Effect of common single nucleotide polymorphisms in *COX-1* gene on related metabolic activity in diabetic patients treated with acetylsalicylic acid

Marek Postula[1,2], Piotr K. Janicki[3], Marek Rosiak[1,2], Agnieszka Kaplon-Cieslicka[4], Agnieszka Kondracka[5], Ewa Trzepla[4], Krzysztof J. Filipiak[4], Dariusz A. Kosior[1,6], Andrzej Czlonkowski[2], Grzegorz Opolski[4]

[1]Department of Cardiology and Hypertension, Central Clinical Hospital, the Ministry of the Interior, Warsaw, Poland
[2]Department of Clinical and Experimental Pharmacology, Medical University of Warsaw, Warsaw, Poland
[3]Perioperative Genomics Laboratory, Department of Anesthesiology, Penn State College of Medicine, Hershey, Pennsylvania, USA
[4]Department of Cardiology, Medical University of Warsaw, Warsaw, Poland
[5]Department of Internal Medicine and Endocrinology, Medical University of Warsaw, Warsaw, Poland
[6]Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland

Corresponding author:
Marek Rosiak
Department of Cardiology
Medical University of Warsaw
1 A Banacha St
02-097 Warsaw, Poland
Phone: +48 22 599 19 58
Fax: +48 22 599 19 57
E-mail:
marek.rosiak@gmail.com

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Abstract

Introduction: The objective of this study was to investigate the effect of common single nucleotide genomic polymorphisms in the cyclooxygenase-1 (COX-1) gene on the thromboxane A_2 (TxA_2) metabolite concentrations in serum and urine, as well as on prostaglandin F2α (PGF2α) urinary excretion in the diabetic population on acetylsalicylic acid (ASA) therapy.
Material and methods: The study cohort consisted of 284 Caucasians with diabetes type 2 who had been taking ASA tablets at the dose of 75 mg/day for at least 3 months. Genotyping for the 4 selected SNPs within the COX-1 gene (two nonsynonymous-coding variants, rs3842787 [C50T, P17L] and rs5789 [C174A, L237M]; and two other synonymous SNPs, rs3842788 [G128A, Q41Q] and rs5788 [C644A]) was performed using the Sequenom iPLEX platform.
Results: No statistically significant results were observed for the investigated SNPs and measured metabolites in the investigated cohort of patients. Statistically significant differences in S-TxB_2 could however be observed for rs5788 in the subgroup of patients with very high S-TxB_2 concentrations. In particular, more patients who were carriers of the minor allele for this polymorphism were observed in the group with S-TxB_2 levels > 95th percentile, when compared with similar carriers in the group with S-TxB_2 < 95th percentile (20% vs. 1.1%, respectively, $p < 0.001$, Mann-Whitney test).
Conclusions: The results of our study suggest that the four investigated common SNPs in the COX1 gene are not associated with obviously altered TxA_2 metabolism and PGF2α synthesis in the investigated diabetic cohort treated with ASA.

Key words: acetylsalicylic acid, cyclooxygenase-1, diabetes mellitus, thromboxane, 8-iso-prostaglandin F2α.

Introduction

Several mechanisms caused by metabolic and cellular abnormalities have been suggested to play a role in increased platelet reactivity. Endogenous

and environmental factors – age, cholesterol and inflammatory markers levels, hypertension, diabetes mellitus, and cigarette smoking – explain only part of the variation in platelet function observed in persons with these conditions [1–9]. Although inherited and genetic factors have known links to cardiovascular disease, the evidence for genetic influences that enhance platelet function is much weaker [10–12].

Patients with type 2 diabetes mellitus (T2DM) are characterized by an elevated risk of recurrent atherothrombotic events. In addition, acetylsalicylic acid (ASA) therapy may be less effective in high-risk populations [11, 13, 14]. Patients with T2DM exhibit platelet hyperreactivity both *in vitro* and *in vivo* coupled with biochemical evidence of persistently increased thromboxane-dependent platelet activation [3–6]. Furthermore, polymorphisms in different genes have been associated with variability for increased platelet reactivity on ASA therapy in T2DM patients [14–16].

