

Clinical and prognostic value of advanced glycation end-products in chronic heart failure

Jasper W.L. Hartog^{1*}, Adriaan A. Voors¹, Casper G. Schalkwijk², Jean Scheijen³, Tom D.J. Smilde¹, Kevin Damman¹, Stephan J.L. Bakker⁴, Andries J. Smit⁴, and Dirk J. van Veldhuisen¹

¹Department of Cardiology, University Medical Center Groningen and University of Groningen, Hanzeplein 1, PO Box 30001, 9700 RB Groningen, The Netherlands; ²Department of Medicine, Academic Hospital Maastricht, Debeyelaan 25, PO Box 5800, 6202 AZ, Maastricht, The Netherlands; ³Department of Clinical Genetics, Academic Hospital Maastricht, Debeyelaan 25, PO Box 5800, 6202 AZ, Maastricht, The Netherlands; and ⁴Department of Medicine, University Medical Center Groningen and University of Groningen, Groningen, The Netherlands

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Aims Advanced glycation end-products (AGEs) have been proposed as a novel factor involved in the development and progression of chronic heart failure (CHF). We aimed to determine whether plasma levels of N^ε-(carboxymethyl)lysine (CML) and N^ε-(carboxyethyl)lysine (CEL), two well-known AGEs, are related to the severity and prognosis of CHF.

Methods and results A total of 102 CHF patients, aged 58 ± 12 years, with an average left ventricular ejection fraction of $28 \pm 9\%$ were followed for 1.7 (1.2–1.9) years. NYHA functional class and NT-pro-BNP were used as estimates of the severity of CHF. CML and CEL were determined by LC-MS/MS. CML levels were associated with NYHA functional class ($P < 0.001$) and NT-pro-BNP levels ($P < 0.001$). Survival analysis for the combined end-point of death, heart transplantation, ischaemic cardiovascular event, and hospitalization for heart failure revealed that CML levels predicted outcome, even after adjustment for age, gender, aetiology of CHF, identified risk modifiers, and several known predictors of outcome in CHF. The predictive value of CML subsided after correction for renal function. CEL was not associated with the severity or prognosis of CHF.

Conclusion Plasma AGEs, in particular CML levels, are related to the severity and prognosis of CHF. The fact that the relation between CML and prognosis subsided after correction for renal function may suggest that AGE accumulation in renal failure explains part of the prognostic value of renal function in CHF. However, further investigation is warranted to exclude the possibility that CML is just an innocent marker of renal function.

Introduction

Chronic heart failure (CHF) poses a significant burden to patients, health care providers, and society. Although mortality rates in CHF patients improved over the years, still roughly 50% of patients die within 5 years from diagnosis.¹ Several factors have been established as independent predictors for survival in CHF patients, among which are left ventricular ejection fraction (LVEF), NYHA functional class, and renal function.^{2,3} In the last few years, advanced glycation end-products (AGEs) have received attention, since they may play a role in the pathophysiology of CHF.

AGEs are end-products of a non-enzymatic reaction of sugar and lipid adducts with proteins called the Maillard reaction.⁴ AGEs form cross-links with long-living tissue proteins, which cause them to accumulate in the body with age.^{5,6} AGE accumulation *in vivo* is found throughout the

body, including in skin, neural, vascular, renal, and cardiac tissue.^{5,6} Enhanced accumulation is found in the presence of diabetes and renal failure.^{5,7} Additionally, AGE precursors in cigarette smoke and food products are possible sources for increased AGE-accumulation.^{8,9} AGE formation affects the physiological properties of proteins in the extracellular matrix, such as turnover, and elasticity.¹⁰ Stimulation of AGE-receptors, such as the RAGE receptor, by AGEs leads to (prolonged) cellular activation and release of inflammatory cytokines.¹⁰ These changes may result in the development and progression of diastolic and systolic dysfunction, and subsequent CHF.^{11,12}

While AGE accumulation has been primarily studied in patients with diabetes and renal failure, the clinical and prognostic value of AGEs in patients with CHF remains unknown. We determined whether plasma levels of N^ε-(carboxymethyl)lysine (CML) and N^ε-(carboxyethyl)lysine (CEL), two well-known AGEs, are related to the severity of CHF and prognosis.

