Clinical research

Elevated serum neopterin predicts future adverse cardiac events in patients with chronic stable angina pectoris

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Aims Serum levels of neopterin, an immune modulator secreted by activated macrophages, are elevated in patients with acute coronary syndromes compared with stable angina patients and control subjects. In unstable angina, serum neopterin levels correlate with the presence of vulnerable coronary stenosis, multiple complex coronary lesions, and patient outcome. The present study assessed the prognostic significance of raised serum neopterin concentrations in patients with stable angina pectoris.

Methods and results We carried out a 1-year follow-up prospective study in 297 patients with chronic stable chest pain undergoing diagnostic coronary angiography. The primary study endpoint was the composite of non-fatal myocardial infarction, unstable angina, and cardiac death. Fifty-one patients (17.2%) had adverse coronary events during follow-up. Mean serum neopterin levels were significantly higher in patients with events compared with those without (P = 0.02). On multiple regression analysis, neopterin levels (P = 0.021), severity of coronary artery disease (P = 0.009), and a history of previous myocardial infarction (P = 0.001) were independent predictors of adverse events.

Conclusions Serum neopterin is an independent predictor of major adverse coronary events in patients with chronic stable angina pectoris. This marker of macrophage activation may be useful for risk stratification in patients with chronic stable angina.

Introduction

Inflammation plays an important role in atherogenesis and plaque vulnerability.† Activated macrophages contribute to both atherosclerotic plaque disruption and intracoronary thrombus formation, often leading to the acute coronary syndrome (ACS), i.e. acute myocardial infarction (AMI), unstable angina, and sudden cardiac death. Plaque disruption generally occurs at sites where the fibrous cap is thinnest and most heavily infiltrated with macrophage-derived foam cells.‡ Activated macrophages synthesize metalloproteinases that contribute to collagen degradation and weakening of the fibrous cap.† Neopterin, a pteridine derivative and a by-product of the guanosine triphosphate–biopterin pathway,§ is also produced by activated macrophages and is thought to represent a marker of immune activation and macrophage activity.‖ Neopterin enhances inflammatory processes within vulnerable plaques and, together with the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α), it stimulates gene transcription for inducible

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nitric oxide synthase (iNOS), which results in the production of cytotoxic NO free radical.

Previous studies from our group showed that serum neopterin levels are elevated in patients with ACS compared with those with stable ischemic heart disease and are also associated with the presence of complex vulnerable coronary lesions in patients with unstable angina. Similarly, we have previously shown that high serum neopterin levels predict the development of acute coronary events in women with chronic stable angina pectoris (CSA) and hypertensive patients without significant obstructive coronary artery disease (CAD). High neopterin concentrations are also associated with rapid angiographic CAD progression in patients with CSA as recently reported by Zouridakis et al. Taken together, these findings indicate that immune activation may play a pathogenic role in the formation and progression of coronary atheroma, and serum neopterin levels may be a useful clinical marker of CAD activity.

In the present study we prospectively assessed the prognostic role of serum neopterin concentrations in patients with CSA. We also assessed the association between neopterin levels and clinical, biochemical, and angiographic variables in these patients.

Methods

Patient selection

From a larger cohort of patients (n = 410) undergoing coronary arteriography for the assessment of chronic stable chest pain, we prospectively investigated 297 patients [mean age 63 ± 9 years, 215 (72%) men]. Eighty-seven patients were not included as they presented with ACS and 26 patients did not give their consent for participation in the study. CSA was defined as typical chest pain brought on by exertion and relieved by rest or sublingual nitrates or both, a positive ECG exercise test response (≥1 mm ST-segment depression), and/or reversible perfusion defects on myocardial perfusion scintigraphy. In all patients, symptoms were stable for at least 3 months before study entry. Patients with coronary artery bypass grafting, life-threatening arrhythmias, cardiac valve disease, acute or chronic liver disease, renal failure (creatinine > 130 μmol/L), infective diseases, recent myocardial infarction (MI) (<12 weeks), or other conditions likely to cause death within 1 year were not entered in the study. None of the patients included in the study had ongoing systemic or cardiac inflammatory processes with the exception of atherosclerosis. All patients gave written informed consent before study entry and the study was approved by the local research ethics committee.

Definition of conventional cardiovascular risk factors

Systemic arterial hypertension was diagnosed in the presence of a systolic blood pressure of ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg as measured on at least two separate occasions. Patients receiving antihypertensive medications were also considered to have systemic hypertension, irrespective of the blood pressure readings at study entry. Hypercholesterolaemia was defined as a documented total cholesterol concentration >5.4 mmol/L. Smokers were defined as those currently smoking any type of tobacco. Patients were considered to have diabetes mellitus if they were receiving active treatment with insulin or oral hypoglycaemic agents. For patients on dietary treatment alone, documentation of abnormal fasting blood glucose or glucose tolerance tests according to World Health Organization criteria were required for the diagnosis of diabetes.

