

Association of common variants in *NPPA* and *NPPB* with circulating natriuretic peptides and blood pressure

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We examined the association of common variants at the *NPPA-NPPB* locus with circulating concentrations of the natriuretic peptides, which have blood pressure-lowering properties. We genotyped SNPs at the *NPPA-NPPB* locus in 14,743 individuals of European ancestry, and identified associations of plasma atrial natriuretic peptide with rs5068 ($P = 8 \times 10^{-70}$), rs198358 ($P = 8 \times 10^{-30}$) and rs632793 ($P = 2 \times 10^{-10}$), and of plasma B-type natriuretic peptide with rs5068 ($P = 3 \times 10^{-12}$), rs198358 ($P = 1 \times 10^{-25}$) and rs632793 ($P = 2 \times 10^{-68}$). In 29,717 individuals, the alleles of rs5068 and rs198358 that showed association with increased circulating natriuretic peptide concentrations were also found to be associated with lower systolic ($P = 2 \times 10^{-6}$ and 6×10^{-5} , respectively) and diastolic blood pressure ($P = 1 \times 10^{-6}$ and 5×10^{-5}), as well as reduced odds of hypertension (OR = 0.85, 95% CI = 0.79–0.92, $P = 4 \times 10^{-5}$; OR = 0.90, 95% CI = 0.85–0.95, $P = 2 \times 10^{-4}$, respectively). Common genetic variants at the *NPPA-NPPB* locus found to be associated with circulating natriuretic peptide concentrations contribute to interindividual variation in blood pressure and hypertension.

Hypertension affects a billion individuals worldwide, and represents a potent risk factor for cardiovascular disease¹. Because the relationship between blood pressure and cardiovascular risk is continuous,

even small increments in blood pressure confer excess hazard^{2,3}. However, the underlying determinants of interindividual variation in blood pressure are poorly defined. Epidemiologic studies have documented substantial heritability of blood pressure, suggesting a role for genetic factors⁴. Although rare genetic variants have been described for monogenic forms of hypertension⁵ and, more recently, for blood pressure in the general population⁶, no common variants associated with blood pressure have been identified. Many pathways, including the renin-angiotensin-aldosterone system and the adrenergic system, have been found to modulate blood pressure in experimental models, but studies have failed to show that genetic variation in these pathways contributes to interindividual differences in blood pressure.

Since the discovery that the heart secretes a family of vasodilatory and natriuretic hormones in response to increased wall stress⁷, it has been speculated that these molecules, known as the natriuretic peptides, might be involved in blood pressure regulation in humans. In mice, knockout of one copy of the atrial natriuretic peptide (ANP) gene, but not the B-type natriuretic peptide (BNP) gene, is associated with salt-sensitive hypertension⁸. Overexpression of ANP through gene therapy in hypertensive mice lowers systolic blood pressure⁹. To date, however, clinical studies have failed to establish a causal link between the natriuretic peptide axis and blood pressure variation in humans.

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Natriuretic peptide production is stimulated by increased systolic blood pressure, via increased cardiac afterload. The competing influences of a potential blood pressure-lowering effect of natriuretic peptides and a natriuretic peptide-raising effect of elevated blood pressure make cross-sectional studies difficult to interpret, and suggest a role for genetic studies, in which directionality is unambiguous. We sought to demonstrate that *cis*-acting genetic variants influence natriuretic peptide concentrations and, in turn, that genetically determined variation in natriuretic peptide concentrations contributes to interindividual variation in blood pressure and hypertension (Supplementary Fig. 1 online).

The *NPPA* (natriuretic peptide precursor A) and *NPPB* (natriuretic peptide precursor B) genes lie in tandem 9.7 kb apart on chromosome 1. We genotyped a set of 13 SNPs that captured the majority of common variation at the *NPPA-NPPB* locus (Fig. 1 and Supplementary Methods and Supplementary Table 1 online). Clinical characteristics of the study samples and covariate relationships to ANP and BNP are presented in Table 1 and Supplementary Table 2 online. Of the 13 SNPs tested in stage 1 in 1,705 unrelated Framingham Heart Study participants, multiple SNPs were nominally associated with ANP concentration (Supplementary Table 3 online), with the strongest association involving a missense SNP in *NPPA* (rs5063, V32M, $P = 7 \times 10^{-55}$). Several SNPs showed nominal association with BNP concentration, with the strongest statistical support for rs632793 ($P = 2 \times 10^{-8}$). Because many of the stage 1 SNPs were

correlated, we carried out additional analyses adjusting for the SNP with the lowest P value (rs5063 for ANP and rs632793 for BNP), in order to determine the statistical support for the remaining SNPs independently (Supplementary Table 4 online). On the basis of these analyses and on the linkage disequilibrium patterns, we selected three SNPs associated with ANP and one SNP associated with BNP for genotyping in stage 2.

