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Authors: Grzegorz Osmenda, Pawel T. Matusik, Tomasz Sliwa, Marta Czesnikiewicz-Guzik, Jan Skupien, Maciej T. Malecki, Mateusz Siedlinski

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Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase p22phox subunit polymorphisms, systemic oxidative stress, endothelial dysfunction, and atherosclerosis in type 2 diabetes mellitus

Short title: CYBA polymorphisms and endothelial function in T2DM

Grzegorz Osmenda¹, Pawel T. Matusik¹,², Tomasz Sliwa¹, Marta Czesnikiewicz-Guzik³,⁴, Jan Skupien⁵, Maciej T. Malecki⁵, Mateusz Siedlinski¹

¹ Department of Internal and Agricultural Medicine, Faculty of Medicine, Jagiellonian University Medical College, Cracow, Poland
² Department of Electrocardiology, Institute of Cardiology, Faculty of Medicine, Jagiellonian University Medical College, Cracow, Poland
³ Department of Periodontology and Oral Sciences Research Group, University of Glasgow Dental School and Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom.
⁴ Department of Dental Prophylaxis and Experimental Dentistry, Faculty of Medicine, Jagiellonian University Medical College, Cracow, Poland
⁵ Department of Metabolic Diseases, Faculty of Medicine, Jagiellonian University Medical College, Cracow, Poland

Correspondence to: Mateusz Siedlinski, PhD, Department of Internal and Agricultural Medicine, Jagiellonian University School of Medicine, J Dietl Hospital, ul. Skarbowa 1, 31-121 Cracow, Poland, email: mateusz.siedlinski@uj.edu.pl

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What’s new?

Current study found a protective effect of the T allele of the missense rs4673 polymorphism in the cytochrome b-245 alpha chain (CYBA), coding a p22phox subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, on Flow-Mediated Dilatation in Type 2 diabetic patients. Growing body of evidence suggests that this particular genetic variation may have important phenotypic consequences in diseases related to endothelial function and redox balance such as diabetes and cardiovascular diseases.
Abstract

**Introduction:** Diabetes mellitus is an important and rapidly increasing problem in public health. It associates with endothelial dysfunction and increased endothelial permeability, which may lead to severe cardiovascular events.

**Objectives:** We aimed to evaluate the relationship between polymorphisms in the *cytochrome b-245 alpha chain (CYBA)* gene encoding p22phox, a key subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, with endothelial function, atherosclerosis and systemic oxidative stress in type 2 diabetes mellitus (T2DM).

**Patients and methods:** Intima-media thickness (IMT), flow- and nitroglycerin-mediated dilatation (FMD and NMD) were measured in 182 patients with T2DM. Assessment of plasma levels of von Willebrand factor (vWF) and malondialdehyde (MDA) levels as well as genotyping of coding sequence C242T (rs4673) and promoter region A-930G (rs9932581) polymorphisms of *CYBA* were performed using standardized protocols.

**Results:** We observed a significant association of the impaired endothelial function, as measured by FMD, with the C allele of C242T, but not with the A-930G polymorphism. Functional relationship of the C242T polymorphism with endothelial dysfunction remained significant following a multivariable adjustment for major risk factors for atherosclerosis. Mean IMT, NMD, concentrations of MDA or vWF were not related to the specific genotypes of the investigated polymorphisms.

**Conclusions:** C242T, but not A-930G, polymorphism of *CYBA* significantly affects endothelial function in T2DM. Thus, it might be a useful marker of endothelial dysfunction in T2DM patients.

**Key words:** atherosclerosis, *cytochrome b-245 alpha chain (CYBA)* polymorphisms, diabetes mellitus, endothelial function, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases
Introduction

Diabetes mellitus is an important and rapidly increasing problem worldwide. In 2013, there were approximately 382 million of people with diabetes mellitus and 90-95% of them suffered from type 2 diabetes mellitus (T2DM) [1, 2]. Diabetes contributes to endothelial dysfunction, increased endothelial permeability, atherosclerosis, as well as disturbance of the epicardial transcriptomic profile and thus leads to severe cardiovascular events, including myocardial infarction and stroke [3-5].

Hyperglycemia causes alterations in the cellular redox state and leads to increased reactive oxygen species (ROS) production [6]. Additionally, autooxidation of glucose and oxidation of glycated protein fragments may be a source of significant amounts of ROS and leads to cellular injury [7]. The main vascular sources of superoxide anion are NOX enzymes and dysfunctional endothelial nitric oxide synthase (eNOS) [8]. Recent studies demonstrate stimulating effect of glucose and advanced glycation end-products (AGE) on NOX activity [9, 10]. High glucose concentration induces NOX expression and superoxide anion production in human endothelial cells leading to endothelial dysfunction [11]. Superoxide anion exaggerates oxidation processes, including oxidation of polyunsaturated fatty acids in LDL particles, which promotes atherosclerosis. Numerous clinical studies have shown that NOX activity correlates with various atherosclerosis risk factors including T2DM [8].