Clinical characterization, follow-up, and study endpoints

Data acquired at study entry included age, gender, height, weight, blood pressure, and risk factor history including MI, systemic hypertension, diabetes mellitus, smoking, hypercholesterolaemia, a family history of CAD, cerebrovascular disease, intermittent claudication, and previous percutaneous transluminal coronary angioplasty (PTCA) or bypass surgery. The New York Heart Association (NYHA) congestive heart failure classification and the Canadian Cardiovascular Society (CCS) functional class, as well as cardiac medications, were recorded at study entry. After recruitment, routine baseline characterization was carried out in all patients, who were then followed for up to 1 year. Prospectively selected study endpoints were: (i) non-fatal AMI defined according to World Health Organization criteria (e.g. raised cardiac enzymes, characteristic ECG changes, and prolonged typical chest pain), (ii) hospital admission with Braunwald’s class IIIb unstable angina requiring medical treatment and/or urgent revascularization, and (iii) cardiac death.

Biochemical measurements

Venous blood was collected from all patients at the time of diagnostic coronary angiography and centrifuged immediately. Both plasma and serum samples were stored at −80°C until assessment was carried out in batches at the end of recruitment. Serum neopterin concentration was measured using a commercially available immunoassay (ELISA Kit, IBL, Hamburg, Germany). The within-coefficient variability was <3% in the 7.7 nmol/L range and <4% in the 20 nmol/L range. C-reactive protein (CRP) measurements were performed with COBAS Integra (Roche Diagnostics Limited, Lewes, East Sussex, UK) using the CRP-Latex assay in both the high-sensitivity application (analytical range 0.2–12 mg/L) and the normal application (analytical range 2–160 mg/L). Analytical precision of the high-sensitivity CRP-Latex assay was 7.6% at a level of 1.02 mg/L, 3.3% at 1.79 mg/L, and 1.3% at a level of 4.36 mg/L. Samples outside the analytical range of the high-sensitivity CRP-Latex assay were analysed by the CRP-Latex assay in the normal application. The analytical precision of the normal CRP-Latex assay was 2.4% at a level of 29.5 mg/L and 1.3% at a level of 113 mg/L. All other biochemical measurements were carried out by the Analytical Unit in our institution.

Angiographic assessment

Coronary angiography was carried out according to the Judkins technique, and images of the coronary tree were obtained in routine, standardized projections with the digital Philips Integris 3000 system (Philips, Holland), using an automated quantitative coronary artery stenosis assessment programme in all patients. Two experienced cardiologists who had no knowledge of the patients’ clinical characteristics and biochemical results reviewed all angiographic images.
Coronary angiograms were scored according to Sullivan et al. and as described in previous studies from our group. Briefly, this includes vessel, stenosis, and CAD extent scores. Vessel score is based on the number of coronary arteries showing >50% stenosis reduction in lumen diameter. Stenosis score is aimed at reflecting the most severe stenosis observed in each of the main coronary vessels assessed. CAD extension score refers to the proportion of the coronary artery tree showing angiographically detectable atheroma. The observed proportion in each vessel is multiplied by a factor that varies according to the artery involved. This total score represents the percentage of the coronary luminal surface area showing the presence of atheroma.

### Statistical analysis

Results for normally distributed continuous variables are expressed as the mean value ± standard deviation (SD), and continuous variables with non-normal distribution are presented as median values (interquartile intervals). Analysis of normality of the continuous variables was performed with the Kolmogorov-Smirnov test. Logistic regression was used to assess the univariate associations between continuous baseline characteristics and the probability of having an event, and χ² testing was used for discrete variables. As CAD extent and severity are related to age, correlations between neopterin concentrations, parameters of severity, and extent of CAD, i.e. vessel, stenosis, and extension scores, were analysed with two-way Pearson or Spearman correlation tests, as appropriate, controlling for age. Information regarding the development of combined endpoint was available for all patients included in the study. We therefore assessed independent predictors of endpoint using a binary logistic regression analysis. Logistic regression analysis parameters were obtained with the Wald test. We also classified patients into three groups according to the tertiles of neopterin values upon study entry. We assessed independent predictors of serum neopterin concentrations using multiple regression analysis. Neopterin serum concentration was logarithm-transformed before any regression analysis was performed in order to fulfill the conditions required for both binary logistic and linear multiple regression analysis. Backward stepwise selection was used in all multivariate models to derive the final model for which significance levels of 0.1 and 0.05 were chosen to exclude and include terms, respectively. Variables included in multivariate analyses were those which showed a correlation in univariate analysis that was significant at the 20% significance level. Differences were considered to be statistically significant if the null hypothesis could be rejected with >95% confidence. The SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for all calculations.

