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Is there an effect of folic acid supplementation on the coagulation factors and C-reactive protein concentrations in subjects with atherosclerosis risk factors?*

Czy suplementacja kwasem foliowym ma wpływ na stężenia czynników krzepnięcia i białka C-reaktywnego u osób z czynnikami ryzyka miażdżycy?

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Folic acid (FA) may delay the formation of atherosclerotic lesions. Increased plasma levels of von Willebrand factor (VWF) are observed in cardiovascular disease, which leads to higher risk of thrombosis. Fibrinogen (Fb) is a well-documented risk factor of cardiovascular disease. The aim of this study was to analyze the effect of FA supplementation on the Fb, VWF and C-reactive protein (CRP) plasma concentrations in subjects with atherosclerosis risk factors.

Material/Methods: The study enrolled 124 Caucasian individuals (60 M, 64 F) with atherosclerosis risk factors – family history of premature ischaemic stroke, arterial hypertension, dyslipidaemia, overweight and obesity, cigarette smoking and low physical activity. The participants were asked to take FA in the low dose of 0.4 mg/24 h for three months.

Results: After FA supplementation a significant reduction of the VWF concentrations in females (76.6 *vs* 72.3%; p=0.028) and in males (75.5 *vs* 66.9%; p=0.001) was observed. Among women and men with dyslipidaemia concentrations of VWF decreased after FA supplementation (76.8% *vs* 69.6%; p=0.003 and 76.7% *vs* 67.8%; p=0.001 respectively). Among females and males with BMI ≥25 kg/m^2 concentrations of VWF decreased only in men (77.6% *vs* 66.5%; p=0.001). In female and male smokers supplementation of FA decreased VWF concentrations (82.5% *vs* 74.4%; p=0.012 and 76.6% *vs* 69.5%; p=0.036 respectively).

Discussion: The results of our study suggest that there is an effect of FA supplementation on VWF concentrations in subjects with atherosclerosis risk factors.

Key words: **atherosclerosis • coagulation factors • CRP • fibrinogen • folic acid • haemostasis • von Willebrand factor**

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Streszczenie

Wprowadzenie: Kwas foliowy (FA) może opóźniać tworzenie się zmian miażdżycowych. W chorobach układu krążenia obserwuje się podwyższone poziomy czynnika von Willebranda (VWF) w osoczu, co zwiększa ryzyko zakrzepicy. Fibrynogen (Fb) jest dobrze udokumentowanym czynnikiem ryzyka chorób układu krążenia. Celem badania była analiza wpływu suplementacji FA na osoczowe stężenia Fb, VWF i białka C-reaktywnego (CRP) u osób z czynnikami ryzyka miażdżycy.

Materiał/Metody: Do badania włączono 124 osoby rasy kaukaskiej (60 M, 64 K) z czynnikami ryzyka miażdżycy – wywiadem rodzinnym przedwczesnego niedokrwiennego udaru mózgu, z nadciśnieniem tętniczym, dyslipidemią, nadwagą i otyłością, nikotynizmem i niewielką aktywnością fizyczną. Uczestnicy zostali poproszeni o przyjmowanie FA w niskiej dawce 0,4 mg/24 h przez trzy miesiące.

Wyniki: Po suplementacji FA zaobserwowano istotną redukcję stężeń VWF u kobiet (76,6 *vs* 72,3%; p=0,028) i u mężczyzn (75,5 *vs* 66,9%; p=0,001). Wśród kobiet i mężczyzn z dyslipidemią stężenia VWF obniżyły się po suplementacji FA (odpowiednio 76,8 *vs* 69,6%; p=0,003 i 76,7 *vs* 67,8%; p=0,001). Wśród kobiet i mężczyzn z BMI ≥25 kg/m^2 stężenia VWF obniżyły się tylko u mężczyzn (77,6 *vs* 66,5%; p=0,001). U palących osobników płci męskiej i żeńskiej suplementacja FA skutkowała obniżeniem stężeń VWF (odpowiednio 82,5 *vs* 74,4%; p=0,012 i 76,6 *vs* 69,5%; p=0,036).