The problems with determining ASA response and the different methodologies available for determining the biochemical and functional effects of ASA have compounded the problems of attempting to define increased platelet reactivity despite ASA therapy, with some investigators suggesting that the term should be used only when production of thromboxane A_2 (TxA_2) (or its breakdown products) is blocked regardless of platelet function [11, 17].

Acetylsalicylic acid irreversibly inhibits cyclooxygenase (COX)-1 in human platelets. COX-1 (prostaglandin endoperoxide G/H synthase) catalyzes the formation of prostaglandin H_2 (PGH_2) from arachidonic acid (AA). This action is the committed step in prostaglandin synthesis, which yields the bioactive eicosanoids, prostaglandin D2, prostaglandin F2α (PGF2α), prostaglandin I_2, and TxA_2 through branching enzymatic pathways (Figure 1) [18]. The effect of ASA repeatedly administered once daily is irreversible, cumulative and saturable, reaching a ceiling effect in the low dose range [19]. The TxA_2 production can be determined by measuring stable metabolites of TxA_2, such as thromboxane B_2 (TxB_2) in the serum and 11-dehydro-TxB_2 (11-dh-TxB_2) in the urine. Because serum TxB_2 production is predominantly dependent on platelet COX-1, it has been used as a primary measure of the inhibitory effects of low-dose ASA on platelets [20]. In addition to TxA_2, platelets release F2-isoprostanes, in particular 8-iso-prostaglandin F2α (8-iso-PGF2α), a chemically stable compound derived from enzymatic and nonenzymatic oxidation of AA [21]. Platelet 8-iso-PGF2α is synthesized *in vivo* as a minor product of the COX-1 enzyme in human platelets and the COX-2 isoform in human monocytes, as well as through the free radical-catalyzed peroxidation of AA in biological membranes [22, 23]. Because 8-iso-PGF2α formation might correlate with the rate of TxA_2 biosynthesis, it was hypothesized that increased oxidant stress in T2DM could induce enhanced generation of 8-iso-PGF2α, which can contribute to platelet activation [24]. Even if it was demonstrated previously that enhanced 8-iso-PGF2α synthesis may be associated with advanced age, cigarette smoking, hypercholesterolemia, and unstable angina, as well as after coronary artery reperfusion, the role of 8-iso-PGF2α in ASA's effect on TxA_2 synthesis remains uncertain [25–33].

Genetic variation in COX-1 may affect enzyme expression, biochemical function or interaction with pharmacological agents [21]. The gene encoding human COX-1 (PTGS1), mapped to chromosome 9q32-q33.3, is approximately 22 kb in length, and contains 11 exons [22, 34]. COX-1 is constitutively expressed and is responsible for the biosynthesis of PGs involved in various functions, such as the regulation of renal, gastrointestinal, and platelet activity [18]. Several single nucleotide polymorphisms (SNPs) in both coding and noncoding regions of human COX-1 have been previously identified and were postulated to change its activity; however, all these studies were performed in healthy individuals or in patients with coronary

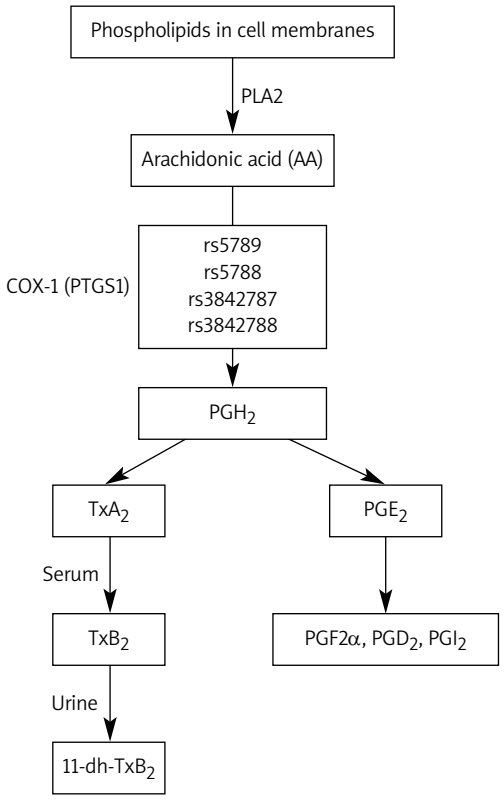

Figure 1. Arachidonic acid cascade and investigated single nucleotide polymorphisms (SNPs)
PLA2 – phospholipase A2, COX-1 – cyclooxygenase-1, PGH_2 – prostaglandin H_2, PGF2α – prostaglandin F2α, PGD_2 – prostaglandin D_2, PGJ_2 – prostaglandin J_2, TxA_2 – thromboxane A_2, TxB_2 – thromboxane B_2, 11-dh-TxB_2 – 11-dehydro-TxB_2