### Results

**Neopterin, CRP levels, and adverse cardiac events during follow-up**

Baseline clinical characteristics, biochemical, and angiographic results in the 297 patients included in the study are shown in Table 1. During the 1-year follow-up, 51 patients (17.2%) suffered events, whereas 246 patients had no adverse events (Tables 2 and 3). The proportion of patients who suffered an adverse event was similar in male and female patients (18.6 vs. 13.4%, P = 0.29). Univariate analysis showed that neopterin serum concentration (P = 0.02), number of diseased vessels (>50% reduction in lumen diameter) (P = 0.003), and a history of previous MI (P < 0.001) were associated with the development of the combined endpoint. We found no significant association between CRP and the occurrence of the combined endpoint. When we assessed the relationship between CRP levels (in tertiles) and the distribution of the endpoint, we found no differences among the three groups (first, second, and third tertiles, 18.8, 19.8, and 14.6% of events, respectively). On binary logistic regression analysis, only neopterin (P = 0.021), number of diseased vessels (P = 0.009), and a history of MI (P = 0.001) remained independent predictors of the combined endpoint (Table 4). Moreover, a significant gradual increase in the number of adverse events was seen during follow-up with increasing neopterin tertiles (Figure 1). After adjustment, on binary logistic regression analysis patients in the highest neopterin tertile (>7 nmol/L) had a three-fold higher risk of developing adverse cardiovascular events (OR 3 (1.25–7.2)

### Table 1 Baseline characteristics of 297 consecutive patients with CSA

| Age, years | 63 ± 9 |
| Male gender, n (%) | 215 (72) |
| Systolic BP, mmHg | 136 ± 20 |
| Diastolic BP, mmHg | 80 ± 11 |
| BMI, kg/m² | 27 ± 4 |
| Previous PTCA, n (%) | 22 (7) |
| Previous MI, n (%) | 65 (22) |
| Previous stroke, n (%) | 15 (5) |
| PVD, n (%) | 41 (14) |

#### Cardiovascular risk factors

| Diabetes mellitus, n (%) | 17 (6) |
| Dyslipidaemia, n (%) | 143 (48) |
| Hypertension, n (%) | 100 (37) |
| Smoking, n (%) | 188 (63) |
| Family history <60 years, n (%) | 173 (58) |
| Number of diseased vessels >50%, n | 2.0 (1.0–3.0) |
| LVEF, % | 67 ± 11 |
| NYHA functional class III or IV, n (%) | 51 (17) |
| CCS functional class III or IV, n (%) | 56 (19) |

#### Treatment

| Aspirin, n (%) | 236 (80) |
| β-Blockers, n (%) | 160 (54) |
| Nitrate, n (%) | 159 (53) |
| ACE inhibitors, n (%) | 43 (14) |
| Diuretics, n (%) | 45 (15) |
| Digoxin, n (%) | 6 (2) |
| HMG-CoA reductase inhibitors, n (%) | 53 (18) |

#### Biochemistry

| Creatinine, mg/dL | 74 ± 30 |
| Total cholesterol, mmol/L | 6 ± 1.2 |
| Neopterin, nmol/L | 5.7 (4–7.6) |
| CRP, mg/L | 2.35 (1–5.1) |

Data are expressed as mean ± standard deviation for normally distributed data, median (interquartile range) for non-normally distributed data, and number (%) for categorical variables. ACE, angiotensin converting enzyme; BMI, body mass index; BP, blood pressure; PVD, peripheral vascular disease.
95% CI; \( P = 0.015 \) than those in the lowest tertile (≤4.5 nmol/L).

**Predictors of serum neopterin level distribution**

Baseline serum neopterin concentrations were higher in patients with CCS functional class III or IV (\( P = 0.009 \)). Interestingly, patients receiving 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors had lower neopterin levels than patients who did not take this medication (\( P = 0.019 \)). A significant correlation was also found between neopterin levels and age (\( r = -0.2, P < 0.0001 \)), hs-CRP levels (\( r = -0.3, P < 0.0001 \)), and left ventricular ejection fraction (LVEF) (\( r = -0.16, P = 0.004 \)). Neopterin levels were not significantly associated with CAD extent or severity scores. When variables with a \( P \)-value of <0.20 in the univariate analysis were introduced into a multiple regression analysis,
age \( (P = 0.047) \), LVEF \( (P = 0.027) \), hs-CRP \( (P = 0.019) \), as well as treatment with HMG-CoA reductase inhibitors \( (P = 0.037) \), were independent predictors of serum neopterin concentrations.

