



Skin autofluorescence is inversely related to HDL anti-oxidative capacity in type 2 diabetes mellitus

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ABSTRACT

Objective: High density lipoprotein (HDL) particles protect apolipoprotein B-containing lipoproteins from oxidative modification. An impaired anti-oxidative functionality of HDL in type 2 diabetes mellitus (T2DM) may contribute to enhanced formation of oxidative stress products, such as Advanced Glycation Endproducts (AGEs). We tested whether in T2DM the HDL anti-oxidative capacity is related to the accumulation of AGEs in the skin.

Methods: Skin autofluorescence (AF), a non-invasive read-out for AGEs, and HDL anti-oxidative capacity, i.e. the ability of HDL to protect against LDL oxidation *in vitro*, were assessed in 67 non-smoking T2DM patients without complications (median age: 60 (53–65), 60% males, 6.5 (5.2–8.5) years of diabetes duration).

Results: In univariate analysis, skin AF correlated inversely with HDL anti-oxidative capacity ($r = -0.305$, $P < 0.02$), but not with HDL cholesterol or apolipoprotein A-I. HDL anti-oxidative capacity correlated inversely with glucose, HbA_{1c}, triglycerides, and insulin resistance (homeostasis model assessment) ($P < 0.05$ to $P \leq 0.001$). Multiple linear regression showed that skin AF remained inversely related to HDL anti-oxidative capacity (partial $r = -0.314$, $P = 0.015$) taking account of age, plasma glucose, non-HDL cholesterol, triglycerides, HOMA_{ir}, and CRP.

Conclusion: These findings suggest that skin AF is inversely related to the HDL anti-oxidative capacity rather than to the HDL cholesterol concentration in T2DM. Impaired anti-oxidative functionality of HDL could contribute to tissue accumulation of AGEs.

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1. Introduction

Type 2 diabetes mellitus (T2DM) is associated with an increased cardiovascular morbidity and mortality [1]. A commonly proposed pathogenetic mechanism is increased oxidative stress *in vivo* [2]. Consequently, oxidation of lipoproteins is enhanced in the vascular wall, promoting inflammation and fatty streak formation, which ultimately leads to clinically manifest atherosclerotic cardiovascular disease [3].

T2DM is hallmarked by high triglycerides and low high density lipoprotein (HDL) cholesterol as well as by oxidative modification of low density lipoprotein (LDL) particles [4]. HDL has the

capacity to inhibit oxidative modification of apolipoprotein (apo) B-containing lipoproteins [5]. Importantly, in early T2DM this capacity may be decreased, thereby contributing to an increased formation of oxidized LDL [6]. An increased oxidative stress burden also accelerates the generation of advanced glycation endproducts (AGEs), i.e. irreversibly modified proteins, lipids, and nucleic acids that are formed by non-enzymatic glycation and oxidation [7]. AGEs are commonly believed to contribute to the pathogenesis of atherosclerosis [8]. Using their characteristic autofluorescence pattern [9], tissue accumulation of AGEs can be non-invasively assessed by measuring skin autofluorescence (AF) [10]. Of note, previous reports documented that increased skin AF in T2DM is related to microvascular and macrovascular complications and represents an independent predictor of incident cardiovascular disease (CVD) [11,12]. Collectively, these findings [6,11,12] raise the possibility that enhanced accumulation of tissue AGEs, as assessed by skin AF, may be associated with decreased HDL functionality.

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The present study was initiated to examine the extent to which the anti-oxidative capacity of HDL is associated with skin AF in T2DM patients without clinically manifest CVD, nephropathy, and (pre)proliferative retinopathy.

2. Materials and methods

The protocol was approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands. T2DM patients (aged >18 years) were recruited by advertisement in local newspapers and provided written informed consent. T2DM had been previously diagnosed by primary care physicians using glucose cut-off values as defined by the World Health Organization (i.e. fasting plasma glucose >7.0 mmol/L, non-fasting glucose >11.1 mmol/L, measured on two independent days). All T2DM patients who responded to the advertisement were considered for participation. Clinically manifest cardiac and pulmonary abnormalities, renal insufficiency (defined as a serum creatinine >100 µg/L), overt proteinuria, microalbuminuria (defined as a urine albumin/creatinine ratio of 2.5–25 mg/mmol for men and 3.5–25 mg/mmol for women), (pre)proliferative retinopathy, thyroid disorders (abnormal thyreotropin level), liver diseases (elevated serum transaminases), pregnancy, primary dyslipidemias, a history of cancer, inflammatory or infectious disease, a dark skin type, current smoking and heavy alcohol consumption (>3 drinks per day) were exclusion criteria. T2DM patients using insulin, thiazolidinediones or lipid lowering drugs were also excluded. These exclusion criteria were applied to avoid possible confounding effects of co-existent clinical conditions, as well as of smoking, heavy alcohol consumption and lipid lowering drugs on HDL function and lipid levels. The use of metformin, sulfonylureas, and antihypertensive drugs was allowed.

Systolic and diastolic blood pressure was measured after 15 min rest with a sphygmomanometer in sitting position. BMI was calculated as weight divided by height squared (in kg/m²). Insulin sensitivity was assessed as homeostasis model assessment (HOMA_{ir}), using the equation: fasting plasma insulin (mU/L) × glucose (mmol/L)/22.5 [13]. A strong correlation of HOMA_{ir} with glucose clamp determined insulin-mediated whole body glucose disposal has been demonstrated in T2DM patients treated with diet and/or oral glucose lowering drugs [14]. All participants were studied after an overnight fast.

2.1. Skin autofluorescence

Skin AF was determined using the Excitation–Emission Matrix Scanner (EEMS), an adapted set-up of the AGE Reader that was used in earlier publications from our laboratory [15]. The EEMS set-up assesses skin AF similar to the AGE Reader, but has the additional potential to discriminate between AF spectra obtained at various excitation wavelengths. AF values obtained with the EEMS are slightly lower than AF values measured with the AGE Reader, for which reference values have been published [16]. This can be explained by some differences between both set-ups. With the EEMS set-up using in the current study, median skin AF amounted to 1.56 (range 0.65–2.58) AU, measured in 68 non-diabetic Caucasian subjects, aged 34–75 years. The EEMS technique and set-up have been described in detail elsewhere [17]. Briefly, approximately 4 cm² of the skin of the ventral site of the lower arm is illuminated by a computer driven system with a 75 W Xenon lamp and a 0.2 m f/4 monochromator (PTI, NJ, USA). A series of measurements is thereby obtained for each subject, varying the peak excitation wavelength from 355 to 405 nm with 5 nm interval

steps. To derive the mean skin AF from the excitation–emission matrices, the AF values for each selected peak excitation wavelength were calculated by dividing the mean emitted intensity per nm in the range between 420 and 600 nm by the mean reflected excitation intensity per nm between 300 and 420 nm for a given excitation wavelength and expressed in arbitrary units (AU). Thereafter, mean skin AF was determined by calculating for each excitation step the contribution in excitation light intensity corresponding to that of a conventional AGE Reader lamp intensity spectrum with a maximum wavelength at 370 nm. The AF value was calculated off-line by automated analysis and is observer-independent. As skin colour can also influence the AF measurement, the skin AF value was corrected for reflection of the skin when reflection was below 12%. Repeated measurements in controls and diabetic patients showed an intra-individual coefficient of variation of 5.0% on a single day and 5.9% for seasonal changes.

2.2. Laboratory analyses

Venous blood samples were collected into EDTA-containing tubes (1.5 mg/mL). Plasma was prepared by centrifugation at 1400 × g for 15 min at 4 °C. Glucose and HbA1c were measured shortly after blood collection. Samples for other assays were kept frozen at –80 °C until analysis.

Plasma cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos. 11876023 and 11875540 respectively, Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. ApoA-I and apoB were quantified by immunoturbidimetry (Roche/Cobas Integra Tinaquant cat nos. 03032566 and 033032574, respectively, Roche Diagnostics).

To determine the anti-oxidative functionality of HDL, first apoB-containing lipoproteins were precipitated by mixing 75 µl polyethylene glycol (PEG) 6000 in 10 mM HEPES (pH 8.0) with 150 µl of plasma followed by a 30 min incubation on ice. Samples were centrifuged at 2000 × g at 4 °C for 30 min and HDL cholesterol concentrations were measured in the supernatants with a commercially available kit (Roche Diagnostics, Mannheim, Germany). The HDL-mediated protection against oxidation of native LDL was assayed following a recently described published method [18]. Briefly, LDL was isolated from a young healthy normolipidemic donor by density gradient ultracentrifugation (1.019 < *d* < 1.063) as described [19]. Then individual HDL samples (0.26 mmol/L cholesterol) were mixed with aliquots of native LDL (1.95 mmol/L cholesterol) followed by the addition of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH, Cayman Chemicals, Ann Arbor, MI, USA; final concentration: 1 mM in a total volume of 110 µL) to induce oxidation. After an incubation for 24 h at 37 °C, TBARS were measured to determine the achieved degree of LDL oxidation exactly as detailed previously [19]. The HDL anti-oxidative capacity was determined as the percent reduction achieved of the respective individual HDL preparations in comparison to the maximum amount of TBARS formation in a reaction without HDL being present. Thereby, higher values indicate more efficient protection against oxidation. In 18 non-diabetic control subjects, aged 28–58 years, the HDL anti-oxidative capacity amounted to 68 (range 57–82)% inhibition. The inter-assay CV was 5.1%.

Insulin was measured with a microparticle enzyme immunoassay (AxSYM insulin assay; Abbott Laboratories, Abbott Park IL, USA). High-sensitive-CRP (CRP) was determined by nephelometry with a lower limit of 0.175 mg/L (BNII N; Dade Behring, Marburg,

Table 1

Clinical characteristics, skin autofluorescence (skin AF), HDL anti-oxidative ability, plasma glucose, HbA_{1c}, insulin, HOMA_{ir}, CRP, lipids and lipoproteins in 67 type 2 diabetic subjects.

	Type 2 diabetic patients (n = 67)
Age (years)	60 (53–65)
Sex (M/F)	40/27
Skin AF (AU)	1.71 (1.43–2.10)
HDL anti-oxidative capacity (%)	66 (59–73)
Systolic blood pressure (mm Hg)	142 (132–158)
Diastolic blood pressure (mm Hg)	86 (82–92)
BMI (kg/m ²)	28.7 (25.7–32.6)
Glucose (mmol/L)	8.6 (7.4–10.2)
HbA _{1c} (%)	6.6 (6.2–7.4)
Insulin (mU/L)	10.9 (7.3–17.0)
HOMA _{ir} (mU × mmol/(L ² × 22.5))	4.01 (2.74–6.96)
CRP (mg/L)	1.74 (1.18–4.15)
Total cholesterol (mmol/L)	5.52 (4.77–6.05)
Non-HDL cholesterol (mmol/L)	4.04 (3.45–4.83)
HDL cholesterol (mmol/L)	1.16 (0.97–1.54)
Triglycerides (mmol/L)	1.79 (1.21–2.30)
Apolipoprotein B (g/L)	0.94 (0.78–1.09)
Apolipoprotein A-I (g/L)	1.28 (1.15–1.52)

Data in median (interquartile range). M: male; F: female; BMI: body mass index; HOMA_{ir}: homeostasis model assessment of insulin sensitivity.

Germany). Glucose was measured with an APEC glucose analyzer (APEC Inc., Danvers, MA, USA). Glycated hemoglobin (HbA_{1c}) was measured by high performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands; reference range 4.6–6.1%).

2.3. Statistical analysis

Data are given in median (interquartile range). Gender differences were determined by Mann–Whitney *U* test. Univariate relationships were calculated using Spearman's rank correlation analysis. Multiple linear regression analysis was performed to disclose independent contributions of variables. Logarithmically transformed values for triglycerides, HOMA_{ir}, and CRP were used because of skewed distribution. Two-sided *P*-values < 0.05 were considered significant.

3. Results

The clinical and laboratory characteristics of the study population which consisted of 67 Caucasian patients are given in Table 1. Diabetes duration was 6.5 (5.2–8.5) years. Eighteen patients had received dietary advice without drug therapy. Fourteen patients were treated with sulfonylurea alone, 14 were treated with metformin alone and 21 were using both types of medication. Other hypoglycemic drugs were not used. Twenty-seven participants were using antihypertensive drugs (most frequently, angiotensin-converting enzyme inhibitors, angiotensin II antagonists, diuretics and β -blockers). Of 27 female patients included, 21 were post-menopausal. One post-menopausal woman used raloxifene. Neither skin AF (1.72 (1.39–2.01) and 1.64 (1.43–2.31) AU in men and women, respectively; *P* = 0.65), nor the HDL anti-oxidative capacity (65 (57–73) and 66 (59–73) % inhibition in men and women, respectively; *P* = 0.85) differed between genders.

