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Endothelial markers may link kidney function to cardiovascular events in type 2 diabetes mellitus

Short running title: Endothelial markers and cardiovascular events

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Objective: The increased cardiovascular risk in diabetes mellitus has been linked to endothelial and renal dysfunction. The aim of this study was to investigate the role of stable fragments of the precursors of adrenomedullin, endothelin-1, vasopressin and atrial natriuretic peptide in progression of cardiovascular disease in patients with diabetes mellitus.

Research design and Methods: Prospective, observational study design with a composite endpoint (death or unexpected admission to hospital due to cardiovascular event) on 781 patients with type 2 diabetes (54 events, median duration of observation 15 months). The four stable precursor peptides MR-proADM, MR-proANP, CT-proET-1 and CT-proAVP (copeptin) were determined at baseline and their association to renal function and cardiovascular events was studied using stepwise linear, and Cox logistic regression analysis and ROC analysis, respectively.

Results: MR-proADM, CT-proET-1, CT-proAVP and MR-proANP were all elevated in patients with future cardiovascular events and independently correlated to serum creatinine. MR-proADM and MR-proANP were significant predictors of a future cardiovascular event, with MR-proANP being the stronger (AUC 0.802 ± 0.034, sensitivity 0.833, specificity 0.576, positive predictive value 0.132, negative predictive value 0.978 with a cutoff value of 75 pmol/L).

Conclusions: The four serum markers of vasoactive and natriuretic peptides are related to both kidney function and cardiovascular events, thus linking two major complications of diabetes mellitus, diabetic nephropathy and cardiovascular disease.

The main cause of death in patients with diabetes mellitus is cardiovascular disease: two out of three diabetes patients develop heart failure, die from myocardial infarction or stroke, and diabetes mellitus is an established independent cardiovascular risk factor (1). There is clear evidence that hyperglycemia as well as hyperinsulinemia – both of which occur in type 2 diabetes – are linked to endothelial dysfunction resulting in atherosclerosis and, eventually, cardiovascular disease (2). On the other hand, in patients with diabetes, other risk factors – such as hypertension, dyslipidemia and central obesity - are frequently present as well, clustered together in the metabolic syndrome. Several risk scores taking into account the above mentioned factors have been developed to estimate cardiovascular risk in diabetes, and additional prognostic parameters including CRP and other inflammatory factors can help identify those at excessive risk for events in the future (2).

One risk factor for cardiovascular events is diabetic nephropathy. The presence of nephropathy is a marker for endothelial damage but neurohumoral factors are thought to contribute to the additional risk (3).

There are several vasoactive peptides with both cardiovascular and renal effects; among them are the natriuretic peptides ANP and BNP, vasopressin (AVP), and the mainly endothelial derived peptides adrenomedullin (ADM) and endothelin-1 (ET-1), which also act as both vasoactive and natriuretic agents.

The main problem in measuring plasma concentrations of these peptides in a clinical setting, is their rapid degradation and binding to receptors or binding proteins: ANP for example has a plasma half-life of 2-3 minutes. Therefore, assays have been developed which detect cleavage products of the preproproteins (4-7). These peptides are released in equimolar concentrations and serve as stable surrogate parameters for their active counterparts.

Recently the following stable plasma markers of vasoactive peptides have been shown to predict outcome after myocardial infarction or in chronic heart failure: mid regional adrenomedullin (MR-proADM)(8), C-terminal provasopressin or copeptin (CT-proAVP)(9; 10) and C-terminal proendothelin-1 (CT-proET-1)(11), Midregional Pro-Atrial Natriuretic Peptide (MR-proANP)(10; 12-14).

It has been shown in patients with type 2 diabetes that MR-proADM is associated to vascular function parameters and increased in those with elevated serum creatinine (15), and that it correlates to BMI in morbid obesity (16). Although CT-proET-1 has not been studied in the context of diabetes, endothelin-1 has been shown to be upregulated in diabetes (17) and to limit insulin action (18). Only little is known about the role of the two other peptides in diabetes: AVP is upregulated in the hypothalamus of diabetic rats(19), and ANP has been proposed to be involved in the hyperfiltration in hyperglycemia, contributing to the development of diabetic nephropathy(20).

The aim of the current study was to study in a population of type 2 diabetic patients the relationship of the four plasma parameters with renal function, and with future cardiovascular events in a prospective manner.

METHODS

Study population: 781 consecutive diabetic patients, mean age 59 ± 0.5 yrs (mean ± s.e.m.), diabetes duration 11.8 ± 0.4 yrs, treated at the diabetes outpatient clinic of the University Clinics of Vienna from Jan 1st 2006 to Feb 17th 2007 were studied. Upon entry in the study, careful medical history with special focus on cardiovascular disease was taken and history of ischemic heart

disease (IHD) was recorded. All patients were asked to complete the Minnesota Living with Heart Failure (MLHF) questionnaire and the Dyspnoe-Score chart. Blood pressure, heart rate, ECG and a blood sample for the determination of serum cholesterol, triglycerides, creatinine, HbA1c, blood glucose and the markers were obtained from each patient and NYHA stage was assessed.

The study was conducted in accordance with the Helsinki II declaration and was approved by the local ethics committee. All participants gave written informed consent.

Laboratory procedures: Total cholesterol, LDL, HDL, triglycerides, blood glucose, HbA1c, and serum creatinine were determined using standard laboratory procedures. GFR was calculated by the Cockroft-Gault and MDRD formulas, respectively. CT-proAVP, CT-proET-1, MR-proADM and MR-proANP were determined from EDTA plasma of all patients at baseline with sandwich immunoluminometric assays (all from B.R.A.H.M.S AG, Hennigsdorf, Berlin, Germany), as described before (4-7).

Endpoints: Based on the short observation period, a composite endpoint consisting of unplanned hospitalisation for cardiovascular disease or death was chosen as the primary endpoint in this study. Unplanned cardiovascular events were defined as follows: Hospitalisation based on heart failure, myocardial infarction or unstable angina, symptomatic bradycardia, atrial fibrillation, ventricular tachycardia, peripheral and central arterial occlusive disease, transistory ischemic attac (TIA) or stroke. All patients were traced through the national registry during 2007. Mortality data were obtained from the Austrian Central Office of Civil Registration ("Zentrales Melderegister"). If a patient had died the date of death was recorded. Hospital reports about hospitalisation were obtained from the regional hospital data network ("Krankenanstaltenverbund"). Information about hospitalisations for cardiovascular disease was obtained from hospital files by a cardiologist, unaware of the results at index time.

Statistical analysis: Variables are expressed as means ± standard error of the mean (s.e.m.), or mean [95% confidence interval], as stated.

Power size calculation was based on the following assumptions: log hazard ratio 0.5 event rate 10%. For $\alpha = 0.05$ and power > 90%, a sample size of 600 patients was obtained.

For group comparisons of continuous variables, a two-tailed Student's t-test was used. Categorical variables were evaluated with the Chi-Square Test.

Associations of variables with NYHA stage were tested using Spearmen's rank test.

ROC analysis was performed to evaluate the predictive performance of MR-proADM, CT-proET-1, MR-proANP, and CT-proAVP.

A forced-entry model was used to evaluate the role of creatinine, and the four peptide markers MR-proADM, CT-proET-1, MR-proANP, and CT-proAVP as independent predictors of reaching the endpoint (unplanned hospitalization due to cardiovascular event and/or death). The following variables: NYHA stage, ischemic heart disease (IHD) history, age, body mass index, systolic blood pressure, HbA_{1c}, LDL-cholesterol, serum triglycerides, serum creatinine were included in the models, with or without the addition of either one of the four peptide markers. Again, HR are given as 1-SD increment. The results were retested in a stepwise cox regression model.

Stepwise logistic regression models were calculated to identify independent variables in order to predict the reaching of the endpoint (unplanned hospitalization due to cardiovascular event and/or death). P-value for entering the stepwise model was set at .05 and for exclusion was set to .10. The stepwise

approach was used to determine the most potent predictors independent of the number of events out of the variables as follows: NYHA stage, ischemic heart disease (IHD) history, age, body mass index, systolic blood pressure, HbA_{1c}, LDL-cholesterol, serum triglycerides, serum creatinine MR-proADM, CT-proET-1, MR-proANP, and CT-proAVP. All results of the regression model are presented using hazard ratios EXP (B). Hazard ratios are given per 1-SD increment. Additionally, five-hundred bootstrap repetitions were done for the Cox regression model, repeating the variable selection for each sample using the same entering and exclusion rules. This bootstrapping procedure is used as a test against over fitting the Cox regression model. It was counted how often a variable was entered into the Cox regression models. The results were retested in a forced in model.

Proportional hazards assumption was assessed and satisfied for all variables based on time interaction test.

