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Plasma Adrenomedullin and Allelic Variation in the *ADM* Gene and Kidney Disease in People With Type 2 Diabetes

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Production of adrenomedullin (ADM), a vasodilator peptide, increases in response to ischemia and hypoxia in the vascular wall and the kidney. This may be an adaptive response providing protection against organ damage. We investigated the hypothesis that ADM has a nephroprotective effect in two prospective cohorts of patients with type 2 diabetes recruited in France. The highest tertile of plasma MR-proADM (a surrogate for ADM) concentration at baseline was associated with the risk of renal outcomes (doubling of plasma creatinine concentration and/or progression to end-stage renal disease) during follow-up in both cohorts. Four SNPs in the *ADM* gene region were associated with plasma MR-proADM concentration at baseline and with eGFR during follow-up in both cohorts. The alleles associated with lower eGFR were also associated with lower plasma MR-proADM level. In conclusion, plasma MR-proADM concentration was associated with renal outcome in patients with type 2 diabetes. Our data suggest that the *ADM* gene modulates the genetic susceptibility to nephropathy progression. Results are consistent with the hypothesis of a reactive rise of ADM in diabetic nephropathy, blunted in risk alleles carriers, and with a nephroprotective effect of ADM. A possible therapeutic effect of ADM receptor agonists in diabetic renal disease would be worth investigating.

The prevalence of chronic kidney disease (CKD) is increasing worldwide (1). Recent data from the Chronic Kidney Disease Surveillance System, Centers for Disease Control and Prevention, U.S., indicate that nearly 15% of the American population aged 20 years or older has CKD (2). Diabetic nephropathy is a frequent complication of both type 1 and type 2 diabetes and is a major cause of end-stage renal disease (ESRD) (3). Diabetes was the primary cause of renal disease in ~40% of the patients with new-onset kidney failure in 2011 in the U.S. (2).

Chronic hyperglycemia is the primary cause of diabetic nephropathy, but the involvement of arterial hypertension is well established. The renin-angiotensin system (RAS) can exert deleterious effects in the diabetic kidney, in part through its renal vasoconstrictor effects. Therapeutic intervention aimed at controlling blood glucose, lowering blood pressure, and blocking the RAS has proven efficient in primary and secondary prevention of CKD in diabetes (4–7). Despite these advances, efficacy of current therapies in preventing the advanced stages of CKD is limited and new therapeutics are needed (6,7). Experimental and epidemiological evidences suggest that other vasoconstrictor systems, such as the endothelin-1 or the vasopressin systems, may play a role in development and/or progression of diabetic nephropathy (8–10). On the other hand, the protective role of vasodilator peptides

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has been suggested but needs to be further documented (11).

Adrenomedullin (ADM) is a 52–amino acid peptide that presents structural homology with the calcitonin gene-related peptide and with amylin (12). At physiological concentration, ADM was shown to have potent vasodilator effect in the systemic and pulmonary circulations (13–15). In many cell types, including endothelial and smooth muscle cells of the vascular wall, production and secretion of ADM are increased in response to cellular strain induced by hypoxia and ischemia (16–18). These two conditions are observed in diabetes, especially at the glomerular and tubular levels in the kidney (19).

ADM is formed from the precursor peptide preproadrenomedullin by enzymatic cleavage. The mid-regional proadrenomedullin (MR-proADM) peptide, a distinct fragment of the precursor, is formed in equimolar amounts to ADM during the cleavage. MR-proADM is easily measurable in blood samples and is considered a stable surrogate marker of ADM (20). High plasma MR-proADM levels were observed in a variety of clinical conditions including chronic airway obstruction, arterial hypertension, occlusive peripheral arterial disease, congestive heart failure, and ischemic heart disease (21–24). Experimental evidence suggests that ADM protects against organ damage during ischemia or hypoxia (16–18), and thus the increase in circulating ADM levels observed in the above mentioned pathological conditions might be an adaptive response to cellular aggression.

Epidemiological data on ADM and diabetic nephropathy are scarce (25–27). Based on what is known of the physiological effects of the peptide, we hypothesized that ADM may have a nephroprotective effect in diabetes. In the present investigation, we assessed the relationship between plasma MR-proADM concentration at baseline and renal outcome (doubling of plasma creatinine concentration and/or progression to ESRD) or all-cause mortality in two cohorts of patients with type 2 diabetes recruited in France. In addition, to address causality between ADM and renal disease, we looked at polymorphisms in the *ADM* locus and their relationship with MR-proADM plasma level and clinical outcomes.

RESEARCH DESIGN AND METHODS

Participants: DIABHYCAR

We studied 3,137 unrelated French patients with type 2 diabetes from the DIABHYCAR cohort. DIABHYCAR was a clinical trial conducted in people with type 2 diabetes selected on the basis of persistent microalbuminuria (urinary albumin concentration [UAC] 20–200 mg/L) or macroalbuminuria (UAC >200 mg/L) without renal failure (plasma creatinine <150 μmol/L) at baseline (28). The trial tested the effect of a low dose of ramipril, an ACE inhibitor, on the incidence of cardiovascular and/or renal events. Renal event was defined as doubling of serum creatinine levels or requirement for hemodialysis or renal transplantation (ESRD) during follow-up. The median duration of follow-up was 4.7 years. Renal events comprised 64 cases of doubling of serum creatinine and 18 cases of ESRD in 77 subjects (2.5%). Mortality from all causes occurred in 436 subjects (13.9%) during follow-up. Clinical characteristics at baseline of individuals who presented a renal event or died during follow-up, as well as of those who did not, are shown in Supplementary Table 1. Results were negative regarding drug effect and were published previously (29). Participants gave written informed consent, and study protocols were approved by the ethics committee of Angers University Hospital, Angers, France.

