

**The skin as a mirror of the aging process** Waaijer, M.E.C.

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## **Chapter 4 Chabter 4**

#### Morphometric skin characteristics dependent on chronological

#### and biological age: the Leiden Longevity Study

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#### Abstract

The effect of chronological age on skin characteristics is readily visible and its underlying histological changes have been a field of study for several years. However, the effect of biological age (i.e. a person's rate of ageing compared to their chronological age) on the skin has so far only been studied in facial photographs. Skin biopsies obtained from middle aged offspring of nonagenarian siblings that are genetically enriched for longevity were compared to their partners who represent the general Dutch population. Though of the same chronological age, the offspring were previously observed to be of a younger biological age than their partners. The biopsies were analysed on several aspects epidermal and elastic fibre morphology. We investigated whether these skin characteristics were dependent on chronological age, familial longevity (the difference between the offspring and partners) and Framingham heart risk scores, adjusted for external stressors. A decreased thickness and flattening of the epidermis as well as an increased amount of elastic fibres in the reticular dermis were observed with chronological age (P<0.001, P<0.001 and P=0.03 respectively), but no effect of familial longevity was found. The Framingham heart risk score was associated with some skin characteristics. A slower rate of skin ageing does not mark offspring from nonagenarian siblings. Epidermal and elastic fibre morphometric characteristics are not a potential marker for familial longevity in middle aged subjects enriched for familial longevity.

#### Introduction

As the largest organ of the human body, the skin constitutes an important functional and cosmetic interface with the exterior world. The disintegration of this protective barrier with age is partly visible as wrinkling and loss of elasticity. Although ageing is associated with a general progressive loss of skin integrity, significant inter-individual differences exist. Apart from the cosmetic aspect, these inter-individual differences provide a potential biomarker for biological age as a lower perceived age based on facial photographs is associated with survival, even after correction for gender and chronological age <sup>1</sup>. The inter-individual differences in skin features can be partly explained by external factors such as smoking, alcohol and sun damage <sup>2</sup>. However, there is increasing evidence that many of these differences in perceived age are genetically determined <sup>1;3</sup>.

Wrinkling and the loss of elasticity are the result of several age related changes in the underlying histological structure. The epidermis shows a flattening at the epidermal-dermal junction and a decreasing thickness with increasing age <sup>4-7</sup>. Within the dermis, the amount and length of mature elastic fibres in the papillary dermis have been found to increase with chronological age <sup>8</sup>. In the reticular dermis the length of elastic fibres and the percentage of elastin at the upper inner arm have all been found to increase with age <sup>8;9</sup>. In addition, smoking has been positively correlated with an increased number and amount of elastic fibres in the reticular dermis of the upper inner arm <sup>10</sup>, high-lighting the importance of controlling for confounding factors in studies of skin morphometric characteristics.

Within the present study we have investigated the effect of chronological and biological age on epidermal histologic characteristics and morphology of elastic fibres in the upper inner arm. To compare with biological age, we used two established markers for biological age, the Framingham cardiovascular disease (CVD) risk score <sup>11</sup> and familial longevity. Familial longevity was defined as differences between middle aged offspring from long lived nonagenarian siblings and, as controls, their partners. Though of the same chronological age, these offspring have been previously observed to be of a younger biological age than their partners as reflected by their lower mortality rate <sup>12</sup>, beneficial glucose and lipid metabolism, preservation of insulin sensitivity and resistance to cellular stress <sup>13-15</sup>. We questioned whether a slower rate of skin ageing marks middle aged offspring of nonagenarian siblings compared to their partners.

#### Methods

#### Study cohort

In the Leiden Longevity Study genetically enriched subjects for familial longevity are compared with their partners to determine the genetic factors contributing to longevity.



Figure 1. Measurements on the epidermis and dermis

a. Thickness based on the epidermal-dermal junction (EDJ): area covered by epidermis (area between the yellow and light blue lines) divided by the EDJ (light blue) length given in  $\mu$ m. Curvature based on the stratum corneum epidermis boundary (SEB): the ratio of the length of the straight horizontal line between the margins (white) and the length of the SEB (yellow). Area covered by elastic fibres (green: in-plane, red: end-on) given in  $\mu$ m<sup>2</sup>. Layer 1: papillary dermis, layers 2 to 5: reticular dermis.

b. Area of a single elastic fibre: amount of area the fibre covers ( $\mu m^2$ ). Length of an elastic fibre: length of the skeleton of the fibre (white) without half of the loop size if present plus for each end-point of the line the distance to the nearest point on the fibre's outline ( $\mu m$ ). Thickness of an elastic fibre: ratio between size and length (nm). Curl of an elastic fibre: the ratio between the fibre's length and the maximum caliper dimension.

