EN-RAGE
A Novel Inflammatory Marker for Incident Coronary Heart Disease

Symen Ligthart, Sanaz Sedaghat, M. Arfan Ikram, Albert Hofman, Oscar H. Franco, Abbas Dehghann

Objective—Inflammation plays a key role in atherosclerosis. We hypothesized that novel inflammatory markers may predict the risk of coronary heart disease (CHD).

Approach and Results—We investigated the association of 16 inflammatory biomarkers with the risk of CHD in a random subset of 839 CHD-free individuals in a prospective population-based cohort study. A Bonferroni corrected P value of 3.1×10⁻³ was used as a threshold of statistical significance. The mean age at baseline was 72.8 years. During a median follow-up of 10.6 years, 99 cases of incident CHD were observed. Among all inflammatory biomarkers, neutrophil-derived human s100a12 (extracellular newly identified receptor for advanced glycation end-products binding protein [EN-RAGE]) showed the strongest association with the risk of CHD (P value 2.0×10⁻³). After multivariable adjustment for established cardiovascular risk factors, each standard deviation increase in the natural log-transformed EN-RAGE was associated with 30% higher risk of incident CHD (hazard ratio, 1.30; 95% confidence interval, 1.06–1.59). Further adjustment for previously studied inflammatory markers did not attenuate the association. Excluding individuals with prevalent type 2 diabetes mellitus, impaired kidney function, or individuals using antihypertensive medication did not change the effect estimates. Cause-specific hazard ratios suggested a stronger association between EN-RAGE and CHD mortality compared with stable CHD.

Conclusions—Our results highlight EN-RAGE as an inflammatory marker for future CHD in a general population, beyond traditional CHD risk factors and inflammatory markers. (Arterioscler Thromb Vasc Biol. 2014;34:2695-2699.)

Key Words: biomarker ■ coronary artery disease ■ cytokines ■ inflammation

With 7.3 million deaths per year globally, coronary heart disease (CHD) is still the world’s leading cause of mortality.1 Inflammation is thought to play a key role in the pathogenesis of atherosclerosis and CHD.2 Accordingly, inflammatory markers have been investigated for predicting the risk of CHD, an effort that has led to the identification and validation of several inflammatory markers for CHD.3-6 However, the inflammatory markers that have been investigated to date only represent a minor part of the diverse molecules that constitute the complex human immune response.7 Exploring prospectively the association of relatively uninvestigated inflammatory markers with CHD may unravel novel inflammatory risk factors for CHD and may shed light on additional pathways that might be involved in the pathogenesis of atherosclerosis and CHD.

We hypothesized that indicators of inflammation which have not been studied previously with the incidence of CHD are associated with incident CHD beyond traditional risk factors and previously studied inflammatory markers. To this end, we studied the association of 16 biomarkers of inflammation with the risk of CHD in the Rotterdam Study, a prospective population-based cohort study.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Table 1 summarizes the baseline characteristics of 839 participants (see Table II in the online-only Data Supplement for baseline characteristics in future CHD cases and noncases). At the start of the study, the mean (±SD) age was 72.8 (7.5), and 58% of the population were female. During a median follow-up of 10.6 years (interquartile range, 6.8–11.9), 2 were lost to follow-up, 353 individuals died (302 unrelated to CHD), and 99 developed CHD (incidence rate, 12.7 per 1000 person years). Out of the 16 inflammatory biomarkers (Figure 1), after Bonferroni correction, only extracellular newly identified receptor for advanced glycation end-products binding protein (EN-RAGE) was significantly associated with CHD when adjusted for age and sex (Table III in the online-only Data Supplement). The risk of CHD was nearly one third increased per standard deviation increase in the natural log-transformed EN-RAGE (hazard ratio [HR], 1.37; 95% confidence interval [CI], 1.12–1.67; Table 2). Compared
with the lowest tertile, participants in the highest tertile experienced approximately a 2.6-fold higher risk of developing CHD compared with participants in the lowest tertile (HR, 2.59; 95% CI, 1.52–4.40). When we further adjusted the association for traditional cardiovascular risk factors, the effect estimates attenuated slightly (HR, 1.30; 95% CI, 1.06–1.59). Additional adjustment for previously studied inflammatory markers yielded slightly increased effect estimates (Table 2; Table IV in the online-only Data Supplement).

Cumulative incidence curves for the tertiles of EN-RAGE adjusted for competing risks are depicted in Figure 2. The 10-year probability of first incident event of CHD was 0.05 (95% CI, 0.03–0.08) for the first tertile, 0.11 (95% CI, 0.07–0.14) for the second tertile, and 0.14 (95% CI, 0.10–0.18) for the third tertile.