Participants: SURDIAGENE

SURDIAGENE is an ongoing prospective monocentric study aiming to identify the genetic and environmental determinants of vascular complications in type 2 diabetes (30,31). Patients were recruited and followed regularly at the University Hospital of Poitiers, France, since 2002. Living status and cardiovascular and renal end points were determined from patients' hospital records and interviews with general practitioners and recorded every other year since 2007. The Poitiers ethics committee (Comité de Protection des Personnes Ouest 3) approved the study protocol, and written informed consent was obtained from each patient. A detailed description of study population, outcome criteria, adjudication procedure, biobanking, and biological procedures was published recently (32). In the present investigation, we studied 1,284 participants. Median duration of follow-up was 5 years. Renal events comprised 54 cases of doubling of serum creatinine and 36 cases of ESRD in 77 subjects (5.9%). Mortality from all causes occurred in 307 subjects (23.9%). Clinical characteristics at baseline of individuals who presented a renal event or died during follow-up, as well as of those who did not, are shown in Supplementary Table 2.

Procedures

MR-proADM concentration was measured in plasma-EDTA samples (DIABHYCAR = 2,962; SURDIAGENE, n = 1,284) by an automated immunofluorescent sandwich immune assay (B·R·A·H·M·S MR-proADM KRYPTOR, Thermo Fisher Scientific, Hennigsdorf, Germany) (20). Urinary albumin was measured by nephelometry.

Five single nucleotide polymorphisms (SNPs) in the *ADM* gene region (chromosome 11p15.4) were analyzed. The SNPs were chosen on the basis of previous studies: rs11042725 was reported to be a functional variant in the promoter of *ADM* (33), rs4399321 and rs7944706 were reported to be in a haplotype block associated with proteinuria in subjects with essential hypertension (34), and rs2957692 and rs2957717 were reported to be associated with MR-proADM levels in a genome-wide association study (35). The chosen SNPs give information on 85% of the allelic variation of SNPs with minor allele frequency (MAF) >5% at $r^2 > 0.8$ in haplotype block containing *ADM* (HapMap, public release no. 23). The structure of the *ADM* gene, location of the SNPs, and linkage disequilibrium

between SNPs are shown in Supplementary Fig. 1. Genotypes were determined by competitive allele-specific PCR genotyping system assays (KASP, LGC Genomics, Hoddeston, U.K.). Genotyping success rate was >95%. Genotypes were in Hardy-Weinberg equilibrium ($P > 0.01$). Estimation of the glomerular filtration rate (eGFR) was calculated according to the MDRD formula (36).

Statistical Analysis

Results are expressed as mean ± SD except where stated otherwise. Tertiles of plasma MR-proADM concentration were computed separately for women and men to take into account sex-related differences in MR-proADM levels. Differences between groups were assessed by Pearson χ^2 test, Wilcoxon/Kruskal-Wallis test, ANOVA, and ANCOVA. When ANOVA or ANCOVA were significant, comparisons between pairs were made using the Tukey-Kramer honestly significant difference (HSD) test. Variation of eGFR during the study was computed as the difference between values at the end of follow-up and at baseline, divided by the duration of follow-up. eGFR values during follow-up were compared between groups in mixed regression models with random effects. The model takes into account that samples from the same individual are not independent. Stepwise regression analyses were performed to evaluate the impact of covariates on the variance of MR-proADM levels. Kaplan-Meier curves (with a log-rank test) were used to plot the relative incidence of renal events over time by tertiles of plasma MR-proADM. Cox proportional hazards survival regression analyses were used to examine the effect of explanatory variables on time-related survival (or event-free) rates in prospective analyses. Hazard ratios (HRs) with their 95% CIs were computed in these analyses. Competing risk regression analysis (Fine and Gray model) was performed to estimate subhazard ratios of risk factors assuming death as a competing risk (37). Adjustments for clinical and biological parameters were carried out by including these parameters as covariates in the regression models. Stratification by UAC baseline status (micro- or macroalbuminuria) of genotype-related effects on the incidence of renal events during the follow-up of DIABHYCAR was performed by nesting the genotype variable within the UAC status variable in the regression model. This results in the computation of statistical effects for participants with micro- or macroalbuminuria separately and adjusted for multiple comparisons owing to the stratification by UAC status. Data were log transformed for all analyses when the normality of the distribution was rejected by the Shapiro-Wilk W test. Correction for multiple comparisons owing to multiple SNP testing took into account the effective number of independent tests (Meff) based on the degree of linkage disequilibrium between SNPs (38). Thus, $P \leq 0.01$ was considered significant for genotype-related comparisons and associations. For other analyses, $P < 0.05$ was considered significant. The power to detect allelic associations with the incidence of renal events in DIABHYCAR and SURDIAGENE participants was 74% and 72%, respectively, for HR ≥1.5 and $\alpha = 0.05$. It was >95% in both cohorts to detect allelic associations with total mortality during follow-up. Statistics were performed with JMP (SAS Institute, Inc., Cary, NC) and Stata (StataCorp, College Station, TX) softwares.

