Measurement of Midregional Proadrenomedullin in Plasma with an Immunoluminometric Assay

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Background: Adrenomedullin (ADM) is a potent vasodilatory peptide, and circulating concentrations have been described for several disease states, including dysfunction of the cardiovascular system and sepsis. Reliable quantification has been hampered by the short half-life, the existence of a binding protein, and physical properties. Here we report the technical evaluation of an assay for midregional pro-ADM (MR-proADM) that does not have these problems.

Methods: MR-proADM was measured in a sandwich immunoluminometric assay using 2 polyclonal antibodies to amino acids 45–92 of proADM. The reference interval was defined in EDTA plasma of 264 healthy individuals (117 male, 147 female), and increased MR-proADM concentrations were found in 95 patients with sepsis and 54 patients with cardiovascular disease.

Results: The assay has an analytical detection limit of 0.08 nmol/L, and the interassay CV was <20% for values >0.12 nmol/L. The assay was linear on dilution with undisturbed recovery of the analyte. EDTA-, heparin-, and citrate-plasma samples were stable (<20% loss of analyte) for at least 3 days at room temperature, 14 days at 4 °C, and 1 year at −20 °C. MR-proADM values followed a gaussian distribution in healthy individuals with a mean (SD) of 0.33 (0.07) nmol/L (range, 0.10–0.64 nmol/L), without significant difference between males or females. The correlation coefficient for MR-proADM vs age was 0.50 (P <0.001). MR-proADM was significantly (P <0.001) increased in patients with cardiovascular disease [median (range), 0.56 (0.08–3.9) nmol/L] and patients with sepsis [3.7 (0.72–25.4) nmol/L].

Conclusions: MR-proADM is stable in plasma of healthy individuals and patients. MR-proADM measurements may be useful for evaluating patients with sepsis, systemic inflammation, or heart failure.
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Adrenomedullin (ADM)[1] is a 52-amino acid peptide described as having a variety of physiologic functions. Its strong vasodilatory activity has been described in several studies *(1–3)*. Quantification of ADM would be helpful in the diagnosis, monitoring, and prognosis of various cardiovascular diseases and sepsis, for which increased plasma concentrations of ADM have been described *(4–7)*. However, the reliable measurement of ADM release in the circulation is difficult. In addition to immediate binding of ADM to receptors in the vicinity of its production, via autocrine and/or paracrine reactions, peripheral measurement is also hampered by the existence of a binding protein *(8)*, the short half-life of ADM (22 min) *(9)*, and technical difficulties *(10)*. ADM is derived from a larger precursor peptide (preproADM; 185 amino acids) by posttranslational processing *(1)* (Fig. 1). During the processing of preproADM, other peptides are generated: another biologically active peptide termed proadrenomedullin N-terminal 20 peptide (PAMP) with a suggested hypotensive effect *(11)*, and 2 peptides flanking ADM: one midregional part of proADM (proADM 45–92) and the COOH terminus of the molecule (proADM 153–185).

We have recently identified this midregional proADM (MR-proADM) in the plasma of patients with septic shock *(12)*. The affinity-purified material was characterized by matrix-assisted laser desorption/ionization mass spectrometry and revealed no smaller peptides, indicating that MR-proADM, in contrast to mature ADM and PAMP, may be stable in human plasma *(12)*. Although MR-proADM might be functionally irrelevant, the lack of a tight feedback control represents a highly interesting diagnostic target. Because of its probable stoichiometric

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[1] Nonstandard abbreviations: ADM, adrenomedullin; PAMP, proadrenomedullin N-terminal 20 peptide; and MR-proADM, midregional proadrenomedullin.
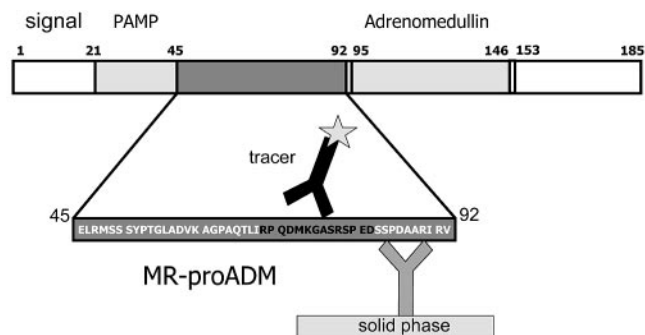
Fig. 1. Sequence of preproADM.

