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/1.73m^2. 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. 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. 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). 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. 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.

References:


