2.

Genome-wide association study on plasma levels of midregional-proadrenomedullin and c-terminal-proendothelin-1
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-proET-1) and midregional proadrenomedullin (MR-proADM), are predictors of cardiac death and heart failure. We studied the association of common genetic variation with MR-proADM and CT-proET-1 by genome-wide association analyses in 3,444 participants of European ancestry. We performed follow-up genotyping of SNPs that showed suggestive or significant association in the discovery stage in an additional 3,230 participants. The minor variant in KLKB1 (rs4253238) and F12 (rs2731672), both part of the kallikrein-kinin system, were associated with higher MR-proADM (P=4.46E-52 and P=5.90E-24, respectively) and higher CT-proET-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-proADM (P=1.05E-12) and a variant in EDN-1 (rs5370) was associated with CT-proET-1 (P=1.49E-27). The total phenotypic variation explained by the genetic variants was 7.2% for MR-proADM and 14.6% for CT-proET-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 demonstrate 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-proET-1 and pre-proADM provides novel insights into the (co-) regulation of these vasoactive peptides in the vascular system.
INTRODUCTION

The endothelium 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 nitric oxide and various vasoactive peptides, including endothelin (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 also involved in the homeostasis of the sodium and water balance.

Reliable measurements of plasma ET-1 and ADM are highly challenging due to 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-proET1) and midregional proadrenomedullin (MR-proADM) which are correlated with ET-1 and ADM in equimolar amounts. Increased plasma levels of CT-proET1 and MR-proADM have both been associated to worse vascular function, cardiac death and heart failure. It is unknown whether increased peptide levels are causal or consequence in disease and only limited knowledge exists on the factors regulating the release and subsequent bioactivation of ET-1 and ADM. Genome wide association (GWA) analyses have been proven a powerful and unbiased tool to identify novel mechanisms and pathways, which might reveal novel targets for therapy. Here we report the results of GWA analyses in 3,444 participants of Prevend and follow-up genotyping in 3,230 participants to identify common genetic variants associated with CT-proET1 and MR-proADM levels, two related vaso-active peptides that have not been assessed by GWAS before.

METHODS

STUDY POPULATION

This study was performed in participants of the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study. Details of PREVEND have been described elsewhere. This study has been approved by the review board of the University Medical Center Groningen. All participants provided informed consent. This study adheres to the principles expressed in the Declaration of Helsinki.

GENOTYPING, QUALITY CONTROL & IMPUATION

Genotyping of 4,016 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 (Online supplement). Replication genotyping of 19 SNPs was performed by KBioscience (KBiosciences, Herts, UK) utilizing the SNPline system in an additional 3,230 independent participants of the PREVEND study.

BIOCHEMICAL MEASUREMENTS

Two commercially available, fully automated sandwich immunoassays were used for the measurement of CT-proET1 and MR-proADM (BRAHMS CT-proET1 KRYPTOR and MR-proADM KRYPTOR; BRAHMS AG, Hennigsdorf, Germany) according to the manufacturer’s instruction manuals. The design of these assays is based on immunoluminometric assays described previously. Assays were performed in EDTA-plasma aliquots taken at baseline. The samples were stored at -80°C prior to analysis. All blood samples were processed by personnel blinded from any patient data. A total of 3,444 samples were available for the discovery analysis and 3,230 for replication.

SNP/GENE BIOLOGY FUNCTIONAL ANNOTATION

For identifying the likely candidate gene in each loci, we used the following information: We considered the nearest gene of the sentinel SNP, all nsSNPs in LD (r² ≥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 to perform a text-based analysis in abstracts on PubMed prior to Dec 2006 (to avoid confounding from GWAS results arising after that date).

STATISTICAL ANALYSIS

We used linear regression on untransformed MR-proADM measures using an additive genetic model with age, sex and body mass index (BMI) as covariates. Linear regression was performed on untransformed CT-proET1 mea-
sures using an additive genetic model with age and sex as covariates. Genotype-phenotype analyses were performed using PLINK (Version 1.07) \(^1\) \(^2\). The most significant (P<5E-5) 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 \(\sim b_0 + b_1.a + b_2.B + b_3.AB + c\). The model uses the allele dosage for each SNP, A and B, the interaction is described by the coefficient \(b_3\). The explained variance of the significant associations was calculated 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 PREPROADRENOMEDULLIN BY IN VITRO TRANSCRIPTION/TRANSLATION SYSTEMS:**

Pre-proADM cDNA without the signal peptide (1,230 base pairs encoding 410 amino acids) was also PCR amplified using a human cDNA clone (Source bioscience life sciences, Nottingham, UK). For amplification, the following primers were used: forward, CACG-GATCACCATGGCTCAGAAACGAGCTTCTAGG and reverse, CTGGCGGCCTACCAATGT-GCTGGTTGGGTGC. 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-proADM and pre-proET-1.