*Corresponding author. Tel: +31 50 361 2355; fax: +31 50 361 4391.
E-mail address: j.w.l.hartog@thorax.umcg.nl

Methods

Patients and study design

Patients and study design have been previously described by Smilde *et al.*¹³ Stable CHF patients aged ≥ 18 years, with LVEF $\leq 45\%$ were asked to participate. All patients had to have an optimal treatment for CHF, including at least a renin–angiotensin system inhibitor. Drug therapy had been stable for at least 1 month. Exclusion criteria were a myocardial infarction within the last 3 months, cardiac surgery or angioplasty within the last 3 months (or scheduled to undergo these procedures), unstable angina pectoris, primary renal disease, prior organ transplantation, or chronic use of renal function compromising medication. Special care was taken to include patients over the full range of severity of CHF. Approximately 121 patients were asked to participate. Between November 2003 and July 2005 in total 110 patients were included in the original analysis by Smilde *et al.*¹³

Samples for CML determination were unavailable in eight patients, which left 102 patients eligible for the present analysis. All patients were Caucasian, except from one patient who was Black.

Baseline measurements included creatinine clearance and albumin excretion from 24 h urine collections, glomerular filtration rate (GFR), effective renal plasma flow (ERPF), and extra cellular volume (ECV) measured as the clearance and the distribution volume of constantly infused ^{131}I -Hippuran and ^{125}I -iothalamate,¹⁴ LVEF measured by radionuclide ventriculography, NT-pro-BNP measured by electrochemiluminescence immunoassay, and levels of plasma CML and CEL by LC–MS/MS. Estimated GFR (eGFR) was calculated using the MDRD formula as described by Smilde *et al.*¹³ The severity of CHF was classified in accordance with the NYHA functional class. A combined clinical outcome parameter was defined as the first occurrence of either death, heart transplantation, ischaemic cardiovascular event (myocardial infarction or primary PTCA), or hospitalization for heart failure. Follow-up data was based upon the patients records available at our out-patient clinic. All patients routinely visited our clinic for heart failure treatment. Information was gathered from local general practitioners when necessary. None of the patients were lost to follow-up. Median follow-up time of event-free patients was 1.7 (1.2–1.9) years (range 1.0–2.4 years). Median time to first event was 0.7 (0.2–1.1) years (range 0.02–1.56 years). This study protocol was approved by the institutional review committee of the University Medical Center Groningen. All patients signed written informed consent.

N^{ϵ} -(carboxymethyl)lysine and N^{ϵ} -(carboxyethyl)lysine by stable-isotope-dilution tandem mass spectrometry

CML and CEL were determined by stable-isotope-dilution tandem mass spectrometry (LC–MS/MS) as described previously.¹⁵ In short, CML and CEL were liberated from plasma proteins by acid hydrolysis after addition of deuterated CML and CEL as internal standards. Chromatographic separation was performed by gradient-elution reversed-phase chromatography with a mobile phase containing $5\text{ }\mu\text{mol/L}$ nonafluoropentanoic acid as ion-pairing agent. Mass transitions of $205.1 \rightarrow 384.1$ and $219.1 \rightarrow 384.1$ for CML and CEL, respectively, and $209.1 \rightarrow 388.1$ and $223.1 \rightarrow 388.1$ for their respective internal standards were monitored in positive-ion mode. CML and CEL were separated by baseline resolution with a total analysis time of 21 min. Within-day and between-day coefficients of variation were <4.4 and $<3.2\%$ for CML, and <6.8 and $<7.3\%$ for CEL.

