Skin Autofluorescence, a Measure of Cumulative Metabolic Stress and Advanced Glycation End Products, Predicts Mortality in Hemodialysis Patients

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Tissue advanced glycation end products (AGE) are a measure of cumulative metabolic stress and trigger cytokines driven inflammatory reactions. AGE are thought to contribute to the chronic complications of diabetes and ESRD. Tissue autofluorescence is related to the accumulation of AGE. Therefore, skin autofluorescence (AF) may provide prognostic information on mortality in hemodialysis (HD) patients. Skin AF was measured noninvasively with an AF reader at baseline in 109 HD patients. Overall and cardiovascular mortality was monitored prospectively during a period of 3 yr. The AF reader was validated against AGE contents in skin biopsies from 29 dialysis patients. Forty-two of the 109 (38.5%) HD patients died. Cox regression analysis showed that AF was an independent predictor of overall and cardiovascular mortality (for overall mortality odds ratio [OR] 3.9), as were pre-existing cardiovascular disease (CVD; OR 3.1), C-reactive protein (OR 1.1), and serum albumin (OR 0.3). Multivariate analysis revealed that 65% of the variance in AF could be attributed to the independent effects of age, dialysis and renal failure duration, presence of diabetes, triglycerides levels, and C-reactive protein. AF was also independently linked to the presence of CVD at baseline (OR 8.8; $P < 0.001$). AF correlated with collagen-linked fluorescence ($r = 0.71$, $P < 0.001$), pentosidine ($r = 0.75$, $P < 0.001$), and carboxy(m)ethyllysine (both $r = 0.45$, $P < 0.01$). Skin AF is a strong and independent predictor of mortality in ESRD. This supports a role for AGE as a contributor to mortality and CVD and warrants interventions specifically aimed at AGE accumulation.

The increased accumulation of tissue advanced glycation end products (AGE) is a series of complex and sequential reactions, collectively called the Maillard reaction. AGE accumulation results from a combination of hyperglycemia, oxidative/carbonyl stress, and/or decreased renal clearance of AGE precursors (1–4). Hyperglycemia is a sufficient but not necessary condition for increased AGE formation. AGE are significantly increased in uremia, even in the absence of hyperglycemia (1). Accumulation of chemically stable AGE on long-lived proteins may serve as a measure of cumulative metabolic stress (5–7) and affects the structure and function of proteins, enhances cytokine production, and activates transcription factors *via* binding to specific receptors (*e.g.*, receptor for AGE) (8).

Because of their biochemical characteristics, AGE have been implicated as a contributing factor in the progression of chronic, age-related diseases, such as atherosclerosis, ESRD, and diabetes (1,3,8–11). In a substudy of the Diabetes Control and Complications Trial, skin AGE levels explained a major part of the variance in diabetic complications, even after adjustment for glycosylated hemoglobin (HbA_{1c}) (12). Inhibition of AGE accumulation experimentally reduces the development of several diabetic complications (13–15). In ESRD, AGE accumulation has been linked to accelerated atherosclerosis, even in euglycemic patients (16–18). Cooper and colleagues (19,20) reported that ACE inhibitors and angiotensin receptor blockers also inhibit AGE formation in animal models of diabetes, further implicating AGE as a biomarker or causative factor in the development of diabetic nephropathy.

The influence of tissue AGE accumulation on survival in diabetes and ESRD is yet unknown. Determination of tissue AGE accumulation is an invasive, expensive procedure, and blood and urine sampling of AGE does not necessarily reflect tissue AGE (21). However, tissue autofluorescence (AF) has been related to the accumulation of AGE and to the progression of chronic complications of diabetes and ESRD (22). Recently, we described a noninvasive optical tool, the AF reader (AFR),

for measuring skin AF (23). Skin AF seemed to be related strongly to collagen-linked fluorescence, pentosidine and carboxy(m)ethyllysine (CML and CEL) accumulation (23) and to long-term complications in patients with diabetes (24).

Mortality rates in hemodialysis (HD) patients are markedly increased, despite measures to improve survival (25). Cardiovascular disease (CVD) is the predominant cause of mortality, and AGE accumulation is severely increased in HD patients (16,26). For these reasons, we analyzed the influence of skin AF on overall and cardiovascular mortality in a population of HD patients. Furthermore, we show preliminary results of the validation of the AFR as a measure of AGE accumulation by comparison with the AGE content in skin biopsies from HD patients.

