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## Accepted Manuscript

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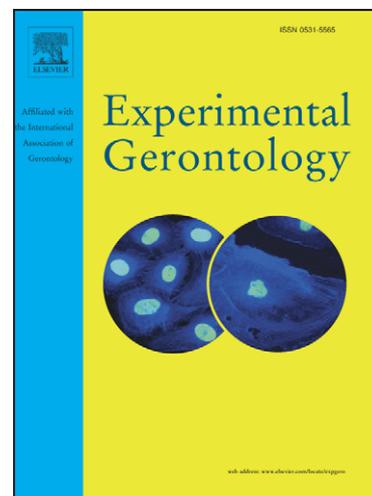
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4 Glycation associated skin autofluorescence and skin elasticity are related to  
5 chronological age and body mass index of healthy subjects  
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32 glycation and elasticity in ageing skin  
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36 skin ageing, glycation, skin elasticity, body mass index  
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4 Glycation is the non-enzymatic reaction between reducing sugars and proteins that  
5 leads to the formation of advanced glycation end products (AGEs). In vivo skin  
6 autofluorescence ( $\lambda_{\text{ex}}/\lambda_{\text{em}} = 370/440\text{nm}$ ) was used as a non-invasive clinical tool to  
7 study skin AGE accumulation in healthy panellists. Using multiple linear regression  
8 analysis, it was shown that for panellists below the age of 40, glycation associated in  
9 vivo skin fluorescence intensity increased as a function of chronological age and body  
10 mass index (BMI). Above the age of 40, the fluorescence was associated to age but  
11 not to BMI, suggesting that the effect of age became dominant over BMI. Since the  
12 accumulation of AGEs is expected to affect the biomechanical properties of the skin,  
13 in vivo skin elasticity data were gathered on a second panel. It was found that skin  
14 elasticity depended on age and BMI in a similar fashion as to what we observed for  
15 the skin fluorescence data. It is hypothesised that skin AGE accumulation contributes  
16 to the loss of skin elasticity in aged and/or overweight people.  
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4 During the process of glycation, reducing sugars react non-enzymatically with target  
5 molecules like proteins, lipids or DNA. During the first stages of this process, early  
6 glycation products are reversibly formed. Side products generated in the intermediate  
7 steps may be oxidising agents and/or reactive carbonyl compounds like  
8 (methyl)glyoxal and glycolaldehyde. Next these early glycation products react further  
9 to give rise to a whole set of modifications collectively termed advanced glycation  
10 endproducts (AGEs), from which some of them are characterised by intra-and  
11 intermolecular cross-linking and typical fluorescence bands (Ulrich and Cerami,  
12 2001).

13  
14 Since the formation of AGEs is a slow process, the body's long-lived proteins such as  
15 collagen are mostly susceptible to AGE accumulation. AGEs have been known to  
16 accumulate in various tissues as a function of chronological age (Hipkiss, 2006). This  
17 build up of glycated modifications happens at an even much higher rate in several  
18 age-related diseases like diabetes (Dyer et al., 1993), vascular (Soldatos and Cooper,  
19 2006) and neurodegenerative (Sasaki et al., 1998) diseases and osteoarthritis (Senolt  
20 et al., 2005).

21  
22 Skin is a tissue in which the formation and accumulation of AGEs has been  
23 extensively studied and the presence of glycation products in the skin has been  
24 proposed as a marker and predictor of the progression of certain diseases (Monnier et  
25 al., 2005). The initial method for quantifying AGEs was based on the measurement of  
26 collagen-linked fluorescence in biopsies (Odetti et al., 1992). The age related trends  
27 observed with this technique were later confirmed using hyphenated analytical tools  
28 that enabled the analysis of specific AGEs like N<sup>ε</sup>-(carboxymethyl)lysine and  
29 pentosidine (Dyer et al., 1993). Recently skin autofluorescence has been proposed as  
30 yet another non-invasive clinical tool to study age related phenomena in human skin  
31 (Kollias et al. 1998). Skin autofluorescence, collected at the excitation and emission  
32 wavelength of 370 and 440nm respectively is assumed to be specific for (collagen  
33 linked) AGEs. Skin autofluorescence has been shown to be correlated with the  
34 presence of specific AGEs (Meerwaldt et al., 2005b) and was associated to  
35 chronological age and diabetes duration (Meerwaldt et al., 2004).

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37 Although the analyses of AGEs in skin has been very often used as a marker of AGE  
38 accumulation, only recently the impact of the glycation process on the structure and  
39 functioning of the skin itself has been explored. In the current study we have used in  
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4 vivo skin autofluorescence measurements to assess skin AGE accumulation in healthy  
5 volunteers as a function of chronological age and body mass index (BMI). Data are  
6 presented to support the link between AGE accumulation and the in vivo loss of skin  
7 elasticity.  
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ACCEPTED MANUSCRIPT

### *Subjects*

Healthy female volunteers, with Fitzpatrick skin type II or III, who were in good general health, free of any dermatologic disorders, and who gave their informed, written consent, were allowed to enter the studies.