Statistical analyses

Data were analysed using SPSS version 12.01 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as

mean \pm SD or as median (25–75% interquartile range) where applicable. Nominal variables are expressed as n (%). P -values for trend were determined by linear regression for continuous variables, and by χ^2 and Jonckheere–Terpstra tests for nominal and ordinal variables, respectively. By calculating P -values for trend over quartiles of CML and CEL, we first identified factors that may influence the prognostic value of CML and CEL (risk-modifiers). Multivariable linear regression analysis was then used on all variables that showed a P -value of at least 0.10 or smaller in trend analysis to determine the variables that showed the strongest association with CML and CEL. Variables which not retained significance in this multivariable analysis were subsequently removed from the model (backward selection). To test whether the model is appropriate and whether the assumptions for linear regression are met, the model has been tested for overall regression, co-linearity, interaction terms, and lack-of-fit with ANOVA. Residuals were tested for normality of distribution. No violations were found. Next, we used Cox regression analysis to evaluate the prognostic value of CML and CEL. After basic adjustments of HR for age, gender, and aetiology of CHF, we made additional corrections for all risk-modifiers that showed a P -value of at least 0.10 or smaller in trend analysis. To further validate our model, we made adjustments for several known predictors of outcome. Linearity of the continuous variables with respect to the response variable was assessed by determining the quartiles of their distribution. Subsequently, hazard ratios for each quartile were calculated. All variables showed a linear trend in the estimated hazard ratios, and were thus introduced in the model as continuous. Log-Minus-log survival curves and time-dependent covariates were used to evaluate adherence of the Cox proportional hazard assumptions. No violations of the proportional hazard assumption were identified. A P -value ≤ 0.05 (two-sided) was considered statistically significant.

Results

Baseline

Baseline characteristics are presented in *Table 1*. We examined 102 CHF patients (77% male), aged 58 ± 12 , with a mean LVEF of $28 \pm 9\%$. Ischaemic heart failure was present in 47% of patients. Cases of non-ischaemic heart failure (54%) were most often patients with an idiopathic dilated cardiomyopathy (69%). Diabetes was present in only a small proportion (9%) of patients, as was a history of hypertension (16%).

Trend analysis revealed that higher CML levels were significantly associated with higher NYHA functional class ($P < 0.001$), higher log NT-pro-BNP ($P < 0.001$), older age ($P = 0.004$), less smoking ($P = 0.04$), lower GFR ($P < 0.001$), increased BMI ($P = 0.003$), and higher ECV (0.04). Additionally, a trend existed for an association between CML and lower diastolic blood pressure (DBP) ($P = 0.06$). Multivariable linear regression analysis showed that GFR ($P < 0.001$) was the most important determinant of CML. Similar analysis revealed that higher CEL levels were significantly associated with older age ($P = 0.03$), less smoking ($P = 0.01$), lower DBP ($P = 0.05$), lower GFR (0.005), and the use of β -blockade ($P = 0.008$). A trend for an association existed between higher CEL and a history of

Table 1 Baseline characteristics

Characteristic	Total (n = 102)
Age (years)	58 ± 12
Sex (male)	78 (77)
Diabetes mellitus, n (%)	9 (9)
History of hypertension, n (%)	16 (16)
Smoking, n (%)	16 (16)
Hypercholesterolaemia, n (%)	55 (54)
History of CVD, n (%)	59 (58)
Body mass index (kg/m ²)	27 ± 4
Systolic blood pressure (mmHg)	119 ± 21
Diastolic blood pressure (mmHg)	69 ± 12
Heart rate (b.p.m.)	65 ± 13
Aetiology of CHF	
Ischaemic, n (%)	47 (46)
Non-ischaemic, n (%)	
Idiopathic dilated cardiomyopathy	37 (36)
Post-viral cardiomyopathy	4 (4)
Heart valve disease	3 (3)
Post-partum cardiomyopathy	2 (2)
Alcoholic cardiomyopathy	1 (1)
Hypertension	1 (1)
Other	6 (6)
LVEF (%)	28 ± 9
NYHA functional class, n (%)	
I	14 (14)
II	47 (46)
III	31 (30)
IV	10 (10)
Medication use, n (%)	
ACEi/ARB	102 (100)
β-Blockers	86 (84)
Diuretics	71 (70)
Calcium antagonists	13 (13)
Anti-arrhythmic	20 (20)
NT-pro-BNP (pg/mL)	634 (1577)
Creatinine (mmol/L)	104 (91–121)
Creatinine clearance (mL/min)	82 ± 34
eGFR with MDRD (mL/min)	63 ± 19
GFR (mL/min/1.73 m ²)	75 ± 27
ERPF (mL/min/1.73 m ²)	275 ± 88
Urinary albumin excretion (mg/24 h)	9.3 (6.6–18.6)
ECV (L/kg body weight)	0.26 ± 0.05
CEL (μmol/L)	1.5 ± 0.5
CML (μmol/L)	1.7 ± 0.5

Parametric parameters are expressed as mean ± SD; non-parametric parameters are expressed as median (25–75% IQR); ordinal parameters are expressed as n (%).

