Chapter 5

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The number of p16INK4a positive cells in human skin reflects biological age.

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Abstract

Cellular senescence is a defense mechanism in response to molecular damage which accumulates with ageing. Correspondingly, the number of senescent cells has been reported to be greater in older than in younger subjects, and furthermore associates with age-related pathologies. Inter-individual differences exist in the rate at which a person ages (biological age). Here, we studied whether younger biological age is related to fewer senescent cells in middle-aged individuals with the propensity for longevity, using p16INK4a as a marker for cellular senescence. We observed that a younger biological age associates with lower levels of p16INK4a positive cells in human skin.

Increasing experimental evidence indicates that the accumulation of molecular damage underlies the ageing process and age-related pathologies [1-3]. Cellular defense mechanisms that occur in response to molecular damage include macromolecule repair, apoptosis and cellular senescence. In tissues, the prevalence of senescent cells, i.e. cells with a permanently arrested cell cycle, has been shown to increase with chronological age, both in animal models [4-7] and in humans [8;9]. Furthermore, increased numbers of senescent cells were found to associate with age-related pathologies such as atherosclerotic lesions [10], diabetes [11] and renal disease [12;13]. Higher levels of p16INK4a were associated as well with higher serum creatinine after renal transplantation [14;15].

Within the Leiden Longevity Study (LLS), we have previously shown that middle aged offspring from long lived nonagenarian siblings are biological younger than their partners, who are age and environmentally matched controls (see [16] for study design details). This is reflected in a lower mortality rate [16], a lower prevalence of cardiometabolic diseases [17], beneficial glucose and lipid metabolism, preservation of insulin sensitivity [18] and resistance to cellular stress *in vitro* [19]. The cyclin dependent kinase inhibitor CDKN2A, commonly referred to as p16INK4a or p16, has been established as a general marker of cellular senescence as p16INK4a was observed to be expressed in most senescent cells in other studies [20-23]. Here, we compared the frequency of p16INK4a positive cells in human skin biopsies from the upper inner arm of 89 middle aged offspring with familial longevity ("better agers") with those of their 89 partners. We hypothesized that the younger biological age of the offspring would be reflected in lower numbers of p16INK4a positive cells when compared to their partners.

The age of the subjects varied from 46 to 81 years, with an average of 63 years. Further characteristics of the subjects are given in Table S1. Figure 1 shows representative figures of p16INK4a staining in both epidermis and dermis. The distribution of tertiles of p16INK4a positive cells differed considerably between offspring and partners in both epidermal cells and dermal fibroblasts. In the lowest tertile of p16INK4a positive cells the offspring significantly outnumbered the partners whereas in the highest tertile the partners significantly outnumbered the offspring (Figure 2). After adjustment for possible confounders such as age, gender and smoking these correlations remained essentially unaltered. Although these correlations were present in both epidermis and dermis, the correlation between the number of p16INK4a positive cells in the epidermis and those in the dermis was low (Pearson's correlation coefficient 0.119, P=0.115).

Next, to confirm that the number of p16INK4a positive cells in human skin is a marker of biological age, we studied the relationship between the number of p16INK4a positive cells and age-related pathologies. The number of cardiovascular diseases (CVD) and medication

use was significantly associated with tertiles of p16INK4a positive cells in epidermal cells (Figure 2), also after adjustment for gender and smoking. There was no relationship between the tertiles of p16INK4a positive dermal fibroblasts and the number of CVD. However, a trend towards higher p16INK4a positivity in dermal fibroblasts and medication use was found.

