VIA MEDICA

ORIGINAL RESEARCH ARTICLE

Acta Haematologica Polonica 2024
Number 4, Volume 55, pages 209–217
DOI: 10.5603/ahp.101636
ISSN 0001–5814
e-ISSN 2300–7117

# Nicotine addiction and an elevated cholesterol level exhibit negative effects on platelet activation in patients with chronic Buerger's Disease

Aleksandra Nowaczyńska[1], Tomasz Zubilewicz[2], Bożena Sokołowska[1], Piotr Terlecki[2], Agnieszka Szymczyk[3], Iwona Hus[3]

[1] The Chair and Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, Poland
[2] The Chair and Department of Vascular Surgery and Angiology, Medical University of Lublin, Poland
[3] Department of Hematology, National Medical Institute of the Ministry of Interior and Administration, Warsaw, Poland

## Abstract

**Introduction:** Buerger's Disease (BD), or thromboangiitis obliterans (TAO), is a vasculo-occlusive disorder of unknown etiology. Cigarette smoking is considered to be the main risk factor for the development of TAO. This study aimed to assess the activation of platelets in patients with BD compared to a control group.

**Material and methods:** Thirty patients (24 men and six women, median age 51.4 years) with TAO were included in the study. In the control group there were 20 healthy adults (16 men and four women, median age 44.0 years). Platelet activation was measured by light aggregometry and flow cytometry methods with and without platelet activators, such as thrombin receptor agonist peptide (TRAP-6) and adenosine diphosphate (ADP).

**Results:** The velocity and intensity of aggregation increased in patients with TAO ($p$ <0.05). MFI (mean fluorescence intensity) of P-selectin, CD63 (GP53-component of lysosomal membrane), and PAC-1 (platelet activation complex-1) on platelets increased in patients ($p$ <0.05). Significantly higher aggregation velocities were noted in smoking patients in response to collagen (Coll) and to arachidonic acid (AA) compared to non-smoking ones. Aggregation velocity in response to AA was higher in heavy smokers compared to all smokers. A significantly higher intensity of aggregation was found in moderate smokers in response to epinephrine (EPI) and AA compared to non-smoking ones. A positive correlation between cholesterol concentrations and its fractions (LGL, TG), and platelet aggregation and the MFI of platelets, was found in patients with an increased cholesterol level. The aggregation velocity with EPI and AA was negatively correlated with HDL level.

**Keywords:** platelet activation, P-selectin, Buerger's Disease

Acta Haematologica Polonica 2024; 55, 4: 209–217

## Introduction

Thromboangiitis obliterans [TAO, also known as Buerger's Disease (BD)] is a nonatherosclerotic segmental occlusive inflammatory disorder of small and medium-sized arteries and veins, characterized by thrombosis and the recanalization of the affected vessels. It occurs mostly in young, male cigarette smokers [1]. Despite many published diagnostic criteria for BD, none has been universally accepted as the gold standard. In 2023, the

*Address for correspondence: Agnieszka Szymczyk, Department of Hematology, National Medical Institute of the Ministry of Interior and Administration, ul. Wołoska 137, 02–507 Warsaw, Poland; tel: (+48 22) 534 54 96, e-mail: agnieszka.szymczyk.med@wp.pl

Received: 03.12.2023 Accepted: 07.07.2024 Early publication date: XX.08.2024

Copyright © 2024
The Polish Society of Haematologists and Transfusiologists, Insitute of Haematology and Transfusion Medicine.

This article is available in open access under Creative Common Attribution-Non-Commercial-No Derivatives 4.0 International (CC BY-NC-ND 4.0) license, allowing to download articles and share them with others as long as they credit the authors and the publisher, but without permission to change them in any way or use them commercially.

International Working Group on Buerger's Disease of the VAS-European Independent Foundation in Angiology/ /Vascular Medicine published the newest diagnostic criteria for BD. They recommended that the definitive diagnosis of BD must require the presence of three features i.e. a history of smoking, typical angiographic features, and typical histopathological features, plus the use of a combination of major and minor criteria. The major criterion is a history of active tobacco smoking. The five minor criteria are disease onset at age less than 45 years, ischemic involvement of the lower limbs, ischemic involvement of one or both of the upper limbs, thrombophlebitis migrans, and red-blue or purple discoloration on edematous toes and/or fingers. According to these criteria, a diagnosis of BD is confirmed in the presence of the major criterion plus four or more minor criteria. If fewer than four minor criteria are met, imaging and laboratory data could also certify the diagnosis [2]. 116 years after Buerger's original description of TAO, there is still no consensus on the etiology and pathogenesis of this disease [3]. Use or exposure to tobacco is crucial for the initiation and progression of BD, most likely via autoimmune mechanisms causing vascular inflammation [4]. Tobacco extract increases cellular sensitivity to type I and II collagens, which are the constituents of blood vessels, and raise anti-endothelial cellular antibody titers [5]. These factors lead to endothelium damage and dysfunction, especially impaired endothelium-dependent vasorelaxation which is the first manifestation of TAO [6]. The presence of certain Human Leukocyte Antigens (HLAs) i.e. HLA-A9, HLA-A54 and HLA-B5, has been associated with increased development of BD [5]. Hemostatic risk factors such as higher levels of coagulation factors VII, VIII, XI, fibrinogen, as well as hyperhomocysteinemia, also play important roles in the pathogenesis of TAO [7]. Moreover, in patients with an aggressive clinical course, disturbance in serum lipids has been noted [7].

