Original Article

Genome-Wide Association Study on Plasma Levels of Midregional-Proadrenomedullin and C-Terminal-Pro-Endothelin-1


Abstract—Endothelin-1 (ET-1) and adrenomedullin (ADM) are circulating vasoactive peptides involved in vascular homeostasis and endothelial function. Elevated levels of plasma ET-1 and ADM, and their biologically stable surrogates, C-terminal-pro-endothelin-1 (CT-pro-ET-1) and midregional proadrenomedullin (MR-pro-ADM), are predictors of cardiac death and heart failure. We studied the association of common genetic variation with MR-pro-ADM and CT-pro-ET-1 by genome-wide association analyses in 3444 participants of European ancestry. We performed follow-up genotyping of single nucleotide polymorphisms (SNPs) that showed suggestive or significant association in the discovery stage in additional 3230 participants. The minor variants in KLKB1 (rs4253238) and F12 (rs2731672), both part of the kallikrein-kinin system, were associated with higher MR-pro-ADM (P=4.46E-52 and P=5.90E-24, respectively) and higher CT-pro-ET-1 levels (P=1.23E-122 and P=1.26E-67, respectively). Epistasis analyses showed a significant interaction between the sentinel SNP of F12 and KLKB1 for both traits. In addition, a variant near the ADM gene (rs2957692) was associated with MR-pro-ADM (P=1.05E-12) and a variant in EDN-1 (rs5370) was associated with CT-pro-ET-1 (P=1.49E-27). The total phenotypic variation explained by the genetic variants was 7.2% for MR-pro-ADM and 14.6% for CT-pro-ET-1. KLKB1 encodes plasma kallikrein, a proteolytic enzyme known to cleave high-molecular-weight kininogen to bradykinin and prorenin to renin. We cloned the precursors of ADM and ET-1 and demonstrated that purified plasma kallikrein can cleave these recombinant proteins into multiple smaller peptides. The discovery of genetic variants in the kallikrein-kinin system and in the genes encoding pre-pro-ET-1 and pro-pre-ADM provides novel insights into the (co-)regulation of these vasoactive peptides in the vascular system. (Hypertension. 2013;61:xxx-xxx.)

Key Words: adrenomedullin ■ blood pressure ■ cardiovascular ■ endothelin-1 ■ genome-wide association study ■ kallikrein

The endothelium (ET) plays a major role in maintaining vascular homeostasis by controlling blood fluidity, platelet aggregation, and (local) vascular tone. Control of vascular tone is mediated by the release of NO and various vasoactive peptides, including ET-1 and adrenomedullin (ADM). ET-1 is a 21-amino acid peptide secreted by endothelial cells and is known to be one of the most potent vasoconstrictors. Conversely, ADM, a 52-amino acid peptide hormone, is secreted by a variety of different cells and is a potent vasodilator. In addition to their direct effects on vascular tonus, both ADM and ET-1 are involved in the homeostasis of the sodium and water balance. Reliable measurements of plasma ET-1 and ADM are highly challenging because of their short half-life, existence of binding proteins, and other technical difficulties. Recently, assays have been developed to measure the biologically stable surrogates, C-terminal-pro-endothelin-1 (CT-pro-ET-1) and midregional-proadrenomedullin (MR-pro-ADM), which
are correlated with ET-1 and ADM in equimolar amounts.\textsuperscript{3,4} Increased plasma levels of both CT-pro-ET-1 and MR-pro-ADM have been associated with worse vascular function, cardiac death, and heart failure.\textsuperscript{5–9} It is unknown whether ADM have been associated with worse vascular function, Helsinki. This study adheres to the principles expressed in the Declaration of Center Groningen. All participants provided informed consent. This study adheres to the principles expressed in the Declaration of Helsinki.

Genotyping, Quality Control, and Imputation
Genotyping of 4016 participants in PREVEND was carried out using Illumina HumanCytoSNP-12 arrays. SNPs were called using Illumina Genome Studio software, and quality control was applied before and after imputation (see online-only Data Supplement). Replication genotyping of 19 SNPs was performed by KBiosciences (KBiosciences, Herts, UK) using the SNPline supplement. Replication genotyping of 19 SNPs was performed by KBiosciences (KBiosciences, Herts, UK) using the SNPline system in an additional 3230 independent participants of the PREVEND study.

Biochemical Measurements
Two commercially available, fully automated sandwich immunoassays were used for the measurement of CT-pro-ET-1 and MR-pro-ADM (BRAHMS CT-pro-ET-1 KRYPTOR and MR-pro-ADM KRYPTOR; BRAHMS AG, Hennigsdorf, Germany), according to the manufacturer’s instruction manuals. The design of these assays is based on immunoluminometric assays described previously.\textsuperscript{3,4} Assays were performed in EDTA-plasma aliquots taken at baseline. The samples were stored at −80°C before analysis. All blood samples were processed by personnel blinded from any patient data. A total of 3444 samples were available for the discovery analysis and 3230 for replication.

