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Serum Brain-Derived Neurotrophic Factor is Related to Platelet Reactivity but not to Genetic Polymorphisms within BDNF Encoding Gene in Patients with Type 2 Diabetes

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Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to investigate the association between serum concentrations of the brain-derived neurotrophic factor (BDNF), platelet reactivity and inflammatory markers, as well as its association with BDNF encoding gene variants in type 2 diabetic patients (T2DM) during acetylsalicylic acid (ASA) therapy.





Material/Methods: This retrospective, open-label study enrolled 91 patients. Serum BDNF, genotype variants, hematological, biochemical, and inflammatory markers were measured. Blood samples were taken in the morning 2–3 h after the last ASA dose. The BDNF genotypes for selected variants were analyzed by use of the iPLEX Sequenom assay.

Results: In multivariate linear regression analysis, CADP-CT >74 sec ($p < 0.001$) and sP-selectin concentration ($p = 0.03$) were predictive of high serum BDNF. In multivariate logistic regression analysis, CADP-CT >74 sec ($p = 0.02$) and IL-6 concentration ($p = 0.03$) were risk factors for serum BDNF above the median. Non-significant differences were observed between intronic SNP rs925946, missense SNP rs6265, and intronic SNP rs4923463 allelic groups and BDNF concentrations in the investigated cohort.

Conclusions: Chronic inflammatory condition and enhanced immune system are associated with the production of BDNF, which may be why the serum BDNF level in T2DM patients with high platelet reactivity was higher compared to subjects with normal platelet reactivity in this study.

MeSH Keywords: **Aspirin • Brain-Derived Neurotrophic Factor • Diabetes Mellitus • Inflammation • Platelet Activation**

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Background

Brain-derived neurotrophic factor (BDNF) plays a key role in regulating neuronal outgrowth, differentiation, synaptic connections, and neuronal repair [1]. Apart from nervous system disorders, several reports documented an association between plasma BDNF and systemic or peripheral inflammatory conditions, such as diabetes, acute coronary syndrome, atherosclerosis, and rheumatoid arthritis [2–5].

It has been shown that peripheral BDNF is stored in large amounts in platelets, and plasma BDNF concentrations can be attributed to its release into the plasma from platelets through activation or clotting process [6–8]. However, the regulation of BDNF in peripheral blood remains poorly understood [6]. It has been reported that age and sex have an impact on BDNF, both circulating and stored in platelets [9]. Previous studies investigated the impact of different antiplatelet drugs on BDNF concentrations in serum and plasma and on the release of BDNF from platelets in healthy volunteers [10]. Moreover, several polymorphisms variants in the BDNF gene were associated with differences in BDNF concentrations and prognosis after stroke or response to psychotropic drugs, in particular in psychiatric disorders and asthma [11–14].

Type 2 diabetes (T2DM) is a hypercoagulable state and is associated with platelet hyper-reactivity [15]. The etiology of high platelet reactivity (HPR) is complex, and is related to metabolic disturbances, hyperglycemia, coexisting inflammation, and oxidative stress [16,17]. In particular, inflammatory and coagulation markers have higher concentrations in T2DM patients, and platelet reactivity is increased in comparison to healthy subjects [18,19].

The primary aim of this study was to investigate the correlation between platelet reactivity and BDNF serum level in T2DM in ASA therapy and its association with selected inflammatory and platelet activation mediators (hsCRP, IL-6, TNF- α , sCD40L, and sP-selectin). The secondary aim of the project was to investigate whether several common single nucleotide polymorphisms (SNPs) in the BDNF gene are associated with the observed serum BDNF concentrations. The following 3 SNPs were investigated: intronic SNP rs925946 (BDNF-AS or BDNF antisense RNA), missense SNP rs6265 (G1281A or V66M), and intronic SNP rs4923463 (BDNF-AS or BDNF antisense RNA).

