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## Increased accumulation of skin advanced glycation end-products precedes and correlates with clinical manifestation of diabetic neuropathy

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**Abstract** *Aims/hypothesis:* The accumulation of AGE is related to the progression of the renal, retinal and vascular complications of diabetes. However, the relationship with diabetic neuropathy remains unclear. We recently showed that skin autofluorescence, measured non-invasively with an AutoFluorescence Reader (AFR), could be used to assess skin AGE accumulation. We evaluated the relationship between skin autofluorescence and the severity of diabetic neuropathy. *Materials and methods:* Skin autofluorescence in arbitrary units (AU) was assessed in 24 diabetic patients with a history of neuropathic foot ulceration (NP<sup>+</sup>), 23 diabetic patients without clinical neuropathy (NP<sup>-</sup>) and 21 control subjects, using the AFR. Arterial occlusive disease was excluded in all. The severity of foot ulceration was assessed by the Wagner score. Peripheral nerve function was assessed by neurography, measuring motor and sensory nerve conduction velocity and amplitude of the median, peroneal and sural nerves. Heart rate variability (HRV) and baroreflex sensitivity

(BRS) were measured by Finapres to assess autonomic nervous function. *Results:* Autofluorescence was increased in NP<sup>-</sup> compared with control subjects. In NP<sup>+</sup> patients, autofluorescence was further increased and correlated with the Wagner score. Autofluorescence correlated negatively with nerve conduction velocity and amplitude, HRV and BRS in both NP<sup>+</sup> and NP<sup>-</sup> groups. Autofluorescence correlated with age, diabetes duration, mean HbA<sub>1c</sub> of the previous year, serum creatinine level, presence of microalbuminuria and severity of diabetic retinopathy. *Conclusions/interpretation:* Skin autofluorescence correlates with the severity of peripheral and autonomic nerve abnormalities in diabetes, even before being clinically manifest. The AFR may be a convenient and rapid clinical tool for assessing risk of progression of long-term diabetic complications.

**Keywords** Advanced glycation end-product (AGE) · Autofluorescence · Autonomic · Nerve conduction · Neuropathy

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**Abbreviations** AFR: autofluorescence reader · AU: arbitrary units · BRS: baroreflex sensitivity · DNS: Dutch Diabetic Neuropathy Symptoms · DNE: Dutch Diabetic Neuropathy Examination · HRV: heart rate variability · NCV/A: nerve conduction velocity/amplitude · NP<sup>+</sup>: patients with a history of neuropathic foot ulceration · NP<sup>-</sup>: patients without clinical neuropathy · RAGE: receptor for advanced glycation end-products

### Introduction

Peripheral sensorimotor neuropathy and autonomic neuropathy are the most frequent manifestations of diabetic neuropathy and both are independent predictors of foot ulceration, lower extremity amputation and mortality [1–4]. Several risk factors for the development of diabetic neuropathy have been identified (e.g. glycaemic status, microalbuminuria), but recent evidence has drawn attention to the accumulation of AGE [5–8].

Glycation of proteins is a series of complex and sequential reactions, collectively called the Maillard reaction, that occur in all tissues and body fluids. Early-stage reactions lead to the formation of the early glycation adducts (e.g. fructosyl-lysine) and later-stage reactions form AGE. Fructosyl-lysine slowly degrades to form AGE. Glyoxal, methylglyoxal and 3-deoxyglucosone are also potent glycating agents formed by the degradation of glycolytic intermediates, glycated proteins and lipid peroxidation. They react with proteins to form AGE [9–12]. Accumulation of chemically stable AGE adducts on long-lived proteins reflects the cumulative effects of oxidative stress and hyperglycaemia, and both are strongly related to the development of diabetic neuropathy. In a substudy of the Diabetes Control and Complications Trial (DCCT), skin AGE levels explained a major part of the variance in diabetic complications, even after adjustment for HbA<sub>1c</sub> [13]. Prevention of AGE accumulation, in predominantly experimental or animal studies, reduced the development of a wide range of diabetic complications, including nerve dysfunction [14–17]. However, little clinical evidence is available on the relationship between AGE accumulation and diabetic neuropathy. Tissue AGE assays are invasive and expensive, and have not reached the clinic. Analysis of AGE in blood proteins, total serum or plasma does not necessarily reflect tissue AGE concentrations [17].