The NOX complex consists of two membrane subunits, p22phox and gp91phox/NOX2 (or one of its homologues: NOX1, NOX3, NOX4, and NOX5), three cytoplasmatic subunits (gp67phox, p47phox, p40phox) and G protein (rac) [12]. While vascular subunits associated with p22phox, NOX1 and NOX2, are thought to possess deleterious effects on the cardiovascular system due to inactivation of NO, the role of NOX4 is more complex [13]. Presence of the p22phox subunit is crucial for enzyme activity, and two polymorphisms of this gene, i.e. C242T in the coding region (substitution of Histidine-72 for
Tyrosine) and A-930G in the promoter region have been intensively studied as modulators of oxidative stress and atherosclerosis progression [14-20]. Importantly, the functional consequences of genetic polymorphisms may be revealed particularly under “stress” conditions, i.e. in the presence of factors that overload cardiovascular system such as T2DM [21].

The present study aimed to evaluate the relationship between a missense C242T (rs4673) and promoter region A-930G (rs9932581) polymorphisms of the p22phox subunit of NADPH oxidase, encoded by the cytochrome b-245 alpha chain (CYBA) gene, with clinically assessed endothelial function and atherosclerosis progression, as well as biochemical parameters related to endothelial function and systemic oxidative stress in type 2 diabetics.

Patients and methods

Study participants and definitions

Consecutive, Caucasian patients, diagnosed with T2DM (see table 1 for detailed clinical characteristic and medication use in the studied cohort) were enrolled and followed up in the Department of Metabolic Diseases, Jagiellonian University Medical College, University Hospital (Krakow, Poland). Detailed medical history and physical examination of all 182 patients were described before [22, 23]. In particular, 71, 30, and 62 patients presented microangiopathic complications related to diabetic retinopathy, nephropathy, and/or neuropathy, respectively. Diabetes was diagnosed according to the World Health Organization criteria [24, 25]. To maximize the homogeneity of the studied group, we included patients with T2DM who were over 30 years old and diabetes diagnosis was made at least 2 years earlier. Hypertension was defined as antihypertensive treatment or systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg. Dyslipidemia was defined as total cholesterol level>5mM or triglycerides level>1.7mM or LDL cholesterol
Peripheral blood for DNA isolation and biochemical parameters assessment was obtained by antecubital vein puncture. The study was approved by the Jagiellonian University Bioethics Committee. All patients provided informed consent.

**Genotyping and biochemical tests**

DNA was isolated using QiaAmp Mini Blood Kit (QIAGEN) according to the manufacturer’s recommendations. DNA amplification was performed using T3 Thermocycler (Biometra) and Hot Star Taq Polymerase (QIAGEN). For amplification the following primers were used, i.e.: for C242T: Forward (F): 5’ TGC TTG TGG GTA AAC CAA GGC CGG TG -3’ and Reverse (R): 5’- AAC ACT GAG GTA AGT GGG GGT GGC TCC TGT -3’ [28] and for -930A>G: F: 5’ AAA CCA CCA AGT GCC TCG GAT GGT GGC T -3’; R: 5’- CCA GCG CCC ATG GGA AGA CTT TAG ACC T -3’. PCR reaction consisted of 15 min. denaturation (95°C), 40 cycles of denaturation (94°C, 45 sec.), annealing (60°C, 45 sec.) and elongation (72°C, 45 sec.) and final 10 min. elongation step in 72°C. Products of PCR reaction were subsequently incubated with restriction enzymes, Rsal (Fermentas, 0.1 U/ul przez 16h, 37°C) or BbvI (Fermentas, 0.06 U/ul, 16h, 65°C), to identify genotypes of the coding sequence (C242T) or promoter region (A-930G) polymorphisms, respectively. Identification of genotypes was performed following agarose gel electrophoresis by 2 independent researchers in the following way: 348 bp band (allele C) and 160+188 bp bands (allele T) for C242T polymorphism and 318 bp band (allele A) and 232 bp band (allele G) for A-930G polymorphism.

Standard laboratory tests including glucose level, lipid profile, creatinine, C peptide, and HbA1c levels were performed. Measurement of plasma vWF levels was performed using ELISA method utilizing rabbit antibodies (DAKO, Glostrup, Denmark). Assessments of MDA plasma levels were performed by a modified method described by Sim et al. using
high-performance liquid chromatography – mass spectrometry (HPLC / MS; LCQ Finnigan Matt) [29]. Samples were incubated with NaOH to liberate bound MDA and with perchloric acid to precipitate proteins. Afterwards, the supernatant was extracted two times with n-hexane and the organic phase was analyzed.

