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Advanced glycation end product associated skin autofluorescence: A mirror of vascular function?

Britt Hofmann*, Anne-Catrin Adam, Kathleen Jacobs, Marcus Riemer, Christian Erbs, Hasan Bushnaq, Andreas Simm, Rolf-Edgar Silber, Alexander Navarrete Santos

Department of Cardiothoracic Surgery, Martin-Luther-University, Halle (Saale), Germany

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ABSTRACT

Advanced glycation end products (AGEs) seem to be involved in aging as well as in the development of cardiovascular diseases. During aging, AGEs accumulate in extracellular matrix proteins like collagen and contribute to vessel stiffness. Whether non-invasive measurement of AGE accumulation in the skin may reflect vessel function and vessel protein modification is unknown. Herein we set out to analyze the AGE-modifications in the collagens extracted from residual bypass graft material, the skin autofluorescence reflecting the accumulation of AGEs in the body as well as the pulse wave velocity reflecting vessel stiffness.

Collagen types I and III (pepsin digestible collagen fraction) were isolated from the veins of 52 patients by proteolysis. The residual collagen fraction was further extracted by collagenase digestion. Collagen was quantified by hydroxyproline assay and AGEs by the AGE intrinsic fluorescence. Skin autofluorescence was measured with an autofluorescence reader; pulse wave velocity with the VICORDER®.

The collagen AGE autofluorescence in patient vein graft material increased with patient age. The pepsin digestible collagen fraction was significantly less modified in comparison to the collagenase digestible fraction. Decreasing amounts of extracted collagenase digestible collagen correspond with increasing AGE autofluorescence. Skin autofluorescence and vessel stiffness were significantly linked to the AGE autofluorescence of the collagenase digestible collagen fraction from graft material. In conclusion we have found that skin autofluorescence and pulse wave velocity as non-invasive parameters significantly correlate with the AGE contained in graft material and therefore are strong predictors of vessel AGE modifications in patients with coronary heart disease. Whether the analysis of the skin autofluorescence leads to an improvement of the risk stratification in patients suffering from cardiovascular disease has to be further tested.

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1. Introduction

Cardiovascular diseases are, according to the World health organization, the leading cause of morbidity and mortality of the aging population in industrial countries. Major age-associated tissue modifications contribute to the progression of such diseases. Since the initial description of the non-enzymatic Maillard reaction finally resulting in the production of advanced glycation end products (AGEs), there is increasing evidence that these bioactive molecules accumulate in tissue and vessels with age and are involved in cardiovascular disease development (Araszkiewicz et al., 2011; Aronson, 2002; Baumann et al., 2009; Semba et al., 2009; Yamagishi et al., 2007). It is known that aging of the vascular system is characterized by decreasing elasticity, endothelial dysfunction, intimal thickening and accumulation of interstitial collagen (Greenwald, 2007; Najjar et al., 2005). Especially collagen of the fibrillary

types I and III is important for the elasticity of the blood vessel system (Maurel et al., 1990). Physiological cross-links of the collagen fibers are important for the functionality of the matrix. However in aging, long-lived proteins like collagens are, due to slow turnover rates, especially susceptible to non-enzymatic glycation resulting in accumulation of AGEs (Reiser, 1998). These AGEs seem to establish additional cross-links resulting in loss of vessel elasticity. This mechanism so far was shown in a diabetic rat model (Reddy, 2004). Although a number of studies confirmed the correlation between AGE accumulation and increasing vascular stiffness the mechanism that may explain this association remains unclear (Campbell et al., 2011; Goldin et al., 2006; Semba et al., 2009).

The accumulation of advanced glycation end products is a general feature of the aging tissue due to glycation and oxidation reactions (Dunn et al., 1991). As shown by Sakata et al. the modification of collagen by AGEs decreases the susceptibility of collagen to enzymatic digestion (Sakata et al., 1995). In order to extract collagen from tissues a subsequent treatment with pepsin under acidic conditions followed by the digestion of the remaining collagen with enzymes like collagenase and proteinase seems to be the biochemical gold

* Corresponding author at: Department of Cardiothoracic Surgery, Martin Luther University Hospital Halle, Ernst-Grube-Straße 40, 06120 Halle (Saale), Germany. Tel.: +49 345 557 4990; fax: +49 345 557 2131.

E-mail address: britt.hofmann@medizin.uni-halle.de (B. Hofmann).

standard (Monnier et al., 1986; Sakata et al., 1995; Turk et al., 1999). Pepsin digestion causes solubilization of a part of the collagen, presumably by degrading the nonhelical telopeptide regions of the collagen molecule, where functional crosslinking is known to occur (Schnider and Kohn, 1981). The result of this treatment is a pepsin digestible collagen fraction (PDCF). Further collagen degradation of the pepsin insoluble fraction is achieved by collagenase treatment resulting in the isolation of a collagenase digestible collagen fraction (CDCF). The PDCF represents the less and the CDCF the more AGE modified collagen fraction.

The non-invasive measurement of skin autofluorescence (SAF) to estimate the skin tissue AGE burden is widely established and was shown to be a strong and independent predictor of cardiovascular mortality in diabetic and hemodialysis patients (Lutgers et al., 2009; Maury et al., 2011; McIntyre et al., 2011; Meerwaldt et al., 2007; Mulder et al., 2009; Noordzij et al., 2012; Samborski et al., 2011; Ueno et al., 2008). For evaluation of central arterial changes the measurement of the carotid to femoral (aortic) pulse wave velocity (aPWV) is an accepted procedure. The aPWV is used as an indicator of central arterial stiffness and seems to be a good predictor of increased cardiovascular risk (Baulmann et al., 2010; Mortensen et al., 2010; Nuernberger et al., 2007). Normally the pressure wave reflected from the periphery reaches the heart during diastole. But with increasing arterial stiffness due to aging or diseases, the velocity of the wave increases and the reflected pressure wave returns to the heart earlier. The pressure wave then reaches the heart during systole, resulting in increased cardiac afterload, elevated systolic and decreased diastolic blood pressure. Especially the decrease in diastolic pressure compromises the coronary blood flow and increases the risk for myocardial ischemia (Sutton-Tyrrell et al., 2005).

However so far it is unknown, whether the measurement of the skin autofluorescence as a non-invasive predictor of AGE burden and the measurement of the aPWV as a non-invasive marker for arterial stiffness do reflect the vascular modifications in situ. We hypothesized that these non-invasive parameters are related to vascular AGE accumulation and therefore studied the association among skin AGE fluorescence, aPWV and vascular collagen modification in graft material from patients with coronary heart disease.

2. Materials and methods

2.1. Patients

In the present study we assessed 52 male patients with diagnosed coronary heart disease. For all patients height, weight and waist circumference were measured. BMI was determined as kg/m². Smoking status was classified as a non-smoker, former smoker or current smoker (smoking within the last 2 months). Diabetes was defined as previously diagnosed diabetes. At the preoperative visit, all the study parameters including skin autofluorescence, aPWV, blood for laboratory analysis and the use of medication were determined. The study was approved by the local medical Ethics Committee and was carried out in accordance with the Declaration of Helsinki guidelines. Written informed consent was given from all study participants.

2.2. Skin autofluorescence

Skin autofluorescence was assessed using the validated SAF reader (AGE reader, DiagnOptics, Groningen, The Netherlands) as previously described (Meerwaldt et al., 2004). Three repeated measurements were performed at a healthy pale skin site (i.e. no scars or hyperpigmentation or other skin abnormalities) of the volar lower arm and an average was calculated. AF was measured in arbitrary units (a.u.). According to Meerwaldt et al. (2004) repeated SAF measurements on the same day show an overall Altman error rate of 5.03%

and intra-individual seasonal variance has an Altman error rate of 5.87%.

2.3. Measurement of aPWV

The carotid–femoral PWV and oscillometric blood pressure were measured after the patients had rested in a supine position (~30°) for 10 min. The VICORDER (Skidmore Medical Limited, Bristol, United Kingdom), a relatively new oscillometric device with FDA approval in 2007, was used for simultaneous recording of carotid and femoral pulse waves according to the manufacturer instruction (<http://www.dopstudio.co.uk/VicorderManual.pdf>) (Kracht et al., 2011). To measure aPWV, a carotid pressure cuff is applied over the right common carotid artery and a femoral pressure cuff is placed around the right upper thigh, as proximal as possible. Then the distance between the suprasternal notch and the middle of the femoral cuff was measured with a tape and this value was entered into the computer. Both cuffs were inflated simultaneously up to 65 mm Hg and the corresponding oscillometric signal from each cuff was digitally analyzed to extract in real time the pulse time delay. After acquiring 10 to 15 steady pulses the investigator saved the recording and the pulse transit time in milliseconds was reported. The aPWV was calculated from the VICORDER software by dividing traveled pulse wave distance by pulse transit time. All measurements were made in triplicate and averaged for the analyses.

2.4. Isolation of collagen types I and III (pepsin digestible collagen fraction – PDCF)

Collagen types I and III were isolated from leftover vein graft material from the coronary bypass graft operation. Equal amounts of vein material (100 mg wet weight) were cut into 10 strips and digested (0.3% pepsin in 0.5 M acetic acid) at room temperature for 16–18 h. After centrifugation the supernatant was taken as pepsin digestible collagen fraction. This fraction was then precipitated with sodium chloride (0.7 M NaCl). After resuspension of the pellet with PBS, the collagen was desalted using a Zeba™ Desalt Spin Column (Thermo Scientific GmbH) according to the manufacturer instruction.

2.5. Isolation of the collagenase digestible collagen fraction (CDCF)

From the residual tissue after acid and pepsin digestion the CDCF was extracted by digestion with collagenase type I (0.1 mg/ml) and proteinase K (0.1 mg/ml) by gentle shaking for 18 h at 37 °C according to Turk et al. (1999). It was then centrifuged and the obtained supernatant (CDCF) was used for further measurements.

2.6. Collagen quantification by 4-hydroxyproline assay

Collagen was estimated by measuring the amount of hydroxyproline. According to the literature it was assumed that hydroxyproline makes up 14% of collagen by weight. The amount of PDCF and CDCF was quantified by the 4-hydroxyproline assay according to Lin and Kuan (Lin and K., 2010).

2.7. Quantification of AGEs in collagen by fluorescence

For the measurement of the AGE autofluorescence, the PDCF and CDCF from each vein graft were diluted up to a total volume of 200 µl with PBS and in duplicate brought on a 96-well microtiterplate. The AGE intrinsic fluorescence (360 nm excitation and 440 nm emission) was measured with a plate reader (FluoStar Optima). For calculation of the concentration of the AGE modification in vitro modified human plasma was used as standard (0.001, 0.003, 0.01, 0.03, 0.11, 0.33, 1, 3, 10 µg modified plasma protein/ml). The measured fluorescence was normalized to the collagen concentration of the isolated collagen solutions.

2.8. Statistical analysis

Results were expressed as mean \pm sd (standard deviation). Statistical analysis was done using MedCalc® 8.0.1.0 program. When the parameter was normally distributed differences were analyzed using a *t*-test, when normality was not met Mann–Whitney *U*-test was used. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Patient diagnosis, BMI, smoking and diabetes state

In the present study we assessed 52 male patients (mean age: 68.7 ± 10.15 years) with diagnosed coronary heart disease. 23.1% of the participants had a BMI (≥ 30 kg/m²). Regarding the smoking history 42.3% were non-smoker, 28.8% were former smoker and 25% were smoking within the last 2 months. 36.5% of the patients had diagnosed type 2 diabetes. From the diabetic participants 15.8% were only on diet, 57.9% were treated with tablets and 23.3% were on insulin therapy. Further demographic and clinical characteristics of the study individuals are shown in Table 1.