Four hundred and twenty one families consisting of 943 long-lived Caucasian siblings with 1671 of their offspring and 745 of the partners thereof were included in this cohort. The offspring carries on average 50% of the genetic propensity of their long lived parent <sup>12</sup>. Their partners, with whom most have shared the same environment for decades, were included as matched controls. There was no selection on demographic or health characteristics <sup>12;13</sup>. The current study includes 150 offspring and 150 partners thereof, from whom skin biopsies were taken in the period from November 2006 to May 2008. Of these 300 subjects, one subject eventually refused to have a biopsy taken and the biopsies of 13 subjects were excluded after quality check. Therefore, for the epidermis and layer 1 of the dermis data was available on 286 subjects. Nine subjects lacked data on layers 2 to 5 of the dermis and were therefore excluded from that analysis. The Medical Ethical Committee of the Leiden University Medical Center approved the study. Declaration of Helsinki protocols were followed and written informed consent was obtained from all participants.

#### Morphometric analysis of the skin

Skin biopsies (4mm) were taken from the sun-protected site of the inner upper arm, and fixed in formalin (Sigma) overnight (18-24 hours). After fixation, the biopsies were washed in fresh phosphate buffered saline twice and then dehydrated in 70% alcohol and stored at room temperature. The samples were embedded in paraffin wax and cut into sections of 4µm. Two sections of each biopsy were stained with Orcein dye. Hematoxylin and eosin staining (Sigma) was performed for quality checking purposes. A Leica DM 1000 microscope, with a Leica DFC420 Camera and Leica Application Suite Software was used to capture images of the slides with an objective magnification of x10. Image capture and analysis were performed blind to age, gender and the offspring/partner status of the subjects. To analyse the image data semiautomatically, image analysis software was constructed. The stratum corneum and epidermaldermal boundary in the image were automatically delineated by the software. The epidermis was identified by its darker nature using an intensity threshold. The stratum corneum epidermis boundary was identified through absolute intensity and degree of striation (due to its layered nature) which was quantified using a variance filter. Thereafter, manual refinement was performed to ensure accurate delineation of the epidermal boundaries. In addition, manual exclusion of regions within the images were performed if they contained large structures (hair follicles, blood vessels, glands) or large areas of non-dermis i.e. blank areas, large gaps or tears in tissue and clusters of small non-dermis areas. Morphometric measurements of the epidermis and elastic fibres are depicted in Fig. 1. Epidermal measurements included the thickness of the epidermis derived from the area covered by epidermis divided by the length of the epidermal-dermal junction (EDJ). Furthermore the curvature in the epidermis was defined as the ratio of a straight line between the horizontal margins of the epidermis over the length of the stratum corneum-epidermis boundary (SEB). Within the dermis, the area covered by the elastic fibres, their number (both corrected for the size of image area they were measured in) and the morphometrical characteristics of individual elastic fibres were measured. These morphometrical characteristics included the area ( $\mu$ m<sup>2</sup>), length ( $\mu$ m), thickness ( $\mu$ m) and the curl of an elastic fibre (defined by the ratio between its length and the caliper dimension). The elastic fibres were identified by applying an intensity threshold on the green channel of the red-green-blue color image. Resolving the measurements by depth was dealt with by using the epidermal-dermal junction as the zero-depth reference point. The dermis was divided into depth layers of 100 $\mu$ m. For each pixel on the epidermaldermal junction the corresponding pixel 100 $\mu$ m lower on the y-axis was determined. Layer 1 represents the papillary dermis, layers 2 to 5 the reticular dermis. 9 biopsies had too little depth to supply data for layer 2 to 5.

#### Potential confounders

For each subject, demographic characteristics and facial photographs as well as factors known to influence skin characteristics were available (e.g. smoking and alcohol use). To control for potential sun-exposure effects on the arm, the photographs of the face were assessed by a dermatologist blind to age, gender and offspring or partner status to determine photo/sun-damage scores <sup>3;16</sup>. This nine-point (no photodamage to severe photodamage) photonumeric scale assesses the severity of cutaneous photodamage. Information on medical history was obtained from the participants' treating physicians, including history of myocardial infarction, stroke, hypertension, diabetes, malignancy, rheumatoid arthritis and chronic obstructive pulmonary disease (COPD). Information on medication use was obtained from the participants' and divided in categories composed of anti-thrombotic, anti-COPD, anti-diabetic, other endocrine and nervous system and cardiovascular drugs including lipid lowering medication. Total cholesterol and HDL-cholesterol used for the Framingham CVD risk score were measured in non-fasted serum samples using the Hitachi Modular P 800 from Roche, Almere, Netherlands.

#### Statistics

All analyses were performed using SPSS 16.0 data editor software. The association of skin morphometric characteristics with chronological age and with offspring and partners status was analysed by linear regression using three different models. Chronological age: model 1 was adjusted for gender, model 2 was adjusted for the same covariates as for model 1 with further adjustment for external factors (smoking, alcohol and sun damage), model 3 additionally for the number of co-morbidities plus the number of medication categories. Familial longevity, comparing offspring (0) and partners (1): model 1 was adjusted for age and gender, models 2 and 3 were similar to those of chronological age.

