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Increased concentrations of neopterin in carotid atherosclerosis

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Abstract

Activation of T-cells and macrophages may play a role in the pathogenesis of atherosclerosis. Therefore, serum concentrations of the immune activation markers neopterin and soluble interleukin-2 receptor were compared with routine laboratory parameters, candidate risk variables and degree of carotid atherosclerosis. Study subjects were 561 individuals (293 men and 268 women) aged between 50 and 79 years who were enrolled in a cross-sectional community based study (Ischemic Heart Disease and Stroke Prevention Study, Bruneck, Italy). Extent of carotid atherosclerosis was quantitated by an ultrasound B-mode procedure based scoring system. Detailed physical examination and quantification of laboratory and candidate risk variables were performed. By univariate as well as multivariate statistical analyses, serum concentrations of neopterin but not soluble interleukin-2 receptor were significantly higher in subjects with carotid atherosclerosis (men, 8.5 ± 2.7 nmol/l neopterin; women, 9.6 ± 3.3) than in those without (men, 6.7 ± 2.3, $P < 0.0001$; women, 7.5 ± 2.3, $P < 0.0001$). The data show that the macrophage-derived immune activation marker neopterin is closely correlated with the extent of carotid atherosclerosis. Chronic activation of immune cells, preferentially of macrophages, may play a key role in atherogenesis and/or progression of atherosclerosis.

Key words: Carotid atherosclerosis; Immune activation; Neopterin; Activated macrophage

1. Introduction

A complicated network of different factors and phenomena, influencing each other, has been found to be responsible for the initiation and progression of atherosclerosis [1–4]. Among them, the involvement of cell mediated immunity and cytokines in the pathogenesis of atherosclerosis has attracted great interest in the past few years [5]. Recent studies suggest that macrophages may be activated in patients with atherosclerosis [6,7]. However, only limited information is available

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about the immune activation status in such patients. To study this problem on a large scale, in vitro functional assays and/or counting of immune cell subsets are difficult to manage. Serum soluble markers for immune function may therefore provide an appropriate alternative.

In a cross-sectional study we investigated the amounts of soluble immune activation markers in serum of a randomized population. In addition to conventional laboratory variables known to be associated with the risk for atherosclerosis, we measured serum concentrations of neopterin, a pyrazino-pyrimidine derivative, which is synthesized by macrophages upon stimulation with the T-cell derived cytokine interferon-gamma [8]. Neopterin is a sensitive indicator for monitoring patients with activated cellular immunity in various clinical conditions [9]. Further, serum concentrations of soluble interleukin-2 receptor (sIL-2R) were determined as an additional parameter indicating immune response. sIL-2R is synthesized and released by several human immune cells upon activation, such as B-cells, T-cells and monocytic cells, and has therefore been established as a useful in vivo parameter for measurement of disease activity under conditions associated with T- and/or B-cell immune activation [10,11].

2. Methods

2.1. Study population

Blood specimens were obtained from 293 men and 268 women, who were drawn from a community based study (Bruneck Ischemic Heart Disease and Stroke Prevention Study). Population recruitment and baseline examination were performed in the community of Bruneck (province of Bozen-Alto Adige, Northern Italy) from July to November 1990. The study population (n = 1000) was selected as a 21% random sample of all inhabitants aged between 40 and 79 years (decade age groups). Men and women were selected equally for inclusion in the study on the basis of an age stratified random sampling strategy. The high participation rate of 93.6% provided a safeguard against potential bias. Men and women aged between 40 and 49 years were excluded from the study because carotid atherosclerosis is almost absent in this decade. All remaining subjects (n = 605) were free from any clinical symptoms of cerebrovascular disease. Since neopterin is known to be increased in patients with viral infections, malignancies or autoimmune diseases [9], we excluded such individuals from further evaluation; i.e., 6 subjects with chronic viral infections, 6 with malignancies and 5 with autoimmune diseases. One subject was excluded from the study because of renal failure and 26 individuals because of missing laboratory data.