In stage 2, we attempted to validate the association of the four SNPs with natriuretic peptide concentrations, using four study samples totaling 14,743 individuals (Framingham Unrelated and Related, Malmö Diet and Cancer, Finrisk97). SNP rs5063 showed a highly significant association with ANP in the Framingham Related sample ($P = 2 \times 10^{-8}$), but no association in Malmö Diet and Cancer ($P = 0.24$) or Finrisk97 ($P = 0.07$), despite the greater than sixfold higher sample sizes in the latter two studies. Because rs5063 is a missense SNP encoding an amino acid change within the binding site of the N-terminal pro-ANP assay used in Framingham but not in Malmö or Finrisk97 (ref. 10), we hypothesized that the association in Framingham was artifactual (Supplementary Methods). When mature ANP was measured in Framingham samples, it failed to show association with rs5063 genotype ($P = 0.97$, Supplementary Note online). Missense SNPs have received particular attention because of their greater likelihood of being functional compared with noncoding SNPs. This example illustrates a potential pitfall of relating missense variants in a gene with the concentration of its protein product.

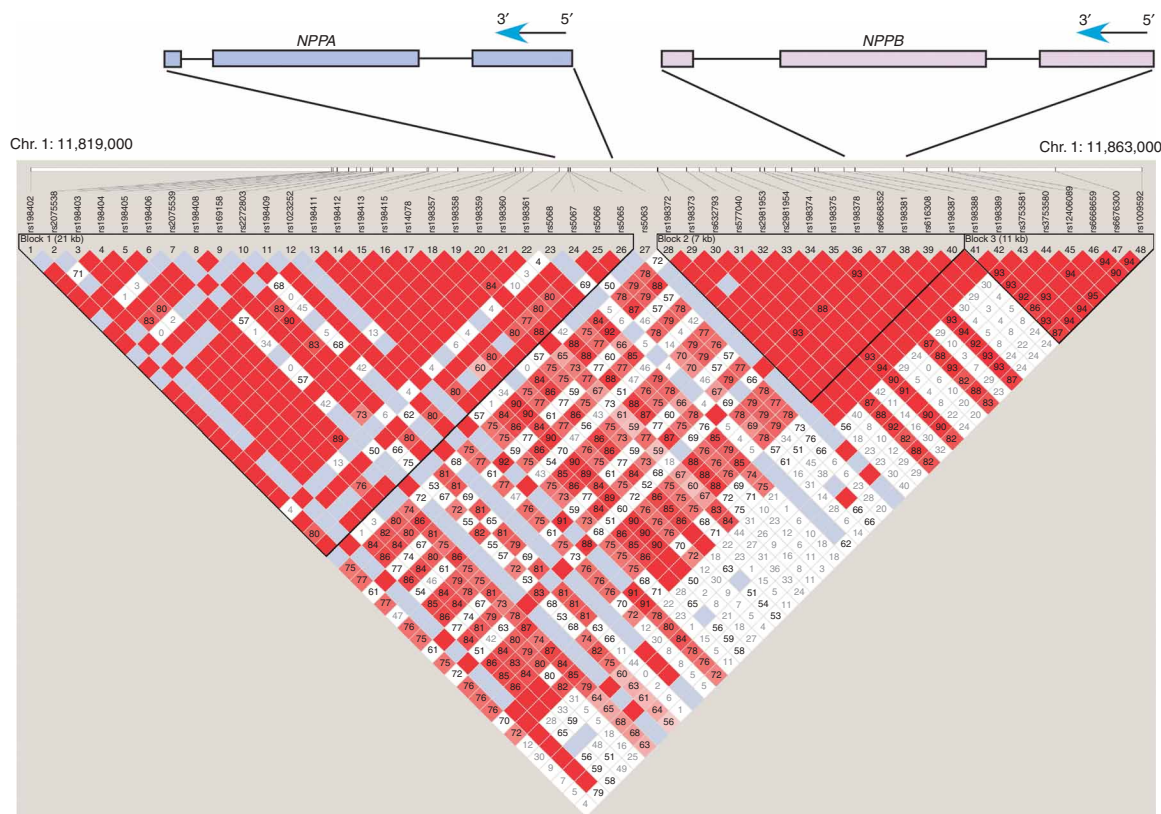


Figure 1 Linkage disequilibrium map across *NPPA-NPPB* locus in CEPH reference sample. The exon structures of *NPPA* and *NPPB* are shown schematically relative to the SNPs genotyped in the reference samples. The two genes fall on the negative strand of the human genome reference sequence and thus are in 3'-to-5' order from left to right as shown. All SNP alleles in the text are shown according to the coding (negative) strand sequence. The pairwise linkage disequilibrium (LD) relationships among the 48 polymorphic (minor allele frequency $\geq 5\%$) SNPs passing quality control in the CEPH reference sample are shown. The linkage disequilibrium between all pairs of SNPs tested is represented at the bottom of the figure: red indicates significant LD, white indicates weak LD and light blue reflects inadequate power to determine LD. Figure prepared using HaploView v2.0.3, defining blocks using the 'spine of LD' method. The genomic position on chromosome 1 is indicated with reference to the hg17 assembly of the human reference sequence (May 2004, NCBI build 35). The SNP numbering 1–48 corresponds to the SNPs as ordered in Supplementary Table 1.