**Clinical tests**

FMD was evaluated as a marker of endothelial function through the measurement of brachial artery diameter before and after acute 5-minute-long occlusion (using the sphygmomanometer cuff) using techniques consistent with the guidelines [30], as described and validated by us before [31]. Briefly, assessments were performed with Toshiba Xario Ultrasound System SSA-340A ultrasonograph, type BF (Toshiba, Japan) and a 8 MHz linear transducer. Before the examination, patients were maintained in a supine position for 15 minutes in a calm and darkened room. The arm was immobilized and a blood pressure cuff was placed and inflated around the right arm to at least 200 mmHg (50 mmHg above the systolic blood pressure level to achieve brachial artery occlusion, for 5 min). Diastolic measurements were performed before cuff inflation and then subsequently 1, 2, and 5 min after cuff deflation.

NMD was measured to study non-endothelium-dependent vasodilation. Vessel diameter was assessed before and 1, 2, and 5 min after sublingual 400 µg of nitroglycerin application. Vessel diameter was measured as a distance between the two M-lines, according to the original Celermajer’s methodology. Maximum FMD (FMDmax%) and NMD (NMDmax%) were calculated as 100 x (peak vascular diameter after hyperemia or nitroglycerin application – baseline vascular diameter) / baseline vascular diameter [32, 33].

IMT measurements were performed using standard method and the same device as described previously [31]. Briefly, assessments were performed at 12 different points on the
right and left common carotid arteries, measuring the distance between the border between the artery lumen and the carotid artery intima and the second bright M-line (the border between the media and adventitia). On the basis of the measurements, the mean and maximum IMT (IMTmean and IMTmax) were calculated.

**Statistical analyses**

Correlations between two continuous variables were evaluated by Spearman test, while associations between categorical variables were tested using Pearson’s \( \chi^2 \) test. Between-genotype differences, as well as recessive and dominant effects of analysed SNPs, were tested using Kruskal-Wallis or Mann-Whitney U tests, respectively. Logistic regression was used to test the effect of SNPs on dichotomized FMDmax (based on median value) or ischemic heart disease with additional adjustment for potential confounders. Concordance of genotypes with Hardy-Weinberg equilibrium was tested with the Pearson’s \( \chi^2 \) test. A significance level of \( P<0.05 \) was assumed for statistical tests. Statistical analyses were performed using Statistica (ver. 7.1) and SPSS (ver. 25.1).

**Results**

**Patients’ characteristics**

The study group included 182 T2DM patients (Table 1). The most common atherosclerosis risk factors among the investigated patients were hypertension and dyslipidemia, while ischaemic heart disease was diagnosed in nearly half of the subjects (Table 1). As expected [31] we observed a negative correlation between mean IMT and maximal FMD, which provides additional validation to our protocol (R= -0.44; p<0.001). Interestingly, a significant correlation between maximal NMD and mean IMT (R= -0.37; p<0.001) was observed as well.
Association between CYBA polymorphisms and intima media thickness, endothelial function, or ischemic heart disease

Based on the frequencies of minor T242 and A-930 alleles (respectively, 37.9% and 39.8%), studied SNPs were common in the T2DM population. Distribution of both polymorphisms did not significantly deviate from the expected based on Hardy-Weinberg Equilibrium. Moreover, no linkage disequilibrium was observed between C242T and A-930G polymorphisms (D’=0.12; R²=0.005).

We found no significant differences in mean IMT between the genotypes of both polymorphisms studied (Figures 1A and 1B). We observed a significant association of C242T polymorphism with maximal FMD in diabetic patients, i.e. TT homozygotes were characterized by a significantly elevated FMDmax compared to CC homozygotes (median in % (IQR): 11.1 (8.5-13.1) vs. 8.0 (5.7-12.1) respectively; Figure 2A). The observed association was confirmed in a logistic regression model estimating odds for low, i.e. below median, FMDmax additionally adjusted for mean IMT (OR (95% C.I.): 0.25 (0.08-0.80), 0.55 (0.33-0.93) and 0.31 (0.11-0.91) for tests involving TT vs CC genotype comparison, additive and recessive models respectively). These associations remained robust after additional adjustment for age, current smoking status, or BMI (data not shown). No significant association between C242T polymorphism and NMD level was observed (Figure 2C).

Genotypes of A-930G polymorphism were not significantly associated with maximal FMD (Figure 2B). Moreover, no significant association was found when recessive or dominant models of inheritance were tested. Similarly, no significant relationship was found between the genotypes of A-930G polymorphism and NMD (Figure 2D). To check whether CYBA polymorphisms correlate with clinical disease outcome, we tested their association with ischemic heart disease, however, no significant results were observed (OR=1.42 (95% C.I.:
and OR=1.20 (95% C.I.: 0.76-1.90) for an additive (i.e. per-minor allele) model testing A-930G and C242T polymorphisms respectively).

