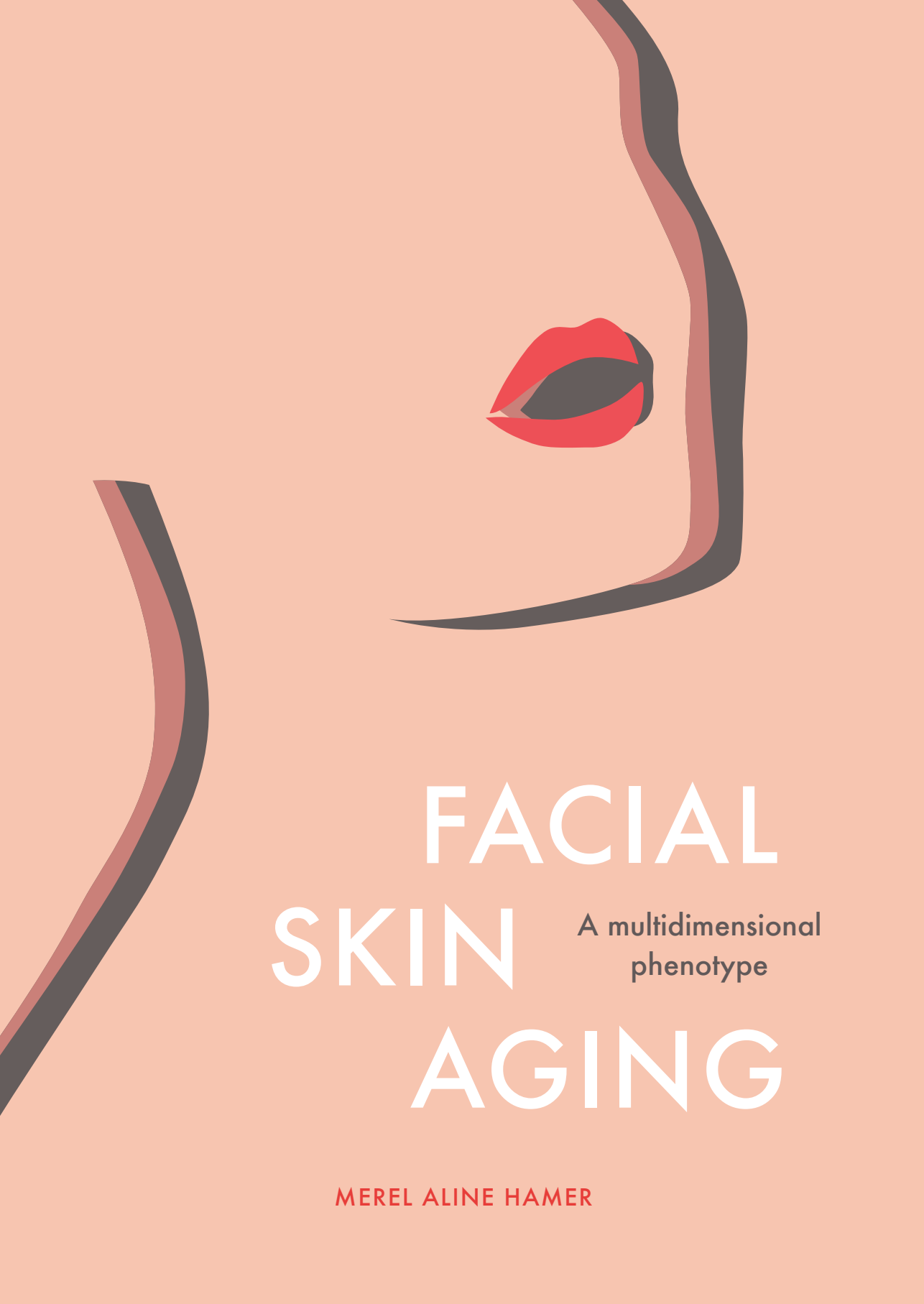

FACIAL SKIN AGING

A multidimensional phenotype

MEREL ALINE HAMER

Facial Skin Aging

A multidimensional phenotype

Huidveroudering
een veelzijdig fenotype

Merel Aline Hamer

Financial support for the publication of this thesis was generously provided by:

FitForMe
Tobrix
La Roche-Posay / L'Oréal
Olmed
Eucerin / Beiersdorf
LEO Pharma
UCB

ISBN: 978-94-6361-431-3
Lay-out and printing by Optima Grafische Communicatie (www.ogc.nl)
Cover design: Marry Teeuwen - de Jong

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Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr. R.C.M.E. Engels
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 1 juli 2020 om 11:30 uur

door

Merel Aline Hamer
geboren te Dordrecht

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotor:
Prof. dr. T.E.C. Nijsten

Overige leden:
Prof. dr. M. Kayser
Prof. dr. E.P. Prens
Prof. dr. B. van der Lei

Copromotor:
Dr. L.M. Pardo Cortes

CONTENTS

Chapter 1

General introduction

FACIAL SKIN AGING

There is a social obsession with youthfulness, which is deeply rooted in many cultures. The appreciation of youthfulness dates back to early Greek civilization, but especially in modern society appearance plays an important role. Many large cosmetic and personal hygiene companies invest astronomic amounts of money in cosmetic products focused on facial skin. In the Netherlands alone, an estimated 400.000 injectable treatments take place yearly[1]. At the other end of the scale of youthfulness, is aging. Facial aging is associated with changes in appearance as well as with declined function of the body; it reflects a person's general health[2] and emotional well-being[3]. Facial aging therefore has large biological, social and medical implications. Perceived age – the estimated age of a person – predicts survival and correlates with physical and cognitive functioning and with leucocyte telomere length[2]. Shorter telomere length has been associated with diseases related to aging and also with mortality. Thus, the importance of skin aging research reaches further than just a youthful appearance. Furthermore, understanding skin aging will help to unravel aging in general. Focusing on (healthy) aging can eventually result in a better understanding of many aging-related diseases. Besides being the largest organ of the human body, the skin is easily accessible. It is therefore the perfect target to understand aging as it may even be seen as a mirror of the internal organs.

There are different ways of assessing skin aging, although it is best to use a standardized approach. Below we describe main definitions of skin aging.

Intrinsic and extrinsic skin aging

Facial skin aging can be divided into intrinsic and extrinsic aging with clinical and pathophysiological differences[4]. Intrinsic (or innate) aging can be regarded as the 'biological clock', slowly progressing independent of external factors, but programmed in the genetic build of an individual[5,6]. It affects the skin as it affects other organs, namely by slow, irreversible tissue degeneration. Intrinsic aging gives rise to changes in the skin which decrease the functional capacity (decreased epidermal turnover, barrier function, sensory perception, vitamin D production, immunosurveillance, inflammatory response, thermoregulation, and mechanical protection) and thus cause skin vulnerability[7]. It is characterized mainly by subtle morphologic changes, such as dry skin, fine wrinkles, lax appearance and sagging[8].

Extrinsic (or acquired) skin aging results from the impact of external factors (e.g. UV-radiation, smoking and other yet to be discovered factors) and gives rise to more striking morphologic and physiologic changes. Extrinsic aging is characterized by coarse wrinkles, coarseness of the skin in general, sallow color, irregular pigmentation and telangiectasia. In an extrinsically aged skin we see more benign, but also pre-malignant and malignant neoplasms[7,9]. The term "photoaging" is also used for extrinsic aging, but this reflects only aging caused by repeated sun exposure. Examples of typically UV-related skin features are Favre Racouchot (nodular elastosis with cysts and comedones), cutis rhomboidalis nuchae (coarse wrinkling at the back of the neck) and poiki-

loderm of Civatte (mottled discoloration and dilated red veins, typically located on the chest and neck area, sparing the area under the chin).

Pathophysiology of facial skin aging

Histologically, intrinsic aging is characterized by flattening of the epidermal-dermal junction and a progressive loss of extracellular matrix (ECM) in the dermis. Increased levels of matrix metalloproteinases (MMPs) cause the breakdown of collagen[10], causing less firmness of the skin. Also, hyaluronic acid synthesis is decreased, leading to a less hydrated skin and therefore a weaker collagen network[11]. There is also a loss of fibroblasts (which produce collagen), melanocytes and Langerhans cells[12]. Moreover, the vascular network is reduced, so there is less supply of nutrients and growth factors to the skin. Decreased activity of growth factor associated protein kinases and increased activity of stress-associated kinases also lead to cell aging[13]. Not only the skin itself, but also the subcutaneous tissues show age-related changes. For example changes in the superficial muscular aponeurotic system (SMAS), loss or redistribution of fat compartments and bone resorption can ultimately lead to sagging of the skin, along with gravity[14].

Damaging environmental exposures cause the generation of reactive oxygen species (ROS)[15]. ROS cause direct deleterious effects on DNA and proteins, leading to the activation of MMPs and thus degenerative changes in the ECM (resulting in coarse wrinkling), superficial vessels (resulting in telangiectasia) and melanocytes (resulting in pigmented spots)[16]. In photodamaged skin, histology shows damaged collagen and dermal elastosis; the deposition of non-functional elastic material in upper dermis. There is an abnormal maturation of keratinocytes in the epidermis and often inflammatory cells are present due to activation of cytokines and growth factor receptors (e.g. epidermal growth factor (EGF), interleukin (IL) 1, tumor necrosis factor-alpha (TNF-α))[15].

SKIN AGING PHENOTYPES FOR EPIDEMIOLOGICAL RESEARCH

Skin aging seems a fairly straightforward endpoint, but it is actually quite complex. It is an umbrella under which many different processes take place and a concept which can be defined in many different ways. For example, skin aging can be divided into intrinsic vs. extrinsic aging. There are distinctive characteristics between intrinsic and extrinsic aging, but in practice it is difficult to separate these two in UV-exposed areas such as the face. The combined effects of both intrinsic and extrinsic facial aging result in a wide range of observable physical characteristics, which can be divided into four major phenotypes: wrinkles, pigmented spots, telangiectasia and sagging. Wrinkling is undoubtedly the most notable feature. However, all of them have an important place in the aging face.

SKIN AGING MEASUREMENTS

As mentioned above, skin aging is difficult to define and therefore measuring it is challenging as well. Many different assessments have been used in literature to investigate skin aging; most are manual photonumeric scales and consider skin aging as a compound phenotype consisting of wrinkles, pigmented spots, telangiectasia, and sagging together[17-19]. There are scarce examples of scales focusing only on one phenotype, including one for pigmented spots[20] and a skin aging atlas with photonumeric severity scales for winkles and sagging per facial site[21].

Another way of grading skin aging is by differentiating between intrinsic and extrinsic factors[8,18]. For this, the skin aging score "SCINEXA" was developed, comprising 5 items indicative of intrinsic and 18 items indicative of extrinsic skin aging[8]. These items were used to define an index allowing to quantify intrinsic versus extrinsic skin aging.

These manual photonumeric scales however, are based on subject experience and therefore prone to bias. In addition, skin aging is a continuous process, rather than a categorical one. Digital scales have also been described. In wrinkle measurement, three-dimensional (3D) skin replicas[22,23], as well as in-vivo skin surfaces[24,25], were mapped using light reflection to measure wrinkle severity on a continuous scale. In pigmented spots measurement, the affected facial area can be assessed by measuring color differences of the skin and the spots[26-28]. For sagging and telangiectasia no digital scales have yet been composed.

Another approach to investigate skin aging is by using the term perceived age: how old a person looks – as opposed to chronological age. Besides being socially relevant, perceived age has been shown to be associated with mortality, independent of chronological age[29-31]. Thus, it may be a relevant biomarker of aging.

EPIDEMIOLOGY OF FACIAL SKIN AGING

Lifestyle and physiological determinants

The four different phenotypes are associated with slightly different risk factors (Table 1). Wrinkling is the best studied phenotype of the four. Smoking and ultraviolet (UV) radiation are the most well known risk factors[32,33]. High body mass index (BMI) accounts for less wrinkles[34], most probably because facial fat has an expanding/filler effect on the skin. Other determinants that have been linked to wrinkles include education[35], alcohol[36] and female sex-steroids[37] but these findings are controversial as they have not all been replicated consistently in other studies. Less studies than for wrinkling investigated risk factors for pigmented spots. Most of them found age, cumulative UV-exposure[20,38-40], and skin color[20,38] as important determinants. In addition, in a cross-sectional study of a middle-aged white population (N=623), insulin-like growth factor (IGF-1), diagnosis of diabetes and hypertension were independently associated with facial pigmented spots[40]. These results are yet to be replicated in other studies.

Table 1. Common risk factors for skin aging (numbers in brackets are the references)

Risk factor	Wrinkles	Pigmented spots	Telangiectasia	Sagging
Male sex	(33, 35)	(35, 39)	(33, 35, 42)	(43)
Skin color	(33)	(20, 33, 38)	(33)	(43)
Smoking	(32, 33)	(20)	(33, 41, 42)	
UV	(32, 33)	(20, 38-40)	(42)	
Low BMI	(34)			
High BMI				(43)
Education	(35)			
Alcohol	(36)			
Female sex steroids	(37)			

Only few studies have specifically focused on telangiectasia. In one cross-sectional study of 1,400 subjects (aged 20-54 years), this phenotype has been associated with increasing age, male sex, fair skin, smoking and mainly outdoor occupations[33]. Smoking has repeatedly been associated with telangiectasia[41,42]. Literature on the phenotype sagging is very scarce. A study on sagging eyelids (which presumably has the same etiology and thus risk factors as sagging of the whole face) showed that male sex, lighter skin color, and higher body mass index were important determinants[43].

Genetics

Knowledge of the genetic risk factors of skin aging is quite scarce and genetic research investigating separate skin aging phenotypes even more so. One genome-wide association study (GWAS) investigated SNPs in relation to photoaging (composed of wrinkling, sagging and pigmented spots severity) in 500 French women. However, this study was too small to find genes for such a heterogeneous phenotype as photoaging; their hit only just reached the significance threshold, without replication[44]. Another small GWAS (N=428) investigating skin youthfulness in Ashkenazi jews[45] showed different hits which also were not all replicated.