Materials and Methods
Study Population
All 109 patients who were on chronic HD treatment at the Dialysis Centre Groningen were observed prospectively, and in 29 of them, skin biopsies were retrieved. Minimum duration of HD was 30 d. No further exclusion criteria were used. Patients received standard medical care as appropriate for HD patients. Medication of patients included antihypertensives (47%), epoetin and iron gluconate (21%), aspirin (36%), vitamins (49%), lipid-lowering agents (19%), and phosphate binders (40%). Patients were dialyzed three times weekly for 4 h with biocompatible low-flux dialyzers (cellulose diacetate and polysulphone). Equilibrated fractional urea clearance (Kt/V) was aimed at 1.2 per dialysis according to Kidney Disease Outcomes Quality Initiative guidelines.

Reference data on normal AFR values were obtained from a group of 43 nonsmoking, age-matched control subjects. In control subjects, diabetes and renal failure were excluded by conventional criteria (American Diabetes Association) and a serum creatinine <120 μmol/L, respectively. HD patients who smoked were not excluded for the analysis of risk factors related to mortality.

Patient and control characteristics are given in Table 1. All patients and control subjects gave informed consent, according tot the rules of the local ethics committee.

Baseline
An independent physician, who was unaware of AFR results, determined before the beginning of the study whether a patient had CVD. A patient was considered to have CVD when coronary heart disease, peripheral vascular disease, or cerebrovascular disease was present (*International Classification of Diseases, Ninth Revision, Clinical Modification* codes I20, I21, I63, I70, and I73). Hypertension was defined as a predialysis systolic BP of >140 mmHg or a diastolic pressure >90 mmHg on at least three occasions or when receiving antihypertensive medication. Diabetes was defined by conventional American Diabetes Association criteria. Duration of renal failure was defined from the date that serum creatinine was >120 μmol/L to the start of the study. Dialysis duration was defined from the initiation of long-term HD treatment to the start of the study. European Dialysis and Transplant Association classification was used to define the primary diagnosis of renal failure (27). Primary diagnosis classification was simplified into four groups: A, Diabetes, B, hypertension/renovascular disease, C, primary glomerular disorders, and D, other primary diagnosis. Laboratory information was collected prospectively.

Table 1. Characteristics of HD patients and control subjects[a]

Characteristics	HD Patients (n = 109)	Control Subjects (n = 43)
Age (yr)	57 ± 16	53 ± 16
Gender (M/F)	68/41	20/23
BMI (kg/m^2)	23.9 ± 3.4	24.1 ± 1.8
Creatinine (μmol/L)	983.8 ± 238.6	88 ± 8.0[b]
BUN (mmol/L)	27.7 ± 6.9	5.1 ± 1.0[b]
Albumin (g/L)	38.3 ± 2.6	41.1 ± 2.6[b]
CRP (mg/L)	4.8 ± 5.6	—
LDL (mmol/L)	3.2 ± 0.8	3.4 ± 1.1
HDL (mmol/L)	1.0 ± 0.2	1.3 ± 0.3[b]
Triglycerides (mmol/L)	2.3 ± 0.9	1.4 ± 0.9[b]
Glucose (mmol/L)	5.7 ± 0.8	4.8 ± 0.6
EDTA (A/B/C/D)	23/41/20/25	

[a]HD, hemodialysis; BMI, body mass index; BUN, blood urea nitrogen; CRP, C-reactive protein. In HD patients, BUN and creatinine levels are predialysis levels. European Dialysis and Transplant Association (EDTA) classification of primary diagnosis: A, diabetes; B, hypertension/renovascular disease; C, primary glomerular disorders; D, other primary disorders.
[b]$P < 0.01$.

Follow-Up
Date and cause of death were obtained from medical records after a follow-up period of 3 yr. Causes of death were certified and classified as cardiovascular mortality (myocardial infarction, sudden death and stroke, and congestive heart failure) or noncardiovascular death (neoplasm, infection, and unknown) according to usual *International Classification of Diseases* coding criteria.

