

# Skin Autofluorescence Is a Strong Predictor of Cardiac Mortality in Diabetes

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**OBJECTIVE** — Advanced glycation end products (AGEs) are biomarkers of metabolic stress and are thought to contribute to the increase of coronary heart disease (CHD) in diabetes. Tissue autofluorescence is related to the accumulation of AGEs. The aim of the present study was to evaluate the relationship between skin autofluorescence and metabolic burden (hyperglycemia and hyperlipidemia) and its relationship with CHD and mortality.

**RESEARCH DESIGN AND METHODS** — Skin autofluorescence was measured noninvasively with an autofluorescence reader in 48 type 1 and 69 type 2 diabetic patients and 43 control subjects. The presence of CHD was observed at baseline and mortality during a follow-up period of 5 years.

**RESULTS** — Autofluorescence correlated with mean A1C, triglycerides, and LDL. Autofluorescence values further increased with age, microalbuminuria, dialysis treatment, and diabetes duration. Autofluorescence was strongly related to the presence of CHD (odds ratio 7.9) and predicted mortality (3.0). Multivariate analysis showed that autofluorescence was more strongly associated with CHD and mortality compared with A1C, triglycerides, and LDL.

**CONCLUSIONS** — Skin autofluorescence is strongly related to cumulative metabolic burden. Skin autofluorescence seems strongly associated with cardiac mortality and may provide important clinical information for risk assessment.

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The accumulation of advanced glycation end products (AGEs) on tissue proteins has been implicated as a contributing factor in the progression of diabetes complications (1–5). Nonenzymatic glycation of proteins is a series of complex and sequential reactions collectively called the Maillard reaction. Early-stage reactions lead to the formation of the early glycation adducts, and later-stage reactions subsequently form AGEs. Fructose lysine undergoes rearrangement and autooxidation reactions to form AGEs,

while compounds, such as glyoxal and methylglyoxal, which may be formed during metabolism or by carbohydrate and lipid peroxidation reactions, also contribute to the formation of AGEs and advanced lipoxidation end products (ALEs). Some compounds, such as carboxymethyllysine (CML), may be derived from either carbohydrate or lipids retermed AGE/ALEs (6,7). Decreased renal clearance of AGE/ALE precursors may further increase the accumulation of end products (7–9). Accumulation of chemi-

cally stable AGEs/ALEs on long-lived proteins may serve as a measure of cumulative metabolic stress. In a sub-study of the Diabetes Control and Complications Trial, skin AGE levels explained a major part of the variance in diabetes complications, even after adjustment for A1C (10). Inhibition of AGE formation, in experimental animal models of diabetes, reduces the development of a wide range of diabetes complications (11–13). Unfortunately, AGE and ALE assays are not widely used in clinical settings because they are a complex mixture of trace compounds in tissue proteins, and their specific and precise measurement relies on sophisticated and expensive techniques, such as gas and liquid chromatography and mass spectrometry (14).

Recently, we described a noninvasive technique, the autofluorescence reader (AFR), for measuring skin autofluorescence (15). Skin autofluorescence correlated with tissue levels of pentosidine, CML, measures of long-term glycemic control (15), and presence of long-term complications in diabetic patients and in hemodialysis patients (15–18). Coronary heart disease (CHD) and mortality rates are markedly increased in diabetic patients, and metabolic burden is a major risk factor for CHD in these patients. We hypothesize that skin autofluorescence, as a measure of AGE/ALE accumulation, may be strongly related to the cumulative effect of the metabolic burden by hyperglycemia and hyperlipidemia and, thus, may predict cardiac mortality in diabetes.

