

## Original papers

# Indexes of inflammation and other secondary risk factors in the progression of coronary artery disease

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### Summary

**Objectives:** The aim of this study was to examine quantitative changes of neopterin (N), high sensitivity C-reactive protein (hsCRP), total homocysteine (H), pyridoxal-5-phosphate (P-5-P) and phospholipids (PL) concentrations in blood serum of patients with different degree of coronary artery disease (CAD) as well as to evaluate the observed changes to prognosticate the course of CAD.

**Design and Methods:** 30 healthy individuals and 84 patients with CAD verified by coronary angiography were examined in this study. Patients with diabetes and/or renal complications as well as patients who received statins in their treatment were excluded. In total, 43 patients with 1-artery disease, 24 patients with 2- or 3-artery disease, and 17 patients with restenosis have been included. Indexes of inflammation (N, hsCRP) and H were measured by commercial available immunoassay according to manufacturer's instructions while manual chemical methods were used for P-5-P and PL determination.

**Results:** We have observed statistically significant increase of N, hsCRP and H concentrations in the patients with CAD while P-5-P and PL concentrations were lowered.

**Conclusions:** The analysis and evaluation of the results allow us to conclude that the changes of indexes observed in this study are significant in prognosticating the course of CAD. The increase of serum H concentrations in the patients with restenosis and positive correlation between N and H ( $r = 0.484$ ;  $p < 0.05$ ) as well as the presumption that this correlation predicts adverse events of restenosis, points to a call for further investigation to reveal this question.

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Atherosclerosis in general as well as atherosclerosis of coronary arteries resembles chronic inflammation in several regards and it is now recognised that cellular components of the immune system are involved in its genesis [1]. Endothelial function is critical for the maintenance of blood flow and vascular integrity. So, the healthy endothelium tends to favour vasodilatation, antithrombosis, fibrinolysis and monocyte disadhesion. Endothelial cells are one of the only surfaces that during a protracted contact can maintain blood in a liquid state. Acute and especially chronic minimal endothelial injury can result in

the dysfunctional endothelium characterised by increased uptake of low density lipoprotein (LDL) and monocyte recruitment into the blood vessel wall, which are both pivotal initiating events in atherosclerosis [2]. Neopterin (N) is a product of interferon-gamma activated monocyte-derived macrophages and is a sensitive marker for Th-1 type immune response [3]. C-reactive protein (CRP) is rapidly secreted by the liver in response to trauma, inflammation and infection, and decreases immediately after the resolution of the conditions [4]. Despite of many well characterised anti-inflammatory and phagocytosis mediated reactions of CRP [5–8], the results of the further investigation have shown that CRP can activate blood monocytes and stimulate the production of inflammatory cytokines: interleukine

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(IL)-1 and tumor necrosis factor (TNF)-alpha in human monocytes [9]. These monocytes are moved to the place of inflammation, infection or traumatic injury and localised in the intima of the vessel wall, where they are transformed into macrophages within 7–8 days [10]. Macrophages produce not only IL-1 and TNF-alpha [11], but interferon-gamma as well. TNF-alpha together with interferon-gamma activate cyclohydrolase I that cleaves guanosine triphosphate (GTP) to 7,8-dihydroneopterin triphosphate in macrophages. From this key metabolite of pterin biosynthesis both, 7,8-dihydroneopterin and 5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) can be formed. Due to low activity of enzyme 6-pyrovyl-tetrahydropterin synthase in macrophages, 7,8-dihydroneopterin is formed by phosphatases. It is converted into neopterin, which may be found in biological fluids of such patients [12]. BH<sub>4</sub> is a cofactor necessary for the activation of nitric oxide (NO) synthase that cleaves L-arginine and forms NO or endothelium-derived-relaxic-factor (EDRF) [13,14]. Contrary to macrophages, human vascular endothelial cells express all enzymes for de novo BH<sub>4</sub> biosynthesis. The bioavailability of BH<sub>4</sub> is pivotal for endothelial function, and intracellular BH<sub>4</sub> levels potentially regulate endothelial NO synthesis and vascular tone [15–17]. The inhibition of BH<sub>4</sub> synthesis is followed by the reduction of endothelial NO production [18]. Since neither inducible NO synthase, nor endothelial NO synthase mRNA is detected in cultivated human smooth muscle cells in the resting state [19,20], it can be assumed that BH<sub>4</sub> should not direct NO-mediated relaxing effect on smooth muscle cells under physiological conditions. It is suggested that BH<sub>4</sub> produced endogenously in endothelium cells or elsewhere in a human body, migrates into smooth muscle cells of the body vessels and relaxation is followed by dose dependent increases of intracellular cGMP levels [21]. Under inflammatory conditions, after the increase of NO synthesis in endothelial cells of the human blood vessels, the concomitant dramatic decrease of endothelial NO synthase mRNA makes this increase only transient, and significantly reduced NO production is expected to occur after certain time of ongoing inflammation in a man [22]. The level of intracellular BH<sub>4</sub> is critical for the degree of reactive oxygen intermediate production by endothelial NO synthase: such as superoxide anions, hydrogen peroxide and hydroxyl radicals. It in turn can interact with vascular signalling systems and potentially oxidises any molecule in a cell, causing DNA nicking and disruption, lipid peroxidation, and protein cross-linking and degradation [23–25]. After the observation that homocysteine promotes vascu-