Recently, we described a non-invasive technique for measuring skin autofluorescence using an Autofluorescence Reader (AFR) [18]. Skin autofluorescence measured with the AFR was correlated strongly with collagen-linked fluorescence and tissue accumulation of the AGE pentosidine, carboxymethyl-lysine (CML) and carboxyethyl-lysine (CEL) in diabetic patients and healthy subjects [19]. If skin autofluorescence reflects tissue AGE accumulation, the AFR may be a convenient tool for assessment of the risk of AGE-related complications. The cross-sectional study described below was designed to test the hypothesis that skin autofluorescence, an indirect measure of skin AGE accumulation, correlates with the severity of both peripheral and autonomic neuropathy in diabetic patients.

## Subjects and methods

### Subjects

Twenty-four diabetic patients with a history of foot neuropathic ulceration, 23 diabetic patients without clinical neuropathy and 21 control subjects were studied. Exclusion criteria were factors that may interfere with nerve function, such as peripheral vascular disease (ankle–arm indices <0.90, toe–arm indices <0.70), electrocardiographic abnormalities, and use of  $\beta$ -blockers or calcium antagonists. Both type 1 (age at diagnosis of diabetes <30 years and insulin-dependent from onset) and type 2 diabetic patients were recruited randomly from our diabetes outpatient clinic. In control subjects, diabetes mellitus and renal failure were excluded by the respective conventional Amer-

ican Diabetes Association criteria and (for renal failure) a serum creatinine of <120  $\mu\text{mol/l}$ .

The presence or absence of clinical neuropathy in diabetic patients was defined using the Dutch Diabetic Neuropathy Symptoms (DNS) scale, the Dutch Diabetic Neuropathy Examination (DNE) scale, and quantitative sensory function testing with Semmes–Weinstein monofilaments. In the group of diabetic patients without neuropathy, DNS and DNE scores (see below) had to be 0 points and  $\leq 3$  points, respectively, with the 10-g Semmes–Weinstein monofilament felt on all occasions. In the patients with foot ulcers and definite neuropathy, the DNS and DNE scores were  $\geq 1$  point and  $\geq 4$  points, respectively, while the 10-g Semmes–Weinstein filament was not felt on all occasions. In diabetic patients, data collected from chart reviews included known duration of diabetes mellitus, mean HbA<sub>1c</sub> during the previous year, blood pressure and presence of microalbuminuria. Microalbuminuria was defined as a urinary albumin excretion rate between 20 and 200  $\mu\text{g/min}$  in at least two out of three overnight urine collections in the preceding year. Clinical characteristics of the subjects are given in Table 1.

Autofluorescence measurements, nerve conduction studies, heart rate variability and baroreflex sensitivity were measured in all subjects. Wagner scores were used to quantify the severity of ulcers in diabetic patients with foot ulcers. The participants were studied in the morning, 1.5 h after breakfast, during which the diabetic patients had taken their oral hypoglycaemic drugs or regular insulin injections. Subjects were instructed not to smoke or consume caffeine drinks 24 h before the study. The local ethics committee approved this study and informed consent was obtained prior to the measurements in all subjects.

### Skin autofluorescence

Skin autofluorescence was assessed by the AFR (patent PCT/NL99/00607; prototype of current AGE Reader I, Diagnostics BV, Groningen, The Netherlands). In short, the AFR illuminates a skin surface of 1  $\text{cm}^2$ , guarded against surrounding light, with an excitation light source between 300 and 420 nm (peak excitation  $\sim 350$  nm). Only light from the skin is measured with the spectrometer (Ocean Optics PC-1000 fiber optic spectrometer; Ocean Optics, Dunedin, FL, USA) in the 300–600 nm range, using 200- $\mu\text{m}$  glass fibre (Farnell, Leeds UK). The measure of autofluorescence was defined as the average light intensity per nm in the range between 420 and 600 nm, divided by the average light intensity per nanometer in the range between 300 and 420 nm (autofluorescence).