SNP/Gene Biology Functional Annotation
For identifying the likely candidate gene in each locus, we used the following information: we considered the nearest gene of the sentinel SNP, all nsSNPs in LD ($r^2$≥0.8 in the HapMap phase II CEU or 1000 Genomes) with the sentinel SNP, conservation among species (GERP and 29 mammals), and biological function. We used GRAIL analysis\textsuperscript{11} to perform a text-based analysis in abstracts on PubMed before December Analysis (to avoid confounding from GWAS results arising after that date).

Statistical Analysis
We used linear regression on untransformed MR-pro-ADM measures using an additive genetic model with age, sex, and body mass index as covariates. Linear regression was performed on untransformed CT-pro-ET-1 measures using an additive genetic model with age and sex as covariates. Genotype-phenotype analyses were performed using PLINK (version 1.07).\textsuperscript{12} The most significant (P<0.05) SNP (sentinel SNP) at each locus was taken forward into replication. For conditional analysis, the sentinel SNPs representing the replicated loci were added as covariates in the original association analysis. Epistasis between the sentinel SNPs of each trait were tested based on the model $Y=b_0+b_1.A+b_2.B+b_3.AB+. The model uses the allele dosage for each SNP, A and B, the interaction is described by the coefficient b3. The explained variance of the significant associations was analyzed using the directly genotyped SNPs from the replication phase. Fixed-effects meta-analysis was performed using the inverse-variance weighting method of the METAL package.

Synthesis of Pre-pro-adrenomedullin by In Vitro Transcription/Translation Systems
Pre-pro-ADM cDNA without the signal peptide (1230 base pairs encoding 410 amino acids) was PCR amplified using a human cDNA clone (Source Bioscience Life Sciences, Nottingham, UK). For amplification, the following primers were used: forward, CGTGAGATCCACCCTGAGCCGTCCTGGTGGATGTC and reverse, CGTGGGCGCGCCTAAAGAAAGTGGGGAGCACTTC. Pre-pro-ET-1 cDNA without the signal peptide (2061 base pairs encoding 687 amino acids) was also PCR amplified using a human cDNA clone (Source Bioscience Life Sciences). For amplification, the following primers were used: forward, CAGCGATCCACCCTGAGCCGTCCTGGTGGATGTC and reverse, CGTGGGCGCGCCTAAAGAAAGTGGGGAGCACTTC. The PCR fragments were cloned into the BamHI and NotI sites of a pcDNA3.1 expression vector (Invitrogen, Karlsruhe, Germany) containing a triple myc-tag. The myc-tag replaced the signal peptide (pre) sequence of pre-pro-ADM and pre-pro-ET-1. Recombinant tagged pre-pro-ADM, pre-pro-ET-1, and luciferase protein was produced using a TNT T7–coupled reticulocyte lysate system (Promega Corporation, Madison, WI) according to the manufacturer’s instructions. Luciferase control DNA (4331 base pairs) was included with the TNT T7–coupled reticulocyte lysate system as a control for the in vitro translation. 20 μCi [35S] methionine (Perkin Elmer NEN, Waltham) was added during transcription/translation reaction to generate radiolabeled myc-pro-ADM, myc-pro-ET-1, and luciferase. For purification of myc-pro-ADM and myc-pro-ET-1, anti-myc 9E10 agarose resin was used (Santa Cruz Biotechnology, Santa Cruz).

In Vitro Assay of Kallikrein Cleavage
Activated human plasma kallikrein was purified by Coachrom Diagnostica (Vienna, Austria) as previously described.\textsuperscript{13} D-Phe-Phe-Arg-chloromethylketone (PPACKII, Calbiochem, Darmstadt, Germany) was used as kallikrein inhibitor. PPACK II also possesses cross-reactivity with factor XII, but the inhibitory action is strongest for plasma kallikrein.\textsuperscript{14} As plasma kallikrein is separated from other proteases in plasma that could copurify, such as factors XI and XII, we do not expect this cross-reactivity to occur. Radiolabeled pre-ADM, pre-pro-ET-1, and luciferase were incubated with or without PPACKII (50 μg/mL) for 0, 5, 30, and 60 minutes at 37°C with 5 μg/mL activated purified plasma kallikrein. The reactions were stopped by adding sample buffer and subsequent heating for 5 minutes at 95°C. Proteins were separated by SDS-PAGE. The gel was exposed to a phosphorimager screen after drying. The radiolabeled protein fragments in the gel were visualized using a Cyclone PhosphorImager (Packard Instruments, Meriden).

