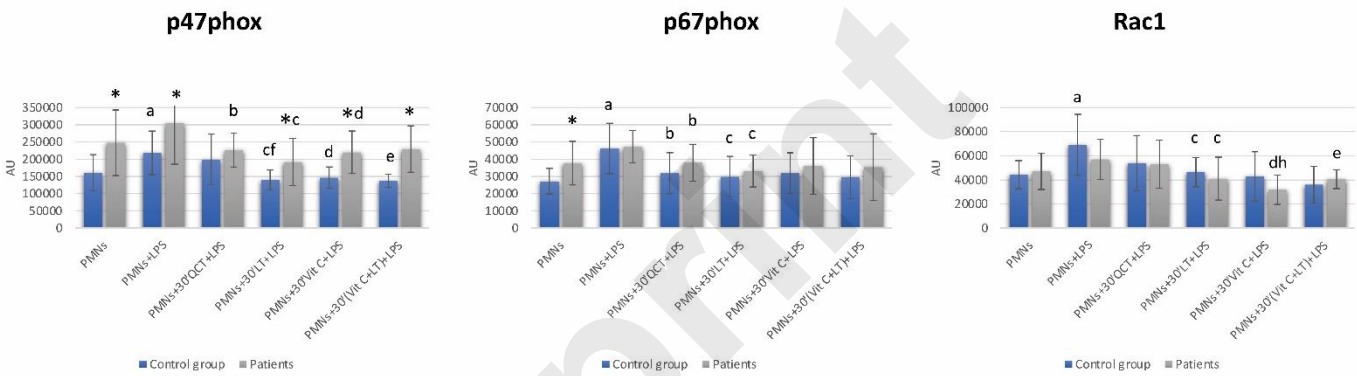


Figure 3. Expression of NADPH oxidase subunits in neutrophils of patients and healthy individuals.

Statistically significant differences ($p<0.5$): * – with control group, a – with unstimulated PMNs, b – between PMNs+LPS and PMNs+30'QCT+LPS, c – between PMNs+LPS and PMNs+30'LT+LPS, d – between PMNs+LPS and PMNs+30'VitC+LPS, e – between PMNs+LPS and PMNs+30'(VitC+LT)+LPS, f – between PMNs+30'LT+LPS and PMNs+30'QCT+LPS, g – between PMNs+30'LT+LPS and PMNs+30'VitC+LPS, h – between PMNs+30'QCT+LPS and PMNs+30'VitC+LPS. Abbreviation: PMNs, neutrophils; LPS, lipopolysaccharide; QCT, quercetin; LT, luteolin; Vit C, vitamin C

Assessment of Parameter Correlations

A positive correlation has been observed between the concentrations of cfDNA and MPO in the supernatants of unstimulated neutrophils in the control group (r=0,63; p=0,002) [Figure 4A]. No such correlation has been observed in patients with atherosclerosis.

A positive correlation has been found between the amount of cfDNA and MPO in the serum of healthy individuals (r=0,77; p=0,001) [Figure 4B]. No such correlation has been found in the group of patients with atherosclerosis.

A positive correlation has been found in the healthy group between the concentration of free circulating DNA in the supernatants of PMNs and its concentration in serum (r=0,749; p=0,001) [Figure 4C]. No such correlation has been observed in the patient group.

In healthy individuals, a positive correlation has been found between the concentrations of myeloperoxidase in the supernatants of PMNs and in the serum (r=0,751; p=0,0001) [Figure 4D]. In contrast, in the group of patients with atherosclerosis, an inverse correlation has been found between the concentration of MPO in the neutrophil supernatants and the concentration of that protein in the serum (r=-0,588; p=0,005) [Figure 4E].

No correlation between age and the analyzed parameters was found in any of the study groups.