Several skin aging studies have identified the melanocortin 1 receptor gene (*MC1R*) to associate with skin aging, perceived age and pigmented spots as a separate feature of skin aging[35,46]. The *MC1R* gene is well known as “the red hair color” gene and is also important in defining freckles and a light skin color. Other genetic variants associated with (features of) skin aging are scarce and have not been replicated (Table 2)[43-45,47]. This is surprising, as wrinkle variation has been shown to be a heritable trait, with a heritability of up to 55%[48]. For pigmented spots, candidate gene studies have been performed; gene variants in the pigmentation genes *SLC45A2* in Asians[49] and *MC1R* in Europeans[50] have been found to be associated with the presence of pigmented spots. To date, there have not been any studies on the genetics of telangiectasia.

Table 2. Suggestive SNPs from GWAS of skin aging

SNP	Chromosome	Position*	Gene**	Published P-value	Associated phenotype
rs7616661[a]	3	5965543	*EDEM1*	4.8×10^{-8}	Photoaging
rs6975107[a]	7	120380907	*KCND2*	4.2×10^{-9}	Photoaging
rs11863929[a]	16	88304433	*ZNF469*	1.8×10^{-8}	Photoaging
rs322458[b]	3	120585315	*STXBP5L*	1.5×10^{-8}	Photoaging
rs11876749[c]	18	3942902	*TGIF1*	1.7×10^{-8}	Sagging eyelids
rs185146[d]	5	33952106	*SLC45A2*	4.1×10^{-9}	Microtopography score
rs12203592[d]	6	396321	*IRF4*	8.8×10^{-13}	Microtopography score
rs4268748[d]	16	90026512	*MC1R*	1.2×10^{-15}	Microtopography score
rs1805007[d]	16	89986117	*MC1R*	1.2×10^{-10}	Microtopography score
rs1805008[d]	16	89986144	*MC1R*	1.1×10^{-5}	Microtopography score

Abbreviation: SNP, single-nucleotide polymorphism.
*based on GRCh37/hg19; **relationship of SNP with gene: either in, near, or in linkage disequilibrium.
[a]SNPs found by Chang et al[45]; [b]SNPs found by Le Clerc et al[44]; [c]SNP found by Jacobs et al for sagging eyelids[43]; [d]SNPs found bij Law et al in a genome-wide meta-analysis for microtopography score of the back of the hand[47].

For sagging eyelids, heritability was estimated to be 61%[43]. A GWAS showed one genome-wide significant hit; this variant is located close to *TGIF1* (an inducer of transforming growth factor ß, which is a known gene associated with skin aging)[43].

AIMS OF THIS THESIS

Most previous skin aging studies were not population based and used suboptimal measures of skin aging. As presented above, facial skin aging is a complex concept acted upon by multiple lifestyle and physiological factors. Many different phenotypes have been used to investigate risk factors associated with skin aging. However, in observational studies, it is important to use phenotypes that are relatively easy to measure accurately in large groups. Measurements derived from digital photographs are solid phenotypes because of their objectivity and easy implementation for epidemiological and genetic skin aging studies.

Given the complexity of facial aging, we decided to investigate determinants for different features of skin aging instead of focusing on a single phenotype. In this thesis, I have investigated wrinkles, pigmented spots and telangiectasia, using digital grading. In addition, the phenotype perceived age was studied. Sagging reflects mainly subcutaneous changes and has proved difficult to grade, therefore this feature was not added. The following topics are described:

PART I – VALIDATION

Since a new digital method for quantifying different skin aging subtypes was used, validation of the methods was necessary. In **Chapter 2**, we performed a validation study for the measurements used to quantify the different skin aging phenotypes.

PART II – WRINKLES

Wrinkles are the largest and most important subtype of skin aging. In the second part of this thesis, we investigated main determinants for wrinkles as assessed within the Rotterdam Study (RS). In **Chapter 3,** we investigated main epidemiological determinants of facial wrinkling. In **Chapter 4,** we studied genetic factors of facial wrinkling in the RS and the Leiden Longevity Study (LLS).

PART III – OTHER SKIN AGING PHENOTYPES

As mentioned above, other phenotypes associated with skin aging were also available, including pigmented spots, telangiectasia and perceived age. As of today, not much is revealed about these features. Therefore, we aimed to define genetic determinants of pigmented spots in the RS (**Chapter 5**), epidemiological factors of telangiectasia in the RS and the SALIA cohort (**Chapter 6**), and genetic factors of perceived age in the RS, the LLS and TwinsUK (**Chapter 7**). In **Chapter 8**, we investigated the relationship between vitamin D and skin aging in the RS and LLS. Finally, in **Chapter 9**, we investigated the relationships between the different features of skin aging using principal component analysis.

STUDY DESIGN

We performed epidemiological and genetic studies using data from the RS, a large population-based cohort study in which genotypes and many different phenotypes are prospectively collected[51]. Fully standardized 3D photographs of the face have been derived from the facial photos to assess the different skin aging phenotypes. For replication purposes, we also used data from other cohorts: Leiden Longevity Study (a family-based study), TwinsUK (a nation-wide twin registry), and SALIA (middle-aged women from the urban Ruhr area and two rural northern counties in Germany).

FUNDING

The studies in this thesis were funded by Unilever. The Rotterdam Study is funded by the Erasmus Medical Center and Erasmus University Rotterdam; the Netherlands Organization for the Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); the Ministry of Education, Culture and Science; the Ministry of Health, Welfare and Sports; and

the European Commission (DG XII). The generation and management of GWAS genotype data for the Rotterdam Study is supported by the Netherlands Organization of Scientific Research NWO Investments (nr. 175.010.2005.011, 911-03-012). Although no products were tested, it is possible that this thesis could promote products that reduce the appearance of wrinkles, which could lead to financial gain for Unilever.

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PART I

VALIDATION

Chapter 2

Validation of image analysis techniques to measure skin aging features from facial photographs

M.A. Hamer
L.C. Jacobs
J.S. Lall
A. Wollstein
L.M. Hollestein
A.R. Rae
K.W. Gossage
A. Hofman
F. Liu
M. Kayser
T. Nijsten
D.A. Gunn

Skin Res Technol. 2015 Nov;21(4):392-402

ABSTRACT

Background: Accurate measurement of the extent of skin aging is challenging, but crucial for research. Image analysis offers a quick and consistent approach for quantifying skin aging features from photographs, but is prone to technical bias and requires proper validation.

Methods: Facial photographs of 75 male and 75 female northwestern European participants, randomly selected from the Rotterdam Study, were graded by two physicians using photonumeric scales for wrinkles (full face, forehead, crow's feet, nasolabial fold and upper lip), pigmented spots and telangiectasia. Image analysis measurements of the same features were optimized using photonumeric grades from 50 participants, then compared to photonumeric grading in the 100 remaining participants stratified by sex.

Results: The inter-rater reliability of the photonumeric grades was good to excellent (intraclass correlation coefficients 0.65-0.93). Correlations between the digital measures and the photonumeric grading were moderate to excellent for all the wrinkle comparisons (Spearman's rho ρ=0.52-0.89) bar the upper lip wrinkles in the men (fair, ρ=0.30). Correlations were moderate to good for pigmented spots and telangiectasia (ρ=0.60-0.75).