3.2. Association between the 360/440 nm fluorescence and collagen amount of the PDCF and CDCF from vein graft material and the age, BMI, HbA_{1c}, blood glucose levels and creatinin levels

The typical AGE fluorescence (360/440 nm) was linked to the age of the patients and showed a specific trend for each fraction. The AGE autofluorescence of the CDCF positively correlated with the age (Fig. 1A, Table 2), whereas the AGE autofluorescence of the PDCF negatively correlated with the age (Fig. 1B, Table 2). A significant connection of the AGE fluorescence with the body mass index was only found for the PDCF. There was no correlation between the fluorescence of the fractions and the glycated HbA_{1c}, blood glucose and creatinin levels (Table 2).

3.3. Association between the glycated HbA_{1c}, the glucose levels and the collagen amount of the fractions

Glycated HbA_{1c} and blood glucose levels showed a significant connection with the amount of PDCF (Table 2). Furthermore the percentage of glycated HbA_{1c} inversely correlated with the amount of collagen in the PDCF and with the ratio of PDCF/CDCF (Fig. 2A, B; Table 2).

Table 1

Demographic and clinical characteristics of the included patients.

Variable	Male patients (n = 52)
Age, y	68.7 \pm 10.15
BMI, kg/m ²	27.8 \pm 4
Waist circumference	97.2 \pm 11.6
LVEF, %	54 \pm 13
Systolic blood pressure, mm Hg	139 \pm 21
Diastolic blood pressure, mm Hg	73 \pm 12
Heart rate, beats/min	67 \pm 14
HbA _{1c} levels, %	6.3 \pm 0.69
Blood glucose levels, mmol/l	6.35 \pm 1.98
Creatinin levels, μ mol/l	99 \pm 27.7
GFR, %	57 \pm 27.7
Ratio HDL/LDL	0.43 \pm 0.17
EuroSCORE additive	6.19 \pm 2.62
EuroSCORE logistic, %	8.15 \pm 8.37

Demographic and clinical data are provided as mean \pm standard deviation (SD).

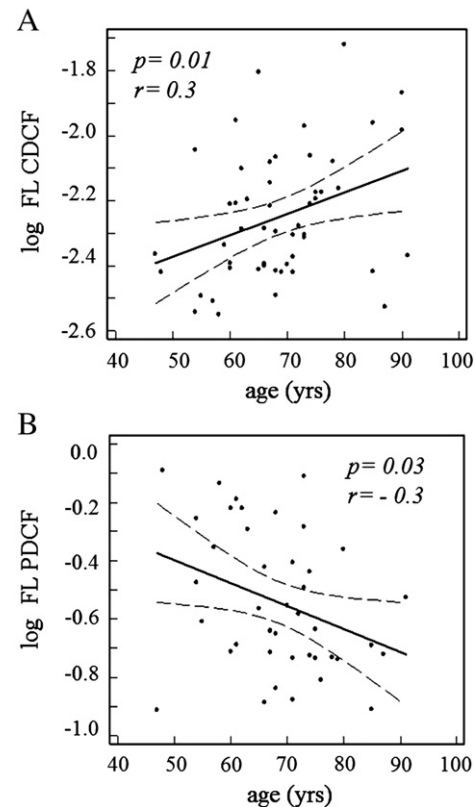


Fig. 1. Correlation between age and autofluorescence (FL) of the collagenase (CDCF) (A) and pepsin (PDCF) (B) digestible collagen fraction from vein graft material. The fluorescence of the CDCF correlates with the age positively. The fluorescence of the PDCF negatively correlates with the age.

3.4. Association between the 360/440 nm fluorescence and extractable collagen (PDCF and CDCF) from vein graft material and diagnosed type 2 diabetes or smoking

Regarding the association to type 2 diabetes and smoking the CDCF collagen fraction fluorescence was significantly higher in non-smokers (Table 3). Comparing the collagen amounts a significantly lower amount of the PDCF was noticed in diabetic patients (Table 4 and Fig. 3).