As demonstrated in Table 2, skin AF was inversely correlated with the HDL anti-oxidative capacity in univariate analysis. Skin AF tended to be correlated positively with age and inversely with plasma non-HDL cholesterol, triglycerides, apoB and CRP, but was not associated with HDL cholesterol and apoA-I. The HDL anti-oxidative capacity on the other hand was inversely correlated with plasma glucose, HbA_{1c}, triglycerides and HOMA_{ir}. HDL anti-oxidative capacity was also unrelated to HDL cholesterol and

Table 2

Univariate relationships of skin autofluorescence (skin AF) and HDL anti-oxidative capacity with clinical variables, glucose, HbA_{1c}, lipids, insulin, HOMA_{ir}, and apolipoproteins in 67 type 2 diabetic subjects.

	Skin AF	HDL anti-oxidative capacity
HDL anti-oxidative capacity	−0.305***	
Age	0.247*	−0.028
Diabetes duration	0.156	0.045
BMI	−0.037	−0.030
Systolic blood pressure	0.090	0.024
Diastolic blood pressure	0.010	−0.144
Glucose	−0.021	−0.400****
HbA _{1c}	−0.028	−0.328***
Insulin	0.070	−0.09
HOMA _{ir}	0.069	−0.264**
CRP	−0.235*	−0.067
Total cholesterol	−0.179	−0.130
Non-HDL cholesterol	−0.209*	−0.120
HDL cholesterol	0.024	0.175
Triglycerides	−0.215*	−0.308**
ApoA-I	−0.085	−0.067
ApoB	−0.240*	−0.048

Spearman's rank correlation coefficients are shown. BMI: body mass index. HOMA_{ir}: homeostasis model assessment of insulin sensitivity.

* *P* < 0.10.

** *P* < 0.05.

*** *P* < 0.02.

**** *P* ≤ 0.001.

apoA-I. Neither skin AF, nor HDL anti-oxidative capacity was significantly correlated with plasma insulin.

In age-adjusted multiple linear regression analysis it was first demonstrated that the inverse relationship of skin AF with the HDL anti-oxidative capacity was unaffected by either HDL cholesterol or apoA-I (β = −0.243, *P* = 0.039 and β = −0.241, *P* = 0.045, respectively; data not shown). To determine which factors were independent predictors of skin AF, multiple linear regression analyses were carried out including those variables to which skin AF or HDL anti-oxidative capacity was correlated at *P* < 0.10 in univariate analysis. In a model, which besides age and HDL anti-oxidative capacity included glucose, non-HDL cholesterol, triglycerides, HOMA_{ir} and CRP, skin AF was only related to HDL anti-oxidative capacity and age (Table 3). Skin AF remained inversely related to HDL anti-oxidative capacity (β = −0.243, *P* = 0.047) and positively with age (β = 0.318, *P* = 0.010) after adjustment for the use of anti-hypertensive drugs (β = 0.140, *P* = 0.25), sulfonylurea (β = −0.263, *P* = 0.031) and metformin (β = 0.177, *P* = 0.145). The relationship of skin AF with HDL anti-oxidative capacity was also significant in alternative models with HbA_{1c} instead of glucose (β = −0.320, *P* = 0.016) and with apoB instead of non-HDL cholesterol (β = −0.317, *P* = 0.016).

Table 3

Multiple linear regression analyses showing relationships of skin autofluorescence (skin AF) with age, HDL anti-oxidative capacity, plasma glucose, Ln HOMA_{ir}, non-HDL cholesterol, Ln triglycerides, and Ln CRP in 67 type 2 diabetic subjects.

Independent variables	Skin AF		
	β	Partial <i>r</i>	<i>P</i> -value
Age	0.299	0.315	0.014
HDL anti-oxidative capacity	−0.324	−0.314	0.015
Glucose	−0.108	−0.096	0.464
Ln HOMA _{ir}	0.135	0.121	0.358
Ln CRP	−0.120	−0.116	0.379
Non-HDL cholesterol	−0.186	−0.162	0.217
Ln triglycerides	−0.106	−0.083	0.526

HOMA_{ir}: homeostasis model assessment of insulin sensitivity. β : standardized regression coefficient. Partial *r*: partial correlation coefficient.

4. Discussion

This cross-sectional study demonstrates for the first time that tissue accumulation of AGEs, as assessed by skin AF, is inversely associated with the anti-oxidative capacity of HDL in non-smoking subjects with T2DM without severe long-term complications. This association was found to be independent of potential confounding factors such as age, glycemic control, plasma lipids, insulin resistance, and CRP levels. Our findings are, therefore, consistent with the possibility that a decreased ability of HDL to protect against LDL oxidation may contribute to the formation of oxidative stress products, such as AGEs. Importantly, analysis of HDL functionality revealed potentially relevant clinical information with respect to skin AF that was not obtained with HDL cholesterol or apoA-I mass measurements as such.