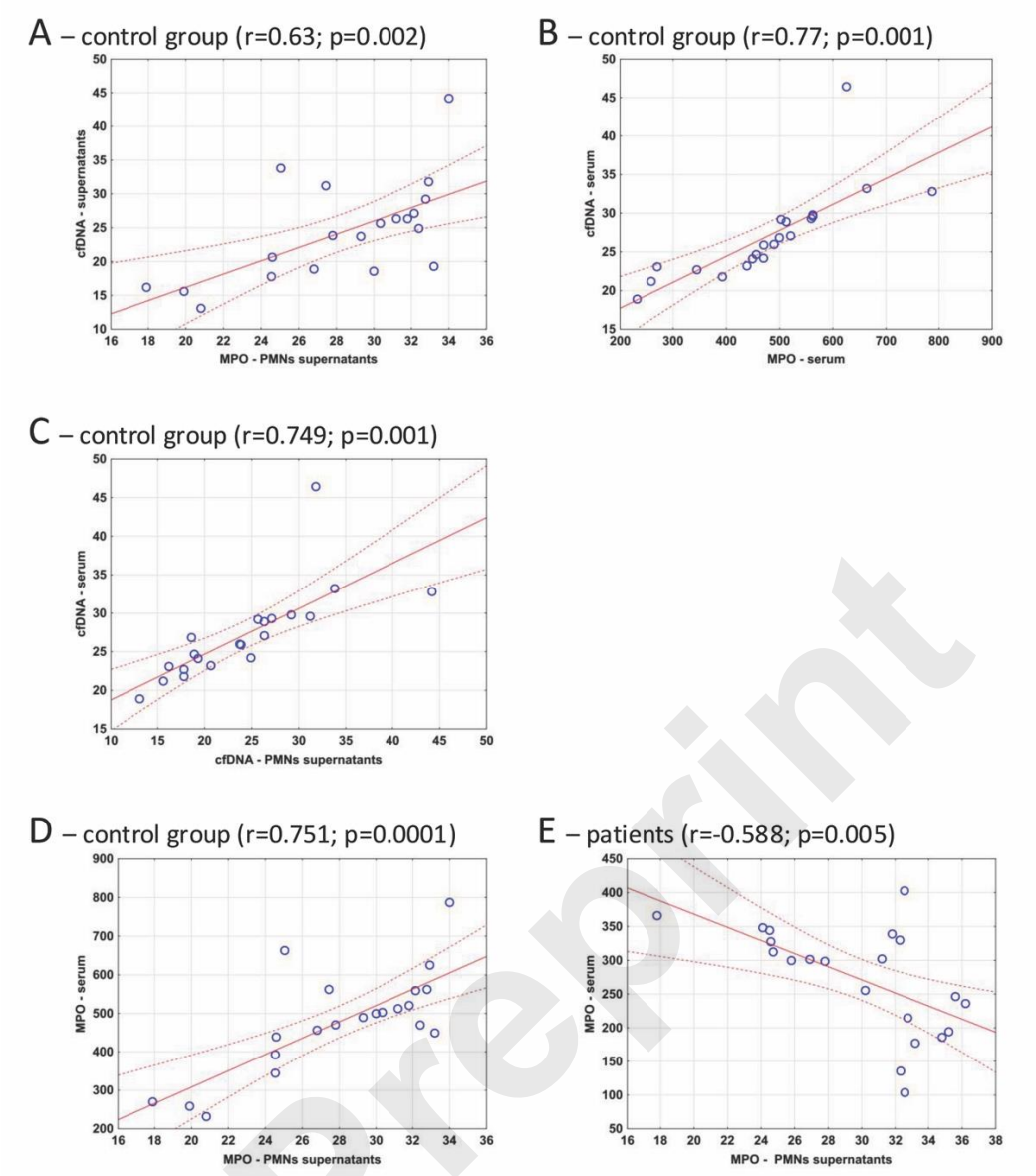


Figure 4. Correlations.

Correlations between concentrations of: A – cfDNA and MPO in supernatants of unstimulated neutrophils in the control group; B – cfDNA and MPO in serum in the control group; C – cfDNA in neutrophil supernatants and cfDNA in serum in the control group; D – MPO in neutrophil supernatants and MPO in serum in the control group; E – MPO in neutrophil supernatants and MPO in serum in patients with atherosclerosis.

DISCUSSION

There are studies indicating that the phenomenon of generating NETs constitutes a potential factor in the development of atherosclerosis[24]. Knight et al. evidenced the neutrophils isolated from mice with atherosclerosis to be more prone to NETs formation than healthy animals[22]. The studies on NET formation in humans were conducted by Megens et al. who detected NETs in patients undergoing endarterectomy[25]. The results of our own studies also suggest a potential role of NETs in the course of atherosclerosis, demonstrated by increased release of NETs observed under the microscope and a rise in the levels of the MPO and cfDNA. The observed changes were not related to the age of the patients, which is confirmed by previous studies[26].

A high number of neutrophils containing MPO and the enhanced ability of those cells to release MPO may directly influence the development of atherosclerosis. It is known that hypochlorous acid, formed in a reaction catalyzed by MPO, is responsible for the oxidative modification of LDL. Those modified LDL particles may be taken up by macrophages, leading to their transformation into foam cells that are part of atherosclerotic plaques[27]. It has also been shown that elevated levels of MPO are associated with an increased risk of coronary artery disease[28], and high MPO levels in plasma may even serve as a biomarker for acute myocardial infarction[29]. Excessive MPO levels may also lead to the increased activation of the NETosis process[30,31,32]. Similar unfavourable consequences may be associated with the significant increase in cfDNA concentrations. Jylhävä et al., showed that increased cfDNA levels correlated with a group of cardiometabolic risk factors, such as high blood pressure, unfavorable lipid metabolism profile, and systemic inflammation[33]. Free circulating DNA may also contribute to the intensification of coagulation processes or impaired fibrinolysis, which is crucial in the pathogenesis of cardiovascular diseases[34].

The role of MPO and cfDNA in the development and progression of atherosclerosis is also supported by the studies of Borissoff et al., who showed that the levels of double-stranded DNA (dsDNA), nucleosomes and MPO-DNA complexes are significantly increased in patients with

severe coronary atherosclerosis[35]. Lower levels of cfDNA and MPO observed in the blood serum of the studied patient group, along with higher levels in the supernatants of PMNs, may be a result of their binding in the atherosclerotic plaque.

The significance of NETs formation in the course of atherosclerosis is also highlighted by the studies indicating vascular damage in atherosclerosis, preceded by intense recruitment and activation of neutrophils[36]. It has been observed that direct co-culture of activated endothelial cells with neutrophils induces NETs formation, and leads to vascular damage[36,37].

Warnatsch et al. proved that NETs may stimulate macrophages to release IL-1β, which activates Th17 lymphocytes[38]. Th17 lymphocytes, by enhancing the recruitment of other immune cells to atherosclerotic plaques may contribute to the development of inflammation in blood vessels. Furthermore, NETs may affect the activation of plasmacytoid dendritic cells present in atherosclerotic plaques that release large amounts of IFN-α, also promoting the development of atherosclerotic lesions[39].

It has been proven that the activity of NADPH oxidase, which is also essential for the formation and release of NETs, is a factor influencing the development of atherosclerosis. The results of the studies on neutrophils from patients with chronic granulomatous disease, characterized by a genetic defect in NADPH oxidase, also confirm this[12,40–42]. There is evidence that ROS cause a series of molecular changes that lead to the infiltration of the endothelium by inflammatory cells, ultimately promoting the formation of an atherosclerotic plaque. ROS also affect arterial dysfunction by inactivating nitric oxide, a potent vasodilator[43]. The results of our own studies confirm the relationship between the increased NET formation and the elevated NADPH oxidase activity in neutrophils from approximately 50% of patients with atherosclerosis.

The causes of the increased NET formation in patients with atherosclerosis may have various origins. One of them seems to be the elevated levels of total cholesterol and LDL, observed in a

significant percentage of the studied group of patients. Warnatsch et al. in a mouse model of atherosclerosis demonstrated that cholesterol crystals influence the induction of NETosis[38]. Brinkmann et al. indicated that the generation of NETs may also be regulated by PMA or LPS[6]. Our own studies confirm that in patients with atherosclerosis LPS activates the NETs production, confirmed by an increase in the number of MPO-positive neutrophils and a rise in cfDNA concentration in the supernatants of cells.