Numbers indicate amino acids. *Signal*, signal peptide. The assay principle for MR-proADM is shown. *Tracer*, labeled antibody; *solid phase*, antibody coated on tubes. Single letter amino acid code for MR-proADM is shown. *Bold font* indicates antibody epitopes.

generation, the released amounts of MR-proADM may directly reflect those of ADM and PAMP.

Here we describe the technical characterization of a new sandwich immunoassay for the measurement of MR-proADM in human plasma, its reference interval in healthy individuals, and the finding of increased plasma concentrations in patients with cardiovascular disease or sepsis.

Materials and Methods

Three peptides related to preproADM were purchased from JERINI AG, where they were chemically synthesized, purified, and quality controlled by standard procedures. The peptides were SPCD19 (sequence CRPQDM-KGASRSPEDSSPD, representing positions 68–86 of preproADM plus an N-terminal cysteine residue), PSR13 (sequence CSSPDAARIRVKR, representing positions 83–94 of preproADM plus an N-terminal cysteine residue), and proADM 45–92 (sequence ELRMSSSYPTGLAD-VKAGPAQTLIRPQDMKGASRSPEDSSPDAARIRV, representing positions 45–92 of preproADM).

Sheep antisera containing antibodies directed against peptides SPCD19 and PSR13 were generated by Micropharm Ltd., according to standard procedures. Briefly, peptides were conjugated with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester to keyhole limpet hemocyanin. Sheep were initially immunized with 100 μg of peptide in its conjugated form and with 50 μg in 4-weekly intervals thereafter. Antisera were purchased from Micropharm starting 3 months after initial immunization. For the purification of peptide-specific antibodies, 5 mg each of peptides SPCD19 and PSR13 were immobilized on SulfoLink gel (Pierce) according to the manufacturer's instructions. Affinity purification was performed as follows: 50 mL of antiserum was diluted with 50 mL of binding buffer [100 mmol/L potassium phosphate containing 1 mL/L Tween (pH 6.8) and 1 tablet of Complete Protease Inhibitor (Roche) per 50 mL] and incubated according to the manufacturer's instructions. The gel was washed with 300 mL of binding buffer. Bound antibodies were eluted

with 50 mmol/L citric acid (pH 2.2) and neutralized with 50 mmol/L potassium phosphate (pH 7.4) and NAP size-exclusion chromatography (Amersham) according to the manufacturer's instructions. The homogeneity of the antibody preparations was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the protein concentrations were measured by the bicinchoninic acid method (Pierce).

A chemiluminescence sandwich immunoassay using coated tubes was set up as follows. Purified anti-SPCD19 antibody (1 g/L) was labeled by incubation with a 1:2 molar ratio of MA70-Akridinium-NHS-Ester (1 g/L; HOECHST Behring) for 15 min at room temperature. The reaction was stopped by addition of a 1:10 volume of 1 mol/L Tris, and labeled antibodies were separated from free label by size-exclusion chromatography on a Protein-Pak SW300 HPLC column (Waters). Tracer was produced by diluting the labeled antibody into assay buffer (300 mmol/L potassium phosphate, 50 mmol/L NaCl, 10 mM sodium EDTA, 1 g/L bovine serum albumin, 1 g/L unspecific sheep IgG, 1 g/L unspecific bovine IgG, 0.9 g/L sodium azide, pH 7.4) to achieve a concentration of 1 000 000 relative light units per 200 μL, determined by a LB952T luminometer (Berthold). Polystyrene tubes (Greiner) were coated with anti-PSR13 antibody (per tube: 0.5 μg in 0.3 mL of 100 mmol/L Tris, 50 mmol/L NaCl, pH 7.8) overnight at room temperature, after which tubes were blocked with 10 mmol/L sodium phosphate (pH 6.5) containing 3 mL/L Karion FP and 3 g/L bovine serum albumin and lyophilized. Dilutions of peptide proADM 45–92 in normal horse serum (Sigma) served as calibrators. The immunoassay was performed by incubating 10 μL of samples/calibrators and 200 μL of tracer in coated tubes under agitation (170–300 rpm) for 2 h at room temperature (18–24 °C). Tubes were washed 4 times with 1 mL of LUMItest wash solution (B·R·A·H·M·S AG), and bound chemiluminescence was measured for 1 s per tube with a LB952T luminometer. The assay was termed B·R·A·H·M·S SEVADIL LIA®.

