Association between tissue human neutrophil peptide 1–3 levels and cardiovascular phenotype: a prospective, longitudinal cohort study

Rami Abu Fanne¹,³, Yaron Arbel², Ehud Chorin², Emad Maraga³, Gabriel M Groisman⁴, Abd Alroof Higazi³ and Shmuel Banai²

Abstract

Objective: Inflammation is associated with atherogenesis. Although a higher neutrophil count is associated with the plaque burden, the role of neutrophil activation is unclear. Human neutrophil peptides 1–3 (HNP1–3) are a risk factor for atherogenesis in bench models and are elevated in human atheromas. This study aimed to examine the association between skin HNP1–3 deposition and the severity of coronary artery disease (CAD), including long-term outcomes.

Methods: HNP1–3 levels were immunohistochemically quantified in skin biopsies, which were prospectively taken from 599 consecutive patients before clinically indicated coronary angiography. Established cardiovascular risk factors and blood markers for atheroinflammation were obtained. CAD severity and the incidence of repeat revascularization and mortality at 48 months of follow-up were assessed in relation to HNP1–3 levels.

Results: The risk of CAD was independently associated with age and HNP1–3 in the entire cohort (F = 0.71 and F = 7.4, respectively). Additionally, HNP1–3 levels were significantly associated with myocardial necrosis (R = 0.26). At the follow-up, high HNP1–3 levels negatively affected mortality (19.54%) and recurrent revascularization (8.05%).

¹Department of Cardiology, Hillel Yaffe Medical Center, Hadera, Israel
²Department of Cardiology, Tel Aviv Medical Center Affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
³Department of Clinical Biochemistry, Hadassah University Hospital, Jerusalem, Israel
⁴Institute of Pathology, Hillel Yaffe Medical Center, Hadera, Israel

Corresponding author:
Rami Abu Fanne, Hillel Yaffe Medical Center, Rappaport Faculty of Medicine, Technion, POB 169, Hashalom Street, Hadera 38100, Israel.
Email: rabufanne@gmail.com
Conclusion: HNP1–3 tissue deposition is positively associated with the severity of CAD, myonecrosis, and long-term sequelae. HNP1–3 levels may be suppressed using colchicine.

Keywords
Inflammation, atherosclerosis, atherothrombosis, human neutrophil peptides 1–3, coronary artery disease, myocardial necrosis

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Introduction
Despite large advances in the treatment of atherosclerotic cardiovascular disease (ASCVD), it remains the leading cause of mortality and disability worldwide.1 CVD risk management revolves around the major risk factors, but a substantial residual unmet risk of cardiovascular events remains, despite optimal control of these factors.2 Compelling data have indicated the role of low-grade chronic nonresolving inflammation in accelerating ASCVD.3–5 A proinflammatory mediator that has received considerable attention in this regard is human neutrophilic peptides 1–3 (HNP1–3). HNP1–3 are the most abundant neutrophilic proteins, with roles in innate and acquired immunity.6 HNPs are hydrophobic and exert various inflammatory effects. Recent studies have shown a pivotal effect of HNP1–3 on the pathogenesis of atherosclerosis. Among others, HNP1–3 enhance platelet activation,7 and negatively affect endothelial function8,9 and tissue-type plasminogen activator-mediated fibrinolysis.10 Our research group was the first to prove an atherogenic causative role of HNP1–3 in a transgenic mice model. We also found that colchicine treatment negated the atherosclerotic phenotype by stabilizing neutrophils and reducing HNP1–3 secretion.11 Recently, we verified robust pro-thrombotic properties of HNP1–3. HNP1–3 accelerate clot formation and alter clot structure by generating compact clots resistant to complete fibrinolysis, dramatically amplifying in vivo thrombus formation.12 These findings suggest that HNP1–3 are an accessible and promising, modifiable risk factor for atherogenesis and atherothrombosis. The normal range of HNP1–3 found in plasma is in the nanomolar range, with a marked elevation during the acute inflammatory processes. HNP1–3 molecules are hydrophobic and are cleared rapidly (t1/2 of 9.7 minutes) from the circulation without being excreted in the urine or feces and are protease resistant.13 Accordingly, single plasma concentrations of HNP1–3 are unlikely to provide an accurate reflection of their cumulative release into the circulation and exposure of the vasculature over time. HNP1–3 accumulate in blood vessel walls,14,15 and in human skin where they are associated with the severity of coronary disease (CAD).16 However, there is shortage of prospective data addressing the interplay between HNP1–3 levels and ASCVD in a large-scale clinical study.