Material and Methods

This was a preliminary, exploratory analysis of the results from the AVOCADO (Aspirin Vs/Or Clopidogrel in Aspirin-resistant Diabetics inflammation Outcomes Study), a multi-center, prospective, randomized, open-label study. 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. In the present analysis we included only fully characterized subjects (i.e., with all biochemical and genetic measurements listed below, including plasma BDNF concentrations) participating in the AVOCADO study as it was previously published [20,21]. Briefly, selected subjects with T2DM were included who, at the time of enrollment, were on a 75-mg daily dose of ASA for at least 3 months for primary or secondary prevention of myocardial infarction (MI).

Blood sample and assay procedures

All blood samples were taken in fasting condition at 9 a.m. at least 2 h after the last dose of ASA. Standard laboratory techniques were used for regular laboratory testing. Serum BDNF concentrations were measured using BDNF Quantikine Immunoassay (R&D Systems, USA) as previously described for our laboratory [14]. ELISA kits were also used to determine concentrations of the following parameters: serum TXB2 (EIA kits, Cayman Chemicals, Ann Arbor, MI, USA), von Willebrand factor (vWF) molecule (vWF: Ag), tumor necrosis factor (TNF)- α (Quantikine[®] HS ELISA Human TNF- α Immunoassay), interleukin (IL)-6 (Quantikine[®] HS ELISA Human IL-6 Immunoassay; both R&D Systems, Inc., Minneapolis, USA), soluble CD40 ligand (sCD40L; Human soluble CD40 Ligand Immunoassay, R&D Systems, Inc., NE, USA), and soluble P-selectin (human P-selectin/CD62P ELISA kit R&D Systems, Inc., Minneapolis, USA). High-sensitivity C-reactive protein (hsCRP) concentrations were assessed using a Cobas Integra 800 device (Roche, Basel, Switzerland), as previously described [21,22]. The compliance with ASA treatment was defined according to previously described criteria [20].

Platelet function analysis

Platelet function analysis was performed using both VerifyNow Aspirin Assay (Accumetrics, San Diego, CA, USA) and PFA-100 assay (Dade-Behring International, Inc., Newark, DE, USA). For VerifyNow ASA, reaction units (ARU) <550 were used to determine platelet dysfunction as the result of ASA [20,21]. Using PFA-100, epinephrine bitartrate (CEPI) and adenosine diphosphate (CADP) closure time (CT) was determined [20,23]. To determine HPR we applied a cut-off value for CEPI-CT \geq 193 sec and for CADP-CT above the lower quartile value (i.e., >74 s) [20,21].

DNA extraction, quality control, and quantification

DNA was obtained from whole blood samples stored frozen until the time of analysis, using the membrane ultrafiltration

Table 1. Demographic and clinical characteristics of the study patients.

	BDNF low* (n=45)	BDNF high* (n=46)	P
Age (years)	68.33±8.045	65.70±9.354	0.179
Female	18 (40%)	24 (52.2%)	0.486
BMI	31.060±5.458	29.403±3.922	0.189
WHR	0.963±0.0768	0.955±0.0804	0.617
SBP	143.53±21.265	137.91±16.616	0.226
DBP	78.93±11.919	77.54±11.519	0.952
Dyslipidemia	39 (86.7%)	39 (84.8%)	0.666
Prior Stroke	5 (22.5%)	4 (8.7%)	0.995
History of smoking	24 (53.3%)	29 (63%)	0.626
Concurrent medications			
Beta-blockers	33 (73.3%)	36 (78.3%)	0.725
ACE inhibitors	33 (73.3%)	32 (69.6%)	0.613
CCB	19 (42.2%)	27 (58.7%)	0.308
Statins	33 (73.3%)	36 (78.3%)	0.458
Fibrates	7 (15.6%)	6 (13%)	0.847
Proton-pump inhibitors	4 (8.9%)	9 (19.6%)	0.105
Sulphonylurea	23 (51.1%)	25 (54.4%)	0.605
Metformin	28 (62.2%)	31 (67.4%)	0.219
Insulin	15 (33.3%)	14 (30.4%)	0.808