artery disease (CAD) [11, 12, 35–37]. In addition, there is no systemic information published about the effects of these common SNPs on the function of COX-1, as measured directly by TxA_2 metabolites and 8-iso-PGF2α concentrations in the diabetic population. To our knowledge, there is also no information about the effects of these common SNPs in the COX-1 gene on the related metabolic activity in the Polish population.

Thus, the objective of this study was to evaluate the impact of the four frequently investigated COX-1 gene polymorphisms in the entirely diabetic population on ASA therapy on TxA_2 synthesis, measured as serum and urinary TxA_2 metabolite concentrations, as well as on 8-iso-PGF2α synthesis measured as its urinary excretion. The genotyping was attempted for four COX-1 SNPs (two missense variants, rs3842787 [C50T, P17L] and rs5789 [C174A, L237M]; and two other synonymous SNPs, rs3842788 [G128A, Q41Q] and rs5788 [C644A]).

Material and methods

Patient population and study design

The ethics committee of the Medical University of Warsaw approved both the study protocol and the informed consent form. The study was conducted in accordance with the current version of the Declaration of Helsinki at the time when the study was designed, and informed written consent was obtained. The genotyping part of the study was reviewed and approved by the Institutional Review Board of Penn State Hershey Medical Center (Hershey, PA, USA). The study subjects were recruited consecutively from patients with T2DM participating in the multi-center, prospective, randomized, and open-label AVOCADO (Aspirin Vs/Or Clopidogrel in Aspirin-resistant Diabetics inflammation Outcomes) study presenting to the outpatient clinic of the Central Teaching Hospital of the Medical University of Warsaw. The full characteristics of the study population, including the inclusion and exclusion criteria, were published previously [14, 38]. Briefly, the study recruited 304 subjects with T2DM who, at the time of enrollment, had been taking ASA tablets at the dose of 75 mg per day for at least 3 months for primary or secondary prevention of myocardial infarction (MI). No clopidogrel or antiplatelet drugs other than ASA were used in any of the investigated patients. All patients had been taking oral antidiabetic agents and/or insulin for at least 6 months; diet-controlled diabetic patients were not included. Compliance with ASA therapy was determined at the study entry based upon the patient's own statement and serum thromboxane B_2 (S-TxB_2) levels below 7.2 ng/ml in all patients as described previously in a diabetic population by Mortensen *et al.* [6].

Blood sample and assay procedures

Blood samples were taken in the morning 2-3 h after the last ASA dose. Blood was taken from the antecubital vein, and the initial 2 ml of blood were discarded to avoid spontaneous platelet activation. Whole blood for S-TxB_2 was allowed to clot at 37°C for 1 h before separating serum by centrifugation. Serum was obtained from venous blood by centrifugation at 1000 g for 15 min at 4°C, and aliquots were stored at –80°C for further analysis. Regular laboratory testing was performed at the laboratory of the Central Teaching Hospital, Medical University of Warsaw, using standard techniques, and included complete blood cell and platelet counts, fasting glycemia, glycosylated hemoglobin (HbA_{1c}), lipid profile, C-reactive protein and serum creatinine concentrations.

Urinary 11-dehydro-thromboxane B_2 (11-dh-TxB_2)

11-dh-TxB_2 was measured using an enzyme immunoassay kit according to the manufacturer's instructions (EIA kit, Cayman Chemical) after extraction and purification on SPE (C18) columns (Waters Associates, Milford, MA, USA). Data were normalized for urinary creatinine concentration.