Physiologically, the platelets are protected from premature activation by the presence of the intact endothelial cell monolayer, by the signal-inhibiting effects of prostaglandin and nitric oxide, and by reducing the accumulation of local platelet agonists [8]. Platelet function disturbances have been detected in many blood-vessel diseases, and platelet activation is the characteristic marker of inflammatory processes developing in vascular diseases [9]. Even so, little is known about platelet function in BD. A better understanding of platelet activation mechanisms in patients with TAO may facilitate better treatment of this disease in the context of antiplatelet therapy.

The aim of this study was to evaluate platelet activation in patients with TAO compared to a control group, and to analyze the effect of smoking and an elevated cholesterol level on platelet activation.

## Material and methods

The study group included 30 patients with TAO (24 men and six women, median age 51.4, range 44.8–58.1) treated in the Department of Vascular Surgery and Angiology, Medical University of Lublin, Poland. All the patients signed informed consent for their participation in the study. The median time from initial diagnosis was 13.2 years (range 8.4 to 18.0). All patients were in a chronic phase of the disease and had second-stage ischemia on Fontaine's scale and presented features of phlebitis migrans. Raynaud's phenomenon occurred only in two patients. In a group of 10 patients, comorbid conditions (coexisting disease) were present, including diabetes, hypertension, coronary artery disease, myocardial infarction, and stroke. All patients received both conservative treatment and surgery. Diagnosis of Buerger's Disease was based on the typical medical history of patients: onset at a young age, exposure to tobacco, and characteristic features in radiological imaging. The main differences between TAO and atherosclerosis are set out in Table I [10].

None of the patients took any medication that might affect platelet functions for seven days before blood collection. The blood samples were used for CBC (complete blood count) testing, flow cytometry and aggregometry analysis, and the determination of standard lipid levels. The control group comprised 20 healthy adults (16 men and four women, aged 44.0 ± 8.4 years) (Tab. II).

Because in the presented group there were no patients who smoked more than 30 cigarettes a day (according to the literature [11] the definition of a heavy smoker) we named the patients who smoked >10 cigarettes a day 'heavy smokers' (HS) and those who smoked <10 cigarettes a day 'moderate smokers' (MS).

Born's turbidimetric method was used to measure platelet aggregation. This was expressed as velocity and intensity of aggregation [12]. The measure of aggregation velocity is based on the angle formed by the intersection of the tangent to the aggregation curve with a horizontal line, expressed as a percentage of the right angle. Formula: $V = (90 - \alpha) \times 100\%$.

Aggregation intensity, i.e. the difference in optical density between the starting point and the end of the second aggregation wave (if two phases occurred), expressed as a percentage of the length of the line connecting the OBP (platelet-rich plasma) and OUP (platelet-poor plasma) optical density points of the control sample. Formula: $I = (ab/ac) \times 100\%$ [12–14].

Four platelet agonists were used to perform this analysis:

- adenosine diphosphate ADP (Trinity Biotech, Bray, Ireland), at a dose of 0.2 mM
- collagen (Trinity Biotech), at a dose of 0.19 mg/mL

**Table I.** Differentiation between TAO and atherosclerosis

| TAO | ATHEROSCLEROSIS |
|---|---|
| Presence of a fresh clot blocking lumen of artery or vein, with an infiltration of multinuclear leukocytes involving all layers of vessel. Clot is characterized by an infiltration consisting of neutrophils, giant cells and, sometimes, granulomas (presence of granulomas is a characteristic feature) | Clot is composed of fibrin, erythrocytes and platelets. There are no granulomatous cells |
| Granulomatous infiltration occurs only in lumen, and is not found in vascular wall | Infiltration includes vascular wall |
| Architecture of wall is maintained. Middle layer and internal elastic membrane remain intact | Blurring of elastic membrane structure |
| There is no necrosis of vascular wall | Presence of necrosis of vascular wall, causing elastic membrane structure to become blurred |

TAO — thromboangiitis obliterans

**Table II.** Main characteristics of patient and control groups

| | Patient group (P): TAO | Control group (C) |
|---|---|---|
| Number of patients | 30 | 20 |
| Sex | Men 24, women 6 | Men 16, women 4 |
| Age (years) | Median 51.4 (44.8–58.1) | Median 44 (33.6–52.4) |
| Number of smokers (S) | 22 | 12 |
| Number of current nonsmokers (NS) | 8 | 8 |
| Number of heavy smokers (HS) (>10 cigarettes a day) | 4 | 6 |
| Number of moderate smokers (MS) (<10 cigarettes a day) | 18 | 6 |
| Number of patients with cholesterol level >200 mg | 18 | 0 |

TAO — thromboangiitis obliterans

- epinephrine (Trinity Biotech), at a dose of 0.1 mM/L
- arachidonic acid (Trinity Biotech), at a dose of 1.5 mM.