Table 1 Characteristics of study samples

| | Framingham men (N = 1,165) | Framingham women (N = 1,291) | Malmö men (N = 2,127) | Malmö women (N = 3,069) | Finrisk97 men (N = 3,711) | Finrisk97 women (N = 3,838) | Malmö Preventive Project men (N = 9,716) | Malmö Preventive Project women (N = 4,800) |
|---|-------------------------------|---------------------------------|--------------------------|----------------------------|------------------------------|--------------------------------|---|---|
| | Samples 1 and 2 | | Sample 3 | | Sample 4 | | Sample 5 | |
| Age, y | 58 ± 10 | 58 ± 10 | 58 ± 6 | 58 ± 6 | 49 ± 13 | 47 ± 13 | 43 ± 6 | 50 ± 7 |
| Body mass index, kg/m ² | 28.7 ± 4.4 | 27.4 ± 5.8 | 26.2 ± 3.5 | 25.5 ± 4.2 | 27.0 ± 3.9 | 26.3 ± 5.0 | 24.5 ± 3.0 | 24.2 ± 3.9 |
| Systolic blood pressure, mm Hg | 130 ± 17 | 127 ± 20 | 143 ± 19 | 140 ± 19 | 140 ± 19 | 132 ± 20 | 128 ± 13 | 126 ± 16 |
| Diastolic blood pressure, mm Hg | 78 ± 9 | 74 ± 9 | 89 ± 10 | 86 ± 9 | 85 ± 11 | 80 ± 11 | 86 ± 9 | 83 ± 9 |
| Anti-hypertensive therapy, % | 30 | 25 | 18 | 16 | 15 | 11 | 3 | 8 |
| Diabetes mellitus, % | 13 | 8 | 12 | 6 | 6 | 5 | 3 | 3 |
| N-terminal proANP, pmol/l | 356 ± 265 | 395 ± 222 | – | – | – | – | – | – |
| Mid-regional N-terminal proANP, pmol/l ^a | – | – | 69 ± 39 | 76 ± 32 | 49 ± 38 | 52 ± 27 | – | – |
| N-terminal proBNP, pg/ml | – | – | 94 ± 261 | 106 ± 143 | – | – | – | – |
| BNP, pg/ml | 14 ± 21 | 16 ± 20 | – | – | 22 ± 63 | 25 ± 31 | – | – |

Shown are the clinical characteristics for the Framingham Heart Study (Unrelated, sample 1, and Related, sample 2, combined), the Malmö Diet and Cancer-Cardiovascular Arm (MDC-CVA, sample 3), the Finrisk97 cohort (sample 4) and the Malmö Preventive Project (sample 5). Values are reported as mean ± s.d. for continuous traits, and as percentages for dichotomous traits.

^aMid-regional N-terminal proANP refers to the assay for N-terminal pro-ANP that uses an antibody against an epitope in the mid-region of the molecule, as opposed to the N-terminal epitopes used in the Framingham assays. In the text, ANP and BNP refer to assays for the mature peptide or N-terminal pro-peptides as described in the **Supplementary Methods**. Dash (–), not available.