	Offspring N=143	Partners N=143
Demographic data		
Female, %	43.4	58
Age, years	63.3 (6.0)	63.2 (7.1)
Body composition		
Height, cm	172.2 (9.1)	170.7 (8.6)
BMI, kg/m <sup>2</sup>	26.6 (3.8)	26.8 (4.1)
Fat, %	28.4 (8.0)	31.4 (8.2)
Lean mass, %	67.5 (7.6)	64.6 (7.7)
Co-morbidities, no. (%) <sup>a</sup>		
MI	1 (0.8)	4 (3.1)
CVA	2 (1.6)	6 (4.7)
Hypertension	28 (22.0)	38 (29.7)
Diabetes mellitus	4 (3.2)	13 (10.2)
Malignancy	4 (3.1)	7 (5.6)
COPD	7 (5.5)	4 (3.1)
Rheumatoid arthritis	0 (0.0)	0 (0.0)
Number of co-morbidities (0-7)	0.3 (0.6)	0.6 (0.8)
Framingham Heart Risk <sup>b</sup>	16.8 (8.2)	16.5 (9.1)
Medication		
Medication categories <sup>a</sup>	0.6 (0.8)	0.8 (1.0)
Hormonal replacement therapy (former and current), no. (%) <sup>e</sup>	8 (14.0)	9 (11.4)
Intoxications <sup>c</sup>		
Users of alcohol, no. (%)	109 (78.4)	110 (79.1)
Former and/or current smoking, no. (%)	85 (61.6)	100 (71.9)
Photodamage score <sup>d</sup>	4.8 (1.2)	4.8 (1.2)
Menopausal status, no. (%) <sup>e</sup>		
Premenopausal	0 (0.0)	7 (8.8)
Perimenopausal	0 (0.0)	5 (6.3)
Postmenopausal	58 (100.0)	68 (85.0)

Table 1. Characteristics of subjects.

<sup>a</sup>N=129, <sup>b</sup>N=182, <sup>c</sup>N=268, <sup>d</sup>N=263, <sup>e</sup>N=58 and 80 (offspring, partners). Values are given as mean (SD) if not otherwise stated. BMI: body mass index, MI: myocardial infarction, CVA: cerebrovascular accident, COPD: chronic obstructive pulmonary disease. SD: standard deviation. Medication categories composed of anti-thrombotic, anti-COPD, anti-diabetic, other endocrine and nervous system and cardiovascular drugs (including lipid lowering medication). Photodamage was determined by scoring photographs of subjects with a nine-point photonumeric standard scale (no photodamage to severe photodamage).

To study the association between cardiovascular disease risk and morphometric characteristics of the skin, the Framingham CVD risk score was calculated as previously described <sup>11</sup>. Model 1 of the linear regression consisted of the 10-year risk prediction of cardiovascular disease, model 2 adjusted for the offspring/partner status, model 3 was additionally adjusted for sun damage. P-values of less than 0.05 were considered to be significant.

#### Results

In total, 286 subjects were included in the analysis after a quality check of the biopsies, consisting of 141 males and 145 females aged between 44 and 81 years. The characteristics of the subjects are summarized in Table 1. The offspring had experienced less age related diseases such as myocardial infarction, hypertension and type 2 diabetes mellitus. Use of hormonal replacement therapy was not different between the offspring and partners, however menopausal state differed significantly. Female partners were more frequently premenopausal compared to female offspring, most likely due to their younger age.

Table 2 shows the association between chronological age and morphometric characteristics of the epidermis and elastic fibres in the dermis with and without adjustment for potential confounders (estimates are given in the supplementary Table 1). With chronological age, the epidermal thickness significantly decreased whereas the curvature parameter increased significantly, indicating a flattening of the epidermis (both P<0.001). The decreasing thickness and the overall flattening of the epidermis with chronological age remained significant after adjustment for gender, external factors (smoking, alcohol and sun damage) and health status (P=0.01 and P=0.001 respectively). In the papillary dermis (layer 1), a trend towards a larger area covered by elastic fibres (P=0.06), a higher number of elastic fibres (P=0.07), a larger area covered by a single elastic fibre (P=0.05), an increased length of an elastic fibre (P=0.08) and more curliness of an elastic fibre (P=0.02) with chronological age was observed. However, these findings were diminished after adjustment for external factors and health status. The area covered by the elastic fibres and the number of elastic fibres in the reticular dermis (layers 2 to 5) were positively correlated with chronological age (P=0.001 and P<0.001 respectively). The increase in number of elastic fibres remained significant after adjustment for potential confounders (P=0.03). None of the other elastic fibre morphometric characteristics were significantly associated with chronological age.

