Circulating Proneurotensin Concentrations and Cardiovascular Disease Events in the Community

The Framingham Heart Study


Objective—Neurotensin is a peptide whose receptor (sortilin receptor 1) is linked to cardiovascular disease (CVD) development. We hypothesized concentrations of proneurotensin (stable profragment of neurotensin) would predict incident cardiovascular events in community-based subjects.

Approach and Results—Blood samples from 3439 participants in the Framingham Heart Study (FHS) Offspring cohort (mean age 59.2 years, 47.1% male) were tested for proneurotensin. Primary outcome of interest was incident hard CVD (myocardial infarction, stroke, and cardiovascular death); interaction between proneurotensin concentration with sex, low-density lipoprotein concentrations, and sortilin receptor 1 single-nucleotide polymorphisms was sought. At baseline, those in the highest log-proneurotensin quartile were younger and heavier (P<0.001); across proneurotensin quartiles, more prevalent hard CVD (from 3% to 7%; P<0.001) and diabetes mellitus (from 6% to 14%; P<0.001) were present. In age- and sex-adjusted models, log-proneurotensin concentrations predicted incident hard CVD (hazard ratio [HR], 1.24 per SD change in log-proneurotensin; 95% confidence intervals [CIs], 1.11–1.39; P<0.001), a finding that remained on adjustment for standard CVD risk factors (HR, 1.13; 95% CI, 1.01–1.27; P=0.03). Elevated log-proneurotensin concentrations were associated with shorter time to first event (P=0.02). We found no effect modification by sex, low-density lipoprotein concentration, or sortilin receptor 1 genotype. (Arterioscler Thromb Vasc Biol. 2016;36:1692-1697. DOI: 10.1161/ATVBAHA.116.307847.)

Key Words: diabetes mellitus ▪ myocardial infarction ▪ nucleotides ▪ risk factors ▪ stroke

Efforts at reducing cardiovascular events rely on the accurate identification of individuals at risk. Unfortunately, patients who experience cardiovascular events often have a paucity of traditional factors predictive of cardiovascular disease (CVD) to facilitate recognition of risk. In this regard, measurement of circulating biomarkers has been examined as an option for assessing risk for cardiovascular events beyond standard risk factors. Although potentially useful to predict and reclassify risk in some cohorts, studies of such testing for predicting cardiovascular events in low to intermediate risk populations have returned mixed results.\(^1\)\(^2\) suggesting further efforts are needed to better understand the role of testing of circulating substances for risk prediction in such patients. Beyond potential clinical application, biomarker measurement has also been leveraged as a tool to understand mechanism of CVD onset. More studies of novel and established circulating markers of disease are thus needed, both to advance understanding of optimal means for risk stratification and supplement knowledge about mechanism of disease.

Neurotensin is a 13-amino acid peptide originally isolated from bovine hypothalamic\(^1\) and later from intestinal tissue.\(^4\) Neurotensin has a wide range of biological roles in the body,\(^5\) notably including a broad range of effects on the cardiovascular system; these include regulation of heart rate, myocardial contractility, and vascular tone.\(^6\) Effects of neurotensin are transduced primarily through 3 receptors: the G-protein–coupled NTS1 and NTS2 receptors and the non–G-protein–coupled NTS3, otherwise known as sortilin receptor 1 (SORT1), a member of the Vps10p-domain receptor family. SORT1 (also known as sortilin) is involved in the binding of several unrelated ligands, and it plays an important role in hepatic secretion of very low–density lipoprotein cholesterol.

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and regulation of circulating LDL cholesterol concentrations. In addition, genetic variation in the 1p13 locus containing the SORT1 gene is also linked to coronary artery disease development. Although SORT1 seems linked to lipid metabolism and CVD risk, it remains unclear if neurotensin plays a role in this association.