2.2. Examination of subjects

All individuals included in the study underwent detailed general examination with cardiological and neurological priority and were given a standardized questionnaire for evaluation of risk factors for atherosclerosis. Systolic and diastolic blood pressure were taken in a seated position using a standard mercury sphygomanometer. Body mass index (BMI) was defined as weight (kg) per body surface (m^2). Average number of cigarettes smoked and the years of abuse (pack-years) were noted for each smoker and ex-smoker. Alcohol consumption was evaluated by estimating daily or weekly average alcohol. A classification into three categories was performed: (1) no alcohol intake or daily alcohol consumption less than 15 g; (2) less than 100 g per day; (3) more than 100 g per day. Physical activity was recorded using a three-category scale: (1) subjects who did not exercise at all; (2) regular physical activity of up to 2 h per week; (3) regular physical activity for more than 2 h per week. Coronary artery disease was diagnosed if the subject had a documented clinical history of myocardial infarction and/or a codable Q/QS pattern in the electrocardiogram and/or a documented history of angina pectoris. Individuals with cardiac insufficiency were categorized into subclasses according to the criteria of the New York Heart Association (NYHA, stages I-IV). Diabetes mellitus was coded as present if subjects were under therapy with insulin or oral antidiabetic drugs and/or if the fasting glucose plasma level exceeded 140 mg/dl (7.77 mmol/l), or if 2 h value after oral glucose load was above 200 mg/dl (11.1 mmol/l).

2.3. Laboratory methods

Venous blood specimens were taken between 07:30 and 09:30 hours after 12 h of fasting and abstinence from smoking. In the case of a known acute infection the definitive samples were drawn up to 6 weeks later. All participants in the study except those receiving antidiabetic therapy underwent an oral glucose tolerance test (75 g of glucose in 10% solution). Determination of serum neopterin concentrations was carried out by means of a commercially available radioimmunoassay (Henning, Berlin, Germany). The accuracy of neopterin radioimmunoassays was proven by solid phase extraction over AASP-SCX cartridges (Analytichem, Harbor City, CA) and on-line-elution HPLC as described in Ref. 12. Coefficients of variation for both techniques lie in the range 5%–10% for both between-run and day-to-day precision. Because neopterin is known to be primarily eliminated by urinary excretion [13], the neopterin per creatinine ratio was calculated, which provides reliable information on activation of cellular immunity independent of renal function. Serum levels of sIL-2R were determined by using an enzyme immunoassay (T Cell Diagnostics, Palo Alto, CA). Commercially available test kits (Merck, Darmstadt, Germany) were used for determination of cholesterol and triglycerides. High density lipoprotein (HDL) cholesterol was measured in supernatants after precipitation of apolipoprotein B containing lipoproteins with phosphotungstic acid and Mg^{2+} ions. Apolipoproteins A-I and B were determined by an immunonephelometric fixed-time method (Behring AG, Marburg, Germany). All other serum parameters investigated were quantified by standard laboratory procedures.

2.4. Assessment of carotid atherosclerosis

Inspection for carotid atherosclerosis was carried out by examination of extracranial carotid arteries using a duplex ultrasound system (UM8 Advanced Technology Laboratories, Bothel, WA) with a 10 MHz imaging probe and a 5 MHz Doppler. The axial resolution in the B-mode was 0.4 mm. All subjects were examined in a supine position. The scanning protocol included imaging of the common (CCA) and internal (ICA) carotid arteries on both sides in multiple longitudinal and transverse planes. A plaque scoring system with slight modifications [14] was used for quantifying the extent of carotid atherosclerosis. The score was calculated by adding the maximum thickness of atherosclerotic plaques (in mm) on the near and far walls at each of four imaging sites of both carotid arteries, with plaques being defined as echo structures encroaching into the lumen of the vessel. Measurements were carried out at the following locations: proximal CCA (15–30 mm proximal to the bifurcation), distal CCA (< 15 mm proximal to the bifurcation), proximal ICA (carotid bulb and the initial 10 mm of the vessel), distal ICA (> 10 mm above the flow divider). All scans were carried out by a single experienced sonographer, and images were recorded on videotape.