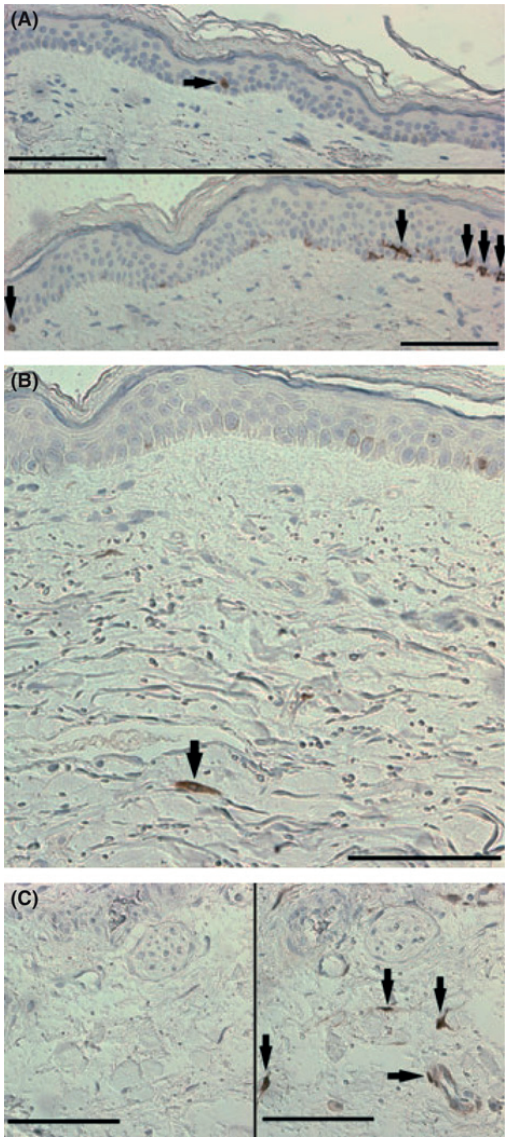

Figure 1. p16INK4a staining in human skin.
Figure 1a: representative p16INK4a staining of the epidermis from a subject in the middle tertile with 1 positive cell visible and an image of a section from a subject in the highest tertile (lower image with 5 positive cells visible). Epidermal staining was located along the basal membrane and mainly nuclear/perinuclear in nature although some cells displayed extensive cytoplasmic staining; note the dendritic nature of the fourth from right positive cell in the lower image characteristic of a melanocyte.
Figure 1b: representative p16INK4a staining of the dermis.
Figure 1c: negative control (no primary antibody, left image) and positive control (right image) of a skin sample used during all staining due to the consistent positive staining seen throughout the tissue. Line-bars represent 100µm (1 dermal counting field was 315µm by 315µm) and black arrows the locations of positively stained cells.

For the first time we have shown here that a marker of cellular senescence, p16INK4a, associates with familial longevity. In addition, we were able to reproduce earlier findings that cellular senescence *in situ* is associated with age-related pathologies. Although further evidence for the link between familial longevity and cellular senescence using other markers of senescence in skin or other tissues would strengthen these findings, the found association between p16INK4a and age-related disease is supported by a recent study. Clearance of p16INK4a positive cells in a mouse model was observed to delay the onset of age-related diseases [21]. Smoking, a well known risk factor for most age-related diseases was previously also found to associate with expression of p16INK4a in peripheral blood T-cells [24]. However, adjustment for smoking did not alter the results.
We have previously shown that skin fibroblasts from the middle-aged offspring respond to chemical stress *in vitro* with lower cellular senescence and higher apoptosis when compared to age-matched controls; this was comparable to the fibroblast response of young relative to old donors [19]. Thus, human familial longevity is not only associated with fewer p16INK4a positive skin cells *in situ*, it is also associated with fewer senescent cells after a cellular stressor *in vitro*.

It is tempting to speculate based on the evidence presented here that the accumulation of senescent cells contributes to tissue failure and ill health. A reduced rate of cellular senescence appears to be a characteristic of offspring from long-lived families both *in vitro* and *in situ*, indicating a role for cellular senescence in the healthy phenotype of familial longevity. We hypothesize that offspring, enriched for genetic effects on longevity, carry also other molecular defense mechanisms (such as repair of damage and apoptosis) of better quality than their partners, resulting in less cellular senescence. Thus, the healthy phenotype of humans enriched for familiar longevity could be related to cellular senescence. However, further work is required to determine if these findings are causative rather than associative in nature to better understand the role senescent cells have *in vivo*.

Methods

Study cohort
In the Leiden Longevity Study genetic factors contributing to longevity are studied by comparing subjects enriched for familial longevity with their partners [16]. On average 50% of the genetic propensity of their long lived parent is carried on to their offspring. Their partners, with whom most have shared the same environment for decades, were included as age and environmentally matched controls. The Medical Ethical Committee of the Leiden University Medical Center approved the study.