## RESEARCH DESIGN AND METHODS

We noninvasively measured skin autofluorescence in 48 type 1 and 69 type 2 diabetic patients and 43 control subjects. Both type 1 (age <30 years at diagnosis of diabetes and insulin dependent from onset) and type 2 diabetic patients were recruited from our diabetes outpatient clinic. In control subjects, diabetes and renal failure were excluded by conventional criteria (American Diabetes Association) and a serum creatinine level <120  $\mu\text{mol/l}$ . Control subjects who smoked were excluded; diabetic patients who smoked were not excluded from the analysis of risk factors

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**Abbreviations:** AFR, autofluorescence reader; AGE, advanced glycation end product; ALE, advanced lipoxidation end product; CHD, coronary heart disease; CML, carboxymethyllysine.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—Group characteristics of the diabetic patients and control subjects

Variables	Type 1 diabetes	Type 2 diabetes	Control
n	48	69	43
Age (years)	45 ± 15	61 ± 13*	53 ± 16
Creatinine (μmol/l)	89 ± 12	97 ± 17	88 ± 8
Triglycerides (mmol/l)	1.65 ± 0.8	1.9 ± 0.8	1.3 ± 0.5†
LDL (mmol/l)	3.2 ± 0.7	3.2 ± 1.0	3.4 ± 1.1
HDL (mmol/l)	1.3 ± 0.5	1.0 ± 0.3*	1.3 ± 0.3
BMI (kg/m <sup>2</sup> )	22.8 ± 1.6	24.4 ± 1.2*	24.1 ± 1.8
Duration diabetes (years)	20 ± 11	10 (5–20)*	—
Mean A1C (%)	7.9 ± 1.0	8.2 ± 0.9	5.5 ± 0.5†
Smoking	11	10	0†
Caucasians (%)	92	95	91
Male:female (%)	48:52	65:35	45:55
ACE inhibitor	15	21	—
Statins	8	32*	—
Antioxidants	9	35*	—
Hypertension	15	33*	—
Hemodialysis	10	14	—
Microalbuminuria	12	22*	—
CHD	13	31*	—
Autofluorescence (AU)	0.016 ± 0.005	0.021 ± 0.003*	0.010 ± 0.001†

Data are n, means ± SD, or median (25–75% range) unless otherwise indicated. ACE inhibitor, statins, and antioxidants describe the number of patients using these medications. Mean creatinine levels are levels in diabetic patients excluding those on dialysis treatment. \* $P < 0.05$  between type 1 and type 2 diabetic patients; † $P < 0.05$  between diabetic patients and control subjects. AU, arbitrary units.

related to CHD. The local ethics committee approved this study, and informed consent was obtained before the measurements.

### Metabolic burden, renal function, and CHD

Measures of metabolic burden (A1C and lipid profiles) were taken from chart review at baseline. A1C levels are analyzed four times a year at our hospital, and mean A1C of the previous year was analyzed in this study at baseline. Lipid profiles are analyzed once a year, and these values were also obtained at baseline. Renal damage was analyzed by the presence of microalbuminuria (urinary albumin excretion rate  $>20 \mu\text{g}/\text{min}$  in at least two of three overnight urine collections) and creatinine clearance ( $<70 \text{ ml/h}$  per  $1.73 \text{ m}^2$  of body surface area), and all diabetic patients on hemodialysis treatment from our Dialysis Centre in Groningen ( $n = 24$ ) were included. Dialysis duration was defined from the initiation of long-term hemodialysis treatment to the start of the study. Hypertension was defined as a systolic blood pressure  $>140 \text{ mmHg}$  or a diastolic pressure  $>90 \text{ mmHg}$  on at least three occasions or when receiving antihypertensive medication. Table 1 shows the subject's characteristics.

The presence of CHD was assessed

from chart review (based on ICD-9-CM [clinical modification] codes I20 and I21, including resting electrocardiogram analysis followed by an exercise electrocardiogram and coronary angiogram in the case of abnormalities). During a follow-up period of 5 years, date and cause of death were obtained from medical records. Causes of death were certified and classified as CHD related according to the usual ICD coding criteria.