Given the adverse consequences of the increased NETs formation within the course of atherosclerosis, it seems important to explore ways to regulate their generation[44–49]. Mohammed et al., demonstrated an increased formation of NETs in animals with a deficiency of ascorbic acid. Furthermore, human neutrophils stimulated with PMA, supplemented with vitamin C, had a reduced ability to generate NETs[48].

There are data regarding the regulation of different neutrophil functions through flavonoids, such as quercetin and luteolin[50-53]. However, there are limited data on the role of these compounds in the regulation of NETs formation. One of the few examples is the study by Yang et al., who observed that luteolin significantly impaired NET formation by human neutrophils[54].

The results of our studies have shown that in patients with atherosclerosis, the presence of both quercetin and luteolin, as well as luteolin combined with vitamin C, led to a reduced formation of NETs by neutrophils stimulated with LPS.

Similar results were obtained by Pečivová et al., who showed that quercetin leads to a reduction in MPO release by neutrophils after PMA stimulation[55]. Yang et al. demonstrated that the anti-inflammatory effect of luteolin is associated with the removal of ROS and inhibition of the Raf1-MEK-1-ERK signaling pathway, which regulates the formation of NETs[54]. It has prompted us to investigate the combined effect of luteolin and vitamin C, which, in the case of superoxide anion generation, has been more effective than the presence of either luteolin or vitamin C alone. It should

be noted here that the effect of flavonoids on human health is not clear; some studies confirm the protective effect of flavonoids on cardiovascular diseases or cancer, other data indicate no effect, and some reports demonstrate potential harm[56].

CONCLUSION

The obtained research results confirm that excessive formation of neutrophil extracellular traps, which may be one of the key causes of development of atherosclerosis. We indicated the beneficial role of quercetin, luteolin and vitamin C in inhibiting the release of NETs and, therefore, their potential importance in limiting the development of this disease. Preventing the excessive formation of NETs seems to be the right direction of research that could be used in practice in the future to bring many beneficial clinical effects in patients suffering from atherosclerosis.

Limitations

This study has several limitations. First, the number of patients included was relatively small, which may limit the statistical power and generalizability of the findings. The recruitment was constrained by the duration of the project and strict inclusion/exclusion criteria. Second, the experimental procedures were conducted exclusively *in vitro*, which, while useful for mechanistic insight, may not fully reflect the complex *in vivo* environment. Therefore, the observed effects of flavonoids and vitamin C on NET formation and oxidative responses in neutrophils should be interpreted with caution. Further research involving larger patient cohorts and *in vivo* models is necessary to validate these findings and assess their clinical relevance.

Reference

1. Cardiovascular diseases (CVDs). Accessed November 26, 2024. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)

2. Netala VR, Teertam SK, Li H, Zhang Z. A Comprehensive Review of Cardiovascular Disease Management: Cardiac Biomarkers, Imaging Modalities, Pharmacotherapy, Surgical Interventions, and Herbal Remedies. *Cells*. 2024;13(17):1471. doi:10.3390/cells13171471

3. Ozdemir E, Stavileci B, Ozdemir B, et al. The association between growth differentiation factor 15 and presence and severity of coronary atherosclerosis. *Adv Med Sci*. 2024;69(1):56-60. doi:10.1016/j.advms.2024.02.003

4. Ionita MG, van den Borne P, Catanzariti LM, et al. High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. *Arterioscler Thromb Vasc Biol*. 2010;30(9):1842-1848. doi:10.1161/ATVBAHA.110.209296

5. Baetta R, Corsini A. Role of polymorphonuclear neutrophils in atherosclerosis: current state and future perspectives. *Atherosclerosis*. 2010;210(1):1-13. doi:10.1016/j.atherosclerosis.2009.10.028

6. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303(5663):1532-1535. doi:10.1126/science.1092385

7. Dwivedi N, Neeli I, Schall N, et al. Deimination of linker histones links neutrophil extracellular trap release with autoantibodies in systemic autoimmunity. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2014;28(7):2840-2851. doi:10.1096/fj.13-247254

8. Urban CF, Ermert D, Schmid M, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog*. 2009;5(10):e1000639. doi:10.1371/journal.ppat.1000639

9. Jaillon S, Peri G, Delneste Y, et al. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J Exp Med*. 2007;204(4):793-804. doi:10.1084/jem.20061301

10. Wartha F, Beiter K, Normark S, Henriques-Normark B. Neutrophil extracellular traps: casting the NET over pathogenesis. *Curr Opin Microbiol*. 2007;10(1):52-56. doi:10.1016/j.mib.2006.12.005

11. Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol*. 2006;8(4):668-676. doi:10.1111/j.1462-5822.2005.00659.x

12. Fuchs TA, Abed U, Goosmann C, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007;176(2):231-241. doi:10.1083/jcb.200606027

13. Hakkim A, Fuchs TA, Martinez NE, et al. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol*. 2011;7(2):75-77. doi:10.1038/nchembio.496

14. McCormick A, Heesemann L, Wagener J, et al. NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. *Microbes Infect*. 2010;12(12-13):928-936. doi:10.1016/j.micinf.2010.06.009

15. Abi Abdallah DS, Lin C, Ball CJ, King MR, Duhamel GE, Denkers EY. Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. *Infect Immun*. 2012;80(2):768-777. doi:10.1128/IAI.05730-11

16. Guimarães-Costa AB, Nascimento MTC, Froment GS, et al. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A*. 2009;106(16):6748-6753. doi:10.1073/pnas.0900226106

17. Saitoh T, Komano J, Saitoh Y, et al. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe*. 2012;12(1):109-116. doi:10.1016/j.chom.2012.05.015