Data are presented as mean ±SD unless otherwise indicated. Abbreviations: BDNF, brain-derived neurotrophic factor (* low <30.6 ng/mL <high); BMI, body mass index (kg/m²); WHR – waist-to-hip ratio; SBP – systolic blood pressure (mmHg); DBP – diastolic blood pressure (mmHg); CAD – coronary artery disease; MI – myocardial infarction; ACE – angiotensin-converting enzyme; CCB – calcium channel blockers.

method with a FujiMiniGene 80 extractor (FujiFilm Life Sciences, distributed by Autogene, Holliston, MA, USA).

Individual SNP genotyping

Genotyping was performed at the Children’s Hospital Boston 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 (Sequenom Inc., La Jolla, CA, USA).

Statistical analysis

The statistical analyses were performed using IBM-SPSS ver. 19 and Stata (Stata Corporation, College Station, TX) software. Normally distributed continuous variables are presented as means ±SD, whereas variables with a highly skewed distribution are presented as medians with corresponding range. Categorical variables were presented as frequencies

(percentages). Normality of distribution was assessed using histograms and quartile plots. All the parametric data were compared by independent *t* test and the nonparametric data by the Mann-Whitney U test between 2 groups with high and low BDNF concentrations, as appropriate. All statistical tests were performed at significance level *p*=0.05 (two-sided).

Univariate and multivariate linear regression analyses were performed to determine the predictive factors of serum BDNF concentration. Moreover, univariate and multivariate logistic regression analyses were performed to determine the predictive factors of serum BDNF concentration above the median. In a multivariate logistic regression model and a multivariate linear regression model, all factors that were found to be significant in univariate analyses were used. Multivariate analysis was performed with adjustment for age, sex, and variables selected using a stepwise procedure. A value of *p*≤0.05 was considered significant for all tests.

Table 2. Hema tological and biochemical parameters.

	BDNF low* (n=45)	BDNF high* (n=46)	p
WBC	6.899±1.777	7.440±2.113	0.284
HGB	14.007±1.252	14.198±1.421	0.498
HCT	42.056±3.592	42.252±3.880	0.803
PLT	213.29±54.078	249.30±66.514	0.004
MPV	9.962±1.224	10.133±1.350	0.530
FG	135.11±39.239	152.24±70.770	0.363
eGFR	66.877±16.703	74.281±21.303	0.069
UA	6.227±1.783	5.528±1.382	0.039
TCh	159.02±37.589	171.17±45.216	0.181
TG	134.78±73.961	146.85±74.564	0.544
LDL	87.25±32.724	90.96±39.656	0.812
HbA1c	6.749±0.928	7.352±1.610	0.098
CEPI-CT	220.73±82.097	209.33±84.652	0.568
CADP-CT	122.53±62.255	108.15±71.126	0.010
VerifyNow ARU	479.67±87.916	477.17±82.697	0.858
sTXB2	0.518±1.025	0.974±1.702	0.223
hsCRP	3.218±2.652	4.372±4.146	0.331
sP-selectin	68.681±24.624	82.385±24.715	0.010
sCD40L	1.0215±1.895	0.601±0.364	0.824
TNF-α	2.064±1.392	2.235±1.519	0.407
IL-6	2.508±1.706	4.069±3.211	0.005
vWF	138.855±55.378	153.282±57.428	0.198