Dyskusja: Wyniki naszego badania wskazują, że suplementacja FA wpływa na stężenia VWF u osób z czynnikami ryzyka miażdżycy.

Słowa kluczowe: **miażdżyca • czynniki krzepnięcia • CRP • fibrynogen • kwas foliowy • hemostaza • czynnik von Willebranda**

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Introduction

Numerous reports confirm preventive effects of folic acid (FA) on developmental birth defects, Alzheimer's disease, megaloblastic anaemia and depressive disorders [18,21]. There is no consensus among researchers on the preventive effect of FA on cardiovascular disease. It has been shown that, through lowering of homocysteine (Hcy) concentrations in the blood, FA may delay the formation of atherosclerotic lesions [1]. The report by Brown et al. indicates that even a slight FA deficiency promotes atherosclerosis formation in arterial endothelium [54]. However, large-scale clinical trials have shown that a preventive use of FA in individuals with established cardiovascular disease or with history of myocardial infarction was not associated with a lower risk of new cardiovascular events [2,3,4,17,28].

Increased concentration of fibrinogen (Fb) promotes hypercoagulability and incidents of thrombosis, leading to myocardial infarction, stroke or critical limb ischaemia. It is classified as an acute-phase protein; therefore Fb levels may also be elevated in inflammation [27]. Fibrinogen is a well-documented risk factor of cardiovascular disease [13]. However, results of studies on the association of FA with coagulation factors are ambiguous. Liem et al. reported lack of association of FA supplementation with Fb concentration [15]. Nevertheless, other reports indicate a beneficial anticoagulative effect of FA through decreasing concentration of Fb and increasing concentrations of plasminogen and antithrombin III [23]. Von Willebrand factor (VWF) has a crucial role in blood coagulation [26]. Increased plasma levels of VWF are observed in cardiovascular, neoplastic and connective tissue diseases, probably because of endothelium dysfunction, which leads to an increased risk of thrombosis [16]. The ARIC study confirmed that VWF is a risk factor of ischaemic stroke [15]. It was found that FA supplementation decreases VWF concentrations [23]. C-reactive protein (CRP) levels rise in response to inflammation. Some research suggest that individuals with elevated basal concentrations of CRP are at an increased risk of diabetes and cardiovascular disease [8,19]. Therefore CRP is not only an inflammation marker, but is also considered as a risk indicator for cardiovascular disease [7].

The aim of this study was to analyse the effect of folic acid supplementation on fibrinogen, von Willebrand factor and C-reactive protein plasma concentrations.

Material and Methods

The study enrolled 124 adult Caucasian individuals (60 males aged 20–39 and 64 females aged 19–39) with atherosclerosis risk factors. A standard interview on the environmental risk factors for atherosclerosis was performed and revealed the presence of family history of premature ischaemic stroke, arterial hypertension, dyslipidaemia, overweight and obesity, cigarette smoking and low physical activity among study participants. The inclusion criteria for the study group were: age ≥18 years, patient's informed consent granted, absence of concurrent inflammation, no hypolipidaemic or metabolism-modulating agents, no administration of B-group vitamins or vitamin preparations within 6 months before the study (use of hypotensive agents and oral contraceptives did not constitute exclusion criteria), young age of parents at the moment of ischaemic stroke (fathers younger than 55 years, mothers younger than 65 years) confirmed by means of computed tomography or magnetic resonance. The study involved an initial assessment through medical history taking, physical examination and blood analysis. Next, the participants were asked to take FA in the low dose of 0.4 mg/24 h for three months [24,29], after which a follow-up examination was performed, again through medical history taking, physical examination and blood analysis. Initially, FA supplementation was offered to 147 subjects, 23 of whom (15.6%) were excluded in the course of the study due to non-compliance (irregular taking of FA, stopping taking it altogether, or not reporting for the follow-up examination at the end of the treatment period). The study was approved by the Bioethics Committee of the Pomeranian Medical University in Szczecin, Poland.