3.5. Association between skin autofluorescence, aPWV and the fluorescence of the insoluble and soluble collagen fraction from vein graft material

In contrast to the AGE autofluorescence of the pepsin digestible collagen fraction compared to the skin autofluorescence, a strong association between the SAF and the AGE autofluorescence of the CDCF was observed (Fig. 4A). Furthermore the AGE fluorescence of the collagenase digestible collagen fraction was highly correlated with aPWV (Fig. 4B). The fluorescence of the PDCF fraction did not correlate with aPWV. Furthermore we noticed a strong association between SAF and aPWV (Fig. 5).

4. Discussion

Coronary heart disease and resulting ischemic heart failure are the leading causes of death among the elderly in western countries. Cardiac surgery has become an accepted therapy for these patients. However, an increasing age of the general population reinforces the urgency for new approaches and strategies to identify people at risk. These strategies should include parameters of basic aging processes to understand age-associated modifications of the cardiovascular system individually. This would have a tremendous impact on the patient outcome and the economy of the health system. Therefore, the analysis of non-invasive

Table 2
Correlation between the fluorescence and the collagen amount of the two collagen fractions with different patient parameters.

	Fluorescence 360/440 nm						Collagen amount					
	PDCF			CDCF			PDCF			CDCF		
	n	r	p	n	r	p	n	r	p	n	r	p
Age	42	−0.33	0.03	51	0.34	0.01	50	−0.13	0.38	51	−0.06	0.65
BMI	43	−0.35	0.02	51	−0.15	0.29	50	0.21	0.15	51	−0.04	0.77
HbA1c	30	0.04	0.84	36	0.16	0.34	35	−0.35	0.04	36	−0.11	0.53
Blood glucose	43	−0.14	0.39	51	0.15	0.31	50	−0.42	0.002	51	−0.12	0.39
Creatinin	43	0.05	0.76	51	0.12	0.36	51	−0.06	0.64	51	0.02	0.88

PDCF = pepsin digestible collagen fraction, CDCF = collagenase digestible collagen fraction, n = number of cases; r = Spearman's rank correlation coefficient; p = probability value. Statistical significance is notated by bold numbers.

parameters to identify people at risk and high risk patients is especially important.

We hypothesized that the non-invasive parameters SAF and aPWV are related to vascular AGE accumulation and therefore studied the association among skin AGE autofluorescence, aPWV and vascular collagen modification in graft material from patients with coronary heart disease.

In the present study we found a strong correlation between SAF/PWV and the specific AGE autofluorescence of the collagenase digestible collagen fraction from vein graft material. Dyer et al. (1992) described the accumulation of glycoxidated proteins in human tissue as a process of chemical aging. Especially long-lived proteins like collagen, due to a slow turnover, are susceptible for non-enzymatic glycation and oxidation resulting in AGE accumulation (Anttinen et al., 1973; Schneider and Kohn, 1981; Turk et al., 1999). These AGEs establish additional cross-links which damage the vascular function resulting in reduced vessel elasticity. The mechanism so far was especially shown in a diabetic rat model by Reddy (2004). It is believed that the same principle is true for the human situation and our data are in line with this report. The chemical reaction underlying this mechanism should be indeed independent of the species; however regarding the in vivo substrates of the reaction, differences cannot be excluded. Due to the slow turnover rate, collagen is a good candidate to reflect the accumulation of AGE

modifications during aging. Measuring the typical AGE fluorescence and the collagen amount of vein graft material and associating these results with patient parameters, a significant correlation of the fluorescence at 360/440 nm with patients age was indeed observed. Regarding other patient characteristics like type 2 diabetes and smoking, we identified interesting correlations which should be further confirmed by studies with bigger subgroups. However, the significant lower amount of pepsin digestible collagen from vein grafts of diabetic patients seems to be of special interest. This result confirms the theory, that collagen of diabetic patients has more modifications and protein cross-links, making isolation of collagen fractions more difficult (Reddy, 2004). In this work we were able to assess two different collagen fractions, PDCF and CDCF, however we still have to admit that due to the accumulation of AGEs during aging on the matrix proteins most of the highly modified collagen remains insoluble (Sakata et al., 1995).