Data are presented as mean ±SD. BDNF, brain-derived neurotrophic factor (* low<30.6 ng/mL<high), WBC – white blood cells ($10^3/\text{mm}^3$); HGB – hemoglobin (g/dL); HCT – hematocrit (%); PLT – platelet count ($10^3/\text{mm}^3$); MPV – mean platelet volume (fL); FG – fasting glucose (mg/dL); eGFR – estimated glomerular filtration rate ($\text{mL}/\text{min}/1.73 \text{ m}^2$); UA – uric acid (mg/dL); TCh – total cholesterol (mg/dL); TG – triglycerides (mg/dL); HDL – high density lipoproteins (mg/dL); LDL – low density lipoproteins (mg/dL); HbA1c – glycosylated hemoglobin (%); CEPI-CT – collagen/epinephrine bitartrate closure time (sec); CADP-CT – collagen/adenosine diphosphate closure time (sec); VerifyNow ARU – VerifyNow aspirin reaction units; sTXB2 – serum thromboxane B2 (ng/mL); hsCRP – high sensitivity C-reactive protein (mg/l); sP-selectin – soluble P-selectin; (ng/mL); sCD40L – soluble CD40 ligand (ng/mL); TNF-α – tumor necrosis factor α (pg/mL); IL-6 – interleukin 6 (pg/mL); vWF – von Willebrand factor (%).

The statistical calculations, aimed at establishing the genetic association between investigated polymorphisms and absolute concentrations of BDNF in serum of subjects, were performed using Golden Helix ver. 8.5.1 platform (Bozeman, MT). The inheritance models used included basic allelic model (D vs. d), genotypic model (DD vs. Dd vs. dd), additive model (DD>Dd>dd), dominant model (dd, dD vs. DD), and recessive model (DD vs. dd, dD).

The analysis was exploratory; therefore, no formal a priori power analysis was performed.

Results

Demographic and basic clinical characteristic

Mean ±SD demographic data, concurrent medications, and biochemical and hematological parameters for the study population are presented in Tables 1 and 2. The subjects were divided into 2 groups based on the observed median value of serum BDNF concentrations (30.6 ng/mL); BDNF ranges (14.15–30.10 ng/mL) in the low BDNF group and BDNF ranges (30.61–64.58 ng/mL) in the high BDNF group.

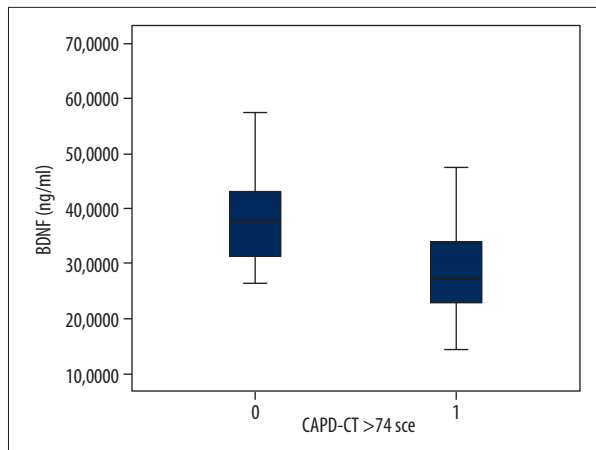


Figure 1. Distribution of serum BDNF concentration across patients with normal platelet reactivity based on CADP-CT (i.e. >74 sec, group 1) and high platelet reactivity (i.e. <74 sec, group 0) in the study group. BDNF – brain-derived neurotrophic factor; CADP-CT – collagen/adenosine diphosphate closure time. The box plot represents the median, the interquartile range, and the minimum and maximum value.

Patients with high BDNF smoked cigarettes more frequently than patients with low BDNF (4.5% vs. 19.6%, $p=0.036$), had higher concentrations of platelet ($213.29 \times 10^3/\text{mm}^3$ vs. $249.30 \times 10^3/\text{mm}^3$, $p=0.005$), lower concentrations of uric acid (6.227 mg/dL vs. 5.528 mg/dL , $p=0.039$), shorter CADP-CT values (122.53 sec vs. 108.15 sec , $p=0.01$), higher concentrations of sP-selectin (68.681 ng/mL vs. 82.385 ng/mL , $p=0.01$), and higher IL-6 concentrations (2.508 pg/mL vs. 4.069 pg/mL , $p=0.005$) (Figure 1).