In order to determine independent predictors of serum creatinine stepwise linear regression was performed. Parameters included in the model were: age (yrs), total and LDL cholesterol (mg/dL), HbA1c (%), systolic and diastolic RR (mmHg), heart rate (min^{-1}), BMI (kg/m^2), CT-proAVP (pmol/L), CT-proET-1 (pmol/L), MR-proADM (nmol/L), MR-proANP (pmol/L)

A p value < 0.05 was considered statistically significant. The statistical software SPSS for Windows (SPSS release 15.0, SPSS Inc., Chicago, IL) was used for analysis.

RESULTS

Metabolic characterization and outcome: An overview of the demographic and metabolic parameters of the study group is given in table 1. Of the entire study population 54 patients reached the composite endpoint during the observation period of up to 22 months (median 15 months, SD 6.9). They were significantly different from their event-free counterparts regarding age ($p<0.0001$), serum creatinine ($p=0.002$), NYHA classification ($p<0.0001$), but not HbA1c, total and LDL cholesterol, systolic and diastolic blood pressure and dyspnoe score .

Plasma markers: In contrast to HbA1c, glucose and serum lipids, MR-proADM (0.93 ± 0.07 vs 0.62 ± 0.01 nmol/l $p<0.0001$), MR-proANP(220.0 ± 25.6 vs 86.8 ± 2.6 pmol/l $p<0.0001$)), CT-proET-197.6 ± 7.0 vs 72.6 ± 0.9 pmol/l $p=0.001$) and CT-proAVP (16.0 ± 1.8 vs 9.5 ± 0.5 pmol/l $p=0.001$) were all significantly higher in patients reaching the composite endpoint than in those who did not .

With increasing NYHA stage, also the serum levels of the four peptides increased significantly (p for trend < 0.001 for all correlations, as assessed by Spearman's rank test).

Also age, serum triglycerides, HbA1c, systolic RR, serum creatinine, and glomerular filtration rate (calculated by both Cockroft-Gault and MDRD formulas) were significantly associated to NYHA stage ($p < 0.001$). In contrast, there was no significant correlation with serum cholesterol, LDL, diastolic blood pressure, plasma glucose, or BMI.

Plasma markers, and cardiovascular events and death: ROC analysis was used to evaluate the ability of the four marker peptides MR-proADM, CT-proET-1, MR-proANP, and CT-proAVP to predict the primary endpoint. Area under the curves (AUCs) of this models were 0.802 ± 0.034 (MR-proANP), 0.698 ± 0.04 (MR-proADM), 0.690 ± 0.038 (CT-proAVP), 0.652 ± 0.048 (CT-proET), ($p < 0.001$ for all four markers). Using a cutoff value of 75 pmol/L, MR-proANP in this sample showed a sensitivity (S) of 0.833, a specificity (SP) of 0.576, a positive predictive value (PPV) of

0.132, and a negative predictive value (NPV) of 0.978. The corresponding values for the three other markers are: MR-proADM (cutoff 0.5 pmol/L): S 0.796, SP 0.395, PPV 0.099, NPV 0.960, CT-proAVP (cutoff 5 pmol/L): S 0.833, SP 0.406, PPV 0.099, NPV 0.972, CT-proET (cutoff 60 pg/mL): S 0.722, SP 0.325, PPV 0.078, NPV 0.942.

Logistic Cox regression forced in analysis was used to identify independent predictors of reaching the composite endpoint. Creatinine was an independent predictor of cardiovascular events, but became insignificant upon addition of any of the four markers. MR-proADM and MR-proANP but not CT-proET or CT-proAVP were significant predictors of an event (table 2 - direct comparison of the five models, see tables A1-5 in the online supplementary materials for the full models—available at http://care.diabetesjournals.org). In a stepwise cox regression models al four hormones remained significant markers of outcome (data not shown).

When stepwise regression analysis was performed including all four hormones beside all risk parameters (as mentioned in the Statistic), only MR-proANP, together with NYHA stage and IHD history, remained a significant predictor of cardiovascular event occurence; a one SD increment of MR-proANP was associated with a 1.564 fold risk (95% CI: 1.360-1.798, p = 0.000, table A6 online). This held also true if a forced in model was calculated (data not shown).

Bootstrap testing supports the importance and robustness of the model (table A7 online). 97,4% of the bootstrap samples include MR-proANP.

Relation of plasma markers to renal parameters: There were significant correlations between serum creatinine (or GFR, as calculated by cockroft-gault or MDRD formulas, respectively) and the plasma markers, as determined by linear regression analysis (coefficients of determination (R^2): MR-proADM 0.401, MR-proANP 0.341, CT-proET 0.319, C-proAVP 0.443, $p < 0.001$ for all parameters). The plasma markers also correlated significantly with each other (not shown).

