Clinical research

The correlation between plasma human neutrophil peptide 1-3 levels and severity of coronary artery disease

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Submitted: 29 October 2016
Accepted: 22 November 2016

Arch Med Sci Atheroscler Dis 2016; 1: e133–e138
DOI: 10.5114/amsad.2016.64164

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Abstract

Introduction: Inflammation plays a key role in atherosclerosis, and discovering new biomarkers of inflammation is becoming important in order to uncover the pathogenesis of atherosclerotic coronary artery disease (CAD). Recent studies have focused on polymorphonuclear neutrophils. It has been suggested that human neutrophil peptide 1-3 (HNP1-3) is proatherogenic. In this study, we aimed to investigate the associations between plasma HNP1-3 levels and the severity of atherosclerosis via a generally accepted scoring system.
Material and methods: This cross-sectional, observational study included 107 consecutive patients suffering from stable angina pectoris and undergoing coronary angiography (CAG). Patients were divided into two groups according to the Gensini scoring (GS) system evaluating disease severity. Group 1 was composed of mild CAD patients with GS < 20 and group 2 consisted of severe CAD patients with GS ≥ 20. Plasma HNP1-3 levels were assessed by the ELISA method.
Results: The mean HNP1-3 levels were found to be lower in group 1 than group 2 (134.7 ng/ml vs. 147.5 ng/ml). HNP1-3 levels were significantly higher in the severe CAD group than the mild CAD group according to GS (p < 0.001). The results of multivariate logistic regression analysis revealed that only age > 62 years and HNP1-3 > 134 ng/ml were independent predictors of the severity of CAD after adjusting for gender, smoking, hypertension, hyperlipidemia, diabetes, family history of CAD and white blood cell count. In predicting the severity of CAD, the sensitivity and specificity of HNP1-3 were 83.9% (p < 0.001) and 58.8% (p < 0.001), respectively.
Conclusions: This study revealed that the plasma levels of HNP1-3 were significantly higher in severe CAD than mild CAD.

Key words: biomarkers, coronary artery disease, Gensini score, HNP1-3, age.

Introduction

Atherosclerosis and coronary artery disease (CAD) are the leading cause of death worldwide [1]. Hypertension, dislipidemia, smoking and diabetes mellitus are well-known traditional risk factors of CAD, but many patients suffering from acute myocardial infarction (AMI) do not have these traditional risk factors [1, 2]. Inflammation plays a key role in atherosclerosis, and discovering new biomarkers of inflammation is

becoming an important issue in order to uncover the pathogenesis of atherosclerosis and CAD. The roles of T-lymphocytes, monocytes, macrophages and platelets in atherosclerosis have been broadly studied, but lately some studies have focused on polymorphonuclear neutrophils (PMNs) [3–5]. Polymorphonuclear neutrophils have an emerging role in atherosclerosis [6]. It has been reported that plasma PMN levels are a predictive and independent risk factor for future cardiovascular events [7]. Coller *et al.* demonstrated an absolute association between PMN count and mortality in over 350,000 atherosclerotic patients [8]. The human neutrophil peptides (HNPs), also known as α-defensins, are being investigated as they are released from PMNs during non-infectious inflammatory conditions such as CAD [9]. The HNPs are antimicrobial peptides that have an emerging role in innate and acquired immunity [6]. They are stored in PMNs and released to the circulation from activated PMNs after inflammation and accumulate in the infected tissue and injured coronary arteries [6]. Maneerat *et al.* recently reported that increased α-defensin expression may be a potential inflammatory marker for the prediction of risk of CAD development in hyperlipidemic patients [10]. Thus, the measurement of HNP1-3 levels may be beneficial for the prediction of CAD risk. In this study, we aimed to investigate the associations between plasma HNP1-3 levels and the severity of coronary atherosclerosis via a generally accepted scoring system.