This study aimed to examine the potential association between HNP1–3 skin levels and coronary atherosclerosis, myocardial infarction (MI) as the admission diagnosis, and long-term outcomes in a cohort of consecutive patients assigned to have coronary angiography.
Materials and methods

Study population

The study was approved by the Tel Aviv Sourasky Medical Center Institutional Review Board (approval number: 07-461, date of approval: June 2009), and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient included in the study. We have de-identified all patients’ details. The reporting of this study conforms to the STROBE guidelines.17 We prospectively recruited 623 consecutive patients who were admitted to Tel Aviv Medical Center and underwent angiography between March 2008 and May 2010. Twenty-four patients were excluded because of inappropriate skin samples. The medical history and conventional CAD risk factors were collected prospectively.

Laboratory tests

The data in this study were collected from the Tel Aviv Prospective Angio Survey (TAPAS).18–20 The TAPAS is a prospective, single-center registry enrolling all patients undergoing cardiac catheterization at the Tel Aviv Medical Center since 2006. All participants signed a written informed consent for participation in the study. Arterial blood was obtained via the femoral sheath as part of the procedure. Fasting blood sugar, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), C-reactive protein (CRP), fibrinogen, homocysteine, and β macroglobulin concentrations, total leukocytes, and the neutrophil counts were measured.

Skin biopsy

A full-thickness skin specimen was attained using the punch biopsy technique. The biopsy site was the exact point assigned for femoral artery puncture. Only healthy appearing skin was taken for the biopsy. After local anesthesia was achieved using lidocaine, a 5 × 2-mm punch biopsy was performed, and the tissue was immersed in 10% formalin.

Immunohistochemistry

Skin biopsies were immunohistochemically analyzed for HNP1–3 content as previously reported.16 Briefly, following antigen unmasking, formaldehyde-fixed 10-μm skin sections were incubated with monoclonal antibody against HNP1–3 (2.5 μg/mL) and stained by the avidin-biotin complex procedure with diamino-benzidine as the substrate (Sigma-Aldrich, St Louis, MO, USA). The primary antibody was replaced by the same concentration of irrelevant immunoglobulin as a negative control. Staining for HNP1–3 was graded by two investigators who were both blinded to the angiographic findings. In case of inter-observer variability, the slides were assessed by a third pathologist, and two similar readings were listed as the designation. Staining for HNP1–3 was scored as negative (0), mild (1), moderate (2), or diffuse (3) (Figure 1).

CAD scoring

Coronary angiography was performed through the transfemoral route. The quantification of CAD severity was based on the findings from conventional angiographic analysis. The presence of a ≥70% narrowing was considered as clinically significant CAD. The severity of CAD was divided into four categories according to the number of diseased vessels (i.e., 0, 1, 2, or 3; groups 1–4). Left main disease ≥50% was considered equivalent to three-vessel CAD. Every patient was graded by an interventional cardiologist who performed the procedure and was unaware of the laboratory results and the nature of the study.
Clinical outcomes and data collection

Prospective data were entered into a database that contained demographic, clinical, angiographic, laboratory, and procedural information, such as the skin HNP1–3 score. Clinical outcomes that were assessed included all-cause mortality at 48 months of follow-up and the occurrence of MI or clinical/imaging ischemia requiring angiography. Long-term follow-up was available only for the first 390 patients who were recruited because of logistic issues. Notably, the demographic, clinical, and angiographic characteristics of this group were similar to the rest of the cohort.

Statistical analysis

Continuous variables are presented as the mean ± standard deviation. Spearman’s correlations were applied to all variables, such as the HNP1–3 score, extent of CAD, and other classical risk factors for CAD. Multinomial logistic regression models were fitted for the severity of HNP1–3 staining, with adjustment for the extent of CAD, age, sex, smoking status, hypertension, diabetes mellitus, hyperlipidemia, chronic renal failure, history of MI, history of coronary artery bypass graft surgery, stroke, and transient ischemic attack. SAS (version 9.2; SAS Institute Inc., Cary,
NC, USA) and Crunch (version 4.0; Crunch Software Corporation, Oakland, CA, USA) statistical software were used for data analysis, and the statistical significance was set at $p < 0.05$.

## Results

A total of 599 patients were enrolled in this study. The characteristics of the patients at baseline are shown in Table 1. The mean age of the patients was $66 \pm 10.6$ years, 23% were women, 42% had diabetes, and 53% had previous coronary revascularization procedures. Nearly half (40.3%) of the patients underwent percutaneous coronary intervention for their index acute coronary event, while 42.3% were catheterized for a positive screening test or planned staged intervention. Patients were divided into two groups of troponin-negative ($n = 502$) and troponin-positive ($n = 97$). Figure 2 shows the CAD score as a function of the HNP1–3 score. HNP1–3 were absent (score $= 0$) in the skin of 12.7% of patients, 46.1% of whom had clinically nonsignificant CAD. The occurrence of clinically nonsignificant CAD decreased as the HNP1–3 level increased.

