Skin Autofluorescence as a Noninvasive Marker of Vascular Damage in Patients With Type 2 Diabetes

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OBJECTIVE — Advanced glycation end products (AGEs) are thought to have a role in the pathogenesis of diabetes complications. We recently reported the association between skin autofluorescence, as a measure of tissue AGE accumulation, and diabetic neuropathy in a selected diabetic population. In this study, we investigated the relation between skin autofluorescence and clinical variables including micro- and macrovascular complications in a type 2 diabetes primary care population.

RESEARCH DESIGN AND METHODS — Clinical data and skin autofluorescence were obtained in the type 2 diabetes group ($n = 973$) and in a control group ($n = 231$). Skin autofluorescence was assessed by illumination of the lower arm with a fluorescent tube (peak intensity ~370 nm).

RESULTS — Skin autofluorescence was significantly higher in type 2 diabetic patients compared with control subjects in each age category. Multiple regression analysis showed significant correlation of skin autofluorescence with age, sex, diabetes duration, BMI, smoking, HbA_{1c}, plasma creatinine, HDL cholesterol, and albumin-to-creatinine ratio in the type 2 diabetes group ($R^2 = 25\%$) and with age and smoking in the control group ($R^2 = 46\%$). Skin autofluorescence was significantly higher in the type 2 diabetes group, with both micro- and macrovascular disease, compared with the group without complications and the group with only microvascular complications.

CONCLUSIONS — This study confirms in a large group of type 2 diabetic patients that skin autofluorescence is higher compared with age-matched control subjects and is associated with the severity of diabetes-related complications. Skin autofluorescence reflecting vascular damage might be a rapid and helpful tool in the diabetes outpatient clinic for identifying diabetic patients who are at risk for developing complications.

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T he formation of advanced glycation end products (AGEs) is increased in diabetes (1–3). Accumulated AGEs have deleterious effects on the vascular wall, contributing to the development of micro- and macrovascular disease, as shown especially in type 1 diabetes (4–8). The Diabetes Control and Complications Trial substudy on skin collagen glycation found strong associations between skin AGE levels and long-term diabetes complications in type 1 diabetes, which persisted after adjustment for HbA_{1c} (A1C) (7). The relation between AGE accumulation and outcome has been studied very little in type 2 diabetes.

Besides, several laboratory assessments of AGEs are used worldwide. In the earlier studies of the 1990s, AGEs were mostly assessed in tissue by collagen-linked fluorescence (CLF) or specific AGE measurements (pentosidine, carboxymethyllysine) (4,9,10). During the last few years, AGEs are increasingly measured in serum or plasma using high-performance liquid chromatography, enzyme-linked immunosorbent assay, or fluorescence. The blood compartment is more practical for repeated measurements than tissue requiring biopsies, but plasma AGE assays are unfortunately less reproducible and less well related to tissue contents of AGEs (11,12). We recently described a tool to noninvasively assess tissue AGEs in vivo using skin autofluorescence (13). This method utilizes the fluorescent properties of AGEs, like the extensively used CLF method, and has been validated with specific AGE measurements and CLF in skin biopsies (13). By linking skin autofluorescence to AGE accumulation and, therefore, to cumulative glycemic and oxidative damage in diabetes, we aim to create a tool that is able to give a rapid impression of the risk for diabetes complications.

Using this tool in a prospective cohort of type 2 diabetic patients, we aim to address the predictive value of skin autofluorescence on diabetes-related complications. This study involves a cross-sectional analysis of the baseline data of this cohort to study the association between skin autofluorescence and clinical variables, including the presence of complications at baseline.

RESEARCH DESIGN AND METHODS — We recruited subjects participating in the ZODIAC (Zwolle Outpatient Diabetes project Integrating Available Care) study, which has previously been described (14). In short, this study investigates the effects of a shared-care project in a primary care population–based cohort of type 2 diabetic patients in

the eastern part of the Netherlands. All patients of 32 general practitioners receiving their diabetes support in the primary care setting and who additionally visited the diabetes outpatient clinic annually were approached. Patients were included from May 2001 to May 2002. Patients with cognitive disability or terminal diseases were excluded from the ZODIAC study and, consequently, also ineligible to the present study. After obtaining informed consent, autofluorescence was measured in 1,121 of the 1,450 patients (77%) that visited the outpatient clinic during the inclusion period.