After excluding participants with chronic kidney disease at baseline, the association between EN-RAGE and incident CHD attenuated slightly (1.28; 95% CI, 1.03–1.59; Table 3). Excluding participants with type 2 diabetes mellitus, the effect estimates of the association between EN-RAGE and CHD did not change: hazard ratio 1.29 (95% CI, 1.04–1.60) in the fully adjusted model. Finally, after excluding participants taking antihypertensive medication, the hazard ratio did not change (HR, 1.40; 95% CI, 1.05–1.87).

Table 4 depicts the results for the associations between EN-RAGE and the different CHD manifestations separately. We observed the strongest association with CHD mortality (HR, 1.56; 95% CI, 1.19–2.04) compared with myocardial infarction and revascularization, which were not significant. Cause-specific HRs were not significantly lower for revascularization compared with myocardial infarction and future major adverse cardiac events. To our knowledge, we are the first to investigate the association between EN-RAGE and CHD in a prospective population-based cohort study with long-term follow-up.

To address the possibility of confounding, we adjusted in the multivariable model for the different traditional CHD risk factors and previously studied inflammatory markers. To address the question whether our results were driven by a certain subgroup, we analyzed the data excluding participants with chronic kidney disease, type 2 diabetes mellitus, and antihypertensive use in the sensitivity analyses. Across all these analyses, there was a consistent effect of EN-RAGE on the risk of CHD, even after adjusting for the established inflammatory markers. These results suggest an effect of EN-RAGE on the risk of CHD beyond well-established metabolic and inflammatory pathways.

We observed a stronger association between EN-RAGE and future myocardial infarction and CHD mortality compared with revascularization. This suggests that EN-RAGE is a more determinant of acute coronary events with plaque instability rather than stable coronary artery disease. This observation that EN-RAGE, a member of the S100 protein family, is a strong determinant of acute coronary events is in line with previous studies that reported higher levels of mRNA and plasma levels of family $100$ proteins ($S100A8/9$) in patients with ST-elevated myocardial infarction compared with stable coronary artery disease cases. Furthermore, a postmortem study in people died from sudden cardiac death has found high expression levels of $S100A12$ in coronary artery smooth muscle in the ruptured plaques, especially in diabetics. However, the cause-specific hazard ratio for the CHD events were not significantly different using the method proposed by Lunn and McNeil. We might have been underpowered to observe a significant difference because of the limited number of cases in this cause-specific analyses.

Studying the added value of EN-RAGE in 10-year CHD risk prediction, we found an improvement in risk prediction when we added EN-RAGE to the Framingham risk score. This suggests that EN-RAGE, as a noninvasive marker of future CHD, could be useful in predicting the risk of CHD in the general population. Although we corrected the change in c-statistic for optimism, we think that further studies are needed to establish the potential role of EN-RAGE in CHD risk prediction.

EN-RAGE, a member of the $S100$ protein family of EF-hand calcium-binding proteins, is an endogenously produced inflammatory ligand of the RAGE and Toll-like receptor 4.
RAGE is a member of the immunoglobulin superfamily of cell surface molecules and is expressed in multiple tissues, including endothelium cells, vascular smooth muscle cells, and monocyte-derived macrophages.23 The binding of RAGE by EN-RAGE activates inflammatory cascades, including the proinflammatory NF-κB signaling pathway, a well-known pathway of the innate immune system involved in the pathogenesis of CHD.21,24 Moreover, intracellular signaling pathways triggered by EN-RAGE may alter gene expression and upregulate the synthesis of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 synthesis.25 Considering atherosclerosis as a chronic inflammatory disease, the engagement of RAGE by EN-RAGE may play an important role in the pathogenesis of atherosclerosis and subsequently CHD. In line with this evidence, the expression of EN-RAGE in vascular smooth muscle cells can modulate the remodeling of the aortic wall and stimulates cytokine production and increases oxidative stress.23 Moreover, EN-RAGE accelerates atherosclerosis and vascular calcification in apolipoprotein E-null mice.26 Recently, EN-RAGE has been shown to accelerate the development of cardiac hypertrophy and diastolic dysfunction in mice with chronic kidney disease.27 The monocyte activation effect of EN-RAGE has also been observed to be Toll-like receptor 4–dependent.22 It was demonstrated that EN-RAGE facilitates inflammatory monocyte activation by Toll-like receptor 4 and that this effect was modulated by RAGE. Toll-like receptors have been investigated extensively in the field of cardiovascular diseases because they are expressed in vascular and myocardial cell membranes.28 The important role of EN-RAGE in the pathogenesis of atherosclerosis is further emphasized by a recent study where pharmacological inhibition of S100A12-mediated atherosclerosis improved atherosclerotic plaque features, including smaller necrotic cores, diminished calcification, and reduced number of inflammatory cells.29 This study has certain strengths and limitations. The prospective population-based study design, the diversity of the available inflammatory biomarkers, and the long-term follow-up of CHD can be marked as the main strengths of the present study. In addition, our findings are robust regarding the strict Bonferroni P value we used as the threshold for significant differences with chronic kidney disease.27