Fasting blood for biochemistry was collected. The folic acid level was determined by an Abbott test kit (Abbott Laboratories, Chicago, Illinois, USA) using the ion capture method on an IMX immunochemical analyser (by Abbott). Total homocysteine (Hcy) was determined by high performance liquid chromatography (HPLC) using test kits from Bio-Rad, on a Hewlett-Packard analyser with a fluorescence detector. Fibrinogen level was determined by the Clauss method on a Hemolab analyser, using bioMérieux test kits. The von Willebrand factor was measured by means of a miniVidas analyser (bioMérieux) and enzyme-linked immunosorbent assay. High-sensitivity C-reactive protein (hsCRP) was measured by the immunonephelometric method using a Dade-Behring BN-100 analyser (Dade Behring Holding GmbH, Liederbach, Germany).

Statistical analysis was performed using the STATISTICA StatSoft Polska v.9.0 package (StatSoft Inc., Tulsa, Oklahoma, USA) and the examined parameters were first evaluated for normal distribution (Shapiro-Wilk test). The significance of statistical differences was assessed with the chi-square test, chi-square test with Yates correction or Fisher's exact test. The paired Student t test was used for the comparison of mean values of measured parameters. The two-way analysis of variance (ANOVA) with statistical significance in the post hoc test (NIR) was carried out. The CRP values were subjected to logarithmic transformation. The significance level was set at $p \leq 0.05$.

Table 1. Subjects' characteristics

Characteristics	Study group n=124
Age (years)	28.4±5.9
Females	51.6%
Family history of PIS	100.0%
Smokers	36.3%
Dyslipidaemia	71.7%
BMI >25 kg/m^2	38.7%
Low physical activity	70.2%
Oral contraception (% of females)	23.4%

PIS – premature ischemic stroke; BMI – body mass index

Results

Table 1 shows the characteristics of examined patients. Low physical activity was adopted according to the Drygas definition [9]. Tables 2 and 3 show that low-dose FA supplementation resulted in statistically significant elevation of FA levels in studied females (6.3 *vs* 12.5 ng/dL; p=0.001) and males (6.4 *vs* 11.4 ng/dL; p=0.001) and concomitantly, a decrease in Hcy levels (10.6 *vs* 8.3 µmol/L; p=0.001 and 11.5 *vs* 9.3; p=0.001 respectively). There were no significant differences in Fb concentrations before and after FA supplementation in both genders. A significant reduction in mean concentration for VWF in females (76.6 *vs* 72.3%; p=0.028) and in males (75.5 *vs* 66.9%; p=0.001) was observed. There were no significant differences in CRP concentrations before and after FA supplementation in both genders. Tables 4 and 5 show that evaluation of FA supplementation in women (n=43) and men (n=46) with dyslipidaemia revealed a significant decrease in VWF concentrations (76.8% *vs* 69.6%; p=0.003 and 76.7% *vs* 67.8%; p=0.001 respectively). Among females (n=19) and males (n=29) with BMI ≥25 kg/m^2 the concentrations of VWF decreased after FA supplementation only in men (77.6% *vs* 66.5%; p=0.001). In female (n=24) and male smokers (n=21) supplementation of FA resulted in decrease of VWF concentrations (82.5% *vs* 74.4%; p=0.012 and 76.6% *vs* 69.5%; p=0.036 respectively).

Discussion

The effect of low-dose FA supplementation (0.4 mg/24 h) on fibrinogen, von Willebrand factor and CRP concentrations was evaluated in this study. FA supplementation resulted in a significant decrease of VWF concentrations in analyzed individuals. Moreover, the decrease of VWF concentrations was especially marked in smoking or dyslipidaemic women and in smoking or dyslipidaemic or overweight and obese men. FA supplementation had no effect on Fb and CRP concentrations in the studied group.