Recombinant tagged proAD-M, proET-1 and luciferase protein was produced using a TNT T7 coupled reticulocytes lysate system (Promega Corporation, Madison, USA) according to the manufacturer instructions. Luciferase control DNA (4331 base pairs) was included with the TNT T7 coupled reticulocytes lysate system as a control for the in vitro translation. 20μCi \(^{35}\)S methionine (Perkin Elmer NEN, Waltham, USA) was added during transcription/translation reaction to generate radiolabeled myc-proADM, myc-proET-1 and luciferase. For purification of myc-proADM and myc-proET-1, anti-myc 9 e10 agarose resin was used (Santa Cruz Biotechnology, Santa Cruz, USA).

**IN VITRO ASSAY OF KALLIKREIN CLEAVAGE**

Activated human plasma kallikrein was purified by Coachrom Diagnostica (Vienna, Austria) as previously described \(^3\). 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 \(^4\). As plasma kallikrein is separated from other proteases in plasma that could co-purify, such as factor XI and XII, we do not expect this cross reactivity to occur. Radiolabeled proADM, proET-1 and luciferase were incubated with or without PPACKII (50 mg/ml) for 0, 5, 30 and 60 minutes at 37°C with 5μg/ml activated purified plasma kallikrein. The reactions were stopped by adding sample buffer and subsequent heating for 5 min at 95°C. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (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, USA).

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**GWAS AND FOLLOW-UP GENOTYPING**

To identify common genetic variants associated with MR-proADM and CT-proET-1 levels we performed a genome-wide analyses of 2,269,099 genotyped or imputed autosomal SNPs catalogued in HapMap CEU panel in 3,444 participants from the PREVEND cohort (Table S1). Test statistic inflation showed no evidence of population stratification or admixture (genomic control \(\lambda_{GC} = 1.039\) CT-proET-1; Figure S1). We observed significant associations at 3 loci for MR-proADM \((P < 5 \times 10^{-4})\) and at 3 loci for CT-proET-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 one additional trait-specific locus, SNPs in EDN-1 for CT-proET-1 and SNPs near ADM for MR-proADM (Figure 1, Table and Figure S2 for corresponding regional plots). At each of the genome wide significant loci we determined the SNP with the lowest P-value (sentinel SNP) and carried out genotyping of these SNPs in 3,230 additional samples of the PREVEND cohort that did not have GWA data available (Table). For each SNP, the total evidence of association was calculated using inverse-variance fixed-effect meta-analysis in METAL \(^5\). The variants at the 4 loci with \(P < 5 \times 10^{-4}\) in the discovery phase were confirmed in further genotyping (Table). The sentinel SNPs at the four loci were included as covariates in conditional analyses for each trait to determine whether there were
Figure 1.
Manhattan plot of CT-proET-1 (A), MR-proADM (B).
**FIGURE 2.**

In-vitro cleavage of recombinant proADM and proET-1 by plasma kallikrein. Recombinant proADM (A), proET-1 (B) and Luciferase (C) were incubated with plasma kallikrein for 0, 5, 30 and 60 minutes with and without the presence of PPACK II, a specific plasma kallikrein inhibitor. The full length proteins are indicated. After 5 minutes of incubation the amount of full length proADM and proET-1 decreases while several smaller products are formed. Luciferase (DNA supplied with the in vitro translation assay as positive control) is not cleaved by plasma kallikrein suggesting that kallikrein is a relatively specific protease for proADM and proET-1.
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-proADM 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-proADM. We observed a significant interaction between the sentinel SNPs of KLKB1 and F12 loci (Table S2). The total phenotypic variation explained slightly increased to 7.2% for MR-proADM 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 x 10^{-8} and P < 1 x 10^{-5}) with MR-proADM or CT-pro-ET-1. However none of these loci became significantly associated after genotyping in the additional 3,230 samples of the PREVEND cohort (Table S3).

**Identification of candidate genes and putative causal genetic variants**

The peptides CT-proET-1 and MR-proADM were both associated to SNPs located in the locus that contained the gene encoding for the precursor of the peptides. CT-proET-1 was associated with SNPs at a locus harboring 5 genes within 1 MB of the sentinel SNP (rs5370). rs5370 is a non-synonymous SNP (nSNP) located in the endothelin-1 (EDN-1) gene. MR-proADM was associated with SNPs at a locus harboring 13 genes within 1 MB of the sentinel SNP (rs2957602), this SNP is closest to the ADM encoding gene (ADM).