### Table 1. Baseline Characteristics of Participants at Risk for CHD

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n=839)</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>72.8±7.5</td>
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<tr>
<td>Men, n (%)</td>
<td>355 (42)</td>
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<tr>
<td>Body mass index, kg/m²</td>
<td>27±4</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td>144±21</td>
<td></td>
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<td></td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>75±11</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Antihypertensive medication use, n (%)</td>
<td>319 (38)</td>
<td></td>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.8±1.0</td>
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<tr>
<td>High-density lipoprotein cholesterol, mmol/L</td>
<td>1.4±0.4</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.5±0.7</td>
<td></td>
<td></td>
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<tr>
<td>Current smokers, n (%)</td>
<td>144 (17.2)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Prevalent type 2 diabetes mellitus, n (%)</td>
<td>107 (12.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated glomerular filtration rate, mL/min/1.73 m²</td>
<td>74±15</td>
<td></td>
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</tr>
<tr>
<td>CD40ligand,* ng/mL</td>
<td>0.028 (0.020–0.039)</td>
<td>0.84±0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement 3, mg/mL</td>
<td>1.43 (0.69–3.28)</td>
<td>9.21 (7.20–12.40)</td>
<td>190 (149–248)</td>
<td>183 (151–225)</td>
</tr>
<tr>
<td>Interleukin 8,* pg/mL</td>
<td>0.056 (0.037–0.082)</td>
<td>0.50 (0.32–0.80)</td>
<td>0.42 (0.31–0.49)</td>
<td>3.54 (2.93–4.34)</td>
</tr>
<tr>
<td>Interleukin 18,* pg/mL</td>
<td>133 (125–225)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor,* ng/mL</td>
<td>0.50 (0.32–0.80)</td>
<td>0.42 (0.31–0.49)</td>
<td>3.54 (2.93–4.34)</td>
<td>6.4±1.7</td>
</tr>
<tr>
<td>Regulated on activation, normal T cell expressed and secreted,* ng/mL</td>
<td>0.50 (0.32–0.80)</td>
<td>0.42 (0.31–0.49)</td>
<td>3.54 (2.93–4.34)</td>
<td>6.4±1.7</td>
</tr>
<tr>
<td>Resistin,* ng/mL</td>
<td>0.42 (0.31–0.49)</td>
<td>3.54 (2.93–4.34)</td>
<td>6.4±1.7</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor receptor 2,* ng/mL</td>
<td>0.50 (0.32–0.80)</td>
<td>0.42 (0.31–0.49)</td>
<td>3.54 (2.93–4.34)</td>
<td></td>
</tr>
<tr>
<td>White blood cell count, ×10⁹/L</td>
<td>6.4±1.7</td>
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</tbody>
</table>

**Plus–minus values are means±SD.**

CHD indicates coronary heart disease.

*Values are presented as median (interquartile range).

### Table 2. The Association Between EN-RAGE Serum Levels and Incident CHD

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EN-RAGE n/N</td>
<td>Model 1</td>
</tr>
<tr>
<td>First</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>Second</td>
<td>1.92 (1.11–3.33)</td>
</tr>
<tr>
<td>Third</td>
<td>2.59 (1.52–4.40)</td>
</tr>
</tbody>
</table>

**P for trend**

<0.001 0.006 0.006

Per SD Ln (EN-RAGE)

99/837 1.37 (1.12–1.67) 1.30 (1.06–1.59) 1.46 (1.11–1.90)

Model 1, adjusted for age and sex; Model 2, adjusted for age, sex, BMI, systolic blood pressure, antihypertensive medication use, HDL cholesterol, total cholesterol, smoking status (current, noncurrent), prevalent type 2 diabetes mellitus, and eGFR; Model 3, additionally adjusted for CD40ligand, Complement 3, C-reactive protein, interleukin 8, interleukin 18, monocyte chemotactic protein 1, macrophage migration inhibitory factor, RANTES, Resistin, TNF receptor 2, and white blood cells.