Stage 2 results for the remaining three SNPs are summarized in **Table 2**; cohort-specific results are shown in **Supplementary Table 5** online. Associations with higher ANP concentration were observed for the minor alleles of rs5068 ($P = 8 \times 10^{-70}$), rs198358 ($P = 8 \times 10^{-30}$) and rs632793 ($P = 2 \times 10^{-10}$). Associations with higher BNP were observed for the minor alleles of rs5068 ($P = 3 \times 10^{-12}$), rs198358 ($P = 9 \times 10^{-25}$) and rs632793 ($P = 2 \times 10^{-68}$). All SNPs were associated with comparable effects on BNP (+0.17 to 0.21 s.d.), but rs5068 was associated with a stronger effect on ANP (+0.42 s.d.) than rs198358 (+0.20 s.d.) or rs632793 (+0.08 s.d.) (**Table 2**). ANP and BNP were highly correlated ($r = 0.71$) but pairwise r^2 values among rs5068, rs198358 and rs632793 were all <0.3. When both rs5068 and rs198358 were entered into a single regression model predicting ANP, the association of each was attenuated but statistically significant in Framingham Unrelated (rs5068 $P = 0.005$, rs198358 $P = 0.02$) and Finrisk97 samples (rs5068 $P = 2 \times 10^{-18}$, rs198358 $P = 0.003$). rs5068 and rs198358 thus seem independent due to their divergent effects on ANP relative to BNP and their significance in a single regression model. A graded relationship of increasing ANP and BNP concentrations with increasing copies of the minor alleles of rs5068, rs198358 and rs632793 was observed in both hypertensive and non-hypertensive groups (**Fig. 2** and **Supplementary Table 6** online).

Although the location of rs5068 in the 3' untranslated region of ANP raises the possibility that it could alter transcript stability, this would not explain its association with BNP. The fact that all three SNPs were associated with both peptides suggests coordinate regulation of the two peptides at the genetic level, perhaps through shared enhancer elements. If a genetic variant directly influenced either ANP or BNP concentration alone, one would expect compensatory changes in concentration of the other peptide (in the opposite direction), as observed in knockout mice⁸. In contrast, we found the same directionality of genetic association for each peptide. Although a genetic influence on natriuretic peptide clearance is also possible, associations were noted with both N-terminal pro-peptides and mature peptides, which have different clearance mechanisms. Additional work will be required to define the precise mechanism of causal genetic variation at the *NPPA-NPPB* locus.

The three SNPs with genome-wide significant associations with natriuretic peptide concentrations in stage 2 (rs5068, rs198358, rs632793) were carried into stage 3, in which we tested for association with blood pressure in the four samples, and combined results using meta-analysis (overall $n = 15,201$). Overall meta-analysis of stage 3 association results are shown in **Table 3**, with cohort-specific blood pressure means by genotype in **Supplementary Table 6** and cohort-specific association results in **Supplementary Table 7** online. Carriers of the rs5068 minor allele had lower systolic blood pressure ($P = 1 \times 10^{-4}$) and lower diastolic blood pressure ($P = 0.002$), compared with major allele homozygotes. Carriers of the rs198358 minor allele had lower systolic blood pressure ($P = 5 \times 10^{-5}$) and lower diastolic blood pressure ($P = 4 \times 10^{-4}$), compared with major allele homozygotes. SNP rs632793 was not significantly associated with either systolic or diastolic blood pressure.

The effects of SNPs on blood pressure roughly paralleled their effects on ANP, suggesting that ANP could have a stronger influence on blood pressure than BNP. This is consistent with the experimental observation that hypertension results from knockout of *Nppa* but not

Table 2 Meta-analysis of natriuretic peptide concentration associations (stage 2)

| SNP ID | Stage 2, samples 1–4, N = 14,473 | | | |
|----------------------|----------------------------------|---------------------|--------------|---------------------|
| | ANP | | BNP | |
| | β (s.e.m.) | P value | β (s.e.m.) | P value |
| rs5068, MAF = 0.06 | +0.42 (0.02) | 8×10^{-70} | +0.17 (0.02) | 3×10^{-12} |
| rs198358, MAF = 0.19 | +0.20 (0.02) | 8×10^{-30} | +0.18 (0.02) | 9×10^{-25} |
| rs632793, MAF = 0.38 | +0.08 (0.01) | 2×10^{-10} | +0.21 (0.01) | 2×10^{-68} |

In stage 2, results are shown from meta-analysis of association results with atrial and B-type natriuretic peptides (ANP, BNP) for rs5068, rs198358 and rs632793 in samples 1–4. Effect sizes (β) are shown on the standard deviation scale. Meta-analysis was performed using inverse variance weights. Association results are shown for the genetic model (dominant, additive, recessive) that yielded the lowest P value for association. Results for all three genetic models in each individual sample are shown in **Supplementary Table 5**. β, effect estimate from linear regression; MAF, minor allele frequency.