ACEi, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; b.p.m., beats per minute; CHF, chronic heart failure; CVD, cardiovascular disease; ECV, extra-cellular volume; GFR, glomerular filtration rate; LVEF, left ventricular ejection fraction; NT-pro-BNP, N-terminal-pro-brain natriuretic peptide; NYHA, New York Heart Association.

hypertension ($P = 0.08$), and a higher NYHA functional class ($P = 0.06$). The most important determinants of CEL levels were GFR ($P = 0.004$), smoking ($P = 0.02$), and use of β-blockade ($P = 0.009$). *Figure 1* illustrates the relation of NYHA functional class with CML and CEL. *Figure 2* depicts a scatter plot of NT-pro-BNP levels vs. CML and CEL levels.

Follow-up

Median follow-up for event-free patients was 1.7 (1.2–1.9) years (range 1.0–2.4 years). Twenty patients reached the

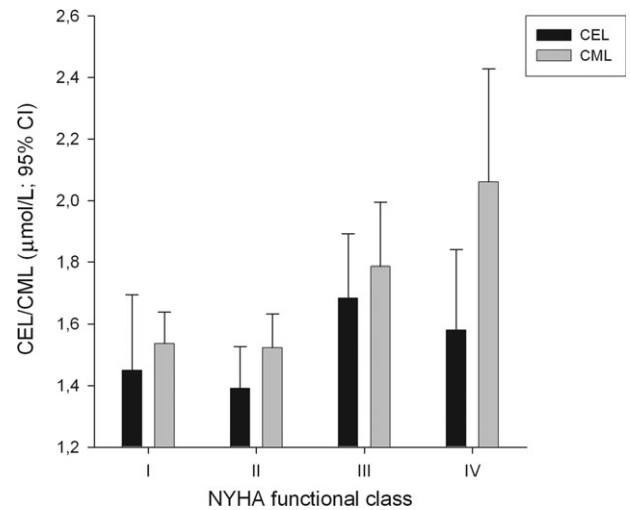


Figure 1 N^ε-(carboxymethyl)lysine and N^ε-(carboxyethyl)lysine levels in plasma divided over New York Heart Association functional class. Error bars indicate 95% confidence intervals. Trend analysis revealed that N^ε-(carboxymethyl)lysine levels significantly increase with New York Heart Association functional class ($P < 0.001$). No significant differences were observed for N^ε-(carboxyethyl)lysine levels over New York Heart Association functional class ($P = 0.06$). CML, N^ε-(carboxymethyl)lysine; CEL, N^ε-(carboxyethyl)lysine.