In our study, SAF and aPWV were strong non-invasive predictors of vascular modifications. We were able to show that collagen modifications of the skin (SAF) are linked to modifications of the collagenase digestible collagen fraction from vein graft material and these modifications are strongly associated to arterial stiffness. Thus we conclude that SAF is a good predictor of vascular collagen modifications.

However, a clear limitation of the study is the usage of saphenous vein graft material and not of arteries to determine the vascular collagen modifications. The reason for this was the problem of getting enough arterial graft material from the CABG operation. One could argue that the modifications in arterial graft material would be even higher because of more extracellular matrix and higher blood glucose levels in the arterial system (Canham et al., 1997; Wahab et al., 1992). On the other hand the internal thoracic artery (ITA) as arterial bypass graft demonstrated in clinical studies a better outcome even in diabetic patients. As Kitamura suggested this may be a result of the special tissue structure of the ITA (Kitamura, 2011). However, because of the limited number of arterial grafts investigated in our study we were not able to compare collagen modifications between both graft materials. In addition, we should recognize that any arterial graft is primarily adjusted to the high pressure system whereas a venous graft needs some remodeling to adapt to this system. This is indeed a disadvantage of a vein as graft material.

Table 3
Correlation between AGE fluorescence of the collagen fractions with diabetes or smoking.

	Fluorescence 360/440 nm					
	PDCF			CDCF		
	Mean ± sem	n	p	Mean ± sem	n	p
Diabetes	0.55 ± 0.051	14	0.71	2.19 ± 0.052	18	0.11
Non-diabetes	0.52 ± 0.052	29		2.28 ± 0.031	33	
Smoker	0.49 ± 0.063	12	0.47	2.36 ± 0.049	13	0.01
Non-smoker	0.56 ± 0.061	18		2.21 ± 0.036	22	

PDCF = pepsin digestible collagen fraction, CDCF = collagenase digestible collagen fraction, fluorescence values presented as logarithmic mean ± standard error of the mean (sem); n = number of cases; p = probability value. Statistical significance is notated by bold numbers.

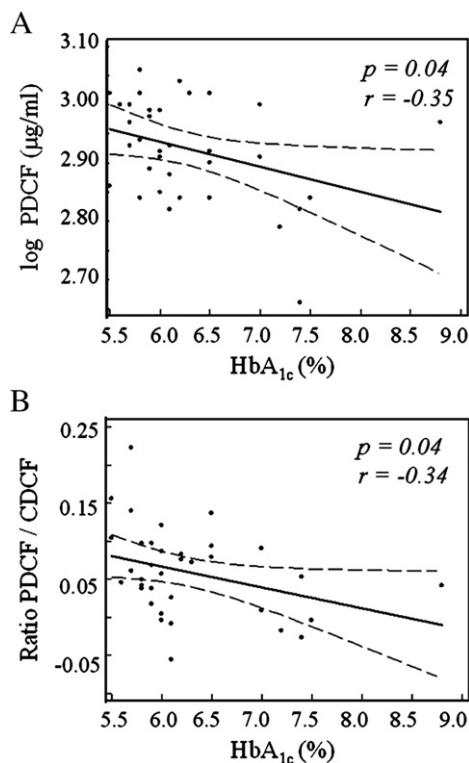
**Fig. 2.** A— Correlation between glycated HbA_{1c} and the amount of collagen in the pepsin digestible collagen fraction (PDCF). B— Ratio PDCF/CDCF amount versus glycated HbA_{1c}.

Table 4

Correlation between collagen amounts of the two fractions with diabetes or smoking.