Skin AF
Skin AF was assessed by the AFR (patent PCT/NL99/00607; prototype of current AGE Reader I; DiagnOptics BV, Groningen, The Netherlands) as described in detail previously (23). In short, the AFR illuminates a skin surface of approximately 1 cm^2, guarded against surrounding light, with an excitation light source between 300 to 420 nm (peak excitation approximately 350 nm). Only light from the skin is measured with a spectrometer (Ocean Optics PC-1000 fiber optic spectrometer; Ocean Optics, Dunedin, FL) in the 300- to 600-nm range, using 200-μm glass fiber (Farnell, Leeds, UK). The measure of AF that we applied was the average light intensity per nanometer in the range between 420 and 600 nm divided by the average light intensity per nanometer in the range between 300 and 420 nm (AF in arbitrary units [AU]).

All measurements were performed at room temperature in a semi-dark environment before dialysis. Repeated AFR measurements on 1 d and intraindividual seasonal variance showed an Altman error percentage of <6%. Pre- and postdialysis measured AF did not differ significantly.

Skin Biopsies
Autofluorescence measurements were followed by full-thickness punch skin biopsies (4 mm), taken from the volar side of the lower arm (same location as AFR measurement) under 2% lidocaine local anesthesia in 29 HD patients. Skin samples were frozen in liquid nitrogen and subsequently stored at −80°C. Skin biopsies were analyzed in a

single batch for collagen-linked fluorescence (excitation at 370 nm, emission at 440 nm) after pepsin digestion and for pentosidine (by HPLC), CML, and CEL (by gas chromatography–mass spectrometry) content as described previously (23,28).

Statistical Analyses

Comparison between groups was performed with t test or Mann-Whitney U test for nonnormally distributed variables, and correlations were analyzed with Spearman rank method. Multivariate regression analyses were performed for determination of independent relationships of variables with AF. The independent effects of variables on the presence of CVD at baseline were assessed by logistic regression analysis. The cumulative incidence of death during follow-up was estimated by the Kaplan-Meier method, and the independent effects and odds ratio (OR) of variables on mortality were estimated with stepwise Cox regression model. The primary analysis of survival included all patients, and data were censored at the time of kidney transplantation. SPSS statistical software (version 11.0; SPSS, Inc., Chicago, IL) was used for the analysis; two-tailed $P < 0.05$ was considered significant. Data are shown as mean ($\pm$ SD), unless otherwise indicated.

Results

Baseline

Table 1 describes baseline characteristics of the HD patients and control subjects. The median duration of renal failure was 4.7 yr (range 9 to 300 mo), and duration on dialysis treatment was 2.3 yr (range 1 to 96 mo). Weekly dialysis time was 10.0 $\pm$ 1.9 h.

Figure 1 shows the AF spectrum of the study populations. Skin AF was 2.4 times increased in HD patients compared with control subjects (0.024 $\pm$ 0.007 *versus* 0.010 $\pm$ 0.001 AU; $P < 0.001$). AF was increased even further in the subgroup of HD patients with diabetes ($n = 23$; 0.029 $\pm$ 0.002 AU). Sixty-five

percent of the variance in AF in HD patients could be explained by the independent effects of age ($P < 0.001$), dialysis ($P < 0.001$) and renal failure duration ($P < 0.001$), diabetes ($P = 0.001$), triglycerides ($P = 0.03$), and C-reactive protein (CRP) levels ($P = 0.002$). In patients with diabetes, AF correlated with age ($r = 0.43$, $P = 0.04$), dialysis duration ($r = 0.54$, $P < 0.01$), LDL ($r = 0.56$, $P < 0.01$), triglycerides ($r = 0.59$, $P < 0.01$), and HbA$_{1c}$ ($r = 0.53$, $P = 0.01$). Gender, body mass index, parathyroid hormone levels, and medication did not have an independent effect on AF values.

Table 2 describes the baseline patient characteristics of the validation substudy. AF correlated strongly with collagen-linked fluorescence (CLF; $r = 0.71$, $P < 0.001$) and with pentosidine skin levels ($r = 0.75$, $P < 0.001$), as shown in Figure 2. Skin biopsy levels of CLF correlated with pentosidine ($r = 0.72$, $P < 0.01$). Furthermore, AF correlated with the nonfluorescent AGE CML and CEL (both $r = 0.45$, $P < 0.01$).