Additionally, skin autofluorescence was measured in a nondiabetic control group of 231 consecutive preoperative-evaluation visitors of the outpatient clinic who did not have a history of diabetes, cardiovascular events, or renal disease (15). The study was approved by the local ethical committee.

Skin autofluorescence was assessed by the autofluorescence reader (AFR; patent PCT/NL99/00607 [prototype of current AGE Reader]; DiagnOptics BV, Groningen, the Netherlands) as described previously (13). In short, the autofluorescence reader illuminates a skin surface of ~4 cm², guarded against surrounding light, with an excitation light source (8-W blacklight; Philips) between 300 and 420 nm (peak excitation ~370 nm). Emission light and reflected excitation light from the skin are measured with a spectrometer (AVS-USB2000; Avantes, Eerbeek, the Netherlands), in the 300- to 600-nm range, using a 50-μm glass fiber (Farnell, Leeds, U.K.). Measurements were performed at room temperature, while patients were in a seated position, at the volar side of the arm ~10 cm below the elbow fold.

Since skin pigmentation may influence autofluorescence by light absorption (16), autofluorescence was calculated by dividing the average emitted light intensity per nanomole in the range 420–600 nm by the average excited light intensity per nanometer in the range 300–420 nm. Autofluorescence was expressed in arbitrary units (AU) and multiplied by 100. Skin reflection was calculated in the range 300–420 nm by dividing the mean intensity reflected from the skin by the mean intensity reflected from a white Teflon block (assuming 100% reflectance).

Autofluorescence measurements were performed in 1,121 type 2 diabetic patients at two different locations, with two identical autofluorescence reader sys-tems, by six diabetes nurses. Measurements in 148 patients (13%) had to be discarded: 74 for improper previous calibration, 36 showed light leakage from surrounding light, 7 because the tube was switched off inadvertently, and 31 because of too dark skin type, resulting in almost complete absorbtion of all the excitation light (skin reflection <7.5%), leaving too little light to measure autofluorescence with the current set up.

Autofluorescence measurements in the control group were performed by one examiner. None of the measurements in the control group were discarded. Thus, autofluorescence measurements in 973 type 2 diabetic patients and 231 control subjects were available for analysis. Overall, the autofluorescence measurements and mean age-corrected autofluorescence per measuring month ($P = 0.3$), per examiner ($P = 0.6$), and per autofluorescence reader system ($P = 0.1$) did not differ significantly.

Clinical data. Clinical data are derived from the Diabetic Electronic Monitoring System containing all data of the diabetic patients since the start of the shared-care project (1998) (17). Clinical data and laboratory results obtained on the date of the autofluorescence measurement were used in the analysis.

The blood pressure was a seated single measurement, obtained after 5 min rest, using an aneroid device. A1C was measured with a Primus CLC-385 using boronate affinity chromatography and high-performance liquid chromatography (reference value 4.0–6.0%). A Roche/Hitachi MODULAR analyzer was used for serum creatinine (kinetic colorimetric assay), nonfasting total cholesterol (cholesterol esterase/peroxidase enzymatic method), nonfasting HDL cholesterol (HDL-Cholesterol, homogeneous enzymatic colorimetric test), nonfasting triglycerides (lipase glycerol kinase enzymatic method), and urinary microalbumin (Tina-quant Albumin, immunoturbidimetric assay).

Retinal photography was scored by an independent ophthalmologist as absent, background, or proliferative retinopathy. Microalbuminuria was defined as an albumin-to-creatinine ratio >3.5 in women or >2.5 in men, in two subsequent samples (at baseline and in the year before baseline) or once in the year before baseline while using an ACE inhibitor at baseline. Diminished sensibility at least at one foot was considered as neuropathy (tested with a 5.07 Semmes Weinstein monofilament, applied on three areas of each foot) (18). The presence of microvascular complications was defined as meeting the criteria of microalbuminuria, neuropathy, and/or at least background retinopathy. Cardiovascular disease was defined as ischemic heart disease (ICD-9 codes 410–414 and/or a history of coronary artery bypass surgery or percutaneous coronary intervention), cerebrovascular accidents, or peripheral vascular disease (surgical intervention, claudication, and/or diminished or absent pulsations of ankle or foot arteries). The presence of macrovascular complications is defined when meeting at least one aspect of cardiovascular disease.