### Skin autofluorescence

Skin autofluorescence was assessed by the AFR (patent PCT/NL99/00607; prototype of the current AGE Reader I; Diagnostica BV, Groningen, the Netherlands) as previously described in detail (15). In short, the AFR illuminates a skin surface of  $\sim 1 \text{ cm}^2$  at the lower arm, guarded against surrounding light, with an excitation light source between 300 and 420 nm (peak excitation  $\sim 350 \text{ nm}$ ). Only light from the skin is measured with a spectrometer (Ocean Optics PC-1000 fiber optic spectrometer; Ocean Optics, Dunedin, FL) in the 300–600 nm range, using 200-μm glass fiber (Farnell, Leeds, U.K.). The measures of autofluorescence we applied were the average light intensity per nanometer in the range 420–600 nm divided by the average light intensity per nanometer in the range 300–420 nm. Repeated

AFR measurements on day 1 showed an overall Altman error percentage  $<5\%$ .

### Statistical analysis

Comparison between groups was performed with Student's  $t$  test or Mann-Whitney  $U$  test. Bivariate regression and correlation were analyzed by the Spearman rank method. Multivariate regression analyses were performed for determination of independent relationships of variables with autofluorescence. Variables shown to be related to CHD in univariate analysis ( $P < 0.01$ ) were tested for their independent effect on CHD by multivariate logistic regression analysis, while always including age as a variable. 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. A two-tailed  $P$  value  $<0.01$  was considered significant because of the large number of correlation tests. Data are shown as mean ± SD, unless otherwise indicated.

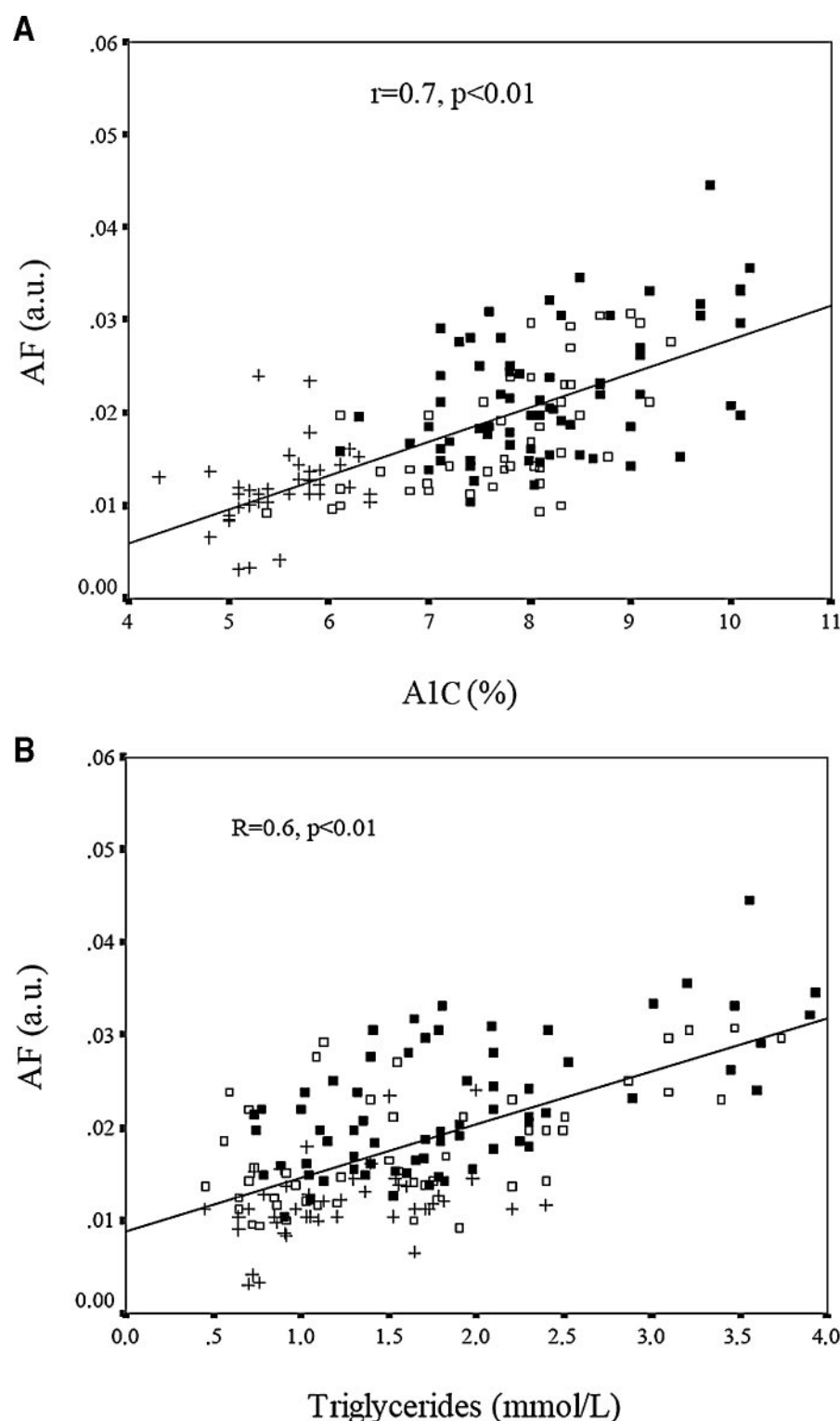
## RESULTS

### Skin autofluorescence, metabolic burden, and renal function

As shown in Table 1, autofluorescence values are significantly increased in diabetic patients compared with control subjects. In type 1 diabetic patients, autofluorescence correlated with age ( $r = 0.6$ ,  $P < 0.01$ ), mean A1C of the previous year ( $r = 0.6$ ,  $P < 0.01$ ) (Fig. 1), triglycerides ( $r = 0.5$ ,  $P < 0.01$ ) (Fig. 1), LDL ( $r = 0.3$ ,  $P = 0.02$ ), and diabetes duration ( $r = 0.4$ ,  $P = 0.05$ ). Multivariate analysis showed that, after correction for A1C, autofluorescence still correlated with triglycerides ( $r = 0.4$ ,  $P < 0.01$ ) but not with LDL.

In type 2 diabetic patients, autofluorescence correlated with age ( $r = 0.4$ ,  $P = 0.01$ ), mean A1C of the previous year ( $r = 0.5$ ,  $P < 0.01$ ) (Fig. 1), triglycerides ( $r = 0.6$ ,  $P < 0.01$ ) (Fig. 1), LDL ( $r = 0.6$ ,  $P < 0.01$ ), and BMI ( $r = 0.3$ ,  $P = 0.03$ ). Multivariate analysis showed that, after correction for A1C, autofluorescence still correlated with triglycerides ( $r = 0.6$ ,  $P < 0.01$ ) and LDL ( $r = 0.6$ ,  $P < 0.01$ ).

In both diabetic populations, autofluorescence correlated with creatinine levels ( $r = 0.5$ ,  $P < 0.01$ ). Multivariate analysis showed that the presence of microalbuminuria ( $P < 0.01$ ) was associ-



**Figure 1**—Type 1 diabetic patients and data for mean A1C (A) and triglycerides (B) from the previous year. AF, autofluorescence.

ated with increased autofluorescence values and also after correction for age, A1C, triglycerides, and LDL. The highest autofluorescence values were observed in diabetic patients on hemodialysis treatment (autofluorescence  $0.029 \pm 0.002$ ).

In diabetic hemodialysis patients, autofluorescence values correlated with age ( $r = 0.4, P = 0.04$ ), A1C ( $r = 0.5, P = 0.01$ ), triglycerides ( $r = 0.6, P < 0.01$ ), LDL ( $r = 0.6, P < 0.01$ ), and dialysis duration ( $r = 0.5, P = 0.01$ ).

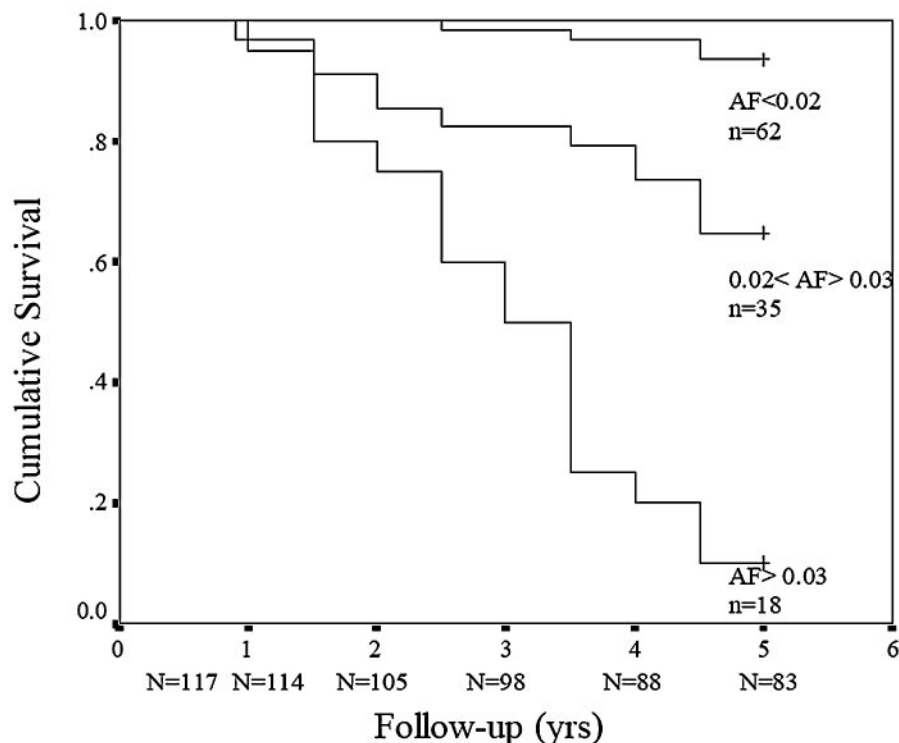
In diabetic patients, no correlations were observed between autofluorescence values and specific medical treatment, sex, HDLs, or smoking habits. In control subjects, autofluorescence correlated with age ( $r = 0.4, P < 0.01$ ). Autofluorescence also correlated, although not statistically significant, with A1C ( $r = 0.4, P = 0.05$ ) and triglycerides ( $r = 0.4, P = 0.06$ ) in control subjects.

### Skin autofluorescence and CHD

CHD was present at baseline in 13 type 1 and 31 type 2 diabetic patients and related to many known risk factors for CHD. Multivariate analysis showed that, in type 1 diabetic patients, autofluorescence (OR 7.8) was independently associated with CHD, besides microalbuminuria (5.3) and hypertension (2.3). In type 2 diabetic patients, autofluorescence (7.9) was independently associated with CHD, besides microalbuminuria (5.9) and hypertension (10.5).

During the follow-up period, 11 type 1 and 23 type 2 diabetic patients died from CHD disease. Mortality was markedly increased in patients with autofluorescence values  $>0.020$  (group mean autofluorescence for all diabetic patients) and further increased in autofluorescence values  $>0.030$  ( $P < 0.001$ ) (Fig. 2). In both diabetic populations, autofluorescence was an independent predictor of mortality (OR 2.0) (Table 2). Mortality was further independently predicted by the presence of CHD at baseline and hemodialysis treatment. A stepwise forward Cox regression analysis was performed to define the “traditional” risk factors (including all variables from Table 2) that were replaced by autofluorescence. Autofluorescence replaced age, mean A1C, triglycerides, LDL, and smoking as independent predictors of mortality. Including autofluorescence in the stepwise Cox regression analysis increased the prognostic power significantly ( $\chi^2$  increased from 80 to 92). The area under a receiver operating curve using autofluorescence to detect mortality was 0.92 and was higher than similar curves using A1C (type 1 diabetes 0.82; type 2 diabetes 0.61).

**CONCLUSIONS**— Our study shows that skin autofluorescence is a measure of (long-term) metabolic burden and seems strongly associated with the presence of CHD and cardiac mortality. These new observations may broaden the use of autofluorescence techniques but need



**Figure 2**—Mortality was markedly increased in patients with increasing autofluorescence (AF) values.

further study to evaluate their (clinical) relevance.

The correlation of skin autofluorescence and AGE/ALE accumulation with measures of long-term glycemic control has been described by others (10). Accumulation of chemically stable AGEs on long-lived proteins may serve as a measure of cumulative glycemic burden. The strong association between fluorescence and hyperlipidemia in our study, and the previous observed correlation with CML, may be indicative of the contribution of ALEs to tissue fluorescence. Dyslipidemia may be as important as hyperglycemia in chemical modification of proteins in diabetes (6). High serum levels of fluorescent AGEs are related to high serum levels of triglycerides and LDL in diabetic patients (19). Future studies of skin autofluorescence, in combination with measures of lipoxidation intermediates (e.g., malondialdehyde), may help in the analysis of the quantitative relationship between AGEs/ALEs and fluorescence in diabetes.

Interestingly, in our (nonsmoking) control population, we observed a trend to higher autofluorescence values in those with increased levels of triglycerides or A1C. It must be made clear that the AFR has never been intended to make a diagnosis of (currently unknown) type 2 dia-

betes. It can be hypothesized that skin autofluorescence also reflects long-term metabolic burden in healthy subjects. These results should be confirmed in a larger study, e.g., in a primary care setting, before conclusions can be drawn.

The potential power of skin autofluorescence as a prognostic factor for mortality is illustrated by the fact that it seemed to serve better in the Cox regression models than the prognostic value of other known risk factors, especially those related to metabolic burden. We hypothesize that by representing the existing tissue damage from cumulative metabolic burden, skin autofluorescence and AGE/ALE accumulation may show the effect of a common pathway more than traditional risk factors alone. Furthermore, skin autofluorescence was more strongly associated with CHD and mortality compared with smoking. Smoking has been recognized as an exogenous source of increased tissue AGE accumulation (20,21). AGEs and ALEs accumulate in the vessel wall and bind to receptors for AGEs and scavenger receptors. Through various pathways, this accelerates coronary atherosclerosis and may induce cardiac remodeling and ventricular dysfunction (22–24).

Since long-term complications of dia-

betes and skin autofluorescence are time-dependent 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 of CHD. The number of type 1 diabetic patients with CHD and mortality was limited (Table 2), and conclusions should be made with caution. Their results were included in this study to show differences in metabolic abnormalities and skin autofluorescence between both diabetic populations. Further prospective research is needed to confirm these results.

Diabetic patients may have a blunted appreciation of ischemic pain, resulting in silent ischemia or silent infarction (25). Not every diabetic patient in our study performed an exercise test; thus, the prevalence of CHD and its relation with skin autofluorescence may be underestimated.

Limitations of the AFR technique have been described previously (15,17,18). Importantly, not all AGEs exhibit fluorescent properties, and fluorescence is a group reactivity, which fails to provide quantitative information on concentrations of individual compounds. There are other fluorescent AGEs besides pentosidine. End products like crosslines and vespersylsines have excitation (380 nm) and emission (440 nm) spectra that may be strongly associated with skin autofluorescence, as measured by AFR, compared with pentosidine (26–28). AGEs may be a major source of skin autofluorescence and probably correlate with pentosidine content of collagen in diabetes. Moreover, some nonfluorescent AGEs, e.g., CML, are thought to have an important pathogenetic role. However, tissue fluorescence itself is associated with the progression of long-term complications in diabetes (4,16,18,29).