The pathogenetic role of AGEs in the development of diabetic complications is well recognized [8]. Based on skin biopsy studies in T2DM, hemodialysis, and healthy subjects it was demonstrated previously that skin AF can be regarded as a proxy for tissue AGE accumulation [10,12,20]. Furthermore, skin AF is related to serum levels of neopterin and to the soluble isoform of the receptor for AGEs, underscoring an intricate relation between skin AGEs and the activation of oxidative and inflammatory pathways [21]. Our current data in T2DM patients without severe micro- and macrovascular complications extend observations that the anti-oxidative capacity of HDL is inversely related to circulating AGE levels in T2DM patients with nephropathy [22]. Taken together, these recent [22] and the present observations agree with the hypothesis that impaired HDL functionality could contribute to the development of long-term diabetic complications.

In this report, plasma glucose as well as HbA_{1c} levels were found to correlate inversely with the ability of HDL to protect against LDL oxidation in univariate analysis. This finding is in line with another report demonstrating an inverse relation between HDL anti-oxidative capacity and hyperglycemia in recent onset T2DM [6]. Of note, actual plasma glucose and HbA_{1c} levels did not correlate with skin AF measurement, in keeping with the possibility that additional oxidative stress generating factors are required for AGE accumulation [7,21]. In other T2DM studies, we also found no or only weak relations between skin AF and short term glycemic control [23]. In fact, skin AF appears to be a better predictor of long term complications than HbA_{1c}, and complements the UKPDS risk engine in identifying those subjects with a particularly high risk for developing cardiovascular events [11].

Population studies have consistently shown that plasma HDL cholesterol levels inversely predict CVD [24–26]. Notably, evidence is accumulating in support of the hypothesis that abnormalities in HDL functionality may be more important than mere changes in HDL cholesterol in the pathogenesis of atherosclerosis [27]. HDL particles carry several proteins with anti-oxidative activity, which endows them to protect LDL against oxidative stress [28]. Indeed, it has been shown that anti-oxidative properties of HDL subfractions correlate negatively with circulating markers of generalized lipid-oxidation, such as plasma 8-isoprostane levels [29]. Validation of our data either in other cross-sectional or in prospective studies would be desirable. Nonetheless, our finding that skin AF correlated inversely with the ability of HDL to protect against LDL oxidation, but not with the plasma HDL cholesterol concentration, would support the potential clinical importance of HDL functionality assessment.

The method that we currently used to assess skin AF (EEMS) is slightly different from AF measurements using the AGE-Reader, which was used another study [17]. Although the physical principles and the optical path and design are similar, the absolute AF values obtained by these set-ups, cannot be directly compared. Subjects with a dark skin type cannot be reliably measured with

the EEMS setup. The current study only included Caucasian subjects with a Fitzpatrick skin type I–IV. Furthermore, since not all AGEs encompass fluorescent properties, skin AF is only representative of part of the total AGE burden. However, in our validation studies, skin AF also correlated strongly with non-fluorescent AGEs, including carboxymethyllysine, carboxyethyllysine [10,30]. Several other methodological issues need to be addressed. The HDL anti-oxidative functionality test does not as such represent a classical clinical chemistry measurement, since this assay system is dependent as starting material on primary LDL from healthy donors. Of note, the relative inhibition of oxidation of native LDL by respective HDL preparations is remarkably stable.

Since we carried out a cross-sectional study, no conclusion is allowed whether an impaired ability of HDL to protect against LDL oxidation contributes to or predicts enhanced tissue AGEs accumulation over time. Skin AF has been consistently found to be higher in previous reports in which diabetic patients were directly compared to control subjects [10,11]. Although a limitation of our study is that we included diabetic patients only, skin AF (also measured using the EEMS setup) was previously reported to be lower in control subjects than the values observed in the current study [31]. Moreover, validation of the current results in large scale cross-sectional and prospective studies is desirable.

In conclusion, skin AF, a non-invasive marker of tissue AGEs, was inversely related to the anti-oxidative capacity of HDL from patients with T2DM. These data raise the possibility that a decreased anti-oxidative capacity of HDL may contribute to tissue accumulation of AGEs, and thereby could be involved in the development of long-term diabetic complications.

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