Measurement of neurotensin in blood is challenging because of its instability and rapid clearance from the circulation. To overcome this issue, immunoassays have been developed for the detection of the propeptide fragment of the peptide, which is released in equimolar amounts to mature neurotensin. Recent data from the Malmö Diet and Cancer Study suggested that concentrations of proneurotensin were independently predictive of diabetes mellitus and CVD, particularly in women. However, beyond these preliminary findings, no other data exist on association of circulating proneurotensin concentrations with the incidence of CVD events, nor are mechanistic analyses available. Accordingly, we sought to examine links between proneurotensin and CVD in a cohort of patients from the Framingham Heart Study (FHS) Offspring study. Our hypothesis was that PNT concentrations are more likely to smoke (P<0.001), prevalent hard CVD (from 3% to 7%; P<0.001), or prevalent hard coronary heart disease (CHD; from 3% to 5%; P=0.06). No association between log-proneurotensin and prevalent cancer (including breast cancer) was observed.

**Materials and Methods**

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

**Results**

Characteristics of the study sample as a function of proneurotensin quartiles are shown in Table 1. The mean age of the study sample was 59.19±10 years, and 53% of participants were women. Compared with subjects in log-proneurotensin quartiles 1 through 3, those in the highest quartile were more likely to be younger (P=0.006), heavier (P<0.001), and more likely to smoke (P<0.001). There was no difference in LDL cholesterol concentrations across log-proneurotensin quartiles; similarly, across quartiles of LDL cholesterol, there was no difference in log-proneurotensin concentrations (P=0.71).

In multivariable linear regression analyses, variables independently correlated with log-proneurotensin concentrations included waist girth (β=0.0044; P=0.005), smoking (β=0.0818; P<0.001), and prevalent diabetes mellitus (β=0.1438; P<0.001); concentrations of LDL cholesterol were not predictive of log-proneurotensin concentrations (β=-0.0002; P=0.39). Furthermore, neither age (β=-0.0011; P=0.10) nor male sex (β=-0.0007; P=0.96) significantly correlated/predicted concentrations of log-proneurotensin.

As detailed in Table 2, at baseline, across proneurotensin quartiles study participants with higher concentrations were more likely to have prevalent diabetes mellitus (from 6% to 14%; P<0.001), prevalent hard CVD (from 3% to 7%; P<0.001), or prevalent hard coronary heart disease (CHD; from 3% to 5%; P=0.06). No association between log-proneurotensin and prevalent cancer (including breast cancer) was observed.

**Biomarker Concentrations and Outcomes**

During a mean follow-up of 14.0 years, 342 (10.5%) individuals had a hard CVD event, with 166 myocardial infarctions, 148 strokes, 27 CHD deaths, and 1 stroke death. During similar follow-up time, 209 (6.3%) individuals had a hard CHD event.

Table 3 details predictive value of log-proneurotensin for hard CVD events. In age- and sex-adjusted Cox proportional hazards models (Table 3), log-proneurotensin concentrations were positively associated with incident hard CVD (HR, 1.242 per 1 SD change in log-proneurotensin; 95% CI, 1.11–1.39; P<0.001). In models adjusted for standard risk factors (including body mass index), log-proneurotensin remained significantly associated with incident hard CVD (HR, 1.13 per 1 SD change in log-proneurotensin; 95% CI, 1.01–1.27; P=0.03). The HR for log-proneurotensin remained significant in models forcing concentrations of LDL (HR, 1.121 per 1 SD change in log-proneurotensin; 95% CI, 1.002–1.254; P=0.05), the interaction factor of male×LDL (HR, 1.121 per 1 SD change in log-proneurotensin; 95% CI, 1.002–1.254; P=0.05), or an LDL cholesterol above the median for the group (HR, 1.122 per 1 SD change in log-proneurotensin; 95% CI, 1.003–1.215; P=0.05).

Examining risk across log-proneurotensin quartiles in age- and sex-adjusted models (Table I in the online-only Data Supplement), greatest risk of incident hard CVD was observed in log-proneurotensin quartile 4 (HR, 1.53 versus quartile 1; P=0.005). Using stepwise selection for prediction of hard CVD, comparable results were found, with higher concentrations of log-proneurotensin predicting risk (Table 3).