Serum thromboxane B_2 (S-TxB_2)

S-TxB_2 was measured also with an enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA). Each lot of TxB_2 EIA kit was tested for the impact of interferences. The correlation of results in three dilutions of five random samples was assessed, as was proposed in the kit protocol. The decision to use the assay without purification was taken after analysis of results, as differences of results did not exceed 20%. Samples with results outside the standard curve were re-assayed with appropriate dilutions.

Urinary isoprostane measurement

Quantification of 8-iso-PGF2α was performed on a morning urine sample with an enzyme immunoassay kit (8-isoprostane EIA Kit, Cayman Chemical, Ann Arbor, MI, USA) and normalized to urinary creatinine concentration.

DNA extraction, quality control, and quantification

DNA was obtained from whole blood samples by the membrane ultrafiltration method using a Fuji MiniGene 80 extractor (FujiFilm Life Sciences distributed by Autogene, Holliston, MA), as described previously [15, 16].

Individual SNP genotyping

Genotyping was performed at Boston Children's Hospital using a custom Sequenom iPLEX assay in conjunction with the Mass ARRAY platform (Sequenom Inc., La Jolla, CA, USA). One panel of SNP markers was designed using Sequenom Assay Design 3.2 software.

Statistical analysis

Power analysis

We planned a prospective, observational study of diabetic patients treated with ASA. The required sample size (n = 265) of the study was designed to have a power of > 80% to detect a change in S-TxB_2 level corresponding to a significant 2-fold increase in S-TxB_2 concentration between alleles of the 4 investigated SNPs at a 0.05/4 = 0.0125 level of significance, with Bonferroni correction applied.

Statistical calculations

The statistical analyses were performed using IBM-SPSS ver. 19 and Stata (Stata Corporation, College Station, TX) software. Deviations from Hardy-Weinberg equilibrium (HWE) were calculated using the χ^2 test. The recorded clinical data, when normally distributed in the analyzed group of patients, are presented as mean and SD, and non-normally distributed data are presented as medians and interquartile range (IQR). In the first stage of the analysis we compared the distribution of median S-TxB_2, 11-dh-TxB_2 and 8-iso-PGF2α levels among all genotypes of successfully genotyped SNPs using the Kruskal-Wallis test. The SNPs with nominal statistically significant (i.e. $p < 0.05$ before applying correction for multiple comparisons) differences in the measurements (S-TxB_2, 11-dh-TxB_2 and 8-iso-PGF2α levels) between medians for each of three genotypes (i.e., homozygotes for minor allele, heterozygotes and homozygotes for major allele) were subjected to further testing based on the dominant, recessive or additive genetic model by Mann-Whitney test.

Results

From the initially enrolled 304 patients, complete clinical data and blood samples finally became available for 298 patients. Subsequently, 8 patients were eliminated from the analysis based on the suspected ASA non-compliance (patients' own statement and S-TxB_2 concentrations > 7.2 ng/ml) and a further 6 patients were eliminated because of the lack of corresponding biochemical and genotype data. Demographic characteristics and clinical data for the remaining 284 patients are summarized in Table I.

The summary results of the allele and genotype frequencies for all genotyped SNPs are summarized in Table II. All 4 SNPs genotyped well (> 86% success rate) and were in Hardy-Weinberg equilibrium. For each of the successfully genotyped SNPs, we initially compared the corresponding S-TxB_2, and urine 11-dh-TxB_2 and 8-iso-PGF2α excretion data between 3 allelic groups (e.g. homozygotes for minor and major alleles, as well as heterozygotes) using the non-parametric Kruskal-Wallis test. The obtained results did not reveal any statistically significant differences in S-TxB_2, 11-dh-TxB_2 and 8-iso-PGF2α concentrations for carriers and non-carriers of the investigated SNP variants.