The following clinical data of the control group were collected: age, sex, current tobacco use, BMI, and blood pressure. An independent anesthesiologist classified the subjects according to the American Society of Anesthesiologists (ASA) classification, ASA class 1–5 (ASA 1 means a healthy patient without medical problems, and ASA 5 means moribund, not expected to live for another 24 h) (19). The large majority of the control group were classified as ASA 1 and 2 (>90%).

Statistical analyses. Multiple linear regression analysis was used to determine independent relations between skin autofluorescence and clinical variables. ANOVA was applied to compare differences between groups. Calculation of sample size of the diabetic population was based on a Cox regression power analysis. As the present cross-sectional study is part of a follow-up study in which cardiovascular end points will be the most critical end point, 741 patients are needed to detect a 6% difference in event-free surviving proportion (0.93 vs. 0.87) with 80% power and $\alpha = 0.05$ in a two-sided test, with 17% lost to follow-up (200 of 1,200).

Sample size of the nondiabetic control population was calculated according to formula of Altman (20). In our previous study, skin autofluorescence was 20–30% higher in diabetic patients (13). This was a heterogeneous group, including type 1 and type 2 diabetic patients. Furthermore, the mean A1C was higher (7.8%) than the mean A1C (7.1%) of the type 2 diabetic patients in this study (calculated in an interim analysis after inclusion of 475 patients). This taken into account, we considered a difference of 10–15% as clinically relevant. According to the nomogram of Altman (20), with 80% power and $\alpha = 0.05$, a sample size of

Table 1—*Clinical characteristics of the type 2 diabetic population and the control group*

Characteristic	Type 2 diabetes	Control subjects
n	973	231
Age (years)	66 ± 11	52 ± 17
Male sex	47	38
Caucasian	97	100
Smoker	19	30
BMI (kg/m^2)	29 ± 5	27 ± 5
Systolic blood pressure (mmHg)	146 ± 20	144 ± 22
Diastolic blood pressure (mmHg)	81 ± 10	82 ± 11
Diabetes duration (years)	4.2 (1.6–8.3)	—
A1C (%)	7.0 ± 1.3	Ref.
Creatinine (μmol/l)	96 ± 20	Ref.
Creatinine clearance (Cockroft-formula) (ml/min)	76 ± 27	Ref.
Urinary albumin-to-creatinine ratio	1.47 (0.8–4.1)	Ref.
Total cholesterol (mmol/l)	5.2 ± 1.0	Ref.
HDL cholesterol (mmol/l)	1.3 ± 0.3	Ref.
LDL cholesterol (mmol/l)	2.9 ± 0.9	Ref.
Triglycerides (mmol/l)	2.3 ± 1.3	Ref.
Microvascular disease	49	—
Macrovascular disease	39	—
Skin autofluorescence (total group) (AU)	2.79 ± 0.8	2.14 ± 0.6
40–49 years (*n* = 64; *n* = 44)*	2.17 ± 0.5†	1.84 ± 0.4
50–59 years (*n* = 199; *n* = 47)*	2.57 ± 0.7†	2.12 ± 0.3
60–69 years (*n* = 278; *n* = 32)*	2.69 ± 0.7	2.50 ± 0.6
70–79 years (*n* = 307; *n* = 37)*	3.04 ± 0.8†	2.76 ± 0.6
>80 years (*n* = 108; *n* = 7)*	3.17 ± 0.7	2.89 ± 0.5

Data are percent, means ± SD, or median (interquartile range). Reference (Ref.) values of the laboratory: A1C 4.0–6.0%, creatinine 70–110 μmol/l, creatinine clearance (Cockroft-formula) 80–120 ml/min, urinary albumin-to-creatinine ratio 0–2.5, total cholesterol 3.5–5.0 mmol/l, HDL cholesterol 0.9–1.7 mmol/l, LDL cholesterol 3.6–4.4 mmol/l, and triglycerides 0.6–2.2 mmol/l. *First *n* represents the number of type 2 diabetic patients, second *n* represents the number of control subjects per age category. †$P < 0.01$ (*t* test in horizontal direction, per age category).