For the highest calibrator (S5), chemically synthesized proADM 45–92 peptide was added to horse serum at a concentration of 25 nmol/L. This was diluted to prepare calibrators (S1 to S4) with final concentrations of 0.2, 0.8, 2.5, and 10.0 nmol/L. As controls, horse sera containing 1 nmol/L (control I) and 5 nmol/L (control II) of calibrator peptide were added at the beginning and end of each run. A typical calibration curve is shown in Fig. 2A.

Plasma samples (EDTA) from healthy individuals were collected from the members of a local health club. Participants had to be without clinical evidence of acute disease or a history of chronic illness. Of ~900 regular members, 326 showed interest in participation, of whom 62 had to be excluded because of a history of cardiovascular disease, diabetes, autoimmune disease, cancer, or infections within the last 3 months. Written consent was obtained from the remaining 264 participants. After peripheral
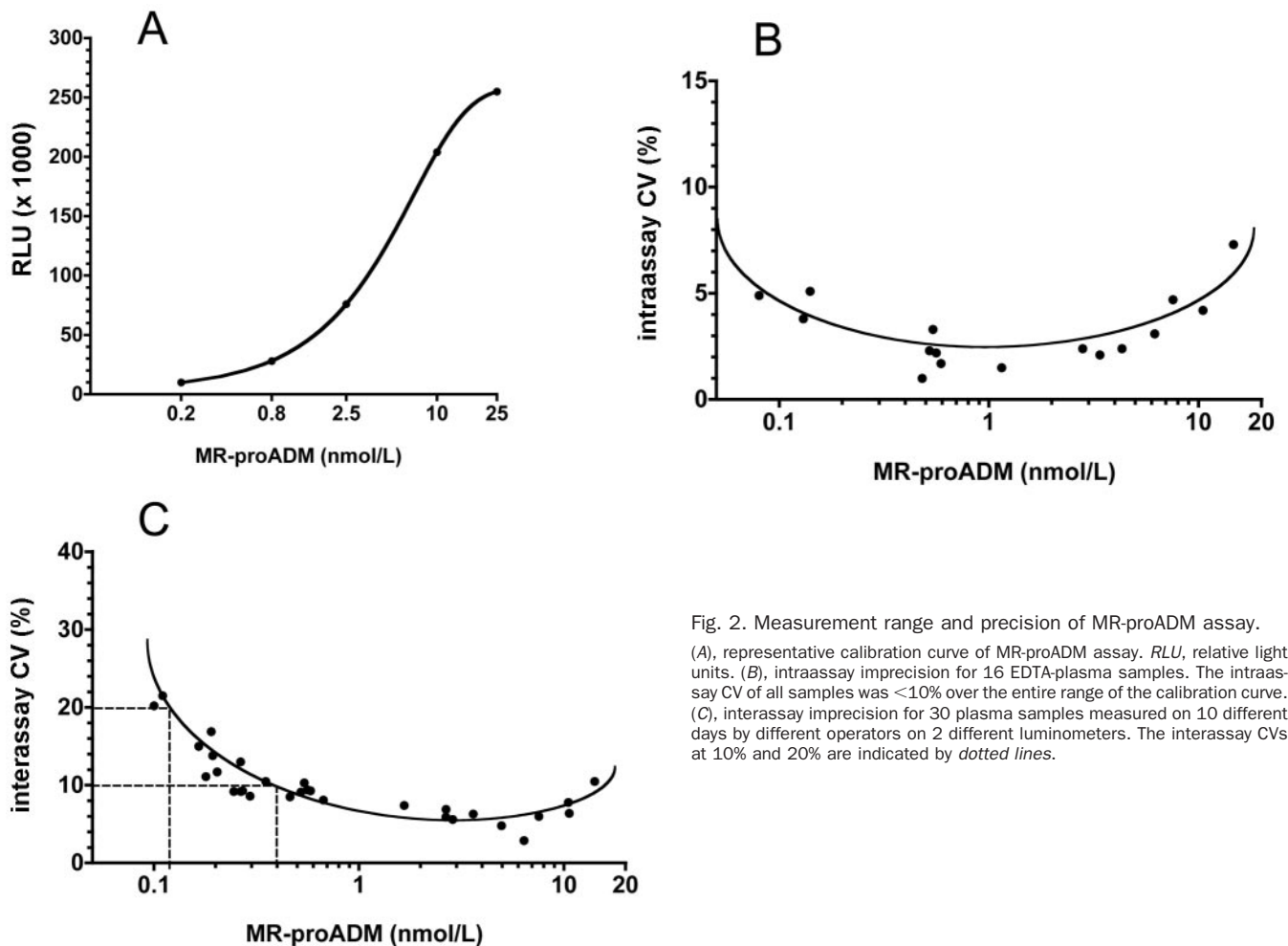
Fig. 2. Measurement range and precision of MR-proADM assay.