Skin reflection measurements in the 300–420-nm range were analysed by comparing them with those of a white Teflon block (assuming 100% reflectance), i.e. the mean intensity reflected from the skin divided by the mean intensity reflected from the white Teflon block in the range 300–420 nm. This correction corrects for effects of skin pigmentation and redness, which may influence autofluorescence by light absorption [20, 21].

**Table 1** Characteristics of the study populations

	Control ( <i>n</i> =21)	Diabetes NP <sup>-</sup> ( <i>n</i> =23)	Diabetes NP <sup>+</sup> ( <i>n</i> =24)
Age (year)	58±10	53±13	57±12
Male : female	10:11	13:10	13:10
Type 1 : type 2 diabetes	–	8:15	5:18
Diabetes duration (year)	–	13±10	17±12
Mean HbA <sub>1c</sub> (%)	5.9±0.72	7.6±0.85	8.2±1.09
Body mass index (kg/m <sup>2</sup> )	27.2±5.1	28.4±5	30±5.1
Blood pressure	138±17/87±8	135±13/82±9	146±20/80±11
Creatinine	88±10	89±12	98±26
Microalbuminuria ( <i>n</i> )	0	3	13*
Coronary heart disease ( <i>n</i> )	0	2	4
Diabetic retinopathy ( <i>n</i> )	–	18:5:0	2:15:6 <sup>a</sup> *
Ankle–arm index	1.20±0.10	1.23±0.12	1.17±0.2
Cholesterol	6.25±1.54	5.04±0.98	4.77±0.87
Triglycerides	1.42±0.9	1.86±1.04	2.13±1.46
Smoking ( <i>n</i> )	6	7	10
ACE inhibitor ( <i>n</i> )	–	7	13*
Diuretics ( <i>n</i> )	–	3	10*
Insulin ( <i>n</i> )	–	19	18
Antioxidants ( <i>n</i> )	–	5	6

NP<sup>-</sup> Diabetic patients without clinical neuropathy, NP<sup>+</sup> diabetic patients with foot neuropathy  
 \**p*<0.05 between NP<sup>+</sup> and NP<sup>-</sup>  
<sup>a</sup>No retinopathy : background retinopathy : proliferative retinopathy

All measurements were performed at room temperature in a dark environment. After control measurements with the lamp off (dark), autofluorescence of the skin was measured six times (every 10 s) at the volar side of the arm approximately 10 cm below the elbow fold. Care was taken to perform the measurement at a normal skin site, thus without visible vessels, scars, lichenification or other skin abnormalities.

AFR measurements on 1 day and intra-individual seasonal variance showed an Altman error percentage of <6%. The differences between repeated measurements did not alter depending on autofluorescence level. The autofluorescence ratio is calculated offline by automated analysis and is observer independent.

### Diabetic neuropathy

The DNS and DNE scales have been described in detail previously [22]. The DNS scale is a 12-item validated-symptom scale modified from earlier neuropathy scores [23], with a high predictive value to screen for distal polyneuropathy and autonomic neuropathy in diabetes. Symptoms of unsteadiness in walking, neuropathic pain, paraesthesia, dizziness and numbness are elicited, and the presence of a symptom is scored as one point.

The DNE scale is a sensitive and validated hierarchical scoring system for diabetic neuropathy. The score includes measures of muscle strength, reflexes and sensation, with a total of eight items, scoring normal, moderate deficit or severely disturbed per item.

Semmes–Weinstein 10-g monofilaments and Vibration Perception Threshold (VPT) using a handheld biothesiometer (Biomedical Instruments, OH, USA) were used as quantitative sensory tests [24, 25]. Deficits in sensing were

measured using a 10-g 5.07 Semmes–Weinstein monofilament applied to eight areas on the dorsal foot skin; deficits in one or more skin areas was defined as impaired sensing. If at VPT the voltage needed for vibration had to be increased to such a level that the mean of three readings at the first toe was above 25 V, the response was defined as certainly defective, and if the mean was between 16 and 24 V it was denoted as intermediately impaired vibration perception.

Nerve conduction studies were performed with standard surface stimulation and recording techniques using an electromyograph (Nicolet Viking IIe and IV) with standard filter settings. All measurements were performed after warming of forearm and lower leg in hot (38°C) water for at least 15 min. Motor nerve conduction velocity was measured on the left forearm segments of the median nerve and the left peroneal nerve. Sensory nerve conduction velocity was measured antidromically with ring electrodes placed around digit 3 (median nerve) and stimulation at the wrist. The sural nerve was tested antidromically after lateral stimulation of the Achilles tendon (sural nerve), 10–12 cm proximal from the active electrode. Peak–peak amplitude values were used.