The flow cytometry analysis was performed by FACS-Calibur (Becton & Dickinson, San Jose, CA, USA) in the CellQuest program. The evaluation of glycoproteins presented on the surface of the platelet was carried out as a 'dot plot' chart and a histogram chart. The results obtained were expressed as mean fluorescence intensity (MFI). Non-activated platelets served as a negative control; non-activated means a platelet in which only CD61 antigen is presented on the platelet surface. During the platelet activation process, other antigens and PAC-1 (platelet activation complex-1) arise on the platelet's surface. To detect these antigens, the following antibodies combined with fluorochromes were used: anti-CD61 antibody, anti-CD62P antibody, anti-CD63 antibody, and anti-PAC-1 antibody.

Cytometric analysis was performed as follows: the platelet population was determined using the anti-CD61 antibody, while activated platelets were determined (on the same graph) using the anti-CD62P, anti-CD63 and anti-PAC-1 antibodies. Therefore, activated platelets showed double positive CD662P+/CD61+ or CD63+/CD61+ or PAC-1/CD61+ expression. To estimate platelet reactivity, two platelet activators were used: thrombin receptor agonist peptide TRAP-6 (SFLLRN, Bachem, Weil am Rhein, Germany) and ADP (Sigma, St. Louis, MO, USA) at doses of 20 µM/mL. Both contained mouse anti-human antibodies.

## Statistical methods

To describe the study group (in relation to continuous variables), the descriptive statistic module of the Statistica StatSoft v.7.1 program was used. Analyzed data was described (depending on the distribution) with median value or statistical average or percentile range or standard deviation. Variable correlations were verified based on Spearman's rank correlations. In relation to intergroup differences, tests from the non-parametric statistics module were used — the Mann-Whitney U test and the Wald-Wolfowitz test, respectively. In order to show the differences in the selection of preferences of individual subpopulations, the Wilcoxon pairwise order test was used. Calculations were performed for a confidence interval of CI = 95%, so statistically significant differences were considered those for which the p-value met the condition $p < 0.05$. An attempt was made to create regression models to capture multi-factor relationships, i.e. environmental conditions, age, and disease duration. The Statistica regression module and

multi-factor exploration were used to build and verify regression models.

## Results

The aggregation velocity and intensity results are set out in Figure 1 and Table III. The patient group is marked as *p*, and the control group as C. Aggregation intensity for all four platelet agonists was significantly higher in the patient group (*p*) compared to the control group (C). Aggregation intensity for ADP was median 63.2% (*p*) vs. median 57.9% (C) ($p = 0.024$), for collagen median 63.6% (*p*) vs. median 54.5% (C) ($p = 0.000081$), for epinephrine median 58.8% (*p*) vs. median 52.4% (C) ($p = 0.000162$), and for arachidonic acid median 65.0% (*p*) vs. median 50.7% (C) ($p = 0.00037$) (Fig. 1).

Aggregation velocity in all patients compared to the control group was only statistically higher in response to collagen: median 66.5% (*p*) vs. median 64% (C) ($p = 0.001033$). In evaluating smoking's impact on platelet aggregation in the group of TAO patients, we achieved some interesting results.

Aggregation velocity in response to collagen and arachidonic acid was higher in smoking (moderate smokers and heavy smokers together) patients compared to nonsmoking ones (Tab. III). What's more, aggregation velocity in response to arachidonic acid was higher in heavy smoking patients compared to all smoking patients: median of 82.0% vs. median of 63.0% ($p = 0.0030$), indicating that the more cigarettes smoked per day, the more pronounced becomes platelet activity. There were no statistically significant differences between moderate smokers and nonsmoking patients.

The intensity of platelet aggregation in response to epinephrine and arachidonic acid was significantly higher in moderate-smoking patients compared to non-smoking ones (Tab. III). There were no statistically significant differences between heavy smokers and nonsmoking patients, or between all smoking patients and nonsmoking ones.

## Mean fluorescence intensity (MFI) of surface platelet antigens

We obtained statistically significant differences according to MFI of surface antigens between not only all groups of patients (P) and all control groups (C), but also comparing nonsmokers (NS), smokers (S), moderate smokers (MS) and heavy smokers (HS) in the TAO group and in the same control groups (nonsmokers; NS), smokers (S), moderate smokers (MS) and heavy smokers (HS). We only reproduce below those results that are statistically significant.

The MFI of surface CD62P+/CD61+ without inductor was statistically significantly higher in all TAO patients compared to all control groups as well in the S, NS, and MS groups compared to the same control groups (S, NS, and MS). The MFI of surface CD62P+/CD61+ with ADP as inductor was statistically significantly higher in the S and MS TAO patient group compared to the same control group (S, MS). The MFI of surface CD62P+/CD61+ with TRAP as an inductor was statistically significantly higher in the NS TAO patient group compared to the same NS control group.