RESULTS

Plasma MR-proADM and Clinical Characteristics of Participants

Plasma MR-proADM concentration in DIABHYCAR and SURDIAGENE participants was significantly and positively correlated with age, age at diagnosis of diabetes, duration of diabetes, systolic blood pressure, and UAC and negatively correlated with eGFR in univariate analyses. It was also significantly and positively correlated with fasting plasma glucose in DIABHYCAR participants and with HbA_{1c}, BMI, and diastolic blood pressure in SURDIAGENE participants (Supplementary Table 3). Plasma MR-proADM concentration was significantly higher in women than in men, in patients treated with diuretics, and in subjects with arterial hypertension or in those with a previous history of angina pectoris or, in SURDIAGENE, of myocardial infarction. It was significantly higher in patients treated with ACE inhibitors or insulin (only tested in SURDIAGENE participants, as use of these medications was an exclusion criterion in DIABHYCAR). Plasma MR-proADM concentration was significantly higher in SURDIAGENE than in DIABHYCAR participants: median 0.74 nmol/L (interquartile range 0.38) vs. 0.30 nmol/L (0.24) ($P < 0.0001$). Clinical characteristics of DIABHYCAR and SURDIAGENE participants by tertiles of plasma MR-proADM reflect the correlations observed in each cohort (Tables 1 and 2). In stepwise regression analyses, correlations with age, BMI, eGFR, UAC, and use of diuretics remained significant in both cohorts, together with blood glucose in DIABHYCAR or HbA_{1c} in SURDIAGENE, and explained ~16% and ~66% of plasma MR-proADM variance in DIABHYCAR and SURDIAGENE, respectively (Supplementary Table 4). The eGFR explained ~9% and ~54% of MR-proADM variance in DIABHYCAR and SURDIAGENE, while the other covariates had a similar impact on the trait in both cohorts.

Plasma MR-proADM and Renal Events During Follow-up

The cumulated incidence of renal events during follow-up by sex-specific tertiles of plasma MR-proADM was 1.01% (T1), 1.93% (T2), and 4.46% (T3) in DIABHYCAR (log-rank test $P < 0.0001$) (Fig. 1*A*) and 1.17% (T1), 2.80% (T2), and 13.79% (T3) in SURDIAGENE (log-rank test $P < 0.0001$) (Fig. 1*B*). Cox proportional hazards survival regression analyses showed a strong positive association of the highest tertile of plasma MR-proADM with the incidence of renal events in both cohorts: HR 5.35 (95% CI 2.78–11.36) for DIABHYCAR and HR 30.81 (95% CI 11.71–81.04) for SURDIAGENE (Table 3) (model 1:

Table 1—DIABHYCAR study: clinical characteristics at baseline by sex-specific tertiles of plasma MR-proADM

	T1	T2	T3	*P*
Men/women (*n*)	726/264	722/263	722/265	
Plasma MR-proADM (nmol/L)§				
Men	0.15 (0.05–0.22)	0.29 (0.23–0.36)†	0.48 (0.37–2.05)†‡	<0.0001
Women	0.16 (0.05–0.25)	0.33 (0.26–0.41)†	0.54 (0.42–1.74)†‡	<0.0001
Age (years)	64 ± 8	65 ± 8†	68 ± 8†‡	<0.0001
Age at diagnosis of diabetes (years)	54 ± 9	55 ± 10†	57 ± 10†‡	<0.0001
Duration of diabetes (years)	10 ± 8	10 ± 7	11 ± 8†‡	0.0004
BMI (kg/m^2)	29.3 ± 4.5	29.2 ± 4.4	29.5 ± 5.0	0.45
Fasting plasma glucose (mmol/L)	9.7 ± 3.0	9.5 ± 2.9	9.1 ± 3.0†‡	<0.0001
HbA_{1c}, % (mmol/mol)	7.9 ± 1.8 (63 ± 8)	7.9 ± 1.7 (63 ± 7)	7.8 ± 1.7 (62 ± 7)	0.26
Systolic BP (mmHg)	144 ± 14	145 ± 14	146 ± 13†	0.006
Diastolic BP (mmHg)	82 ± 8	82 ± 8	82 ± 9	0.43
Arterial hypertension (%)	50	55	64	<0.0001
eGFR (mL/min/1.73 m^2)	81 ± 21	79 ± 20†	72 ± 19†‡	<0.0001
UAC (mg/L)§	59 (111)	65 (137)†	80 (199)†‡	<0.0001
UAC status (%)	80/20	78/22	71/29	<0.0001
Previous myocardial infarction (%)	5	5	7	0.19
Previous angina pectoris (%)	10	11	15	0.001
Use of diuretics (%)	16	21	30	<0.0001

Data expressed as mean ± SD except for MR-proADM (median and range) and UAC (median and interquartile range). UAC status: percentage of subjects with microalbuminuria or macroalbuminuria, respectively. Arterial hypertension: systolic blood pressure (BP) >140 mmHg and/or diastolic blood pressure >90 mmHg or presence of antihypertensive medication and history of hypertension. Statistics for quantitative parameters are ANOVA with log-transformed data except §Wilcoxon test. Tukey-Kramer HSD test following ANOVA or Wilcoxon for each pair: significantly different from †T1 or ‡T2. $P < 0.05$ is significant.