	Collagen amount							
	PDCF		n	p	CDCF		n	p
	Mean ± sem				Mean ± sem			
Diabetes	2.87 ± 0.018	18	0.0007		2.83 ± 0.016	18	0.24	
Non-diabetes	2.95 ± 0.012	32			2.86 ± 0.019	33		
Smoker	2.93 ± 0.028	15			2.88 ± 0.025	15		
Non-smoker	2.92 ± 0.013	21			2.83 ± 0.017	22		

PDCF = pepsin digestible collagen fraction, CDCF = collagenase digestible collagen fraction, collagen amounts presented as logarithmic mean ± standard error of the mean (sem), n = number of cases; p = probability value. Statistical significance is notated by bold numbers.

To our knowledge this is the first study to show that vascular collagen modifications in patient graft material correlate strongly with skin autofluorescence and arterial stiffness. These findings and a number of other published data from humans and animal models suggest that AGEs play a role in the development of vascular stiffness (Baumann et al., 2009; Mulder et al., 2009; Reddy, 2004; Semba et al., 2009; Ueno et al., 2008; Wolffenbuttel et al., 1998; Yamagishi et al., 2007). In humans, Kass et al. have already shown the positive effect of an advanced glycation end product crosslink breaker on the arterial compliance (Kass et al., 2001). This promising data is supported by a number of animal studies (Vaitkevicius et al., 2001; Wolffenbuttel et al., 1998). It is known that AGEs induce cross-linking of collagen in arterial walls on the one hand and promote endothelial dysfunction by reducing nitric oxide on the other hand. A couple of additional mechanisms like increased production of reactive oxygen species, increased oxidation of low density lipoproteins, enhanced macrophage migration and up regulation of inflammation via RAGE are initiated by AGEs and contribute to vascular dysfunction and arterial stiffness (Basta, 2008). AGEs may also induce oxidative stress by binding to receptors like RAGE and thereby promote apoptosis in cells like endothelial cells (Chen et al., 2010; Kaji et al., 2003).

Semba et al. suggested AGEs as a major risk factor for arterial stiffness and found in older disabled community-dwelling women with elevated serum AGEs an increased risk for dying of cardiovascular disease (Semba et al., 2009). We could not find a correlation of serum AGEs to SAF, aPWV or collagen modifications. This may be due to our small sample size. However, Semba et al. also postulated that non-invasive parameters like aPWV could predict persons at risk and this would open up the possibility to direct them earlier to a specific therapy (diet, optimization of antihypertensive treatment, sports e.g.).

For resource-intensive operations like coronary artery bypass grafting, a valid prediction of postoperative mortality, morbidity and prolonged hospital stay has gained increased importance for the health system and the patient as well as the family to weigh the risk and the benefit of the intervention. Since 1999, the EuroSCORE (European System for

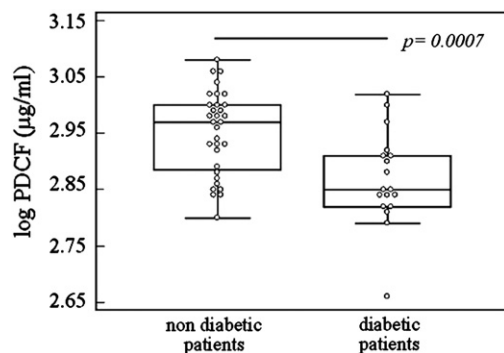


Fig. 3. Comparison between the amount of pepsin digestible collagen (PDCF) from vein graft material of non-diabetic and patients with type-2 diabetes.