lar endothelial [26] and smooth muscle cell [27] growth, this amino acid has been proposed as a link to atherosclerosis development and progression. In mammals, homocysteine is formed from essential amino acid methionine. Methionine adenosine transferase in the presence of ATP forms S-adenosylmethionine, which is the principal biological methyl group donor [28]. Methyl groups are required for numerous methylation reactions, about 100 of which have been identified. Phospholipids (PL), which participate in keeping blood cholesterol in the state of solution and so prevent atherosclerosis development and progression, have been mentioned between them [29]. After giving methyl groups S-adenosylmethionine transforms into S-adenosylhomocysteine and after that into homocysteine. Homocysteine (H) can be cleaved to cysteine by pyridoxal-5-phosphate (P-5-P) dependent enzyme systems [30] and/or remethylated to methionine by betaine-homocysteine methyltransferase in liver cells and by 5,10-methylentetrahydrofolate reductase and methionine synthase in cells of other tissues [29]. S-adenosylhomocysteine is a potent competitor to S-adenosylmethionine at different binding sites and can therefore inhibit methylation [31]. Moreover, an elevation of total homocysteine (homocysteine + S-adenosylhomocysteine) could also probably be an index for delayed methylation and so impaired different pathways of intercellular metabolism. Therefore, the aim of this study was to examine the changes of N, CRP, H, P-5-P and PL concentrations in coronary artery disease (CAD) patients and to evaluate these changes in the progression of CAD.

## Design and Methods

30 healthy individuals and 84 patients with CAD verified by coronary angiography were examined in this study before percutaneous transluminal coronary angioplasty (PTCA). Patients with diabetes and/or renal complications as well as patients who received statins in their treatment were excluded. In total, 43 patients with 1-artery disease, 24 patients with 2- or 3-artery disease and 17 patients with restenosis have been included. Blood serum indexes of inflammation: N (BRAHMS, Berlin, Germany) and hsCRP (Dade Behring) as well as H (Abbot IMx) were measured by commercially available immunoassay according to manufacturer's instructions. The method of Serfontein and others [32] was used for the determination of P-5-P in blood serum. After the extraction of lipids from blood serum by the method of Folch and others [33], the method of Urbach and Raabe [34] was used to determine PL

concentration. Mean values and standard deviations (SD) of means were calculated. Statistical significance between the values obtained from the healthy individuals (control) and the groups of CAD patients as well as between the values obtained from the patients with 1-artery disease and the patients with restenosis were calculated by the Student's *t*-test. *P* values below 0.05 were considered to indicate significant differences. For the calculation of correlation coefficients, the Excel programme for personal computers has been used. This study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethics/scientific committee.

## Results

Table 1 shows the mean values of indexes of inflammation: N and hsCRP as well as other secondary risk factors: H, P-5-P and PL blood serum concentrations in the healthy individuals and in all the 3 groups of CAD patients. It is known that the concentration of N in the healthy individuals oscillated from 2.6 to 8.7 nmol/l [35]. So, in all the CAD patients' groups of this study the mean N concentration was elevated ( $p < 0.001$ ). This elevation in the patients with restenosis was also statistically significant if compared with the values obtained in the patients with 1-artery disease ( $p < 0.001$ ). According to the data of the Dade Behring manufacturer, the concentration of blood serum hsCRP oscillated between 0–5.0 mg/l in healthy individuals. The mean  $\pm$  SD concentration was  $1.2 \pm 0.96$  mg/l in our group of healthy persons. The elevation of mean concentration of hsCRP was observed in all the groups of CAD patients but statistical significance of elevation was found in the patients with 2- or 3-artery disease ( $p < 0.05$ ) as well as in the patients with restenosis ( $p < 0.001$ ). This elevation in the pa-