There was a significant correlation between the severity of CAD and the intensity of skin HNP1–3 staining in the whole cohort ($R = 0.23$, $p < 0.0001$), in men ($R = 0.27$, $p = 0.0002$) and in women ($R = 0.20$, $p < 0.001$). Age was also significantly correlated with the severity of CAD in the whole cohort ($R = 0.24$, $p < 0.0001$), but not in women ($R = 0.1$, $p = 0.24$). There was no significant relationship between the severity of CAD and traditional risk variables such as HbA1c and CRP (Table 2).

We then performed multiple regression analysis to determine the association between HNP1–3 levels and coronary atherosclerosis. We found that skin HNP1–3 levels and age were independent predictors of CAD severity ($F = 7.4$, $p = 0.003$, $F = 7.1$, $p = 0.007$, respectively). Additionally, we assessed the relation between the HNP1–3 score and MI, and found that HNP1–3 deposition independently predicted the occurrence of troponin-positive presentation ($R = 0.26$, $p = 0.023$). At 48 months (Figure 3), after excluding patients with non-interventional repeat angiograms (24% had routine angiography performed before valvular surgery or diagnostic

| Table 1. Patients’ baseline characteristics. |
|--------------------------------------------|
| Age, years | $66 \pm 9.6$ |
| Sex (M/F) | 23/77 |
| Diagnosis, n (%) | |
| ST elevation MI | 4 (0.7) |
| Non-STEMI | 87 (14.5) |
| Unstable angina | 150 (25.1) |
| Angina pectoris | 20 (3.3) |
| Staged PCI | 38 (6.3) |
| Nonspecific chest pain | 32 (5.3) |
| Dyspnea | 16 (2.7) |
| Valvular disease | 16 (2.7) |
| Positive stress test | 46 (7.7) |
| Positive thallium test | 170 (28.3) |
| AF/CPR | 6 (1) |
| Risk factors, n (%) | |
| HTN | 449 (75) |
| Hyperlipidemia | 479 (80) |
| Diabetes | 252 (42) |
| Previous MI | 252 (42) |
| Previous PCI | 317 (53) |
| CABG | 144 (24) |
| Valvular disease | 66 (11) |
| PVD | 60 (10) |
| CRF | 90 (15) |
| Dialysis | 28 (14) |

Normally distributed data are presented as mean ± standard deviation.

MI, myocardial infarction; PCI, percutaneous coronary intervention; AF, atrial fibrillation; CPR, cardiopulmonary resuscitation; HTN, hypertension; CABG, coronary artery bypass grafting; PVD, peripheral vascular disease; CRF, chronic renal failure.
angiography showing non-significant coronary disease), the incidence of recurrent revascularization was significantly higher in the high HNP1–3 group (score: 2 or 3) than in the low HNP1–3 group (score: 0 or 1) (8.05% vs. 5.36%, \( p < 0.05 \)). MI was the diagnosis in 12.5% and 14% in the low and high HNP1–3 groups, respectively. The remaining patients had ischemia as shown by various tests (e.g., radionuclear imaging and stress test/stress echocardiography).

The median time to revascularization was similar in the low and high HNP1–3 groups (40 vs. 42.5 months). The mortality rate was negatively affected by a high HNP1–3 score (19.54%: median time to death was 12 months vs. 8%: median time to death was 33 months, \( p < 0.05 \)). Notably, the incidence of coronary artery bypass graft surgery was 31% in the high HNP1–3 group and 21.4% in the low HNP1–3 group (\( p < 0.05 \)), which indicated more diffuse disease in the high HNP1–3 group.

After applying multiple logistic regression analysis, age and HNP1–3 levels were identified as independent predictors of death (\( F = 7.4, \ p = 0.008; \ F = 7.2, \ p < 0.05 \), respectively).

**Discussion**

Atherosclerosis is a life-threatening disease affecting millions of individuals worldwide. Progress in defining the causative pathways involved in atherosclerosis has traditionally been hindered by this disease’s etiological complexity. Meticulous classification of the individual inflammatory burden could be beneficial in generating a patient-tailored therapeutic approach. Insights from basic research have shown that HNP1–3 levels are a risk factor for atherosclerosis and thrombosis, and are considerably suppressed by colchicine use. HNP1–3 are a marker of active neutrophils and can serve as an innovative surrogate biomarker.
to further refine the classification of atherogenesis. This study examined whether the cumulative inflammatory burden, reflected by skin HNP1–3 levels, is associated with an enhanced ASCVD phenotype. We found a significant correlation between skin HNP1–3 staining and the CAD severity score in a large cohort of consecutive patients referred for coronary angiography. HNP1–3 exert major cardiovascular effects, induce monocyte adhesion and transmigration, accelerate foam cell formation, and amplify the activation and aggregation of human platelets.21 In vitro studies have shown that HNP1–3 are related to the inhibition of fibrinolysis, endothelial dysfunction, lipid metabolism, and platelet activation. Abu-Fanne et al11 were the first to propose HNP1–3 as a risk factor for atherogenesis using a transgenic mouse model for HNP1–3. In their recent publication,12 they further showed a prothrombotic in vivo effect of HNP1–3 using a mouse inferior vena cava model for deep vein thrombosis. HNP1–3 accelerated clot formation and altered clot structure by generating compact clots resistant to complete fibrinolysis.