Table 3 shows the effect of familial longevity status, i.e. offspring and partner comparison, on the epidermal and elastic fibre morphometric characteristics adjusted for potential confounders (estimates are given in the supplementary Table 2). No differences between

	Chronological age (years)						
	1st quartile	2nd quartile	3rd quartile	4th quartile	Draha	Draha	Draha
	M:45.5-59.9	M:59.9-64.6	M:64.6-68.8	M:68.8-81.3	Model	Model	Model
	F:44.1-58.5	F:58.5-62.4	F:62.4- 66.8	F:66.8-78.3	1	2	3
Epidermisª							
Thickness based on EDJ, $\mu m$	42.4 (0.69)	42.6 (0.88)	40.2 (0.76)	38.2 (0.78)	< 0.001	< 0.01	0.01
Curvature based on SEB <sup>c</sup>	803.2 (8.50)	824.5 (6.70)	849.9 (7.52)	839.0 (9.41)	< 0.001	0.001	0.001
Dermis							
Elastic fibres, layer 1ª							
Area covered by fibres <sup>c</sup>	23.7 (1.80)	28.0 (2.33)	27.9 (2.99)	29.1 (2.70)	0.06	0.26	0.67
Number of fibres	43.4 (3.92)	53.6 (5.21)	50.9 (5.01)	52.8 (5.06)	0.07	0.18	0.59
Area of a single fibre, $\mu m^2$	27.5 (0.79)	28.7 (0.96)	29.1 (1.04)	29.9 (1.42)	0.05	0.35	0.68
Length of a fibre, µm	15.3 (0.41)	15.8 (0.43)	16.2 (0.44)	16.0 (0.50)	0.08	0.30	0.66
Thickness of a fibre, nm	1928 (32.6)	1886 (21.4)	1893 (15.6)	1910 (23.0)	0.43	0.31	0.42
Curl of a fibre <sup>c</sup>	926.1 (4.97)	906.1 (8.10)	907.1 (5.73)	907.3 (7.06)	0.02	0.10	0.24
Elastic fibres, layer 2-5°							
Area covered by fibres <sup>c</sup>	57.3 (2.76)	67.1 (3.38)	71.5 (3.92)	72.5 (4.84)	0.001	0.02	0.14
Number of fibres	85.3 (3.47)	95.6 (3.38)	103.2 (4.16)	104.4 (4.84)	< 0.001	0.01	0.03
Area of a single fibre, $\mu m^2$	47.8 (1.50)	51.4 (2.77)	51.3 (1.73)	49.2 (2.61)	0.29	0.31	0.82
Length of a fibre, µm	20.9 (0.47)	21.5 (0.68)	21.8 (0.49)	20.6 (0.76)	0.68	0.70	0.74
Thickness of a fibre, nm	2153 (18.2)	2177 (22.3)	2175 (18.3)	2155 (25.8)	0.35	0.26	0.74
Curl of a fibre <sup>c</sup>	909.4 (4.02)	903.5 (4.73)	902.5 (5.45)	898.3 (8.40)	0.49	0.97	0.98

Table 2. Chronological age and morphometric skin characteristics.

<sup>a</sup>1<sup>st</sup> quartile: N=71, 2<sup>nd</sup> quartile: N= 72, 3<sup>rd</sup> quartile N=72, 4<sup>th</sup> quartile: N=71. <sup>b</sup>1<sup>st</sup> quartile: N=69, 2<sup>nd</sup> quartile: N=72, 3<sup>rd</sup> quartile N=68, 4<sup>th</sup> quartile: N=68. M=male, F=female, <sup>c</sup>=x10^3. Values are given as mean (SE). EDJ=epidermal-dermal junction, SEB=stratum corneum epidermis boundary. P values are derived from linear regression analysis. Model 1: gender adjusted, model 2: as model 1 plus external factors (smoking, alcohol and sun damage), model 3: as model 2 plus health status (no. of co-morbidities, no. of medication categories). Estimates of the different models are given in supplementary table 1.

offspring and partners were observed in any of the epidermal and elastic fibre characteristics before and after adjusting for chronological age, gender, external factors and health status. Stratification by chronological age did not change the results (data not shown).

To determine whether skin morphometric characteristics were associated with cardiovascular disease risk, epidermal and elastic fibre characteristics were related to the Framingham CVD risk scores (10-year risk predictions for cardiovascular disease, FHR). The results are shown in supplementary Tables 3 and 4 for the epidermis and elastic fibres respectively. Epidermal and reticular elastic fibre (layer 2 to 5) morphometric characteristics were not significantly associated with the cardiovascular risk profile. The area covered by elastic fibres and the