Considering hard CHD, similar results were found, with higher concentrations of log-proneurotensin predicting hard CHD in adjusted models that also contained prevalent CVD (Tables I and II in the online-only Data Supplement). In fully adjusted models for hard CHD, we found an HR of 1.156 per 1 SD change in log-proneurotensin (95% CI, 1.005–1.330; P=0.04).

Addition of further biomarker results for highly sensitive troponin I, growth differentiation factor-15, or soluble ST2 (previously reported to be predictive of cardiovascular events in this cohort) in a stepwise model resulted in retention of growth differentiation factor-15 as a predictor of hard CVD (HR, 1.25 per 1 SD change in log-transform; 95% CI, 1.11–1.40; P<0.001), whereas log-proneurotensin became marginally nonsignificant (HR, 1.11 per 1 SD change in log-proneurotensin; 95% CI, 0.99–1.24; P=0.07).

In interaction testing with fully adjusted models, we did not observe a proneurotensin×LDL interaction (P=0.97) for prediction of hard CVD events. Similarly, we found no proneurotensin×SORT1 single-nucleotide polymorphism (SNP) interactions for prognostication of hard CVD (proneurotensin×SORT1).

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>FHS</td>
<td>Framingham Heart Study</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
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<tr>
<td>SORT1</td>
<td>Sortilin receptor 1</td>
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rs629301, P = 0.76; proneurotensin×rs646776, P = 0.56; and proneurotensin×rs12740374, P = 0.65). Similarly, negative results were found in proneurotensin×LDL or proneurotensin×SNP interaction analyses for hard CHD.

In contrast to previous data,8 we found no interaction term with respect to sex- and proneurotensin-based prognostication (Table 4). Although the HR for log-proneurotensin predicting hard CVD was numerically higher in women (HR, 1.175 [95% CI, 0.993–1.390]) compared with men (HR, 1.118 [95% CI, 0.962–1.300]), these differences did not approach statistical significance.

Notably, log-transformed concentrations of proneurotensin were not related to incident change in body mass index or waist girth. Log-proneurotensin did not predict incident diabetes mellitus; during follow up, there were only 32 incident cases. Over a mean follow-up of 8.7
Cardiac Structure and Function

In age-, sex-, and height-adjusted regression, proneurotensin concentrations were associated with LVM (parameter estimate 0.016 per SD of log-transformed proneurotensin; \( P = 0.0002 \)) and the presence of LVSD (\( P = 0.05 \)). Across proneurotensin quartiles (Q), significantly higher mean LVM (\( P = 0.001 \)) was observed; LVSD was least likely in proneurotensin Q1 versus Q4 (\( P = 0.05 \)). In multivariable-adjusted models, these findings were attenuated (\( P = 0.10 \) for LVM; \( P = 0.22 \) for LVSD). Proneurotensin concentrations were associated with extent of CAC in age- and sex-adjusted regression analyses (parameter estimate 0.145 per SD of log-transformed proneurotensin; \( P = 0.02 \)); however, in multivariable-adjusted analyses, this finding was no longer significant (\( P = 0.10 \)). Findings were similar when excluding subjects with prevalent CVD.

Discussion

The principal findings of our analysis were that concentrations of PNT were associated cross-sectionally with a more deleterious cardiometabolic state and function, independent of LDL concentrations or SORT1 genotypes relevant to the development of atherosclerosis. The association of PNT with hard CVD and hard CHD remained robust after adjustment for traditional cardiovascular risk factors, and it provided modest risk reclassification for predicting events. Concentrations of proneurotensin were modestly associated with cardiac structure and function in echocardiographic and CAC imaging, but in rigorously adjusted models, these associations were less obvious. These results support a possible role for the neurotensin system in the development of clinical CVD, but further evaluation is needed.