tients with restenosis was also statistically significant if compared with the values obtained in the patients with 1-artery disease ( $p < 0.01$ ). We have also found a positive correlation between N and hsCRP in all the observed CAD patients ( $r = 0.536$ ;  $p < 0.01$ ). The elevation of N and hsCRP above maximal normal values (Table 2) was found in 30.1% of cases with 1-artery disease, in 54.2% of cases in the patients with 2- or 3-artery disease and in 76.5 % of cases in the patients with restenosis. In the patients with restenosis the elevation of N concentration above maximal normal values was found more frequently than the elevation of hsCRP. From the data of the Abbot Mlx manufacturer the concentration of serum H can oscillate between 5–15  $\mu\text{mol/l}$  [36]. The mean  $\pm$  SD serum H concentration of 29 healthy individuals aged 23–65 examined in Riga was  $8.9 \pm 1.5$   $\mu\text{mol/l}$  [37] that agree with the data of literature too [38]. So, the mean serum total homocysteine concentration (Table 1) was elevated in all the groups of CAD patients ( $p < 0.001$ ). The statistically significant difference in the increase between the groups was not found. Nevertheless, the positive correlation was found ( $r = 0.485$ ;  $p < 0.05$ ) between N and H concentrations in the

**Table 2.**

Percentage of cases with the increase of high sensitivity C-reactive protein and/or neopterin above maximal normal values in different groups of patients with coronary artery disease

Groups of patients	1-artery disease	2- or 3-artery diseases	Restenosis
hsCRP (%)	13.9	16.7	11.8
hsCRP + N (%)	2.3	20.8	23.5
N (%)	13.9	16.7	41.2
hsCRP and/or N in total (%)	30.1	54.2	76.5

hsCRP – high sensitivity C-reactive protein; N – neopterin.

**Table 1.**

Blood serum concentrations (mean  $\pm$  SD) of neopterin, high sensitivity C-reactive protein, total homocysteine, pyridoxal-5-phosphate and total phospholipids in healthy persons and in patients with coronary artery disease

Indexes	Healthy	1-artery disease	2- or 3-artery diseases	Restenosis
N (nmol/l)	$5.34 \pm 2.56$	$7.15 \pm 2.45^*$	$8.80 \pm 5.08^*$	$16.10 \pm 7.84^*$
hsCRP (mg/l)	$1.20 \pm 0.96$	$2.70 \pm 2.90$	$6.90 \pm 9.90^+$	$5.10 \pm 3.90^*$
H ( $\mu\text{mol/l}$ )	$8.90 \pm 1.50$	$13.70 \pm 4.90^*$	$14.80 \pm 6.00^*$	$15.00 \pm 3.90^*$
P-5-P (nmol/l)	$52.40 \pm 14.9$	$21.40 \pm 13.7^*$	$19.30 \pm 12.6^*$	$17.60 \pm 10.3^*$
PL (mmol/l)	$2.12 \pm 0.15$	$1.76 \pm 0.27^*$	$1.67 \pm 0.31^*$	$1.51 \pm 0.26^*$

H – homocysteine; hsCRP – high sensitivity C-reactive protein; N – neopterin; PL – phospholipids; P-5-P – pyridoxal-5-phosphate.

Statistical significance of values observed in different groups of patients is obtained in comparison with values found in healthy persons:  $^*p < 0.001$ ,  $^+p < 0.05$ .

patients with restenosis. P-5-P concentrations (Table 1) were profoundly decreased in all the groups of CAD patients without differences between the groups. The mean  $\pm$  SD serum PL concentration was  $2.12 \pm 0.15$  in our group of healthy individuals (Table 1). So, the mean concentration of serum PL was decreased in all the groups of CAD patients ( $p < 0.001$ ). The decrease of serum PL concentration in the patients with restenosis was also statistically significant if compared with the values obtained in the patients with 1-artery disease ( $p < 0.001$ ). The correlation ( $r = -0.321$ ) between PL and H was negative, but not statistically significant ( $p > 0.05$ ).