**Association between C242T and A-930G polymorphisms and drug use or level of biochemical parameters**

Levels of vWF and MDA (figure 3A-D) or biochemical parameters, except for total cholesterol level (Table 2), did not significantly differ between the genotypes studied. We additionally tested whether C242T and A-930G polymorphisms associated with the use of major medications reported by patients (Table 1), however no significant association was found between these SNPs and oral antidiabetic medications, insulin, ACE inhibitors, diuretics, statins, acetylsalicylic acid, α- or β-blockers, nitrates or calcium channel blockers in genotypic, recessive or dominant models and Pearson’s χ2 test (data not shown).

**Discussion**

The main finding of the current study is demonstration of the protective effect of the T allele of C242T CYBA polymorphism on endothelial function in T2DM patients. This is of importance since, to our knowledge, the association of CYBA gene polymorphisms and endothelial function has not been tested in diabetic patients so far.

T2DM coexists and is an important risk factor for cardiovascular diseases that are often characterized by endothelial dysfunction [3, 34, 35]. Activation of NOX enzymes plays an important role in increased reactive oxygen species (ROS) production and contributes to the loss of nitric oxide (NO) bioavailability, endothelial dysfunction and cardiovascular pathology [8, 36]. Of interest, elevated activation of NOX has been found in an animal model of three comorbidities, including hyperglycaemia, which resulted in an impaired endothelial function of coronary arteries as well as left ventricular dysfunction [37]. Overall, this
implicates a mediating effect of NOX isoforms in the development of vascular dysfunction in T2DM.

Both experimental and clinical studies revealed that endothelial dysfunction is reversible by inhibition of NOX enzymes or by ROS scavengers [38, 39]. Furthermore, mice lacking the functional p22phox gene are protected against leptin resistance and high-fat feeding-induced weight gain, while mice overexpressing p22phox develop characteristics of metabolic syndrome [40]. Induction of T cell infiltrate into perivascular fat in mice overexpressing p22phox [40] additionally points to inflammatory pathways as a link between oxidative stress, obesity, insulin resistance, and diabetes, supported by both human and animal studies [8].

Nair and colleagues demonstrated decreased maximal FMD and increased IMT values in diabetic patients compared to controls [41]. Impairment of these parameters may precede diabetic vascular complications [41] and associate with NOx level in chronic renal failure [42], while FMD negatively correlates with the number of complications in diabetic subjects [43]. The above studies emphasize the need for detailed characterization of both genetic and environmental factors influencing FMD and IMT in T2DM patients.

Letonja and colleagues found that the NADPH C242T polymorphism was not associated with the degree of oxidative stress and carotid atherosclerosis in a group of Slovenian patients with T2DM [44]. On the other hand, Hayaishi-Okano et al. demonstrated that T2DM patients with CT+TT genotype were characterized by significantly lower mean IMT than T2DM patients carrying CC genotype. Interestingly, no such relationship was observed in control subjects [45]. Additionally, lower fasting plasma insulin concentration was observed in patients carrying the 242T allele [45]. The above data suggest that the effect
of C242T CYBA polymorphism on carotid artery atherosclerosis may depend on the ethnic background of T2DM patients.

While the T allele of the C242T CYBA polymorphism has been associated with lower odds for metabolic syndrome [46] and fatal events related to coronary artery disease [47] compared to the C allele, Fan and colleagues observed an improved endothelial function in subjects with CT/TT genotype compared to CC genotype in a population-based sample of young healthy adults [48]. This is in agreement with our results and raises a question about the possible modification of the C242T SNP effect in diseased subjects. Of note, Fan et al. observed an average difference in brachial artery FMD of approximately 0.9% between carriers of CC and TT genotypes. The effect observed in the current study, i.e. 3% difference in median FMD between both genotypes, might suggest a relatively more pronounced association in T2DM patients compared to a healthy population. Among the mechanisms, which could be involved in an observed FMD improvement, are reduced vascular NADPH oxidase activity (independently of other risk factors for atherosclerosis) and decreased vascular superoxide anion production [8]. The 242T allele in CYBA was described as a genetic inhibitor of Nox2 activation in response to high-glucose in vitro [16] and as a predictor of lower risk of recurrence of cardiovascular events in high-risk patients and was associated with reduced systemic oxidative stress, determined by plasma levels of 8-hydroxy-2’-deoxyguanosine (8-OHdG) [17]. In line with these results, functional studies revealed that T allele of the C242T, but not A-930G, SNP was associated with lower respiratory burst in neutrophils of healthy subjects and lower, PMA-induced superoxide anion production in peripheral mononuclear cells of hypertensive subjects [18, 20]. Hypertensive patients exhibited also significantly higher values of vWF than normotensive and this effect was modified by the C242T genotype [18]. It is important to note that the endothelium, and particularly eNOS, acts in a paracrine and endocrine manner in various tissues. For example,
eNOS mediates the protective effects of nitroglycerin in a cardiac ischaemia/reperfusion injury in vivo model [49]. Therefore, the genetic predisposition towards increased ROS production and NO inactivation may have broad implications in cardiovascular and immune-related diseases.