Table I. Demographic and clinical characteristics of the study patients (n = 284)

Demographics	
Age [years]	67.6 ±8.7
Female, *n* (%)	135 (47.5)
BMI [kg/m^2]	31.19 ±12.0
SBP [mm Hg]	142.3 ±18.9
DBP [mm Hg]	80.5 ±11.3
Dyslipidemia	234 (82.4%)
Hypertension	262 (92.3%)
CAD	162 (57.0%)
Prior MI	87 (30.6%)
Prior stroke	23 (8.1%)
Heart failure	107 (37.7%)
History of smoking	160 (56.3%)
Current smoking	28 (9.8%)
Concurrent medications	
Oral hypoglycemic	243 (85.6%)
Insulin	93 (32.7%)
β-Blockers	205 (72.2%)
ACE inhibitors	185 (65.1%)
Statins	206 (72.6%)
Biochemical and hematological parameters	
HGB [g/dl]	13.8 ±1.3
HCT [%]	41.3 ±4.5
WBC [10^3/mm^3]	7.1 ±2.2
PLT [10^3/mm^3]	227.8 ±58.3
MPV [fl]	9.9 ±1.2
eGFR [ml/min/1.73]	70.8 ±20.9
HbA_{1c} [%]	7.0 ±1.3
hsCRP	4.1 ±5.6

Data are presented as mean ± SD unless otherwise indicated. BMI – body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, CAD – coronary artery disease, MI – myocardial infarction, ACE – angiotensin-converting enzyme, HGB – hemoglobin, HCT – hematocrit, WBC – white blood cells, PLT – platelet count, MPV – mean platelet volume, eGFR – estimated glomerular filtration rate, HbA_{1c} – glycosylated hemoglobin, hsCRP – high-sensitivity C-reactive protein.

Table II. The effects of different analyzed genotypes of SNPs in COX-1 on plasma levels of S-TxB2 and urine excretion of 11-dh-TxB_2 and 8-iso-PGF2α in diabetic patients on ASA

Analyzed SNPs in COX-1	S-TxB_2 [ng/ml]	11-dh-TxB_2 [ng/mmol Cr]	8-iso-PGF2α [ng/mg Cr]
Rs5788 C>A (MAF = 0.180)			
Homozygotes for major allele (*n* = 193)	0.155 (0.6)	40.485 (40.82)	0.411 (0.3)
Heterozygotes (*n* = 81)	0.159 (0.6)	38.990 (41.55)	0.410 (0.4)
Homozygotes for minor (variant) allele (*n* = 10)	0.107 (1.7)	61.690 (108.70)	0.405 (0.2)
P* (KW test)	0.945	0.288	0.885
Rs5789 C>A (MAF = 0.017)			
Homozygotes for major allele (*n* = 275)	0.153 (0.6)	40.490 (41.56)	0.410 (0.3)
Heterozygotes (*n* = 8)	0.223 (0.5)	26.200 (38.89)	0.352 (0.5)
Homozygotes for minor (variant) allele (*n* = 1)	n/a	n/a	n/a
P (MW test)	0.509	0.247	0.597
Rs3842787 C>T (MAF = 0.061)			
Homozygotes for major allele (*n* = 250)	0.154 (0.6)	40.430 (41.68)	0.406 (0.3)
Heterozygotes (*n* = 28)	0.157 (0.6)	42.800 (42.07)	0.494 (0.4)
Homozygotes for minor (variant) allele (*n* = 3)	0.033 (–)	36.980 (–)	0.301 (–)
P (MW test)	0.602	0.978	0.284
Rs3842788 G>A (MAF = 0.015)			
Homozygotes for major allele (*n* = 275)	0.154 (0.5)	40.450 (40.45)	0.410 (0.3)
Heterozygotes (*n* = 9)	0.071 (1.1)	39.505 (57.38)	0.342 (0.4)
Homozygotes for minor (variant) allele (*n* = 0)	n/a	n/a	n/a
P (MW test)	0.815	0.897	0.945

*Data are shown as median and interquartile range (IQR). *P using Kruskal-Wallis (KW) test for differences between 3 analyzed genotypes for each SNP or using Mann-Whitney test (MW) for differences between 2 genotypes (carriers of wild-type allele and pooled carriers of variant allele); n – number of carriers for each genotype, MAF – minor allele frequency for each analyzed SNP in investigated cohort, SNPs – single nucleotide polymorphisms, COX-1 – cyclooxygenase-1, S-TxB_2 – serum thromboxane B_2, 11-dh-TxB_2 – urinary 11-dehydro-thromboxane B_2, 8-iso-PGF2α – 8-iso-prostaglandin F2α.*