200 nondiabetic control subjects was needed.

RESULTS — In the analysis of this study, 973 type 2 diabetic patients and 231 control subjects were included. The medical characteristics are depicted in Table 1. All data were distributed normally, except for diabetes duration and albumin-to-creatinine ratio. Mean age in the control group was ~14 years lower compared with the type 2 diabetes group, and there were more male subjects in the type 2 diabetes group (47 vs. 38% in the control group). In the type 2 diabetes group, median diabetes duration was 4.2 years (mean 6.3 years). Mean A1C was 7.0%, while 16% of the diabetic population was treated with insulin and 84% on noninsulin strategies (diet and/or oral agents). Microvascular complications were present in 49% of the patients (prevalence of retinopathy, 20%; microalbuminuria, 21%; and neuropathy, 28%). The prevalence of macrovascular disease was 39% (ischemic heart disease, 21%; cerebrovascular disease, 8%; and peripheral vascular disease, 22%). There were no patients with (near) end-stage renal disease.

Skin autofluorescence. Overall, mean skin autofluorescence was 33% higher (2.79 AU) in the type 2 diabetes group compared with the control group (2.14 AU). In both type 2 diabetic and control subjects, autofluorescence was higher at each increment in age category ($P < 0.01$) (Table 1). Autofluorescence was also higher in the type 2 diabetes group compared with the control group at each specific age category, which was significant ($P < 0.01$) in the age categories 40–49, 50–59, and 70–79 years, and nonsignificant ($P = 0.1$, $P = 0.3$) in the age categories 60–69 and >80 years. Sample sizes of the latter categories were very small in the control group. Multiple regression analysis showed that 25% (R^2) of the variance of skin autofluorescence could be predicted by age (β = 0.35, $P < 0.0001$), female sex (β = 0.17, $P < 0.0001$), current tobacco use (β = 0.14, $P < 0.0001$), diabetes duration (β = 0.10, $P < 0.01$), plasma creatinine (β = 0.15, $P < 0.0001$), A1C (β = 0.10, $P <$ 0.001), albumin-to-creatinine ratio (β = 0.10, $P < 0.001$), BMI (β = 0.08, $P < 0.01$), and HDL cholesterol (β = −0.07, $P < 0.05$). Blood pressure and other nonfasting lipids were not significant in this model.

In the nondiabetic control group, multiple regression analysis showed that 46% (R^2) of the variance of skin autofluorescence could be predicted by age (β = 0.71, $P < 0.001$) and current tobacco use (β = 0.17, $P = 0.001$). In this multivariate analysis, the univariate correlation of autofluorescence with BMI ($r = 0.2$, $P < 0.05$) and systolic blood pressure ($r = 0.3$, $P < 0.001$) disappeared.

Smoking was independently and positively related to autofluorescence in both groups. Smoking was more prevalent at younger age in both groups; in the type 2 diabetes group, the prevalence of smoking was 27% before age 65 years and 14% after age 65 years and in the control group, 37 and 13%, respectively. Figure 1*A* illustrates the effect of smoking on skin autofluorescence in control and type 2 diabetic subjects.

Female sex was independently and positively associated with skin autofluorescence in type 2 diabetes. No sex relation with autofluorescence was found in the control group. Further analysis in the type 2 diabetes group showed that autofluorescence was significantly higher in women under age 56 years compared with men (2.56 vs. 2.30 AU, respectively; $P < 0.01$) (Fig. 1*B*). There was no significant sex-related difference of autofluorescence in the higher age categories.

Figure 2 shows the mean skin autofluorescence in the type 2 diabetic population per complication category with the 95% CI. Mean skin autofluorescence was significantly higher in the group with both micro- and macrovascular disease compared with the group without complications and the group with only microvascular complications (mean 3.12 [95% CI 3.01–3.23] vs. 2.57 [2.50–2.65] and 2.71 AU [2.62–2.80]; $P < 0.001$). Autofluorescence in the group with only macrovascular complications (2.91 AU [2.78–3.03]) was higher than in the group without complications. These differences remained significant when autofluorescence was corrected for age.