Little is known about the effect of A-930G CYBA polymorphism on IMT or FMD in healthy or diseased populations. However, it may possess functional properties such as affecting transcription activity and, ultimately, modulation of gene expression [19]. Promoter CYBA variants and particularly A-930G polymorphism have been associated with coronary artery disease in the Polish population [14, 15]. However, we did not observe a significant association between A-930G polymorphism and FMD, IMT, or ischemic heart disease among T2DM patients investigated. This suggests that C242T, rather than A-930G, possesses functional consequences in diseased subjects and especially in T2DM patients. While deep clinical phenotyping of the studied subjects certainly increased precision of the genetic effect estimates, a moderate sample size might be perceived as a limitation of our study and could result in a lack of significant association concerning promoter CYBA polymorphism.

In conclusion, the C242T, missense polymorphism of CYBA significantly correlates with endothelial function in patients with T2DM. Since endothelial function is a genetically complex trait, this polymorphism, among other environmental and genetic factors, may contribute to a risk score for endothelial dysfunction in type 2 diabetic patients.
**Contribution statement:** GO, MTM and MS conceived the study and interpreted the data. GO, JS, PTM, TS and MCG participated in patients recruitment. GO, MS and JS analysed the data. All authors edited and approved the final version of the manuscript.

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**References:**

1. Egashira K, Hirooka Y, Kai H, et al. Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. Circulation. 1994; 89: 2519-2524.
2. Gajos G. Diabetes and cardiovascular disease: from new mechanisms to new therapies. Polish archives of internal medicine. 2018; 128: 178-186.
3. Mundi S, Massaro M, Scoditti E, et al. Endothelial permeability, LDL deposition, and cardiovascular risk factors-a review. Cardiovascular research. 2018; 114: 35-52.
4. Konduracka E, Cieślik G, Malecki MT, et al. Obstructive and nonobstructive coronary artery disease in long-lasting type 1 diabetes: a 7-year prospective cohort study. Polish archives of internal medicine. 2019; 129: 97-105.
5. Haberka M, Machnik G, Kowalówka A, et al. Epicardial, paracardial, and perivascular fat quantity, gene expressions, and serum cytokines in patients with coronary artery disease and diabetes. Polish archives of internal medicine. 2019; 129: 738-746.
6. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circulation research. 2010; 107: 1058-1070.
7. Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes. 1991; 40: 405-412.
8. Guzik TJ, Skiba DS, Touyz RM, et al. The role of infiltrating immune cells in dysfunctional adipose tissue. Cardiovascular research. 2017; 113: 1009-1023.

9. Ray R, Shah AM. NADPH oxidase and endothelial cell function. Clin Sci (Lond). 2005; 109: 217-226.

10. Schramm A, Matusik P, Osmenda G, et al. Targeting NADPH oxidases in vascular pharmacology. Vasc Pharmacol. 2012; 56: 216-231.

11. Maeda M, Hayashi T, Mizuno N, et al. Intermittent high glucose implements stress-induced senescence in human vascular endothelial cells: role of superoxide production by NADPH oxidase. PloS one. 2015; 10: e0123169.

12. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res. 2000; 86: 494-501.

13. Morawietz H. Cardiovascular protection by Nox4. Cardiovascular research. 2018; 114: 353-355.

14. Niemiec P, Nowak T, Iwanicki T, et al. The -930A>G polymorphism of the CYBA gene is associated with premature coronary artery disease. A case-control study and gene-risk factors interactions. Molecular biology reports. 2014; 41: 3287-3294.

15. Nowak T, Niemiec P, Iwanicki T, et al. Analysis of selected promoter polymorphisms and haplotypes of the CYBA gene encoding the p22phox, subunit of NADPH oxidases, in patients with coronary artery disease. Free radical research. 2018; 52: 1132-1139.

16. Meijles DN, Fan LM, Ghazaly MM, et al. p22phox C242T Single-Nucleotide Polymorphism Inhibits Inflammatory Oxidative Damage to Endothelial Cells and Vessels. Circulation. 2016; 133: 2391-2403.