2.5. Statistical analysis

All analyses were performed separately for men and women. Associations between continuous variables were assessed by Spearman's rank correlation technique. In order to correct for multiple tests, a Bonferroni correction was applied when calculating levels of statistical significance [15]. This method is known to be conservative, and possibly to have low statistical power; however, in our study even correlation coefficients with relatively low absolute value were significantly different from zero because of the large number of subjects studied.

To compare the various laboratory and clinical variables with respect to their ability to discriminate between subjects with and without carotid atherosclerosis, receiver operated characteristics (ROC) curves were computed by calculating the diagnostic sensitivity and specificity using as cut-off limit, one after another, all possible values of a variable. Sensitivity was plotted against specificity. The area between this graph and the coordinate axes is a reliable measure for diagnostic accuracy [16]. A completely useless test has an area value of 0.50 (i.e. its graph is defined by the equation sensitivity + specificity = 1.00); a test with optimal discriminative power has an area of 1.00 (i.e. its graph obeys the two equations: sensitivity = 1.00 for all specificities, and specificity = 1.00 for all sensitivities).

Multivariate judgment of the potential of the variables to discriminate between subjects with and without carotid atherosclerosis was performed by stepwise linear discriminant analysis. For validation of the discriminative power of this multivariate model, the jackknife procedure ('leave-*n*-out' method) was applied, using $n = 1$ [17]. This simple but computationally laborious method proceeds by removing a small number n of observations at a time from the original data and recalculating the classifier for each of the truncated data sets. At each step, the 'truncated' classifier is applied to just the removed measurement vectors which have not been used for the estimation of this classifier. This procedure is repeated until all measurement vectors have been classified by an appropriate truncated classifier; thus, the overall classification power obtained is a reliable estimate for the true value that can be expected for the classifier under consideration when it is applied to a new data set.

ROC analyses were performed using a self-written FORTRAN program; all other statistical analyses were done using the BMDP statistical software (Cork, Ireland).

3. Results

Carotid atherosclerosis was evident in 140 of 268 women and 181 of 293 men. Serum neopterin concentrations in nonsclerotic individuals were significantly lower than in sclerotic subjects. This was true in women (7.5 ± 2.3 nmol/l vs. 9.6 ± 3.3; $P < 0.0001$, *U*-test) as well as in men (6.7 ± 2.3 vs. 8.5 ± 2.7; $P < 0.0001$, *U*-test).

In order to obtain more detailed information on the association of candidate risk variables for atherosclerosis and laboratory parameters to the extent of atherosclerosis, Spearman rank correlation coefficients were computed (Table 1, carotid atherosclerosis score). Table 1 gives only variables that, after Bonferroni correction for multiple tests, were still significantly correlated to atherosclerosis in men or women. Obviously, neopterin as well as neopterin per creatinine ratio were significantly related to the quantitative extent of carotid atherosclerosis in women as well as in men. Besides neopterin, only age, systolic blood pressure, erythrocyte sedimentation rate and fibrinogen were significantly related to atherosclerosis in both sexes while HbA1c, uric acid and serum glucose

Table 1
Spearman rank correlations coefficients between score of carotid atherosclerosis and other variables, and between concentrations of neopterin and other variables in 268 women and 293 men

Correlation between	Carotid atherosclerosis score		Neopterin concentration	
	Men	Women	Men	Women
Atherosclerosis score	(1.000)	(1.000)	0.4579	0.3385
Age	0.4758	0.5249	0.6305	0.5408
Neopterin	0.4579	0.3385	(1.000)	(1.000)
Nicotine (pack years)	0.3624	n.s.	n.s.	n.s.
RRsys	0.3112	0.3542	0.2403	0.2304
ESR	0.2544	0.2249	0.2487	n.s.
Fibrinogen	0.2391	0.2338	0.2855	n.s.
HbA1c	n.s.	0.3342	n.s.	0.2107
Uric acid	n.s.	0.1978	0.2019	0.3255
sIL-2R	n.s.	n.s.	n.s.	0.2797
Glucose	n.s.	0.2337	n.s.	n.s.
Creatinine	n.s.	n.s.	0.2084	0.2385