18. Narasaraju T, Yang E, Samy RP, et al. Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J Pathol*. 2011;179(1):199-210. doi:10.1016/j.ajpath.2011.03.013

19. Kaplan MJ, Radic M. Neutrophil extracellular traps: double-edged swords of innate immunity. *J Immunol Baltim Md 1950*. 2012;189(6):2689-2695. doi:10.4049/jimmunol.1201719

20. Shi D, Huang J, Wu J. Down-regulation of SHP2 promotes neutrophil autophagy and inhibits neutrophil extracellular trap formation to alleviate asthma through the ERK5 pathway. *Cent Eur J Immunol*. 2024;49(1):252-272. doi:10.5114/ceji.2024.143691

21. Dąbrowska D, Jabłońska E, Garley M, Sawicka-Powierza J, Nowak K. The Phenomenon of Neutrophil Extracellular Traps in Vascular Diseases. *Arch Immunol Ther Exp (Warsz).* 2018;66(4):273-281. doi:10.1007/s00005-018-0505-y

22. Knight JS, Luo W, O'Dell AA, et al. Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. *Circ Res*. 2014;114(6):947-956. doi:10.1161/CIRCRESAHA.114.303312

23. Bhuyan KC, Bhuyan DK. Superoxide dismutase of the eye Relative functions of superoxide dismutase and catalase in protecting the ocular lens from oxidative damage. *Biochim Biophys Acta BBA - Gen Subj*. 1978;542(1):28-38. doi:10.1016/0304-4165(78)90229-5

24. Manda A, Pruchniak MP, Araźna M, Demkow UA. Neutrophil extracellular traps in physiology and pathology. *Cent-Eur J Immunol*. 2014;39(1):116-121. doi:10.5114/ceji.2014.42136

25. Megens RTA, Vijayan S, Lievens D, et al. Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thromb Haemost*. 2012;107(3):597-598. doi:10.1160/TH11-09-0650

26. Garley M, Omeljaniuk WJ, Motkowski R, et al. Immunoaging – the effect of age on serum levels of NET biomarkers in men: a pilot study. *Int J Occup Med Environ Health*. 2023;36(3):333-348. doi:10.13075/ijomeh.1896.02125

27. Hazell LJ, Stocker R. Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. *Biochem J*. 1993;290 (Pt 1)(Pt 1):165-172. doi:10.1042/bj2900165

28. Zhang R, Brennan ML, Fu X, et al. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA*. 2001;286(17):2136-2142. doi:10.1001/jama.286.17.2136

29. Brennan ML, Penn MS, Van Lente F, et al. Prognostic value of myeloperoxidase in patients with chest pain. *N Engl J Med*. 2003;349(17):1595-1604. doi:10.1056/NEJMoa035003

30. Parker H, Dragunow M, Hampton MB, Kettle AJ, Winterbourn CC. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J Leukoc Biol*. 2012;92(4):841-849. doi:10.1189/jlb.1211601

31. Palmer LJ, Cooper PR, Ling MR, Wright HJ, Huissoon A, Chapple ILC. Hypochlorous acid regulates neutrophil extracellular trap release in humans. *Clin Exp Immunol*. 2012;167(2):261-268. doi:10.1111/j.1365-2249.2011.04518.x

32. Metzler KD, Fuchs TA, Nauseef WM, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood*. 2011;117(3):953-959. doi:10.1182/blood-2010-06-290171

33. Jylhävä J, Lehtimäki T, Jula A, et al. Circulating cell-free DNA is associated with cardiometabolic risk factors: the Health 2000 Survey. *Atherosclerosis*. 2014;233(1):268-271. doi:10.1016/j.atherosclerosis.2013.12.022

34. Gould TJ, Lysov Z, Liaw PC. Extracellular DNA and histones: double-edged swords in immunothrombosis. *J Thromb Haemost JTH*. 2015;13 Suppl 1:S82-91. doi:10.1111/jth.12977

35. Borissoff JI, Joosen IA, Versteylen MO, et al. Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. *Arterioscler Thromb Vasc Biol*. 2013;33(8):2032-2040. doi:10.1161/ATVBAHA.113.301627

36. Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A*. 2010;107(36):15880-15885. doi:10.1073/pnas.1005743107

37. Gupta AK, Joshi MB, Philippova M, et al. Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. *FEBS Lett*. 2010;584(14):3193-3197. doi:10.1016/j.febslet.2010.06.006

38. Warnatsch A, Ioannou M, Wang Q, Papayannopoulos V. Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science*. 2015;349(6245):316-320. doi:10.1126/science.aaa8064

39. Döring Y, Manthey HD, Drechsler M, et al. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation*. 2012;125(13):1673-1683. doi:10.1161/CIRCULATIONAHA.111.046755

40. Bianchi M, Hakkim A, Brinkmann V, et al. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood*. 2009;114(13):2619-2622. doi:10.1182/blood-2009-05-221606

41. Lim MBH, Kuiper JWP, Katchky A, Goldberg H, Glogauer M. Rac2 is required for the formation of neutrophil extracellular traps. *J Leukoc Biol*. 2011;90(4):771-776. doi:10.1189/jlb.1010549

42. Röhm M, Grimm MJ, D'Auria AC, Almyroudis NG, Segal BH, Urban CF. NADPH oxidase promotes neutrophil extracellular trap formation in pulmonary aspergillosis. *Infect Immun*. 2014;82(5):1766-1777. doi:10.1128/IAI.00096-14

43. Violi F, Basili S, Nigro C, Pignatelli P. Role of NADPH oxidase in atherosclerosis. *Future Cardiol*. 2009;5(1):83-92. doi:10.2217/14796678.5.1.83

44. Levy R, Schlaeffer F. Successful treatment of a patient with recurrent furunculosis by vitamin C: improvement of clinical course and of impaired neutrophil functions. *Int J Dermatol*. 1993;32(11):832-834. doi:10.1111/j.1365-4362.1993.tb02780.x

45. Vohra K, Khan AJ, Telang V, Rosenfeld W, Evans HE. Improvement of neutrophil migration by systemic vitamin C in neonates. *J Perinatol Off J Calif Perinat Assoc*. 1990;10(2):134-136.