In a stepwise linear regression analysis (Table 3), where all hormones beside classical risk factors were included all four plasma markers remained independent predictors of serum creatinine.

DISCUSSION

There are two main findings in this study: 1) In diabetic patients progression to cardiovascular events are associated to the plasma levels of MR-proADM, CT-proAVP, CT-proET-1 and, MR-proANP. 2) MR-proANP and MR-proADM are significant independent predictors of cardiovascular events in this patient group and 3) all four markers are independent from each other predictors of serum levels of creatinine (and of GFR).

Plasma levels of all four markers were significantly higher in patients reaching the composite endpoint and significantly higher in patients with increasing severity of cardiac symptoms at baseline as assessed by NYHA stage. All four markers were useful as predictors of future cardiovascular events, with MR-proANP being the strongest. In this sample, the two markers MR-proADM and MR-proANP proved to be stronger predictors of an event than traditional risk markers. CT-pro-AVP and CT-proET only remained significant in a stepwise, but not in a forced in cox regression model. Noteworthy, all four parameters showed a very good negative predictive quality, a property that would be very valuable were these parameters to be used in a clinical setting. The information that a patient is currently at lower risk for the occurence of a cardiovascular event over the next year helps better targeting aggressive management and close monitoring to those who really are at higher risk.

NT-proBNP, another natriuretic peptide marker showed also a high negative predictive value in a comparable population to the sample presented here. Again, NT-poBNP was superior to traditional risk factors in the prediction of cardiovascular events(21). MR-proANP, the strongest predictor in this sample, has been studied in comparison to NT-proBNP as survival predictor in the setting of chronic heart failure (12) and acute cardiac failure and has been found to be non-inferior to this established parameter.

It has been shown repeatedly that diabetic nephropathy is a main risk factor for future cardiovascular events (22). In confirmation of these observations, in this sample serum creatinine was significantly higher and glomerular filtration rate was significantly lower in those reaching the composite endpoint over the short time frame of little more than a year. Additionally, baseline serum creatinine was strongly associated with all four serum markers of vascular function, and all four remained independent predictors of serum creatinine in the stepwise linear regression model. Although creatinine was a highly significant predictor of outcome in a model including traditional risk markers, it became insignificant if any one of the four markers where added. Both adrenomedullin and endothelin may be produced by renal endothelial cells and have been studied in the context of nephropathy and renal failure (21); in this sample of diabetic patients also the stable serum markers MR-proADM and CT-proET-1 correlated to glomerular filtration rate and serum creatinine. In another sample of Type 2 diabetes patients MR-proADM was increased in the presence of nephropathy and related to insulin resistance(15). The markers MR-proANP and CT-proAVP have been studied in the context of sepsis and myocardial infarction(24), and have been used as outcome parameters regarding morbidity and mortality.

These parameters, to our knowledge, have not been studied in patients with diabetes and in the context of diabetic nephropathy. However, CT-proAVP (copeptin) has been shown to correlate negatively with glomerular filtration rate in chronic heart failure(10) and MR-proANP was negatively correlated with serum creatinine in chronic heart failure(13) . Thus, the close relationship of these parameters both with MR-proADM and CT-proET as well as with serum creatinine is noteworthy. It is not known how the precursor peptides of the vasoactive peptides are cleared from the circulation, and decreased renal clearance would be at least a partial explanation for the correlation of all four peptides with kidney function. Alternatively or additionally, the increase of plasma levels of the four peptides could also be explained by increased production due to endothelial stress.

While the vasoactive properties differ, all four peptides act as natriuretic agents. This natriuretic property is thought to be beneficial, and the fact that patients with heart failure do not have increased natriuresis, but instead fluid retention and edema is thought to be due to (renal) resistance to the effect. Thus, the upregulation of natriuretic peptides in heart failure is thought to be physiologic and cardio- and renopretective. The higher levels of these peptides in patients progressing to an event would therefore represent increased compensating effort of the body. Interestingly, in this study sample all four markers were independent predictors of serum creatinine in the multivariate linear regression analysis. This interesting finding underscores the complex regulation of kidney function with apparently each of the different peptide hormones (vasopressin, adrenomedullin, endothelin and atrial natriuretic peptide) playing a distinct role in the diabetic kidney.