Only correlations with significance level $P < 0.05$ after Bonferroni correction for multiple tests are shown.
Abbreviations: ESR, erythrocyte sedimentation rate; HbA1c, hemoglobin A1c; n.s., not significant; RRsys, systolic blood pressure; sIL-2R, soluble IL-2 receptor.

were significant only in women, and nicotine abuse was significant only in men. Categorized variables such as alcohol consumption, physical activity or diabetes mellitus lacked significant correlation to atherosclerosis, and are therefore not included in Table 1. Upon stratification of subjects by median age (i.e. 65 years for women and 63 years for men), only neopterin and neopterin to creatinine ratio in both age groups of men, and systolic blood pressure in older men, remained significantly correlated to atherosclerosis. In younger women, solely age, systolic blood pressure, neopterin and neopterin to creatinine ratio remained significant, while in older women no variable besides age was significantly correlated with atherosclerosis (data not shown).

The last observation based on correlation analyses was also confirmed by comparing mean neopterin values in male and female subjects with and without carotid atherosclerosis, stratified by median age. In all groups except older women, neopterin levels are significantly lower in non-sclerotic individuals than in those with carotid atherosclerosis. In older women (above 65 years) without atherosclerosis, serum neopterin concentrations were higher (9.8 ± 2.9 nmol/l) than in non-sclerotic older men (8.6 ± 2.9) or younger women (6.6 ± 2.4) and were almost as high as in older men with progressed carotid atherosclerosis (10.0 ± 2.5) (details not shown).

Fig. 1 shows ROC curves. Neopterin had the highest discriminative potential regarding presence vs. absence of carotid atherosclerosis in women as well as in men when compared with sIL-2R and erythrocyte sedimentation rate and systolic blood pressure (Fig. 1). The last variable was chosen as