Predictors of high serum BDNF concentration

In univariate linear regression analyses, predictive factors for serum BDNF level above the median were: coronary artery disease ($p=0.035$), history of MI ($p=0.035$), current smoking ($p=0.037$), platelets number ($p=0.004$), uric acid concentration ($p=0.043$), HDL cholesterol ($p=0.046$), CADP-CT >74 sec ($p>0.001$), sP-selectin concentration ($p=0.008$), and IL-6 concentration ($p=0.05$). In multivariate analysis only CADP-CT >74 sec and sP-selectin concentration were predictive for high serum BDNF (Table 3).

In univariate logistic regression analyses, predictive factors for serum BDNF level above the median were: higher platelets number (odds ratio, OR 1.015, 95% confidence interval, 95% CI 1.005–1.024, $p=0.002$), higher uric acid concentration (OR 0.727, 95% CI 0.549–0.962, $p=0.025$), CADP-CT >74 sec (OR 0.144, 95% CI 0.442–0.466, $p=0.001$), sP-selectin concentration (OR 1.018, 95% CI 1.000–1.036, $p=0.046$) and IL-6 concentration (OR 1.301, 95% CI 1.048–1.616, $p=0.017$). In multivariate analysis only CADP-CT >74 sec and IL-6 concentration were risk factors for serum BDNF above the median (Table 4).

Results of genetic analysis

Frequency of alleles and genotypes of the analyzed polymorphisms in BDNF gene in investigated patients are given in Table 5. We found no differences between 3 allelic groups (e.g., homozygotes and heterozygotes for minor and major alleles) and serum BDNF concentration using the non-parametric Kruskal-Wallis test. No statistically significant differences in the effects of different haplotype blocks on any analyzed dependent variables were observed (data not shown).

Table 3. Predictors of serum BDNF concentration in multivariate linear regression analysis.

Variable	Coef.	95% CI	p
CAD	2.11	-2.25–6.48	0.339
MI	2.76	-1.87–7.41	0.239
Current smoking	3.51	-2.14–9.17	0.220
Platelets	0.005	-.03–0.04	0.759
UA	-0.84	-1.98–0.31	0.149
HDL	0.05	-0.07–0.17	0.396
CADP-CT >74 sec.	-8.69	-13.38–(-4.01)	<0.001
sP-selectin	0.08	0.007–0.15	0.030
IL-6	0.13	-0.57–0.83	0.720

CI – confidence interval; CAD – coronary artery disease; MI – myocardial infarction; UA – uric acid (mg/dL); HDL – high density lipoproteins (mg/dL); CADP-CT – collagen/adenosine diphosphate closure time (sec); sP-selectin – soluble P-selectin; (ng/mL); IL-6 – interleukin 6 (pg/mL).

Table 4. Predictors of high serum BDNF concentration in multivariate logistic regression analysis.

Variable	OR	95% CI	p
PLT	1.01	0.99–1.02	0.09
CADP-CT >74sec.	0.20	0.05–0.8	0.02
IL-6	1.32	1.02–1.69	0.03

OR – odds ratio; CI – confidence interval; PLT – platelet count ($10^3/\text{mm}^3$); CADP-CT – collagen/adenosine diphosphate closure time (sec); IL-6 – interleukin 6 (pg/mL).

Table 5. Frequency of alleles and genotypes of the analyzed polymorphisms in BDNF gene in investigated patients (N=91).

SNP	MAF	Genotypes DD/Dd/dd	Minor allele (D) count	Major allele (d) count
rs925946	0.3	7/41/43	55	127
rs6265	0.1	0/19/72	19	163
rs4923463	0.14	1/23/67	25	157

D – minor allele; d – major allele.