Neurotensin is thought to work as a local hormone on peripheral organs, including the heart; it has a broad range of cardiovascular effects, regulating heart rate, myocardial contractility, and blood pressure.\(^6\) Although measurement of neurotensin had been challenging because of analytic instability, recent development of a propeptide assay to quantify neurotensin has overcome this challenge. We, therefore, measured proneurotensin in the FHS Offspring Study in an effort to examine the prognostic role of the neurotensin system for CVD incidence.

Our results are notable because little information exists presently about neurotensin and risk for heart disease. Data from Melander et al\(^8\) from the Malmö Diet and Cancer Study suggest that concentrations of proneurotensin predicted adverse cardiometabolic outcome, including CVD and incident diabetes mellitus. An intriguing interaction between proneurotensin-based prognostication and sex was found by Melander et al\(^8\), suggesting proneurotensin provided unique prognostic information particularly in women, correctly reclassifying as much as 40% of women for risk of cardiovascular death. In addition, in Malmö, proneurotensin predicted incident breast cancer. Our results are reasonably comparable with those from Malmö with respect to the ability of proneurotensin to predict hard CVD and hard CHD, although such ability to predict is much more modest in our more rigorously adjusted analyses. In addition, we could not confirm a sex-specific value of proneurotensin. Furthermore, although proneurotensin was associated with more deleterious cardiometabolic state at enrollment (with higher values of proneurotensin associated with more prevalent obesity and diabetes mellitus), we could not confirm proneurotensin predicted incident obesity or diabetes mellitus. Finally, no predictive ability to prognosticate incident cancers was seen in our analysis. The differences in results may be explained on the basis of the fact that our study sample was smaller, and participants differ considerably in terms of baseline cardiometabolic risk compared with those in the Malmö analysis. In addition, our statistical models were more rigorously adjusted. Despite more modest results, our findings are important because they clarify those from Malmö.

We initially hypothesized that the deleterious effect(s) of neurotensin might be explainable on the basis of binding to

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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard Ratio (95% CI)</th>
<th>( P )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proneurotensin (standardized log transform)</td>
<td>1.136 (1.017–1.270)</td>
<td>0.03</td>
</tr>
<tr>
<td>Age</td>
<td>1.063 (1.049–1.078)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>1.636 (1.313–2.040)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol/HDL ratio</td>
<td>1.052 (1.018–1.086)</td>
<td>0.002</td>
</tr>
<tr>
<td>Smoker</td>
<td>2.390 (1.825–3.130)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>1.008 (1.002–1.014)</td>
<td>0.007</td>
</tr>
<tr>
<td>Antihypertensive medication use</td>
<td>1.536 (1.213, 1.945)</td>
<td>0.004</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.759 (1.319–2.345)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

CI indicates confidence intervals; CVD, cardiovascular disease; and HDL, high-density lipoprotein.

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Table 2. Prevalent Medical History as a Function of Log-Transformed Proneurotensin Quartiles

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Quartile 1 (n=860)</th>
<th>Quartile 2 (n=860)</th>
<th>Quartile 3 (n=859)</th>
<th>Quartile 4 (n=860)</th>
<th>( P )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes mellitus</td>
<td>6.3% (54/860)</td>
<td>10.7% (92/860)</td>
<td>12.1% (104/859)</td>
<td>14.3% (123/860)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hard CVD</td>
<td>3.0% (26/860)</td>
<td>4.5% (39/860)</td>
<td>6.8% (58/859)</td>
<td>6.6% (57/860)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hard CHD</td>
<td>2.8% (24/860)</td>
<td>3.6% (31/860)</td>
<td>5.0% (43/859)</td>
<td>4.9% (42/860)</td>
<td>0.06</td>
</tr>
<tr>
<td>Cancer</td>
<td>6.4% (55/860)</td>
<td>6.2% (53/860)</td>
<td>5.4% (46/859)</td>
<td>6.5% (56/860)</td>
<td>0.75</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>1.6% (14/860)</td>
<td>1.2% (10/860)</td>
<td>1.3% (11/859)</td>
<td>1.6% (14/860)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