### *Clinical measurements*

In vivo skin autofluorescence measurements were collected with an LS 50B fluorescence spectrometer (PerkinElmer, Waltham, MA, USA) equipped with a fibre optic. The excitation and emission wavelengths were set to 370 and 440 nm respectively and data were collected on the ventral forearm of the panellists. On the same site, skin colour was measured with a chromameter (Minolta, Osaka, Japan) and expressed as Individual Typology Angle (ITA°). Pigmented skin is characterised by a low ITA-value. Height was measured and body weight recorded using a Terrillon TFX70 scale (Terrillon, Paris, France). Body mass index (BMI) was calculated by dividing the weight of the panellist in kilograms by the square of the height in meters. A BMI of 20 to 25 indicate normal weight; a number from 25 to 30 indicate the person is overweight and a number above 30 suggests the person is obese.

Skin elasticity was measured on the outer thigh of the panellists with a Torsional ballistometer (Dia-Stron Ltd., Andover, UK). Elasticity of the skin was expressed as the rate of energy damping  $\alpha$ . A high  $\alpha$ -value is indicative for a reduced elasticity.

### *Statistical analysis*

Statistical calculations were performed using Statistica 6.0 (Statsoft, Tulsa, OK, USA). Data were analysed by single and multiple linear regression analysis. A probability level of 0.05 was chosen as the point at which differences were considered significant.

### *AGE-associated fluorescence as a function of chronological age and BMI*

In the first study (study A) skin autofluorescence data were collected on the ventral forearm of 448 female Caucasian panellists. The group characteristics are summarised in Table 1. Besides skin autofluorescence, the skin colour, expressed as Individual Typology Angle (ITA), was measured on the same site. There existed a strong inverse correlation between the skin colour and the fluorescence intensity ( $n=448$ ;  $r=0.76$ ,  $p<10^{-17}$ ). The dataset was broken up in two groups, depending on the chronological age of the panellist viz. below or above the age of 40. For each of both datasets, multiple linear regression analysis was used to investigate the independent effects of skin colour, chronological age and BMI on the skin autofluorescence. Multicollinearity statistics revealed that tolerance values were typically above 0.9 hence the individual parameters were independent. Pareto charts are shown in Fig. 1 for both the younger panellists (top panel) and for the group of the older panellists (bottom panel). The statistical outcome is summarised in Table 2. For both age groups the bar for the skin colour crossed the dotted line, which marks the critical value for statistical significance (Fig. 1). The fitted parameters for each variable are listed in Table 2 and this indicates that an increase in the ITA value, and thus a less pigmented skin, is associated to a higher autofluorescence value. Out of the three variables studied, ITA was clearly the most dominant one. For the young-age group, both age and BMI were positively associated to the in vivo AGE-associated fluorescence signal (Fig. 1 and Table 2). For the older panellists the dependence of the fluorescence on chronological age was maintained, but there was no statistically significant association with the BMI. This suggests that only for younger panellists, both BMI and chronological age were recognised as being factors positively associated to the autofluorescence signal, while in the old age group the effect of age on the fluorescence was much stronger and became dominant over the BMI effect.

### *Skin elasticity as a function of chronological age and BMI*

Eighty-four female panellists were enrolled in the clinical study (study B) and the group characteristics are summarised in Table 1. Skin elasticity was measured with a torsional ballistometer and expressed as the rate of energy damping  $\alpha$ . Data were collected on the outer thigh, a body site that is prone to fat accumulation in women. The panellists were subdivided in two age groups and the number of panellists