(*A*), representative calibration curve of MR-proADM assay. *RLU*, relative light units. (*B*), intraassay imprecision for 16 EDTA-plasma samples. The intraassay CV of all samples was <10% over the entire range of the calibration curve. (*C*), interassay imprecision for 30 plasma samples measured on 10 different days by different operators on 2 different luminometers. The interassay CVs at 10% and 20% are indicated by *dotted lines*.

venipuncture, all blood samples were centrifuged and frozen in aliquots at −20 °C within 1 h.

Samples from patients with cardiovascular disease, sepsis, severe sepsis, or septic shock [as defined by the American College of Chest Physicians/Society of Critical Care Medicine consensus conference (*13*)] were collected from intensive care unit wards according to ethics guidelines and were stored at −20 °C until further use. Cardiac patients were scheduled for coronary artery bypass operations or mitral or aortic valve replacement and had ejection fractions <50%. Patients with coronary artery disease had stable angina and were not suffering from acute myocardial infarction.

All statistical analyses were performed with Graph Pad Prism 4.0. Comparisons of parametric data were done with an unpaired *t*-test or one-way ANOVA. Nonparametric data were compared with Kruskal–Wallis ANOVA and the Dunn post test. Correlations were calculated as Pearson correlations. *P* values <0.05 were considered significant.

Results

The lower detection limit, as determined with horse serum (mean relative light units of 20 determinations plus 2 SD), was 0.08 nmol/L. The intraassay imprecision (CV) was determined by measuring 16 human EDTA plasma samples covering the range 0.08–14.7 nmol/L in 10 parallel measurements. The intraassay CV was <5% in all samples except for the highest sample, for which it was 7% (Fig. 2B). The interassay imprecision was determined by measuring 30 samples (range, 0.10–14.1 nmol/L) on 10 different days by alternating operators on 2 different luminometers (Fig. 2C). The functional assay sensitivity, defined as the MR-proADM concentration with an interassay CV of 20%, was 0.12 nmol/L, and the MR-proADM concentration for which the interassay CV was 10% was 0.4 nmol/L. A high-dose hook effect was seen when MR-proADM concentrations >500 nmol/L were added to plasma samples. However, this concentration is 20-fold higher than the highest calibrator and would still be found as highly positive in the assay.