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			P-value	P-value	P-value
	Offspring	Partners	Model 1	Model 2	Model 3
Epidermis <sup>a</sup>					
Thickness based on EDJ, μm	40.9 (0.56)	40.9 (0.58)	0.94	0.46	0.87
Curvature based on SEB <sup>c</sup>	832.2 (5.44)	826.2 (6.28)	0.62	0.22	0.86
Dermis					
Elastic fibres, layer 1ª					
Area covered by fibres <sup>c</sup>	27.1 (1.89)	27.2 (1.64)	0.70	0.35	0.56
Number of fibres	49.9 (3.55)	50.5 (3.29)	0.91	0.91	0.91
Area of a single fibre, $\mu m^2$	29.1 (0.83)	28.5 (0.69)	0.31	0.18	0.35
Length of a fibre, µm	16.0 (0.32)	15.6 (0.31)	0.18	0.15	0.32
Thickness of a fibre, nm	1892 (12.1)	1916 (20.5)	0.42	0.64	0.40
Curl of a fibre <sup>c</sup>	909.7 (3.48)	913.5 (5.64)	0.53	0.15	0.16
Elastic fibres, layers 2-5 <sup>b</sup>					
Area covered by fibres <sup>c</sup>	66.3 (2.55)	67.7 (2.64)	0.95	0.65	0.86
Number of fibres	97.1 (2.76)	97.1 (2.99)	0.89	0.32	0.52
Area of a single fibre, $\mu m^2$	50.7 (1.42)	49.2 (1.72)	0.42	0.41	0.35
Length of a fibre, µm	21.5 (0.42)	21.0 (0.45)	0.27	0.31	0.16
Thickness of a fibre, nm	2186 (13.4)	2145 (16.4)	0.15	0.12	0.21
Curl of a fibre <sup>c</sup>	909.1 (3.00)	897.9 (4.94)	0.15	0.14	0.15

Table 3. Comparison of offspring and partners for morphometric skin characteristics.

<sup>a</sup>N=286, <sup>b</sup>N=277, <sup>c</sup>= x10^3. Values are given as mean (SE). P values are derived from linear regression analysis. EDJ=epidermal-dermal junction, SEB=stratum corneum epidermis boundary. Model 1: partner (1) and offspring (0), age and gender, model 2: as model 1 plus external factors (smoking, alcohol and sun damage), model 3: as model 2 plus health status (no. of co-morbidities, no. of medication categories). Estimates of the different models are given in supplementary table 2.

number of elastic fibres of the papillary dermis (layer 1) were negatively associated with the Framingham CVD risk scores (both P=0.10). Furthermore, the length of an elastic fibre and the area covered by an elastic fibre were significantly negatively related to the Framingham CVD risk scores in the papillary dermis after adjustment for potential confounders (layer 1, both P=0.01).

#### Discussion

In the present study we showed changes in morphometric characteristics of the epidermis: a decreased thickness and flattening with chronological age. In the reticular dermis the number of elastic fibres was increased with chronological age. None of the other morphometric characteristics were related to chronological age in our study population. Furthermore,

none of the morphometric epidermal and elastic fibre characteristics differed in offspring from nonagenarian siblings, genetically enriched for familial longevity and their partners as environmentally matched controls. The Framingham CVD risk scores did associate with some characteristics of the papillary dermis, however other epidermal and elastic fibre morphometric characteristics did not clearly associate with the Framingham CVD risk scores.

The Leiden Longevity Study was designed as a case-control study. We have previously demonstrated that the middle aged offspring from long-lived nonagenarian siblings have a healthier metabolic profile, a lower prevalence of cardiovascular disease and a lower mortality rate compared to their partners <sup>12:13</sup>. Thus, this unique study design enables the study of longevity-related factors that are present at middle-age.

We first examined whether skin morphometric parameters of the upper inner arm were dependent on the chronological age of the donor. Consistent with the literature, we were able to show a deterioration of the epidermal structure with chronological age consisting of a reduced thickness and a flatter appearance 4-7. In addition, we have further demonstrated that this relationship remains after adjustment for potential confounders. For elastin morphology, a distinction was made between papillary and reticular dermis, as fibroblasts from upper (papillary), mid and lower (reticular) dermal fibroblasts were observed to differentially express elastin in vitro <sup>17</sup>. An increase in the number, curl, area and length of the fibres were found in the papillary dermis, the latter two findings supporting a previous report on elastin morphology in the upper inner arm<sup>8</sup>. These associations disappeared after adjustment for potential confounders. In the reticular dermis (layers 2 to 5), a significant increase in the area covered by the elastic fibres with chronological age was found, supporting previous findings <sup>7-9;18</sup>. While we found that the number of fibres significantly increased with chronological age, even after adjustment for potential confounders, we did not find a significant difference in fibre length, contradicting previous reports <sup>8;9</sup>. These discrepancies might be due to different methodologies used, another cause might be the limited age range of our subjects.

A higher perceived age has been shown to be partly genetically determined and to be related with mortality <sup>1;3</sup>. In addition, skin wrinkling at the upper inner arm has been linked to health status <sup>19</sup>. Therefore, we next examined whether the histological changes of the skin serve as a marker of biological age, as defined by familial longevity and Framingham CVD risk. The Framingham CVD risk score is one the most established measures of disease outcome <sup>11</sup>. In addition, elastin accumulates in atherosclerotic carotid plaques <sup>20</sup> and it can bind to LDL particles in the arterial wall <sup>21</sup>. Furthermore, increased arterial stiffening has been linked to properties of elastin <sup>22</sup> and elastin morphology at the upper inner arm has been shown to correlate with that in temporal arteries <sup>23</sup>. Thus, we examined whether epidermal or elastic

fibre morphometric characteristics were related to CVD risk. The Framingham CVD risk scores associated with the average area and length of elastin fibres in the papillary dermis, even after adjustment for potential confounders. This is the first time that elastin morphology in the skin has been linked to CVD disease risk. However, the decrease in area and length of elastin fibres with increased CVD risk was opposite to their tendency to increase with chronological age. Thus, although significant associations between elastin morphology in the skin and CVD risk has been found, replication of these results is warranted.