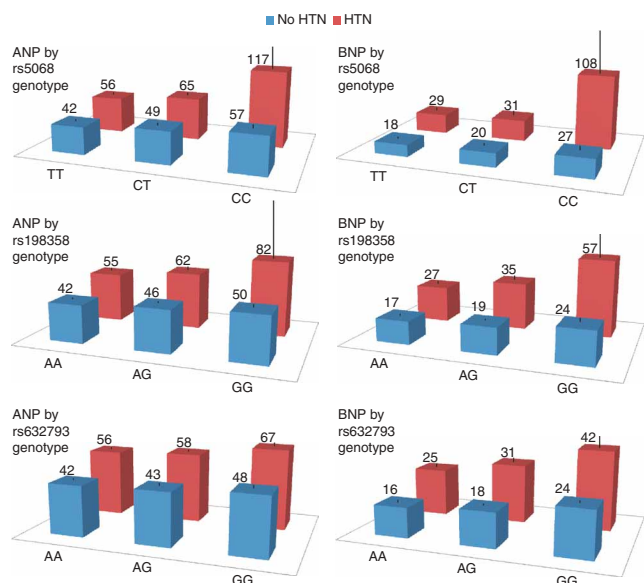


Figure 2 N-terminal proANP and BNP concentration in 7,091 individuals from Finrisk97 by genotype and by hypertension status. Genotype classes are shown from major homozygotes (left) to minor homozygotes (right). Mean plasma concentration of natriuretic peptide is shown by genotype in individuals with (red) and without (blue) hypertension. Hypertension is defined as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg or use of antihypertensive medication. N-terminal proANP units are pmol/l and BNP units are pg/ml. Error bars, s.e.m.

Hg for systolic blood pressure and 0.3–0.8 mm Hg for diastolic blood pressure.

We examined the association of rs5068 and rs198358 with prevalent hypertension, defined as systolic blood pressure ≥ 140 mm Hg, diastolic blood pressure ≥ 90 mm Hg or use of antihypertensive therapy. The alleles associated with higher natriuretic peptide concentrations were associated with reduced risk of hypertension (OR = 0.85, $P = 4 \times 10^{-5}$ for rs5068; OR = 0.90, $P = 2 \times 10^{-4}$ for rs198358; **Table 4**).

In summary, we report the first common genetic variants for blood pressure and hypertension in the general population. Our findings were consistent across multiple, large cohorts, and supported by the demonstration that these variants (or variants in close association) influence circulating concentrations of molecules known to modulate vascular tone and sodium excretion.

It has been difficult to identify common genetic variants influencing blood pressure^{11–13}, which may reflect the complex nature of blood pressure. In contrast to previous studies, we used an intermediate trait, natriuretic peptide concentrations, to select a limited number of SNPs a priori to carry forward into blood pressure analyses. Each positive association satisfied the hypothesis that variants leading to higher natriuretic peptide concentrations would be associated with lower blood pressure.

Although it is known that disruption of the natriuretic peptide axis in genetically engineered animals or infusion of large doses of these molecules in experimental protocols alters blood pressure, relevance to normal human physiology has not been previously established. Indeed, natriuretic peptide concentrations in healthy individuals are orders of magnitude lower than those observed in either experimental models or individuals with heart failure¹⁴, and the effects of small variations in natriuretic peptide concentrations at this low end of the range are unknown.

The present study uses the natural experiment of the random mendelian assortment of alleles to show that genetically determined alterations in natriuretic peptide concentrations are associated with changes in blood pressure. Compared with other studies based on the

Nppb in mice⁸. An alternative explanation is that rs632793 is associated with an altered form of BNP with less biological activity.

Because counter-regulatory effects of blood pressure on natriuretic peptide production could mask the primary effect of genetic variants on blood pressure, we also repeated the blood pressure association analyses after adjustment for concurrent ANP concentrations. In models adjusted for ANP, rs5068 and rs198358 showed stronger association with blood pressure, particularly systolic blood pressure (rs5068 $P = 1 \times 10^{-8}$, rs198358 $P = 7 \times 10^{-7}$, **Table 3**). These models seem to adjust for the counter-regulatory changes in natriuretic peptide concentrations that result from a genetically determined decrease in blood pressure.

