Materials and Methods

Study Sample

In 1971, 5124 individuals were enrolled into the prospective cohort called the Framingham Offspring Study[1]. The sixth examination, which occurred between 1995 and 1998, was used for the present analysis. Of the 3532 attendees, 3439 individuals (97.4% of attendees) had available blood samples for the present investigation. The study protocol was approved by the Partners Healthcare Institutional Review Board, and all participants provided written informed consent.

At each Heart Study examination, participants underwent a standardized evaluation that included a medical history and physician-administered physical examination. Diabetes mellitus was defined by a fasting glucose ≥126 milligrams/deciliter or the use of insulin or other hypoglycemic medication. Participants were considered current cigarette smokers if they reported having smoked cigarettes regularly during the year preceding the Heart Study examination.

pro-NT Measurement

Blood was collected in all participants using morning samples collected after an overnight fast. Participants were supine for approximately five to ten minutes prior to phlebotomy. Blood samples were immediately centrifuged, and plasma and serum were stored at –70° C. The samples did not undergo any freeze-thaw cycles prior to the present study.

Concentrations of pro-NT were measured in 120 µL of citrated plasma in duplicate using a one-step enzyme-linked chemiluminescence immunosorbent assay (SphingoTec, GMBH; Henningsdorf, Germany). The limit of detection of this assay is 10 picomoles/liter with a coefficient of variation <20% for inter-assay comparisons and <10% for intra-assay. The assay has good linearity across the range of expected values of pro-NT, with no described evidence for high-end hook; pro-NT has been previously shown to be resistant to degradation *in vitro*[2].

Single Nucleotide Polymorphisms

We examined relevant SORT 1 single nucleotide polymorphisms (SNP) for this analysis. One of the SNPs, Rs629301 was genotyped directly using the Illumina OMNI5 array. From a total number of 549,781 genotyped SNPs, we used 412,053 of those as input to the MACH program for phasing. A total of 137,728 genotyped SNPs were removed based on the following filtering criteria: 22,018 SNPs for Hardy-Weinberg Equilibrium p-value of less than 0.000001, 48,285 SNPs for a call rate of less than 96.9%, 66,063 SNPs for a minor allele frequency of less than 0.01, 82 SNPs due to not mapping correctly from Build 36 to Build 37 locations, 428 SNPs missing a physical location, 25 SNPs for number of Mendelian errors greater than 1000, 786 SNPs due to not being on chromosomes 1-22 or X and 41 SNPs because they were duplicates.

The other two SNPs (Rs646776 and rs12740374) have been imputed using the November 2010 release of 1000G ALL panel based on all 1,092 individuals. For rs12740374, R^2=0.92265 and for rs646776 R^2=0.93285. MACH/minimac was used in this imputation. The imputation was

done in a two-step process; the first step consist of phasing the input genotypes in the samples to be imputed using MACH. The second step imputes the SNPs using Minimac and a set of reference haplotypes[3].

Outcomes

During follow up, all Heart Study participants are under continuous surveillance for the development of CVD events. All suspected events were reviewed by a committee of three experienced investigators, using hospital records, physician office notes, and pathology reports.

For the purposes of this analysis, we focused on hard CVD, comprised of acute myocardial infarction (MI), stroke (hemorrhagic or ischemic), subarachnoid hemorrhage, and cardiovascular death (both sudden and non-sudden), as detailed previously[4]. In secondary analyses, we examined hard coronary heart disease (CHD; acute MI and death) as well. As in prior studies[4, 5], we classified events that were based on history only (e.g. symptoms of typical chest pain without ECG evidence of ischemia or injury) as "non-major" and did not include them in the primary endpoint or multivariable regression models.

Statistical Analyses

Before inferential analyses, pro-NT concentrations were natural log transformed due to highly skewed distributions. Clinical characteristics were examined as a function of log-pro-NT quartiles; analysis of variance was used for assessing continuous variables, and Cochran-Mantel-Haenszel test was used for categorical variables. Given prior report associating pro-NT concentrations with risk for incident diabetes mellitus and cancer in a population cohort[6], we examined prevalent diabetes mellitus and both prevalent cancer and prevalent breast cancer as a function of log-pro-NT. Lastly, to more thoroughly examine possible associations between log-pro-NT and circulating LDL cholesterol values, we examined concentrations of LDL cholesterol across log-pro-NT quartiles as well as log-pro-NT concentrations across LDL quartiles. Multivariable linear regression was performed using log-pro-NT concentrations as the dependent variable in an effort to examine correlates of log-pro-NT values. The following covariates were used in the linear model for adjustment: age and sex, waist girth, total/high density lipoprotein (HDL) cholesterol ratio, valve disease, smoking status, alcohol consumption, systolic blood pressure, antihypertensive medication use, diabetes mellitus, prevalent MI, and prevalent cancer; following adjustment for covariates noted above, LDL cholesterol was also added to the linear regression model.