In the study sample presented here, the stable markers of all four peptides correlated strongly with each other (data not shown),

despite their different origin, and all of them were higher in patients with a future event than in those who remained event-free. To our knowledge, this is the first report of the four markers MR-proADM, CT-proET-1, MR-proANP and CT-proAVP in a large sample of patients with diabetes. The data presented here describe a close relationship between renal function parameters and the four serum markers, and a relationship especially MR-proANP, and – to a lesser extent – the other three markers and the occurrence of cardiovascular events over a time frame of less than a year in a cohort of diabetes patients. Thus, these markers (or rather, the active peptides, for which they are surrogate markers) could be factors linking renal function to cardiovascular events.

Disclosure: Joachim Struck is employed by BRAHMS AKtiengesellschaft, a company that holds patent rights on and manufactures assays for endothelial markers.

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Table 1 Baseline characteristics of the study population

Age (yrs)	59.05 ± 0.5
Gender distribution (m/f, %)	65.1/43.9
HbA1c (%)	7.82 ± 0.06
History of cardiovascular disease (%)	17.2
NYHA class distribution (I/II/III/IV; %)	71.6/20.9/7.2/0.4
Serum creatinine (mg/dL)	1.05 ± 0.02
GFR (mL/min/1.73m^2)	91.17 ± 1.37
Serum Cholesterol (mg/dL)	197.5 ± 1.8
LDL (mg/dL)	110.3 ± 1.3
RR syst. (mmHg)	141.7 ± 0.8
RR diast. (mmHg)	83.9 ± 0.5
MR-proANP (pmol/L)	96.63 ± 1.02
MR-proADM (nmol/L)	0.64 ± 0.01
CT-proET (pmol/L)	74.63 ± 1.02
CT-proAVP (pmol/L)	9.88 ± 0.43

Table 2 Comparison of association of known risk factors and the four markers with cardiovascular events. Logistic regression models using the following parameters (forced entry): NYHA, age, serum creatinine, LDL cholesterol, serum triglycerides, HbA1c, RR syst., BMI (model 1), and the same parameters plus CT-proAVP (model 2), CT-proET (model 3), MR-proADM (model 4), or MR-proANP (model 5), respectively. Only parameters reaching significance are shown (see supplementary material for the full models) Exp(B) are given per 1-SD increment with the exception of NYHA

Model 1	**NYHA**	**Age**	**Creatinine**	
Significance	0.004	0.000	0.042	
Wald	8.275	13.025	4.116	
Exp(B) [95% CI]	1.689 [1.182-2.413]	1.833 [1.319-2.547]	1.125 [1.004-1.260]	
Model 2	**NYHA**	**Age**	**Creatinine**	**CT-proAVP**
Significance	0.004	0.000	n.s.	n.s.
Wald	8.253	12.434	-	-
Exp(B) [95% CI]	1.687 [1.181-2.411]	1.819 [1.304-2.537]	-	-
Model 3	**NYHA**	**Age**	**Creatinine**	**CT-proET**
Significance	0.022	0.001	n.s.	n.s.
Wald	5.208	11.187	-	-
Exp(B) [95% CI]	1.546 [1.063-2.247]	1.752 [1.261-2.434]	-	-
Model 4	**NYHA**	**Age**	**Creatinine**	**MR-proADM**
Significance	0.028	0.004	n.s.	0.018
Wald	4.810	8.278	-	5.553
Exp(B) [95% CI]	1.526 [1.046-2.226]	1.658 [1.175-2.341]	-	1.346 [1.051-1.724]
Model 5	**NYHA**	**Age**	**Creatinine**	**MR-proANP**
Significance	n.s.	n.s.	n.s.	0.000
Wald	-	-	-	31.191
Exp(B) [95% CI]	-	-	-	1.850 [1.491-2.297]

Table 3 Association of variable with serum creatinine using stepwise linear regression analysis Adjusted R^2 of the model was 0.61, variables not included in the model were age, total and LDL cholesterol, triglycerides, HbA1c, fasting glucose, BMI and diastolic RR

Variable	Standardized coefficient	T	P
CT-proAVP (pmol/L)	0.443	13.335	< 0.0001
MR-proADM (pmol/L)	0.177	4.005	< 0.0001
MR-proANP (pmol/L)	0.163	4.102	< 0.0001
CT-proET (pg/mL)	0.166	4.457	< 0.0001
Heart rate (min^{-1})	-0.092	-3.390	0.001
RR syst. (mmHg)	-0.054	-1.987	0.047