46. de la Fuente M, Ferrández MD, Burgos MS, Soler A, Prieto A, Miquel J. Immune function in aged women is improved by ingestion of vitamins C and E. *Can J Physiol Pharmacol*. 1998;76(4):373-380.

47. Bozonet SM, Carr AC, Pullar JM, Vissers MCM. Enhanced human neutrophil vitamin C status, chemotaxis and oxidant generation following dietary supplementation with vitamin C-rich SunGold kiwifruit. *Nutrients*. 2015;7(4):2574-2588. doi:10.3390/nu7042574

48. Mohammed BM, Fisher BJ, Kraskauskas D, et al. Vitamin C: a novel regulator of neutrophil extracellular trap formation. *Nutrients*. 2013;5(8):3131-3151. doi:10.3390/nu5083131

49. Kirchner T, Hermann E, Möller S, et al. Flavonoids and 5-aminosalicylic acid inhibit the formation of neutrophil extracellular traps. *Mediators Inflamm*. 2013;2013:710239. doi:10.1155/2013/710239

50. Santos EOL, Kabeya LM, Figueiredo-Rinhel ASG, et al. Flavonols modulate the effector functions of healthy individuals' immune complex-stimulated neutrophils: a therapeutic perspective for rheumatoid arthritis. *Int Immunopharmacol*. 2014;21(1):102-111. doi:10.1016/j.intimp.2014.04.014

51. Liu G, Wang W, Masuoka N, et al. Effect of three flavonoids isolated from Japanese Polygonum species on superoxide generation in human neutrophils. *Planta Med*. 2005;71(10):933-937. doi:10.1055/s-2005-871283

52. Lu HW, Sugahara K, Sagara Y, et al. Effect of three flavonoids, 5,7,3',4'-tetrahydroxy-3-methoxy flavone, luteolin, and quercetin, on the stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophil. *Arch Biochem Biophys*. 2001;393(1):73-77. doi:10.1006/abbi.2001.2457

53. Lu J, Feng X, Sun Q, et al. Effect of six flavonoid compounds from Ixeris sonchifolia on stimulus-induced superoxide generation and tyrosyl phosphorylation in human neutrophils. *Clin Chim Acta Int J Clin Chem*. 2002;316(1-2):95-99. doi:10.1016/s0009-8981(01)00725-2

54. Yang SC, Chen PJ, Chang SH, et al. Luteolin attenuates neutrophilic oxidative stress and inflammatory arthritis by inhibiting Raf1 activity. *Biochem Pharmacol*. 2018;154:384-396. doi:10.1016/j.bcp.2018.06.003

55. Pečivová J, Mačičková T, Sviteková K, Nosáľ R. Quercetin inhibits degranulation and superoxide generation in PMA stimulated neutrophils. *Interdiscip Toxicol*. 2012;5(2):81-83. doi:10.2478/v10102-012-0014-5

56. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr*. 2002;22:19-34. doi:10.1146/annurev.nutr.22.111401.144957