adjusted for sex and age at baseline plus study treatment in the original DIABHYCAR cohort). The associations remained significant when further adjusted for blood glucose (DIABHYCAR), HbA_{1c} (SURDIAGENE), eGFR, arterial hypertension, and coronary artery disease (previous history of angina pectoris or myocardial infarction) at baseline (Table 3) (model 2). The associations between plasma MR-proADM and incidence of renal events evaluated with the Cox model might be biased by the association between plasma MR-proADM and mortality if many patients died before achieving the renal end points (see below). Consequently, we performed competing risk regression analyses to estimate subhazard ratios of plasma MR-proADM tertiles as a risk for renal events assuming death as a competing risk. The association of the highest tertile of plasma MR-proADM with the incidence of renal events remained highly significant in both cohorts, indicating that death was not a competing risk (Table 3) (model 3).

The yearly variation of eGFR during follow-up by tertiles of plasma MR-proADM is shown in Supplementary Fig. 2. It was −0.77 ± 0.23 (T1), −1.14 ± 0.23 (T2), and −1.78 ± 0.23 mL/min/1.73 m^2 (T3) for DIABHYCAR (means ± SEM, ANCOVA $P = 0.008$, adjusted for sex, age, and study treatment in the original DIABHYCAR trial). It was −1.44 ± 0.29 (T1), −2.33 ± 0.28 (T2), and −3.51 ± 0.32 mL/min/1.73 m^2 (T3) for SURDIAGENE ($P < 0.0001$, adjusted for sex and age).

Plasma MR-proADM and Mortality During Follow-up

Total mortality during follow-up by sex-specific tertiles of plasma MR-proADM was 10.6% (T1), 14.1% (T2), and 19.5% (T3) in DIABHYCAR (log-rank test $P < 0.0001$) and 11.5% (T1), 16.8% (T2), and 43.5% (T3) in SURDIAGENE (log-rank test $P < 0.0001$). Cox proportional hazards survival regression analyses showed a positive association of the highest tertile of plasma MR-proADM with mortality in both cohorts: HR 1.58 (95% CI 1.24–2.02, $P = 0.0002$) for DIABHYCAR and HR 3.22 (95% CI 2.33–4.55, $P < 0.0001$) for SURDIAGENE (Supplementary Table 5) (model 1: adjusted for sex and age at baseline plus study treatment in the original DIABHYCAR cohort). The associations remained significant when further adjusted for eGFR, arterial hypertension, and coronary artery disease (previous history of angina pectoris or myocardial infarction) at baseline (Supplementary Table 5) (model 2).

Plasma MR-proADM and *ADM* Genotypes

In both cohorts, plasma MR-proADM was significantly higher in carriers of the minor allele of rs4399321, rs11042725, and rs2957692 and was significantly lower in homozygous carriers of the minor allele of rs7944706 (Table 4). No genotype-related difference in plasma MR-proADM was observed for rs2957717. The allelic associations with plasma MR-proADM levels remained statistically significant in stepwise regression analyses when each of the

Table 2—SURDIAGENE study: clinical characteristics at baseline by sex-specific tertiles of plasma MR-proADM

	T1	T2	T3	*P*
Men/women (*n*)	246/182	246/182	246/182	
Plasma MR-proADM (nmol/L)§				
Men	0.53 (0.30–0.62)	0.71 (0.63–0.85)†	1.10 (0.86–6.48)†‡	<0.0001
Women	0.59 (0.34–0.67)	0.77 (0.68–0.88)†	1.20 (0.89–6.71)†‡	<0.0001
Age (years)	60 ± 10	65 ± 10†	71 ± 8†‡	<0.0001
Age at diagnosis of diabetes (years)	47 ± 10	51 ± 12†	53 ± 12†‡	<0.0001
Duration of diabetes (years)	12 ± 9	14 ± 10†	18 ± 11†‡	<0.0001
BMI (kg/m^2)	30.0 ± 5.1	31.7 ± 6.1†	32.1 ± 7.1†	<0.0001
HbA_{1c}, % (mmol/mol)	7.9 ± 1.5 (63 ± 17)	7.9 ± 1.6 (62 ± 18)	7.7 ± 1.5 (60 ± 16)†	0.04
Systolic BP (mmHg)	130 ± 16	132 ± 18	136 ± 20†‡	<0.0001
Diastolic BP (mmHg)	74 ± 10	72 ± 11	71 ± 12†	0.004
Arterial hypertension (%)	68	87	93	<0.0001
eGFR (mL/min/1.73 m^2)	93 ± 21	83 ± 22†	54 ± 31†‡	<0.0001
UAC (mg/L)§	16 (45)	17 (55)	71 (511)†‡	<0.0001
UAC status (%)	56/38/6	53/35/12	31/35/34	<0.0001
Previous myocardial infarction (%)	9	16	22	<0.0001
Previous angina pectoris (%)	11	19	24	<0.0001
Use of diuretics (%)	30	43	61	<0.0001
Use of ACE inhibitors (%)	28	40	46	<0.0001
Use of insulin (%)	55	59	71	<0.0001

Data are mean ± SD except for MR-proADM (median and range) and UAC (median and interquartile range). Arterial hypertension: systolic blood pressure (BP) >140 mmHg and/or diastolic blood pressure >90 mmHg or presence of antihypertensive medication and history of hypertension. UAC status: percentage of subjects with normoalbuminuria, microalbuminuria, or macroalbuminuria, respectively. Statistics for quantitative parameters are ANOVA with log-transformed data except §Wilcoxon test. Tukey-Kramer HSD test following ANOVA or Wilcoxon for each pair: significantly different from †T1 or ‡T2. $P < 0.05$ is significant.