Forty-eight HD patients had CVD at baseline, 40 (83%) of whom had coronary heart disease. Table 3 shows the variables related to CVD at baseline. AF was independently related to the presence of CVD at baseline, as were serum albumin level and CRP.

Follow-Up

During the follow-up period of 3 yr, 42 (38.5%) patients had died, which indicates an annual mortality rate of approximately 15%. Fifty-five percent of these patients died of CVD. Seven patients received a kidney transplant during the follow-up period.

Overall and cardiovascular mortality were markedly increased in patients with AF values above the group mean AF compared with those with values below the group mean AF at
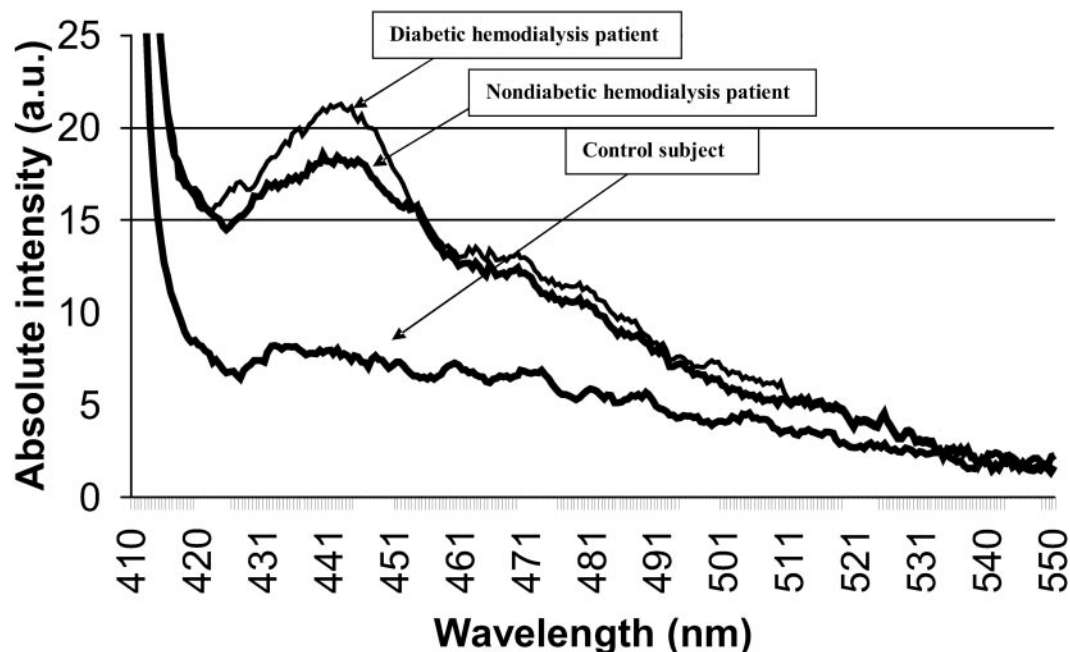

Figure 1. Autofluorescence (AF) spectrum (intensity, arbitrary units [AU]) measured with the AF reader (AFR) in a control subject, a hemodialysis (HD) patient without diabetes, and an HD patient with diabetes; comparable in age, duration of renal failure, and dialysis duration.

Table 2. Characteristics of HD patients from the validation substudy[a]

Characteristics	HD Patients ($n = 29$)
Age (yr)	57 ± 16
Gender (M/F)	16/13
BMI (kg/m^2)	22.2 ± 2.8
Creatinine (μmol/L)	1003.4 ± 247.1
BUN (mmol/L)	26.8 ± 7.9
Albumin (g/L)	39.0 ± 2.0
CRP (mg/L)	5.2 ± 4.8
LDL (mmol/L)	3.2 ± 0.8
HDL (mmol/L)	1.1 ± 0.3
Triglycerides (mmol/L)	2.4 ± 0.8
Glucose (mmol/L)	5.9 ± 0.9
EDTA (A/B/C/D)	5/11/6/7

[a]In HD patients, BUN and creatinine levels are predialysis levels. EDTA classification of primary diagnosis: A, diabetes; B, hypertension/renovascular disease; C, primary glomerular disorders; D, other primary disorders.

baseline (60 *versus* 18%, and 48 *versus* 8%, respectively; $P < 0.001$; Figure 3). Patients with CVD at baseline but with AF values below group mean died predominantly within the first year. Although their AF values were below group mean, these patients had high AF values (0.022 ± 0.001).