## Discussion

The initial lesion of atherosclerosis develops when monocytes cross the endothelial barrier to accumulate in the intima [39]. The migration of monocytes is induced by CRP [4]. Monocytes localized in the intima are transformed into macrophages within 7–8 days [10]. CRP also activates the induction of inflammatory cytokines: IL-1 and TNF-alpha in human monocytes [9] and macrophages [11]. These cytokines together with interferon-gamma stimulate an increase of 7,8-dihydroneopterin and N formation in macrophages due to low activity of 6-pyrovyl-tetrahydropterin synthase necessary for BH<sub>4</sub> biosynthesis [12]. The results of this study have shown an increase of N and hsCRP concentrations (Tables 1 and 2) in all the groups of CAD patients as well as positive correlation between these indexes ( $r = 0.536$ ). Moreover, the increase of serum N and hsCRP concentrations above normal maximal values was found more frequently in the patients with 2- or 3-artery disease and, especially, in the patients with restenosis than in the patients with 1-artery disease (Table 2). So, our data points to the significance of inflammation in the progression of CAD. If increased hsCRP concentration characterises only acute processes, then elevated serum N concentration has also been observed 1 year after non Q-wave myocardial infarction. Elevated N concentration predicts adverse events in patients with non Q-wave myocardial infarction. When increased CRP levels are added, the predictive value proves out even stronger [40]. Our observation of more frequent increase of N concentration than hsCRP protein elevation in the patients with restenosis (see Table 2) improves the data of literature. Homocysteine stimulates the growth of blood-vessels' endothelial and smooth muscle cells [26,27]. So, mild elevation of serum homocysteine concentration in all the groups of

CAD (Table 1) shows a possible thickening of the blood-vessels' wall and the narrowing of the lumen. The pathobiology of restenosis after PTCA is characteristic and distinct from that of *de novo* atherosclerosis. As reviewed by Libby [2] the loss of luminal caliber after balloon angioplasty may induce a vessel constriction from the adventitial side (negative remodelling), which has renewed an adventitial inflammation with scar formation, wound contraction and so restenosis. In contrast, in-stent stenosis depends on intimal thickening. In general, it would appear that plasma lipid concentrations are not related to the risk for restenosis [41–43]. Moreover, many clinical trials have shown that statin therapy does not prevent restenosis [44]. Mild increase of serum homocysteine concentration in the restenosis patients of this study and in our previous investigations [45] as well as positive correlation between neopterin and homocysteine concentrations in the patients with restenosis of this study ( $r = 0.485$ ;  $p < 0.05$ ) and in CAD patients of our previous investigations [46] show a call for further investigations to reveal this question. Profound decrease of P-5-P serum concentration in this study (Table 1) as well as in our previous investigations [45,47] shows a possible pathogenesis of increased homocysteine concentration observed in the patients with CAD. The decrease of PL serum concentrations in all the groups of CAD (Table 1), especially in the patients with restenosis, shows a possibly impaired PL biosynthesis due to profoundly decreased P-5-P serum concentration observed in our CAD patients (P-5-P is necessary for decarboxylation of phosphatidylserine [48]) and due to slight increase of serum H (homocysteine + S-adenosylhomocysteine), and so delayed methylation processes in an organism necessary for phosphatidylcholine formation [29,36]. However, the decrease of serum PL concentration may also be due to the increased level of hsCRP in the CAD patients. It is known that CRP interacts with phosphatidylcholine and so prepares lipids for phagocytosis. Recently it has been found that low-density lipoproteins (LDL) which play a pivotal role in atherogenesis contain negatively charged phospholipids. The binding of CRP to these phospholipids increases uptake of oxidised LDL by macrophages providing a basis for atherogenesis [49]. Therefore, the determination of PL, especially phosphatidylcholine serum concentrations in the course of CAD has the same significance as the determination of total cholesterol, high-density lipoprotein cholesterol and LDL-cholesterol widely used at present.

## Conclusions

The analysis and evaluation of the results allow to conclude that:

- (1) Acute and chronic inflammation has a pivotal role in the progression of coronary artery disease.
- (2) The examination of both neopterin and high sensitivity C-reactive protein indexes is necessary to find the presence of inflammation, infection or trauma and so prognosticate the course of coronary artery disease.
- (3) Mild increase of homocysteine concentration points to possible thickening of the blood vessels' wall and the narrowing of the lumen.
- (4) The increase of serum homocysteine concentrations in patients with restenosis and positive correlation between neopterin and homocysteine ( $r = 0.484$ ;  $p < 0.05$ ) as well as suggestion that this correlation predicts adverse events of restenosis, points to a call for further investigation to reveal this question.
- (5) Profound decrease of pyridoxal-5-phosphate serum concentrations in all the groups of coronary artery disease patients points to a possible pathogenesis of increased serum homocysteine observed in this study.
- (6) The determination of phospholipids, especially phosphatidylcholine, concentrations in the course of coronary artery disease has the same significance as the determination of total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol widely used at present.

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