Fibrinogen induces reversible aggregation of erythrocytes, reduces the fluidity of blood and affects clot formation through thrombocyte aggregation [6,13,27]. Fb also binds to leukocytes due to their surface receptors and acts

Table 2. The effect of three-month diet supplementation with folic acid on Fb, VWF and CRP concentrations in females (n=64)

Factor	Before supplementation		After supplementation		p*
	mean	SD	mean	SD	
Folic acid (ng/dL)	6.3	3.0	12.5	3.9	0.001
Hcy (μmol/L)	10.6	3.8	8.3	2.1	0.001
VWF (%)	76.6	23.0	72.3	22.0	0.028
Fibrinogen (g/L)	3.1	0.6	3.1	0.7	NS
CRP (mg/L)	0.3	1.0	0.2	1.0	NS

* Student's paired t-test. Hcy – homocysteine; VWF – von Willebrand factor; CRP – C-reactive protein

Table 3. The effect of three-month diet supplementation with folic acid on Fb, VWF and CRP concentration in males (n=60)

Factor	Before supplementation		After supplementation		p*
	mean	SD	mean	SD	
Folic acid (ng/dL)	6.4	2.8	11.4	3.0	0.001
Hcy (μmol/L)	11.5	3.9	9.3	1.8	0.001
VWF (%)	75.0	25.2	66.9	18.2	0.001
Fibrinogen (g/L)	2.7	0.6	2.8	0.6	NS
CRP (mg/L)	0.4	0.9	0.4	1.1	NS

* Student's paired t-test. Hcy – homocysteine; VWF – von Willebrand factor; CRP – C-reactive protein

Table 4. The effect of three-month supplementation with folic acid on VWF, Fb and CRP concentrations depending on dyslipidaemia (n=43), BMI ≥25 kg/m^2 (n=19) and smoking (n=24) in females

Parameter in females	Dyslipidaemia (n=43)			BMI ≥25 kg/m^2 (n=19)			Smokers (n=24)		
	Before suppl.	After suppl.	p	Before suppl.	After suppl	p	Before suppl.	After suppl.	p
VWF (%)	76.8	69.6	0.003	80.3	80.7	NS	82.5	74.4	0.012
Fb (g/L)	3.16	3.09	NS	3.4	3.48	NS	3.0	3.1	NS
CRP (mg/L)	0.16	0.14	NS	1.23	1.65	NS	0.83	0.89	NS

pANOVA with statistical significance in the post hoc test (NIR). VWF – von Willebrand factor; Fb – Fibrinogen; CRP – C-reactive protein

Table 5. The effect of three-month supplementation with folic acid on VWF, Fb and CRP concentrations depending on dyslipidaemia (n=46), BMI ≥25 kg/m^2 (n=29) and smoking (n=21) in males

Parameter in males	Dyslipidaemia (n=46)			BMI ≥25 kg/m^2 (n=29)			Smokers (n=21)		
	Before suppl.	After suppl.	p	Before suppl.	After suppl	p	Before suppl.	After suppl.	p
VWF (%)	76.7	67.8	0.001	77.6	66.5	0.001	76.6	69.5	0.036
Fb (g/L)	2.76	2.85	NS	2.77	2.90	NS	2.79	2.6	NS
CRP (mg/L)	0.25	0.31	NS	1.53	1.38	NS	1.4	1.4	NS

pANOVA with statistical significance in the post hoc test (NIR). VWF – von Willebrand factor; Fb – Fibrinogen; CRP – C-reactive protein

as a ligand for ICAM-1 (intercellular adhesion molecule-1) located on the thrombocytes and endothelium. This interaction leads to adhesion of platelets and leukocytes to the endothelial cells and plays a significant role in atherothrombosis [12]. A meta-analysis of prospective clinical trials regarding the effect of Fb on the cardiovascular incidents revealed that Fb concentrations were correlated with risk of death as a result of coronary heart disease, stroke (both ischaemic and haemorrhagic) or other vascular causes [10]. We found no correlation between FA supplementation and Fb concentrations. Liem et al. also reported a lack of impact of FA (0.5 mg/24 h) supplementation on