SNPs was included as a covariate together with age, use of diuretics, BMI, blood glucose (DIABHYCAR), HbA_{1c} (SURDIAGENE), and eGFR levels at baseline (data not shown). The SNPs explained ~1% of the variance of plasma MR-proADM in both cohorts.

ADM Genotypes and Clinical Events During Follow-up

Average of consecutive eGFR during DIABHYCAR follow-up was significantly higher in homozygous carriers of the minor allele of rs4399321 and in carriers of the minor allele of rs11042725 and rs2957692 (Table 5). It was significantly lower in homozygous carriers of the minor allele of rs7944706. We observed a nominal ($0.01 < P \leq 0.05$) inverse association and a nominal positive association of the minor alleles of rs11042725 and rs7944706, respectively, with the incidence of renal events during the follow-up of DIABHYCAR (Table 6). When we considered the subset of patients with macroalbuminuria at baseline, robust inverse association with the incidence of renal events was observed for the minor alleles of rs4399321, rs11042725, and rs2957692 and again a positive association for the minor allele of rs7944706. Average eGFR during SURDIAGENE follow-up was significantly higher in carriers of the minor allele of rs11042725 and rs2957692 and significantly lower in homozygous carriers of the minor allele of rs7944706 and rs2957717 (Table 5). A nominal inverse association of the minor allele rs2957692 and a nominal positive association of the minor allele of rs7944706 with incidence of renal events were observed in SURDIAGENE (Table 6). Only a trend toward association was observed for rs11042725. An association of the minor allele of rs2957717 with total mortality during follow-up was observed in SURDIAGENE (data not shown). No other allelic association with mortality was observed in any of the cohorts.

DISCUSSION

In this study, we demonstrated that high plasma MR-proADM concentration was strongly associated with the risk of severe renal outcomes (doubling of plasma creatinine concentration and/or ESRD) and with all-cause mortality in two prospective cohorts of patients with type 2 diabetes. These associations were independent of baseline UAC and eGFR, as well as of other relevant covariates. We observed already at baseline a strong inverse correlation between plasma MR-proADM levels and eGFR. This correlation might result, at least in part, from reduced renal clearance of the peptide. It is noteworthy that eGFR was the main predictor of plasma MR-proADM concentration in both cohorts. It explained ~9% of MR-proADM variance in DIABHYCAR but as much as 54% of the variance in SURDIAGENE. The lower eGFR in SURDIAGENE than in DIABHYCAR participants may account for the

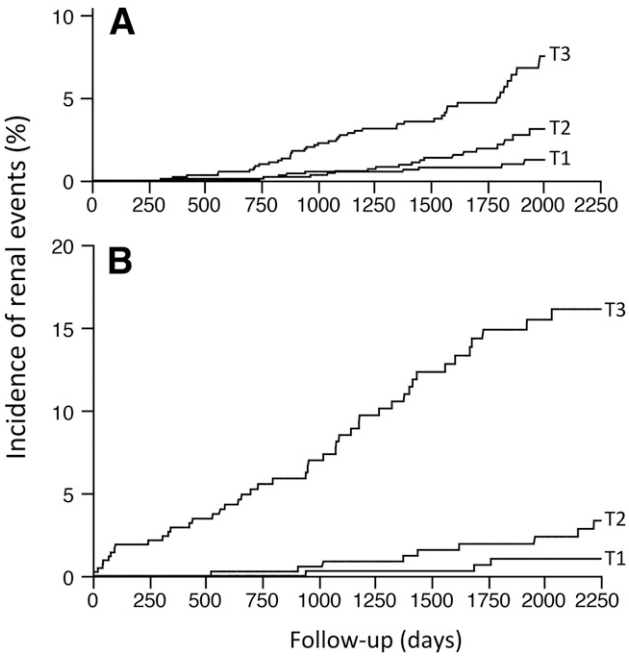

Figure 1—Kaplan-Meir curves: incidence of renal events during follow-up by tertiles of plasma MR-proADM in DIABHYCAR (*A*) and SURDIAGENE (*B*) studies. Renal events defined as doubling of serum creatinine levels or requirement of hemodialysis or renal transplantation. Log rank $P < 0.0001$ in both cohorts.

higher levels of plasma MR-proADM in SURDIAGENE than in DIABHYCAR participants.

Four SNPs located in a single haplotype block containing the *ADM* gene were associated with plasma MR-proADM concentration in both cohorts. The SNPs were also associated with eGFR during follow-up in both cohorts and with the incidence of renal events during follow-up in the subset of DIABHYCAR presenting with macroalbuminuria at baseline. Nominal associations were observed for two of the SNPs when we considered the whole DIABHYCAR cohort or the SURDIAGENE cohort. For each of the SNPs, the allele associated with lower eGFR and higher incidence of renal events was also associated with lower plasma MR-proADM levels. The direction of these associations is consistent with our working hypothesis of a protective effect of ADM on vascular and kidney function. In other words, the higher levels of plasma MR-proADM observed in our patients with severe clinical outcomes might result from an adaptive response to hypoxia, ischemia, and renal disease. We speculate that the risk alleles at the *ADM* locus are associated with a less efficient adaptive response, which is consistent with the observation that carriers of risk alleles display lower plasma levels of MR-proADM.