Material and methods

Study population

This cross-sectional, observational study was conducted between September 2011 and April 2012 and included 107 consecutive patients (52 female, 55 male) suffering from stable angina pectoris according to the history and undergoing coronary angiography. Demographic features such as age, sex, smoking, family and disease history and medications were recorded. Body mass index (BMI) was calculated according to World Health Organization criteria [11]. Patients with known CAD were excluded from the study for *de novo* patient inclusion to facilitate the calculation of disease severity. Patients older than 75 years and those with estimated glomerular filtration rate (eGFR) < 60 ml/min, serious valvular disease, uncontrolled hypertension, heart failure, hepatic failure, acute or chronic infection, fever, immunologic system disease, rheumatic disease, malignancy or osteoporosis were also excluded. All patients gave written informed consent. The study protocol was approved by the local Institutional Review Board and Ethics Committee. The study was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice (GCP) and International Conference on Harmonization (ICH) guidelines.

Laboratory measurements and HNP1-3 analysis

We collected venous blood samples from all patients 2 h before CAG to measure HNP1-3 levels, blood count and other biochemical parameters. Venous blood samples were collected into tubes with EDTA in order to measure HNP1-3 from all patients. Blood samples were centrifuged at 25°C, 3500 rpm for 10 min to separate the serum and stored in polypropylene tubes until analysis at –70°C. Hemolyzed and lipemic samples were excluded. HNP1-3 levels were measured by ready-to-use solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle with a HK317 kit (HyCult biotechnology, Uden, Holland). Assays were used according to the manufacturer's protocol and absorbance was measured at 450 nanometer via a microplate reader.

Electrocardiogram (ECG) and transthoracic echocardiography (TTE) were performed in all patients before CAG.

Coronary angiography and disease severity scoring

All CAG procedures were performed using the standard Judkins method with a cineangiography device (Axiom Artis, Siemens, Germany). All of the angiograms were recorded to compact discs in DICOM format and examined visually later by two experienced interventional cardiologists blinded to the study. The severity and extent of CAD were evaluated according to the Gensini score (GS), which depends on the degree of the coronary artery stenosis and its regional importance [12]. The degree of lumen narrowing, concentricity and eccentricity of the plaques were evaluated. According to the Gensini scoring system, 1 point is given for 1–25% stenosis, 2 points for 26–50%, 4 points for 51–75%, 8 points for 76–90%, 16 points for 91–99% and 32 points for 100% stenosis. Then, each lesion's number of points is multiplied by the coefficient which is given for each principal vascular segment according to the functional significance of the vessel (the left main coronary artery × 5; the proximal segment of the left anterior descending coronary artery (LAD) × 2.5; the proximal segment of the circumflex artery × 2.5; the mid-segment of the LAD × 1.5; the right coronary artery, the distal segment of the LAD, the posterolateral artery and the obtuse marginal artery × 1; and others × 0.5), and the sum of all points constitutes the total score [12]. Scoring was performed

together with two observers and averaged. The cut-off point of the Gensini score for the severity of disease was accepted as 20 points so as to maximize the differences in plasma HNP1-3 levels between the severe and mild stenosis groups. The GS < 20 was considered as mild CAD (group 1) and GS ≥ 20 was accepted as severe CAD (group 2).

Statistical analysis

The statistical analyses of the study were performed using Statistical Package for the Social Sciences (SPSS) software version 19.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The Kolmogorov-Smirnov test and coefficient of variation were used for the compatibility of quantitative data with a normal distribution. Normally distributed data were analyzed by parametric methods, and abnormally distributed data were analyzed by nonparametric methods. The independent *t*-test was used for the comparison of two independent groups. Categorical data were analyzed by Pearson's χ^2 test. Logistic regression analysis was performed for the evaluation of cause and effect relationships of binomial and multinomial categories. The receiver operating characteristic (ROC) curve was used for calculating the cut-off values. Quantitative data were expressed as mean ± standard deviation and median ± OR. Categorical variables were expressed as *n* (number) and percentages (%). Confidence intervals were constructed at the 95% confidence level and *p*-values lower than 0.05 were considered as significant.