BMI indicates body mass index; CHD, coronary heart disease; CI, confidence interval; eGFR, estimated glomerular filtration rate; EN-RAGE, extracellular new identified receptor for advanced glycation end-products binding protein; HDL, high-density lipoprotein; HR, hazard ratio; SD, standard deviation; and TNF, tumor necrosis factor.

*Hazard ratios are represented per standard deviation increase in log-transformed EN-RAGE.

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**Figure 1. Flow chart of inflammatory biomarker inclusion.**
transformed EN-RAGE.

T2D, type 2 diabetes mellitus.

products binding protein; HDL, high-density lipoprotein; HR, hazard ratio.

interval; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; EN-RAGE, extracellular newly identified receptor for advanced glycation end-products binding protein.

associations in the first step. Several limitations should also be acknowledged. First, although we adjusted our analysis for different potential confounders, we cannot exclude the effect of unknown or unmeasured confounders. However, because we adjusted for the traditional and commonly used risk factors for CHD and inflammatory pathways, we think that EN-RAGE as a novel inflammatory marker for CHD is interesting because it might reflect other pathways that lead to CHD. Second, we had to exclude inflammatory biomarkers with low serum concentrations. Nonetheless, the selected biomarkers have >60% completeness of measurements, indicating acceptable quality of quantification. Third, our population is ≥55 years. Therefore, generalization of the results to a younger age category should be with caution. Our study only indicates an association; we think that further studies are needed to establish the causal role of EN-RAGE in the pathogenesis of CHD.

Table 3. The Association of EN-RAGE With CHD in Absence of Patients With CKD, T2D, or Antihypertensive Use

<table>
<thead>
<tr>
<th>n/N</th>
<th>HR (95% CI)* Model 1</th>
<th>HR (95% CI)* Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR&lt;60 excluded</td>
<td>1.34 (1.09–1.65)</td>
<td>1.28 (1.03–1.59)</td>
</tr>
<tr>
<td>Prevalent diabetes mellitus excluded</td>
<td>1.34 (1.09–1.64)</td>
<td>1.29 (1.04–1.60)</td>
</tr>
<tr>
<td>Anti-hypertensive use excluded</td>
<td>1.45 (1.10–1.92)</td>
<td>1.40 (1.05–1.87)</td>
</tr>
</tbody>
</table>

Model 1, adjusted for age and sex; Model 2, adjusted for age, sex, BMI, systolic blood pressure, antihypertensive medication use, HDL cholesterol, total cholesterol, smoking status (current and noncurrent), prevalent type 2 diabetes mellitus, and eGFR.

BMI indicates body mass index; CHD, coronary heart disease; CI, confidence interval; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; EN-RAGE, extracellular newly identified receptor for advanced glycation end-products binding protein; HDL, high-density lipoprotein; HR, hazard ratio; and T2D, type 2 diabetes mellitus.

*Hazard ratios are represented per standard deviation increase in log-transformed EN-RAGE.

In conclusion, our study suggests that higher levels of serum EN-RAGE are associated with incidence of CHD beyond conventional cardiovascular risk factors and inflammatory markers. These results provide evidence for a role of EN-RAGE in the development of CHD and suggest this marker as a potential target for drug therapy and risk prediction.

Acknowledgments

We are grateful to the study participants, the staff from the Rotterdam Study, and the participating general practitioners and pharmacists.

Sources of Funding

Abbas Dehghan is supported by Netherlands Organisation for Scientific Research (NWO) grant (veni, 916.12.154) and the EUR Fellowship. The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. O.H. Franco works in ErasmusAGE, a center for aging research across the life course funded by Nestlé Nutrition (Nestec Ltd.); Metagenics Inc.; and AXA, Nestlé Nutrition (Nestec Ltd.); Metagenics Inc.; and AXA had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the article.

Disclosures

None.

References

Coronary heart disease is the most common cause of mortality worldwide. Inflammation plays an important role in atherosclerosis and the development of coronary heart disease. We aimed to identify new inflammatory markers associated with the risk of future coronary heart disease events. We propose extracellular newly identified receptor for advanced glycation end-products binding protein (EN-RAGE) as a new inflammatory marker for incident coronary heart disease in the general population. These results highlight the role of EN-RAGE in the development of coronary heart disease and suggest EN-RAGE as a potential target for drug therapy and risk prediction.
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Arterioscler Thromb Vasc Biol. 2014;34:2695-2699; originally published online October 23, 2014;
doi: 10.1161/ATVBAHA.114.304306
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/12/2695