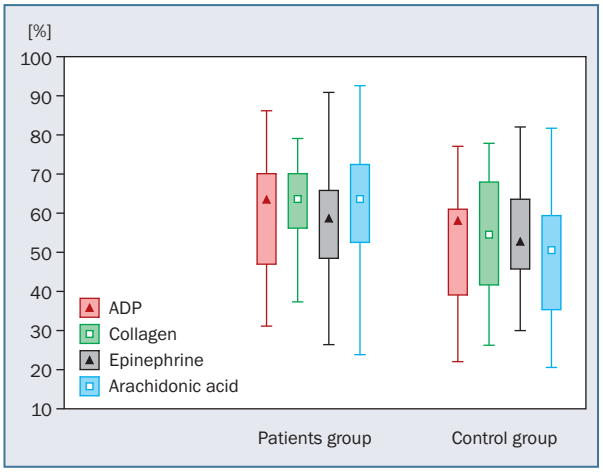


**Figure 1.** Platelet aggregation intensity inducted by ADP, collagen, epinephrine and arachidonic acid in all patients and in control group; ADP — adenosine diphosphate

The MFI of surface PAC-1+/CD61+ without inductor was statistically significantly higher in all TAO patients group compared to all control groups as well as in the NS, MS TAO patient group compared to the same control groups (NS, MS). The MFI of surface PAC-1+/CD61+ with ADP as an inductor was statistically significantly higher in NS TAO patients compared to the NS control group. The MFI of surface PAC-1+/CD61+ with TRAP as an inductor was statistically significantly higher in NS, HS, and MS TAO patients compared to the same control groups (NS, HS, MS).

The MFI of surface CD63+/CD61+ without inductor was statistically significantly higher in all patients compared to all control groups and in NS and MS TAO patients compared to the same control groups (NS, MS). The MFI of surface CD63+/CD61+ with ADP and TRAP as inductors was statistically significantly higher only in NS TAO patients compared to the NS control group. All these results are set out in Table IV.

## Correlations between total cholesterol and its fraction levels and platelet activation

In 16 patients with TAO, the total cholesterol level was elevated to >200 mg/dL. In this group of patients, we found positive correlations seen only on unstimulated platelets between MFI of surface CD62P+/CD61+ and TG level ($p < 0.05$), between MFI of surface PAC-1+/CD61+ and total cholesterol and LDL levels ($p < 0.05$), and between MFI of

**Table III.** Aggregation velocity (%) and intensity of aggregation (%) depending on intensity of smoking in response to collagen and arachidonic acid in patients with TAO and in control group (differences between two means — unilateral test)

| | | N | agonist | median | min/max | *p* |
|---|---|---|---|---|---|---|
| Aggregation velocity | NS | 8 | | 64.50 | 44.00/80.00 | |
| | S | 22 | Coll | 71.00 | 62.00/77.00 | *p*1 = 0.0307 |
| | control | 20 | | 69.00 | 40.00/70.00 | |
| | NS | 8 | | 63.00 | 38.00/79.00 | |
| | S | 22 | AA | 69.50 | 56.00/82.00 | *p*1 = 0.0148 |
| | HS | 6 | | 82.00 | 82.00/82.00 | *p*2 = 0.0030 |
| | control | 20 | | 67.00 | 60.00/76.00 | |
| Intensity of aggregation | NS | 8 | | 55.55 | 20.51/68.29 | |
| | MS | 17 | EPI | 61.11 | 48.68/90.91 | *p*1 = 0.0108 |
| | control | 20 | | 52.38 | 30.21/81.81 | |
| | NS | 8 | | 62.13 | 17.64/83.33 | |
| | MS | 17 | AA | 65.00 | 52.27/92.86 | *p*1 = 0.0322 |
| | control | 20 | | 50.74 | 20.83/81.74 | |

AA — arachidonic acid; EPI — epinephrine; HS — heavy smokers; MS — moderate smokers; N — number; NS — nonsmokers; p — patient group; S — smokers

CD63+/CD61+ and LDL level ($p$ <0.05). In Figure 2, we see a positive correlation between the MFI of PAC-1+/CD61+ and cholesterol level.

In this group of patients (with elevated cholesterol levels), we also observed a negative correlation between the HDL level and aggregation velocity induced by epinephrine ($p$ <0.05) and arachidonic acid ($p$ <0.05), as well as between HDL level and aggregation intensity induced by collagen ($p$ <0.05). We also saw a positive correlation between aggregation intensity induced by collagen and TG level ($p$ <0.05). In Figure 3, we see a negative correlation between aggregation intensity induced by collagen and HDL levels.

We noted statistically significant differences using the Mann-Whitney U test between patients with elevated cholesterol level >200 mg/dL) and normal cholesterol level (150–200 mg/dL) according to the: MFI of surface antigens CD63+/CD61+on non-stimulated platelets, MFI of surface antigens CD63+/CD61+ on platelets stimulated by ADP 20 µM, MFI of surface antigens CD62+/CD61+ on platelets stimulated by TRAP 20 µM, and MFI of surface antigens PAC-1+/CD61+ on platelets stimulated by TRAP 20 µM (Tab. V).