Results

The baseline demographic characteristics, clinical features and laboratory findings of the patients are given in Table I. Gender, history of hypertension, diabetes, hyperlipidemia, family history of CAD, smoking and white blood cell count (WBC) were not different among groups ($p > 0.05$). The mean age of the patients was lower in the mild CAD group than the severe CAD group (56.6 ±5.9 vs. 59.5 ±6.8, $p = 0.015$). The optimal cut-off values of HNP1-3 level and age to predict high GS were 134 ng/ml and 62 years, respectively (Table II and Figure 1). The mean HNP1-3 levels were lower in the mild CAD group than the severe CAD group (134.7 ng/ml vs. 147.5 ng/ml, $p < 0.001$) (Figure 2). In predicting disease severity, the sensitivity and specificity for HNP1-3 level were 83.9% and 58.8%, $p < 0.001$, respectively, and the sensitivity and specificity for age were 34% and 83.6%, $p = 0.034$, respectively (Table II). The cut-off value calculated for WBC count to distinguish the groups was found to be not statistically significant ($p = 0.113$). Categorized evaluation of HNP1-3 level revealed that 76.9% of patients in the mild CAD group and 23.1% of patients in the severe CAD group had HNP1-3 ≤ 134, and 30.9% of patients in the mild CAD group and 69.1% of patients in the severe CAD group had HNP1-3 > 134 ($p < 0.001$). The results of multivariate logistic regression analysis in predicting high GS suggested that only age > 62 years and HNP1-3 > 134 ng/ml were independent predictors of the severity of CAD after adjusting for gender, smoking, hypertension, hyperlipidemia, diabetes, family history of CAD and WBC count (OR = 5.16, 95% CI: 1.59–16.71, $p = 0.006$ and OR = 6.82, 95% CI: 2.53–18.37, $p < 0.001$, respectively) (Table III).

Table I. Demographic features and HNP1-3 levels among groups

Parameter	Mild CAD group	Severe CAD group	*P*-value
Sex:			
Female	29 (56%)	23 (41%)	0.103
Male	22 (44%)	33 (59%)	
Hypertension:			
No	34 (67%)	37 (66%)	0.948
Yes	17 (33%)	19 (34%)	
Diabetes:			
No	42 (82%)	39 (70%)	0.126
Yes	9 (8%)	17 (30%)	
Smoking:			
No	27 (53%)	27 (48%)	0.625
Yes	24 (47%)	29 (52%)	
Hyperlipidemia:			
No	34 (67%)	37 (66%)	0.948
Yes	17 (33%)	19 (34%)	
Family history:			
No	31 (61%)	32 (57%)	0.702
Yes	20 (39%)	24 (43%)	
Age [years]:			
< 62	44 (86%)	37 (66%)	0.015
> 62	7 (14%)	19 (34%)	
WBC [× 10^3/ml]	8.78 ±1.19	9.23 ±1.45	0.08
HNP1-3 [ng/ml]:	134.78 ±12.69	147.55 ±13.31	< 0.001
≤ 134	30 (59%)	9 (16%)	< 0.001
> 134	21 (41%)	47 (84%)	

CAD – coronary artery disease, WBC – white blood cell count, HNP – human neutrophil peptide. Pearson χ^2 test was used.

Table II. Cut-off values of age, HNP1-3 and WBC according to ROC analysis

Parameter	AUC ± SD	Cut-off value	Sensitivity (%)	Specificity (%)	*P*-value
HNP 1-3	0.756 ±0.046	134	83.9	58.8	< 0.001
WBC	0.589 ±0.055	9	46.4	72.5	0.113
Age	0.619 ±0.054	62	34.0	86.3	0.034

AUC – area under the ROC curve, SD – standard deviation, HNP – human neutrophil peptide, WBC – white blood cell.