We assessed assay linearity by dilution experiments; we also performed pooling and recovery studies. Linear dilutions (up to 1:32) of 8 EDTA-plasma samples were tested. Measured concentrations were multiplied by the dilution factor and compared with the original undiluted concentrations. None of the 8 samples showed a deviation during dilution >20% of the original value. Pooling of 5 plasma samples with low MR-proADM concentrations with 5 plasma samples with high MR-proADM concentrations in 10 different combinations gave a mean measured concentration that was 98% of the expected concentration (range, 93%–104%). We performed recovery experiments by adding the calibrator peptide in 2 concentrations to 5 different plasma samples. Recovery of the analyte was 89%–105% of the calculated value in all 10 experiments.

We measured the interference of several biological substances by adding the potential interferents to samples, according to Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines. The assay was not influenced by albumin concentrations up to 10 g/L, bilirubin up to 0.4 g/L, hemoglobin up to 5.46 g/L, triglycerides up to 6.34 g/L, or heparin up to 8000 IU/L. The SD between the samples without added interferents and those with added interferents was always within the ±1 SD range of the interassay precision profile, and the deviation of the values for samples without added interferents was always <20%.

We evaluated the stability of the analyte at room temperature in citrate-, EDTA-, and heparin-plasma samples from 5 different patients. All samples for stability studies contained only endogenous MR-proADM and contained no added peptide. The analyte was stable in all 3 matrices for at least 72 h at room temperature. Mean measured concentrations were between 92% and 104% of the original value (Fig. 3). We evaluated the stability of MR-proADM at 4 °C in 11 different EDTA-plasma samples containing endogenous MR-proADM (range, 1.5–11.3 nmol/L) without added MR-proADM peptide. After 14 days of storage, measured MR-proADM values were between 78% and 129% of the original values (mean, 98%). The same 11 samples were subjected to 4 freeze–thaw cycles, which had no influence on the analyte. The mean measured concentration after the first freeze–thaw cycle was 101% of the original value (range, 80%–115%), and that after the fourth cycle was also 101% of the original value (range, 77%–122%). The mean measured concentration for 11 samples stored for 12 months at −20 °C was 99% of the original value.

Matrices other than EDTA plasma were tested with 50 matched samples obtained from healthy controls. Serum consistently gave values that were >30% lower than those obtained with EDTA plasma, and a high percentage of healthy control samples had values below the functional assay sensitivity (interassay CV = 20%). The assay therefore is not suitable for measurement of MR-proADM in serum samples. Heparin plasma gave higher values
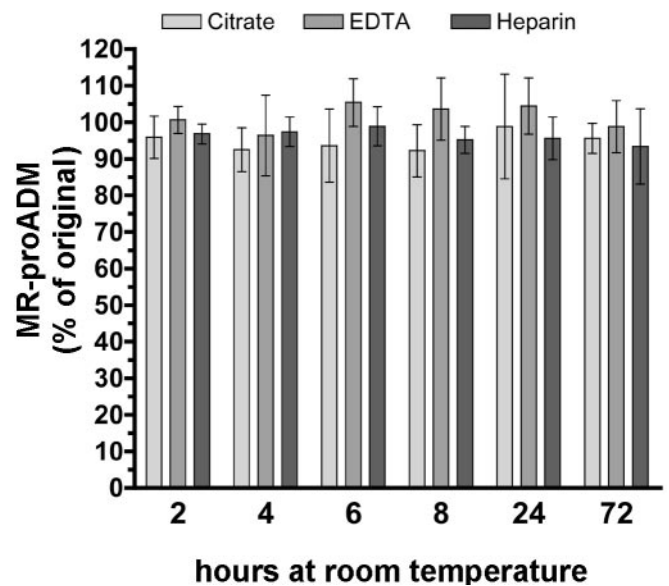

Fig. 3. Stability of the MR-proADM assay.

Stability of MR-proADM in citrate-, EDTA-, and heparin plasma at room temperature. Shown is the mean (SD) MR-proADM concentration as a percentage of the original value in 5 different samples over 72 h at room temperature.

(mean of 10% higher); however, a few heparin-plasma samples had very low values compared with the matched EDTA-plasma sample. We therefore do not recommend use of heparin plasma. Citrate plasma gave consistently lower values (mean of 7% lower) than EDTA plasma. It therefore is possible to use citrate plasma if the laboratory defines reference intervals for this specimen type. All values presented in the remainder of this study were obtained with EDTA plasma.