Results
GWAS and Follow-up Genotyping
To identify common genetic variants associated with MR-pro-ADM and CT-pro-ET-1 levels, we performed a genome-wide analyses of 2 269 099 genotyped or imputed autosomal SNPs catalogued in HapMap CEU panel in 3444 participants from
the PREVEND cohort (Table S1 in the online-only Data Supplement). Test statistic inflation showed no evidence for population stratification or admixture (genomic control $\lambda_{GC}=1.021$ MR-pro-ADM, $\lambda_{GC}=1.039$ CT-pro-ET-1; Figure S1). We observed significant associations at 3 loci for MR-pro-ADM ($P<5\times10^{-8}$) and at 3 loci for CT-pro-ET-1. Two of the loci (SNPs in or near KLKB1 and F12) were the same for both traits. Each trait also had significant associations at 1 additional trait-specific locus, SNPs in EDN-1 for CT-pro-ET-1 and SNPs near ADM for MR-pro-ADM (Figure 1, Table; and Figure S2 for corresponding regional plots). At each of the genome-wide significant locus, we determined the SNP with the lowest $P$ value (sentinel SNP) and carried out genotyping of these SNPs in 3230 additional samples of the PREVEND cohort that did not have GW A data available (Table). For each SNP, the total evidence of association was calculated using inverse-variance fixed-effect meta-analysis in METAL.15 The variants at the 4 loci with $P<5\times10^{-8}$ in the discovery phase were confirmed in further genotyping (Table). The sentinel SNPs at the 4 loci were included as covariates in conditional analyses for each trait to determine whether there were independent associations at these loci. These analyses identified no secondary signals. The total variance explained by the genome-wide significant variants was 6.7% for MR-pro-ADM and 14.3% for CT-pro-ET-1 levels (Table 3). Additional epistasis analyses were carried out by testing pairwise combinations between the 3 sentinel SNPs of CT-pro-ET-1 and between the 3 sentinel SNPs of MR-pro-ADM. We observed a significant interaction between the sentinel SNPs of the KLKB1 and F12 loci (Table S2). The total phenotypic variation explained

<table>
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<tr>
<th>Locus</th>
<th>Trait</th>
<th>CHR</th>
<th>SNP</th>
<th>A1/A2</th>
<th>Discovery, $n=3230$</th>
<th>Replication, $n=3444$</th>
<th>Combined, $n=6674$</th>
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<tbody>
<tr>
<td>1</td>
<td>ADM</td>
<td>4</td>
<td>rs4253238</td>
<td>C/T</td>
<td>0.027 (0.003) 6.93E-24</td>
<td>0.034 (0.003) 2.76E-34</td>
<td>4.46E-52 KLKB1</td>
</tr>
<tr>
<td>2</td>
<td>ET</td>
<td>4</td>
<td>rs4253238</td>
<td>C/T</td>
<td>4.811 (0.305) 4.07E-54</td>
<td>5.476 (0.312) 5.49E-66</td>
<td>1.23E-122 KLKB1</td>
</tr>
<tr>
<td>3</td>
<td>ADM</td>
<td>5</td>
<td>rs2731672</td>
<td>T/C</td>
<td>0.024 (0.003) 7.07E-14</td>
<td>0.020 (0.003) 6.19E-10</td>
<td>5.90E-24 F12</td>
</tr>
<tr>
<td>4</td>
<td>ET</td>
<td>6</td>
<td>rs5370*</td>
<td>T/G</td>
<td>2.928 (0.379) 1.38E-14</td>
<td>2.983 (0.390) 2.49E-14</td>
<td>1.49E-27 EDN-1</td>
</tr>
</tbody>
</table>

$\beta$ values estimate the difference in concentrations (C-terminal-pro-endothelin-1 [CT-pro-ET-1] in pmol/L, midregional-proadrenomedullin [MR-pro-ADM] in nmol/L) per copy of the coded allele, adjusted for the covariates in the model. SNP indicates single nucleotide polymorphism.

*Nonsynonymous SNP.

Figure 1. Manhattan plot of (A) C-terminal-pro-endothelin-1 (CT-pro-ET-1) and (B) midregional-proadrenomedullin (MR-pro-ADM).
slightly increased to 7.2% for MR-pro-ADM and 14.6% for CT-pro-ET-1 by including the interaction term of the KLKB1 and F12 SNPs.

We also brought 12 loci forward for replication that showed suggestive evidence for association ($P >5\times10^{-8}$ and $P <1\times10^{-4}$) with MR-pro-ADM or CT-pro-ET-1. However, none of these loci became significantly associated after genotyping in the additional 3230 samples of the PREVEND cohort (Table S3).

Identification of Candidate Genes and Putative Causal Genetic Variants

The peptides CT-pro-ET-1 and MR-pro-ADM were both associated with SNPs located in the locus that contained the gene encoding for the precursor of the peptides. CT-pro-ET-1 was associated with SNPs at a locus harboring 5 genes within 1 MB of the sentinel SNP (rs3570). rs3570 is a nonsynonymous SNP (nsSNP) located in the endothelin-1 (EDN-1) gene. MR-pro-ADM was associated with SNPs at a locus harboring 13 genes within 1 MB of the sentinel SNP (rs2957692); this SNP is closest to the ADAM encoding gene (ADM).

We examined all nsSNPs that are in LD ($r^2>0.8$) with one or more of the sentinel SNPs in the HapMap phase II or 1000 genome CEU data sets. We identified 1 nsSNP (rs3733402) in the KLKB1 gene in full LD ($r^2=1.00$) with the sentinel SNP. We performed wet-laboratory genotyping of the rs3733402 variant in 3230 participants and confirmed its strong association with both CT-pro-ET-1 and MR-pro-ADM levels (Table). The nsSNP rs3733402 is located in KLKB1 providing a potential biological mechanism. The nsSNP (rs3733402) in KLKB1 by itself explains $4.7\%$ of variance of MR-pro-ADM and $8.6\%$ of CT-pro-ET-1 plasma levels. rs2731672 lays 5.8 kb from F12 and is in LD ($r^2=1$) with rs1801020 that is located in a highly conserved region within the 5’ untranslated region part of F12, making it a potential candidate. F12 is also a biological plausible candidate when considering the KLKB1 locus because both genes are part of the kallikrein-kinin system (KKS) and have interactions with each other on a molecular level. GRAIL literature mining tool based on publications before 2006 also suggested ADM, EDN-1, KLKB1, and F12 as candidate genes ($P<0.01$).