Heart rate variability was measured in accordance with the guidelines of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology [26]. ECG signals were sampled at 100 Hz and stored, and analysed offline by using the CARSPAN program as described previously [27]. After artefact correction and stationary check, discrete Fourier transformation of the RR interval (ms) was performed. To assess baroreflex sensitivity (BRS), blood pressure and heart rate were measured beat to beat by Finapres. BRS was determined by the transfer function method using the CARSPAN program, as previously described in detail [28].

**Table 2** Diabetic neuropathy parameters in our study populations

	Control ( <i>n</i> =21)	Diabetes NP <sup>-</sup> ( <i>n</i> =23)	Diabetes NP <sup>+</sup> ( <i>n</i> =24)
SNCV median nerve (m/s)	42.6±6.7	39.5±6.0	32.9±6.5*
SNCA median nerve (mV)	34 (23–45)	35 (17–53)	12 (3–9)*
SNCV sural nerve (m/s)	46.4±3.9	44.4±5.2	36.6±5.0*
SNCA sural nerve (mV)	9.7±4.4	9.1±4.4	1.9 (1.3–2.6)*
MNCV median nerve (m/s)	57.7±4.3	54.5±2.9	47.1±5.6*
MNCA median nerve (mV)	9.8±3.0	10.1±4.9	7.7±2.5
MNCV peroneal nerve (m/s)	47.3±5.0	44.2±4.6	31.6±4.5*
MNCA peroneal nerve (mV)	9.1 (4–14)	10.4 (5–15)	1.9 (0.9–3.1)*
ln HRV (ms)	9.3±1.0	8.8±1.0	7.4±1.1*
ln BRS (ms/mmHg)	1.9±0.7	1.8±0.4	1.3±0.6*
Wagner score	–	–	5:8:7:4

Data shown as means±SD, or median (25–75% CI)

Wagner score=1:2:3:4

NP<sup>-</sup> Diabetic patients without clinical neuropathy, NP<sup>+</sup> diabetic patients with foot neuropathy, SNCV sensory nerve conduction velocity, MNCV motor nerve conduction velocity, SNCA sensory nerve conduction amplitude, MNCA motor nerve conduction amplitude

\**p*<0.05 between NP<sup>+</sup> and NP<sup>-</sup>

### Statistical analyses

Differences in autofluorescence between the three studied groups were analysed using ANOVA with post hoc Bonferroni correction. For HRV and BRS, a logarithmic transformation was used to obtain normal distribution for further analysis. In the case of normal distribution of variables, Pearson's correlation was assessed, in the case of non-normal distribution or of categorical values Spearman's correlation was assessed. Multivariable stepwise regression analyses were performed for determination of independent effects. A two-tailed *p* value <0.05 was considered significant. Data are shown as means±SD.

## Results

### Skin autofluorescence in general

Autofluorescence was increased in diabetic patients without clinical neuropathy compared with control subjects (respectively, 0.017±0.004 arbitrary units [AU] vs 0.011±0.001 AU, *p*<0.01) and further increased in diabetic patients with neuropathic foot ulcers (0.025±0.007 AU, *p*<0.001). In diabetic patients, autofluorescence correlated

with age (*r*=0.46, *p*<0.01), diabetes duration (*r*=0.47, *p*<0.01), mean HbA<sub>1c</sub> of the previous year (*r*=0.61, *p*<0.01), serum creatinine level (*r*=0.50, *p*<0.01), the severity of diabetic retinopathy (*r*=0.42, *p*<0.01) and urinary albumin excretion (*r*=0.37, *p*<0.01). No correlations were observed between autofluorescence and specific medical treatment, insulin dosage, body mass index, sex or smoking habits. Approximately 60% of the variance in autofluorescence could be explained by the independent effects of age (*p*=0.03), mean HbA<sub>1c</sub> of the previous year (*p*=0.01) and the presence of microalbuminuria (*p*=0.001) in diabetic patients (overall *r*=0.77, *p*<0.001). In control subjects, autofluorescence correlated with age (*r*=0.53, *p*=0.01) and smoking habits (*p*<0.01).