In 264 healthy individuals (117 male, 147 female) MR-proADM values followed a gaussian distribution (Fig. 4A) with mean (SD) values of 0.33 (0.07) nmol/L (95% confidence interval of the mean, 0.32–0.34 nmol/L) and a range of 0.10–0.64 nmol/L. The 99th percentile of the healthy population was 0.52 nmol/L, the 97.5th percentile was 0.49 nmol/L, 2.5th percentile was 0.17 nmol/L, and the 1st percentile was 0.14 nmol/L (based on a gaussian distribution). There was no significant difference in mean MR-proADM values between males and females (0.32 nmol/L for males; 0.32 nmol/L for female; Fig. 4B). Further stratification of the population by age is shown in Table 1. The mean (SD) increased from 0.29 (0.04) nmol/L in the age group <25 years of age, to 0.41 (0.08) nmol/L in the group 55–64 years of age, and to 0.41 (0.06) nmol/L in the age group >65 years of age ($P < 0.001$, ANOVA). Post hoc analysis by the Newman–Keuls multiple comparison test revealed significant differences among all groups except between the 18–24 and 25–34 groups, between the 25–34 and 35–44 groups, and between the 2 oldest groups. Correlation analysis revealed a significant correlation of MR-proADM concentrations and age ($r = 0.50$; 95% confidence interval, 0.34–0.58; $P < 0.001$).
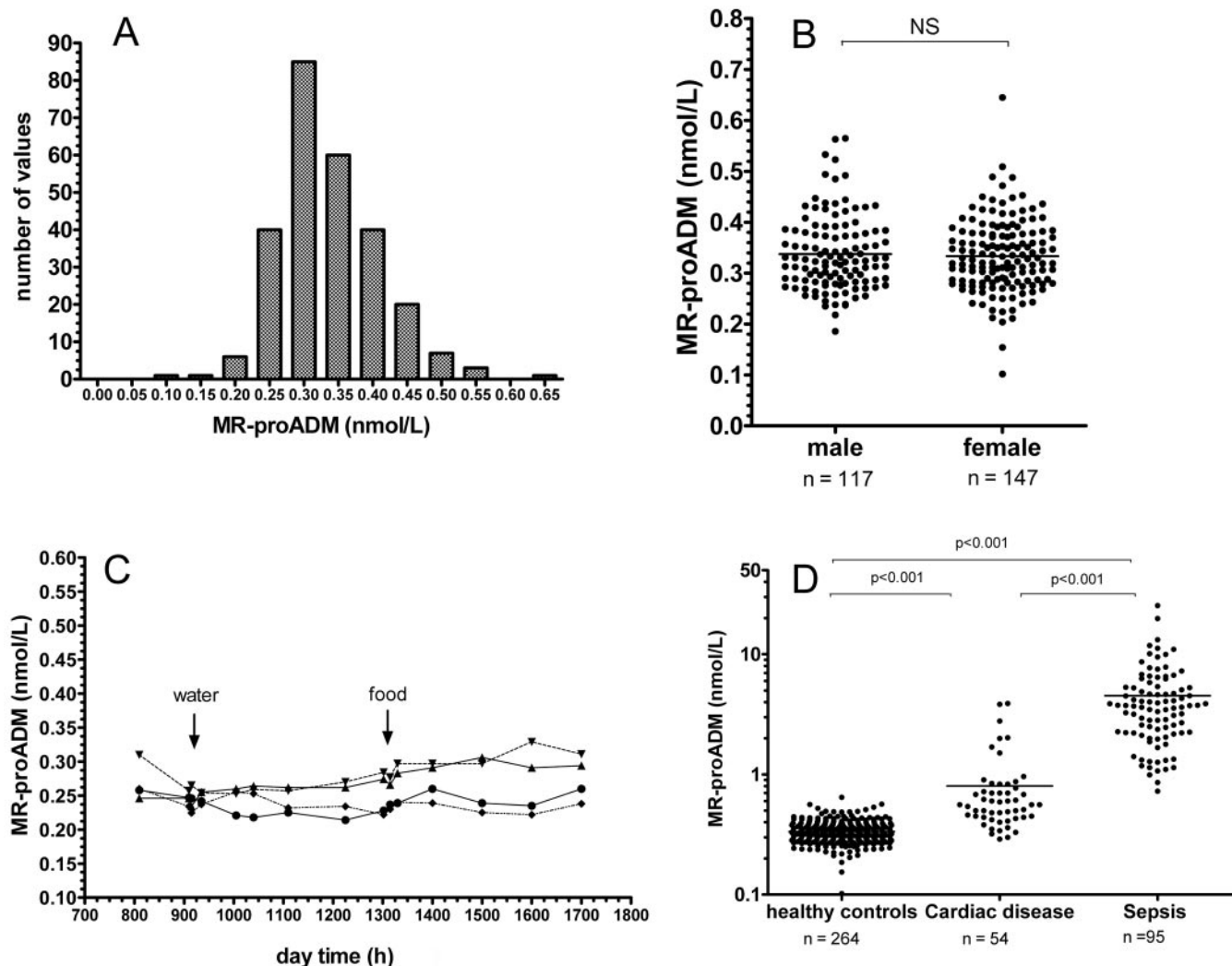
Fig. 4. MR-proADM in healthy individuals and patients.