**Discussion**

The results of our study show that neopterin, a marker of macrophage activation, predicts adverse cardiovascular events during follow-up in patients with CSA. After multivariate analysis, patients in the highest tertile of neopterin distribution had a three-fold higher risk of developing adverse cardiovascular events than those in the lowest tertile, a finding that was independent of the severity of CAD. Patients with events had significantly higher levels of serum neopterin compared with patients without cardiovascular events at the 1-year follow-up. Thus, serum neopterin may be a useful marker of risk in patients with CSA. Our results confirm and expand previous findings from our group and others regarding the predictive role of neopterin in ischaemic heart disease. Results in the present study also suggest a role for monocyte/macrophage activation in the pathogenesis of acute coronary events.

**Neopterin, inflammation, and immune system activation**

Neopterin has been suggested to be a marker of atherosomatous plaque activity. The relationship between serum neopterin concentration and angiographically complex coronary artery stenosis in patients with unstable angina was previously assessed by our group. We reported a significant association between circulating neopterin levels and the number of angiographically complex stenoses in patients with ACS. Complex stenoses are known to represent vulnerable or disrupted atherosomatous plaques. Moreover, we found that serum neopterin levels were higher in patients with chronic CAD and normal control subjects. Schumacher et al. also reported elevated serum neopterin levels in patients with AMI, compared with patients with CSA and healthy control subjects. The relation of serum neopterin concentrations with adverse cardiovascular events during follow-up in patients with CSA in the present study supports the notion that clinical stability does not always indicate atheromatous plaque stability. It has been reported that CSA patients have unstable coronary stenoses. In several atherectomy studies, considerable amounts of inflammatory cells were found in culprit lesions of stable patients and fragments of thrombus were also documented in up to 20% of apparently stable plaques. Ongoing inflammation in stable plaques may predispose the individual to rapid CAD progression and the development of unstable syndromes. The prognostic value shown by other markers of inflammation, such as CRP, in patients with CSA, may be a reflection of ongoing inflammation in the atheromatous plaques. Findings in previous studies from our group and others with CRP suggest that the inflammatory state of the coronary atheromatous lesions determines the clinical outcome in patients with CAD. As also suggested for CRP previously, it is likely that the predictive ability of neopterin as observed in our study and others may also indicate that neopterin is not only a marker of CAD activity, but may also play a pathogenic role in CAD. Neopterin has been shown to be involved in the activation of both constitutive and iNOS. Neopterin stimulates nuclear factor-κB translocation to the nucleus, promoting the expression of proinflammatory genes, adhesion molecules, tissue factor, and other substances implicated in the
inflammatory processes that take place within the arterial wall in atherogenesis and atheromatous plaque disruption.

Predictors of serum neopterin concentrations
Recent findings indicate that HMG-CoA reductase inhibitors have anti-inflammatory properties, in addition to their lipid-lowering effects. HMG-CoA reductase inhibitors have been shown to modulate immune responses and recent reports showed that lipid-lowering therapy suppressed macrophage growth and macrophage accumulation in atheromatous lesions, probably by reducing oxidative stress. HMG-CoA reductase inhibitors have also been shown to down-regulate neopterin production by monocytes in vitro. However, only two small clinical studies have assessed the relationship between neopterin levels and the use of statins, with controversial results. Our study shows that HMG-CoA reductase inhibitors are associated with lower serum neopterin levels in patients with CAD, even after adjustment for confounding factors. This finding is consistent with the known anti-inflammatory effect of statins and also suggests that lower neopterin levels in patients receiving statin treatment may be a marker of the beneficial effects of these pharmacological agents on monocyte/macrophage function.

Besides the observed correlation of neopterin levels with both increasing age and serum CRP levels, we have also found an association between neopterin levels and impaired LVEF. This finding is in agreement with previous studies, which showed a significant relationship between circulating levels of neopterin and left ventricle function parameters. As suggested by animal models, enhanced oxidative stress induced by neopterin may result in LV dysfunction.

Neopterin and severity/extent of CAD
Although studies have reported an association between neopterin and the extent of atherosclerosis, i.e. peripheral vascular occlusions and carotid atherosclerosis, serum neopterin levels in our study did not correlate with severity or extent of CAD. Our data suggest that neopterin may be a marker of inflammatory coronary disease activity rather than a measure of the anatomical extent of the coronary atheromatous process. This finding is also in agreement with Schumacher et al.'s observations that serum neopterin levels in patients with CSA did not correlate with the number of diseased coronary vessels.

Limitations of the study
A limitation of this study is the relatively small size of the patient population investigated. The small sample size may also explain why, in the present study, hs-CRP levels did not differ significantly between patients with events and those without events. However, even with the relatively small sample size, neopterin levels were significantly higher in patients who developed serious events compared with those who had an uneventful clinical course during follow-up.

Conclusions
Our study shows that serum neopterin concentrations are associated with the development of adverse cardiovascular events in patients with CSA. This association was independent of the severity of CAD. Neopterin may thus be a useful marker of risk of future coronary events in these patients.

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