CHD indicates coronary heart disease; and CVD, cardiovascular disease.
SORT1. This receptor is intracellular and non–G-protein–coupled and plays a role in endocytosis and trafficking of several molecules (including various cholesterol particles). SORT1 has been implicated in LDL cholesterol metabolism and in very low–density lipoprotein and proprotein convertase subtilisin/kexin type 9 secretion. Genetic variation of SORT1 is pivotally linked to coronary artery disease development in humans, in part, through its effects on lipoprotein metabolism. In theory, therefore, higher values of proneurotensin could influence cardiac risk through interactions at the level of the SORT1 receptor via interference with normal lipid processing. In our analysis, however, we found no association between proneurotensin concentrations and LDL cholesterol values. In addition, in evaluating various SORT1 SNPs important to CVD development, we could not detect any proneurotensin×SORT1 interaction for prognosis; this lack of significant interaction is not because of solely lack of power as the outcome HR per 1 SD increase of log-PNT for participants above and below median rs629301 were 1.23 and 1.21, respectively, indicating consistent log-PNT effect across the values of this SNP. As another example, a similar trend was seen for rs1274074 (HR was 1.20 for participants above the median and 1.24 for participants below it). Indeed, with the current sample size/event rates, we have ≈90% power to detect an interaction with SNP if HR for participants below median SNP was approximately twice that for participants above the median SNP or vice-versa. Finally, although we did not perform interaction testing between proneurotensin and other variables influenced by the SORT1 receptor (such as proprotein convertase subtilisin/kexin type 9), our results suggest that the deleterious link(s) between proneurotensin and CVD may be mediated via effects of neurotensin on receptors other than SORT1.

Although neurotensin binds both NTS1 and NTS2, substantial differences exist between the 2 receptors. Although both are G-protein coupled, NTS1 has considerably higher affinity for neurotensin. Furthermore, although both receptors are found in cardiovascular tissue, NTS1 is currently suspected to be more involved in regulation of cardiovascular effects of neurotensin. Manipulation of NTS1 receptor function with SR48692 (a selective nonpeptide NTS1 inhibitor) resulted in dose-dependent effects on blood pressure, heart rate, myocardial contractility, vascular tone, permeability, and endothelial cell survival. Hypothetically, therefore, higher concentrations of neurotensin may result in cardiac stimulation, increased vascular tone, and accelerated atherogenesis as a consequence of binding to the NTS1 receptor. In contrast, NTS2—a low affinity receptor for neurotensin—is not currently thought to play a role in cardiovascular responses. Of importance, it remains unclear if circulating proneurotensin concentrations reflect tissue-based concentrations of neurotensin. More studies are needed to better understand the role of both NTS1 and tissue-based neurotensin as participants in the development of CVD.

Table 4. Multivariable-Adjusted Cox Proportional Hazards Model for Proneurotensin Prediction of Hard CVD Events in Males and Females

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard Ratio, per SD (95% CI)</th>
<th>P Value</th>
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<tr>
<td>Proneurotensin (standardized log transform)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>1.118 (0.962–1.300)</td>
<td>0.15</td>
</tr>
<tr>
<td>Females</td>
<td>1.175 (0.993–1.390)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

CI indicates confidence intervals; and CVD, cardiovascular disease.

*Model adjusted for age, sex, waist girth, total cholesterol/high-density lipoprotein ratio, valve disease, smoking, number of alcoholic beverages per week, systolic blood pressure, antihypertensive medication use, diabetes mellitus, and prevalent cancer.