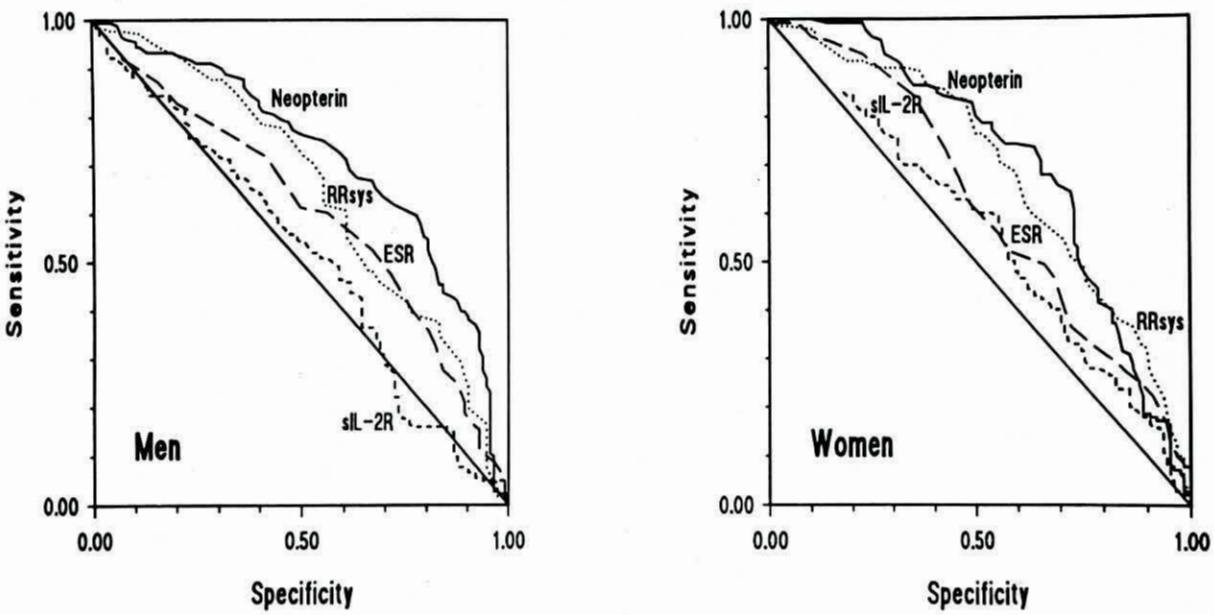

Fig. 1. Receiver operated characteristics (ROC) curves for selected variables. 'Sensitivity' means fraction of subjects with carotid atherosclerosis that have a value of the respective variable above a chosen cutoff-value. 'Specificity' denotes the fraction of subjects without carotid atherosclerosis who have a result below this chosen cutoff-value. The cutoff-values are varied systematically over the whole range of observed values of the variable under consideration. The straight line bisecting the whole area denotes the ROC curve of a hypothetical diagnostic test which is absolutely non-discriminative; the more an ROC curve deviates from the line, the better is the discriminative power of the test in question. Abbreviations: ESR, erythrocyte sedimentation rate; RRsys, systolic blood pressure; sIL-2R, soluble IL-2 receptor.

it was one of the best other indicators of carotid atherosclerosis in our study (Table 1). While systolic blood pressure and erythrocyte sedimentation rate were able to distinguish between presence and absence of atherosclerosis, albeit to a lesser extent than neopterin, this was not the case for sIL-2R.

Additionally, the correlations between neopterin and all other variables including atherosclerosis score were calculated (Table 1, neopterin concentration). After Bonferroni correction, neopterin was significantly correlated with atherosclerosis score, age, systolic blood pressure, uric acid and creatinine in men and women, additionally with erythrocyte sedimentation rate and fibrinogen in men, and with HbA1c and sIL-2R in women.

Thus it was evident that neopterin was correlated with indicators of renal function, namely creatinine and uric acid, which suggested that elevation of serum neopterin concentration was primarily due to mere impairment of renal function because of damage of the kidneys associated with atherosclerosis, hypertonia or diabetes. To take this into account, neopterin concentrations were also related to serum creatinine levels (neopterin to creatinine ratio); the correlation with atherosclerotic score was the same or even better (Table 1).

Since neopterin and atherosclerosis score were closely correlated with age, we investigated the joint discriminant power of all variables by multivariate discriminant analysis. Presence vs. absence of carotid atherosclerosis was chosen as the group-defining independent variable (Table 2). When age was excluded from the model, the ratio of neopterin to creatinine was the variable contributing most to correct classification in men, and in addition to systolic blood pressure in men and women. Besides the neopterin to creatinine ratio and systolic blood pressure, among all other variables only diastolic blood pressure, degree of cardiac insufficiency and LDL concentrations were included in the model in women, and diastolic blood pressure, nicotine abuse, triiodothyronine concentrations and BMI in men. By these models

Table 2
Discriminant analytical model for classification of women and men according to presence vs. absence of carotid atherosclerosis

Variable	Model 1 (without age)		Model 2 (with age)	
	F-value	Classification	*F*-value	Classification
Women				
Age	—		26.5	
RRsys	25.4		21.5	
N/C ratio	12.9		7.9	
RRdiast	9.8	74% correct	7.2	74% correct
HI	8.3	73% (jackknife)	n.s.	72% (jackknife)
LDL	7.7		4.3	
Men				
Age	—		25.4	
N/C ratio	20.2		11.1	
RRsys	18.6		10.6	
Nicotine (pack years)	12.4	74% correct	16.9	75% correct
RRdiast	5.7	72% (jackknife)	n.s.	73% (jackknife)
BMI	5.6		5.6	
T3	5.5		4.5	

Abbreviations: BMI, body mass index; HI, heart insufficiency; LDL, low density lipoprotein; N/C ratio, neopterin to creatinine ratio; n.s., not significant; RRdiast, RRsys, diastolic and systolic blood pressure; T3, free triiodothyronine.

72% of men and 73% of women with or without atherosclerosis were correctly classified applying the jackknife validation (Table 2).

When age was included in the model, *F*-to-remove-values of all parameters loaded into the described multivariate models were reduced, with age being the variable contributing most to correct classification of subjects (Table 2). This demonstrates that all other parameters included in the model depend to a certain degree on age.