Novel Role of KLKB1 on pro-ADM and pro-ET-1 Cleavage

We tested the hypothesis that KLKB1 is the causal gene related to the observed association with MR-pro-ADM and CT-pro-ET-1 trough cleavage because (1) KLKB1 encodes a cleavage protein with cleavage sites that overlap with cleavage sites of the precursors of MR-pro-ADM and CT-pro-ET-1, (2) previous GWA publications associated bradykinin19 and renin levels20 to the same variant, and (3) the sentinel SNP is in full LD with a coding SNP21 affecting the proteolytic activity of plasma kallikrein. For this purpose, we designed an in vitro assay. The ADM cDNA and EDN-1 cDNA were cloned into a pcDNA3.1 expression vector and generated [35S]-methionine-labeled recombinant proteins by in vitro transcription/translation. We incubated recombinant pro-ADM and pro-ET-1 proteins with active purified plasma kallikrein (human) and observed a time-dependent cleavage of pro-ADM and pro-ET-1 to multiple smaller peptides. This reaction was completely inhibited by a kallikrein inhibitor (Figure 2). Cleavage kinetics was also dependent on the kallikrein concentration (data not shown). In contrast, luciferase was used as a positive control DNA for the in vitro translation assay was not cleaved by plasma kallikrein (Figure 2).

Discussion

Using GWA analyses and functional in vitro follow-up, we identified a novel role for plasma kallikrein in the regulation of both MR-pro-ADM and CT-pro-ET-1 (Figure 1). We identified 2 loci harboring components of the KKS: KLKB1 on chromosome 4 and F12 on chromosome 5. In addition, we observed a strong epistatic effect between the genetic variants at these loci. We also found an association with a SNP near ADM with MR-pro-ADM and an nsSNP in EDN-1 with CT-pro-ET-1. The total phenotypic variation explained by all the significant genetic variants was high; 7.2% for MR-pro-ADM and 14.6% for CT-pro-ET-1.

KLKB1 encodes plasma prekallikrein and is activated to plasma kallikrein by factor XIIa. Factor XIIa is the active form of factor XII and is encoded by F12. Plasma kallikrein and factor XII colocalize to endothelial cells.
Plasma kallikrein plays a key role in the precursor maturation of other cardiovascular peptides, such as bradykinin and renin by proteolysis. The KLKB1 locus has also been associated with circulating bradykinin and renin levels by GWA studies (\(r^2=1.00\) with our lead SNP). We found the sentinel SNP at the KLKB1 locus to be in complete LD (\(r^2=1.00\)), with an nsSNP (rs3733402) causing an asparaginase to serine amino acid substitution (at position 124), located within the functional catalytic domain of plasma kallikrein. This amino acid substitution is known to modify the proteolytic activity of kallikrein, suggesting that the KLKB1 locus is associated with MR-pro-ADM and CT-pro-ET-1 because of differences in cleavage, similar to bradykinin and renin.

Human pre-pro-ET-1, consisting of 212 amino acids, is cleaved into various peptides: pre-pro-ET-17 to 53, Big endothelin (pre-pro-ET43-92), pre-pro-ET90-168, and CT-pro-ET-1 (pre-pro-ET-168 to 203). Pre-pro-ADM consists of 185 amino acids, which can be cleaved into 4 known peptides: proadrenomedullin N-terminal 20 peptide (pre-pro-ADM22–41), MR-pro-ADM (pre-pro-ADM45–91), ADM (pre-pro-ADM94–143), and ADT (adrenotensin; pre-pro-ADM150–185). The cleavage sites involved in the bioprocessing of pre-pro-ET-1 and pre-pro-ADM-derived peptides include arg-arg and lys-arg sequences (see Figure 3 for a schematic overview), which are among the specific recognition sites of plasma kallikrein. On these grounds, we hypothesized that plasma kallikrein is involved in the bioprocessing of pre-pro-ET-1 and pre-pro-ADM derived peptides. Using a custom designed in vitro assay, we provide preliminary data that purified plasma kallikrein cleaves pro-ADM and pro-ET-1 into smaller fragments (Figure 2). The exact cleavage products remain to be identified, and future studies are warranted to clarify putative physiological mechanisms between plasma kallikrein and these peptides. Luciferase was not cleaved by plasma kallikrein, indicating plasma kallikrein substrate specificity.

The KKS is tightly coordinated through multiple complex interactions with the renin-angiotensin-aldosterone system. Interestingly, ADM and ET-1 are also suspected to interact with the renin-angiotensin-aldosterone system. The KKS and renin-angiotensin-aldosterone system are involved in a multitude of physiological and pathophysiological actions that impact salt sensitivity, blood flow, and vascular reactivity, similar to the actions of ADM and ET-1. It is suggested that the plasma KKS operates at the level of individual tissues and is dependent on the local production of its substrates, which is also in concordance with the local production and action of ADM and ET-1.