Table 4 shows that AF (OR 3.9), pre-existing CVD (OR 3.1), and albumin (OR 0.3) were independent predictors of overall mortality. AF was a stronger predictor for cardiovascular mortality (OR 6.8; 95% CI 2.6 to 17.5) compared with overall mortality. CRP showed a borderline significance as predictor of mortality. AF replaced age, diabetes, duration of renal failure, triglycerides, LDL, and parathyroid hormone levels as independent predictors of mortality. The area under the curve for a receiver operating characteristic curve using AF to detect overall mortality was 0.89, and this was higher compared with other measures of metabolic stress (*e.g.*, HbA$_{1c}$, triglycerides).

Discussion

Skin AF is a strong and independent predictor of overall and cardiovascular mortality and is associated with CVD in HD patients. Our data indicate that increased skin AF reflects increased AGE accumulation (CLF and pentosidine). Thus, this study is the first to show the predictive value of tissue AGE (pentosidine) accumulation for mortality. Our results confirm the clinical correlates of CVD with recognized risk factors, such as diabetes and albumin levels (26,29,30). The noninvasive AFR may become a clinical desktop tool for risk assessment in ESRD.

The power of skin AF as a prognostic factor for mortality is illustrated by the fact that it was found to serve better in the Cox regression models than the prognostic value of other known risk factors. In this and previous studies, skin AF was related to metabolic stress (HbA$_{1c}$ and hyperlipidemia) and the accumulation of pentosidine, CML, and CEL (21,23). We hypothesize that by representing the existing tissue damage from
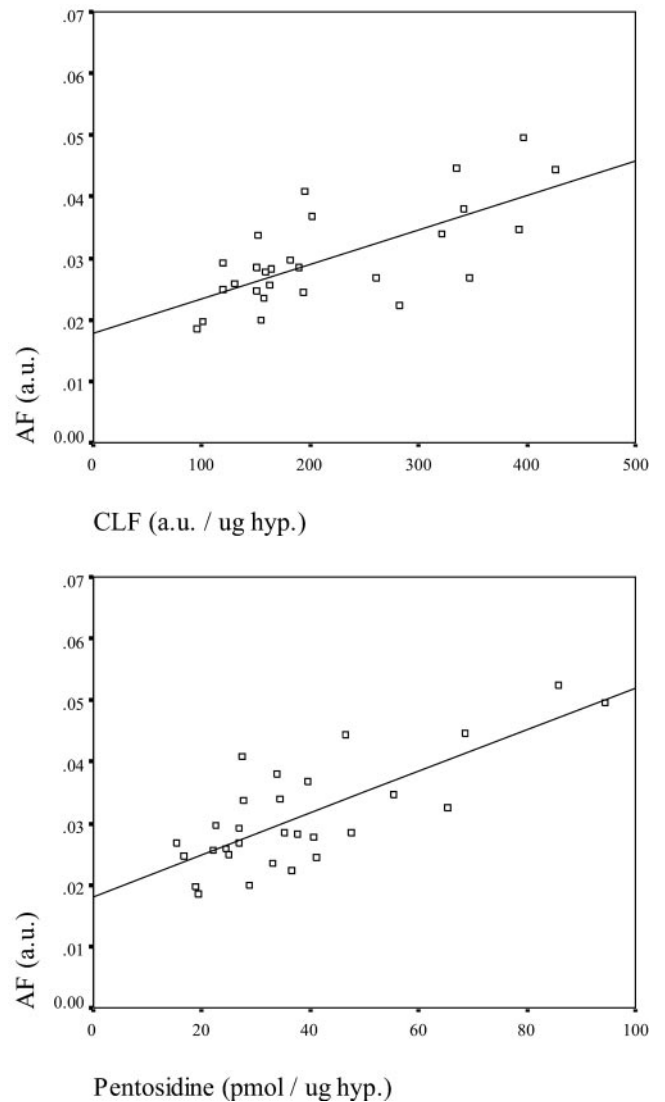

Figure 2. The relationship between noninvasive measurement of skin AF (in AU) and skin collagen-linked fluorescence (CLF; AU/μg hyp), and pentosidine (pmol/μg hyp) levels of skin biopsies from 29 HD patients. Hyp, hydroxyproline content of collagen.