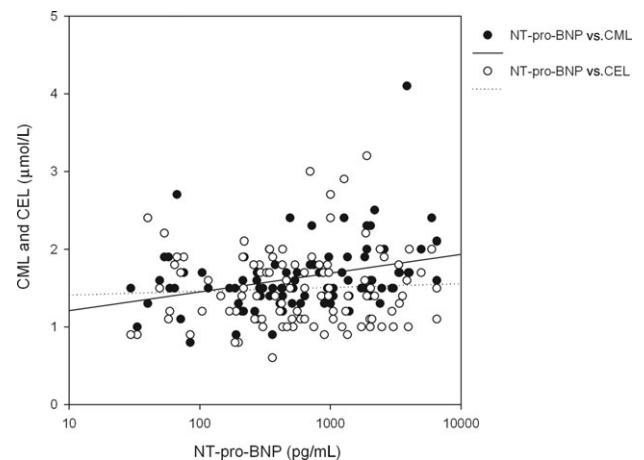


Figure 2 Scatter plot of N^ε-(carboxymethyl)lysine and N^ε-(carboxyethyl)lysine levels in plasma vs. log NT-pro-BNP levels. N^ε-(carboxymethyl)lysine levels were significantly correlated with log NT-pro-BNP ($R = 0.40$, $P < 0.001$). No significant correlation was observed for N^ε-(carboxyethyl)lysine levels with log NT-pro-BNP ($R = 0.07$, $P = 0.47$). CML, N^ε-(carboxymethyl)lysine; CEL, N^ε-(carboxyethyl)lysine.

combined end-point, of whom six died, one underwent HTx, and 13 patients were hospitalized for heart failure. None of the patients had an ischaemic cardiovascular event. *Figure 3A and B* depict the results of Cox regression analysis. Univariate Cox regression analysis revealed that CML was a significant predictor of the combined endpoint. The prospective value of CML persisted after basic adjustments were made for age, sex, and aetiology of CHF. Next, we made additional corrections for all risk-modifiers that showed a P -value of at least 0.10 or smaller in trend analysis. The relation between CML levels and outcome remained significant after adjustments were made for NYHA functional class, NT-pro-BNP, DBP, smoking, and ECV. However, the predictive value of CML subsided after adjustments for GFR