4. Discussion

Data presented here demonstrate that serum neopterin levels are significantly correlated with the extent of carotid atherosclerosis. Neopterin was correlated even more strongly to atherosclerosis than all other candidate risk variables studied. Furthermore, there exists a close correlation between neopterin and age (Table 1). By using multivariate models we showed that neopterin as well as all other candidate risk variables depend to a certain degree on age. The correlation between neopterin and carotid atherosclerosis remaining after correction by age is close and significant in men and women and is prominent over most other candidate risk variables (Table 2). There is little danger that the results are biased by few outliers, because there were no exceptionally high neopterin levels in our data material, and moreover, the main part of statistical analyses was by non-parametric methods.

The correlation with atherosclerosis was nearly the same for neopterin and for the neopterin to creatinine ratio. From this it is evident that elevation of neopterin concentrations in serum of atherosclerotic subjects reflected immune activation, but was not due to impaired renal excretion. Our results agree well with a recent pilot study [18] where elevated serum neopterin levels were observed in patients with severe atherosclerosis irrespective of the anatomical site. These authors, however, could not substantiate their primary findings in a second small study [19] involving 13 subjects with severe coronary artery disease compared with 11 controls. However, the lack of significance for neopterin differences in the latter report is not surprising: in both studies [18,19] as well as in the present work, only comparatively small differences of neopterin mean levels are seen between subjects with and without atherosclerosis. Given these small differences, relatively large subject groups are required to detect significant correlations between atherosclerosis and neopterin. On the other hand, in our study we did not merely find a difference between subjects with and without atherosclerosis; rather, we observed a significant correlation between atherosclerosis and neopterin also on a quantitative basis, i.e. some sort of 'dose–response' relationship which clearly underscores the reliability of our findings. Of course, the small absolute differences found seem to rule out neopterin as an early marker for clinical diagnosis of atherosclerosis; rather, the observation of significant neopterin elevations in atherosclerosis adds to the existing evidence that immune activation may play a role in atherosclerosis.

Large amounts of neopterin are synthesized by human monocytes/macrophages upon stimulation with interferon-gamma [8], a cytokine originating from activated T-cells. This is due to direct stimulation by interferon-gamma of GTP-cyclohydrolase I, the first enzyme in the biosynthesis of neopterin and 5,6,7,8-tetrahydrobiopterin, cleaving GTP to form 7,8-dihydroneopterin triphosphate. Neopterin has been shown to be a sensitive indicator for monitoring activity of cell mediated immunity in vivo [9,20].

Our results suggest that chronic activation of cellular immunity is present in atherosclerotic patients. The role of activated macrophages and circulating cytokines in the pathogenesis and progression of atherosclerosis, e.g., by influencing lipid metabolism via uptake and conversion of certain lipids, has attracted great interest [1–7,21–23]. Besides other cytokines, interferon-gamma was suggested to play a crucial role in atherogenesis [5,24]. Interferon-gamma was shown to increase MHC class II antigen expression on human smooth muscle and vascular endothelial cells, which indicates the presence of activated T cells and macrophages in atheroma lesions [5,25]. In addition, interferon-gamma is known to stimulate monocytes/macrophages to produce cytotoxic free radicals [26], a mechanism that may contribute to the progression of athero-

sclerosis. It is not surprising that the extent of carotid atherosclerosis positively correlates with serum concentrations of the interferon-gamma inducible macrophage product neopterin, suggesting that cellular immune activation may play a role for atherogenesis also in vivo. Most recent results demonstrate that neopterin is able to enhance radical-induced chemiluminescence as well as radical-induced cytotoxicity [27], and thus, neopterin might play an important functional role in enhancing the cytotoxic potential of activated macrophages.

Concentrations of sIL-2R, another serum soluble marker of immune activation, were only slightly increased in atherosclerotic patients. The association is far less significant than that between neopterin levels and carotid atherosclerosis. This may be due to the fact that concentrations of sIL-2R reflect activation of multiple immune cells, since increased expression of IL-2R is known to occur on many activated immune cells such as T-cells, B-cells, natural killer cells, blood monocytes or thymocytes [10,11,28–31].

Our data indicate that continuous activation of cellular immunity, in particular activation of macrophages, takes place in atherosclerotic patients. This is supported by the fact that the interferon-gamma inducible macrophage factor neopterin was correlated even more strongly with carotid atherosclerosis than all other classical risk factors for atherosclerosis investigated in this study.

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