In addition to the KLKB1 and F12 loci, we identified genetic variants at the ADM locus to be associated with MR-pro-ADM levels and SNPs at the END-1 locus with CT-pro-ET-1 levels. The variant (rs2957692) identified near the ADM locus is not in LD (\(r^2=0.00\)) with a previously reported variant in ADM (rs4910118) identified in an earlier candidate gene study. Recently, the ADM locus was reported to be associated with systolic blood pressure, our sentinel SNP is low LD (\(r^2<0.2\)) with this locus. Whether this locus is independent requires further conditional analyses. Our sentinel SNP at the END-1 locus (rs5370) is an nsSNP located in the CT-pro-ET-1 part of the ET-1 coding gene. We cannot exclude this is a false-positive association as the nsSNP is located 2 amino acids downstream from the epitope of the CT-pro-ET-1-tracer, this may affect the antibody-epitope affinity, \(r^2=0.00\) with this Locus. Whether this locus is independent requires further conditional analyses.

In summary, using GWAS and in vitro functional follow-up, we report the involvement of the KKS system in the regulation of MR-pro-ADM and CT-pro-ET-1 plasma levels. Preliminary functional data support the hypothesis that the precursors are ligands for plasma kallikrein. Future functional studies should further characterize the potentially complex regulations of these peptides by the KKS. We also found associations between MR-pro-ADM and CT-pro-ET-1 plasma levels and the genes encoding their precursors.

**Perspectives**

ET-1 and ADM are circulating vasoactive peptides involved in vascular homeostasis and endothelial function. In this study, we identified 4 common variants to influence plasma levels of MR-pro-ADM and CT-pro-ET-1. The common variants in ADM and END-1 should be evaluated further to assess how MR-pro-ADM and CT-pro-ET-1 levels are related to disease.
and whether they should be considered a target for therapy. In addition, the variants of the KKS (KLKB1 and F12) could be used in a Mendelian randomization study to assess the potential of plasma kallikrein as a novel therapeutic target, as suggested recently.11

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Disclosures
J. Struck is employed by BRAHMS GmbH, a company manufacturing and holding patent rights on the MR-pro-ADM and CT-pro-ET-1 assays. The authors have no conflicts to report.

References
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**Novelty and Significance**

**What Is New?**
- We performed a genome-wide association study for genetic variants influencing C-terminal-pro-endothelin-1 and midregional-proadrenomedullin levels.

**What Is Relevant?**
- Single nucleotide polymorphism located in *EDN1* and near *ADM* were associated with C-terminal-pro-endothelin-1 and midregional-proadrenomedullin as might be expected.

**Summary**
- Genetic variants at *KLKB1* and *F12* were associated with both C-terminal-pro-endothelin-1 and midregional-proadrenomedullin. *KLKB1* and *F12* are components of the kallikrein-kinin system, known to be involved in the bioprocessing of other vasoactive peptides.

The discovery of genetic variants in the kallikrein-kinin system and in the genes encoding pre-pro-ET-1 and pre-pro-ADM provides novel insight on how vasoactive peptides are being (co-)regulated within the vascular system.
Genome-Wide Association Study on Plasma Levels of Midregional-Proadrenomedullin and C-Terminal-Pro-Endothelin-1


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ONLINE SUPPLEMENT

PREKALLIKREIN AND FACTOR 12 ARE ASSOCIATED WITH PLASMA LEVELS OF MID-REGIONAL-
PROADRENOMEDULLIN AND C-TERMINAL-PROENDOTHELIN-1

Short title: GWAS on MR-proADM and CT-proET-1 peptides

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Online Methods

Genotyping, quality control & imputation

Genotyping of 4,016 participants in PREVEND was carried out using Illumina HumanCytoSNP-12 arrays. SNPs were called using Illumina Genome Studio software. 47 samples with call rates <0.95 were excluded. We excluded another 65 closely related participants based on Identity-By-Descent estimated using PLINK v1.07. Population structure was assessed using PCA based on 16,842 independent SNPs. Based on this analysis, an additional 2 samples were excluded that diverged from the mean with at least 3 standard deviations (Z-score > 3) for the first 5 PCAs. Another 35 participants were excluded based on sex inconsistencies. Of 421 participants no phenotype was available, as a result 3,444 (1,663 men, 1,781 women) were available for GWAS analysis. SNPs were excluded with a minor allele frequency of <0.01, call rate <0.95, or deviation from Hardy Weinberg equilibrium (P<5E). Genome wide genotype imputation was performed using Beagle v. 3.3.1 30, 232,571 genotyped SNPs were imputed up to 2,269,099 million autosomal SNPs with NCBI build 36 of Phase II HapMap CEU data (release 22) as reference panel.

Software

Genome Wide Association analysis was performed using PLINK (Version 1.07, http://pngu.mgh.harvard.edu/~purcell/plink/), Manhattan and QQ plots were plotted in R (v2.11.1, http://www.r-project.org/), additional regression analyses were done using STATA MP 11. (Texas, USA). Regional association plots were generated using LocusZoom Stand-alone package (http://genome.sph.umich.edu/wiki/LocusZoom_Standalone) and LD structures were visualized in Haploview 4.2 utilizing LD information from HapMap phase II CEU (r22). METAL (version 2011-03-25, www.sph.umich.edu/csg/abecasis/metal) was used for the meta analysis.
Table S1: Baseline characteristics of the PREVEND population, divided into GWAS discovery and replication groups.