In a human cohort of patients with stable angina, plasma HNP1–3 levels were significantly associated with advanced CAD.22 Paulin et al challenged the proatherosclerotic properties of HNP1–3.23 They claimed a potent atheroprotective effect of HNP ascribed to a reduction in plasma LDL-cholesterol levels by facilitating the clearance of LDL particles in the liver via the LDL receptor. In a follow-up study using ApoE<sup>−/−</sup> mice from our laboratory,24 we showed a similar cholesterol-lowering effect of HNP and cholestyramine in mice, which led to a lower aortic lesion size than in ApoE<sup>−/−</sup> mice. However, the lesion size was larger in mice administered HNP than those administered cholestyramine. Similar conclusions were made by Paulin et al23 who studied ApoE<sup>−/−</sup> mice fed a high fat diet. These mice were exposed to exogenous HNP1–3 with cholesterol levels of 25.86 to 38.8 mmol/L, and they developed larger lesions than untreated mice with comparable serum levels.
Overall, HNP1–3 lowers plasma LDL levels and enhances lipid deposition in the vasculature. Therefore, although colchicine, which reduces HNP1–3 release from neutrophils,\textsuperscript{11} increases plasma oxidized LDL,\textsuperscript{25} it eventually reduces the incidence of cardiovascular events.\textsuperscript{26} The recently published COLCOT study\textsuperscript{27} reinforced the protective effect of long-term (median: 22.6 months), low-dose colchicine therapy. The COLCOT study enrolled patients who developed recent MI, and showed that low-dose colchicine was effective at preventing major adverse cardiovascular events compared with placebo, primarily by attenuating the incidence of stroke (number needed to treat: 171, fragility index: 3) and urgent hospitalization for unstable angina requiring revascularization (number needed to treat: 96, fragility index: 7). The protective cardiovascular effect of colchicine was attributed to general anti-inflammatory properties of this agent. Maneerat et al\textsuperscript{28} proposed increased HNP1–3 expression as a potential inflammatory marker for predicting the risk of developing ASCVD in Thai patients with hyperlipidemia. On the basis of basic research data, the LoDoCo\textsuperscript{27} and COLCOT\textsuperscript{26} clinical studies, and the current study, we believe that the positive cardio-protective effect of colchicine is at least partially achieved by stabilizing neutrophils, inhibiting neutrophil degranulation, and reducing circulatory HNP1–3 levels.

**Study limitations**

This study has some limitations. First, this was an observational study. Therefore, we are currently unable to generate causal conclusions. Second, we had a rate of 39% for the loss of follow-up, which may have introduced bias. However, the baseline characteristics of the remaining patients were similar to those who were lost to follow-up. Third, most (73%) of the study population were men, which limited the study generalizability to both sexes. Nonetheless, because our study included all patients admitted for coronary angiography, this allows overall generalizability of our results. Finally, we failed to establish a robust correlation between the severity of CAD and HNP scores of 1 and 2. However, overall, there was still a positive correlation between the severity of CAD and HNP1–3 levels.

**Conclusion**

Clinical pharmacotherapy is trending towards customized medicine. The goal is to enhance the chances of successful outcomes, while limiting risks posed by unnecessary or ineffectual therapies. The concept of inflammatory endotyping of atherosclerosis has gained considerable attention. Accordingly, stratifying patients with ASCVD by means of plasma and/or skin HNP1–3 levels might potentially improve stratification and intervention practices. Theoretically, HNP1–3-based endotyping could enable personalized colchicine prescription to achieve a lower number needed to treat in patients and more effective pharmaco-intervention.

**Author contributions**

Rami Abu Fanne – Conceptualization, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – Review & Editing, Visualization, Supervision, Project administration. Yaron Arbel – Software, Validation, Formal analysis, Data curation, Writing – Review & Editing. Ehud Chorin – Formal analysis, Investigation, Data curation. Emad Maraga – Formal analysis, Investigation, Data curation. Abd Alroof Higazi – Conceptualization, Validation, Formal analysis, Resources, Data curation, Writing – Review & Editing, Funding acquisition. Shmuel Banai – Conceptualization, Validation, Resources, Data curation, Writing – Review & Editing, Supervision, Funding acquisition. Groisman
Declaration of conflicting interests
The authors declare that there is no conflict of interest.

Data availability statement
All data generated or analyzed during this study are included in this published article.

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ORCID iD
Rami Abu Fanne https://orcid.org/0000-0001-9350-6614

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