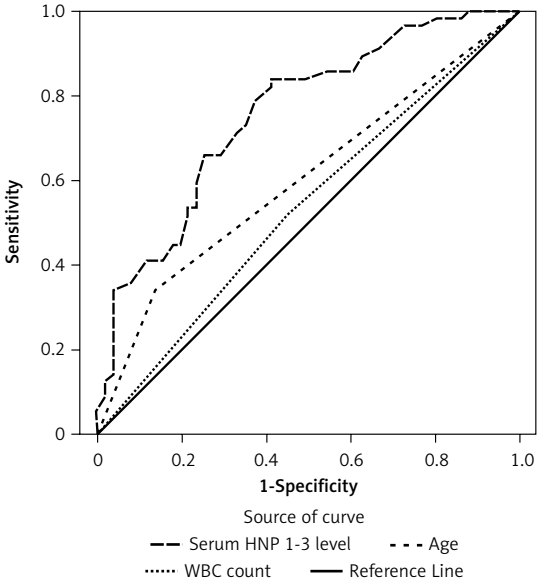

Figure 1. ROC analysis for the predictive power of the HNP 1-3, age, WBC and Gensini score

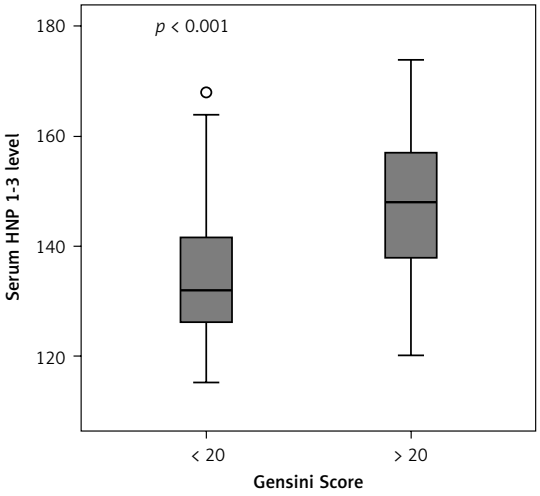

Figure 2. The results of HNP1-3 levels according to the Gensini score

Table III. Multivariate logistic regression analysis for the prediction of severe CAD

Parameter	*P*-value	Odds ratio	Odds ratio 95% CI	
			Lower limit	Upper limit
Age (> 62)	0.006	5.161	1.593	16.718
Sex (male)	0.441	1.440	0.569	3.641
Hypertension	0.674	0.809	0.302	2.171
Diabetes	0.630	1.305	0.442	3.852
Smoking	0.444	1.448	0.561	3.740
HNP 1-3 (> 134)	< 0.001	6.820	2.531	18.377
Hyperlipidemia	0.574	1.321	0.500	3.494
Family history of CAD	0.416	1.478	0.576	3.793
WBC count	0.811	0.893	0.351	2.268

CI – confidence interval, HNP – human neutrophil peptide, CAD – coronary artery disease, WBC – white blood cell.

Discussion

This study revealed that the plasma levels of HNP1-3 were significantly higher in severe CAD than mild CAD, and HNP1-3 levels and age were well correlated with the severity of CAD. Using biomarkers is of great importance for the prediction of the severity and prognosis of CAD. High-sensitivity C-reactive protein (hs-CRP), PMNs and interleukin-6 have been widely investigated for this application and have shown promising results [6]. Recent studies have examined the contribution of HNPs to cardiovascular atherosclerosis in an indirect fashion, so HNPs are released from PMNs during acute and chronic inflammatory situations such as atherosclerosis and form complexes with