<table>
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<tr>
<th>Characteristics</th>
<th>Total N=6,674</th>
<th>GWAS N=3,444</th>
<th>Replication N=3,230</th>
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<tbody>
<tr>
<td>MR-proADM (nmol/l), min-max</td>
<td>0.01-1.34</td>
<td>0.01-1.14</td>
<td>0.01-1.34</td>
</tr>
<tr>
<td>MR-proADM (nmol/l)</td>
<td>0.39+0.13</td>
<td>0.39+0.13</td>
<td>0.38+0.14</td>
</tr>
<tr>
<td>CT-proET (pmol/l), min-max</td>
<td>0.6-103.1</td>
<td>1.2-103.1</td>
<td>0.6-90.6</td>
</tr>
<tr>
<td>CT-proET (pmol/l)</td>
<td>34.9+13.9</td>
<td>35.0+13.8</td>
<td>34.7+14.1</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>49+12</td>
<td>49+12</td>
<td>49+13</td>
</tr>
<tr>
<td>Men (%)</td>
<td>47.2</td>
<td>48.3</td>
<td>46.0</td>
</tr>
<tr>
<td>Caucasians (%)</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>BMI</td>
<td>26+4</td>
<td>26+4</td>
<td>26+4</td>
</tr>
<tr>
<td>Waist-Hip Ratio</td>
<td>0.88+0.09</td>
<td>0.88+0.10</td>
<td>0.87+0.09</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>128+20</td>
<td>128+19</td>
<td>128+20</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>74+10</td>
<td>74+10</td>
<td>74+09</td>
</tr>
</tbody>
</table>
Table S2: Explained variance of genotyped SNPs on MR-proADM and CT-proET-1 levels in 3230 participants. Epistasis analysis between the leads SNPs KLKB1 and F12 showed significant addition to the explained variance, EDN-1 and ADM did not show any interaction with one of the other sentinel SNPs. Beta values estimate the difference in concentrations (CT-proET-1 in pmol/l, MR-proADM in nmol/l) per copy of the coded allele, adjusted for the covariates in the model.

<table>
<thead>
<tr>
<th>Description</th>
<th>Gene</th>
<th>MR-proADM R2</th>
<th>P</th>
<th>Beta(se)</th>
<th>CT-proET-1 R2</th>
<th>P</th>
<th>Beta(se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4253238</td>
<td>KLKB1</td>
<td>0.045</td>
<td>2.76E-34</td>
<td>0.034(0.003)</td>
<td>0.086</td>
<td>5.49E-66</td>
<td>5.476(0.312)</td>
</tr>
<tr>
<td>rs3733402 (coding snp)</td>
<td>KLKB1</td>
<td>0.047</td>
<td>1.88E-35</td>
<td>0.035(0.003)</td>
<td>0.086</td>
<td>1.37E-65</td>
<td>5.501(0.314)</td>
</tr>
<tr>
<td>rs2731672</td>
<td>F12</td>
<td>0.012</td>
<td>6.19E-10</td>
<td>0.020(0.003)</td>
<td>0.037</td>
<td>2.92E-28</td>
<td>4.178(0.375)</td>
</tr>
<tr>
<td>rs5370</td>
<td>EDN-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.018</td>
<td>2.49E-14</td>
<td>2.983(0.390)</td>
</tr>
<tr>
<td>rs2957692</td>
<td>ADM</td>
<td>0.006</td>
<td>9.87E-06</td>
<td>0.013(0.003)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total variance explained without epistasis</td>
<td>6.67%</td>
<td>14.28%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4253238 x rs2731672</td>
<td>KLKB1 x F12</td>
<td>0.063</td>
<td>7.56E-05</td>
<td>-0.018(0.005)</td>
<td>0.127</td>
<td>3.22E-04</td>
<td>-1.823(0.506)</td>
</tr>
<tr>
<td>rs3733402 x rs2731672</td>
<td>KLKB1 x F12</td>
<td>0.064</td>
<td>1.67E-04</td>
<td>-0.017(0.005)</td>
<td>0.127</td>
<td>1.03E-03</td>
<td>-1.678(0.623)</td>
</tr>
<tr>
<td>Total variance explained with epistasis</td>
<td>7.16%</td>
<td>14.61%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table S3:** Summary of all genetic variants from the discovery phase that were selected to be genotyped in the replication phase. Beta values estimate the difference in concentrations (CT-proET-1 in pmol/l, MR-proADM in nmol/l) per copy of the coded allele, adjusted for the covariates in the model.