### Skin autofluorescence and neuropathy

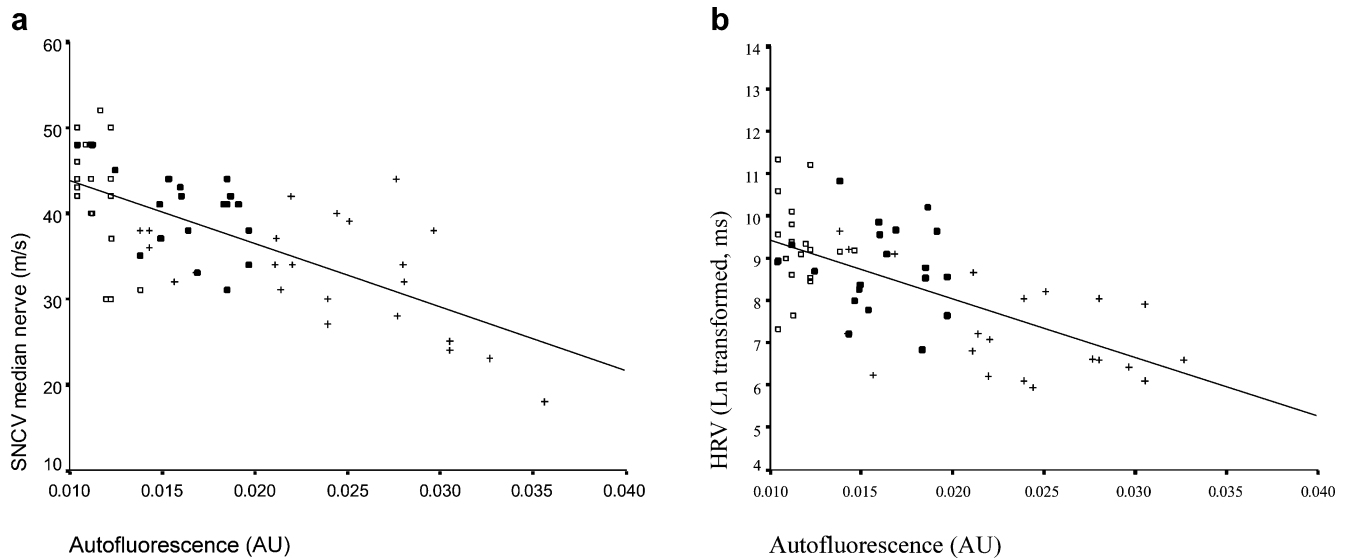
Table 2 describes the analysis of diabetic neuropathy parameters in our study populations. Autofluorescence correlated with the DNS (*r*=0.34, *p*=0.02) and DNE scale (*r*=0.50, *p*=0.001). Table 3 shows the correlations for autofluorescence with nerve conduction velocity and amplitude of the median, peroneal and sural nerve in both diabetic groups and control subjects. Autofluorescence correlated