The functional variant or variants behind the allelic associations are not clearly identified. The haplotype block containing *ADM* extends for more than 40 kb with strong linkage disequilibrium. It is noteworthy that the four variants associated with plasma MR-proADM level and with eGFR are inside the haplotype block ($D' \geq 0.90$ between consecutive SNPs) (Supplementary Figure 1), while the variant not associated with the traits is located outside the block at 3′. Data from the literature suggest that rs11042725, located 1,923 bp upstream the translation start site, has a functional effect on *ADM* transcription and/or translation that could account for the association

Table 3—Risk of renal events during the follow-up by tertiles of plasma MR-proADM at baseline in DIABHYCAR and SURDIAGENE studies

	DIABHYCAR		SURDIAGENE	
Model 1	HR (95% CI)	*P*	HR (95% CI)	*P*
T3 vs. T1	5.35 (2.78–11.36)	<0.0001	30.81 (11.71–81.04)	<0.0001
T3 vs. T2	2.55 (1.51–4.49)	0.0004	9.34 (4.88–17.88)	<0.0001
T2 vs. T1	2.10 (1.00–4.70)	0.05	3.30 (1.15–9.45)	0.03
Model 2	HR (95% CI)	*P*	HR (95% CI)	*P*
T3 vs. T1	4.40 (2.27–9.40)	<0.0001	25.86 (10.51–78.77)	<0.0001
T3 vs. T2	2.16 (1.26–3.82)	0.004	9.43 (5.07–18.98)	<0.0001
T2 vs. T1	2.04 (0.97–4.58)	0.06	2.74 (1.00–8.78)	0.05
Model 3	Subhazard ratio (95% CI)	*P*	Subhazard ratio (95% CI)	*P*
T3 vs. T1	5.01 (2.47–10.14)	<0.0001	24.02 (8.15–70.76)	<0.0001
T3 vs. T2	2.45 (1.42–4.22)	0.001	7.38 (3.95–13.77)	<0.0001
T2 vs. T1	2.05 (0.95–4.41)	0.07	3.25 (1.08–9.83)	0.04

Renal event during follow-up defined as the doubling of the serum creatinine levels or ESRD. Model 1: adjusted for sex and age at baseline. Model 2: adjusted for sex, age, duration of diabetes, blood glucose (DIABHYCAR), HbA_{1c} (SURDIAGENE), eGFR, arterial hypertension, and coronary artery disease (previous history of angina pectoris or myocardial infarction) at baseline. Model 3: adjusted for sex and age at baseline and with death as a competing risk. In the DIABHYCAR cohort, all models were further adjusted by study treatment (randomization group in the original DIABHYCAR study: ramipril vs. placebo). HRs (models 1 and 2) computed by Cox proportional hazards survival regression analyses. Subhazard ratios (model 3) computed by competing risk regression analysis. $P < 0.05$ is significant.

Table 4—Plasma MR-proADM at baseline by *ADM* genotype in DIABHYCAR and SURDIAGENE studies

	DIABHYCAR			SURDIAGENE		
SNP	*N*	MR-proADM (nmol/L)	*P*	*N*	MR-proADM (nmol/L)	*P*
rs4399321						
AA	1,202	0.32 ± 0.01	0.006	544	0.77 ± 0.02	0.008
AG	1,304	0.35 ± 0.01		535	0.79 ± 0.02	
GG	219	0.36 ± 0.01		137	0.82 ± 0.03	
rs11042725						
CC	788	0.32 ± 0.01	0.008	355	0.76 ± 0.02	0.01*
CA	1,402	0.34 ± 0.01		603	0.79 ± 0.02	
AA	662	0.35 ± 0.01		285	0.80 ± 0.02	
rs7944706						
GG	929	0.34 ± 0.01	0.03†	388	0.80 ± 0.02	0.002†
GA	1,394	0.34 ± 0.01		620	0.79 ± 0.02	
AA	542	0.32 ± 0.01		255	0.75 ± 0.02	
rs2957692						
AA	971	0.32 ± 0.01	0.006*	428	0.76 ± 0.02	0.006*
AG	1,298	0.34 ± 0.01		559	0.80 ± 0.02	
GG	568	0.35 ± 0.01		247	0.80 ± 0.02	
rs2957717						
CC	1,255	0.34 ± 0.01	0.58	580	0.80 ± 0.02	0.13
TC	1,274	0.34 ± 0.01		522	0.79 ± 0.02	
TT	277	0.33 ± 0.01		134	0.77 ± 0.03	

Data are means ± SEM. Comparisons between genotypes are ANCOVA, adjusted for sex, age, BMI, blood glucose (DIABHYCAR), HbA_{1c} (SURDIAGENE), eGFR, and arterial hypertension status. Statistics computed for the minor alleles in a codominant, *dominant, or †recessive model. $P \leq 0.01$ is significant.

of the haplotype block with MR-proADM levels we have observed. In a luciferase gene reporter assay, constructs containing the A or the C allele of rs11042725 were transiently transfected into RN46A cells (33). Luciferase gene expression was significantly higher under the control of the A allele (associated in our study with higher plasma MR-proADM

