Plasma Adrenomedullin, Allelic Variations in the *ADM* Gene, and Risk for Lower-Limb Amputation in People With Type 2 Diabetes

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PATHOPHYSIOLOGY/COMPLICATIONS

OBJECTIVE

Patients with diabetes have an increased risk for lower-limb amputation (LLA), but biomarkers to assess risk of LLA are lacking. Adrenomedullin (ADM) is a vasodilator peptide that also plays a role in fluid and electrolyte homeostasis in the kidney, increasing natriuresis and diuresis. ADM was shown to be associated with cardiovascular and renal events in diabetes, but it was not investigated in terms of LLA risk. We investigated the hypothesis that ADM is associated with LLA in people with type 2 diabetes.

RESEARCH DESIGN AND METHODS

We studied 4,375 participants in the DIABHYCAR and SURDIAGENE cohorts (men, 68%; mean 66 years of age; mean duration of diabetes 12 years; and median follow-up 5.3 years). Plasma midregional proadrenomedullin (MR-proADM; a surrogate for ADM) was measured by immunofluorescence. Five single nucleotide polymorphisms (SNPs) in the *ADM* gene region were genotyped.

RESULTS

LLA requirement during follow-up by increasing tertiles of plasma MR-proADM distribution was 1.0% (tertile 1 [T1]), 2.3% (T2), and 4.4% (T3) ($P < 0.0001$). In Cox multivariate analysis, the adjusted hazard ratio (95% CI) for LLA was 4.40 (2.30–8.88) ($P < 0.0001$) for T3 versus T1. Moreover, MR-proADM significantly improved indices for risk stratification of LLA. Four SNPs were associated with plasma MR-proADM concentration at baseline and with LLA during follow-up. Alleles associated with higher MR-proADM were associated with increased LLA risk.

CONCLUSIONS

We observed associations of plasma MR-proADM with LLA and of *ADM* SNPs with plasma MR-proADM and with LLA in people with type 2 diabetes. This pattern of Mendelian randomization supports the causality of the association of ADM with LLA.

Diabetes is the leading cause of nontraumatic lower-limb amputation (LLA) (1). LLA is a severe complication of diabetes (2), associated with excess risk of cardiovascular and noncardiovascular diseases, and resulting in subsequent loss of quality of life and a significant reduction in life expectancy (3–5). LLA in diabetes is related to the presence of foot ulcers leading to ischemia and necrosis and results from a

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range of conditions, including peripheral arterial disease (PAD), diabetic neuropathy, impaired wound healing, and susceptibility to infection (6). However, despite these well-known causal factors, biomarkers able to predict the risk of LLA are lacking.

Adrenomedullin (ADM) is a 52-amino acid peptide expressed and secreted in many cell types, including endothelial and vascular smooth muscle cells, cardiomyocytes, fibroblasts, monocytes, leukocytes, and the kidney cortex and medullary collecting ducts (7). Production and secretion of ADM are increased in response to hypoxia and ischemia (7,8). At physiological concentrations, ADM was shown to have potent vasodilator and hypotensive effects in the systemic and pulmonary circulations (9). In addition, ADM plays a role in fluid and electrolyte homeostasis, increasing natriuresis and diuresis (10,11). ADM is formed from the precursor peptide preproadrenomedullin by enzymatic cleavage. The midregional 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 considered a stable surrogate of ADM (12). High plasma MR-proADM levels have been observed in many clinical conditions associated with hypoxia and ischemia, including PAD, coronary heart disease, and chronic heart failure (13–16).

In the present investigation, we assessed the relationship between baseline plasma MR-proADM concentration and the risk of LLA and the requirement of lower-limb revascularization (LLRV) during follow-up in two independent cohorts of patients with type 2 diabetes. In addition, to address causality between ADM and LLA, we looked at polymorphisms in the *ADM* locus and their relationship with circulating MR-proADM and LLA risk.

RESEARCH DESIGN AND METHODS

Study Population

DIABHYCAR was a multinational, multicenter clinical trial conducted in people with type 2 diabetes selected on the basis of persistent microalbuminuria or macroalbuminuria without renal failure (plasma creatinine <150 μmol/L) at baseline (17). The trial tested the effect of a low dose of ramipril, an ACE inhibitor (ACE-I) on the incidence of cardiovascular and/or renal events. The median duration of follow-up was 4.7 years. Results were negative regarding the drug effect and were published previously (18). SURDIAGENE is a prospective monocenter study aiming to identify the genetic and environmental determinants of vascular complications in type 2 diabetes (19). Patients have been recruited and followed regularly at the Diabetes Department of the University Hospital of Poitiers (Poitiers, France). Living status and cardiovascular and kidney end points were determined from hospital records and interviews with general practitioners. Median duration of follow-up was 7.1 years. Detailed description of study population, outcome criteria, and adjudication procedure was published previously for both cohorts (18–20). In the present investigation, we studied 2,962 and 1,413 participants with type 2 diabetes from the DIABHYCAR and SURDIAGENE cohorts, respectively, for whom plasma MR-proADM at baseline and LLA information during follow-up were available. Participants from both cohorts provided written informed consent, and study protocols were approved by the ethics committee of Angers University Hospital, Angers, France (DIABHYCAR) and the CCP Ouest III Ethics Committee, Poitiers, France (SURDIAGENE).