cumulative metabolic stress, skin AF and AGE accumulation may show the effect of a common pathway more than traditional risk factors alone. AGE accumulate as a result of nonenzymatic glycation, oxidative/carbonyl stress, and/or diminished clearance of AGE precursors (3,4,31). Hyperlipidemia may contribute in the tissue accumulation of advanced lipoxidation end products (ALE), which may contribute to tissue and skin AF (32). Indeed, in our study, skin AF correlated strongly with triglycerides and LDL levels. The quantitative relationship between AGE and ALE in diabetes and ESRD is still unknown, but dyslipidemia may be as important as hyperglycemia in chemical modification of proteins (32).

Tissue AGE accumulation was also independently associated with the presence of CVD at baseline in our study. In ESRD, AGE/ALE accumulate in the vessel wall and contribute to the

Table 3. Variables related to the presence of CVD in HD patients at baseline by logistic regression analysis[a]

	Univariate		Multivariate	
	P	OR (± 95% CI)	*P*	OR (± 95% CI)
Age (yr)	<0.01	1.8 (1.1 to 2.5)	NS	—
Albumin (g/L)	<0.01	0.2 (0.1 to 0.2)	<0.01	0.2 (0.1 to 0.3)
CRP (mg/L)	<0.01	1.2 (1.1 to 1.3)	0.04	1.1 (1.0 to 1.3)
Diabetes	<0.01	5.0 (1.8 to 14.1)	NS	—
Dialysis duration (yr)	<0.01	1.4 (1.1 to 1.7)	NS	—
Duration of CRF (yr)	0.01	1.1 (1.0 to 1.1)	NS	—
HT	0.07	2.0 (0.9 to 4.3)	NS	—
Triglycerides (mmol/L)	0.02	1.8 (1.0 to 2.5)	NS	—
LDL (mmol/L)	<0.01	1.9 (1.2 to 3.0)	NS	—
Smoking	0.07	1.6 (1.0 to 3.0)	NS	—
PTH (pmol/L)	<0.01	8.1 (6.0 to 10.1)	NS	—
AF (AU)	<0.01	17.2 (5.7 to 51.9)	<0.01	8.8 (2.8 to 28.1)

[a]OR, odds ratio; CI, confidence interval; CVD, cardiovascular disease; HT, patients with primary diagnosis of hypertension/renovascular disease; CRF, chronic renal failure; PTH, parathyroid hormone; AF, skin autofluorescence; AU, arbitrary units.
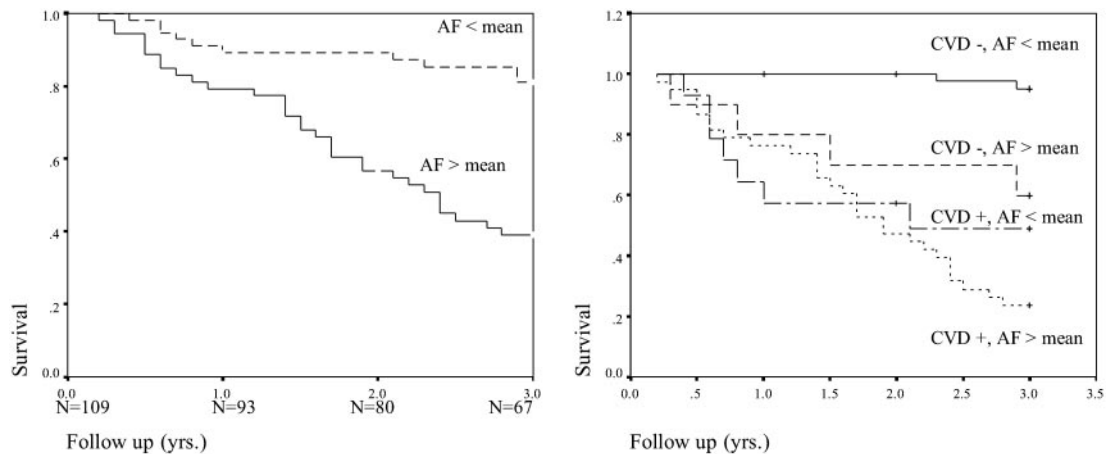