In order to more closely analyze the relationship between S-TxB_2 and COX-1 polymorphisms (in particular in the outlier sub-cohort with S-TxB_2 > 75th percentile), we also established several empirical cut-off criteria (e.g., at 75th percentile 0.612 ng/ml, 1.5 × 75th percentile 0.918 ng/ml, and at 95th percentile 4.05 ng/ml), which divided the investigated patients into 2 categories, based on S-TxB_2 concentration. No effects of any investigated polymorphisms in COX-1 were observed for the analyzed patient cohort with the S-TxB_2 cut-off at the 75th percentile or 1.5 × 75th percentile (not shown). When the investigated cohort was subsequently divided into two parts, based on the 95th percentile, a statistically significant difference could be established for the rs5788 polymorphism, based on the recessive model, i.e., in carriers of a variant minor allele. In particular, more patients who were carriers of the minor allele for this polymorphism were observed in the group with S-TxB_2 levels > 95th percentile, when compared with similar carriers in the group with S-TxB_2 < 95th percentile (20% vs. 1.1%, respectively, $p < 0.001$, Mann-Whitney test). No changes were observed for the analyzed outliers in S-TxB_2, 11-dh-TxB_2 or 8-iso-PGF2α or for the other three investigated SNPs in COX-1.

The presented statistical analysis was performed on 284 patients with observed S-TxB_2 levels above the non-compliance threshold (i.e. S-TxB_2 > 7.2 ng/ml). Very similar results were obtained when this analysis was repeated in all 292 patients (i.e. without removal of any of them for suspected non-compliance), including the effect of rs5788 polymorphism on outlier S-TxB_2 levels (data not shown).

Discussion

The current genetic study of platelet reactivity during chronic ASA therapy represents, to our knowledge, the first comprehensive analysis of SNPs in the *COX-1* gene during chronic ASA therapy in diabetic patients and aims to directly correlate genetic variants within the *COX-1* gene with AA metabolites (i.e. TxB_2, 11-dehydro TxB_2, 8-iso-PGF2α) in an ethnically homogeneous and entire-

ly diabetic population from central Poland. In our study COX-1 inhibition was based on TxA_2-related measurements, such as serum TxB_2 levels (index of the maximum biosynthetic capacity of platelet COX-1 *ex vivo*) and urinary 11-dehydro TxB_2 excretion (index of *in vivo* TxA_2 biosynthesis) [39, 40].

We found that four common COX-1 gene polymorphisms (two missense variants, rs3842787 [C50T, P17L] and rs5789 [C174A, L237M]; and two other synonymous SNPs, rs3842788 [G128A, Q41Q] and rs5788 [C644A]) (Table II) had no statistically significant effects on TxA_2-related indexes and 8-iso-PGF2α excretion in the investigated diabetic patients treated with ASA. A possible exception was observed for SNP rs5788, for which the carriers of the minor allele for this polymorphism were more frequently observed in the group with S-TxB_2 levels > 95th percentile, when compared with similar carriers in the group with S-TxB_2 < 95th percentile (20% vs. 1.1%, respectively, $p < 0.001$, Mann-Whitney test). The clinical significance of this last finding remains unclear, but may indicate that this SNP could be responsible for the inter-individual differences in the S-TxB_2 concentrations in at least a small portion of the patients with high COX-1 activity or may be due to known or unknown genetic variants in linkage disequilibrium.

Several genetic variants in human COX-1 were found previously to alter COX-1-mediated AA metabolism in the non-diabetic population; however, only the rs3842787 (or so-called C50T) polymorphism in COX-1 was repeatedly shown to be of functional significance in relation to the antiplatelet effect of ASA [11, 12, 41, 42]. Some studies demonstrated an association between this SNP and reduced ASA effect on platelet aggregation and TxB_2 formation [21, 43, 44]. Gonzalez-Conejero *et al*. demonstrated that ASA-treated healthy subjects with the COX-1 rs3842787 polymorphism had an almost two-fold increase *in-vivo* TxB_2 production [43]. Similar findings were observed in a trial including 144 patients with stable CAD, which showed higher AA-induced platelet aggregation and serum TxB_2 synthesis in carriers with a haplotype containing the variant of rs3842787 [21]. Lepantalo *et al*. examined the polymorphisms of COX-1 on antiplatelet effect of ASA in coronary artery disease patients and observed a correlation with AA-induced aggregometry and PFA-100, but not with serum TxB_2 levels [44]. However, a recent comprehensive systematic review revealed no significant association of rs3842787 with so-called "ASA resistance", as the number of studies and of subjects used was small, making it difficult to exclude definitively any contribution of these polymorphisms to the effect of ASA (OR for aspirin resistance 1.07; 95% CI 0.41, 2.77; $p = 0.89$) [11].