Definition of Clinical Parameters and Outcomes

In both cohorts, an ad hoc event committee reviewed the case record of each patient to validate the baseline data and outcomes during follow-up (17,19). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration study equation for serum creatinine (21). Microalbuminuria was defined as urinary albumin concentration (UAC) between 30 and 300 mg/24 h or 20 and 200 μg/min or 20 and 200 mg/L and macroalbuminuria as UAC >300 mg/24 h or >200 μg/min or >200 mg/L. The primary outcome was the first occurrence of LLA during follow-up, defined as a nontraumatic amputation at or above the metatarsophalangeal joint. The requirement of LLRV (angioplasty or bypass surgery) during follow-up was considered as a secondary outcome. Information regarding the level of amputation (transmetatarsal, transtibial, or transfemoral) was available only for SURDIAGENE participants.

Laboratory Procedures

MR-proADM concentration was measured in plasma-EDTA samples by an automated immunofluorescent sandwich immune assay (B·R·A·H·M·S MR-proADM KRYPTOR; Thermo Fisher Scientific, Hennigsdorf, Germany) (12). 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* (22), rs4399321 and rs7944706 to be in a haplotype block associated with proteinuria in subjects with essential hypertension (23), and rs2957692 and rs2957717 to be associated with MR-proADM levels in a genome-wide association study (24). 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 the haplotype block containing *ADM*. Location of the SNPs and linkage disequilibrium between SNPs in our cohorts were published previously (25) and are summarized in Supplementary Fig. 1. Genotypes were determined by competitive allele-specific PCR genotyping system assays (KASP; LGC Biosearch Technologies, Hoddeston, U.K.). Genotyping success rate was $>95\%$. Genotypes were in Hardy-Weinberg equilibrium ($P > 0.01$).

Computations and Statistical Analyses

Categorical variables were expressed as number of participants with corresponding percentage. Continuous variables were expressed as mean ± SD, mean ± SEM when regressed in adjustment models, or median and interquartile range (IQR) for those with skewed distribution. Data from SURDIAGENE and DIABHYCAR were pooled to increase sample size and the number of events at baseline (LLA) and during follow-up (LLA or LLRV) and thus the statistical power of the analyses. Characteristics of participants at baseline were compared using Pearson χ^2 test, Fisher exact test, Student *t* test, ANOVA, or Wilcoxon test. Associations of MR-proADM with the prevalence of LLA at baseline were assessed by logistic regression analyses, adjusted for relevant confounding covariates (see regression

model 1 below), with odds ratios (OR) and associated 95% CI computed for cohort- and sex-specific tertiles of baseline plasma MR-proADM distribution and for 1 SD of log[MR-proADM]. For the computations of OR and hazard ratios (HR; see below) for 1 SD of log[MR-proADM], a *z* score of log[MR-proADM] was calculated for each participant, taking into account the mean and SD of log[MR-proADM] of pooled cohorts.

Kaplan-Meier curves were used to plot the incidence of outcomes over time. Difference of incidence between groups was compared by log-rank test. Cox proportional hazards regression models were fitted to estimate associations of baseline plasma MR-proADM or *ADM* genotypes with the outcomes. HR with associated 95% CI were computed in these analyses for *ADM* genotypes, for cohort and sex-specific tertiles of baseline plasma MR-proADM distribution, and for 1 SD of log[MR-proADM]. MR-proADM–related risk of LLA and LLRV was adjusted for cohort membership, sex, age, BMI, duration of diabetes, arterial hypertension, tobacco smoking, HbA_{1c}, total cholesterol, HDL-cholesterol, eGFR, UAC, and use of insulin, ACE-I or angiotensin receptor blocker (ARB), diuretics, antiplatelet or anticoagulation drugs, and lipid-lowering drugs at baseline (model 1) plus previous history of LLA at baseline (model 2). Continuous covariates with skewed distribution were included in the regression models as a *z* score of log-transformed data and UAC as a categorical variable (normo-, micro-, or macroalbuminuria). Cohort membership was included as a covariate in the regression models to take into account cohort-related differences.