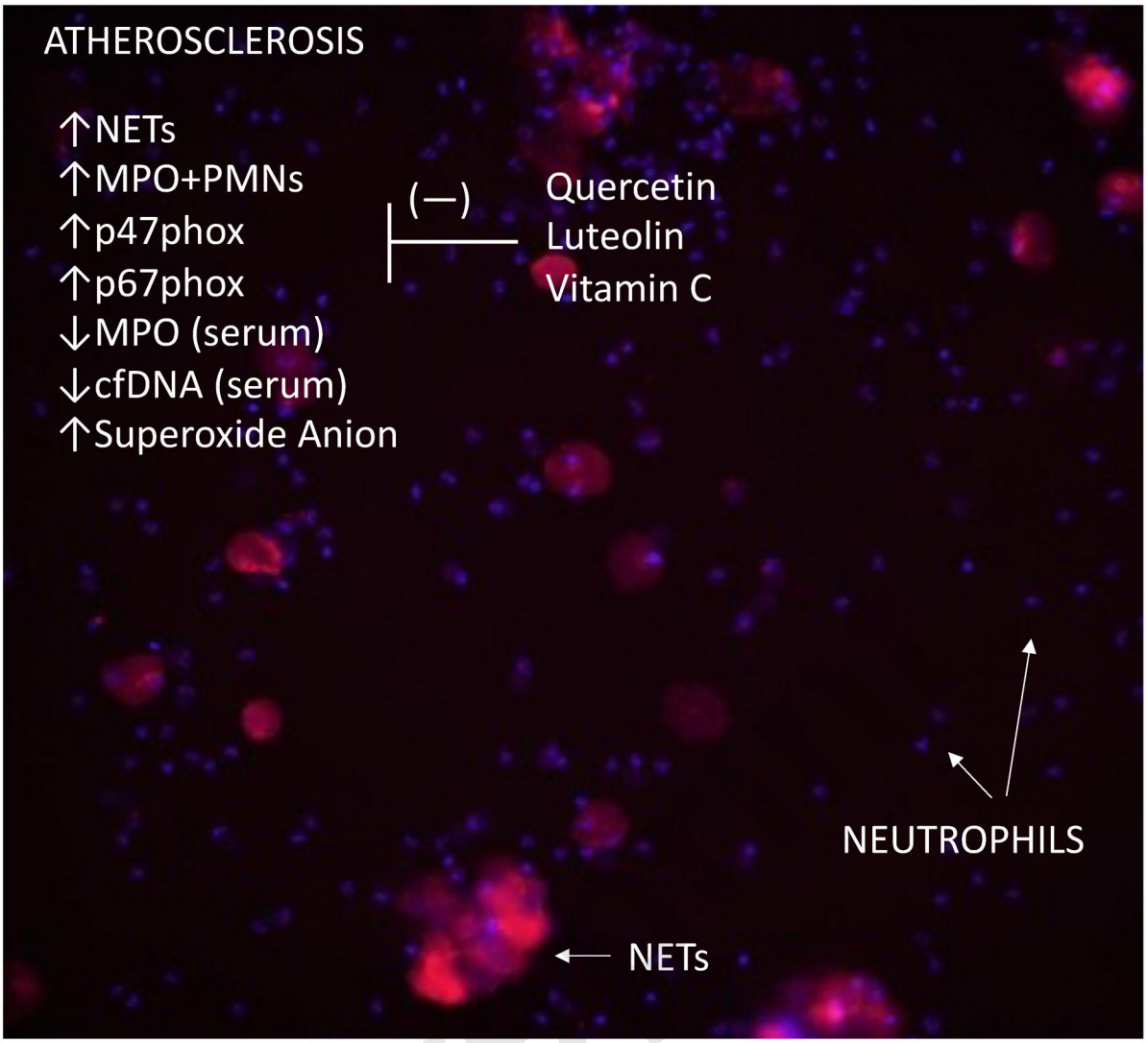

ATHEROSCLEROSIS
↑NETs
↑MPO+PMNs
↑p47phox
↑p67phox
↓MPO (serum)
↓cfDNA (serum)
↑Superoxide Anion
(—)
Quercetin
Luteolin
Vitamin C
NEUTROPHILS
NETs

Table 1. Basic diagnostic parameters of patients and the control group.

Group	**Control** *n*=21				**Patients** *n*=22			
Sex	Women *n*=10		Men *n*=11		Women *n*=11		Men *n*=11	
Parameter	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*
WBC [x10^3/μL]	**6.20**	*1.13*	**6.19**	*1.07*	**6.44**	*1.65*	**7.40**	*1.61*
Blood smear [%]:								
neutrophils	**49**	*10*	**57**	*6*	**59**	*11*	**57**	*6*
lymphocytes	**44**	*10*	**34**	*6*	**31**	*10*	**35**	*5*
monocytes	**5**	*2*	**6**	*2*	**7**	*2*	**6**	*2*
eosinophils	**2**	*1*	**2**	*1*	**4**	*2*	**2**	*1*
basophils	**0**	-	**0**	-	**0**	-	**0**	-
TC [mg/dL]	References <190				**213.67***	*42.84*	**173**	*35.87*
LDL-C [mg/dL]	< 115 in the absence of risk factors; < 100 severe risk factors, diabetes without organ complications; < 70 with risk: disease				**121.33***	*42.22*	**101.11**	*25.37*
HDL-C [mg/dL]	>40				**64.33**	*13.83*	**56.56**	*15.34*
TG [mg/dL]	<150				**116.33**	*65.78*	**88.67**	*21.01*
Glc [mg/dL]	70-100				**105***	*14.35*	**105***	*9.03*
ALP [IU/L]	37-123				**58.89**	*14.95*	**53.63**	*17.38*
tCa [mmol/L]	2.25-2.75				**2.45**	*0.11*	**2.48**	*0.08*
PHOS [mmol/L]	0.74-1.52				**1.15**	*0.07*	**1.11**	*0.11*
HCY [μmol/L]	4.44-13.56				**16.49***	*6.42*	**13.24**	*4.37*

* - exceeding reference values

Abbreviations: TC, total cholesterol; LDL-C: low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglyceride; Glc, glucose; ALP, alkaline phenyl phosphatase; tCa, total calcium; PHOS, phosphorus; HCY, homocysteine.

Table 2. Percentage of neutrophils forming NETs.

Control	**Mean** [%] *SD*	**5.03** *1.95*	**9.07**[a] *2.09*	**5.53**[b] *1.18*	**3.51**[cf] *0.57*	**5.55**[dg] *1.32*	**4.93**[e] *2.26*
Patients	**Mean** [%] *SD*	**8.66*** *1.47*	**14.85***[a] *1.58*	**9.78**[b] *5.14*	**9.69***[c] *4.82*	**10.24** *5.9*	**11.57** *8.69*
Incubation procedure	PMNs	+	+	+	+	+	+
	LPS	–	+	+	+	+	+
	QCT	–	–	+	–	–	–
	LT	–	–	–	+	–	+
	Vit C	–	–	–	–	+	+

Statistically significant differences ($p<0.5$): * – with control group, a – with unstimulated PMNs, b – between PMNs+LPS and PMNs+30'QCT+LPS, c – between PMNs+LPS and PMNs+30'LT+LPS, d – between PMNs+LPS and PMNs+30'VitC+LPS, e – between PMNs+LPS and PMNs+30'(VitC+LT)+LPS, f – between PMNs+30'LT+LPS and PMNs+30'QCT+LPS, g – between PMNs+30'LT+LPS and PMNs+30'VitC+LPS.

Abbreviation: PMNs, neutrophils; LPS, lipopolysaccharide; QCT, quercetin; LT, luteolin; Vit C, vitamin C

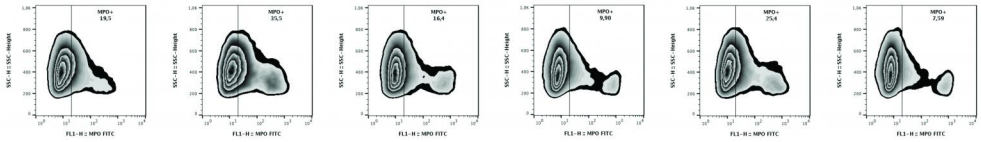
Table 3. Concentration of MPO, cfDNA, and superoxide anion in patient and control samples.