Conclusion: These comparisons demonstrate that all the image analysis measures, bar the upper lip measure in the men, are suitable for use in skin aging research and highlight areas of improvement for future refinements of the techniques.

INTRODUCTION

Skin aging is a heterogeneous phenotype, which includes features such as wrinkles, pigmented spots, and telangiectasia (i.e. red veins). During the last few decades, people have become increasingly concerned about their appearance, with facial skin aging being a critical component[1]. Consequently, basic and clinical research on this topic has expanded rapidly. To measure the degree that skin has visibly aged, several different photonumeric scales have been published, which are feature specific or a combination of different skin aging features[2-5]. However, a recognized gold standard scale for skin aging is still lacking.

Griffiths et al[2] introduced one of the first facial skin aging scales, assessing photoaging as a single entity, combining wrinkles, pigmented spots and telangiectasia in a 9-point scale. Larnier et al[3] also created a photonumeric scale, but introduced three different photographs per grade to cover the variable nature of photodamage. Subsequently, photonumeric scales for wrinkles at different facial sites were created to evaluate aesthetic procedures, either using photographs[4] or computer-simulated images[6]. Other scales differentiated between the relative contribution of intrinsic vs. extrinsic factors to facial skin aging[7,8]. For pigmented spots, a few photonumeric severity scales are available for Caucasian[9-11] and non-Caucasian populations[12]. For telangiectasia, available scales mainly capture improvement after cosmetic procedures[13]. Only a few scales have been published for epidemiological purposes, either descriptive[8,14] or photonumeric[11]. However, the inter-observer agreement for the photonumeric scale was rather low and only telangiectasia in the crow's feet area were taken into account[11].

In addition to these categorical scales, there are quantitative rating scales that measure three-dimensional (3D) details of the skin surface using skin replicas[4,15] or computer-assisted skin surface topography[16]. Raking light optical profilometry applied directly to facial photography[17] is another method to quantitatively measure wrinkles, providing multiple wrinkle parameters, including wrinkle number, length, width, area and depth. Correlations with photonumeric grading of crow's feet were good, although correlations for the other facial sites were not mentioned[17]. Recently, a 3D fringe projection method was used to measure facial wrinkles[18,19]. It was utilized to estimate the likelihood of the lifetime development of wrinkles, based on wrinkle differences between age groups[18]. Digital measures previously developed for pigmented spots measure the affected skin area using various image analysis techniques[20-22]. However, none of these techniques, nor image analysis techniques for measuring telangiectasia, have been validated against photonumeric grading.

The potential advantages of digital measurements are their sensitivity, reliability and generation of continuous outcomes. In contrast to digital methods, photonumeric grading can be unwittingly influenced by other features of aging such as hair graying or facial sagging. In addition, it seems plausible although speculative that digital measurement is more sensitive to subtle pre-clinical aging, which is not always visible to the human eye. Digital measurement is also time-saving which is of benefit for research, particularly in large cohorts. Furthermore, a continuous digital measure

may detect smaller differences between individuals and, therefore, have more power to detect associations in observational studies compared to photonumeric categorical scales. However, technical influences (e.g. variations in lighting) affect image analysis techniques and hence blinded tests are required to determine the similarity of the digital measures with human expert assessment.

The aim of this study is to create and validate digital measurements for wrinkles, pigmented spots and telangiectasia, using high-resolution digital photographs.

METHODS

Study population

The Rotterdam Study (RS) is a prospective population-based cohort study conducted in Ommoord, a suburb of Rotterdam, the Netherlands. Details of the study design and objectives have been described elsewhere[23]. From August 2010 onwards, standardized high-resolution digital 3D facial photographs were collected on participants at the RS center (N=4648 to date). The current study included images of 150 participants, all of northwestern European ancestry. The RS has been approved by the medical ethics committee according to the Wet Bevolkingsonderzoek ERGO (Population Study Act Rotterdam Study), executed by the Ministry of Health, Welfare and Sports of the Netherlands and all participants provided written informed consent.

Image acquisition

For all participants, high resolution standardized full face photographs were obtained with a Premier 3dMD face3-plus UHD camera (3dMD, Atlanta, GA, USA), in a room without daylight. Participants focused on a standardized viewpoint and were asked not to wear any make-up, facial cream, or jewelry. Three two-dimensional (2D) photographs (2452 × 2056 pixels, 14.7MB in BMP format) were taken simultaneously from three prefixed angles (one upper frontal and two 45° lateral photos). By combining these photos, the 3dMD software (www.3dmd.com) created an image containing 3D information of the whole face. The machine was calibrated daily to control for camera position and environmental light intensity.

Photonumeric grading

We created new 5-point scales for full face wrinkles, pigmented spots and telangiectasia. Full facial wrinkles have different patterns in men and women[18,24,25] but there are no sex-specific scales available in the literature. Therefore, we established new sex-specific scales for full face wrinkling, based on photodamage grading scales by Griffiths et al.[2] and Larnier et al.[3], using images from the RS. For pigmented spots and telangiectasia, there was no accessible photonumeric scale available beyond the crow's feet area. Therefore, we created new scales as for global wrinkles, but these were not sex-specific because there seemed to be little difference in facial location of pigmented spots and telangiectasia between men and women. Pigmented spots included both solar lentigines

and seborrheic keratoses. Freckles, nevi and actinic keratoses were not considered as pigmented spots. For telangiectasia grading, we took into account only red and purple-blue vein like structures as well as spider nevi. Erythema, red papules and other reddish structures in the face were ignored.

For the photonumeric grading of the forehead, crow's feet, nasolabial fold and upper lip wrinkles, we used the Skin Aging Atlas book[26], which is based on several published scales[2-4,27]. The scales within the book are focused solely on the depth of the deepest wrinkle but for the crow's feet area, a scale for the number of wrinkles was also available. Hence, for the crow's feet we also generated an overall wrinkle severity score ((number + depth)/2). The location-specific scales consisted of either 6 or 7 grades[26]. In order to create uniformity, we only used six grades and in case of seven, we discarded the lowest one, considering that our study was conducted in an elderly population.

For all skin aging features (full face wrinkles, forehead wrinkles, crow's feet, nasolabial fold, upper lip wrinkles, pigmented spots and telangiectasia), an optimization set of 50 photos was graded by two independent physicians (MAH and LCJ) for all seven features. Subsequently the two physicians discussed any grading differences and reached a consensus grade; these grades were also used to optimize the digital measurements. A validation set of 100 photos was then graded blindly by the two physicians for the same seven features.

Masking of photographs

Full face wrinkles

For quantification of wrinkles on the whole face, standardized 2D front and side images were generated from the 3D rendering (1920×1080 pixels, 1MB in TIF format) using Blender (http://www.blender.org/v2.7) as the original front 2D photographs were taken from above the participants, causing the chin to be tilted away from the camera reducing the area of skin visible. The photographs were masked to isolate the skin areas in the image using semi-automated masking (MATLAB, The MathWorks, Inc, Natick, MA, USA, version 2013a), Figure 1A.

Wrinkles per localized facial site

The original 2D photographs of the left-hand side of the face were used to measure wrinkle severity at localized facial sites as they had a higher resolution than their 3D equivalent. A bespoke semi-automated program cropped localized sites (forehead, crow's feet, nasolabial fold and upper lip) from each image, Figure 1B.