A set of sensitivity or additional analyses was performed. First, associations between MR-proADM with the incidence of minor (transmetatarsal) or major (transtibial or transfemoral) LLA, individually, were assessed in the SURDIAGENE cohort. Second, since the risk of LLA is higher in patients using diuretics (26), we compared MR-proADM–related risk of LLA in subgroups of patients stratified by diuretics use at baseline. Third, to assess the impact of renal function on MR-proADM–related risk of LLA, Cox analyses were performed independently in subgroups of patients stratified by eGFR below or equal/above the median of eGFR distribution. We also assessed interactions of MR-proADM with use of diuretics and with eGFR in MR-proADM–related risk of LLA by including multiplicative interaction terms in the regression models. Fourth, correlations of clinical parameters at baseline (those included as covariates in the Cox analyses) with incident LLA were assessed in univariate regressions and in a stepwise regression analysis with backward selection. To standardize comparisons of coefficient of determination (R^2), continuous parameters were normalized as *z* scores of log-transformed data. Fifth, Harrell C-statistic, integrated discrimination improvement, and continuous net reclassification improvement indices were computed to evaluate prognostic value of baseline plasma MR-proADM on top of traditional LLA risk factors (sex, age, duration of diabetes, HbA_{1c}, systolic and diastolic blood pressure, total cholesterol, HDL-cholesterol, eGFR, UAC, and tobacco smoking) in the discrimination and classification of incident LLA as assessed by survival methodology (27). Statistics were performed with JMP (Cry, NC) (https://www.jmp.com), SAS (https://www.sas.com), and Stata (College Station, Texas) (https://www.stata.com) software. A *P* value <0.05 was considered significant.

RESULTS

MR-proADM and Clinical Characteristics at Baseline

Baseline characteristics of participants by cohort- and sex-specific tertiles of MR-proADM are shown in Table 1. Participants in the higher tertile of plasma MR-proADM were older, had a longer duration of diabetes, higher BMI, systolic blood pressure, HbA_{1c}, and UAC, and had a lower eGFR. They were less likely to be active smokers, more likely to have a history of arterial hypertension, myocardial infarction, and stroke, and more likely to use renin-angiotensin system blockers, diuretics, antihypertensive drugs, antiplatelet or anticoagulation drugs, and insulin. They also had higher plasma copeptin, a surrogate of vasopressin and a marker of the hydration status. The prevalence of LLA at baseline was lower in the first tertile, intermediate in the second tertile, and higher in the third tertile of MR-proADM (Pearson χ^2 test, $P < 0.0001$). In logistic regression analyses, the higher tertiles of baseline plasma MR-proADM and log[MR-proADM] were significantly and positively associated with the prevalence of LLA at baseline (Table 2).

Baseline MR-proADM and Risk of LLA During Follow-up

The median (IQR) duration of follow-up was 5.3 (1.8) years. The cumulative incidence of LLA during follow-up was 2.5% (n = 111), and its incidence rate was 4.8/1,000 person-years. Baseline characteristics of participants by the occurrence of LLA during follow-up were previously reported (28) and are summarized in Supplementary Table 1. Briefly, incident cases of LLA, compared with participants not presenting the outcome, were more likely to be men, had a longer duration of diabetes, had higher concentrations of MR-proADM, total cholesterol, and UAC, had lower eGFR and HDL cholesterol, and were more likely to be taking renin-angiotensin system blockers, diuretics, antihypertensive drugs, antiplatelet or anticoagulation drugs, lipid-lowering drugs, and insulin. A history of LLA at baseline was more frequent in incident cases. Transmetatarsal, transtibial, or transfemoral amputation accounted for 47%, 35%, and 18%, respectively, of the 77 incident cases of LLA in SURDIAGENE participants.

The Kaplan-Meier curve for the incidence of LLA during follow-up by tertiles of baseline plasma MR-proADM is shown in Fig. 1. The cumulative incidence of LLA was 1.0% for the first tertile (T1), 2.3% for the second tertile (T2), and 4.4% for the third tertile (T3; log-rank test χ^2 = 57.6; $P < 0.0001$), and the incidence rate was 1.6 (T1), 4.2 (T2), and 9.8 (T3)/1,000 person-years. In Cox regression analyses, the higher tertiles of baseline plasma MR-proADM and log[MR-proADM] were significantly and positively associated with increased risk of LLA during follow-up in all regression models that were tested (Table 2). In sensitivity analysis in the SURDIAGENE cohort, the highest tertile of baseline plasma MR-proADM was significantly associated with increased risk of both minor (transmetatarsal) and major (transtibial or transfemoral) amputations during follow-up (Supplementary Table 2).