We sought additional validation by genotyping rs5068 and rs198358 in 14,516 subjects in the Malmö Preventive Project (stage 4). The minor alleles of rs5068 and rs198358 were associated with lower systolic and diastolic blood pressures in this cohort (**Table 3**), with clear replication of the rs5068 association (cohort-specific $P = 0.005$ for systolic blood pressure, $P = 1 \times 10^{-4}$ for diastolic blood pressure). Incorporation of the results into the overall meta-analysis led to strengthened associations for rs5068 (systolic, $P = 2 \times 10^{-6}$; diastolic, $P = 1 \times 10^{-6}$). The minor alleles of rs5068 and rs198358 were associated with reductions of 0.9–1.5 mm

Table 3 Association of confirmed natriuretic peptide variants with blood pressure (stages 3 and 4)

| SNP ID | Stage 3 Blood pressure, samples 1–4 <i>N</i> = 15,201 | | | | Stage 4 Blood pressure, sample 5 <i>N</i> = 14,516 | | | | Stage 3 + Stage 4 Blood pressure, samples 1–5 <i>N</i> = 29,717 | | | |
|------------------|---|--------------------|------------------|--------------------|--|----------------|------------------|--------------------|---|--------------------|------------------|--------------------|
| | SBP | | DBP | | SBP | | DBP | | SBP | | DBP | |
| | β (s.e.m.) | <i>P</i> value | β (s.e.m.) | <i>P</i> value | β (s.e.m.) | <i>P</i> value | β (s.e.m.) | <i>P</i> value | β (s.e.m.) | <i>P</i> value | β (s.e.m.) | <i>P</i> value |
| rs5068 | –0.09 (0.02) | 1×10^{-4} | –0.07 (0.02) | 0.002 | –0.07 (0.02) | 0.005 | –0.09 (0.02) | 1×10^{-4} | –0.08 (0.02) | 2×10^{-6} | –0.08 (0.02) | 1×10^{-6} |
| Adjusted for ANP | | 1×10^{-8} | | 0.001 | – | | – | | – | | – | |
| rs198358 | –0.07 (0.02) | 5×10^{-5} | –0.06 (0.02) | 4×10^{-4} | –0.03 (0.02) | 0.10 | –0.04 (0.02) | 0.03 | –0.05 (0.01) | 6×10^{-5} | –0.05 (0.01) | 5×10^{-5} |
| Adjusted for ANP | | 7×10^{-8} | | 4×10^{-4} | – | | – | | – | | – | |
| rs632793 | –0.02 (0.01) | 0.16 | –0.02 (0.01) | 0.12 | – | | – | | – | | – | |

Association results for systolic and diastolic blood pressure (SBP, DBP) adjusted for age, sex and body mass index, are shown for rs5068, rs198358 and rs632793 in stage 3 (samples 1–4), and for rs5068 and rs198358 in an independent sample in stage 4 (sample 5, $n = 14,516$), as well as summary meta-analysis results in the combined stage 3 and stage 4 samples (samples 1–5). In stage 3 samples, analyses were repeated with additional adjustment for ANP. Blood pressure associations (stages 3 and 4) were tested under the genetic model most supported by the natriuretic peptide concentration association results (**Table 2**). For rs5068 and rs198358 a dominant model was used, and for rs632793 an additive model was used. β , effect estimate from linear regression; dash (–), not available.

Table 4 Association of confirmed natriuretic peptide variants with hypertension

| SNP | Hypertension FHS, MDC, Finrisk97, N = 15,201 | | | Hypertension FHS, MDC, Finrisk97, MPP, N = 29,717 | | |
|----------|--|-----------|--------------------|---|-----------|--------------------|
| | OR | 95% CI | P value | OR | 95% CI | P value |
| rs5068 | 0.84 | 0.76–0.93 | 7×10^{-4} | 0.85 | 0.79–0.92 | 4×10^{-5} |
| | With adjustment for ANP | | 2×10^{-5} | | | |
| rs198358 | 0.87 | 0.80–0.94 | 2×10^{-4} | 0.90 | 0.85–0.95 | 2×10^{-4} |
| | With adjustment for ANP | | 4×10^{-5} | | | |