Previously we have described that skin fibroblasts derived from the offspring of nonagenarian siblings resembled the responses to cellular stress of fibroblast strains from young subjects, i.e. were biologically younger compared to their partners representing the general population <sup>15</sup>. Based on these findings we expected that the biological differences would have been visible in morphometric skin characteristics comparing the skin of offspring from nonagenarian siblings to their partners. However, none of the morphometric epidermal and elastic fibre characteristics differed dependent on familial longevity status.

To the best of our knowledge our study is the first to address skin morphometric characteristics in relation to familial longevity including 286 middle aged subjects. The large number of very well characterized subjects is a major strength of this study. However, the chronological age range was rather limited, ranging from 44 to 81 years, with most subjects in their 50<sup>th</sup> or 60<sup>th</sup> decade of life. This possibly explains the relatively small effects of chronological age on morphometric skin characteristics and could explain why the more subtle effects of familiar longevity were not found. Another strength of the study is the method used to analyse the slides quantitatively to reduce observer variation. However, it cannot be excluded, even after optimal adjustment of the software settings that measurements such as the morphology of elastic fibres were determined without some technical variability. It is therefore possible that the subtle effects dependent on biological age could not be detected. To minimize any technical influences all measurements were performed in batches including equal numbers of offspring and partner as well as young and old subjects. The sampling of biopsies was performed under highly standardized conditions. However, we cannot fully exclude that skin tissue may have been slightly altered due to the excision procedure. Indeed, during the quality checking procedure it was noted that some biopsies contained tears, which was suggestive of thin weak skin typically associated with elderly individuals <sup>24</sup>. Ethnic differences in skin morphometric characteristics have mainly been linked to photo ageing due to differences in pigmentation <sup>25</sup>. In a morphological study of facial skin comparing black and white women differences in several histological aspects of the skin such as epidermal focal atrophy and more dermal elastic fibres in skin obtained from white women compared to black women have been described <sup>26</sup>. Another study has reported a more curled epidermal-dermal junction and a slightly thicker epidermis in black skin but no apparent differences in collagen and elastic fibre architectural organization in the dermis <sup>27</sup>. In order to rule out these possible influences of ethnicity on our results only Caucasian subjects were included in our study. All biopsies were taken from the inner site of the upper arm in a highly standardized procedure to ensure maximal comparability of the data. Although seasonal effects on thickness of the epidermis have been previously described <sup>28</sup> these effects did not apply on the analysis of biological age (familial longevity), as skin biopsies of the offspring – partner couples were taken on the same day.

Unlike many previous reports examining skin epidermal and elastic fibre morphology, a number of potential confounders were taken into consideration in our analysis. Skin biopsies were taken from the upper inner arm to ensure minimal UV damage. Since the skin might still have been exposed to some UV light during its lifetime, we additionally adjusted for sundamage scores obtained from facial photographs of the subjects. The effect of sun damage are also termed extrinsic ageing and are superimposed on the effects of intrinsic (chronological) ageing. Photo-exposed skin shows a broad band of elastic fibres in the papillary dermis including the upper part of the reticular dermis late in life. In the mid and deep reticular dermis the effect of sun damage is neglible <sup>29</sup>. Smoking is also a known possible confounder of skin morphometric characteristics, as the relative area, thickness and number of elastic fibres was observed to be increased in non sun-exposed skin of smokers compared to non-smokers <sup>10;30</sup>. We indeed observed changes in the association of chronological or biological age and skin morphometric characteristics when adjusting for external factors, indicating that external factors are closely linked to these morphometric characteristics. Moreover, several diseases have previously been linked to altered skin integrity such as the systemic disease diabetes mellitus type 2 due to increased cross-linking of proteins such as collagen <sup>31</sup>. Facial wrinkling has also been associated with COPD and smoking <sup>32</sup> and skin wrinkling at the upper inner arm to health status <sup>19</sup>. Since the association between morphometric characteristics and chronological age weakened after adjustment for disease status, chronological age might not be the most important driver for deterioration of measured epidermal and elastic fibre characteristics; these may largely been driven by external factors and health status. In support of this, in healthy middle aged subjects an association between cardiovascular disease risk and several histological skin characteristics was found.

In conclusion, typical changes of skin morphometric parameters were seen with chronological age. A slower rate of skin ageing was not found in offspring from nonagenarian siblings compared to their partners. Epidermal and elastic fibre morphometric characteristics, as measured from skin biopsies, are therefore not a potential biomarker for familial longevity in middle aged subjects. However, a link between elastic fibre morphology in the papillary

dermis and CVD risk indicates that some aspects of skin morphometric characteristics are markers of health status. Further studies should concentrate on which particular aspects of health and CVD risk relate to skin morphometric characteristics to identify the mechanisms responsible for these links.