The incidence of LLA in users and nonusers of diuretics at baseline was 4.3% vs. 1.8%, respectively ($P < 0.0001$). MR-proADM was 0.59 (0.52) vs. 0.36

Table 1—Baseline characteristics of participant by tertiles of MR-proADM

	T1	T2	T3	*P*
N DIABHYCAR/SURDIAGENE	990/471	986/471	986/471	—
MR-proADM				
DIABHYCAR, nmol/L*	0.15 (0.08)	0.30 (0.08)	0.50 (0.17)	<0.0001
SURDIAGENE, nmol/L*	0.55 (0.10)	0.75 (0.10)	1.16 (0.56)	<0.0001
Sex (male), *n* (%)	997 (68)	993 (68)	993 (68)	0.99
Age, years	62 ± 9	65 ± 8	69 ± 9	<0.0001
Duration of diabetes, years	11 ± 8	11 ± 9	13 ± 9	<0.0001
BMI, kg/m^2	29.5 ± 4.7	30.0 ± 5.2	30.4 ± 5.9	<0.0001
Systolic blood pressure, mmHg	140 ± 16	141 ± 16	142 ± 17	<0.0001
Diastolic blood pressure, mmHg	79 ± 10	79 ± 10	78 ± 11	0.14
Arterial hypertension, *n* (%)	824 (56)	954 (65)	1076 (73)	<0.0001
Current tobacco smoking, *n* (%)	218 (15)	199 (14)	162 (11)	0.008
Previous myocardial infarction, *n* (%)	92 (6.3)	125 (8.6)	166 (11.4)	<0.0001
Previous stroke, *n* (%)	45 (3.1)	65 (4.5)	87 (6.0)	0.0008
Previous LLA, *n* (%)	7 (0.5)	24 (1.7)	41 (2.8)	<0.0001
HbA_{1c}, %	7.9 ± 1.7	7.9 ± 1.7	7.7 ± 1.7	0.01
HbA_{1c}, mmol/mol	63 ± 18	62 ± 19	61 ± 18	0.01
Total cholesterol, mmol/L	5.48 ± 1.16	5.44 ± 1.16	5.46 ± 1.26	0.67
HDL cholesterol, mmol/L	1.28 ± 0.36	1.28 ± 0.37	1.29 ± 0.40	0.70
Triglycerides, mmol/L*	1.76 (1.43)	1.75 (1.25)	1.76 (1.26)	0.90
Plasma copeptin, pmol/L*	5.85 (5.58)	6.77 (6.60)	9.71 (10.26)	<0.0001
Plasma creatinine, μmol/L*	80 (22)	84 (26)	97 (39)	<0.0001
eGFR, mL/min/1.73 m^2	82 ± 17	77 ± 17	63 ± 21	<0.0001
UAC, mg/L*	50 (90)	58 (112)	85 (256)	<0.0001
UAC stages, *n* (%)				
Normoalbuminuria	267 (18)	244 (17)	128 (9)	
Microalbuminuria	957 (66)	923 (63)	842 (58)	<0.0001
Macroalbuminuria	237 (16)	287 (20)	484 (33)	
Use of antiplatelet or anticoagulation drugs, *n* (%)	339 (24)	409 (28)	552 (38)	<0.0001
Use of lipid-lowering drugs, *n* (%)	375 (26)	362 (25)	416 (29)	0.06
Use of blood pressure–lowering drugs, *n* (%)	808 (55)	963 (66)	1,083 (74)	<0.0001
Use of ACE-I or ARB, *n* (%)	291 (20)	362 (25)	413 (28)	<0.0001
Use of diuretics, *n* (%)	311 (21)	418 (29)	589 (40)	<0.0001
Use of insulin, *n* (%)	255 (17)	275 (19)	326 (22)	0.003

Quantitative data expressed as mean ± SD unless otherwise indicated. Statistics are ANOVA or Pearson χ^2 test. $P < 0.05$ was significant. *Data are median (IQR), Wilcoxon test.

(0.35) nmol/L, respectively (median [IQR]; Wilcoxon test, $P < 0.0001$). The hazard risk of LLA during follow-up for the T3 versus the T1 of MR-proADM distribution (model 1) was 6.70 (2.07–30.25; $P = 0.0009$) in users and 3.08 (1.39–7.22; $P = 0.005$) in nonusers of diuretics (P for interaction = 0.70). The median (IQR) of baseline eGFR distribution was 75 (47) mL/min/1.73 m^2. The incidence of LLA in participants with baseline eGFR below and equal/above the median was 3.1% vs. 2.0%, respectively ($P = 0.02$). MR-proADM was 0.46 (0.53) vs. 0.37 (0.36) nmol/L, respectively (median [IQR]; Wilcoxon test, $P < 0.0001$). The hazard risk of LLA during follow-up for the T3 versus T1 of MR-proADM distribution was 3.68 (1.38–12.76; $P = 0.007$) for participants with baseline eGFR below the median and 3.76 (1.47–9.82; $P = 0.006$) for those with baseline eGFR equal/above the median (P for interaction = 0.40).