17. Arca M, Conti B, Montali A, et al. C242T polymorphism of NADPH oxidase p22phox and recurrence of cardiovascular events in coronary artery disease. Arteriosclerosis, thrombosis, and vascular biology. 2008; 28: 752-757.
18. Moreno MU, San Jose G, Fortuno A, et al. The C242T CYBA polymorphism of NADPH oxidase is associated with essential hypertension. Journal of hypertension. 2006; 24: 1299-1306.

19. San Jose G, Moreno MU, Olivan S, et al. Functional effect of the p22phox -930A/G polymorphism on p22phox expression and NADPH oxidase activity in hypertension. Hypertension (Dallas, Tex : 1979). 2004; 44: 163-169.

20. Wyche KE, Wang SS, Griendling KK, et al. C242T CYBA polymorphism of the NADPH oxidase is associated with reduced respiratory burst in human neutrophils. Hypertension (Dallas, Tex : 1979). 2004; 43: 1246-1251.

21. Kojda G, Cheng YC, Burchfield J, et al. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. Circulation. 2001; 103: 2839-2844.

22. Malecki MT, Osmenda G, Walus-Miarka M, et al. Retinopathy in type 2 diabetes mellitus is associated with increased intima-media thickness and endothelial dysfunction. Eur J Clin Invest. 2008; 38: 925-930.

23. Malecki MT, Undas A, Cyganek K, et al. Plasma asymmetric dimethylarginine (ADMA) is associated with retinopathy in type 2 diabetes. Diabetes Care. 2007; 30: 2899-2901.

24. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med. 1998; 15: 539-553.

25. Unwin N, Alberti KG, Bhopal R, et al. Comparison of the current WHO and new ADA criteria for the diagnosis of diabetes mellitus in three ethnic groups in the UK. American Diabetes Association. Diabet Med. 1998; 15: 554-557.
26. Szymanski FM, Barylski M, Cybulska B, et al. Recommendation for the management of dyslipidemia in Poland - Third Declaration of Sopot. Interdisciplinary Expert Position Statement endorsed by the Polish Cardiac Society Working Group on Cardiovascular Pharmacotherapy. Cardiology journal. 2018; 25: 655-665.

27. Catapano AL, Graham I, De Backer G, et al. 2016 ESC/EAS Guidelines for the Management of Dyslipidaemias. Eur Heart J. 2016; 37: 2999-3058.

28. Inoue N, Kawashima S, Kanazawa K, et al. Polymorphism of the NADH/NADPH oxidase p22 phox gene in patients with coronary artery disease. Circulation. 1998; 97: 135-137.

29. Sim AS, Salonikas C, Naidoo D, et al. Improved method for plasma malondialdehyde measurement by high-performance liquid chromatography using methyl malondialdehyde as an internal standard. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2003; 785: 337-344.

30. Corretti MC, Anderson TJ, Benjamin EJ, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. Journal of the American College of Cardiology. 2002; 39: 257-265.

31. Wilk G, Osmenda G, Matusik P, et al. Endothelial function assessment in atherosclerosis: comparison of brachial artery flowmediated vasodilation and peripheral arterial tonometry. Pol Arch Med Wewn. 2013; 123: 443-452.

32. Verma S, Anderson TJ. Fundamentals of endothelial function for the clinical cardiologist. Circulation. 2002; 105: 546-549.

33. Harris RA, Nishiyama SK, Wray DW, et al. Ultrasound assessment of flow-mediated dilation. Hypertension. 2010; 55: 1075-1085.
34. Siedlecki Ł, Szygula-Jurkiewicz B, Szczurek W, et al. Mortality risk factors in patients with advanced heart failure and diabetes mellitus. Kardiologia polska. 2019; 77: 604-609.

35. Trzeciak P, Desperak P, Duda-Pyszny D, et al. Long-term outcomes of 11 021 patients with chronic coronary syndromes and after coronary angiography: the PRESAGE registry. Polish archives of internal medicine. 2020; 130: 1043-1052.

36. Guzik B, Chwala M, Matusik P, et al. Mechanisms of increased vascular superoxide production in human varicose veins. Pol Arch Med Wewn. 2011; 121: 279-286.

37. Sorop O, Heinonen I, van Kranenburg M, et al. Multiple common comorbidities produce left ventricular diastolic dysfunction associated with coronary microvascular dysfunction, oxidative stress, and myocardial stiffening. Cardiovascular research. 2018; 114: 954-964.

38. Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. Int J Vasc Med. 2012; 2012: 918267.

39. Sukumar P, Viswambharan H, Imrie H, et al. Nox2 NADPH oxidase has a critical role in insulin resistance-related endothelial cell dysfunction. Diabetes. 2013; 62: 2130-2134.