We then examined the association between pro-NT and the risk of hard CVD using multivariable Cox proportional hazards models[7] after excluding pre-existing hard CVD, expressing the hazard ratio (HR) and 95% confidence intervals (CI) for outcome events per standard deviation (SD) of change in log-pro-NT. The proportionality of hazards assumption was met for all models. We first assessed pro-NT in models containing age and sex; following, standard CV risk factors and relevant covariates (including age, sex, BMI, waist girth, total cholesterol, high density lipoprotein cholesterol, valve disease, current tobacco use, number of alcoholic beverages/week, systolic blood pressure, antihypertensive medication use, prevalent diabetes mellitus, and prevalent cancer) were then considered in a fitted model. Because of a prior report suggesting specific ability of pro-NT to predict risk in women[6], models were then stratified by sex to evaluate for differences in prognostication between groups. Following these steps, we then repeated the models above using the stepwise selection with the cut-off alpha level of 0.10 for retention in the multivariable model. Hard CHD was also examined in this same fashion, including prevalent CVD in the models. Following, Cox Proportional Hazard models to predict hard CVD and hard CHD events were examined for interaction between pro-NT concentrations and both LDL cholesterol values as well as relevant SORT1 SNP rs629301-G, rs646776, and

rs12740374[8]; to do so, we specifically sought an age, sex and LDL*log-pro-NT or SNP*log-pro-NT interaction relative to outcomes. The p-value for the interaction was reported. Across pro-NT quartiles, we examined time to first event using Kaplan-Meier curves, assessed using the log-rank test.

Routine echocardiography was performed at the same exam as the blood draw; 2596 subjects had both pro-NT and echocardiography results. Between 2002 and 2005, these same participants underwent CAC scanning; 1257 had available pro-NT and CAC results. We evaluated associations between log-transformed pro-NT and log-transformed left ventricular mass (LVM), hypertrophy (LVH, defined as LVM/height^2 >80th sex specific percentile), ≥mild systolic dysfunction (LVSD; defined as an LV ejection fraction ≤50%), left atrial dimension (LAD), and extent of CAC.

All analyses were performed using SAS software, version 9.4 (SAS Institute, Inc., Cary, NC). A two-sided p-value < 0.05 was considered statistically significant.

References, materials and methods:

1. Kannel WB, Feinleib M, McNamara PM, Garrison RJ and Castelli WP. An investigation of coronary heart disease in families: the Framingham Offspring Study. *Am J Epidemiol*. 1979;110:281-290.
2. Ernst A, Hellmich S and Bergmann A. Proneurotensin 1-117, a stable neurotensin precursor fragment identified in human circulation. *Peptides*. 2006;27:1787-93.
3. Howie B, Fuchsberger C, Stephens M, Marchini J and Abecasis GR. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat Genet*. 2012;44:955-9.
4. Wang TJ, Gona P, Larson MG, Tofler GH, Levy D, Newton-Cheh C, Jacques PF, Rifai N, Selhub J, Robins SJ, Benjamin EJ, D'Agostino RB and Vasan RS. Multiple biomarkers for the prediction of first major cardiovascular events and death. *N Engl J Med*. 2006;355:2631-9.
5. Wang TJ, Larson MG, Levy D, Benjamin EJ, Leip EP, Omland T, Wolf PA and Vasan RS. Plasma natriuretic peptide levels and the risk of cardiovascular events and death. *NEnglJ Med*. 2004;350:655-663.
6. Melander O, Maisel AS, Almgren P, Manjer J, Belting M, Hedblad B, Engstrom G, Kilger U, Nilsson P, Bergmann A and Orho-Melander M. Plasma proneurotensin and incidence of diabetes, cardiovascular disease, breast cancer, and mortality. *JAMA*. 2012;308:1469-75.
7. Cox DR. Regression models and life tables. *J R Stat Soc [B]*. 1972;34 (Series B):187-220.
8. Musunuru K, Strong A, Frank-Kamenetsky M, Lee NE, Ahfeldt T, Sachs KV, Li X, Li H, Kuperwasser N, Ruda VM, Pirruccello JP, Muchmore B, Prokunina-Olsson L, Hall JL, Schadt EE, Morales CR, Lund-Katz S, Phillips MC, Wong J, Cantley W, Racie T, Ejebe KG, Orho-Melander M, Melander O, Koteliansky V, Fitzgerald K, Krauss RM, Cowan CA, Kathiresan S and Rader DJ. From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus. *Nature*. 2010;466:714-9.