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Materials and methods

Study Population

The Rotterdam Study is a prospective population-based cohort study in Ommoord, a district of Rotterdam, the Netherlands. The design of the Rotterdam Study has been described in more detail elsewhere. Briefly, in 1989 all residents of Ommoord aged 55 years or older were invited to participate of whom 78% (7,983 out of 10,275) agreed. The first examination round was completed between 1990 and 1993, after which follow-up examinations were conducted in 1993-1994, 1997-1999, 2002-2004 and 2009-2011. This study was based on data collected during the third visit (1997-1999). Among 5990 (80%) eligible individuals, 4797 individuals visited the research center. A random subset of 971 participants was selected as part of a separate case-cohort study to investigate biomarkers in association with dementia. Given the random sampling these persons can be considered representative of the source population. We excluded 132 participants with history of CHD (defined as clinically manifest myocardial infarction, coronary artery bypass grafting, or percutaneous trans luminal coronary angioplasty), resulting in 839 participants for analysis. The Rotterdam Study has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. A written informed consent was obtained from all participants.

Measurement of Inflammatory Biomarkers

In the third center visit, fasting blood samples were collected at the research center. Plasma was isolated and immediately put on ice and stored at -80°C. Citrate plasma (200 µL) was sent in July 2008 to Rules-Based Medicine, Austin, Texas (www.myriadrbm.com). Fifty inflammatory biomarkers were quantified using multiplex immunoassay on a custom designed human multianalyte profile. The intra-assay variability was less than 4% and the inter-assay variability was less than 13%. Biomarkers with more than 60% completeness of measurements were selected for imputation and further analysis (Figure 1). Among the 26 eligible biomarkers, 10 were excluded since they have previously been investigated prospectively with the incidence of CHD (Supplementary Table 1). This resulted in a final set of 16 novel inflammatory biomarkers that were selected to investigate with incidence of CHD (Supplementary Table 2). The inflammatory markers investigated in the current study have no standard international calibration reference, therefore interpretation of the absolute values should be with caution. Since the current study is conducted within one set of individuals, the use of relative measures does not affect the effect estimates.

Coronary Heart Disease Diagnosis

Information on the incidence of CHD was obtained from general practitioners and from letters and discharge reports of medical specialists. Two independent study physicians coded all reported events and in case of disagreement, consensus was sought. Subsequently, a medical specialist validated all events. Incident CHD was defined as myocardial revascularization, fatal and non-fatal myocardial infarction and CHD mortality. Definition and coding of CHD events within the Rotterdam Study is described in more detail elsewhere. Follow-up data until January 1st 2011 was used.
**Covariates**

Anthropometric measures were obtained during the visit to the research center. Body mass index (BMI) was defined as weight in kilogram divided by the square of height in meters. Blood pressure was measured during research center visit at the right brachial artery, with participants in sitting position. The mean of two consecutive measurements was used. Total and high-density lipoprotein cholesterol (HDL-cholesterol) levels, creatinine and white blood cell counts were measured in fasting blood samples with standard laboratory techniques. The glomerular filtration rate (GFR) was estimated by the abbreviated modification of diet in renal disease (MDRD) equation which is recommended by the National Kidney Foundation. Chronic kidney disease was defined as an eGFR < 60 ml/min/1.73m². Prevalent diabetes mellitus was defined as a fasting plasma glucose level ≥ 7.0 mmol/L or use of anti-diabetic medication. Information on medication use, medical history and smoking behavior was collected via computerized questionnaires during home visits. Smoking was classified as current versus non-current smokers. The previously studied inflammatory markers were measured using the same multiplex immunoassay that was also used for the novel inflammatory biomarkers.

**Statistical Analyses**

In the first step, we used Cox proportional hazard models to investigate the age and sex adjusted association between each inflammatory biomarker and the incidence of CHD. All models met the proportional hazards assumption. Markers with a right-skewed distribution were transformed to the natural logarithmic scale (Supplementary Table 2). For a better comparison between the biomarkers, all markers were standardized by dividing the measured value by the standard deviation. We defined biomarker values as an outlier when the value was > 4 standard deviations higher or lower than the mean. Participants were excluded from the analysis when the biomarker value for this person was an outlier. The maximum number of excluded individuals was 3 among all biomarkers. We selected the significant biomarkers from the first step to further assess their association with CHD in multivariable analyses. In this second step, we additionally adjusted the association for BMI, serum total cholesterol, HDL-cholesterol, systolic blood pressure, use of anti-hypertensive medication (defined as diuretics, anti-adrenergic agents, β blockers, calcium channel blockers and RAAS inhibitor), eGFR, prevalent type 2 diabetes and smoking. The hazard ratios were also calculated for the two upper tertiles with the first tertile as reference. In the third model, we additionally adjusted for the inflammatory markers that have previously been studied. In a sensitivity analysis, we excluded individuals with prevalent type 2 diabetes, chronic kidney disease and individuals using anti-hypertensive medication. Participants were censored at the time of occurrence of CHD, death, loss to follow-up or the end of the study period on January 1, 2011. We estimated 10-year risks for first-incident CHD for different tertiles of the identified biomarker(s). The cumulative incidence curves were created taking into account competing events.

In addition, we analyzed EN-RAGE with the different CHD outcomes separately (myocardial infarction, coronary revascularization and CHD mortality). To compare directly the effect estimates on these specific first CHD events using Cox regression, we
applied the data augmentation method proposed by Lunn and McNeil \(^7\). This method estimates the difference in cause-specific hazard ratios of EN-RAGE on the specific CHD events when competing CHD events and non-CHD events are present \(^5\). We presented the results for the model in which we adjusted for the traditional CHD risk factors.

The measures of association are presented with 95% confidence intervals (CI). We hypothesized that inflammatory markers may predict the incidence of CHD. To this end, we tested the association between 16 markers of inflammation with the incidence of CHD. To avoid false positive findings, we applied a Bonferroni corrected p-value of \(3.1 \times 10^{-3}\) (0.05/16) as a robust threshold of significance. All other statistical tests were considered significant with a p-value < 0.05.

We compared the 10-year CHD risk prediction of the traditional Framingham risk score model to the new model that additionally included EN-RAGE using the c-statistic difference, continuous net reclassification improvement (NRI) and integrated discrimination improvement (IDI) \(^8-10\). The difference in c-statistic between the base model and the model with EN-RAGE was corrected for optimism using 100 bootstraps.

Approximately 5% of the participants lacked data on one or more of the cardiovascular covariates, except for the covariate “use of antihypertensive medication”, where 9% of the values were missing. Missing data for these covariates was imputed by multiple imputation where 5 datasets were pooled to obtain the risk estimates for the association between EN-RAGE and incident CHD \(^11,12\). Biomarkers with missing data due to values under the lower detection limit were imputed with the lower detection limit. Data were handled and analyzed using the IBM SPSS Statistics version 21.0.0.1 (IBM Corp., Somers, NY, USA) and R version 3.0.0 \(^13\).

References:


Supplement Material

Supplementary Table I. List of previously investigated inflammatory markers with incident Coronary Heart Disease.

<table>
<thead>
<tr>
<th>Biomarker</th>
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<tbody>
<tr>
<td>- C-reactive protein[^1]</td>
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<tr>
<td>- CD40 Ligand[^2,3]</td>
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<tr>
<td>- Complement factor 3[^4]</td>
</tr>
<tr>
<td>- Interleukin 18[^5]</td>
</tr>
<tr>
<td>- Monocyte chemotactic protein-1[^6]</td>
</tr>
<tr>
<td>- Regulated on activation, normal T cell expressed and secreted[^7]</td>
</tr>
<tr>
<td>- Resistin[^8]</td>
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<tr>
<td>- TNF receptor II[^9]</td>
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<tr>
<td>- Interleukin 8[^10]</td>
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**Supplementary Table II.** Differences in baseline characteristics between future CHD cases and CHD non-cases.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CHD cases (n=99)</th>
<th>CHD non-cases (740)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>74.0±7.9</td>
<td>72.6±7.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Men, n (%)</td>
<td>48(49)</td>
<td>307(42)</td>
<td>0.19</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27±4</td>
<td>27±4</td>
<td>0.06</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>148±20</td>
<td>144±22</td>
<td>0.06</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>75±12</td>
<td>75±11</td>
<td>0.66</td>
</tr>
<tr>
<td>Antihypertensive medication use, n (%)</td>
<td>56 (57)</td>
<td>260 (35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.9±1.2</td>
<td>5.8±0.9</td>
<td>0.48</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.3±0.3</td>
<td>1.4±0.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.6±0.7</td>
<td>1.5±0.8</td>
<td>0.18</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>20 (20.2)</td>
<td>124 (16.8)</td>
<td>0.35</td>
</tr>
<tr>
<td>Prevalent type 2 diabetes, n (%)</td>
<td>13 (13.1)</td>
<td>94 (12.7)</td>
<td>0.90</td>
</tr>
<tr>
<td>eGFR, ml/min/1.73m²</td>
<td>74±14</td>
<td>74±15</td>
<td>0.63</td>
</tr>
<tr>
<td>CD40ligand*, ng/mL</td>
<td>0.028 (0.020 – 0.038)</td>
<td>0.028(020 – 0.038)</td>
<td>0.87</td>
</tr>
<tr>
<td>Complement 3, mg/mL</td>
<td>0.85±0.14</td>
<td>0.84±0.14</td>
<td>0.38</td>
</tr>
<tr>
<td>C-reactive protein*, mg/L</td>
<td>1.64 (0.78 – 3.73)</td>
<td>1.42 (0.68 – 3.13)</td>
<td>0.36</td>
</tr>
<tr>
<td>Interleukin 8*, pg/mL</td>
<td>10.40 (7.36 – 13.40)</td>
<td>9.15 (7.01 – 12.40)</td>
<td>0.06</td>
</tr>
<tr>
<td>Interleukin 18*, pg/mL</td>
<td>206 (150 – 270)</td>
<td>188 (149 – 245)</td>
<td>0.12</td>
</tr>
<tr>
<td>Monocyte chemotactic protein 1*, pg/mL</td>
<td>189 (151 – 236)</td>
<td>183 (151 – 225)</td>
<td>0.50</td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor*, ng/mL</td>
<td>0.059 (0.032 – 0.091)</td>
<td>0.055 (0.037 – 0.082)</td>
<td>0.79</td>
</tr>
<tr>
<td>RANTES*, ng/mL</td>
<td>0.46 (0.33 – 0.75)</td>
<td>0.51 (0.32 – 0.81)</td>
<td>0.51</td>
</tr>
<tr>
<td>Resistin*, ng/mL</td>
<td>0.40 (0.27 – 0.64)</td>
<td>0.43 (0.31 – 0.59)</td>
<td>0.68</td>
</tr>
<tr>
<td>TNFR-II*, ng/mL</td>
<td>3.64 (2.97 – 4.45)</td>
<td>3.52 (2.91 – 4.33)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Plus-minus values are means ±SD