Mean / *SD* / Group	PMNs	PMNs+LPS	PMNs+30'QCT+LPS	PMNs+30'LT+LPS	PMNs+30'Vit C+LPS	PMNs+30'(Vit C+LT)+LPS	Serum
MPO [ng/mL]							
Control	**28.08** *4.25*	**38.57**[a] *6.23*	**27.89**[b] *3.68*	**28.19**[c] *4.11*	**28.09**[d] *4.20*	**27.73**[e] *4.36*	**556.4** *339.2*
Patients	**30.28** *3.65*	**35.97**[a] *8.01*	**27.69**[b] *4.62*	**29.11**[c] *4.17*	**28.76**[d] *4.07*	**27.71**[e] *4.04*	**273.3*** *108.2*
cfDNA [ng/mL]							
Control	**24.36** *5.93*	**39.20**[a] *17.01*	**28.52** *8.50*	**27.90**[c] *11.91*	**28.87**[d] *7.40*	**28.10** *16.51*	**27.91** *6.29*
Patients	**30.42*** *7.82*	**36.39**[a] *10.19*	**27.33**[b] *10.19*	**29.57** *12.72*	**27.27**[d] *7.06*	**31.25** *7.70*	**24.30*** *2.94*
Superoxide anion radical [mmol/$L^{-1}cm^{-1}$]							
Control	**2.62** *1.14*	**4.52**[a] *1.42*	**2.87** *1.86*	**3.68** *0.19*	**1.27**[dg] *0.48*	**3.13** *2.35*	-
Patients	**2.89** *0.60*	**7.63***[a] *2.38*	**4.34**[b] *1.73*	**4.48**[c] *1.54*	**4.06***[d] *1.51*	**1.82**[eijk] *0.61*	-

Statistically significant differences ($p<0.5$): * – with control group, [a] – with unstimulated PMNs, [b] – between PMNs+LPS and PMNs+30'QCT+LPS, [c] – between PMNs+LPS and PMNs+30'LT+LPS, [d] – between PMNs+LPS and PMNs+30'Vit C+LPS, [e] – between PMNs+LPS and PMNs+30'(LT+Vit C)+LPS, [g] – between PMNs+30'LT+LPS and PMNs+30'Vit C+LPS, [i] – between PMNs+30'QCT+LPS and PMNs+30'(Vit C+LT)+LPS, [j] – between PMNs+30'LT+LPS and PMNs+30'(Vit C+LT)+LPS, [k] – between PMNs+30'Vit C+LPS and PMNs+30'(Vit C+LT)+LPS.

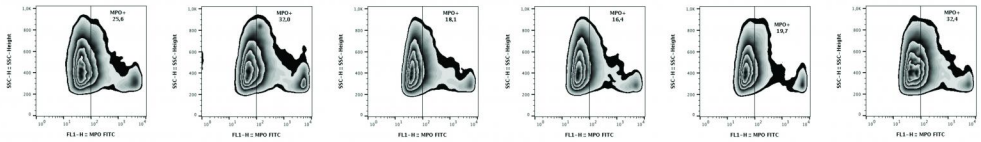
Abbreviation: PMNs, neutrophils; LPS, lipopolysaccharide; QCT, quercetin; LT, luteolin; Vit C, vitamin C; MPO, myeloperoxidase; cfDNA, cell free DNA.

Control



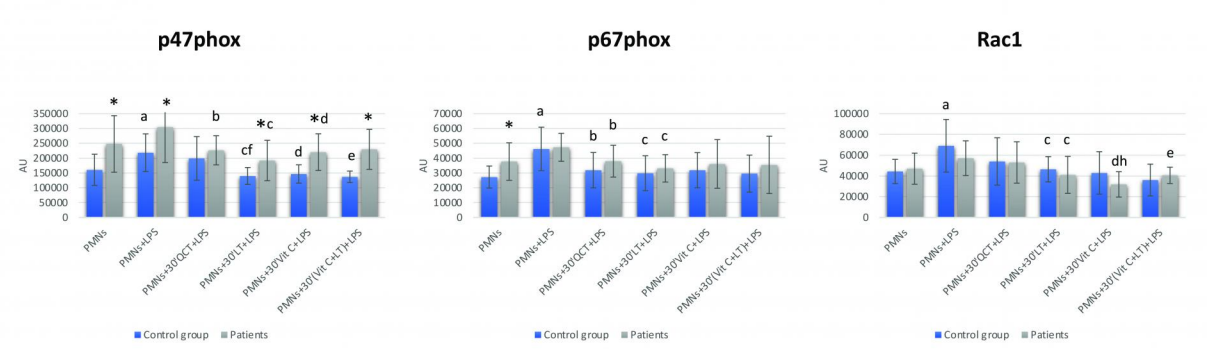
Mean [%]	**17.00**	**25.05**[a]	**20.42**	**19.00**	**21.36**	**19.63**
SD	*2.94*	*7.14*	*2.85*	*8.43*	*6.16*	*6.44*

Patients

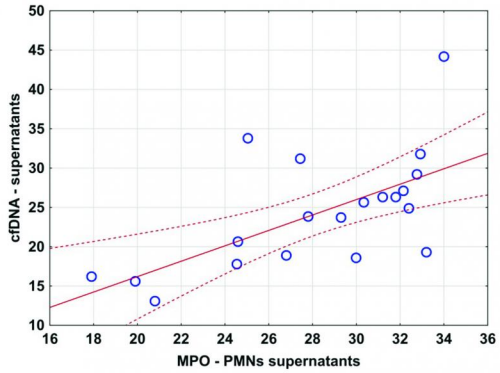


Mean [%]	**26.92***	**30.64**[a]	**24.15**[b]	**24.92**[c]	**21.52**[d]	**23.08**[e]
SD	*3.66*	*4.52*	*5.96*	*4.12*	*6.58*	*9.25*