**Table 3** The correlations between skin autofluorescence and peripheral nerve function

	Skin autofluorescence		
	Control ( <i>n</i> =21)	Diabetes NP <sup>-</sup> ( <i>n</i> =23)	Diabetes NP <sup>+</sup> ( <i>n</i> =24)
SNCV median nerve (m/s)	<i>r</i> =−0.52 ( <i>p</i> =0.02)	<i>r</i> =−0.53 ( <i>p</i> =0.02)	<i>r</i> =−0.52 ( <i>p</i> =0.01)
SNCA median nerve (mV)	<i>r</i> =−0.21 ( <i>p</i> =0.3)	<i>r</i> =−0.69 ( <i>p</i> <0.01)	<i>r</i> =−0.43 ( <i>p</i> =0.03)
SNCV sural nerve (m/s)	<i>r</i> =−0.53 ( <i>p</i> =0.02)	<i>r</i> =−0.57 ( <i>p</i> =0.01)	<i>r</i> =−0.62 ( <i>p</i> =0.03)*
SNCA sural nerve (mV)	<i>r</i> =−0.18 ( <i>p</i> =0.3)	<i>r</i> =−0.82 ( <i>p</i> <0.01)	<i>r</i> =−0.23 ( <i>p</i> =0.5)*
MNCV median nerve (m/s)	<i>r</i> =−0.33 ( <i>p</i> =0.1)	<i>r</i> =−0.50 ( <i>p</i> =0.03)	<i>r</i> =−0.56 ( <i>p</i> =0.01)
MNCA median nerve (mV)	<i>r</i> =−0.25 ( <i>p</i> =0.1)	<i>r</i> =−0.60 ( <i>p</i> =0.01)	<i>r</i> =−0.44 ( <i>p</i> =0.03)
MNCV peroneal nerve (m/s)	<i>r</i> =−0.46 ( <i>p</i> =0.03)	<i>r</i> =−0.54 ( <i>p</i> =0.01)	<i>r</i> =−0.48 ( <i>p</i> =0.09)*
MNCA peroneal nerve (mV)	<i>r</i> =−0.56 ( <i>p</i> =0.02)	<i>r</i> =−0.75 ( <i>p</i> =0.01)	<i>r</i> =−0.36 ( <i>p</i> =0.1)*

SNCV sensory nerve conduction velocity, MNCV motor nerve conduction velocity, SNCA sensory nerve conduction amplitude, MNCA motor nerve conduction amplitude

\**n*=14 (in nine patients, the diabetic neuropathy resulted in absent nerve conduction)



**Fig. 1** The relationship between skin autofluorescence and sensory nerve conduction velocity (SNCV) of the median nerve and heart rate variability total power (HRV). *Open squares*, control subjects;

*filled squares*, diabetic patients without clinical neuropathy; *+ sign*, diabetic patients with foot neuropathy

negatively with both sensory and motor nerve conduction velocities and amplitude in both diabetic groups. In control subjects, autofluorescence showed a negative trend with nerve conduction velocity. Figure 1 shows the relationship between autofluorescence and sensory nerve conduction velocity of the median nerve.

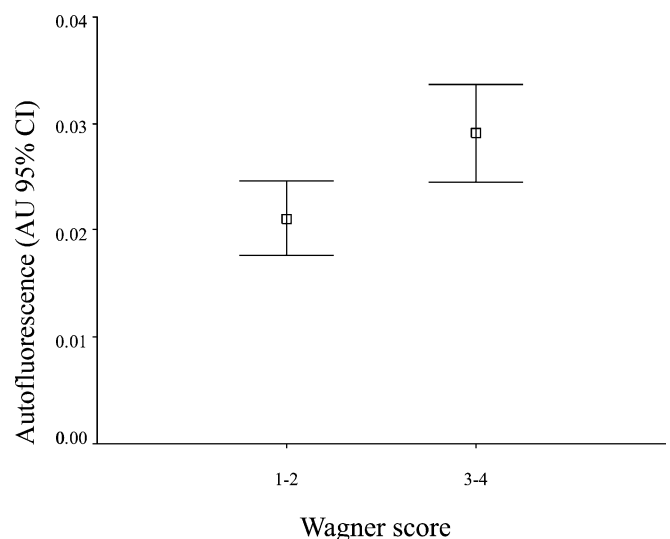
Nerve conduction velocity and amplitude further correlated with age ( $r=-0.4$ ,  $p=0.03$ ), mean HbA<sub>1c</sub> of the previous year ( $r=-0.5$ ,  $p<0.01$ ), diabetes duration ( $r=-0.36$ ,  $p=0.02$ ), serum creatinine level ( $r=-0.4$ ,  $p=0.04$ ) and presence of microalbuminuria ( $p=0.02$ ). Stepwise multivariate analysis showed that autofluorescence independently explained 55% of the variance in nerve conduction velocity and amplitude ( $r=0.74$ ,  $p<0.01$ ).