*Values are presented as median (inter-quartile range)

CD40ligand, cluster of differentiation 40 ligand; eGFR, estimated glomerular filtration rate; HDL-cholesterol, high-density lipoprotein cholesterol; RANTES, regulated on activation, normal T cell expressed and secreted; TNFR-II, Tumor necrosis factor receptor 2.
Supplementary Table III. Biomarkers and their age and sex adjusted association results with incident Coronary Heart Disease.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Median (SD/IQR)</th>
<th>N</th>
<th>Beta (95% CI)</th>
<th>P-value</th>
<th>nr. under LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40, ng/mL</td>
<td>0.70 (0.58 - 0.83)</td>
<td>836</td>
<td>1.11 (0.89 - 1.39)</td>
<td>0.38</td>
<td>0</td>
</tr>
<tr>
<td>CFH, μg/mL</td>
<td>2455.6 (838.9)</td>
<td>839</td>
<td>1.00 (1.00 - 1.00)</td>
<td>0.27</td>
<td>90&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>EN-RAGE, ng/mL</td>
<td>10.80 (7.66 - 14.70)</td>
<td>837</td>
<td>1.37 (1.12 - 1.67)</td>
<td>0.002</td>
<td>0</td>
</tr>
<tr>
<td>Eotaxin, pg/mL</td>
<td>161 (116 - 217)</td>
<td>838</td>
<td>1.11 (0.90 - 1.37)</td>
<td>0.34</td>
<td>3</td>
</tr>
<tr>
<td>FASLR, ng/mL</td>
<td>4.69 (3.97 - 5.54)</td>
<td>832</td>
<td>1.06 (0.85 - 1.31)</td>
<td>0.62</td>
<td>0</td>
</tr>
<tr>
<td>HCC4, ng/mL</td>
<td>4.90 (1.96)</td>
<td>838</td>
<td>1.17 (0.97 - 1.41)</td>
<td>0.11</td>
<td>0</td>
</tr>
<tr>
<td>IL13, pg/mL</td>
<td>4.32 (4.09 – 4.52)</td>
<td>838</td>
<td>0.97 (0.79 – 1.19)</td>
<td>0.76</td>
<td>30</td>
</tr>
<tr>
<td>IL16, pg/mL</td>
<td>5.94 (5.74 - 6.08)</td>
<td>837</td>
<td>1.03 (0.83 – 1.27)</td>
<td>0.79</td>
<td>0</td>
</tr>
<tr>
<td>IL17, pg/mL</td>
<td>13.67 (5.20)</td>
<td>838</td>
<td>0.88 (0.72 – 1.08)</td>
<td>0.23</td>
<td>47</td>
</tr>
<tr>
<td>IL1ra, pg/mL</td>
<td>68.5 (47.75 - 102.00)</td>
<td>838</td>
<td>1.19 (0.97 - 1.46)</td>
<td>0.10</td>
<td>20</td>
</tr>
<tr>
<td>MDC, pg/mL</td>
<td>352 (294 - 419)</td>
<td>836</td>
<td>1.09 (0.89 - 1.33)</td>
<td>0.42</td>
<td>0</td>
</tr>
<tr>
<td>MIP1alpha, pg/mL</td>
<td>45 (38 - 56)</td>
<td>835</td>
<td>1.21 (0.98 - 1.49)</td>
<td>0.07</td>
<td>4</td>
</tr>
<tr>
<td>MIP1beta, pg/mL</td>
<td>122 (95 - 153)</td>
<td>828</td>
<td>0.92 (0.73 - 1.17)</td>
<td>0.51</td>
<td>0</td>
</tr>
<tr>
<td>PARC, ng/mL</td>
<td>3.38 (3.18 - 3.56)</td>
<td>834</td>
<td>0.97 (0.78 - 1.20)</td>
<td>0.75</td>
<td>0</td>
</tr>
<tr>
<td>sRAGE, ng/mL</td>
<td>2.66 (1.94 - 3.63)</td>
<td>839</td>
<td>0.99 (0.81 - 1.21)</td>
<td>0.92</td>
<td>0</td>
</tr>
<tr>
<td>TRAILR3, mg/mL</td>
<td>6.62 (5.16 - 8.41)</td>
<td>837</td>
<td>0.90 (0.73 - 1.10)</td>
<td>0.28</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Markers that were not following a normal distribution were log transformed and presented as median and interquartile range. Measures are presented based on non-imputed values.