(*A*), gaussian distribution of the MR-proADM values in all 264 healthy individuals. (*B*), distribution of MR-proADM values in 117 male and 147 female healthy individuals. There was no significant difference (*NS*) in mean values (unpaired *t*-test), as indicated by the *horizontal bracket*. (*C*), proADM values for 4 of 6 representative healthy individuals over a 9-h period. *Arrows* indicate the intake of water (1 L within 5 min) and a standardized meal (~1200 kcal over 45 min). ♦, 33-year-old male, body mass index = 21 kg/m^2; ▼, 44-year-old male, body mass index = 24 kg/m^2; ▲, 50-year-old female, body mass index = 23 kg/m^2; ●, 31-year-old male, body mass index = 30 kg/m^2. (*D*), distribution of MR-proADM values in 54 patients with cardiac disease and 95 patients with sepsis compared with the 264 healthy individuals. The median value for each group is indicated by a *line*. Differences between all 3 groups are significant by Kruskal–Wallis ANOVA.

To evaluate intraday variations in healthy individuals, we monitored 6 participants (3 male, 3 female) from 0800 to 1700, taking 15 consecutive samples for MR-proADM over this period. At the first blood sample, all participants had been without water or food for 14 h. Although values among individuals varied between 0.16 and 0.28 nmol/L, the values for each participant remained very stable (SD

of 15 measurements, 0.01–0.05 nmol/L) over the observation period and were not influenced by water (1 L) or food intake (standardized lunch of ~1200 kcal) at defined time points (Fig. 4C).

MR-proADM was increased in patients undergoing heart surgery because of an underlying cardiac disease and in patients in the Intensive Treatment Unit with

Table 1. MR-proADM in 264 healthy blood donors stratified by age.

Age of blood donors, years	18–24	25–34	35–44	45–54	55–64	65–80
n	47	67	75	40	18	17
Mean (SD), nmol/L	0.29 (0.04)	0.31 (0.06)	0.33 (0.06)	0.37 (0.07)	0.41 (0.08)	0.41 (0.06)
Range, nmol/L	0.18–0.38	0.10–0.52	0.21–0.48	0.25–0.64	0.26–0.56	0.28–0.51
No significant difference[a]	b	b,c	c		d	d

[a] All differences are significant ($P < 0.05$, ANOVA with Newman–Keuls post test), except for column pairs indicated by letters b to d.

sepsis, severe sepsis, or septic shock. Cardiac patients were scheduled for coronary artery bypass surgery or mitral or aortic valve replacement and had ejection fractions <50%. Median (range) values were 0.56 (0.08–3.9) nmol/L for cardiac patients and 3.7 (0.72–25.4) nmol/) for sepsis patients. The difference, compared with healthy individuals, was significant for both groups ($P <0.001$, Kruskal–Wallis ANOVA with Dunn post test). The data distributions in all 3 groups are shown in Fig. 4D.