Shown are results of association of SNPs with dichotomous hypertension, defined as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg or use of antihypertensive therapy. Hypertension was adjusted in logistic regression models for age, sex and body mass index, with or without adjustment for ANP. ANP was not available in the Malmö Preventive Project, so results including this sample are shown separately.

concept of mendelian randomization¹⁵, our investigation is unique in that the biomarker (ANP) and the clinical trait (blood pressure) lie in a feedback loop, such that natriuretic peptides exert a negative influence on systolic blood pressure, but systolic blood pressure has a positive effect on natriuretic peptide release. Use of genetic variants, clearly upstream of both natriuretic peptide concentrations and blood pressure, eliminates the risk of confounding or an inference of reverse causality from this feedback loop.

Although most of the previous studies relating natriuretic peptide-encoding genes to human blood pressure variation have been small and inconclusive, recently two larger studies reported associations between missense variants in *NPPA* and incident hypertension¹⁶ and response to diuretic therapy¹⁷, but statistical significance was modest and the studies lacked replication. Another study has reported that a missense variant in *CORIN* (which cleaves pro-ANP and pro-BNP) is associated with blood pressure in African American samples¹⁸.

Although the reductions in blood pressure associated with the minor alleles of rs5068 and rs198358 may seem modest, even a 1 mm Hg decrement in systolic blood pressure is associated with an 8% lower risk of death from stroke or ischemic heart disease in observational studies³. Furthermore, potentially lifelong exposure to changes in blood pressure, as can occur due to differences in genotype, could magnify these effects.

Therapeutic agents that chronically activate the natriuretic peptide system are under active development. Our finding that genetic variation found associated with natriuretic peptide concentrations was also associated with blood pressure and hypertension suggests that these agents might prove useful for the treatment of hypertension. However, clinical and mechanistic studies with in-depth phenotyping are necessary to explore the role of these genetic variants in blood pressure regulation and cardiovascular physiology.

METHODS

Study design. The Framingham Heart Study is an epidemiologic study that includes an offspring cohort enrolled in 1971 (ref. 19). The Framingham participants are predominantly of European ancestry. DNA from 1,809 unrelated participants attending a routine examination of the offspring cohort (1995–1998) was available for genetic analyses in the first stage (sample 1, 'Framingham Unrelated'). Selected SNPs with nominal evidence of association with natriuretic peptide concentrations in the first stage underwent genotyping in a second stage, comprising three independent study samples: a family-based sample from Framingham (sample 2, 'Framingham Related')²⁰, unrelated participants in the Malmö Diet and Cancer-Cardiovascular Arm (sample 3)²¹ and unrelated participants from the Finrisk97 cohort (sample 4)²². All stage 2 Framingham individuals were unrelated to stage 1 individuals. For stage 3, three SNPs were carried over from stage 2 to examine the association with blood pressure in all four samples. In stage 4, an additional set of individuals from the Malmö Preventive Project (sample 5) was added to samples 1–4 to test the relationship of two SNPs with blood pressure and hypertension²³. The Malmö Preventive Project baseline examination (1974–1992) was used in

continuous blood pressure analyses because of the lower rates of treatment (4%), and the follow-up examination (2002–2006) was used in hypertension analyses because of the higher prevalence of hypertension.

Subjects in stages 1 and 2 were excluded if they had prior heart failure, missing data on natriuretic peptide concentrations or DNA or, in Framingham, serum creatinine > 2.0 mg/dl. After exclusions, sample sizes were 1,705 (sample 1), 751 (sample 2), 5,196 (sample 3), 7,091 (sample 4) and 14,516 (sample 5). An additional 458 subjects in Finrisk97 with data on blood pressure but not natriuretic peptide concentrations were available for stage 3. A study design overview is shown in **Supplementary Figure 1**. Studies were approved by local institutional review boards. All subjects gave written informed consent.

Clinical evaluation. Natriuretic peptide assays are described in the **Supplementary Methods**. Risk factor ascertainment in the Framingham Heart Study, Malmö Diet and Cancer, Finrisk97 and Malmö Preventive Project is described elsewhere^{22–25}. Clinical variables were measured contemporaneously with the blood draws for natriuretic peptides. Blood pressures in Framingham and Finrisk97 were averaged from two measures using a mercury column sphygmomanometer in seated participants resting for at least 5 min²⁶. In Malmö Diet and Cancer, blood pressure was measured once using a mercury column sphygmomanometer in supine individuals after 10 min of rest²⁷. In the Malmö Preventive Project, blood pressure was the average of two supine and two standing measures, 10 min apart²³.