Prognostic Value of MR-proADM as a Marker of LLA

In univariate correlation analyses of baseline clinical parameters with incident LLA, the highest coefficients of correlation (R^2) were observed for a history of previous LLA and for plasma MR-proADM (Supplementary Table 3). In a stepwise

Table 2—LLA at baseline and outcomes during follow-up by baseline plasma MR-proADM

	Crude model		Adjusted model 1		Adjusted model 2	
	OR or HR (95% CI)	*P*	OR or HR (95% CI)	*P*	HR (95% CI)	*P*
LLA at baseline						
T3 vs. T1	6.01 (2.87–14.70)	<0.0001	6.79 (2.42–20.94)	0.0002		
T2 vs. T1	3.48 (1.57–8.77)	0.002	3.09 (1.27–8.36)	0.01		
T3 vs. T2	1.73 (1.05–2.92)	0.03	2.20 (1.09–4.49)	0.03		
Log[MR-proADM]	3.89 (3.05–5.02)	<0.0001	2.52 (1.33–4.76)	0.005		
Primary outcome: LLA						
T3 vs. T1	6.55 (3.78–12.21)	<0.0001	4.40 (2.30–8.88)	<0.0001	3.80 (1.98–7.67)	<0.0001
T2 vs. T1	2.60 (1.42–5.02)	0.002	2.17 (1.16–4.26)	0.01	1.97 (1.05–3.87)	0.03
T3 vs. T2	2.52 (1.66–3.89)	<0.0001	2.03 (1.26–3.30)	0.003	1.93 (1.19–3.15)	0.007
Log[MR-proADM]	2.42 (1.96–2.99)	<0.0001	2.07 (1.41–3.05)	0.0002	1.82 (1.24–2.67)	0.002
Secondary outcome: LLRV						
T3 vs. T1	2.69 (1.90–3.84)	<0.0001	1.83 (1.22–2.77)	0.003		
T2 vs. T1	1.41 (0.97–2.10)	0.07	1.28 (0.87–1.89)	0.25		
T3 vs. T2	1.90 (1.38–2.64)	<0.0001	1.43 (1.01–2.04)	0.04		
Log[MR-proADM]	1.38 (1.18–1.62)	<0.0001	1.33 (1.05–1.69)	0.02		

OR for LLA at baseline computed by logistic regression analysis and HR for LLA and LLRV during follow-up computed by Cox proportional hazards survival regression analysis for tertiles of plasma MR-proADM and for 1 SD of log[MR-proADM]. Model 1: adjusted for cohort membership, sex, age, BMI, duration of diabetes, arterial hypertension, tobacco smoking, HbA_{1c}, total cholesterol, HDL-cholesterol, eGFR, UAC, and use of insulin, ACE-I or ARB, diuretics, antiplatelet or anticoagulation drugs, and lipid-lowering drugs at baseline. Model 2: model 1 plus adjustment for previous history of LLA at baseline. Number of participants with/without LLA at baseline by tertiles of MR-proADM: 7/1,454 (T1), 24/1,433 (T2), and 41/1,416 (T3). Number of participants with/without incident LLA during follow-up (primary outcome) by tertiles of MR-proADM: 14/1,447 (T1), 33/1,424 (T2), and 64/1,393 (T3). Number of participants with/without LLRV during follow-up (secondary outcome) by tertiles of MR-proADM: 48/1,413 (T1), 62/1,395 (T2), and 94/1,363 (T3).

regression analysis, a history of previous LLA and plasma MR-proADM at baseline remained the most important contributors to the outcome variation during follow-up (Supplementary Table 3).

The prognostic value of baseline plasma MR-proADM for discrimination and classification of LLA was assessed. When added to a basic model of traditional risk factors, log[MR-proADM] significantly improved Harrell C-statistic index ($P = 0.009$), relative integrated discrimination improvement ($P < 0.001$), and categorical net reclassification improvement ($P < 0.0001$) for risk stratification of LLA (Supplementary Table 4).

Baseline MR-proADM and LLRV During Follow-up

LLRV was performed in 204 (4.7%) participants during follow-up (incidence rate 8.9/1,000 person-years). Characteristics of participants who had a revascularization as compared with those who had not are shown in Supplementary Table 1. The cumulative incidence of LLRV during follow-up by tertiles of baseline plasma MR-proADM was 3.3% (T1), 4.3% (T2), and 6.5% (T3; log-rank test $\chi^2 = 36.5$; $P < 0.0001$) (Fig. 1), and the incidence rate was 5.6 (T1), 7.9 (T2), and 14.5 (T3)/1,000 person-years. The highest tertile of baseline plasma MR-proADM and log[MR-proADM] was significantly and positively associated with the requirement of LLRV during follow-up in Cox regression analyses (Table 2).