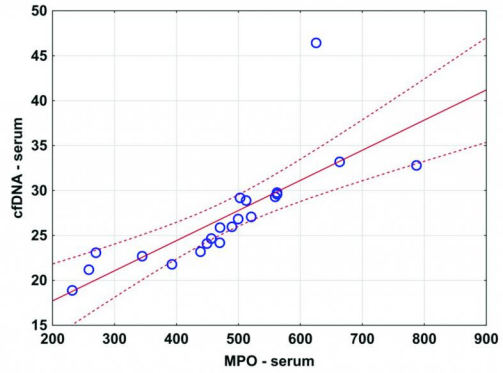
Incubation procedure							
	PMNs	+	+	+	+	+	+
	LPS	–	+	+	+	+	+
	QCT	–	–	+	–	–	–
	LT	–	–	–	+	–	+
	Vit C	–	–	–	–	+	+


p47phox
p67phox
Rac1
AU
PMNs
PMNs+LPS
PMNs+30'OCT+LPS
PMNs+30'LT+LPS
PMNs+30'Vit C+LPS
PMNs+30'(Vit C+LT)+LPS
Control group
Patients

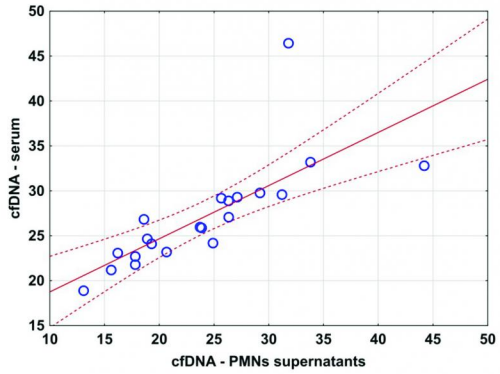
A



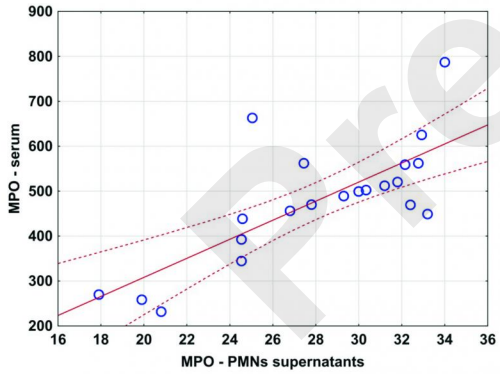
B



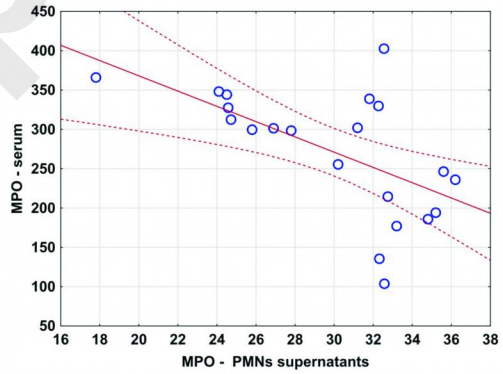
C

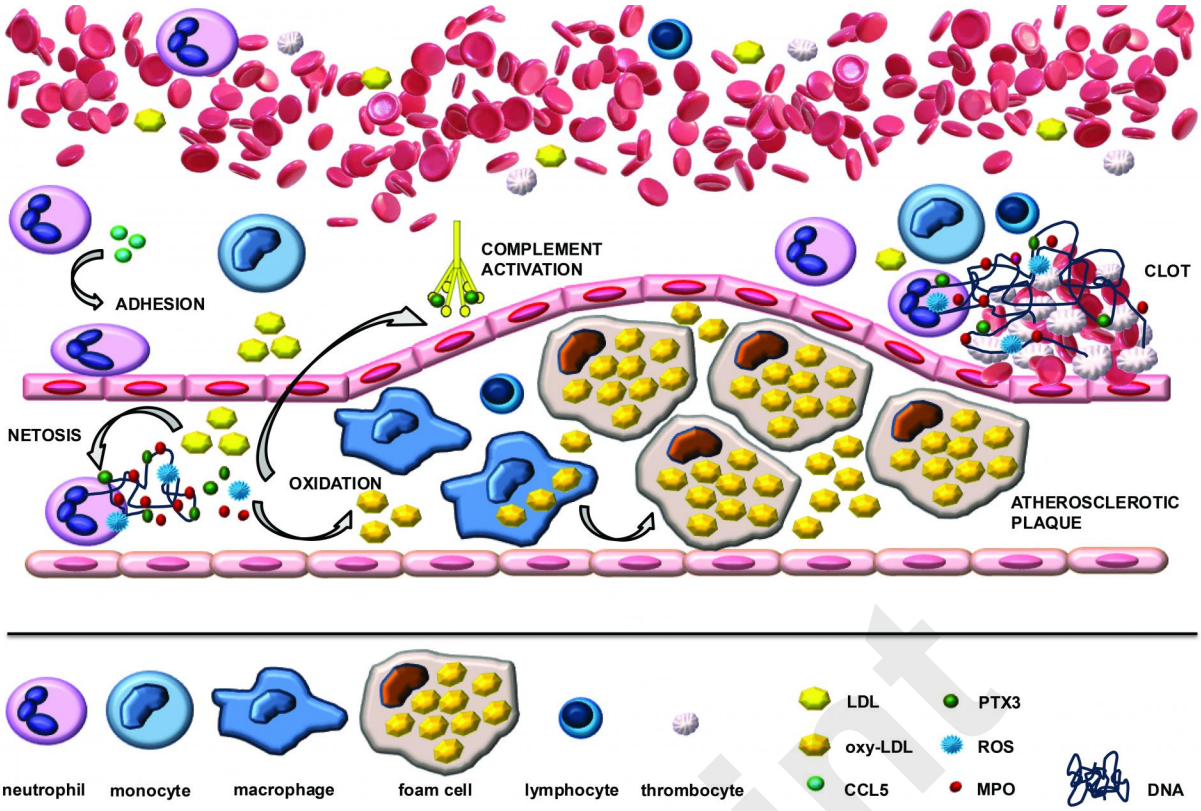


D



E




COMPLEMENT
ACTIVATION
ADHESION
CLOT
NETOSIS
OXIDATION
ATHEROSCLEROTIC
PLAQUE
neutrophil
monocyte
macrophage
foam cell
lymphocyte
thrombocyte
LDL
oxy-LDL
CCL5
PTX3
ROS
MPO
DNA