We examined all nSNPs 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 datasets. We identified one nSNP (rs3733402) in the KLKB1 gene in full LD (r^2 = 1.00) with the sentinel SNP. We performed wet-lab genotyping of the rs3733402 variant in 3,230 participants and confirmed its strong association with both CT-proET-1 and MR-proADM levels (Table). The nSNP rs3733402 is located in KLKB1 providing a potential biological mechanism. The nSNP (rs3733402) in KLKB1 by itself explains approximately 4.7% of variance of MR-proADM and 8.6% of CT-pro-ET-1 plasma levels. rs2731672 lays 5.8kb from F12 and is in LD (r^2 = 1) with rs1801020 that is located in a highly conserved region within the 5’UTR part of F12, making it a potential candidate. F12 is also a biological plausible candidate when considering the KLKB1 locus as both genes are part of the kallikrein-kinin system (KKS) and have interactions with each other on a molecular level16. Grail literature mining tool based on publications prior 2006 also suggested ADM, EDN-1, KLKB1 and F12 as candidate genes (P<0.01).

**Novel role of KLKB1 on proADM and proET-1 cleavage**

We tested the hypothesis that KLKB1 is the causal gene related to the observed association with MR-proADM and CT-proET-1 trough cleavage because 1) KLKB1 encodes a cleavage protein with cleavage sites that overlap with cleavage sites of the precursors of MR-proADM and CT-proET-114,18,19,2.) previous GWA publications associated bradykinin17 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 proADM and proET-1 proteins with active purified plasma kallikrein (human) and observed a time-dependent cleavage of proADM and proET-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).
endothelin converting enzyme (ECE)

Pre-proET-1

Pre-proADM

**Figure 3A**

**Figure 3B**

*Figure 3A / 3B. Structural features of pre-proET-1 and pre-proADM. Numbers indicate amino acids.*
by GWA studies (r²=1.00 with our lead SNP). We found the sentinel SNP at the KLKB1 locus to be in complete LD (r² = 1.00) with a nsSNP (rs3733402) causing an asparagine 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 31 suggesting that the KLKB1 locus is associated to MR-proADM and CT-proET-1 due to differences in cleavage, similar to bradykinin and renin.

Human pre-proET1, consisting of 212 amino acids 17 and is cleaved into various peptides: pre-proET-17-53, Big-endothelin (pre-proET43-92), pre-proET90-168 and CT-proET-1 (pre-proET168-203). Pre-proADM consists of 185 amino acids, which can be cleaved into four known peptides: PAMP (proadrenomedullin N-terminal 20 peptide; pre-proADM22–41), MR-proADM (pre-proADM45–91), ADM (preproADM94–143) and ADT (adrenotensin; pre-proADM150–185). The cleavage sites involved in the bioprocessing of pre-proET1 and pre-proADM derived peptides include arg-arg and lys-arg sequences (see Figure 3a / 3b for a schematic overview), which are among the specific recognition sites of plasma kallikrein 18. On these grounds, we hypothesized that plasma kallikrein is involved in the bioprocessing of pre-proET1 and pre-proADM derived peptides. Using a custom designed ‘in vitro’ assay we provide preliminary data that purified plasma kallikrein cleaves proADM and pro-ET1 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 (RAAS) 22,23. Interestingly, ADM and ET-1 are also suspected to interact with the RAAS 40. The KKS and RAAS are involved in a multitude of physiologic and pathophysiologic actions that impacts 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 1,22,25,26.

In addition to the KLKB1 and F12 loci, we identified genetic variants at the ADM locus to be associated with MR-proADM levels and SNPs at the EDN-I locus with CT-proET-1 levels. The variant (rs2957692) identified near the ADM locus is not in LD (r² = 0.00) with a previously reported variant in ADM (rs4910118) identified in an earlier candidate gene study 27. Recently, the ADM locus was reported to be associated with systolic blood pressure, our sentinel SNP is low LD (r²<0.2) with this Locus 28. Whether this locus is independent requires further conditional analyses. Our sentinel SNP at the EDN-I locus (rs5370) is a nsSNP located in the CT-proET-1 part of the endorhelmin-1 coding gene. We cannot exclude this is a false positive association as the nsSNP is located two amino acids downstream from the epitope of the CT-proET-1-tracer, this may affect the antibody-epitope affinity 3. rs5370 has previously been associated to idopathic pulmonary arterial hypertension 39 and risk for hypertension 10.

In summary, using GWAS and in vitro functional follow-up we report the involvement of the KKS system in the regulation of MR-proADM and CT-proET-1 plasma levels. Preliminary functional data supports 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 kallikrein-kinin system. We also found associations between MR-proADM and CT-proET-1 plasma levels and the genes encoding their precursors.

Perspectives

Endothelin-1 (ET-1) and adrenomedullin (ADM) are circulating vasactive peptides involved in vascular homeostasis and endothelial function. In this study we identified 4 common variants to influence plasma levels of MR-proADM and CT-proET-1. The common variants in ADM and EDN-I should be evaluated further to assess how MR-proADM and CT-proET-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 31.

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DISCLOSURES

J Struck is employed by BRAHMS GmbH, a company manufacturing and holding patent rights on the MR-proADM and CT-proET-1 assays. The authors have declared that no competing interests exist.


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Table: Summary of the loci associated with MR-proADM and CT-proET-1 levels, discovery, replication and combined. 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.

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*non synonymous SNP