The same result was found by categorizing the type 2 diabetes group with both micro- and macrovascular disease and the type 2 diabetes group without complications in age decades. In each age decade >60 years, skin autofluorescence was sig-
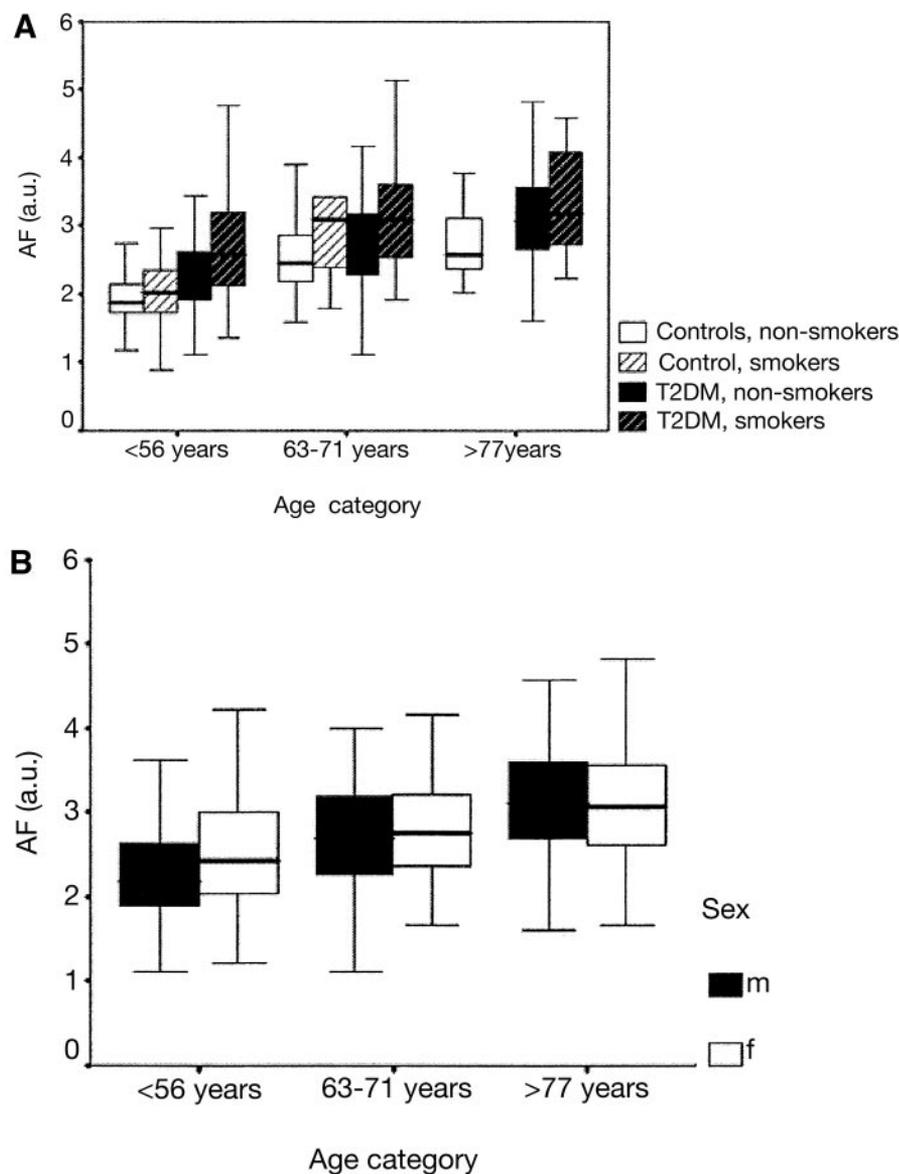
Figure 1—These boxplots show the distribution of skin autofluorescence (AF) per quintile of age in the type 2 diabetic (T2DM) population. Only the first, third, and fifth age quintile are shown. A: Effect of smoking in control compared with type 2 diabetic subjects. The boxplot of the smoking control subjects in the age category >77 years has not been depicted because of the group size was too small. B: Differences in autofluorescence due to sex at different age categories in the type 2 diabetic population. Skin autofluorescence is significantly higher in female patients aged <56 years (P < 0.01). The horizontal line within a box represents the median, and the lower and upper end of a box are the first and third quartile, respectively. The lower line and the upper line outside the box represent the 5th and 95th percentile, respectively.