Pigmented spots

The 2D front photographs were used to generate the pigmented spots digital measure, since the higher resolution was necessary to detect subtle color differences of the skin between small objects (e.g. pores versus senile lentigines). Masking was applied to each image similar to the full face wrinkle masking but additionally excluding the jaw and mouth area (Figure 1C), because stubble in men can influence the measurement.

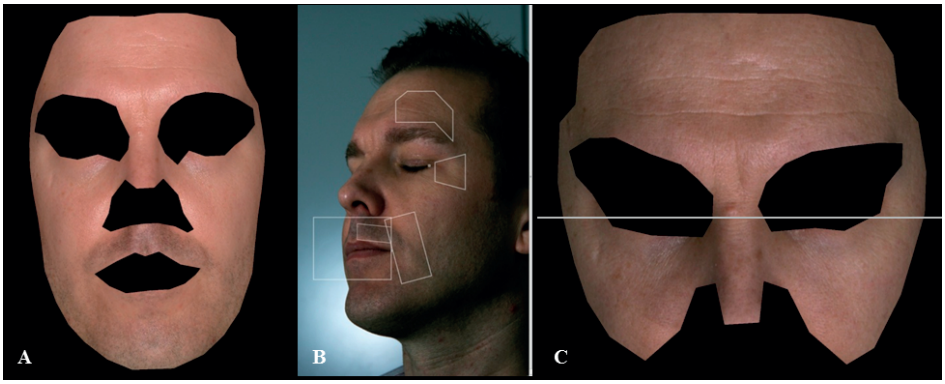

Figure 1. Examples of masking and the delineation of localized sites in images. (**A**) Masking of an image produced from the 3D rendering for full face wrinkle measurement. Non-skin features that could be detected as wrinkles (i.e. eyes, eyebrows, hair, ears, nostrils, and lips) were masked as well as the shadowing that was present along outer most lateral sides of the face. A mask was placed onto the image using the position of the eyes and mid-upper lip vermilion border, with mask position refinement performed manually. (**B**) Lateral left side 2D photo prepared for wrinkle measurement at different regions. New site images were delineated via positioning of points at the lateral canthus of the left eye and the left corner of the mouth; the distance from the eye to the mouth was used to ensure correct sizing and positioning of each box. The upper lip was further segmented from the surrounding features in the box region surrounding the mouth using a point at the mid-upper lip vermilion border. (**C**) A masked image prepared for pigmented spot digital measurement, the line across the image represents where the image was additionally cropped for telangiectasia measurement on the cheeks and nose.

Telangiectasia

The 2D front photographs that had been previously masked for the pigmented spots measurement were used to measure red veins on the nose and cheeks. The images were further cropped down the face, removing the forehead (Figure 1C), using Adobe Photoshop CS4 (www.adobe.com). Differently to pigmented spots, telangiectasia almost solely present on the nose and cheeks.

Image analysis

All image analyses were conducted using MATLAB.

Wrinkles

First, large scale shading in the image was removed by flat-fielding the image – dividing the original image by a Gaussian filtered version of the image and then rescaling; the image was smoothed using Gaussian and median filters to remove fine skin texture and very small objects such as pores (Figure 2A-B). The 2nd derivative (which highlights dark ridges, Figure 2C) was used for a dual threshold technique inspired by the Canny Edge Detector algorithm. Low and high thresholds were applied separately using the red green channels for the high threshold and the red channel for the low threshold. Two new binary images containing candidate wrinkle areas were generated, with smaller finer wrinkles more commonly present in the low threshold image (Figure 2D-E). The candidate wrinkles in both images were accepted or rejected based on shape

(eccentricity and solidarity), intensity, and direction metrics. A line connection algorithm on the high threshold binary image was additionally performed (Figure 2E) to prevent rejection by the size of wrinkles broken into sections. Hence, line sections were connected if they were close to each other and pointing in a similar direction. The final detected wrinkles were taken from the low threshold binary image if they overlapped with part of a wrinkle in the high threshold image (Figure 2F). Wrinkles in the low threshold image were also included if they were not detected by the high threshold filtering but were very linear in nature (eccentricity threshold) and above a certain size.

Finally, a number of wrinkle variables were outputted: (1) Area, consisting of the cumulative number of pixels detected as wrinkles as a percentage of total skin area (i.e. the unmasked skin for full face wrinkles and the box area for localized site wrinkles). (2) Number, consisting of the total number of individual detected wrinkle lines, corrected for total skin area. (3) Length, consisting of the cumulative length of (skeletonized) areas detected as wrinkles, normalized by the square root of the total skin area. (4) Mean width, the average width of the detected wrinkles. (5) Depth, average of the 2nd derivative values for the pixels detected as wrinkles.

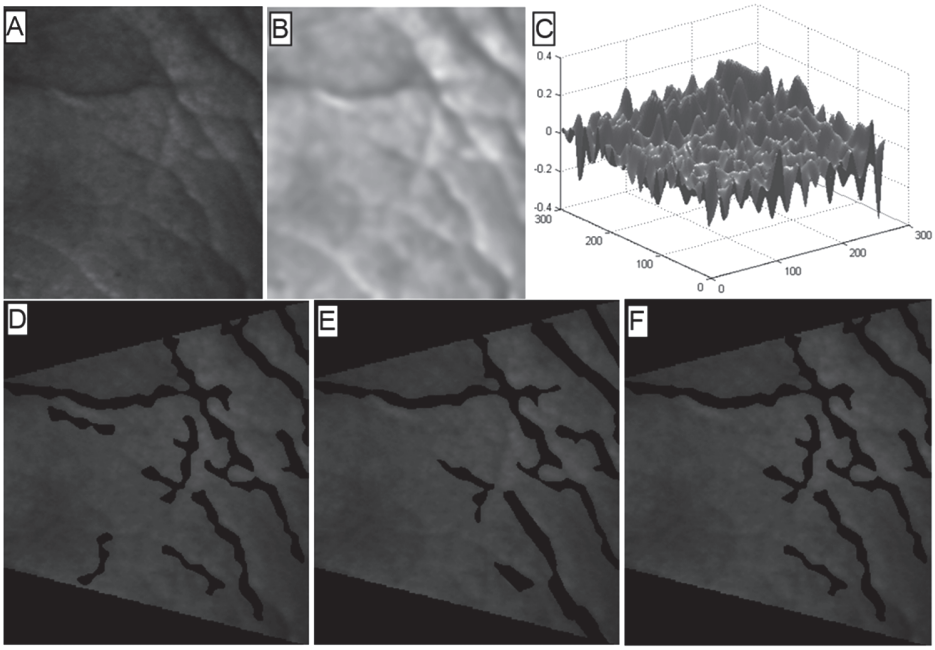

Figure 2. Illustration of dual threshold wrinkle detection on a crow's feet image. (**A**) Shows the original image; (**B**) is a flat-fielded and smoothed image; (**C**) a 3D representation of (B) which is an approximation of the 2nd derivative. The 2nd derivative detects bright and dark ridges in the image; dark ridges have positive values and correspond to wrinkles in the image. (**D**) Wrinkles detected by the low threshold (black lines), (**E**) wrinkles detected by the high threshold detection and (**F**) the final detected wrinkles – i.e. wrinkles in the low threshold image that intersect those in the high threshold image.