## Discussion

In this study, we analyzed the correlations between platelet activation parameters and smoking and cholesterol levels in patients with TAO. Compared to the control group, we found an increased expression of three major antigens: P-selectin-CD62, fibrinogen receptor PAC-1, and glycoprotein (GP-53) CD63 on the surface of the activated platelets, as well as increased platelet velocity and intensity of aggregation in response to ADP, collagen, epinephrine and arachidonic acid in patients with TAO. Data published so far on platelet activation in patients with TAO is very limited. In contrast to the results of this study, Pietraszek et al. found no differences in aggregation markers expressed in platelets stimulated with 4.0 µM ADP or 2.0 µg/mL of collagen in 12 patients with TAO. The authors found increased platelet response to serotonin, which was not used in the present study [15]. Some interesting results that should also be considered in the context of patients with Buerger's Disease were described in 2005 by Yee et al. [16]. In a group of 359 healthy volunteers, they showed that increased platelet response to one of the activators used i.e. ADP, epinephrine, collagen, collagen-related peptide, and ristocetin, correlated with an increased platelet response (over 60%) with other activators. Yee et al. postulate that this feature may be defined as platelet hyperreactivity and is predisposed to platelet thrombus formation. According to these results, patients with increased platelet activation showed an increased prothrombotic tendency.

Our results demonstrated that aggregation intensity was significantly higher in patients compared to the control group using all four platelet agonists i.e. ADP, collagen, epinephrine, and arachidonic acid, which might indicate platelet hyperactivity in patients with Buerger's Disease. In 2006, Yee et al. [14] confirmed again (in a group of healthy volunteers) that some people have the thrombocyte phenotype, predisposing them to thrombotic diseases. The platelets of these individuals exhibit increased aggregation in response to maximum concentrations of ADP and epinephrine; there is a positive correlation between these results and the expression (MFI) of P-selectin and PAC-1. One can speculate that patients with BD have a specific thrombotic phenotype predisposing them to thrombotic

**Table IV.** MFI of glycoproteins CD62P+/CD61+, PAC-1+/CD61+ and CD63+/CD61+ in all specified groups of patients (P) (depending on intensity of smoking) compared to control group (C). Some results were statistically significant in Wald-Wolfowitz test*, but others were statistically significant in Mann-Whitney U-test

| | Study group | | Inductor | Median | Min/max | p |
|---|---|---|---|---|---|---|
| Glycoproteins CD62P/CD61 | All | P | Without | 4.96 | 2.75/141.81 | |
| | | C | Inductor | 3.89 | 1.62/10.04 | 0.000869* |
| | Smokers | P | Without | 4.35 | 2.75/141.81 | |
| | | C | Inductor | 4.29 | 1.86/10.04 | 0.0211* |
| | | P | ADP | 50.38 | 17.96/176.28 | |
| | | C | 20 µM | 40.24 | 19.81/116.05 | 0.000554* |
| | Non smokers | P | Without | 5.86 | 2.93/43.40 | |
| | | C | Inductor | 3.89 | 1.62/6.83 | 0.000104 |
| | | P | TRAP | 82.17 | 25.04/256.70 | |
| | | C | 20 µM | 58.23 | 24.52/209.46 | 0.000071* |
| | Moderate smokers | P | Without | 4.48 | 2.75/8.07 | |
| | | C | Inductor | 3.53 | 1.86/10.04 | 0.010979 |
| | | P | ADP | 50.38 | 17.96/88.15 | |
| | | C | 20 µM | 45.84 | 34.63/104.58 | 0.001880 |
| Glycoproteins PAC-1/CD61 | All | P | Without | 95.87 | 12.22/327.90 | |
| | | C | Inductor | 47.45 | 9.61/292.54 | 0.004027 |
| | Non smokers | P | Without | 96.33 | 16.71/327.90 | |
| | | C | Inductor | 39.83 | 9.61/163.30 | 0.003839 |
| | | P | ADP | 248.47 | 67.83/524.61 | |
| | | C | 20 µM | 186.63 | 27.34/405.42 | 0.035694 |
| | | P | TRAP | 285.86 | 58.03/430.68 | |
| | | C | 20 µM | 254.34 | 24.74/404.40 | 0.016533 |
| | Heavy smokers | P | TRAP | 138.99 | 43.42/234.50 | |
| | | C | 20 µM | 254.34 | 92.50/617.35 | 0.047909* |
| | Moderate smokers | P | Without | 100.85 | 12.25/179.49 | |
| | | C | Inductor | 91.52 | 13.53/292.54 | 0.047909* |
| | | P | TRAP | 288.89 | 59.46/437.63 | |
| | | C | 20 µM | 128.46 | 116.36/292.19 | 0.010979* |
| Glycoproteins CD63/CD61 | All | P | Without | 37.17 | 6.08/117.49 | |
| | | C | Inductor | 13.32 | 4.71/158.86 | 0.004806 |
| | Non smokers | P | Without | 38.37 | 6.08/117.49 | |
| | | C | Inductor | 13.32 | 4.71/158.86 | 0.002790 |
| | | P | ADP | 88.04 | 26.55/228.75 | |
| | | C | 20 µM | 64.76 | 16.54/143.71 | 0.027686 |
| | | P | TRAP | 155.00 | 33.34/338.26 | |
| | | C | 20 µM | 123.50 | 22.89/249.91 | 0.0000* |
| | Moderate Smokers | P | Without | 46.98 | 6.89/71.90 | |
| | | C | Inductor | 22.53 | 10.93/48.25 | 0.010979 |