Table 5—eGFR during follow-up by *ADM* genotype in DIABHYCAR and SURDIAGENE studies

	DIABHYCAR				SURDIAGENE			
SNP	Subjects	Samples	eGFR (mL/min)	*P*	Subjects	Samples	eGFR (mL/min)	*P*
rs4399321								
AA	1,202	5,960	70 ± 1	0.006*	544	10,079	54 ± 1	0.42
AG	1,304	6,193	70 ± 1		535	10,602	55 ± 1	
GG	219	1,545	73 ± 1		137	2,634	56 ± 1	
rs11042725								
CC	788	3,964	68 ± 1	0.002	355	6,400	52 ± 1	0.007†
CA	1,402	6,673	71 ± 1		603	11,976	55 ± 1	
AA	662	3,194	72 ± 1		285	5,879	56 ± 1	
rs7944706								
GG	929	4,468	72 ± 1	0.002*	388	8,004	57 ± 1	0.005
GA	1,394	6,672	70 ± 1		620	12,047	55 ± 1	
AA	542	2,720	68 ± 1		255	4,579	51 ± 1	
rs2957692								
AA	971	4,833	69 ± 1	0.0001†	428	8,134	52 ± 1	0.0007†
AG	1,298	6,239	72 ± 1		559	10,998	56 ± 1	
GG	568	2,694	71 ± 1		247	5,081	57 ± 1	
rs2957717								
CC	1,255	6,175	70 ± 1	0.47	580	10,999	55 ± 1	0.01*
TC	1,274	6,037	71 ± 1		522	10,524	55 ± 1	
TT	277	1,393	70 ± 1		134	2,616	51 ± 1	

Data are means ± SEM. Average values of eGFR throughout the study were compared between groups in mixed regression models with random effects, including as covariates age, sex, duration of diabetes, duration of follow-up at eGFR measurement, arterial hypertension, plasma MR-proADM concentration, subject identification, and randomization group in the original DIABHYCAR study (ramipril vs. placebo). The model takes into account that samples from the same individual are not independent. Statistics computed for the minor alleles in a codominant, *recessive, or †dominant model. $P \leq 0.01$ is significant.

Table 6—*ADM* genotype frequencies by the incidence of renal events during follow-up

	DIABHYCAR: all subjects		DIABHYCAR: subjects with macroalbuminuria at baseline		SURDIAGENE: all subjects	
SNP	No events (n = 3,060)	Renal events (n = 77)	No events (n = 686)	Renal events (n = 52)	No events (n = 1,208)	Renal events (n = 76)
rs4399321						
AA	0.426	0.471	0.399	0.600	0.447	0.457
AG	0.460	0.485	0.482	0.400	0.440	0.443
GG	0.114	0.044	0.119	0	0.113	0.100
MAF	0.344	0.287	0.360	0.200	0.333	0.321
HR (95% CI)	0.77 (0.53–1.11)		0.45 (0.26–0.74)		0.86 (0.59–1.23)	
P	0.17		0.001		0.40	
rs11042725						
CC	0.275	0.384	0.257	0.500	0.286	0.288
CA	0.491	0.452	0.495	0.396	0.482	0.534
AA	0.234	0.164	0.248	0.104	0.232	0.178
MAF	0.479	0.390	0.495	0.302	0.474	0.445
HR (95% CI)	0.70 (0.50–0.97)		0.48 (0.31–0.72)		0.58 (0.30–1.03)†	
P	0.03		0.0004		0.06	
rs7944706						
GG	0.326	0.229	0.344	0.192	0.310	0.257
GA	0.486	0.500	0.486	0.425	0.489	0.527
AA	0.188	0.271	0.170	0.383	0.201	0.216
MAF	0.431	0.521	0.413	0.596	0.445	0.480
HR (95% CI)	1.40 (1.01–1.95)		1.91 (1.28–2.87)		1.69 (1.02–2.96)	
P	0.04		0.001		0.04	
rs2957692						
AA	0.342	0.443	0.340	0.553	0.343	0.414
AG	0.458	0.371	0.452	0.319	0.455	0.414
GG	0.200	0.186	0.208	0.128	0.202	0.172
MAF	0.429	0.371	0.434	0.287	0.430	0.379
HR (95% CI)	0.68 (0.43–1.10)*		0.46 (0.26–0.82)*		0.58 (0.36–0.95)*	
P	0.12		0.008		0.03	
rs2957717						
CC	0.450	0.420	0.443	0.378	0.462	0.589
TC	0.451	0.449	0.448	0.467	0.431	0.288
TT	0.099	0.131	0.109	0.155	0.107	0.123
MAF	0.324	0.355	0.333	0.389	0.323	0.267
HR (95% CI)	1.20 (0.84–1.70)		1.22 (0.79–1.85)		0.61 (0.31–1.33)†	
P	0.31		0.36		0.20	

SNPs are sorted in 5′ to 3′ order. DIABHYCAR analyses adjusted for sex, age, duration of diabetes, HbA_{1c} levels, arterial hypertension, and randomization group in the original DIABHYCAR study (ramipril vs. placebo). SURDIAGENE analyses adjusted for sex, age, duration of diabetes, HbA_{1c} levels, plasma MR-proADM concentration, arterial hypertension, and treatment by ACE inhibitors. $P \leq 0.01$ is significant. HR for the minor allele in a codominant, *dominant, or †recessive model computed by Cox proportional hazards survival regression analyses.

levels and less severe kidney phenotypes) than under the control of the C allele. Several missense variants were described in the *ADM* gene (www.ncbi.nlm.nih.gov/variation/view/?q=ADM&filters=source:dbsnp&assm=GCF_000001405.25), but their effect on ADM circulating levels is unknown. Given their low frequency in Caucasians (MAF <0.001), linkage disequilibrium with these variants could not explain the associations we have observed.