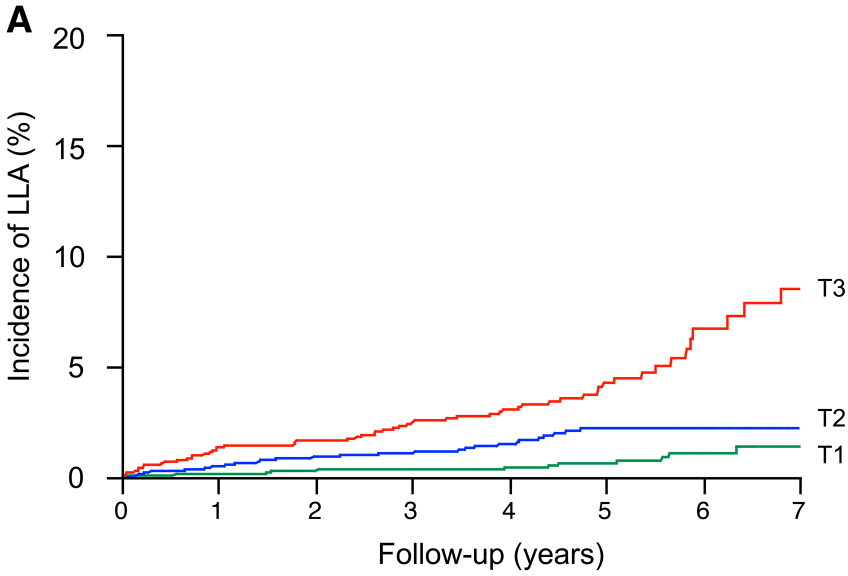
ADM Variants, Plasma MR-proADM, and LLA Risk

Associations of all SNPs except rs2957717 with MR-proADM levels both in DIABHYCAR and SURDIAGENE were reported previously (25). Results from pooled cohorts are shown in Supplementary Table 5. The A-allele of rs11042725 and the G-alleles of rs4399321, rs7944706, and rs2957692 were significantly associated with higher plasma MR-proADM levels. Genotype frequency and MAF for incident and nonincident cases of LLA are shown in Supplementary Table 6. The Kaplan-Meier curves for the incidence of LLA during follow-up by *ADM* SNPs are shown in Supplementary Fig. 2. Alleles or genotypes associated with high plasma MR-proADM levels were also associated with increased risk of LLA during follow-up (Table 3).

CONCLUSIONS

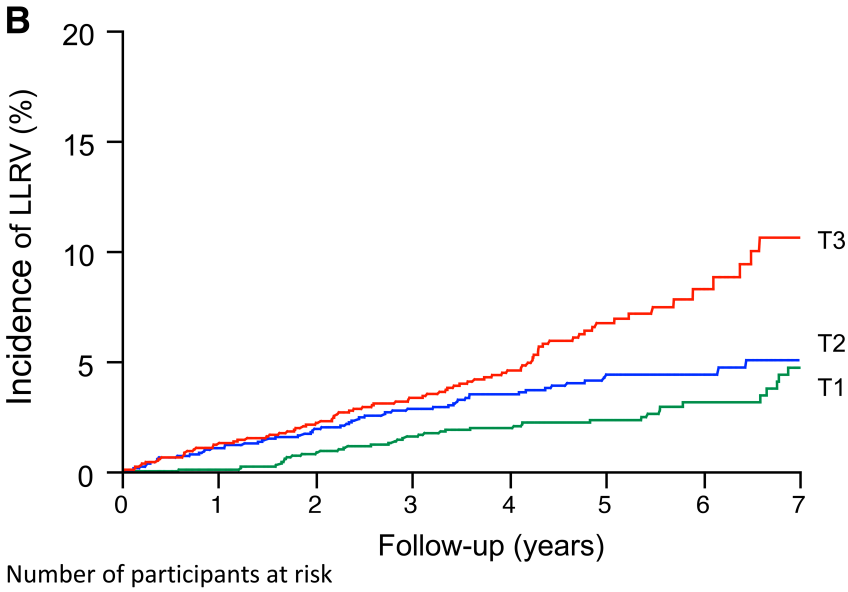
In the present investigation, high MR-proADM levels were strongly associated with the prevalence of LLA at baseline and with the incidence of LLA and the requirement of LLRV during a median follow-up of 5 years in two cohorts of people with type 2 diabetes. These associations were independent of other traditional risk factors of LLA, including duration and severity of diabetes, tobacco smoking, a history of arterial hypertension and cardiovascular disease or chronic kidney disease, and use of diuretics, as well as of other relevant covariates. A history of previous LLA and high MR-proADM at baseline were the most important contributors to the outcome variation during follow-up. Moreover, when added on top of a regression model of traditional risk factors of LLA, MR-proADM significantly improved the indices for risk stratification of the outcome.