<table>
<thead>
<tr>
<th>Trait</th>
<th>CHR</th>
<th>SNP</th>
<th>A1/A2</th>
<th>Nearest gene</th>
<th>Discovery Beta (se)</th>
<th>P</th>
<th>Replication Beta (se)</th>
<th>P</th>
<th>FRQ</th>
<th>Combined Beta (se)</th>
<th>P-value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM 1</td>
<td>1</td>
<td>rs1501550</td>
<td>A/G</td>
<td>KCNH1</td>
<td>-0.014(0.003)</td>
<td>2.31E-06</td>
<td>-0.047(0.039)</td>
<td>9.06E-01</td>
<td>0.21</td>
<td>-0.011(0.002)</td>
<td>2.41E-06</td>
<td>6,550</td>
</tr>
<tr>
<td>ADM 2</td>
<td>1</td>
<td>rs10176670</td>
<td>G/T</td>
<td>TRIB2</td>
<td>-0.014(0.003)</td>
<td>6.91E-06</td>
<td>-0.003(0.003)</td>
<td>2.88E-01</td>
<td>0.49</td>
<td>-0.008(0.002)</td>
<td>7.38E-05</td>
<td>6,604</td>
</tr>
<tr>
<td>ET</td>
<td>3</td>
<td>rs1583673</td>
<td>T/C</td>
<td>GBE1</td>
<td>-1.974(0.438)</td>
<td>6.91E-06</td>
<td>-0.298(0.346)</td>
<td>3.88E-01</td>
<td>0.35</td>
<td>-0.941(0.271)</td>
<td>5.22E-04</td>
<td>6,687</td>
</tr>
<tr>
<td>ADM 4</td>
<td>4</td>
<td>rs4253238</td>
<td>C/T</td>
<td>KLKB1</td>
<td>0.027(0.003)</td>
<td>6.93E-24</td>
<td>0.034(0.003)</td>
<td>2.76E-34</td>
<td>0.46</td>
<td>0.031(0.002)</td>
<td>4.46E-52</td>
<td>6,673</td>
</tr>
<tr>
<td>ET</td>
<td>4</td>
<td>rs4253238</td>
<td>C/T</td>
<td>KLKB1</td>
<td>4.811(0.305)</td>
<td>4.07E-54</td>
<td>5.476(0.312)</td>
<td>5.49E-66</td>
<td>0.46</td>
<td>5.136(0.218)</td>
<td>1.23E-12</td>
<td>6,692</td>
</tr>
<tr>
<td>ADM 4</td>
<td>4</td>
<td>rs1912826</td>
<td>G/A</td>
<td>KLKB1</td>
<td>0.027(0.003)</td>
<td>6.61E-24</td>
<td>0.034(0.003)</td>
<td>1.37E-33</td>
<td>0.46</td>
<td>0.031(0.002)</td>
<td>2.07E-51</td>
<td>6,630</td>
</tr>
<tr>
<td>ET</td>
<td>4</td>
<td>rs1912826</td>
<td>G/A</td>
<td>KLKB1</td>
<td>4.813(0.306)</td>
<td>5.06E-54</td>
<td>5.429(0.313)</td>
<td>1.03E-64</td>
<td>0.46</td>
<td>5.114(0.219)</td>
<td>5.13E-12</td>
<td>6,650</td>
</tr>
<tr>
<td>ADM 4</td>
<td>4</td>
<td>rs3733402</td>
<td>NA</td>
<td>KLKB1</td>
<td>NA</td>
<td>NA</td>
<td>0.035(0.003)</td>
<td>1.88E-35</td>
<td>0.46</td>
<td>0.035(0.003)</td>
<td>2.71E-36</td>
<td>3,216</td>
</tr>
<tr>
<td>ET</td>
<td>4</td>
<td>rs3733402</td>
<td>NA</td>
<td>KLKB1</td>
<td>NA</td>
<td>NA</td>
<td>5.501(0.314)</td>
<td>1.37E-65</td>
<td>0.46</td>
<td>5.501(0.314)</td>
<td>1.41E-68</td>
<td>3,236</td>
</tr>
<tr>
<td>ADM 5</td>
<td>5</td>
<td>rs2545801</td>
<td>T/C</td>
<td>F12</td>
<td>0.024(0.003)</td>
<td>6.38E-14</td>
<td>0.02(0.003)</td>
<td>1.16E-09</td>
<td>0.24</td>
<td>0.022(0.002)</td>
<td>4.32E-22</td>
<td>6,569</td>
</tr>
<tr>
<td>ET</td>
<td>5</td>
<td>rs2545801</td>
<td>T/C</td>
<td>F12</td>
<td>4.99(0.372)</td>
<td>4.13E-40</td>
<td>4.192(0.379)</td>
<td>6.18E-28</td>
<td>0.24</td>
<td>4.599(0.265)</td>
<td>2.67E-67</td>
<td>6,618</td>
</tr>
<tr>
<td>ADM 5</td>
<td>5</td>
<td>rs2731672</td>
<td>T/C</td>
<td>F12</td>
<td>0.024(0.003)</td>
<td>7.07E-14</td>
<td>0.02(0.003)</td>
<td>6.19E-10</td>
<td>0.24</td>
<td>0.022(0.002)</td>
<td>5.90E-24</td>
<td>6,658</td>
</tr>
<tr>
<td>ET</td>
<td>5</td>
<td>rs2731672</td>
<td>T/C</td>
<td>F12</td>
<td>5.041(0.375)</td>
<td>3.85E-40</td>
<td>4.178(0.375)</td>
<td>2.92E-28</td>
<td>0.24</td>
<td>4.61(0.265)</td>
<td>1.26E-67</td>
<td>6,677</td>
</tr>
<tr>
<td>ET</td>
<td>6</td>