Our results extend and complement those of a few studies that investigated associations of plasma MR-proADM with nephropathy in patients with type 2 diabetes. Higher plasma levels of ADM or of MR-proADM were observed in patients with diabetic nephropathy than in patients without nephropathy in small cross-sectional Japanese and Chinese studies (25,26). However, no independent association was observed with the incidence of new cases of albuminuria in a larger Dutch study (ZODIAC) with a median follow-up of 5.6 years (27). Association of high plasma MR-proADM with cardiovascular morbidity and mortality has also been reported in diabetic and nondiabetic subjects (21–24,27). Associations of rs2957692 and rs2957717 with plasma MR-proADM have been reported in other populations (35,39).

The strengths of our study are the detailed phenotype assessment of renal function during a 5-year follow-up, the replication of findings in an independent cohort, and the genotyping of SNPs covering the haplotype block containing *ADM*. However, our study has limitations. First, the design did not allow any firm conclusion on

causality between circulating ADM, *ADM* variants, and disease evolution. Second, we have not measured the true GFR but used an estimation index based on plasma creatinine levels. Third, the number of renal events during follow-up in both cohorts was relatively small. Statistical power was adequate to detect SNP effects with HR $\geq$1.5 in the subset of DIABHYCAR participants with macroalbuminuria but might have been insufficient to detect effects of smaller magnitude. On the other hand, it is unlikely that these genetic findings only reflect type 1 error (false positive results). A type 1 error due to population stratification is less likely to occur in a prospective study, less prone to selection bias, than in case control studies. Moreover, genetic findings were consistent in two independent cohorts, and allelic associations in both cohorts were also observed during follow-up with the evolution of eGFR, an independent trait. Finally, we studied two Caucasian cohorts with a mixed, largely European background, and the allelic associations we have observed may not apply to people from other ethnic backgrounds.

ADM is expressed and secreted in many cell types including endothelial and vascular smooth muscle cells, cardiomyocytes, fibroblasts, monocytes, and leukocytes (40). At the kidney level, ADM mRNA was detected in the glomeruli, distal tubules, and cortical and medullary collecting ducts of rat nephrons (41). The effects of chronic modulation of ADM action on kidney damage were assessed in a few experimental studies. Exogenous ADM attenuated tubular damage in rats with streptozotocin-induced diabetes (42) and prevented tubulointerstitial fibrosis and cell proliferation in a rat model of renal fibrosis (43). Reciprocally, a small decrease in ADM expression aggravated renal fibrosis in mice with a renin transgene (44). These results support the hypothesis that ADM protects against renal damage. Several mechanisms might be implicated in the renoprotective effect of ADM, including modulation of renal hemodynamics (45) and inhibition of reticulum endoplasmic stress (46) and of oxidative stress (47), a key driver of development of renal damage in diabetes (48). Moreover, ADM has antagonistic effects on the RAS (15), inhibiting both the production and the actions of angiotensin II (49,50). ADM was shown to inhibit angiotensin II–induced migration and proliferation of mesangial cells in the rat kidney (49), to inhibit the generation of reactive oxygen species induced by angiotensin II, and to decrease oxidative stress in the rat vascular wall (47). In contrast, experimental evidences suggest that the effects of ADM on renal hemodynamics are not mediated by RAS inhibition. ADM infusion in the renal artery of anesthetized dogs induced a drop in the filtration fraction and a rise in the sodium fractional excretion, with no effect on blood pressure or glomerular filtration rate (45). This is suggestive of a vasodilatory effect on both afferent and efferent arterioles, associated with an effect on glomerular permeability, tubular function, or both. In contrast, the known renoprotective effect of RAS blockers is based, at least in part, on a decreased intraglomerular pressure and reduced filtration. These observations argue for independent effects of the RAS blockers and ADM on renal hemodynamics. In support of this hypothesis, we did not observe any interaction between use of RAS blockers or ACE insertion/deletion genotype and plasma MR-proADM on the associations with clinical outcomes and phenotypes (data not shown).

In conclusion, we showed strong associations between plasma MR-proADM levels and risk of severe renal outcomes and mortality in patients with type 2 diabetes, as well as associations between SNPs at the *ADM* locus and both plasma MR-proADM and renal outcomes. The risk alleles of the SNPs for the clinical outcomes were associated with lower MR-proADM levels. We propose as an explanation for our results that the higher levels of MR-proADM observed in patients with pejorative clinical outcome might result from an adaptive response, which is less efficient in carriers of the risk alleles. These results are consistent with a protective role of ADM against diabetic nephropathy, a hypothesis supported by data reported in experimental models of kidney disease that deserves further clinical investigation. Possible therapeutic effect of ADM or ADM receptor agonists in diabetic renal disease would also be worth investigating.

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