Autofluorescence correlated negatively with heart rate variability (HRV;  $r=-0.61$ ,  $p<0.01$ ; Fig. 2) and with baroreflex sensitivity (BRS;  $r=-0.63$ ,  $p<0.01$ ) in the group of diabetic patients, but not in control subjects. Both HRV and BRS also correlated with age ( $r=-0.57$ ,  $p<0.01$ ), mean HbA<sub>1c</sub> ( $r=-0.41$ ,  $p<0.01$ ), diabetes duration ( $r=-0.33$ ,  $p=0.03$ ), serum creatinine level ( $r=-0.39$ ,  $p<0.01$ ), systolic blood pressure ( $r=-0.44$ ,  $p<0.01$ ) and the presence of microalbuminuria ( $p<0.01$ ). Approximately 50% of the variance in heart rate variability and baroreflex sensitivity could be explained by the independent effects of autofluorescence and the presence of microalbuminuria in diabetic patients (overall  $r=0.68$ ,  $p<0.01$ ). There was no, or only weak, correlation ( $r<0.2$ ) of BRS and HRV with nerve conduction velocity or amplitude.

In diabetic patients with neuropathic foot ulcers, the Wagner score correlated with autofluorescence ( $r=0.64$ ,  $p<0.01$ ), mean HbA<sub>1c</sub> of the previous year ( $r=0.45$ ,  $p=0.03$ ), systolic blood pressure although not significantly ( $r=0.42$ ,  $p=0.06$ ), presence of microalbuminuria ( $p=0.01$ ), BRS ( $r=-0.46$ ,  $p=0.04$ ), sensory nerve conduction velocity and amplitude ( $r=-0.61$ ,  $p=0.01$ ) and serum creatinine

level ( $r=0.50$ ,  $p=0.01$ ). Autofluorescence was related independently to the Wagner score, and Fig. 2 shows autofluorescence for diabetic patients with higher vs lower Wagner scores. The stepwise multivariate analysis showed that autofluorescence explained independently approximately 50% of the variance in the Wagner score ( $r=0.69$ ,  $p<0.01$ ).

Over half of the diabetic patients with neuropathy had microalbuminuria. The multivariate analysis showed that the presence of microalbuminuria was related strongly to nerve dysfunction. Importantly, the above-mentioned correlations were, to a lesser extent, also observed in diabetic patients without microalbuminuria.



**Fig. 2** Skin autofluorescence in diabetic patients with Wagner score of 3 or 4 ( $n=11$ ) vs Wagner score of 1 or 2 ( $n=13$ ).  $p=0.02$



## Discussion

Our study shows that skin autofluorescence is increased in diabetic patients in relation to the severity of diabetic neuropathy and foot ulceration. But even before clinical symptoms of diabetic neuropathy exist, skin autofluorescence is closely related to nerve conduction and to markers of autonomic nerve function. As skin autofluorescence reflects AGE accumulation [19], our study supports the idea that AGE accumulation is an important risk factor for neuropathy in diabetic patients. Skin autofluorescence reflects accumulation independently of other well-known risk factors for neuropathy such as age, hyperglycaemia and microalbuminuria.

The power of skin autofluorescence as the best indicator for risk factors related to diabetic neuropathy was illustrated in the multivariate analysis in comparison with risk factors like hyperglycaemia. As we have previously shown that increased skin autofluorescence reflects increased AGE accumulation, it may represent the cumulative effect of hyperglycaemia, oxidative stress and renal dysfunction. Furthermore, AGE accumulation may be increased due to exogenous sources, such as smoking [29, 30]—as shown in our control subjects—and nutrition [31]. Importantly, tissue AGE accumulation may represent the long-term effects of a final common pathway for various risk factors. Thus, based on the present work, skin autofluorescence may integrate these factors and be more informative than the actual levels of these risk factors in themselves.

AGE accumulation has been related to the development of diabetic neuropathy [32]. The peripheral nervous system and, to a lesser extent, the central nervous system have been shown to be vulnerable to AGE accumulation in diabetes [16, 33]. Both intensive metabolic control of diabetic patients [13] and treatment with aminoguanidine (AGE inhibitor) in diabetic rats decrease the accumulation of AGE and the progression of diabetic neuropathy; they also reduce abnormal nerve conduction [16, 34]. Interestingly, we found increased skin autofluorescence in parallel with reduced nerve conduction velocity and amplitude in diabetic patients without clinical manifestations of diabetic neuropathy (Fig. 2, Table 3). Nerve conduction abnormalities of both peripheral and central nerves have been shown in diabetic patients without clinical neuropathy [35], but not as of yet in relation to AGE accumulation. Even in our control subjects, skin autofluorescence was related to nerve conduction velocity (Fig. 2, Table 3).