Pigmented spots

For the detection of pigmented spots and telangiectasia, we used the Difference of Gaussians technique on all three RGB channels. This algorithm uses a 2D Gaussian filter at two sizes to create new "contrast" images. A low-pass filter is used with a large standard deviation and a high-pass filter is used with a small standard deviation. The two filtered images from each RGB channel were subtracted and the resultant difference used to generate a contrast image (Figure 3B). Pigmented spots in the contrast image appear as blue spots (as the greatest contrast in their appearance to surrounding skin is in the blue channel). To further filter out spurious artifacts an intensity ratio threshold (targeting pixels with high blue values relative to their green and red values), a minimum pixel size (to remove noise), a solidarity threshold (to remove branched objects) and an eccentricity threshold (to remove linear objects – e.g. wrinkles) were applied to the contrast image, Figure 3B. The digital output of the detected blue spots consisted of two measures: (1) Area, consisting of the cumulative detected bluish and roundish areas, as a percentage of total skin area. (2) Number, consisting of the total number of individual detected areas, corrected for total skin area.

Telangiectasia

A contrast image was also created for detecting telangiectasia. Red/purple veins would appear green in color in the contrast weighted image, so an algorithm and threshold was applied to detect pixels with high green relative to red and blue values; additionally filtering was applied to target linear (eccentricity) and branched structures (solidarity), Figure 3C. As for pigmented spots, the digital output consisted of two measures: (1) Area, consisting of the cumulative detected greenish linear areas, as a percentage of total skin area. (2) Number, consisting of the total number of individual detected areas, corrected for total skin area.

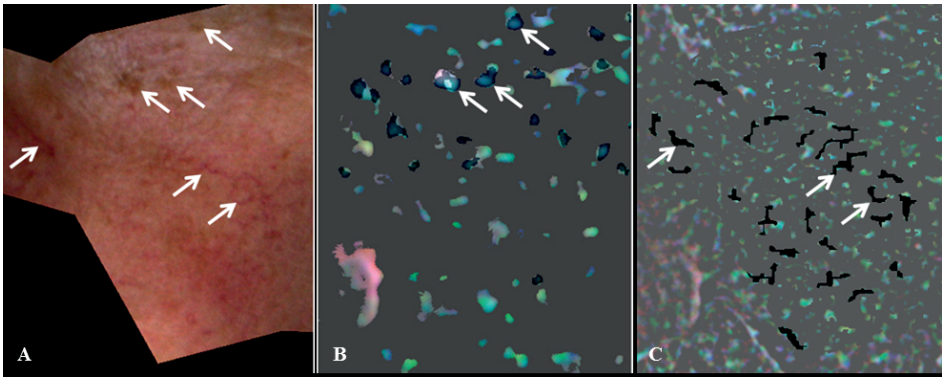

Figure 3. Illustration of pigmented spot and telangiectasia detection. (**A**) Shows the original image with pigmented spots (left facing arrows) and telangiectasia (right facing arrows); (**B**) is the contrasted image targeted to features approximate in size to pigmented spots, brown features appear blue. Detected spots are shaded; (**C**) is the contrasted weighted image targeted to features approximate in size to telangiectasia, red features appear as green and branched green objects were detected (black lines in image).

Statistical analysis

The intraclass correlation coefficient (ICC) was used to determine inter-rater reliability between the two investigators. A Two-Way Mixed model with the participants as a random factor and the raters as a fixed factor was used, with the ICC representing the reliability of the raters in the sample[28]. In case of a significant systematic difference in means between the two graders (i.e., someone graded consistently lower or higher), as tested by the paired-samples t-test, we used the absolute agreement type. Otherwise, we used the consistency type[28]. A correlation coefficient of ≥0.70 indicates a high reliability, 0.40–0.60 represents a moderate reliability and ≤0.3 a low reliability[29-31].

We calculated the Spearman's correlation coefficient (ρ) to describe the agreement between the average photonumeric grades (ordinal categorical variable) and the digital measurements (continuous variable). To interpret the similarity between the image analysis measures and photonumeric grading we used Colton's[32] recommendation of 0.25-0.50 to be fair, 0.50-0.75 to be moderate to good and >0.75 as very good to excellent. Men and women were analyzed separately as there appeared to be considerable differences between sexes. All analyses were performed using SPSS for Windows version 21.0 (SPSS, Chicago, IL, USA). A two-sided P-value of <0.05 was considered statistically significant.

RESULTS

Study population

All participants (N=150) were of northwestern European origin; the blinded comparisons between photonumeric and digital grading were based on a subgroup of 100 participants, with a mean age of 72.2 ±4.3 for the men and 71.4 ±3.7 for the women.

Photonumeric grading

The blinded inter-rater reliability of the photonumeric grading scales was good to excellent for all seven features. Full face wrinkles, pigmented spots and telangiectasia showed excellent ICCs (0.78-0.93). For wrinkle severity per site, the ICC was excellent for the forehead, crow's feet in men, nasolabial fold and upper lip (0.79-0.93), and good for crow's feet in women (0.65).

Digital measures

For the seven skin aging features, the mean affected area varied greatly, ranging from 0.6% for telangiectasia to 8.4% for crow's feet in men (Table 1). Detected wrinkles covered on average 5% of the face in both men and women, and covered more area on the forehead, crow's feet, and female upper lip (5.6% - 8.4%). However, upper lip wrinkles in men covered a notably smaller area (2.0%). Compared to the wrinkle features, the affected area of pigmented spots and telangiectasia was up to 10 times smaller. Although the photonumeric wrinkle grading for the localized facial

sites (i.e. forehead, crow's feet, nasolabial fold and upper lip) focused on the depth of the deepest wrinkle, the digital measure of depth (average depth of all wrinkles) did not give notably higher correlations than the digital area measure (e.g. Table 2).

Table 1. Means for the digital measures for all seven skin aging features and their correlations with average photonumeric grading, in men and women

	Men (N=50)		Women (N=50)	
Skin aging feature	**Mean ± SD**	**ρ**	**Mean ± SD**	**ρ**
Full face wrinkles	5.3 ± 2.2	0.79	5.2 ± 2.8	0.89
Forehead wrinkles	8.2 ± 6.5	0.63	6.9 ± 6.2	0.63
Crow's feet wrinkles	8.4 ± 5.0	0.52	5.6 ± 4.8	0.81
Nasolabial fold wrinkle	1.2 ± 1.0	0.86	0.6 ± 0.7	0.58
Upper lip wrinkles	2.0 ± 2.5	0.30	6.1 ± 6.4	0.76
Pigmented spots	0.8 ± 0.5	0.70	2.1 ± 1.0	0.69
Telangiectasia	0.6 ± 0.3	0.75	0.8 ± 0.5	0.60

Abbreviations: ρ, Spearman's correlation coefficient; SD, standard deviation.
Digital measures represent mean percentages of the affected area per total skin area. Spearman's correlation coefficients between the digital measures and photonumeric grading for each feature are given.