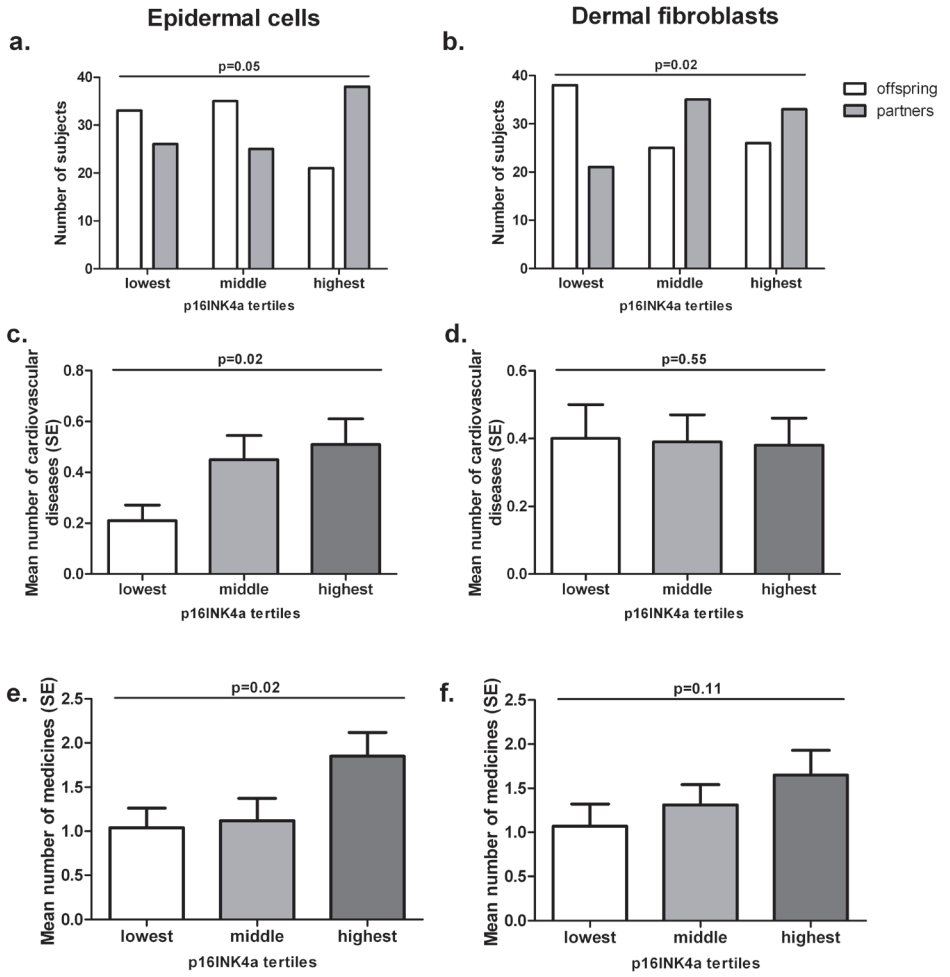

Figure 2. Tertiles of p16INK4a positivity in human skin.
Left column (a, c, e) presents epidermal cells, the right column (b, d, f) presents dermal fibroblasts. (a, b) A comparison of distribution of tertiles of p16INK4a positive cells between offspring from long-lived families and their partners, N=178. (c, d) Average number ± standard error (SE) of cardiovascular diseases over tertiles of p16INK4a positive cells, N=155. (e, f) Average number ± SE of medicines over tertiles of p16INK4a positive cells, N=136.
Tertiles of p16INK4a positive epidermal cells: lowest ≤0.30 (median=0.00), middle 0.30-1.30 (median=0.55), highest ≥1.30 (median=3.09) cells per mm length of the epidermal-dermal junction. Tertiles of p16INK4a positive dermal fibroblasts: lowest ≤0.72 (median=0.00), middle 0.72-2.05 (median=1.29), highest ≥2.05 (median 3.20) cells per 1mm2 dermis. P-values are adjusted for age, gender and smoking in a and b; for gender and smoking in c, d, e and f.

Skin biopsies

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.