SNP selection and genotyping. We characterized linkage disequilibrium patterns by genotyping SNPs across the *NPPA-NPPB* locus in 96 independent CEPH chromosomes and selected 13 tag SNPs for genotyping in stage 1 (**Fig. 1**, **Supplementary Methods** and **Supplementary Table 1**). All SNPs genotyped in Framingham had call rates $> 85\%$ (average 96.8%). Genotyping in Framingham and Finrisk97 was done on the Sequenom platform, and in the Malmö cohorts with Taqman using 'assays by design'. After quality control, genotyping call rates for rs5068, rs198358 and rs632793 were $\geq 99.8\%$, 99.9% and 99.9% in Finrisk97, 96.9%, 96.4% and 97.6% in Malmö Diet and Cancer, and 93.1% and 98.1% in the Malmö Preventive Project (rs632793 was not genotyped in the Malmö Preventive Project). All SNPs were in Hardy-Weinberg equilibrium.

Statistical analysis. Natriuretic peptide concentrations were log-transformed. Residuals were obtained using sex-specific regression models in which natriuretic peptide concentrations were adjusted for age, body mass index, diabetes, systolic and diastolic blood pressure, antihypertensive therapy, myocardial infarction, atrial fibrillation (Framingham) and serum creatinine (Framingham)^{20,28}. As previously described²⁹, Tobit regression was used to generate residuals in Framingham, to account for left-censoring of BNP by the assay detection limit (4 pg/ml). Linear regression was used in Malmö Diet and Cancer and Finrisk97, because N-terminal proBNP distributions had minimal left-censoring. Covariate relations are shown in **Supplementary Table 2**.

The natriuretic peptide residuals were standardized (mean = 0, s.d. = 1). Using linear regression, residuals were screened for association in the Framingham Unrelated sample with individual SNPs using a 2 degree-of-freedom test (general model). Four SNPs were chosen for validation in stage 2, based on the strength of association and linkage disequilibrium patterns. Association testing was done in sample 2 using SAS PROC MIXED to account for familial correlations. In the other samples, which consisted of unrelated subjects, linear

regression was used. Data from all four samples were combined using fixed-effects meta-analysis with inverse-variance weights. Dominant, additive and recessive models were tested for rs198358 and rs632793, whereas only a dominant model was examined for rs5068 because of the small number of minor homozygotes.

For stage 3, association testing with blood pressure, we selected the three SNPs with convincing association with ANP, BNP or both in the first two stages. For each SNP, we identified the genetic model (dominant, additive or recessive) for the blood pressure analysis according to the model that showed the strongest association with natriuretic peptide concentrations. Blood pressure in treated individuals was imputed using an approach described previously⁴. Systolic and diastolic blood pressure residuals from regression on age, sex and body mass index were standardized, and results from all five samples were meta-analyzed using inverse-variance weights. The average weights for each study sample were 4% (sample 1), 1% (sample 2), 18% (sample 3), 34% (sample 4) and 43% (sample 5).

We used SAS v8.1 for analyses in Framingham and Finrisk97, and SPSS v15.0 for analyses in Malmö Diet and Cancer and Malmö Preventive Project. Two-sided *P* values are shown. Because cumulative meta-analysis is more powerful than a derivation-validation design, results at successive stages include meta-analysis of all samples tested cumulatively³⁰. In stage 1, we carried out 26 tests (13 SNPs, 1 genetic model, 2 natriuretic peptides) and therefore considered *P* < 0.002 (0.05/26) to be significant. In stage 2, we conducted 24 tests (4 SNPs, 3 genetic models, 2 natriuretic peptides) and considered *P* < 0.002 (0.05/24) to be significant. In stages 3 and 4, we did up to 6 tests (3 SNPs, 1 genetic model, 2 blood pressure traits) and considered *P* < 0.008 (0.05/6) to be significant.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

C.N.-C., M.G.L., O.M. and T.J.W. designed the study. C.N.-C., K.D.B., A.S., C.G., J.S., N.G.M., A.B., S.B., F.K. and O.M. generated the data. C.N.-C., M.G.L., X.Y. and O.M. were responsible for the analyses. C.N.-C. and T.J.W. drafted the manuscript. All authors contributed to interpretation of the data and critical review of the manuscript and approved the final version.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturegenetics/>.

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