Figure. Kaplan–Meier survival curves for hard cardiovascular disease (CVD) events in the Framingham Heart Study Offspring Study as a function of log-proneurotensin quartiles. Those with higher proneurotensin values had shorter time to first hard CVD event.
Limitations of our analysis include the fact that our sample size is relatively small and our event rates are modest. Nonetheless, our results support the primary hypothesis that proneurotensin is predictive of cardiovascular events in the community, although in a manner somewhat more modest than that demonstrated by Melander et al. Our results provide balance to the literature. Although the more modest prognostic implication of proneurotensin in our study may be because of limited event rates, for both hard CVD and hard CHD outcomes, we had >85% power to detect a difference between each of the upper and the reference quartiles of log-proneurotensin. The highly skewed nature of proneurotensin makes association between graded variables (such as age or body mass index) somewhat challenging. We have log-transformed proneurotensin to best address this fact. Although binding of neurotensin to NTS1 remains a potential mechanistic explanation for the increased cardiovascular risk associated with higher values of proneurotensin, we cannot directly inform mechanism of how the neurotensin system predicts CVD or CHD. Finally, unfortunately, we lack SNP data on NTS1 polymorphisms.

In summary, concentrations of proneurotensin predicted onset of hard CVD and hard CHD events in a community-based cohort. The ability of proneurotensin to prognosticate such events seems to be independent of LDL cholesterol values or SORT1 genotypes, suggesting deleterious cardiovascular effects of neurotensin are mediated via another mechanism, such as binding to the NTS1 receptor. Further data are needed on the role(s) played by the neurotensin system in cardiovascular risk.

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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 $\geq 126$ 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 $-70^\circ$ 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.

**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. In secondary analyses, we examined hard coronary heart disease (CHD; acute MI and death) as well. As in prior studies, 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, 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 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, 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
rs127403746; 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/height² >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:**

Supplemental Table I: Risk for A) hard CVD or B) hard CHD across log-pro-NT quartiles.

A)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proneurotensin (Quartile 2 of log transform)</td>
<td>0.924</td>
<td>0.668, 1.277</td>
<td>0.63</td>
</tr>
<tr>
<td>Proneurotensin (Quartile 3 of log transform)</td>
<td>1.217</td>
<td>0.900, 1.644</td>
<td>0.20</td>
</tr>
<tr>
<td>Proneurotensin (Quartile 4 of log transform)</td>
<td>1.529</td>
<td>1.135, 2.059</td>
<td>0.005</td>
</tr>
<tr>
<td>Age</td>
<td>1.072</td>
<td>1.059, 1.085</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Male</td>
<td>1.750</td>
<td>1.412, 2.167</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proneurotensin (Quartile 2 of log transform)</td>
<td>0.920</td>
<td>0.592, 1.431</td>
<td>0.72</td>
</tr>
<tr>
<td>Proneurotensin (Quartile 3 of log transform)</td>
<td>1.613</td>
<td>1.092, 2.381</td>
<td>0.02</td>
</tr>
<tr>
<td>Proneurotensin (Quartile 4 of log transform)</td>
<td>1.745</td>
<td>1.178, 2.586</td>
<td>0.006</td>
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<tr>
<td>Age</td>
<td>1.058</td>
<td>1.042, 1.074</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Male</td>
<td>2.151</td>
<td>1.627, 2.845</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

CI denotes: confidence intervals.
**Supplemental Table II**: Cox proportional hazards model for pro-NT prediction of hard CHD events. pro-NT predicted hard CHD in both age and sex adjusted and fully adjusted models.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard Ratio per SD</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proneurotensin (Standardized log transform)</td>
<td>1.294</td>
<td>1.126, 1.486</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Model 2: Multivariable adjusted***

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard Ratio per SD</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proneurotensin (Standardized log transform)</td>
<td>1.156</td>
<td>1.005, 1.330</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Model adjusted for age, sex, waist girth, total cholesterol/HDL ratio, valve disease, smoking, number of alcoholic beverages per week, systolic blood pressure, antihypertensive medication use, diabetes mellitus, prevalent CVD, and prevalent cancer. SD denotes: standard deviation; CI denotes: confidence intervals.