Discussion

We describe the technical characterization of a new MR-proADM sandwich immunoassay. The assay has a functional assay sensitivity (defined as an interassay CV <20%) of 0.12 nmol/L and allows the measurement of MR-proADM in a range between 0.12 and 25 nmol/L. Studies using either pooled samples or addition of the chemically synthesized analyte as well as dilution studies showed good performance. In contrast to mature ADM, MR-proADM is stable in plasma at room temperature for at least 72 h, at 4 °C for at least 14 days, and at −20 °C for at least 12 months. Although the choice of anticoagulant had no influence on the stability of the analyte after the sample was taken, the use of heparin as anticoagulant gave, in a few matched samples, lower MR-proADM values compared with EDTA (<50% of EDTA value). We therefore do not recommend the use of heparin plasma or serum for this assay, whereas citrate plasma can be used.

The released amounts of MR-proADM may directly reflect those of ADM and PAMP, and measurement of the latter molecules is technically challenging because they can be cleared rapidly as a result of their autocrine/paracrine effects; i.e., because their receptors are located very close to the site of release (2, 3, 14, 15). In addition, circulating ADM could be influenced by a binding protein (complement factor H), making it less accessible for immunometric analysis (8). It has also been reported that ADM tends to stick to surfaces and thus might not be quantitatively recovered in an immunoassay (10). In a study comparing different ADM gene-derived peptides (16), MR-proADM had no physiologic effect, whereas all other peptides (ADM, PAMP, and adrenotensin) exhibited activity. The apparent stability may thus be attributable to this lack of function because only bioactive substances require tight regulation by proteolysis.

Because of a lack of readily available routine assays for the measurement of ADM, we could not demonstrate a direct correlation between MR-proADM and mature ADM. Although it is fair to speculate on a stoichiometric release of both peptides, there are reports concerning ADM and PAMP in which this stoichiometric release was found in patients with heart failure (17) but not in patients on hemodialysis (18). At present it is unclear whether this discrepancy is attributable to the respective assays or whether it reflects a distinct release pattern of the 2 peptides.

In 264 healthy individuals, MR-proADM followed a gaussian distribution with a mean (SD) of 0.33 (0.07) nmol/L. There was no difference between the male and female cohort, and all healthy individuals had detectable MR-proADM. There was a significant trend to higher MR-proADM values in older individuals, whereas in individual volunteers, values were very stable during the day and not influenced by either food or water intake. The concentration of MR-proADM found in this study is more than 1000-fold higher than that reported for mature ADM in healthy individuals (2.7–10.1 pmol/L) (10).

The concept of replacing the problematic measurement of a bioactive, rapidly cleared peptide by measuring a nonfunctional, stable peptide derived from the cognate precursor is well known and has been applied with great success for the A- and B-type natriuretic peptides (19–23). Our finding of such a kind of peptide derived from the ADM precursor opens the door to better assessment of the actual release of ADM gene products under pathologic conditions involving dysfunctions of the cardiovascular system and, thus, to improving the diagnosis, monitoring, and prognosis of these diseases.

One practical example in this study is the measurement of MR-proADM in patients with sepsis, severe sepsis, or septic shock. Although it is well known that ADM is increased in these conditions (7), the previously reported concentrations of ADM (median, 0.194 nmol/L) are much lower than the MR-proADM concentrations reported here (median, 3.7 nmol/L). The ADM concentration in a patient may be of relevance for clinical intervention (24); therefore, the actual true release of ADM should be known. Because the influences on ADM measurement may vary considerably among individuals and among different pathologies, measurements of mature ADM may underestimate the true release rate of this potent vasoactive peptide in patients. It will be up to further studies to evaluate whether MR-proADM has technical and clinical advantages over the measurement of mature ADM and whether this peptide is influenced by a certain medications.

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