Immunohistochemistry

Mouse monoclonal antibody clone E6H4 (CINtec Histology Kit, MTM Laboratories) raised against human p16INK4a protein was used, which has been previously validated as specific to the p16INK4a protein in human tissue samples [25]. CINtec clinical test reagents were used according to the manufacturers' instructions (CINtec Histology Kit, MTM Laboratories) to detect p16INK4a in the formalin fixed paraffin embedded skin tissue. Serial sections were treated for 30 minutes with one of the following, negative controls: no antibody solution (CINtec kit) or CINtec anti-p16INK4a antibody. Sections were counterstained with Mayer's hematoxylin, washed and mounted in glycergel (DAKO).

Cell counting

For dermal fibroblast counts, cell identification and counting was carried out in the papillary and upper to mid reticular areas using a x40 objective. Counts were restricted to nucleated linear or oval cells; thus, stained small circular bodies which may have been cross-sections of fibroblasts were omitted. The area of dermis available for counting varied greatly between sections due to variations in dermal depth and the presence of blood vessels, hair follicles and sebaceous glands. Thus, to control for the size of area examined for positive cells, we used a graticule (Zeis, Netzmikrometer 12) in the x10 eye piece which covered a 0.1 mm^2 area under the x40 objective. As the graticule was divided into 10x10 squares it enabled counting to be carried out in quarter graticule fields where appropriate (e.g. a hair follicle covered some of the graticule field). The total number of graticule areas (termed fields) screened per section was captured.

For positive p16INK4a cells in the epidermis, positive cells were counted along the full length of the 4 mm epidermis, this number was then corrected for the length of the epidermal-dermal junction as all cells were located in or immediately above the basal membrane.

Demographic and medical information

Demographic characteristics and medical information were available for each subject. Information on medical history was obtained from the participants' treating physicians, information on medication use from the participants' pharmacists. Cardiovascular diseases included cerebrovascular accident, myocardial infarction, hypertension and diabetes mellitus. For the number of medicines, the absolute number of different medicines was used.

Statistics

All analyses were performed using SPSS 16.0 data editor software. The data was divided into three in principle equal-sized data subsets. These tertiles of p16INK4a positivity were computed for the epidermis and dermis separately. The tertiles of p16INK4a positive epidermal cells were distributed as following: lowest ≤0.30, middle 0.30-1.30, highest ≥1.30 cells per mm length of the epidermal-dermal junction. For the tertiles of p16INK4a positive dermal fibroblasts: lowest ≤0.72, middle 0.72-2.05, highest ≥2.05 cells per 1mm^2 dermis. The association between p16INK4a positivity in both epidermal cells and dermal fibroblasts with offspring or partner status was analysed by using the Pearson chi-square test on a cross table based on p16INK4a positivity tertiles. Logistic regression was used to adjust the p-values for age and gender. Linear regression was used to calculate the association between p16INK4a positivity and number of cardiovascular diseases and number of medicines.

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Supplementary table 1. Characteristics of subjects.

	Offspring (N=89)	Partners (N=89)
Demographic data		
Female, %	50.6	50.6
Age, years	63.4 (6.17)	63.4 (7.07)
Body composition		
Height, cm	172 (8.34)	171 (8.98)
Body mass index, kg/m²	26.6 (4.23)	26.5 (3.45)
Fat, %	28.8 (8.53)	30.3 (7.7)
Lean mass, %	67.0 (8.10)	65.7 (7.32)
Co-morbidities, no. (%) [b]		
Myocardial infarction	1 (1.2)	2 (2.4)
Cerebrovascular accident	2 (2.4)	5 (6.1)
Hypertension	19 (23.5)	24 (29.3)
Diabetes mellitus	3 (3.7)	8 (9.9)
Malignancy	2 (2.5)	4 (5.0)
Chronic obstructive pulmonary disease	3 (3.7)	3 (3.7)
Rheumatoid arthritis	0 (0)	0 (0)
Number of cardiovascular diseases [b]	0.30 (0.56)	0.48 (0.72)
Number of medicines [c]	1.24 (1.68)	1.46 (1.74)
Intoxications [a]		
Users of alcohol (>1 unit/week), no. (%)	63 (75.0)	68 (80.0)
Former and/or current smoking, no. (%)	46 (54.8)	59 (69.4)

[a] N=169, [b] N=164, [c] N=136 Values are given as mean (SD) if not otherwise stated. SD: standard deviation.

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