Fb concentrations [15]. The authors stated that low-dose folic acid supplementation in regard to cardiovascular risk should be treated with caution. However, two reports indicate a beneficial effect of FA supplementation. Oral administration of FA (10 mg/24 h) decreased Fb and VWF concentrations and increased plasminogen and antithrombin III concentrations in studied subjects [23]. The second report showed a decrease of Fb concentrations and an increase of antithrombin concentrations after FA supplementation (dose of 5.0 or 10.0 mg/24 h) in patients with hyperhomocysteinaemia [22]. Mangoni et al. observed an anti-thrombotic effect of FA [20]. CRP along with Fb is a marker of an inflammatory response and is classified as an acute-phase protein. The role of CRP is binding to phosphocholine on microbes and damaged cells and enhancing phagocytosis by macrophages [31]. Therefore CRP takes part in the clearance of necrotic and apoptotic cells [25]. There are some reports on the association between FA supplementation and inflammatory markers. As in our study, some research carried out by other authors showed no effect of FA supplementation on CRP concentrations in analysed subjects [15,20,31].

Von Willebrand factor is a multimeric blood glycoprotein, produced by endothelial cells, subendothelial connective tissue and partly by megakaryocytes [26]. It is involved in primary haemostasis as an adhesive protein through binding platelets to the collagen and secondary haemostasis due to forming a complex with factor VIII, protecting it against proteolysis [26]. VWF is considered to be a marker of endothelial dysfunction in vascular disorders [16]. The ARIC study analysed VWF as an ischaemic stroke risk factor through comparison of the highest and the lowest quartile of VWF concentrations in analysed subjects. The relative risk of an ischaemic stroke, after amendments to the conventional risk factors, was 1.71 (without regarding CRP) and 1.21 (after correction for CRP) [11]. We observed a significant decrease of VWF concentrations among studied subjects. Our observations confirm the results obtained by Mayer et al., who achieved a 6.8% reduction in VWF concentrations among individuals with high cardiovascular risk, after FA supplementation in the dose of 10 mg/24 h [23]. However, it should be noted that we achieved a similar reduction in VWF concentrations using a 25 times lower dose of FA than Mayer et al. Moreover, we observed a significant reduction of VWF serum concentrations especially in subjects with dyslipidaemia, obese males and cigarette smoking individuals. The decrease of VWF levels was demonstrated probably because low doses of FA improve vascular endothelium function, which is involved in the synthesis, storage and release of VWF. When endothelial cells are damaged, the VWF release is increased. Therefore VWF levels are useful as an atherosclerosis/thrombosis indicator. Endothelial damage is secondary to arterial hypertension, hyperlipidaemia, smoking, diabetes or metabolic disorders and results in activation of the coagulation cascade [26]. Hcy, levels of which are increased in those diseases, is linked to oxidation and endothelial injury. Administration of FA improves endothelial cell function through Hcy levels lowering and oxidative stress reduction [23]. Nevertheless, not all authors have obtained similar results. Mangoni et al., Vermuelen et al. and Klerk et al., applying doses of 5.0 mg/24 h or 0.8 mg/24 h, observed no correlation between FA supplementation and VWF concentrations [14,20,30]. Reported differences are associated with studied subjects and methods of FA supplementation. Mangoni et al. analysed a group which consisted of 100% smokers, while 36.3% of our group were smokers. Vermeuelen et al. supplemented not only FA, but also vitamin B6. Klerk applied FA during a 1-year period, but in our study the supplementation lasted for 12 weeks because of earlier reported observations that FA supplementation effects are noticeable from the 4th week of oral intake [24,29].

The results of our study suggest that there is an effect of folic acid supplementation on von Willebrand factor concentrations in subjects with atherosclerosis risk factors. However, to confirm this observation further studies are needed.

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