Platelet 8-iso-PGF2α is synthesized *in vivo* as a minor product of the COX-1 enzyme in human platelets and the COX-2 isoform in human monocytes and mostly independently of the activity of COX-1, through the free radical- catalyzed peroxidation of AA in biological membranes [22, 23]. Contrary to TxA_2, 8-iso-PGF2α does not directly induce platelet aggregation, but it elicits shape change, amplifies the platelet response to common agonists, and enhances platelet recruitment, and thus can modulate platelet aggregation through the thromboxane receptor (TP) or a closely related receptor, regardless of COX-1 inhibition by ASA [23, 24, 32]. As thromboxane synthase inhibitors do not reliably inhibit AA-induced platelet aggregation, it was postulated that the measurement of 8-iso-PGF2α can serve as a better marker than TxB_2 for analysis of the correlation between functional outcomes and genetic variants at the COX-1 locus. Because platelet COX activity can also produce small (when compared with TxB_2) amounts of 8-iso-PGF2α, we tried to establish an association between enhanced urinary excretion of 8-iso-PGF2α and COX-1 polymorphisms that could influence its enzymatic activity. Halushka *et al*. reported that ASA has an enhanced inhibitory effect on *in vitro* PGH2 formation by platelets (measured as 8-iso-PGF2α) from healthy carriers of variant allele rs3842787 [35]. In contrast to those results, we did not find any association between rs3842787 (C50T) SNP and 8-iso-PGF2α levels in the diabetic population. These differences could be, in part, explained by the previous report that compared 8-iso-PGF2α excretion in the presence and absence of platelet COX blockade by low-dose aspirin. In this study COX-1 inhibition, as reflected by suppression of TxB_2 biosynthesis, was not associated with any detectable difference in F_2-isoprostane formation [32]. In another study it was also shown that urinary 8-iso-PGF2α excretion did not contribute to impaired platelet responsiveness to ASA in a COX-1 dependent mechanism [33].

According to Lee *et al*., the COX-1 rs5789 (L237M) variant can presumably influence catalytic activity of COX-1 through its predicted impact on dimerization [42]. Moreover, they found that COX-1 metabolic activity *in vitro* was significantly lower with the rs5789 (L237M) variant relative to wild type after inhibition with indomethacin as the carriers of the rs5789 (L237M) variant also had significantly lower basal activity [42]. However, this observation may not extend to all NSAIDs, and thus TxB2 formation on ASA therapy *in vivo*.

The inherent limitations of observational design apply to this study. The candidate SNPs within the COX-1 gene were chosen because of their established or suspected influence on platelet function, as assessed by numerous studies in several laboratories. However, it cannot be ruled out that some other non-analyzed SNPs (or other genetic variants)

can alter COX-1 activity. As such, our current findings should be interpreted with caution and await further confirmation from more detailed studies. Moreover, pretreatment measurements (i.e. before beginning ASA treatment) could not be conducted because all patients included in this study had diagnosed CAD or multiple risk factors for CAD and therefore were on ASA therapy at the time of enrollment. Other weaknesses of our study include the lack of external replication in an independent cohort of diabetic patients. Lastly, our association might be either random or associated with the particular patient population (e.g. diabetes), and this question could not be answered in this study.

In conclusion, we were unable to confirm an association between investigated common COX-1 gene SNPs and COX-1 activity in the diabetic population treated with a low dose of ASA. Additional investigation is warranted to establish whether COX-1 activity in the T2DM population may be influenced by other, less frequent, genetic variants of COX-1, which were not investigated in this study. Further studies in larger and different ethnic diabetic populations are needed to validate and possibly extend the conclusions from this relatively small, but ethnically homogeneous, cohort.

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Marek Postula and Piotr K. Janicki contributed equally to this work.

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