ADP — adenosine diphosphate; C — control group; MFI — mean fluorescence intensity; P — patient group; TRAP — thrombin receptor agonist peptide

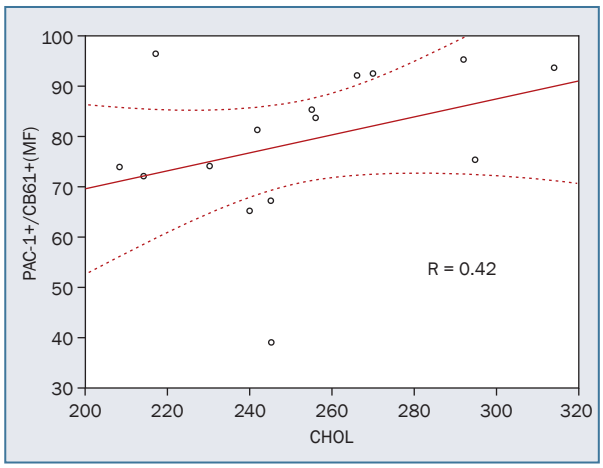


**Figure 2.** Positive correlation between MFI of PAC-1+/CD61+ and cholesterol level; CD61 — platelet glycoprotein IIIa; CHOL — cholesterol; MFI — mean fluorescence intensity; PAC-1 — platelet activation complex-1

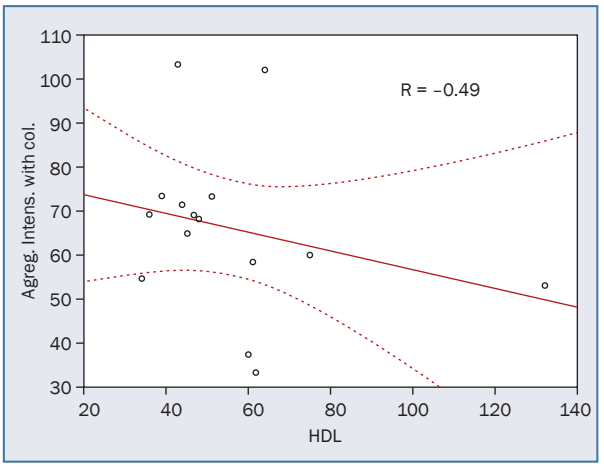


**Figure 3.** Negative correlation between aggregation intensity induced by collagen and HDL level; HDL — high-density lipoprotein

**Table V.** Comparison of platelet activation markers and statistical characteristics between a group of patients with elevated total cholesterol levels (above 200 mg/dL) and a group of patients with cholesterol levels within normal range (150–200 mg/dL) in Mann-Whitney U test

| Platelet activation marker | Patients with Cholesterol level [mg%] | Median | min/max | p |
|---|---|---|---|---|
| MFI | Higher than 200 | 38.77 | 12.85/71.90 | |
| CD63+/CD61+ | Normal range | 20.04 | 6.08/117.49 | 0.00344 |
| MFI ADP 20μM | Higher than 200 | 98.97 | 29.80/228.75 | |
| CD63+/CD61+ | Normal range | 46.84 | 26.55/201.03 | 0.000057 |
| MFI TRAP 20μM | Higher than 200 | 91.46 | 25.11/256.70 | |
| CD62+/CD61+ | Normal range | 45.68 | 25.00/202.77 | 0.00262 |
| MFI TRAP 20μM | Higher than 200 | 297.68 | 213.65/430.68 | |
| PAC-1/CD61+ | Normal range | 180.76 | 43.42/437.63 | 0.00002 |

ADP — adenosine diphosphate; MFI — mean fluorescence intensity; TRAP — thrombin receptor agonist peptide

incidents. Such an explanation could be helpful in indicating the consequences of increased platelet activation in patients with TAO, suggesting a potentially important role of activated platelets in the pathogenesis of this disorder. Carr et al. [17] found that reduced platelet aggregation was obtained in two men with TAO after the implementation of antiplatelet therapy. Carr et al.'s study demonstrated the need for antiplatelet therapy and proved its effectiveness.

To further study platelet reactivity, we used TRAP and ADP to stimulate platelets and evaluate the expression of P-selectin, PAC-1 and CD63 with and without these two activators. The MFI of surface CD62P/CD61, PAC-1/CD61 and CD63/CD61 without an activator (TRAP, ADP) was higher in all the patients compared to the control group. Using TRAP or ADP to stimulate platelets, the same tendency was observed: in non-smokers and in moderate smoking patients, the expression of P-selectin, PAC and CD63 was higher in patients with TAO than in the control group. Only in heavy smokers, after TRAP stimulation, was the expression of PAC lower than in the control group.

This means that the platelets of BD patients are more susceptible to TRAP and ADP stimulation than are the platelets of healthy subjects.