40. Youn JY, Siu KL, Lob HE, et al. Role of vascular oxidative stress in obesity and metabolic syndrome. Diabetes. 2014; 63: 2344-2355.

41. Nair BM, Viswanathan V, Snegalatha C, et al. Flow mediated dilatation and carotid intimal media thickness in South Indian type 2 diabetic subjects. Diabetes Res Clin Pract. 2004; 65: 13-19.

42. Batko K, Krzanowski M, Pietrzycka A, et al. Interplay of nitric oxide metabolites and markers of endothelial injury, inflammation, and vascular disease in the spectrum of advanced chronic kidney disease. Kardiologia polska. 2020; 78: 51-58.
43. Kimura Y, Matsumoto M, Miyauchi E, et al. Noninvasive detection of endothelial dysfunction in elderly with NIDDM by ultrasonography. Echocardiography. 2001; 18: 559-564.

44. Letonja MS, Nikolajevic-Starcevic J, Batista DC, et al. Association of the C242T polymorphism in the NADPH oxidase p22 phox gene with carotid atherosclerosis in Slovenian patients with type 2 diabetes. Mol Biol Rep. 2012; 39: 10121-10130.

45. Hayaishi-Okano R, Yamasaki Y, Kajimoto Y, et al. Association of NAD(P)H oxidase p22 phox gene variation with advanced carotid atherosclerosis in Japanese type 2 diabetes. Diabetes Care. 2003; 26: 458-463.

46. Pourgholi L, Pourgholi F, Ziaee S, et al. The association between CYBA gene C242T variant and risk of metabolic syndrome. European journal of clinical investigation. 2020; 50: e13275.

47. Racis M, Sobiczewski W, Stanisławska-Sachady A, et al. NADPH Oxidase Gene Polymorphism is Associated with Mortality and Cardiovascular Events in 7-Year Follow-Up. Journal of clinical medicine. 2020; 9.

48. Fan M, Raitakari OT, Kahonen M, et al. CYBA C242T gene polymorphism and flow-mediated vasodilation in a population of young adults: the Cardiovascular Risk in Young Finns Study. J Hypertens. 2007; 25: 1381-1387.

49. Bibli SI, Papapetroupolos A, Iliodromitis EK, et al. Nitroglycerine limits infarct size through S-nitrosation of cyclophilin D: a novel mechanism for an old drug. Cardiovascular research. 2019; 115: 625-636.
**Table 1. Clinical characteristics of type 2 diabetic patients studied (N=182)**

| Clinical characteristics                              |       |
|--------------------------------------------------------|-------|
| Age in years, mean (SD)                                | 56 (7) |
| Male sex, n (%)                                        | 91 (50) |
| Arterial hypertension, n (%)                           | 161 (88.4) |
| Dyslipidemia, n (%)                                    | 179 (98.3) |
| Ischaemic heart disease, n (%)                         | 86 (47.2) |
| History of myocardial infarction, n (%)                | 22 (12.1) |
| Obesity (BMI ≥30 kg/m\(^2\)), n (%)                   | 109 (59.8) |
| BMI in kg/m\(^2\), mean (SD)                           | 32.7 (6.5) |
| Microangiopathic complication, n (%)                   | 104 (57.1) |
| Current smoking, n(%)                                   | 39 (21.4) |
| FMD max in % median (IQR)                              | 8.8 (6.0-12.3) |
| NMD max in %, median (IQR)                             | 15.2 (11.8-18.9) |
| IMT mean in mm, median (IQR)                           | 0.84 (0.73-0.93) |

| Level of biochemical parameters                        |       |
|--------------------------------------------------------|-------|
| Total cholesterol in mmol/l, median (IQR)              | 5.1 (4.5-5.9) |
| LDL in mmol/l, median (IQR)                            | 2.9 (2.4-3.6) |
| HDL in mmol/l, median (IQR)                            | 1.1 (1.0-1.3) |
| Triglycerides in mmol/l, median (IQR)                  | 1.9 (1.4-2.9) |
| Peptide C in mg/ml, median (IQR)                       | 3.0 (1.7-4.0) |
| Creatinine in µm/l, median (IQR)                       | 75.3 (65.7-87.6) |
| HbA1c in %, median (IQR)                               | 7.6 (6.8-8.8) |

| Treatment or medication use                             |       |
|--------------------------------------------------------|-------|
| Only diabetic diet, n(%)                                | 13 (7.1%) |
| Oral anti-diabetic medication, n(%)                     | 115 (63.2%) |
| Insulin, n(%)                                           | 96 (52.7%) |
| Angiotensin-converting enzyme inhibitor, n(%)           | 140 (76.4%) |
|                     | n(%)  |
|---------------------|-------|
| Diuretic, n(%)      | 91 (50.0%) |
| Statin, n(%)        | 90 (49.5%) |
| Acetylsalicylic acid, n(%) | 79 (43.4%) |
| β-blocker, n(%)     | 67 (36.3%) |
| Calcium channel blocker, n(%) | 43 (23.1%) |
| Nitrate, n(%)       | 27 (14.8%) |
| α-blocker, n(%)     | 14 (7.7%) |