Discussion

In the present study we evaluated the association between serum BDNF concentrations and both platelet reactivity and inflammatory markers. We showed that serum BDNF concentration could be at least in part related to platelet reactivity and inflammation in patients with T2DM on 75 mg of ASA therapy. We found that concentration of BDNF is related to platelet reactivity measured with PFA-100 CADP-CT and sP-selectin concentration. Adenosine diphosphate is one of the most important pro-aggregating substances released and interacts with purinergic receptors (P2X, P2Y1, and P2Y12) on the platelet surface [24]. The PFA-100 CADP-CT reflects the combined effects of platelet activation by collagen, ADP, and high shear [25]. It was previously demonstrated that increased residual platelet reactivity measured by PFA-100 CADP-CT consistently predicts the occurrence of cardiovascular events [25–27]. Because of the general insensitivity of CADP for platelet inhibition induced by aspirin, it was hypothesized that the correlation of CADP-CT with the composite events would have been observed in these patients whether or not they were taking aspirin therapy [28]. If so, this suggests that CADP-CT is detecting an underlying ADP-dependent platelet hypersensitivity that is clinically significant. Both surface expression of P-selectin and formation of monocyte-platelet aggregates were significantly enhanced by stimulation with ADP in these patients when compared to normal controls [29].

The strong correlation of serum BDNF levels with the platelet alpha-granule marker transforming growth factor- β 1 (TGF- β 1) in comparison to the platelet dense-core granule marker serotonin (5-HT) indicates that BDNF and TGF- β 1 could be anatomically and functionally related in the platelets [9]. However, BDNF is not produced in human megakaryocyte precursor cells, but

is actively acquired by human platelets from external sources [6]. A number of platelet function agonists, like thrombin, collagen, or shear stress, induce a rapid release of BDNF from platelets [6]. Our results are in agreement with previous observation that showed reduced BDNF concentrations in serum and the BDNF release from platelets in 24 volunteers after a single loading dose (600 mg) of clopidogrel, but not after aspirin (500 mg), because clopidogrel inhibits platelet activation by acting on P2Y12 receptor, which is activated by ADP. They also found that serum BDNF is correlated with TGF- β 1 level, which is a marker of platelet alpha-granules degranulation [10]. Moreover, it was described that serum BDNF levels were higher in the MI group and were correlated with sP-selectin, which is a biomarker of platelet activation and inflammation [5]. It strongly supports our findings that BDNF release from platelets depends on the ADP-dependent pathway and increased activation of platelets.

Moreover, in our study we found that IL-6 is associated with increased BDNF concentration, which is in accordance with some previous studies that described similar observations in different clinical conditions [30–32]. It has been shown that activated antigen-specific T cells, B cells, and monocytes produce BDNF and IL-6, and TNF- α represents a specific link between monocyte infiltration and neuronal changes in inflammatory diseases [33,34]. In obese individuals, peripheral blood mononuclear cells produce a greater BDNF production, which is associated with an increased IL-6 response *ex vivo* [35].

There are 3 main limitations of our study. First, it was observational and it is impossible to account for all possible confounding influences. Second, this study is limited by its small sample size and lack of controls without T2DM. Further study with a large T2DM population with known platelet reactivity

status would add further information about the role of platelet activation in BDNF. Third, we measured only the serum levels of BDNF, and this was the major limitation of the study.

Conclusions

Chronic inflammatory condition and enhanced immune system are associated with the increased production of BDNF, which may explain why serum BDNF level in T2DM patients with HPR was higher than in subjects with normal platelet reactivity. Further research is needed to determine which source of BDNF – platelets, plasma, serum, or whole blood – provides the most reliable biological marker of patients with T2DM and HPR. A prospective, randomized trial would provide

definitive evidence of the platelet reactivity status of BDNF in this group of patients and would help to elucidate the underlying mechanisms.

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Authors disclosure statement

No competing financial interests exist.

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