nificantly higher in type 2 diabetic patients with complications versus type 2 diabetic patients without complications (40–49 years: 2.27 vs. 2.08 AU [P = NS]; 50–59 years: 2.77 vs. 2.55 AU [P = NS]; 60–69 years: 2.83 vs. 2.53 AU; 70–79 years: 3.24 vs. 2.86 AU; and >80 years: 3.47 vs. 3.01 AU [P < 0.05]).

CONCLUSIONS — This study confirms that in a large group of type 2 diabetic patients, skin autofluorescence is higher compared with age-matched control subjects. Moreover, skin autofluorescence was associated with a graded increase in the presence and severity of diabetes-related complications.

Known diabetes duration was relatively short, and, moreover, metabolic control was good (mean A1C 7.0%) compared with the previously reported smaller group with increased skin

autofluorescence (13). Thus, our type 2 diabetes group might be expected to represent a more or less "healthy" diabetic population relatively unaffected by chronic complications. However, about half of this group was already diagnosed with micro- or macrovascular complications.

A1C had a small independent contribution to autofluorescence in our study. Sharp et al. (21) found no correlation with A1C and serum low–molecular weight AGEs (LMW-AGEs). They suggest that glycemia itself may not be responsible for increased AGE accumulation in diabetes, but other factors are involved. However, it can also be hypothesized that the restricted relation between glycemia (A1C) and AGEs is caused by the short turnover time of A1C. A1C represents metabolic control of the last ~8 weeks, which implies a rather "short-term memory" of glycemic stress. AGEs can also link to hemoglobin (Hb-AGE). Hb-AGE was slightly superior to A1C as a measure for metabolic control, which was attributed to the irreversible nature of AGEs (22). However, the relatively short turnover time of hemoglobin remains the limiting factor for the use of Hb-AGE or A1C as a real long-term glycemic index. An earlier study that confirmed the importance of protein turnover in AGE accumulation estimated the half-life of skin collagen on 15 years (23). Thus, skin AGE levels might provide a more "long-term memory" of glycemic stress and, therefore, be better in predicting complications. The Diabetes Control and Complications Trial substudy already demonstrated in type 1 diabetes that skin collagen glycation was a better predictor for diabetes complications compared with A1C (7,24).

Sex was an independent variable of skin autofluorescence in the type 2 diabetes group, with a higher autofluorescence in women (aged <56 years). This suggests an estrogen-related effect. The loss of sex difference in autofluorescence at an older age may be due to a decrease in skin collagen content in postmenopausal women (25). Sex hormones influence the collagen turnover rate and is important in the rate of AGE accumulation. Another large study in type 2 diabetes found a similar independent positive relation between female sex and levels of LMW-AGEs (26).