SD - standard deviation; BMI – body mass index; IQR - interquartile range; LDL - low-density lipoprotein; HDL - high-density lipoprotein; HbA1c - glycated haemoglobin; FMD= flow-mediated dilatation; NMD= nitroglycerin-mediated dilatation; IMT= Intima-media thickness
Table 2. Level of biochemical parameters according to genotypes of CYBA polymorphisms studied

| SNP | C242T | A-930G |
|-----|-------|--------|
|     | CC    | CT     | TT    | CC    | CT     | TT    | CC    | CT     | TT    | CC    | CT     | TT    |
| Genotype | Percentile 25<sup>th</sup> | median | 75<sup>th</sup> | Percentile 25<sup>th</sup> | median | 75<sup>th</sup> | Percentile 25<sup>th</sup> | median | 75<sup>th</sup> | Percentile 25<sup>th</sup> | median | 75<sup>th</sup> | Percentile 25<sup>th</sup> | median | 75<sup>th</sup> |
| Total cholesterol in mM | 4.47 | 5.05 | 5.67 | 4.33 | 4.89 | 5.93 | 4.95 | 5.45<sup>*</sup> | 6.50 | 4.63 | 5.12 | 6.01 | 4.44 | 5.09 | 5.73 | 4.33 | 4.72 | 5.50 |
| Triglycerides in mM | 1.27 | 1.79 | 2.95 | 1.44 | 1.94 | 2.60 | 1.56 | 2.35 | 3.13 | 1.33 | 2.01 | 2.93 | 1.34 | 1.90 | 2.65 | 1.52 | 2.00 | 2.91 |
| LDL in mM | 2.42 | 2.88 | 3.44 | 2.28 | 2.87 | 3.63 | 2.49 | 3.08 | 3.92 | 2.55 | 3.00 | 3.67 | 2.26 | 2.86 | 3.63 | 2.30 | 2.73 | 3.03 |
| HDL in mM | 1.00 | 1.10 | 1.34 | 0.95 | 1.13 | 1.29 | 0.94 | 1.24 | 1.43 | 0.94 | 1.10 | 1.33 | 1.00 | 1.18 | 1.37 | 0.93 | 1.10 | 1.28 |
| Peptide C in mg/ml | 1.60 | 3.31 | 4.44 | 1.79 | 2.90 | 3.74 | 1.83 | 2.82 | 4.04 | 2.04 | 2.99 | 4.00 | 1.59 | 2.74 | 3.94 | 2.08 | 3.23 | 4.34 |
| Creatinine in µM | 68.38 | 78.55 | 94.53 | 64.20 | 75.20 | 87.35 | 62.5 | 70.70 | 81.03 | 63.80 | 76.50 | 93.40 | 65.80 | 75.10 | 87.20 | 69.80 | 75.30 | 84.30 |
| HbA1c in % | 6.80 | 7.60 | 8.85 | 6.30 | 7.40 | 8.70 | 6.80 | 7.80 | 8.85 | 6.58 | 7.40 | 8.88 | 6.80 | 7.95 | 9.05 | 6.00 | 7.40 | 8.10 |

SNP=Single Nucleotide Polymorphism; CYBA= *cytochrome b-245 alpha chain*

*<sup>p=0.03</sup> as compared to CC/CT genotypes
Figure 1 - Mean intima-media thickness (IMT) in carotid arteries, among patients with different genotypes of C242T (Panel A) and A-930G (Panel B) polymorphisms.

Boxes represent 25%-75%, while whiskers 10%-90% of the mean Intima-media thickness (IMT) values.
Figure 2 - Flow-mediated dilatation (FMDmax; Panels A and B) and nitroglycerin-induced dilatation (NMDmax; Panels C and D) of brachial arteries, among patients with different genotypes of C242T (Panels A and C) and A-930G (Panels B and D) polymorphisms.

Boxes represent 25%-75%, while whiskers 10%-90% of the flow-mediated dilatation (FMDmax) or nitroglycerin-mediated dilatation (NMDmax) values.
Figure 3 - Association of C242T (Panels A and C) and A-930G (Panels B and D) CYBA polymorphisms with von Willebrand factor (vWF; Panels A and B) and malondialdehyde (MDA; Panels C and D) level.

Boxes represent 25%-75%, while whiskers 10%-90% of the von Willebrand factor (vWF) or malondialdehyde (MDA) level values.