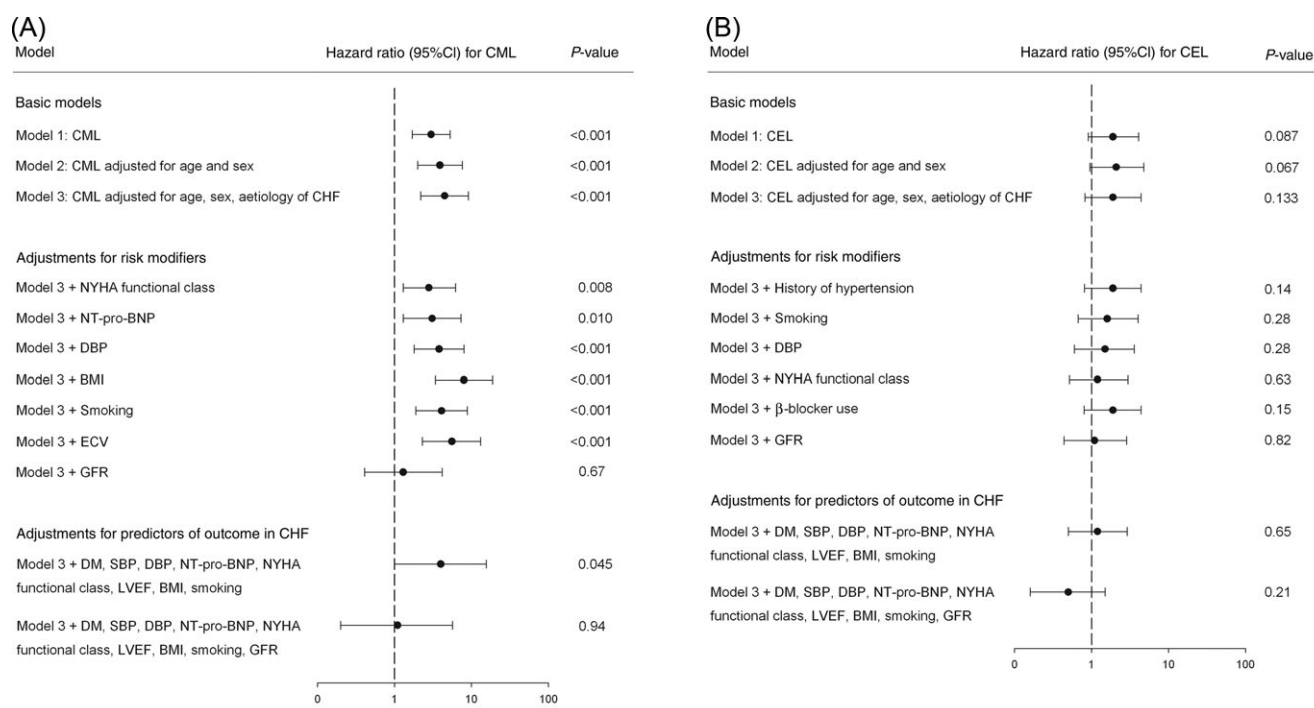


Figure 3 (A) Results from the survival analysis of N^c-(carboxymethyl)lysine for the combined endpoint. Firstly, basic adjustments were made for age, sex, and aetiology of chronic heart failure. Secondly, the relation between N^c-(carboxymethyl)lysine and the combined endpoint was adjusted for risk-modifiers identified with trend analysis, including New York Heart Association function class, NT-pro-BNP, diastolic blood pressure, body mass index, smoking, extracellular volume, and glomerular filtration rate. Thirdly, our basic model was corrected for important predictors of outcome in chronic heart failure, namely diabetes mellitus, systolic blood pressure, diastolic blood pressure, NT-pro-BNP, New York Heart Association functional class, left ventricular ejection fraction, body mass index, smoking, and glomerular filtration rate. (B) Results from the survival analysis of N^c-(carboxyethyl)lysine for the combined endpoint. Firstly, basic adjustments were made for age, sex, and aetiology of chronic heart failure. Secondly, the relation between N^c-(carboxyethyl)lysine and the combined endpoint was adjusted for risk-modifiers identified with trend analysis, including history of hypertension, smoking, diastolic blood pressure, NYHA functional class, β -blocker use, and glomerular filtration rate. Thirdly, our basic model was corrected for important predictors of outcome in chronic heart failure, namely diabetes mellitus, systolic blood pressure, diastolic blood pressure, NT-pro-BNP, New York Heart Association functional class, left ventricular ejection fraction, body mass index, smoking, and glomerular filtration rate. Abbreviations: DBP, diastolic blood pressure; BMI, body mass index; ECV, extracellular volume; GFR, glomerular filtration rate; DM, diabetes mellitus; SBP, systolic blood pressure; LVEF, left ventricular ejection fraction; CML, N^c-(carboxymethyl)lysine; CEL, N^c-(carboxyethyl)lysine; CHF, chronic heart failure.

were made. To discriminate whether the latter results implies an effect of hypoperfusion secondary to a more compromised systolic LVEF or an effect of actual renal damage, we evaluated the effect of adjustments for ERPF and urinary albumin excretion as risk modifiers as well. Adding ERPF to our model instead of GFR resulted in an HR (95% CI, *P*-values) for CML of 2.2 (0.9–5.5, *P* = 0.099), while correction for urinary albumin excretion resulted in an HR for CML 4.42 (2–9.0, *P* < 0.001). To further validate our model, we also made adjustments for important predictors of outcome in CHF. CML remained an independent predictor of outcome after adjustments for diabetes mellitus (DM), systolic blood pressure, DBP, NT-pro-BNP, NYHA functional class, LVEF, body mass index (BMI), and smoking. Again, the predictive value of CML subsided after additional adjustment for GFR. Although a trend exists for CEL to be associated with outcome, it did not reach statistical significance. In additional analysis it remained insignificant, also after corrections for the risk-modifiers identified in trend analysis, and important predictors of outcome in CHF. The survival curves for the combined endpoint show a decrease in survival with higher quartiles of CML and CEL, respectively (Figure 4A and B).

Baseline and follow-up results did not substantially change after correction of CML and CEL for plasma protein concentrations or ECV. Additionally, we evaluated the effect of

other parameters of renal function as risk modifiers. Replacing GFR with creatinine, 24 h creatinine clearance, and eGFR with MDRD, resulted in hazard ratio (95% CI, *P*-values) for CML levels of 2.6 (1.0–7.2, *P* = 0.05), 2.5 (1.1–5.9, *P* = 0.033), and 1.7 (0.6–5.0, *P* = 0.33), respectively.