In this study, we also evaluated the relationship between platelet aggregation and smoking and cholesterol levels. Aggregation velocity in response to collagen and arachidonic acid was significantly higher in smoking patients compared to non-smoking ones. Furthermore, we detected that aggregation velocity in response to arachidonic acid was significantly higher in heavy smoking patients than in all smoking patients. The association between smoking and platelet aggregation was shown previously both in a healthy population [18, 19] as well as in patients with TAO [20, 21]. Nair et al. [19] state that there are some ways in which tobacco can influence the cardiovascular system. The authors proved that increased platelet activation was

associated with the intensity of smoking and was higher in chronic smokers. They used the aggregometry method with different agonists i.e. ristocetin, ADP and collagen both before and after smoking in acute and chronic smokers. Flow cytometry analysis involved the following antigens: CD61, CD62P, and CD42b. They found that the increase in activated platelets occurred immediately after smoking, and that a significant number of circulating platelets are in an activated state in chronic smokers. These studies clearly demonstrate that smoking has an activating effect on platelet function. Smoking appears to have an activation effect on platelet function due to thromboxane metabolism, and results in an altered susceptibility to antiplatelet therapy. Weber et al. [22] found that there was a significantly stronger inhibition of collagen and ADP-induced platelet aggregation by aspirin in smokers compared to non-smokers in a complete inhibition of thromboxane $A_2$ synthesis.

The result of this study and of Weber's study suggest that physicians should pay more attention to prophylaxis using antiplatelet therapy in patients with BD, especially those who smoke [22].

In patients with TAO with an elevated cholesterol level, we found positive correlations seen only on unstimulated platelets between the MFI of CD62P/CD61 and TG level, the MFI of PAC-1/CD61 and total cholesterol and LDL level, and the MFI of CD63/CD61 and LDL level. On the other hand, there were no such correlations in the group of BD patients with normal cholesterol levels. This may suggest that, in patients with a higher cholesterol level (>200 mg/dL), there is an additional platelet-activating effect of LDL and TG. A higher TG or LDL level may contribute to an increased expression of CD62P/CD61, PAC-1/CD61 and CD63/CD61, leading to more pronounced platelet activation. Chan at al. [23] have described a mechanism by which L5, the most electronegative of the five recognized fractions of LDL, activates both platelets and endothelium in a manner that supports thrombosis [23]. In both groups of TAO patients (those with elevated and those with normal cholesterol levels), we observed a negative correlation between the HDL level and platelet aggregation. Aggregation intensity with collagen becomes especially higher in patients with a lower level of HDL.

In summary, lipid disturbance such as higher total cholesterol levels, higher TG and LDL levels, and a lower HDL level, may play an indirect but still important role in the pathogenesis of Buerger's Disease, causing platelet activation responsible for the hypercoagulation state, even though BD is a nonatherosclerotic disorder.

In conclusion, our results show that:

1) The increase of the mean fluorescence intensity of P-selectin (CD62P), an active form of the receptor for fibrinogen (PAC-1), and the lysosomal protein (CD63) on platelets in patients with TAO suggests their participation in the pathogenesis of the hypercoagulable state observed in Buerger's Disease.
2) Smoking increases platelet activation in patients with TAO expressed by platelet aggregation, especially in response to arachidonic acid. This might explain the contribution of smoking to pathogenesis in TAO.
3) Lipid disturbances play an indirect but important role in the pathogenesis of TAO, causing platelet activation even though TAO is a nonatherosclerotic disorder.

## Article information and declarations


### Acknowledgments
None.


### Authors' contributions
AN, TZ, BS, PT, IH — design of study; AN, TZ, BS, PT, IH, AS — provision of clinical data, analysis of clinical data, editorial preparation of manuscript; AN, BS, PT, IH, AS — writing manuscript. All authors — critical revision and final approval.

### Conflict of interest
The authors declare no conflict of interest.

### Ethics statement
The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.
The ethics committee of Medical University of Lublin approved this study (REC number: KE-0254/93/2005).


### Funding
This study was funded by the Polish Ministry of Education, grant No 2P0 5B 075 30.


### Supplementary material
None.

### References


1. Thromboangiitis Obliterans (Buerger Disease). In: Sindawy AN, Perler BA. ed. Rutherford's vascular surgery and endovascular therapy. Vol. 1. 9th edition. Elsevier 2019, Philadelphia, PA 2019.
2. Fazeli B, Poredos P, Kozak M, et al. Diagnostic criteria for Buerger's disease: International Consensus of VAS - European Independent Foundation in Angiology/Vascular Medicine. Int Angiol. 2023; 42(5): 396–401, doi: 10.23736/S0392-9590.23.05098-8, indexed in Pubmed: 38010012.
3. Olin JW. Thromboangiitis obliterans: 110 years old and little progress made. J Am Heart Assoc. 2018; 7(23): e011214, doi: 10.1161/JAHA.118.011214, indexed in Pubmed: 30571606.