Table 2. Correlations between the different digital wrinkle measures outputted by the image analysis and average manual photonumeric grading for the crow's feet region

		Photonumeric grades	
Digital measures		**Depth**	**Number and depth**
Men	Number	0.40	0.62
	Depth	0.57	0.55
	Width	0.49	0.47
	Length	0.48	0.67
	Area	0.52	0.70
Women	Number	0.71	0.77
	Depth	0.58	0.59
	Width	0.55	0.53
	Length	0.80	0.86
	Area	0.81	0.86

Abbreviations: ρ, Spearman's correlation coefficient; Depth, average of 2 graders; Number and depth = (average number + average depth)/2.
The inclusions of wrinkle number as well as depth to the photonumeric scores increased the correlations, particularly for the digital number, length, and area measures.

Photonumeric grading vs. digital measures

Overall, the correlations between the photonumeric grading and digital measures were moderate to excellent for both sexes (ρ>0.50, P-value<0.001), except for upper lip wrinkles in men (ρ_m=0.30, P-value=0.035), Table 1. Full face wrinkle area gave excellent correlations with the photonumeric

grading in men and women (ρ_m=0.79 and ρ_w=0.89). The correlations between the photonumeric grading and the localized wrinkle area measures were excellent for nasolabial fold in the men, and upper lip and crow's feet in the women (ρ_m=0.86; ρ_w=0.76 and ρ_w=0.81, respectively), moderate-to-good for the forehead and crow's feet in the men, and for the forehead and nasolabial fold in the women (ρ_m=0.63 and 0.52, ρ_w=0.63 and 0.58, respectively), but fair for the upper lip in the men (ρ_m=0.30). A combined photonumeric score of wrinkle number and depth increased the correlations with the crow's feet digital area measure (ρ_m =0.52 to 0.70 and ρ_w=0.81 to 0.86, Table 2). For pigmented spots, there was a good correlation between photonumeric grading and the digital measures in both men and women – both approximately 0.7 (Table 1). The correlations for the digital telangiectasia area with the photonumeric grading were also good, particularly in the men (ρ_m=0.75 and ρ_w=0.60, Table 1).

The increase in the digital measures per increase in photonumeric grade was consistent for full face wrinkles, pigmented spots and telangiectasia (Figure 4). Per photonumeric grade, the digital measures significantly increased (Figure 4A) bar for pigmented spots grades 4-5 (Figure 4B) and telangiectasia grades 1-2 (Figure 4C).

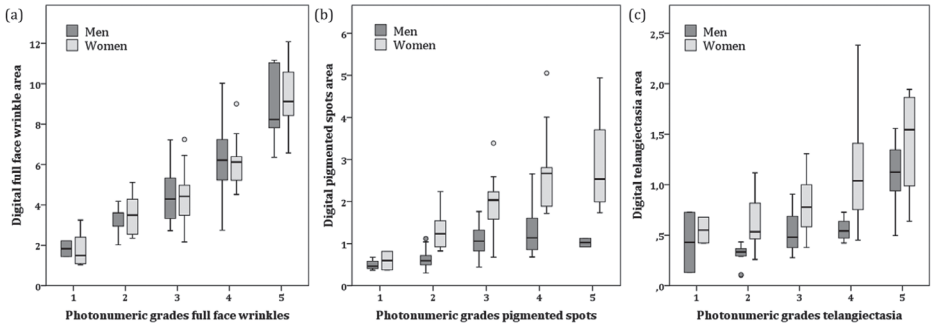

Figure 4. Boxplots of photonumeric vs. digital measures for three skin aging features, separately for men (N=50) and women (N=50). The average photonumeric grades (rounded up for half values) are shown on the x-axis, digital measures on the y-axis. The band in the box represents the median, with the bottom and top parts the first and third quartile. The bottom vertical line indicates data within 1.5 of the interquartile (IQR) range of the 1st quartile, and the top vertical line represents data within 1.5 IQR of the 3rd quartile. (**A**) Full face wrinkle measurement; (**B**) pigmented spots measurement; (**C**) telangiectasia measurement.

DISCUSSION

The digital area measures for wrinkles, pigmented spots and telangiectasia had moderate to excellent correlations with photonumeric grading, with the correlation for the upper lip wrinkle measure in men being the only exception.

Although there is no gold standard for photonumeric grading of the different components of skin aging, the good to excellent inter-rater reliability of our photonumeric scales suggests they are a valid comparative measurement for digital measures. The photonumeric full face wrinkle

scale, which was based on a combination of different wrinkle severity characteristics (i.e. number, length, width and depth), had higher correlations with the digital area measure than the photonumeric wrinkle grading from the localized wrinkle sites. This was likely due to the fact that the photonumeric grading for the localized sites graded the depth of the deepest wrinkle rather than overall wrinkle severity. A combined photonumeric score for crow's feet wrinkle number and depth gave higher correlations with the digital area measure than photonumeric depth alone, indicating that area was indeed a better measure of overall wrinkle severity than wrinkle depth. However, the digital depth measure did not have consistently higher correlations with photonumeric depth than the digital area did. This could be due to the fact that digital depth represented the average depth across all detected wrinkles rather than the depth of the deepest wrinkle. Hence, for future validation studies we recommend comparing the area of wrinkles detected with a photonumeric scale of overall wrinkle severity or, if depth of the deepest wrinkle is a research interest, adapting the image analysis techniques to generate a more similar digital measure.

All outcomes were stratified by sex because visible skin aging differs between men and women[18,24,25]. Although evaluating sex differences in skin aging warrants investigation in larger studies, we found sex differences in the correlations between the digital measures and photonumeric grading. The crow's feet and upper lip wrinkle measures in the men showed a much lower correlation than in the women. Male sex is an independent risk factor for sagging of upper eyelids[33], which can merge with crow's feet wrinkles. On inspection, eyelid sagging was found to be detected by the image analysis in some images, but was ignored by the graders. Hence, sagging eyelids in men could be reducing the correlation between digital wrinkle area and the photonumeric grading. As eyelid sagging and crow's feet wrinkles are likely two distinct phenotypes, distinguishing between the two features in future image analysis techniques will help isolate the risk factors specific to each.

The lowest correlation between the image analysis and photonumeric grading was for the upper lip in the men. On visual inspection of the images, we identified three main reasons. First, the men had very few wrinkles on the upper lip compared to the women; this sex difference has been confirmed in other studies[25]. This means that any error in the image analysis (e.g. missing the only wrinkle present) has a much larger impact on the digital measure. Second, the region of the upper lip used for digital measurement was small (see Figure 1B) compared to that used by the graders (full upper lip region) and the deepest wrinkle (which was the only one graded) lay outside the digital area for some male participants. Third, the presence of stubble in this region meant there were a few individuals where the darkness of the stubble facilitated the odd erroneous wrinkle detection. Hence, further optimization and validation of the upper lip wrinkle detection in men is required (e.g. to eliminate stubble effects and enlarge the lip area analyzed).