The severity of diabetic foot ulceration was also related to skin autofluorescence in our study. Although the presence of peripheral macrovascular disease was an exclusion criterion in our study, a complex inter-relationship between neuropathy and microvascular disease exists in diabetic patients. Microvascular disease may worsen ulceration, and endothelial dysfunction has been demonstrated in relation to both diabetic neuropathy and foot ulceration [36, 37]. AGE accumulation has also been reported to worsen endothelial function, and endothelial RAGE has been proposed as the major key in such an interaction. Blockade

of RAGE accelerates wound closure in diabetic mice and suppresses levels of cytokines such as tumour necrosis factor [38]. AGE activation of inflammatory cells and a chronic inflammatory state can be established by the ability of cytokines to secrete proteases in excess of their inhibitors [39]. Therefore AGE accumulation may play a role in diabetic foot ulceration by its effects on neuropathy, on vascular disease and on delayed wound repair.

Our observation that skin autofluorescence increases in concert with autonomic dysfunction in diabetic patients has not been reported before. Different pathophysiological mechanisms in the development of autonomic and peripheral neuropathies have been suggested, as both types of neuropathy do not develop in concert in type 2 diabetic patients [40]. Importantly, HRV parameters in part normalise after intensive glycaemic control. We have also shown previously that the relationship between glycaemic status and autonomic function extends to the non-diabetic range, and suggested that, besides the glucose levels, oxidative stress might be involved in this correlation [41–44].

One limitation of the AFR is that not all AGE exhibit fluorescent properties. ELISA-based studies have shown increased accumulation of specific AGE before increased autofluorescence in diabetes [45]. Fluorescence represents group reactivity, which fails to provide quantitative information on concentrations of individual compounds. Importantly, the current understanding of physiological AGE indicates that the major AGE are not fluorescent cumulatively. However, our previous results in diabetic patients showed that skin autofluorescence may function as a marker of the AGE pool, based on the strong correlations with both fluorescent and non-fluorescent skin AGE levels [19]. Another limitation in our study is that we do not yet know the degree to which the relationship of autofluorescence with AGE accumulation is affected by other skin fluorophores, such as the oxidation degradation product of tryptophan, *N*-formylkynurenine, or elastin-linked fluorescence. In particular, advanced lipoxidation end-products (ALE) may also contribute to tissue and skin autofluorescence. The quantitative relationship between AGE and ALE in diabetes is still unknown, but dyslipidaemia may be as important as hyperglycaemia in chemical modification of proteins in diabetes [46]. Apparent changes in skin fluorescence as a consequence of light absorption by haemoglobin or NADH have been described [20]. Despite these theoretical limitations in AFR assays, skin biopsy fluorescence itself is strongly related to long-term diabetic complications [13].

Sunlight exposure as a source of skin photo-ageing might be considered as a confounding factor in skin AGE accumulation, but previous results with the AFR suggest that this effect is limited [19]. The present study was performed in Caucasian patients and controls, and, for the moment, our conclusions on the value of skin autofluorescence in diabetic neuropathy should be limited to non-pigmented skin.

The relevance of our non-invasive autofluorescence measurements in predicting progression of diabetic complications and mortality is now being investigated in a

larger prospective study. Our results in diabetic patients with subclinical neuropathy show that the AGE accumulation as assessed by the AFR is associated with accepted early, preclinical markers of long-term complications, such as delayed nerve conduction velocity, and HRV and BRS abnormalities.

In conclusion, our results show that skin autofluorescence is increased during early stages of diabetic neuropathy and correlates with the severity of nerve dysfunction and foot ulceration. Assuming that autofluorescence reflects AGE accumulation, our study supports the important clinical impact of AGE accumulation in the pathogenesis of diabetic neuropathy. Long-term prospective studies must reveal the predictive value of AGE accumulation, or skin autofluorescence, in the progression of neuropathy and other diabetic complications.

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#### Duality of Interest:

R. Graaff and A. J. Smit are founders of Diagnostics, The Netherlands, who manufacture the autofluorescence readers (<http://www.diagnostics.com>).

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