Discussion

The main finding of this study is that CML, a well-known AGE, is associated with the severity, and prognosis of patients with CHF. The relation between CML and outcome was independent of age, sex, aetiology of CHF, and identified risk modifiers, including NYHA functional class, NT-pro-BNP, DBP, BMI, smoking, and ECV. Furthermore, it retained significance after adjustment for several known predictors of outcome in CHF. However, it subsided after adjustment for GFR. This is one of the first studies that analysed the relation between AGE levels and the severity and prognosis of CHF.

Several lines of evidence indicate that AGEs may play an active role in the development and progression of CHF. AGEs may be increased in CHF via the progression of renal failure, smoking, DM, age, and increased oxidative stress. Increased AGE accumulation is associated with the development of diastolic and systolic dysfunction in experimental animal models.^{16,17} Moreover, in preliminary human

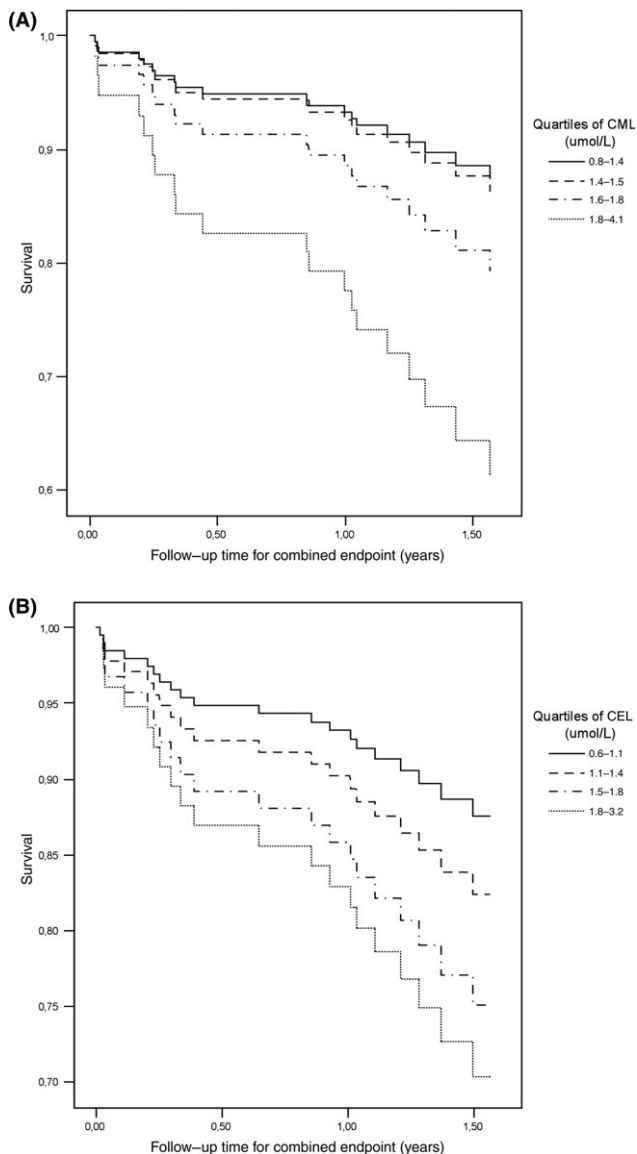


Figure 4 (A and B) Survival for the combined endpoint over quartiles of N $^{\epsilon}$ -(carboxymethyl)lysine and N-(carboxyethyl)lysine. Survival curves were corrected for age and gender. CML, N $^{\epsilon}$ -(carboxymethyl)lysine; CEL, N $^{\epsilon}$ -(carboxyethyl)lysine.

intervention studies, CHF patients seem to benefit from AGE breaking medication.^{18,19}

The predictive value of CML found in this study was independent of age, sex, aetiology of CHF, identified risk-modifiers, and several predictors of outcome in CHF, but dependent of renal function. To validate this result, we performed additional analysis using other parameters for renal function as risk modifiers. Although CML remained significantly associated with outcome after adjustment for creatinine, and 24 h creatinine clearance, adjustment for eGFR calculated using the MDRD formula resulted in more similar findings as for GFR. We feel that the latter result more reliably reflects the truth, a feeling which is strengthened by the results of Smilde *et al.*,¹³ who found that the MDRD formula is the most accurate indirect measurement of renal function in CHF.