Whether our data indicate an association or causal relationship between skin autofluorescence, AGEs, and the progression of CHD has to be decided by intervention studies aimed at reducing AGE accumulation. At least our results show that skin autofluorescence may be more strongly associated with CHD compared with other measures of metabolic stress (e.g., A1C). Skin autofluorescence also seems to be related to microvascular complications in diabetes and to long-term complications and mortality in nondiabetic patients with end-stage renal disease (16). However, these results were beyond the scope of the present study. The development and severity of CHD in diabetic patients without cardiovascular disease at



**Table 2—Variables related to coronary heart mortality in diabetic patients by Cox regression analysis**

Variables	Univariate		Multivariate	
	P value	OR (95% CI)	P value	OR (95% CI)
Type 1 diabetic patients				
Age (years)	<0.01	1.1 (1.0–1.2)	NS	—
Mean A1C (%)	<0.01	3.9 (1.8–9.8)	NS	—
Diabetes duration (years)	NS		NS	—
Creatinine ( $\mu\text{mol/l}$ )	0.01	1.1 (1.0–1.3)	NS	—
Hypertension	<0.01	3.9 (1.8–8.3)	NS	—
Microalbuminuria	NS		NS	—
Hemodialysis treatment	<0.01	7.3 (2.2–24.0)	<0.01	4.7 (1.4–12.4)
Triglycerides (mmol/l)	<0.01	2.4 (1.5–3.6)	NS	—
LDL (mmol/l)	NS		NS	—
BMI ( $\text{kg/m}^2$ )	NS		NS	—
Smoking	0.05	2.0 (0.9–14.1)	NS	—
CHD at baseline	<0.01	9.3 (2.5–25.5)	<0.01	4.8 (1.9–18.3)
AF (AU)	<0.01	6.0 (2.5–14.2)	<0.01	2.0 (1.3–2.7)
Type 2 diabetic patients				
Age (years)	NS		NS	—
Mean A1C (%)	0.03	1.6 (1.0–2.3)	NS	—
Diabetes duration (years)	NS		NS	—
Creatinine ( $\mu\text{mol/l}$ )	0.01	1.1 (1.0–1.2)	NS	—
Hypertension	0.01	1.7 (1.1–2.7)	NS	—
Microalbuminuria	NS		NS	—
Hemodialysis treatment	0.01	5.9 (2.5–13.7)	<0.01	4.9 (1.9–12.5)
Triglycerides (mmol/l)	<0.01	3.8 (1.8–7.8)	NS	—
LDL (mmol/l)	<0.01	3.0 (1.9–4.8)	NS	—
BMI ( $\text{kg/m}^2$ )	0.01	1.2 (1.0–1.5)	NS	—
Smoking	0.02	3.3 (1.1–9.5)	NS	—
CHD at baseline	<0.01	8.6 (2.9–25.3)	<0.01	5.0 (1.5–16.0)
AF (AU)	<0.01	2.9 (1.8–6.1)	<0.01	2.9 (1.3–4.4)

AF, skin autofluorescence measured with the AFR (see RESEARCH DESIGN AND METHODS); AU, arbitrary units.

baseline in relation to skin autofluorescence is under investigation in a group of 1,000 type 2 diabetic patients.

In conclusion, our study shows that skin autofluorescence is related to (long-term) metabolic burden and the progression of coronary heart disease in diabetes. The new observation that skin autofluorescence is strongly related to hyperlipidemia in diabetic patients deserves further analysis, and the larger cohort study in type 2 diabetic patients offers such an opportunity. The noninvasive AFR may become a clinical desktop tool for follow-up in metabolic control and risk assessment but also provides a novel approach for monitoring the role of AGEs in disease.

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