BMI and HDL cholesterol also contributed significantly to autofluorescence in the regression model. Although the coefficients were low, these relations can be valid. It is possible that skin autofluorescence is affected by the skin content of
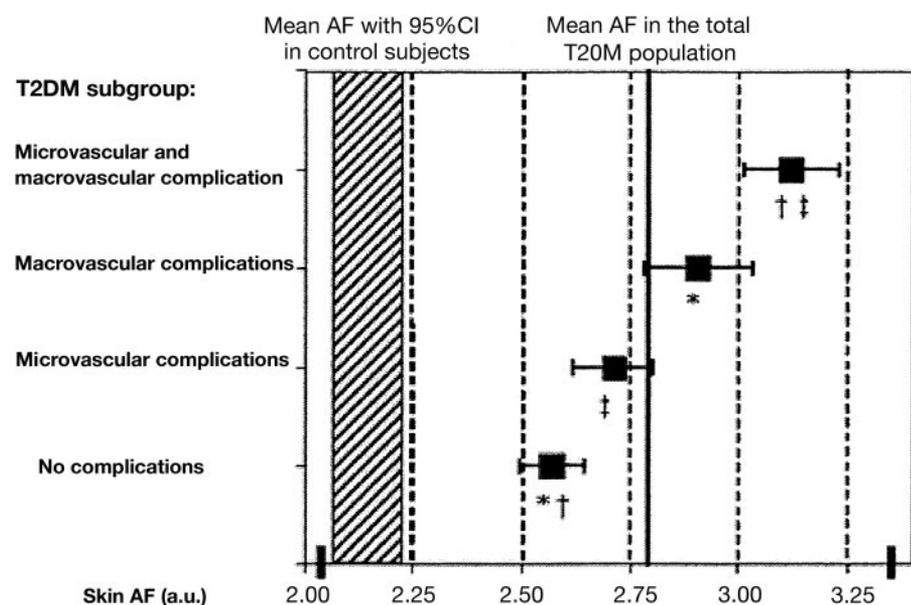
Figure 2—*Mean skin autofluorescence (AF) with 95% CIs in different categories of complications in the type 2 diabetic (T2DM) population. Matching symbols represent significant differences between the groups (P < 0.001). These differences remained significant after age correction of skin autofluorescence. The lined column represents the mean skin autofluorescence with 95% CIs of the control group.*

lipoxidation products, as some of these products are fluorescent (27). These advanced lipoxidation end products are formed analogous to AGEs from reactive carbonyl intermediates through oxidation of polyunsaturated fatty acids instead of sugars. If an increase in subcutaneous fat mass and a decrease in HDL cholesterol are associated with increased formation of advanced lipoxidation end products, this might explain the contribution to skin autofluorescence.

This study further supports the earlier finding that smoking results in increased AGE accumulation, as we showed that smoking independently increases autofluorescence in both type 2 diabetic and control subjects in the multivariate analysis (28).

Skin autofluorescence had an explained variance, with the tested clinical variables in the regression analysis of 46% in the control group and 25% in the type 2 diabetes group. It should be noted that the total variance (SD^2) was approximately two times higher in type 2 diabetes, which implies that the explained variances in the control group and the type 2 diabetes group are about the same $[SD^2(1 - R^2)]$. Sharp et al. (21), who measured fluorescent LMW-AGEs in plasma, also found an R^2 value of 25% and increased total variance in type 2 diabetic compared with control subjects.

One of the reasons for a higher total variance in the diabetic population might be that other "disturbing" disease-related factors exist. For example, interindividual differences in actual hyperglycemic induced oxidative stress during the autofluorescence measurement (2). It is also possible that genetic factors are involved in the individual susceptibility to glycation, as has been described previously (29).

There are some restrictions of the skin autofluorescence technique. First, nonfluorescent AGEs are not measured with our tool. In our previous study, however, skin autofluorescence was well correlated with levels of both fluorescent (pentosidine) and nonfluorescent AGEs (e.g., carboxymethyllisine) assessed in skin biopsies, which suggests a parallel trend formation of AGEs. It is unclear, so far, whether skin autofluorescence represents some intracellular-produced AGEs, which may be nonfluorescent.

Second, other fluorophores might be measured (e.g., NADH, which has an overlapping excitation spectrum [350–370 nm]) (16). Finally, the large majority of our study population was Caucasian. Some non-Caucasian type 2 diabetic patients had to be excluded because of their skin type. The tool is in further development to measure autofluorescence in dark skin. Skin autofluorescence has recently shown clinical relevance as a strong and independent predictor of total and car-

diovascular mortality in a study in patients with end-stage renal disease undergoing hemodialysis (30).

In this study, we showed that increased levels of skin autofluorescence was related to the extent of diabetes-related complications. In a 4-year follow-up study, the progression of micro- and macrovascular complications, as well as mortality, is now evaluated in the current study group of type 2 diabetic patients to analyze whether skin autofluorescence contributes to the prediction of the development or progression of diabetes complications. If this hypothesis is confirmed, skin autofluorescence, reflecting vascular damage, will become an easy tool for clinical practice to identify diabetic patients who are at increased risk for developing vascular complications.

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