Four SNPs located in a single haplotype block containing the *ADM* gene were associated both with plasma MR-proADM concentration and with the incidence of LLA during follow-up. For each of the SNPs, the allele associated with higher plasma MR-proADM levels was also associated with higher incidence of LLA. These associations suggest a pattern of Mendelian randomization and are consistent with the hypothesis of a causal effect of ADM on the pathophysiology of LLA.



Number of participants at risk

T1	1,461	1,339	862	303
T2	1,457	1,258	721	263
T3	1,457	1,138	511	140



Number of participants at risk

T1	1,461	1,324	847	297
T2	1,457	1,234	710	259
T3	1,457	1,124	500	138

Figure 1—Kaplan-Meier curves for the cumulative incidence of outcomes during follow-up by tertiles of baseline plasma MR-proADM. *A*: Primary outcome: LLA (log-rank test $\chi^2 = 57.6$; $P < 0.0001$). *B*: Secondary outcome: LLRV (log-rank test $\chi^2 = 23.6$; $P < 0.0001$).

The functional variant or variants backing the allelic associations are not clearly identified. The haplotype block containing *ADM* extends for >40 kb with strong linkage disequilibrium. There are ~130 variants with an MAF >5% in the *ADM* haplotype block between SNPs rs4399321 and rs2957692 (https://www.ensembl.org/Homo_sapiens/Location/Variant/Table? r=11:10301931-10346572). However, data from the literature suggest that rs11042725, located 1,923 bp upstream of the translation start site, has a functional effect on *ADM* transcription and/or translation that could account for the association 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 (22). Luciferase gene expression was significantly higher under the control of the A-allele (associated in our study with higher plasma MR-proADM levels and with LLA) than under the control of the C-allele. It is noteworthy that the other three SNPs (rs4399321, rs7944706, and rs2957692) associated with baseline plasma MR-proADM levels and LLA during follow-up are in strong linkage disequilibrium with rs11042725 (Supplementary Fig. 1). Those four SNPs are inside the *ADM* haplotype block ($D' \geq 0.90$ between consecutive SNPs), while rs2957717, not associated with the traits, is located outside the block at 3′.

The pathophysiological mechanisms behind the associations of MR-proADM and *ADM* SNPs with the outcomes are unclear. High circulating levels of MR-proADM were observed in cardiovascular complications of diabetes (16,29), but are not specific to diabetes complications. They are also observed in a variety of clinical conditions in populations without diabetes, including chronic airway obstruction, arterial hypertension, congestive heart failure, occlusive peripheral arterial disease, and ischemic heart disease (13–15,30). Experimental evidence suggests that ADM protects against organ damage during ischemia or hypoxia (31–33), and thus, the increase in circulating ADM levels observed in the abovementioned pathological conditions might be an adaptive response to cellular aggression. Kidney hypoxia, especially at the glomerular and tubular levels, was observed in diabetes (34), and the ADM protective effect in the kidney was well documented by experimental studies (35–37). In this regard, we have previously observed in the same cohorts of the present investigation that high plasma MR-proADM concentration was associated with renal function decline and risk of severe renal outcomes (25). The SNP risk alleles associated with higher incidence of renal outcomes were associated with lower plasma MR-proADM levels. The direction of these associations was consistent with the hypothesis of a protective effect of ADM on kidney function, with a less efficient adaptive response in carriers of the risk alleles. However, in the present investigation,

Table 3—Risk of LLA during follow-up by *ADM* genotype

SNP	MAF		Crude model		Adjusted model 1		Adjusted model 2		Genetic model
	LLA	No LLA	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	
rs4399321	0.406	0.339	1.83 (1.05–3.17)	0.04	1.48 (1.11–1.96)	0.008	1.39 (1.05–1.84)	0.02	Codominant G
rs11042725	0.536	0.474	1.53 (1.01–2.26)	0.04	1.86 (1.23–2.78)	0.004	1.71 (1.12–2.56)	0.01	Recessive A
rs7944706	0.391	0.438	1.43 (0.97–2.08)	0.07	1.78 (1.20–2.63)	0.005	1.70 (1.14–2.51)	0.01	Recessive G
rs2957692	0.500	0.427	1.59 (1.03–2.39)	0.03	1.83 (1.18–2.78)	0.007	1.84 (1.18–2.81)	0.008	Recessive G
rs2957717	0.384	0.323	1.70 (0.98–2.92)	0.06	1.24 (0.94–1.63)	0.13	1.26 (0.95–1.66)	0.11	Codominant T