<sup>2</sup>Samples included in analysis, outliers excluded.

<sup>3</sup>CFH values are missing due to insufficient quantity of serum.

Abbreviations: CD40, cluster of differentiation 40; CFH, Complement Factor H; EN-RAGE, Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein; FASLR, Fas Ligand Receptor; HCC4, Human CC chemokine-4; IL13, interleukin 13; IL17, interleukin 16; IL17, interleukin 17; IL1ra, interleukin 1 receptor antagonist; LOD, limit of detection; MDC, Monocyte Derived Chemokine; MIP1alpha, Macrophage Inflammatory Protein 1 alpha; MIP1beta, Macrophage Inflammatory Protein 1 beta; PARC, Pulmonary and Activation-Regulated Chemokine; sRAGE, soluble Receptor of Advanced Glycation End-products; TRAILR3, TNF-Related Apoptosis-Inducing Ligand Receptor.
**Supplementary Table IV.** Effect estimates for all covariates included in model 3 per standard deviation.

<table>
<thead>
<tr>
<th>Covariate</th>
<th>HR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.05 (1.01 – 1.08)</td>
<td>0.004</td>
</tr>
<tr>
<td>Gender</td>
<td>0.66 (0.43 – 1.01)</td>
<td>0.05</td>
</tr>
<tr>
<td>EN-RAGE*</td>
<td>1.41 (1.13 – 1.76)</td>
<td>0.002</td>
</tr>
<tr>
<td>CD40*</td>
<td>1.06 (0.80 – 1.40)</td>
<td>0.68</td>
</tr>
<tr>
<td>Complement 3</td>
<td>1.08 (0.86 – 1.37)</td>
<td>0.51</td>
</tr>
<tr>
<td>C-reactive protein*</td>
<td>0.93 (0.73 – 1.19)</td>
<td>0.57</td>
</tr>
<tr>
<td>Interleukin 18*</td>
<td>1.11 (0.90 – 1.37)</td>
<td>0.33</td>
</tr>
<tr>
<td>Interleukin 8*</td>
<td>1.17 (0.94 – 1.46)</td>
<td>0.15</td>
</tr>
<tr>
<td>Monocyte chemotactic protein 1*</td>
<td>0.93 (0.75 – 1.16)</td>
<td>0.52</td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor*</td>
<td>0.97 (0.77 – 1.22)</td>
<td>0.79</td>
</tr>
<tr>
<td>RANTES*</td>
<td>0.84 (0.68 – 1.04)</td>
<td>0.11</td>
</tr>
<tr>
<td>Resistin*</td>
<td>0.85 (0.68 – 1.06)</td>
<td>0.14</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor II*</td>
<td>1.08 (0.81 – 1.43)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

EN-RAGE, extracellular newly identified receptor for advanced glycation end-products binding protein; RANTES, regulated on activation, normal T cell expressed and secreted.

*Markers were natural log-transformed.
References