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4 assigned to the young age ( $< 40$  years) and older age ( $\geq 40$  years) group was 30 and  
5 54 respectively. Statistical evaluation of the data proceeded in a similar fashion as for  
6 the first study, except that skin colour was not considered as being a relevant  
7 parameter for skin elasticity. Data are summarised in Fig. 2 and in Table 3. For the  
8 young age group, a decrease in skin elasticity was mainly associated with high BMI  
9 values but there was no significant correlation with panellist age. For the older age  
10 group, the opposite was observed, and the skin elasticity was largely affected by the  
11 age of the panellist but not by the BMI.  
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4 Several methods exist for the measurement of AGEs in skin samples. Some of these  
5 methods require the sampling of biopsies and are invasive in nature. Recently skin  
6 autofluorescence has been proposed as a non-invasive tool to study AGEs in human  
7 skin (Meerwaldt et al., 2004).  
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10 A clinical study was conducted in which the glycation associated fluorescence and  
11 skin colour were measured on the ventral forearm of 448 female healthy Caucasian  
12 panellists. Their Fitzpatrick skin type was II to III and they had similar skin tone with  
13 a median ITA value of 48.0 (Table 1). Nevertheless there existed a very strong inverse  
14 correlation between skin colour and fluorescence ( $p < 10^{-17}$ ). This agrees with  
15 observations already described in the literature (Na et al., 2001). Pigmented skin most  
16 probably contains compounds that absorb or scatter the fluorescent light and as a  
17 result the fluorescence intensity decreases. Various methods have been proposed to  
18 anticipate for this strong dependence of the fluorescence intensity on skin colour  
19 (Sandby-Moller et al., 2003; Meerwaldt et al., 2005a; Stamatatos et al., 2006). We have  
20 used multiple linear regression analysis tools to study the skin autofluorescence as a  
21 function of skin colour, chronological age and BMI. The latter, which is a measure for  
22 the panellist's overweight was considered here since an increase in overweight and  
23 obesity prevalence is evident in the Western societies over the last decades (Hedley et  
24 al., 2004; Tzotzas and Krassas, 2004). In this study the median BMI of the panellists  
25 was  $26 \pm 6.3$  and the majority of the panellists had a BMI between 20 and 30,  
26 indicating that this study was primarily focussed towards normal to overweight  
27 people. The age of the panellists ranged from 19 up to 70 years, with about hundred  
28 panellists for each decade of age. Formerly it was shown that pentosidine in skin  
29 collagen increased in an age dependent manner (Sell et al. 1996) but at relatively  
30 young age, AGE levels were low and remained fairly constant. Only above the age of  
31 40 the rate at which these AGEs accumulated strongly increased. Immunostaining  
32 experiments of skin samples showed similar trends (Jeanmaire et al., 2001). The same  
33 observation was made in human cartilage collagen where during the first decades of  
34 life, levels of AGEs remained low but at higher age the concentration of the AGEs  
35 increased up to 30 fold (Verzijl et al., 2000). This age dependence of AGE  
36 accumulation urged us to break up the dataset in two, based on the age of the  
37 panellist. Statistical calculations were then performed independently for the young  
38 age group ( $< 40$  years) and for the older age group ( $\geq 40$  years). Within a population of  
39 normal to overweight, young ( $< 40$  years) and healthy panellists, both chronological  
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4 age and BMI contributed in a statistically significant way to the increase in the  
5 glycation associated fluorescence (Table 2 and figure 1). For the older age panellists  
6 the age effect was maintained but the effect of BMI on the glycation associated  
7 fluorescence was not conserved. These data suggest that skin AGEs accumulated as a  
8 function of chronological age, which agrees with many other studies (Sell et al. 1996;  
9 Jeanmaire et al., 2001; Dyer et al., 1991). BMI however only seemed to be associated  
10 with the glycation associated fluorescence in the young age group. This might be  
11 explained by an increase in the rate of accumulation of skin AGEs at higher  
12 chronological age. Within the older age group, the effect of age on the fluorescence  
13 became dominant over BMI.  
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16 A second clinical study was conducted in which skin elasticity of 84 female panellists  
17 was measured. Mechanical properties of the skin and elasticity in particular can be  
18 evaluated with a number of instruments based on torsion or extension methods. In this  
19 study a torsional ballistometer was used. This instrument records the bouncing profile  
20 of a small hammer that is dropped on the skin surface with a constant force. Earlier it  
21 has been reported that skin elasticity measured with the ballistometer decreased in an  
22 old (50-67 years) versus a young population (18-28 years) (Agarwal et al., 2007). In  
23 this study an additional parameter viz. BMI was considered. The impact of obesity on  
24 skin physiology has been recognised before (Yosipovitch et al., 2007), but the  
25 evaluation of normal to slightly overweight panellists forms the focus of this study.  
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28 From Table 1 it follows that the age and BMI distribution was very similar to the first  
29 study. Statistical treatment of skin elasticity data was identical to study A, except that  
30 the skin colour, which was not associated to skin elasticity, was omitted as an  
31 independent variable in these calculations. The dependence of the skin elasticity,  
32 expressed as the  $\alpha$ -value, on chronological age and BMI was different for both age-  
33 groups. For the young age group, skin elasticity was negatively associated to BMI but  
34 not to chronological age. The opposite was observed in the older age panellists (Fig. 2  
35 and Table 3).  
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38 There was similarity in the way that both glycation associated autofluorescence and  
39 skin elasticity depended on chronological age and BMI in both age groups. For both  
40 fluorescence and elasticity, BMI was a statistically significant parameter only in the  
41 group of young panellists (under the age of 40 years). Chronological age affected the  
42 fluorescence in both age groups but with respect to the elasticity measurements, age  
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4 seemed to be more important in the older age group. Based on this analogy it is  
5 hypothesised that the accumulation of AGEs as measured with the in vivo skin  
6 autofluorescence technique is one of the factors that contributes to the progressive  
7 decline in skin elasticity. Similar findings have been presented in a diabetic  
8 population in which it was shown that glycation of plantar epidermal proteins could  
9 play an important role in the stiffening of plantar skin in diabetes (Hashmi et al.,  
10 2006). This also agrees with data showing that in vitro glycated skin samples are  
11 characterised by a loss of biomechanical properties (Reihnsner et al., 2000).  
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18 In summary measurement of in vivo skin autofluorescence is proposed as a non-  
19 invasive clinical tool that enables the measurement of skin AGE accumulation in  
20 healthy panellists. This method was used to evaluate skin AGE accumulation as a  
21 function of chronological age and BMI of the panellist. Furthermore skin elasticity  
22 data also showed a dependence on chronological age and BMI, leading to the  
23 hypothesis that collagen glycation or skin AGE accumulation in general is one of the  
24 parameters that contributes to the loss of skin elasticity.  
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4 Table 1: Group characteristics for both clinical studies  
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7 Table 2: Statistical outcome of the multiple linear regression analysis on the  
8 association between in vivo skin fluorescence and skin colour (ITA), chronological  
9 age and BMI for the two age groups. Statistical significant parameters are shown in  
10 bold.  
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14 ♦ A probability p-value less than 0.05 is deemed statistically significant