4. Ribieras AJ, Ortiz YY, Liu ZJ, et al. Therapeutic angiogenesis in Buerger's disease: reviewing the treatment landscape. Ther Adv Rare Dis. 2022; 3: 26330040211070295, doi: 10.1177/26330040211070295, indexed in Pubmed: 37180424.
5. Naiem N. Thromboangiitis Obliterans (Buerger Disease): Practice Essentials, Pathophysiology and Etiology, Epidemiology. https://emedicine.medscape.com/article/460027-overview?form=fpf (14 April 2024).
6. Rivera-Chavarría IJ, Brenes-Gutiérrez JD. Thromboangiitis obliterans (Buerger's disease). Ann Med Surg (Lond). 2016; 7: 79–82, doi: 10.1016/j.amsu.2016.03.028, indexed in Pubmed: 27144003.
7. Hus I, Sokolowska B, Walter-Croneck A, et al. Assessment of plasma prothrombotic factors in patients with Buerger's disease. Blood Coagul Fibrinolysis. 2013; 24(2): 133–139, doi: 10.1097/MBC.0b013e32835b7272, indexed in Pubmed: 23358197.
8. Brass LF. Thrombin and platelet activation. Chest. 2003; 124 (3 Suppl): 18S–25S, doi: 10.1378/chest.124.3_suppl.18s, indexed in Pubmed: 12970120.
9. Rubenstein DA, Yin W. Platelet-Activation Mechanisms and Vascular Remodeling. Compr Physiol. 2018; 8(3): 1117–1156, doi: 10.1002/cphy.c170049, indexed in Pubmed: 29978900.
10. Arkkila PET. Thromboangiitis obliterans (Buerger's disease). Orphanet J Rare Dis. 2006; 1: 14, doi: 10.1186/1750-1172-1-14, indexed in Pubmed: 16722538.
11. Seebald J, Gritters L. Thromboangiitis obliterans (Buerger disease). Radiol Case Rep. 2015; 10(3): 9–11, doi: 10.1016/j.radcr.2015.06.003, indexed in Pubmed: 26649109.
12. Born GV, Dearnley R, Foulks JG, et al. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature. 1962; 194: 927–929, doi: 10.1038/194927b0, indexed in Pubmed: 13871375.
13. Zawilska K, Komarnicki M, Mańka B, et al. [Effect of beta-blocking agents on platelet aggregation]. Pol Arch Med Wewn. 1975; 54(2): 203–208, indexed in Pubmed: 240155.
14. Yee DL, Bergeron AL, Sun CW, et al. Platelet hyperreactivity generalizes to multiple forms of stimulation. J Thromb Haemost. 2006; 4(9): 2043–2050, doi: 10.1111/j.1538-7836.2006.02089.x, indexed in Pubmed: 16961612.
15. Pietraszek MH, Choudhury NA, Koyano K, et al. Enhanced platelet response to serotonin in Buerger's Disease. Thromb Res. 1990; 60(3): 241–246, doi: 10.1016/0049-3848(90)90186-g, indexed in Pubmed: 2084953.
16. Yee DL, Sun CW, Bergeron AL, et al. Aggregometry detects platelet hyperreactivity in healthy individuals. Blood. 2005; 106(8): 2723–2729, doi: 10.1182/blood-2005-03-1290, indexed in Pubmed: 15972447.
17. Carr ME, Hackney MH, Hines SJ, et al. Enhanced platelet force development despite drug-induced inhibition of platelet aggregation in patients with thromboangiitis obliterans–two case reports. Vasc Endovascular Surg. 2002; 36(6): 473–480, doi: 10.1177/153857440203600610, indexed in Pubmed: 12476239.
18. Inoue T, Hayashi M, Uchida T, et al. Significance of platelet aggregability immediately after blood sampling and effect of cigarette smoking. Platelets. 2001; 12(7): 415–418, doi: 10.1080/09537100120071068, indexed in Pubmed: 11674858.
19. Nair S, Kulkarni S, Camoens HM, et al. Changes in platelet glycoprotein receptors after smoking–a flow cytometric study. Platelets. 2001; 12(1): 20–26, doi: 10.1080/09537100120046020, indexed in Pubmed: 11281626.
20. Papa M, Bass A, Adar R, et al. Autoimmune mechanisms in thromboangiitis obliterans (Buerger's Disease): the role of tobacco antigen and the major histocompatibility complex. Surgery. 1992; 111(5): 527–531, indexed in Pubmed: 1598672.
21. Rahman M, Chowdhury AS, Fukui T, et al. Association of thromboangiitis obliterans with cigarette and bidi smoking in Bangladesh: a case-control study. Int J Epidemiol. 2000; 29(2): 266–270, doi: 10.1093/ije/29.2.266, indexed in Pubmed: 10817123.
22. Weber AA, Liesener S, Schanz A, et al. Habitual smoking causes an abnormality in platelet thromboxane A2 metabolism and results in an altered susceptibility to aspirin effects. Platelets. 2000; 11(3): 177–182, doi: 10.1080/095371000403125, indexed in Pubmed: 10938895.
23. Chan HC, Ke LY, Chu CS, et al. Highly electronegative LDL from patients with ST-elevation myocardial infarction triggers platelet activation and aggregation. Blood. 2013; 122(22): 3632–3641, doi: 10.1182/blood-2013-05-504639, indexed in Pubmed: 24030386.