SNPs are sorted in 5′ to 3′ order. Genotype frequencies are shown in Supplementary Table 6. HR computed by Cox proportional hazards survival regression analyses. Model 1: adjusted for cohort membership, sex, age, duration of diabetes, arterial hypertension, tobacco smoking, HbA_{1c}, total cholesterol, HDL cholesterol, eGFR, UAC, and use of insulin, ACE-I or ARB, diuretics, antiplatelet or anticoagulation drugs, and lipid-lowering drugs at baseline. Model 2: model 1 plus adjustment for previous history of LLA at baseline. $P \leq 0.05$ is significant. Recessive A model for rs11042725: AA vs. CA or AA genotype. Recessive G model for rs7944706: GG vs. GA or AA genotype. Recessive G model for rs2957692: GG vs. AG or AA genotype. MAF is for incident cases of LLA and for participants without LLA during follow-up.

the risk alleles for LLA are associated with higher plasma MR-proADM levels, suggesting that ADM might have a deleterious effect on the outcome.

An increasing body of recent data supports the hypothesis that volume depletion and associated hypovolemia in people with diabetes, especially in subjects with diabetic foot ulcers or PAD, could worsen hypoperfusion of distal lower extremities, triggering ischemia and necrosis, eventually leading to amputation (38). We observed in a previous investigation a significantly increased risk of lower-limb events (including LLA and LLRV) in users of diuretics as compared with nonusers in the SURDIAGENE cohort (26). We have also observed a significant association between high baseline levels of plasma copeptin, a marker of the hydration status, and increased risk of LLA in cohorts of people with type 1 and type 2 diabetes (28). It is noteworthy that in the present investigation, there was a significant positive correlation between circulating levels of MR-proADM and copeptin, suggesting that high levels of MR-proADM were associated with relative dehydration. Moreover, the risk of LLA associated with MR-proADM was about twice as high in users of diuretics than in nonusers. The kidney is a major producer of ADM, with renal and urinary levels being much higher than circulating levels (11). ADM and ADM receptors are expressed in the cortex and in the medullary collecting ducts. In physiological concentrations, ADM increases GFR and decreases distal tubular sodium reabsorption, increasing natriuresis and diuresis (11). ADM regulates tubular water reabsorption by activating the phospholipase C–protein kinase C signaling pathway. The decrease in water permeability results from a decrease in aquaporin 2 (AQP2) phosphorylation, which hinders AQP2 trafficking to the plasma membrane (11). Thus, it is possible to speculate that the association of ADM with LLA risk might be driven, at least in part, by its diuretic effects, leading to relative hypovolemia and hypoperfusion of lower limbs in susceptible individuals. Thus, ADM might not only be a marker of LLA, but also might contribute to the pathophysiological mechanisms leading to LLA.

The main strengths of our work are the collection of a comprehensive range of demographic, clinical, and biological features of two prospective cohorts of patients with type 2 diabetes, the investigation of two prespecified end points (LLA and LLRV) with consistent results across end points, and the genotyping of SNPs covering the haplotype block containing *ADM*. There are limitations of our study to acknowledge. First, the design did not allow any firm conclusion on causality between circulating ADM, *ADM* variants, and disease evolution. Second, the number of LLA events during follow-up was relatively small. Statistical power was adequate to detect associations in pooled cohorts but was insufficient to detect effects in individual cohorts. Third, Mendelian randomization could not be properly tested in the present investigation conducted with a set of SNPs from a single locus, in strong linkage disequilibrium, and presenting pleiotropic effects on MR-proADM levels. In a preliminary Mendelian randomization analysis, we observed that the inverse variance weighting effect and the weighted median effect were statistically significant, but the MR-Egger regression was not (data not shown). This suggests a lack of statistical power due to the relatively small number of LLA cases during follow-up and/or the presence of pleiotropy (SNPs influencing MR-proADM and LLA through multiple independent pathways). The SNPs had both an independent effect on MR-proADM and an effect via an association with eGFR, which is a major determinant of MR-proADM levels in our cohorts (25). However, there is clearly a pattern of Mendelian randomization in our data: exposure (MR-proADM) associated with trait (LLA) and instrumental variable (SNPs) associated with both exposure and trait. Finally, we studied two cohorts consisting predominantly of people of European descent, and the allelic associations we have observed may not apply to people from other ethnic backgrounds.

In conclusion, we showed associations between plasma MR-proADM levels and risk of LLA in patients with type 2 diabetes, as well as associations between SNPs at the *ADM* locus and both plasma MR-proADM and LLA. The risk alleles of the SNPs for the outcome were associated with higher MR-proADM levels. The pathophysiological mechanisms by which high ADM levels increase LLA risk might be related to the diuretic effect of ADM, but further investigations are needed to confirm this hypothesis.

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