<td>rs5370</td>
<td>T/G</td>
<td>EDN1</td>
<td>2.928(0.379)</td>
<td>1.38E-14</td>
<td>2.983(0.39)</td>
<td>2.49E-14</td>
<td>0.22</td>
<td>2.955(0.272)</td>
<td>1.49E-27</td>
<td>6,696</td>
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<tr>
<td>ET</td>
<td>6</td>
<td>rs4236146</td>
<td>T/G</td>
<td>ZNF451</td>
<td>-2.397(0.474)</td>
<td>4.40E-07</td>
<td>-0.047(0.039)</td>
<td>9.06E-01</td>
<td>0.21</td>
<td>-1.023(0.035)</td>
<td>8.05E-04</td>
<td>6,581</td>
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<tr>
<td>ET</td>
<td>6</td>
<td>rs9505932</td>
<td>A/C</td>
<td>THBS2</td>
<td>1.564(0.351)</td>
<td>8.59E-06</td>
<td>0.462(0.355)</td>
<td>1.92E-01</td>
<td>0.31</td>
<td>1.019(0.249)</td>
<td>4.43E-05</td>
<td>6,678</td>
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<tr>
<td>ADM 9</td>
<td>9</td>
<td>rs2169324</td>
<td>A/G</td>
<td>.</td>
<td>-0.023(0.005)</td>
<td>6.46E-06</td>
<td>0.0(0.004)</td>
<td>9.29E-01</td>
<td>0.16</td>
<td>-0.008(0.003)</td>
<td>6.28E-03</td>
<td>6,614</td>
</tr>
<tr>
<td>ADM 11</td>
<td>11</td>
<td>rs2957692</td>
<td>G/A</td>
<td>ADM</td>
<td>0.017(0.003)</td>
<td>2.46E-08</td>
<td>0.013(0.003)</td>
<td>9.87E-06</td>
<td>0.40</td>
<td>-0.015(0.002)</td>
<td>1.05E-12</td>
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</tr>
<tr>
<td>ADM 11</td>
<td>11</td>
<td>rs1503436</td>
<td>C/G</td>
<td>SOX6</td>
<td>-0.032(0.007)</td>
<td>4.22E-06</td>
<td>0.003(0.005)</td>
<td>5.28E-01</td>
<td>0.08</td>
<td>-0.009(0.004)</td>
<td>2.99E-02</td>
<td>6,636</td>
</tr>
<tr>
<td>ET</td>
<td>11</td>
<td>rs10836389</td>
<td>A/G</td>
<td>SLC1A2</td>
<td>-1.897(0.425)</td>
<td>8.40E-06</td>
<td>0.351(0.441)</td>
<td>4.26E-01</td>
<td>0.17</td>
<td>-0.815(0.360)</td>
<td>7.74E-03</td>
<td>6,686</td>
</tr>
<tr>
<td>ADM 12</td>
<td>12</td>
<td>rs17761268</td>
<td>A/G</td>
<td>GRIN2B</td>
<td>0.029(0.006)</td>
<td>5.83E-06</td>
<td>-0.002(0.007)</td>
<td>7.25E-01</td>
<td>0.05</td>
<td>0.015(0.005)</td>
<td>7.17E-04</td>
<td>6,634</td>
</tr>
<tr>
<td>ADM 13</td>
<td>13</td>
<td>rs12858120</td>
<td>A/G</td>
<td>SMAD9</td>
<td>-0.04(0.009)</td>
<td>5.17E-06</td>
<td>-0.012(0.011)</td>
<td>2.55E-01</td>
<td>0.02</td>
<td>-0.028(0.007)</td>
<td>3.70E-05</td>
<td>6,637</td>
</tr>
<tr>
<td>ADM 13</td>
<td>13</td>
<td>rs1239706</td>
<td>G/A</td>
<td>DLEU7</td>
<td>-0.016(0.004)</td>
<td>2.68E-06</td>
<td>-0.001(0.003)</td>
<td>7.75E-01</td>
<td>0.41</td>
<td>0.006(0.002)</td>
<td>1.06E-02</td>
<td>6,621</td>
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<tr>
<td>ADM 19</td>
<td>19</td>
<td>rs2665601</td>
<td>A/G</td>
<td>SULT2B1</td>
<td>-0.039(0.008)</td>
<td>1.36E-06</td>
<td>-0.002(0.004)</td>
<td>5.45E-01</td>
<td>0.14</td>
<td>-0.01(0.004)</td>
<td>6.13E-03</td>
<td>6,635</td>
</tr>
</tbody>
</table>
Figure S1. Quantile-quantile plots of observed versus expected p-values for MR-proADM (A) and CT-proET-1 (B).
Figure S2. Regional plots of all the loci that are significantly associated to CT-proET1 and MR-proADM levels (P < 5E-8).

Trait: MR-ProADM

![Graph showing regional plots of CT-proET1 and MR-proADM loci](image-url)
Trait: MR-ProADM

rs4253238

$-\log_{10}(p\text{-value})$

recombination rate (cM/Mb)

position on chr4 (Mb)

SORBS2 → TLR3 → CYP4V2 → MTNR1A

FAM149A → F11 → FAT1

KLKB1