15 # Regression coefficient after standardisation of all variables  
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19 Table 3: Statistical outcome of the multiple linear regression analysis on the  
20 association between skin elasticity and chronological age and BMI for the two age  
21 groups. Statistical significant parameters are shown in bold.  
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24 ♦ A probability p-value less than 0.05 is deemed statistically significant

25 # Regression coefficient after standardisation of all variables  
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29 Fig. 1 Pareto charts of the multiple linear regression analyses on the association  
30 between in vivo skin fluorescence and skin colour (ITA), chronological age and BMI.  
31 Results for the young age and old age group are shown in the top and bottom panel  
32 respectively.  
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38 Fig. 2 Pareto charts of the multiple linear regression analyses on the association  
39 between skin elasticity and chronological age and BMI. Results for the young age and  
40 old age group are shown in the top and bottom panel respectively.  
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ACCEPTED MANUSCRIPT

Characteristics	Study A	Study B
N	448	84
(below / above 40 years of age)	(147 / 301)	(30 / 54)
Fitzpatrick skin type	II / III	II / III
Age (median $\pm$ st. dev.)	47.0 $\pm$ 13.9	43.0 $\pm$ 8.7
BMI (median $\pm$ st. dev.)	26.0 $\pm$ 6.3	24.5 $\pm$ 3.9
ITA (median $\pm$ st. dev.)	48.0 $\pm$ 7.6	/

Age category	Variable	Regression coefficient	t-value	p-value <sup>♦</sup>	Standardised regression coefficient $\beta^{\#}$	95% confidence limits
< 40 years	<b>Age</b>	<b>1.85</b>	<b>4.11</b>	<b><math>6.6 \cdot 10^{-5}</math></b>	<b>0.186</b>	<b>0.096 - 0.275</b>
	<b>BMI</b>	<b>1.22</b>	<b>2.53</b>	<b>0.012</b>	<b>0.119</b>	<b>0.026 - 0.211</b>
	<b>ITA</b>	<b>6.16</b>	<b>16.73</b>	<b><math>&lt;10^{-10}</math></b>	<b>0.777</b>	<b>0.685 - 0.869</b>
$\geq$ 40 years	<b>Age</b>	<b>1.32</b>	<b>4.35</b>	<b><math>1.9 \cdot 10^{-5}</math></b>	<b>0.159</b>	<b>0.087 - 0.231</b>
	<b>BMI</b>	0.34	0.81	0.417	0.030	-0.043 - 0.103
	<b>ITA</b>	<b>7.28</b>	<b>20.25</b>	<b><math>&lt;10^{-10}</math></b>	<b>0.752</b>	<b>0.679 - 0.825</b>

Age category	Variable	Regression coefficient	t-value	p-value <sup>†</sup>	Standardised regression coefficient $\beta^{\#}$	95% confidence limits
< 40 years	age	3.70E-05	0.52	0.61	0.091	-0.266 - 0.447
	<b>BMI</b>	<b>2.59E-04</b>	<b>2.43</b>	<b>0.022</b>	<b>0.422</b>	<b>0.066 - 0.779</b>
≥ 40 years	<b>age</b>	<b>4.96E-04</b>	<b>4.57</b>	<b>3*10<sup>-5</sup></b>	<b>0.540</b>	<b>0.303 - 0.777</b>
	BMI	2.30E-05	0.17	0.86	0.021	-0.217 - 0.258