LDL and alter LDL metabolism [6, 13–15]. Kougias *et al.* first mentioned the role of HNPs in atherosclerosis in 2005 [16]. Increased HNP levels were found in atherosclerotic coronary arteries, and intimal and medial smooth muscle cells of carotid arteries [17]. Maneerat *et al.* recently found that α-defensin mRNA expression increased in hyperlipidemic and CAD patients compared with controls, and CAD development moderately correlated with α-defensin mRNA expression and with plasma HNP1-3 levels [10]. Nassar *et al.* investigated HNP levels in skin biopsies of patients with CAD and revealed a correlation between HNP levels and the extent of CAD [18]. However, they stated severe CAD as 2 or more vessel disease on angiogram, simply [18]. This simple classification of CAD does not project the severity and extent of CAD adequately, and is outdated. Nowadays, more detailed scoring systems such as SYNTAX and Gensini scores have become important for the evaluation of the severity and extent of CAD. The present study supports their results. Christensen *et al.* observed higher HNP levels in patients with advanced heart failure (New York Heart Association (NYHA) stage III–IV) than stable heart failure (NYHA stage I–II) and reported the incremental prognostic value of HNPs with regard to mortality [19]. Subramaniam *et al.* observed an association between HNP1-3 levels and thromboembolic complications in patients with heart failure [20]. HNP1-3 inhibits plasmin formation by inhibiting tissue plasminogen activator (t-PA) and increases the tendency of blood coagulation [6]. Joseph *et al.* reported higher plasma HNP levels in patients with type 1 diabetes with cardiovascular disease (CVD) than without CVD, and they indicate HNPs as a risk marker for CVD-related morbidity and mortality in diabetics [21]. Zhao *et al.* noted the highest HNP1-3 levels in patients with acute ST elevation myocardial infarction compared with stable CAD and without CAD [22]. Their results suggest that HNP1-3 levels are chronically increased in stable CAD and rise even more in the acute state of myocardial infarction [22]. Several studies have demonstrated that the severity of CAD is related to the WBC count [23, 24]. Although total WBC count was higher in the severe CAD group than the mild CAD group in our study, the result was not statistically significant ($p = 0.08$), and multiple regression analysis revealed that WBC count was not predictive for severe CAD.

The present study does have considerable limitations. The main limitations of our study were its single-center basis and relatively small patient population size. We mentioned above that history of hypertension, diabetes, hyperlipidemia, family history of CAD, smoking and WBC were not different among groups ($p > 0.05$). However, these comparisons were performed only by categorizing these major cardiovascular risk factors as dichotomous variables, so we were not able to compare the two groups by giving these data in continuous forms (i.e. severity of blood pressure, LDL cholesterol levels, etc). Also we were not able to compare the groups in terms of antihypertensive medications, statins, etc. We were able to take one measurement of HNP1-3 level, so we may have missed the biological and individual variations of HNP1-3 levels in plasma, which need to be investigated. One parameter was assayed in our study, whereas comparisons with other inflammatory parameters would increase the scientific value of the work. The results would be more reliable if we had used two methods instead of one. Maneerat *et al.* compared α-defensin expression among groups using real-time quantitative polymerase chain reaction to determine α-defensin mRNA expression and ELISA to determine plasma HNP1-3 levels to increase the power of the study [10]. It has previously been reported that reduced kidney function increases HNP1-3 levels independently [19, 21]. Hence, we excluded patients with e-GFR < 60 ml/min. We only calculated WBC count, but absolute neutrophil count would be more valuable as HNPs are released from PMNs.

Despite the above limitations, as far as we know, HNP1-3 levels have not previously been studied for the prediction of the severity of CAD with an eligible and comprehensive scoring system. We evaluated the severity of CAD using GS. However, there are other scoring systems, such as the Syntax score and CAD score. Future studies evaluating and comparing these scoring systems may provide more comprehensive information.

In conclusion, this study revealed that the plasma levels of HNP1-3 were significantly higher in severe CAD than mild CAD patients, and HNP1-3 levels and age were well correlated with the severity of CAD. In predicting the severity of CAD, the sensitivity and specificity of HNP1-3 levels were 83.9% ($p < 0.001$) and 58.8% ($p < 0.001$), respectively. Plasma HNP1-3 levels may be a promising marker for the prediction of the severity and extent of CAD.

Conflict of interest

The authors declare no conflict of interest.

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