Figure 3. Kaplan-Meier estimates of survival during follow-up with regard to overall mortality in HD patients in relation to skin AF above and below mean values, and the presence (±) of cardiovascular disease (CVD) at baseline.

progression of CVD by several mechanisms, including cross-linking of extracellular proteins (*e.g.*, LDL), binding to receptor for AGE inducing oxidative stress, inflammation, and endothelial dysfunction (5,8,9,33). Indeed, we observed a strong correlation between skin autofluorescence and CRP. Altogether, this may accelerate coronary atherosclerosis and induce cardiac remodeling and ventricular dysfunction (34,35). Intervention studies with an AGE breaker have demonstrated improved vascular and ventricular compliance (13).

A limitation of our study is that we cannot exclude completely the influence of other uremic toxins or skin fluorophores on skin AF measurements. Furthermore, the current understanding of physiologic AGE indicates that most AGE are not fluorescent. Importantly, fluorescence represents group reactivity, which fails to provide quantitative information on concentrations of individual compounds. However, our previous results in patients with diabetes and these results in HD patients

show that skin AF may function as a marker of the AGE pool, on the basis of the strong correlations with both fluorescence and nonfluorescent skin AGE levels (23). This study was neither of sufficient size nor intended to define further the relationship and kinetics of skin AF with tissue and serum AGE accumulation in relation to specific modalities of renal replacement therapies. A larger validation study is now in progress, including data of skin AF and skin/serum AGE accumulation in the follow-up after kidney transplantation.

Schwedler *et al.* (36) and Busch *et al.* (37) reported that circulating AGE do not predict mortality in HD patients. However, serum AGE may be influenced by dialysis modalities, absorption from food, and smoking (38–40). High serum AGE might even reflect a better nutritional support, associated with improved survival (36). An alternative explanation is that serum AGE may not adequately reflect tissue AGE accumulation (21).

Because long-term complications and skin AF are time-de-

Table 4. Predictors of 3-yr overall mortality in HD patients by forward Cox regression analysis[a]

	Univariate		Multivariate	
	P	OR	P	OR
Pre-existing CVD	<0.01	15.7 (8.3 to 28.9)	0.03	3.1 (1.1 to 9.2)
Age (yr)	<0.01	1.6 (1.2 to 2.0)	NS	—
Albumin (g/L)	<0.01	0.1 (0.1 to 0.2)	0.016	0.3 (0.1 to 0.8)
CRP (mg/L)	<0.01	1.3 (1.1 to 1.5)	<0.01	1.1 (1.0 to 1.1)
Diabetes	0.06	1.8 (1.4 to 2.2)	NS	—
Dialysis duration (yr)	0.02	1.2 (1.0 to 1.4)	NS	—
Duration CRF (yr)	<0.01	1.1 (1.0 to 1.1)	NS	—
PTH (pmol/L)	<0.01	6.0 (2.7 to 10.9)	NS	—
HT	0.03	2.0 (1.1 to 4.7)	NS	—
Triglycerides (mmol/L)	<0.01	2.0 (1.2 to 3.0)	NS	—
LDL (mmol/L)	<0.01	1.6 (1.2 to 2.3)	NS	—
Smoking	0.09	1.3 (1.0 to 2.1)	NS	—
AF (AU)	<0.01	9.0 (2.6 to 23.8)	<0.01	3.9 (1.9 to 8.1)

[a]OR (± 95% CI).

pendent processes, the results of our study could be biased by age. To reduce such a bias, we always included age as a variable in the multivariate analysis. Furthermore, our study shows a correlation between skin AF and mortality. Whether this concerns a causal relationship has to be decided by interventions aimed at reducing AGE accumulation.

In conclusion, our study shows that skin AF is an independent predictor of mortality and is associated with CVD in HD patients. As AF was related to AGE accumulation, our study supports the important clinical impact of AGE accumulation in the pathogenesis of vascular disease and is the first to show the prognostic power of AGE accumulation in renal failure. A growing body of evidence on the role of AGE in chronic age-related diseases warrants interventions that specifically are aimed at AGE accumulation. The noninvasive AFR may become a rapid clinical desktop tool for risk assessment but also provides a novel approach for monitoring the role of AGE in disease.

Acknowledgments

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