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	Chronological age (years)					
	Model 1		Model 2		Model 3	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
Epidermisª						
Thickness based on EDJ, µm	-0.26 (0.06)	< 0.001	-0.21 (0.08)	< 0.01	-0.22 (0.09)	0.01
Curvature based on SEB <sup>c</sup>	2.56 (0.62)	< 0.001	2.62 (0.78)	0.001	2.96 (0.91)	0.001
Dermis						
Elastic fibres, layer 1ª						
Area covered by fibres <sup>c</sup>	0.35 (0.17)	0.06	0.27 (0.24)	0.26	0.12 (0.27)	0.67
Number of fibres	0.66 (0.37)	0.07	0.62 (0.46)	0.18	0.28 (0.51)	0.59
Area of a single fibre, µm <sup>2</sup>	0.16 (0.08)	0.05	0.10 (0.10)	0.35	0.05 (0.12)	0.68
Length of a fibre, µm	0.06 (0.03)	0.08	0.04 (0.04)	0.30	0.02 (0.05)	0.66
Thickness of a fibre, nm	-1.46 (1.83)	0.43	-2.26 (2.22)	0.31	-2.16 (2.67)	0.42
Curl of an elastic fibre <sup>c</sup>	-1.21 (0.51)	0.02	-0.88 (0.53)	0.10	-0.71 (0.61)	0.24
Elastic fibres, layers 2-5 <sup>b</sup>						
Area covered by fibres <sup>c</sup>	0.93 (0.28)	0.001	0.78 (0.34)	0.02	0.58 (0.40)	0.14
Number of fibres	1.17 (0.31)	< 0.001	1.03 (0.38)	0.01	0.95 (0.44)	0.03
Area of a single fibre, $\mu m^2$	0.18 (0.17)	0.29	0.22 (0.22)	0.31	0.06 (0.26)	0.82
Length of a fibre, µm	0.02 (0.05)	0.68	0.02 (0.06)	0.70	-0.02 (0.07)	0.74
Thickness of a fibre, nm	1.51 (1.60)	0.35	2.26 (2.02)	0.26	0.80 (2.37)	0.74
Curl of a fibre <sup>c</sup>	-0.30 (0.44)	0.49	-0.02 (0.56)	0.97	-0.02 (0.65)	0.98

Supplementary table 1. Chronological age and morphometric skin characteristics.

 $^{a}N=286$ ,  $^{b}N=277$ ,  $^{c}=x10^{3}$ . EDJ=epidermal-dermal junction, SEB=stratum corneum epidermis boundary. Linear regression analysis:  $\beta$ =Estimate; SE=Standard error. Model 1: gender adjusted, model 2: as model 1 plus external factors (smoking, alcohol and sun damage), model 3: as model 2 plus health status (no. of co-morbidities, no. of medication categories). **Supplementray table 2.** Comparison of offspring and partner status and morphometric skin characteristics

	Familial longevity					
	Model	1	Model 2		Model 3	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
Epidermis, mean (SD)ª						
Thickness based on EDJ, µm	-0.06 (0.79)	0.94	0.63 (0.85)	0.46	-0.16 (0.95)	0.87
Curvature based on SEB <sup>c</sup>	-4.00 (8.13)	0.62	-10.7 (8.76)	0.22	-1.80 (10.1)	0.86
Dermis, mean (SD)						
Elastic fibres, layer 1ª						
Area covered by fibres <sup>c</sup>	-0.95 (2.49)	0.70	-2.54 (2.71)	0.35	-1.75 (3.03)	0.56
Number of fibres	-1.24 (4.83)	0.91	-5.24 (5.20)	0.91	-3.49 (5.67)	0.91
Area of a single fibre, µm <sup>2</sup>	-1.10 (1.07)	0.31	-1.54 (1.15)	0.18	-1.22 (1.30)	0.35
Length of a fibre, µm	-0.60 (0.44)	0.18	-0.67 (0.47)	0.15	-0.52 (0.52)	0.32
Thickness of a fibre, nm	19.4 (24.1)	0.42	11.9 (25.1)	0.64	25.1 (23.4)	0.40
Curl of a fibre <sup>c</sup>	4.17 (6.66)	0.53	8.69 (5.96)	0.15	9.48 (6.66)	0.16
Elastic fibres, layers 2-5 <sup>b</sup>						
Area covered by fibres <sup>c</sup>	0.21 (3.64)	0.95	-1.79 (3.90)	0.65	-0.76 (4.37)	0.86
Number of fibres	-0.57 (4.03)	0.89	-4.25 (4.27)	0.32	-3.12 (4.81)	0.52
Area of a single fibre, µm <sup>2</sup>	-1.81 (2.26)	0.42	-2.02 (2.45)	0.41	-2.64 (2.83)	0.35
Length of a fibre, µm	-0.69 (0.62)	0.27	-0.68 (0.67)	0.31	-1.09 (0.76)	0.16
Thickness of a fibre, nm	-30.6 (21.0)	0.15	-36.0 (22.9)	0.12	-32.7 (26.1)	0.21
Curl of a fibre <sup>c</sup>	-8.44 (5.80)	0.15	-9.36 (6.38)	0.14	-10.3 (7.15)	0.15