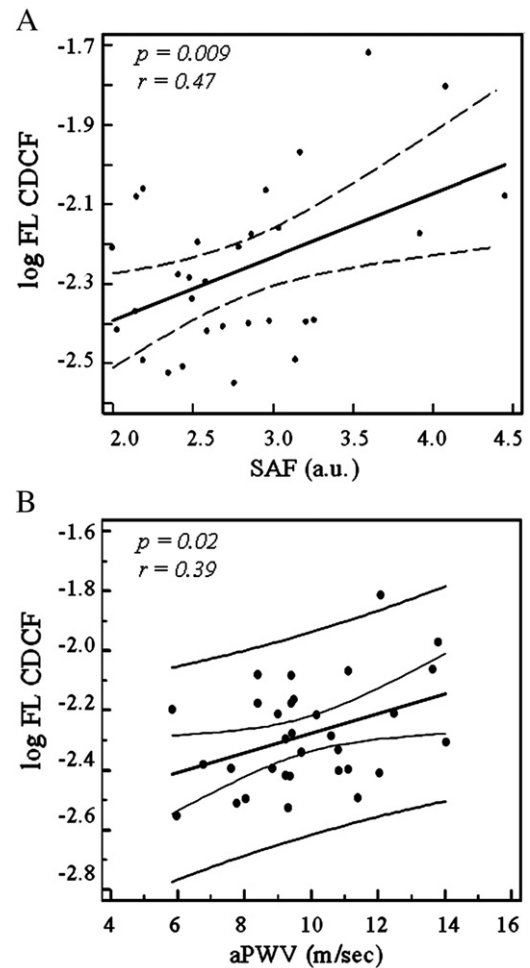


Fig. 4. A— Correlation between skin autofluorescence (SAF) and autofluorescence (FL) of the collagenase digestible collagen fraction (CDCF) from vein graft material. B— The pulse wave velocity (aPWV) positively correlates with the autofluorescence (FL) of the CDCF.

Cardiac Operative Risk Evaluation) is used as a simple and predictive overall score throughout Europe and a number of other countries (Lebreton et al., 2011). However, especially in older patients the EuroSCORE seems to have limitations and overestimates the mortality rate by far (Danner et al., 2009; Iyem, 2009). As we know that vascular function is crucial for patients with coronary heart diseases, our data suggests that the non-invasive parameter SAF could be beside the aPWV an interesting candidate for a better risk stratification of CHD patients (Fig. 6). For the proof of the reliability of SAF and aPWV as

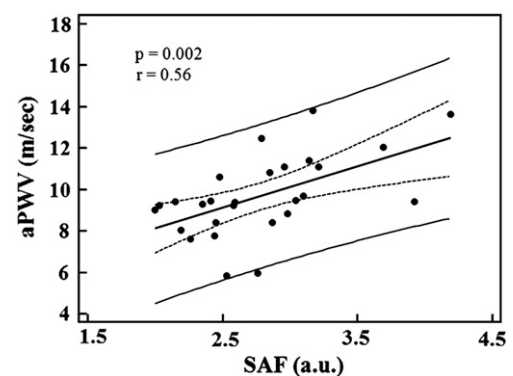


Fig. 5. Correlation between skin autofluorescence (SAF) and pulse wave velocity (aPWV).

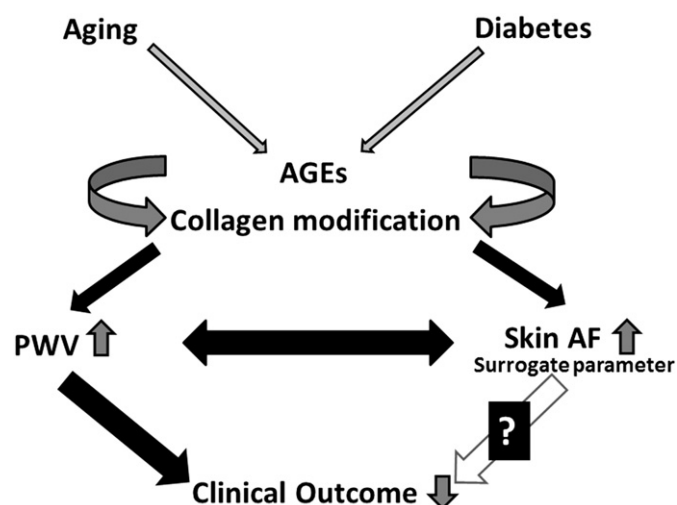


Fig. 6. Hypothetic scheme of skin autofluorescence as a mirror of vascular function.

predictive non-invasive parameters for the outcome in cardiac surgery patients further studies are needed.

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Disclosures

None.

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