Renal function is a clinically significant risk factor for mortality in CHF patients, independent of traditional prognostic

factors, such as LVEF, and NYHA functional class.^{3,20} AGEs are known to increase in renal failure due to decreased clearance.²¹ Therefore, our results may indicate that CML levels explain (part of) the prognostic value of renal function in patients with CHF. This is further supported by our finding, that CML was not only related to prognosis, but also to a functional classification as well as a biochemical marker for the severity of CHF. However, due to the observational nature of our study, we cannot rule out the possibility that CML acts as a marker for impaired renal function, and as such might have predictive value in CHF.

By adding ERPF and urinary albumin excretion as risk modifiers to our model, we made an attempt to discriminate whether the relations with GFR implies an effect of hypoperfusion secondary to a more compromised systolic LVF or an effect of actual renal damage. The fact that the HR for CML decreased substantially after adding ERPF to our model instead of GFR, but not changed after addition of urinary albumin excretion suggests that hypoperfusion is far more likely than renal damage.

While the chemical structures of CML and CEL are quite similar, the results we obtained for both are not. One reason may be that CML originates from different pathways than CEL. CML can be formed through lipid peroxidation and glycooxidation pathways, while CEL is mainly formed through glycooxidation pathways.²²

AGE levels were previously evaluated in CHF by Heidland *et al.*²³ They evaluated plasma AGEs in a small group of patients with severe CHF, heart transplant recipients, and normal controls. Paradoxically, they found a decrease in CML and AGE-fluorescence in patients with CHF when compared with controls. Heart transplant recipient did, however, show an increase in measured AGE data. Unfortunately, data on NYHA functional class, NT-pro-BNP levels, and prognosis were not provided. The authors suggested that their results were possibly biased by hypervolemia, lowered plasma protein concentrations, and decreased dietary intake of AGEs in CHF. While we cannot correct our data for dietary intake of AGEs, correction of our data for plasma protein concentrations did not substantially change our results. Moreover, correction for ECV which could be estimated from the distribution volume of ¹²⁵I-iothalamate, did not change our results either.

Recently, Koyama *et al.*²⁴ for the first time evaluated the prognostic value of serum AGEs as risk factor in CHF. They found that serum pentosidine levels were a significant predictor of cardiac death and re-hospitalization. Although they corrected their findings for other known risk factors in CHF, like BNP, renal function, age, and NYHA functional class they may have introduced a possible co-linearity problem by simultaneously introducing creatinine levels and estimated GFR in the multivariable model. Therefore, their data should be interpreted with caution.

The prognostic value of AGEs has been studied in other populations than CHF as well. Kilhovd *et al.*²⁵ showed that high levels of circulating AGEs predicted cardiovascular mortality in non-diabetic women. However, they presented their data uncorrected for renal function, and their results may, therefore, be biased. In patients with renal failure results vary widely. While Schwedler *et al.*²⁶ and Busch *et al.*^{27,28} reported that circulating AGE levels were not related to prognosis, Wagner *et al.*²⁹ and Roberts *et al.*³⁰ did find prognostic value of circulating AGEs. Our group previously demonstrated

that AGE accumulation measured by skin-autofluorescence, was a strong and independent determinant of prognosis in both dialysis and diabetic patients.^{31,32}

In many of the above-mentioned studies, enzyme-linked immunosorbent assay (ELISA) was used to determine AGE levels. Several difficulties exist with standardization of ELISA methods to assess AGE levels. Therefore, the results of this studies should be interpreted with caution. In contrast, in the present study, we used LC-MS/MS to determine CML and CEL levels, which is currently seen as the most accurate method to assess plasma AGE levels.

Conclusion

Plasma AGEs, in particular CML levels, are related to the severity and prognosis of CHF. The fact that the relation between CML and prognosis subsided after correction for renal function may suggest that AGE accumulation in renal failure explains part of the prognostic value of renal function in CHF. However, further investigation is warranted to exclude the possibility that CML is just an innocent marker of renal function.

Conflict of interest: none declared.

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