<sup>a</sup>N=286, <sup>b</sup>N=277, <sup>c</sup>= x10^3. EDJ=epidermal-dermal junction, SEB=stratum corneum epidermis boundary. Linear regression analysis:  $\beta$ =Estimate; SE=Standard error. Model 1: partner (1) and offspring (0), age and gender, model 2: as model 1 plus external factors (smoking, alcohol and sun damage), model 3: as model 2 plus health status (no. of co- morbidities, no. of medication categories).

	FHR score	
	β (SE)	P-value
Thickness based on EDJ, μm		
Model 1: logFHR	-0.95 (0.64)	0.14
Model 2: as model 1 offspring/partner	-0.95 (0.64)	0.14
Model 3: as model 2 plus sun damage	-0.56 (0.65)	0.39
Curvature based on SEB <sup>a</sup>		
Model 1: logFHR	9.88 (7.36)	0.18
Model 2: as model 1 offspring/partner	9.92 (7.37)	0.18
Model 3: as model 2 plus sun damage	8.83 (7.65)	0.25

**Supplementary table 3** Framingham heart risk prediction and epidermal morphometric characteristics.

Models 1 and 2: N=182, Model 3: N=173. EDJ=epidermal-dermal junction, SEB=stratum corneum epidermis boundary. <sup>a</sup>=x10^3. Linear regression analysis:  $\beta$ =Estimate; SE=Standard error. Model 1: the log of Framingham Heart Risk, FHR (%), 10-year risk prediction of cardiovascular disease, model 2: as model 1 plus offspring/partner, model 3: as model 2 plus sun damage.

**Supplementary table 4** Framingham heart risk prediction and elastic fibre morphometric characteristics.

	FHR score			
	Layer	Layer 1 <sup>a</sup> Layers 2-5 <sup>b</sup>		
	β (SE)	P-value	β (SE)	P-value
Area of dermis covered by elastic fibres <sup>c</sup>				
Model 1: logFHR	-2.97 (2.36)	0.21	-1.70 (3.33)	0.61
Model 2: as model 1 offspring/partner	-2.99 (2.36)	0.21	-1.78 (3.33)	0.59
Model 3: as model 2 and sun damage	-4.06 (2.43)	0.10	-3.80 (3.37)	0.26
Number of elastic fibres				
Model 1: logFHR	-5.68 (4.33)	0.19	0.73 (3.59)	0.84
Model 2: as model 1 offspring/partner	-5.70 (4.34)	0.19	0.68 (3.60)	0.85
Model 3: as model 2 and sun damage	-7.51 (4.47)	0.10	-1.73 (3.60)	0.63
Area of a single elastic fibre, $\mu m^2$				
Model 1: logFHR	-2.00 (0.91)	0.03	-1.43 (2.02)	0.48
Model 2: as model 1 offspring/partner	-2.01 (0.91)	0.03	-1.41 (2.03)	0.49
Model 3: as model 2 and sun damage	-2.37 (0.93)	0.01	-1.79 (2.13)	0.40
Length of an elastic fibre, µm				
Model 1: logFHR	-0.97 (0.38)	0.01	-0.68 (0.54)	0.21
Model 2: as model 1 offspring/partner	-0.97 (0.38)	0.01	-0.67 (0.54)	0.22
Model 3: as model 2 and sun damage	-1.01 (0.40)	0.01	-0.77 (0.57)	0.18
Thickness of an elastic fibre, nm				
Model 1: logFHR	-15.6 (22.6)	0.49	20.1 (17.3)	0.25
Model 2: as model 1 offspring/partner	-16.0 (22.5)	0.48	20.5 (17.3)	0.24
Model 3: as model 2 and sun damage	-21.4 (23.1)	0.36	20.1 (18.0)	0.27
Curl of an elastic fibre <sup>c</sup>				
Model 1: logFHR	-1.78 (5.30)	0.74	3.61 (4.98)	0.47
Model 2: as model 1 offspring/partner	-1.83 (5.30)	0.73	3.79 (4.96)	0.45
Model 3: as model 2 and sun damage	1.61 (5.11)	0.75	6.11 (5.12)	0.23

<sup>a</sup> Models 1 and 2: N=182, Model 3: N=173, <sup>b</sup> Models 1, 2 and 3: N=178, Model 4: N=169.

<sup>c</sup>=x10^3. Linear regression analysis:  $\beta$ =Estimate; SE=Standard error. Model 1: the log of Framingham Heart Risk, FHR (%), 10-year risk prediction of cardiovascular disease, model 2: as model 1 plus off-spring/partner, model 3: as model 2 plus sun damage.

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