For the women, the nasolabial fold area correlation with the photonumeric grading was lower than for the men. On visual inspection of the detected nasolabial fold in the participant images, the women were found to have more surrounding wrinkles, which were occasionally detected by the image analysis as being part of the nasolabial fold; in such situations the human graders

would have excluded the presence of such wrinkles in their grading. To remove the influence of wrinkles in the region, images were filtered on position and angle of the nasolabial fold. This caused the lower percentage coverage of the nasolabial fold in this region compared to wrinkle coverage in other regions. However, refinement of the technique to further remove the influence of surrounding wrinkles would help improve this measure further, particularly for measurements in women.

Limitations to the study here include a lack of heterogeneity in the sample population, which was a middle-aged to elderly northwestern European sample. There was no corresponding increase in the digital measures between the highest two grades for pigmented spots or the lowest two grades for telangiectasia. Although the number of participants in the extreme grades was very low (<5), it suggests the digital measures might not be discriminating appropriately between these grades, which will be more common in older (for the pigmented spots) or younger (for telangiectasia) individuals. Hence, further image analysis optimization and validation are required before these techniques can be utilized with confidence in older or younger cohorts, and additionally for darker skinned individuals. The image analysis of wrinkles at the localized sites was only performed on the left side of the face. Hence, there may have been under- or overestimation of the amount of wrinkles due to asymmetry in facial photoaging[34,35]. However, at a population level it probably does not radically influence the results. Finally, although image analysis techniques are consistently applied to every image, technical variation in the images can bias the outcomes. The Premier 3dMD face3-plus UHD camera was designed for analysis of facial structure via 3D rendering rather than image analysis on the 2D camera images. Hence, there was no face rest resulting in skin luminance variability across participants. To counteract such effects, the image analysis methods incorporated compensatory algorithms such as utilizing the contrast in color and lightening (e.g. 2nd derivative) within the images rather than absolute color or lightening values. Thus, the digital measures should have been unaffected by differences in lightening levels, although they would still be affected by variations in color balance and the total contrast in light intensity. Hence, more standardized camera set-ups and greater image resolution should improve the reproducibility of the image analysis techniques in the future.

Although previously the measurement of skin aging has been mainly based on photonumeric scales[2,6,7,36], digital measurement has enough advantages over photonumeric grading to suggest it will become the main choice in the future. First, there were good to excellent correlations for the majority of digital measures with photonumeric grading. Second, digital measurement generates a continuous outcome giving more statistical power to detect risk factor associations[37]. Third, better quality images, more automated masking, improved lightening consistency etc. will further improve the utility of image analysis techniques in the future. Finally, digital measurement is less time consuming once an image analysis system is built as it can calculate multiple outcomes per aging component and measure multiple features almost simultaneously.

In conclusion, our digital grading system has proven to be a suitable scale for the measurement of wrinkles (with upper lip wrinkles in men being the exception), pigmented spots and telangi-

ectasia. Digital measurement provides continuous outcomes for different aspects of skin aging, which makes it useful for unbiased discrimination of feature differences in photographic images. Thus, these digital measurement systems for skin aging features demonstrate potential for use in observational and experimental skin aging research.

ACKNOWLEDGMENTS

The authors are grateful to the study participants, the staff from the Rotterdam Study and the participating general practitioners and pharmacists. We thank Sophie Flohil, Emmilia Dowlatshahi, Robert van der Leest, Joris Verkouteren, Ella van der Voort and Shmaila Talib for collecting the phenotypes. Additionally we thank Sophie van den Berg for masking and reviewing all the photographs. We would like to acknowledge Peter Murray for advice around statistical analyses and Arthur Weightman for building the software to segment the localized facial sites.

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PART II

WRINKLES

Chapter 3

Lifestyle and physiological factors associated with facial wrinkling in men and women

M.A. Hamer
L.M. Pardo Cortes
L.C. Jacobs
M.A. Ikram
J.S. Laven
M. Kayser
L.M. Hollestein
D.A. Gunn
T. Nijsten

J Invest Dermatol. 2017 Aug;137(8):1692-1699

ABSTRACT

Facial wrinkling is one of the most notable signs of skin aging. Men and women show different wrinkling patterns yet the lifestyle and physiological factors underlying these sex-specific patterns are relatively unknown. Here, we investigated sex-specific determinants for facial wrinkles. Wrinkle area was quantified digitally using facial photographs of 3831 northwestern Europeans (51-98 years, 58% female). Effect estimates from multivariable linear regressions are presented as the percentage difference in the mean value of wrinkle area per unit increase of a determinant (%Δ). Wrinkle area was higher in men (median 4.5%, interquartile range (IQR) 2.9-6.3) than in women (3.6%, IQR 2.2-5.6). Age was the strongest determinant, and current smoking (men: 15.5%Δ; women: 30.9%Δ) and lower body mass index (men: 1.7%Δ; women: 1.8%Δ) were also statistically significantly associated with increased wrinkling. Pale skin color showed a protective effect (men: -21.0%Δ; women: -28.5%Δ) and, in men, sunburn tendency was associated with less wrinkling. In women, low educational levels and alcohol use were associated with more wrinkling, whereas female pattern hair loss and a higher free androgen index were associated with less wrinkling. In summary, we validated known and identified additional determinants for wrinkling. Skin aging-reducing strategies should incorporate the sex differences found in this study.

INTRODUCTION

Skin aging is an ongoing process associated with declined skin function and changes in its appearance. It reflects a person's general health[1] and emotional well-being[2]. Skin aging is a complex phenotype and includes different features, of which facial wrinkles are arguably the most notable. There is large interest in understanding the pathophysiology of wrinkles because it is one of the most obvious targets for improving skin appearance and is a key anti-aging target for the cosmetic market.

Both intrinsic and extrinsic factors[3] contribute to skin aging; smoking and ultraviolet (UV) radiation are the most well known extrinsic risk factors[4,5]. High body mass index (BMI) accounts for less wrinkles[6], most probably because facial fat has an expanding/filler effect on the skin. Other determinants (associative factors) that have been linked to wrinkles include education[7], alcohol[8], and female sex steroids[9] but these findings are controversial as they have not all been replicated consistently in other studies.

The extent and characteristics of facial wrinkles differ between men and women, regarding localization and depth[10,11] and could in part be due to hormonal differences[12,13]. A possible explanation for the observed perioral skin wrinkling difference is that women have less sebaceous glands and sweat glands and a lower ratio between vessel area and connective tissue area in the dermis[14]. The impact of lifestyle and physiological factors on sex-specific skin wrinkling is not well documented.

Although three-dimensional (3D) microtopography of the dorsum of the hand as an index of actinic skin damage[15] or a digital fringe projection method to assess wrinkle severity[10] have been used, most clinical skin aging studies have used manual photonumeric scales. Most scales regard skin aging as a compound phenotype including wrinkles, telangiectasia, pigmented spots, and sagging together[3,16,17]. Therefore, it is difficult to infer the role of possible determinants specifically linked to wrinkles. Investigating the skin aging aspects separately could lead to the discovery of determinants specific for each aging phenotype. Moreover, photonumeric scales are prone to human grading bias due to scoring of different components of skin aging, for example, sagging or hair graying. A digital measure can provide a more objective, valid, and reliable measurement of skin wrinkles.

In 3831 individuals of northwest European ancestry in the Rotterdam Study (RS), we tested for associations between the main lifestyle and physiological factors and facial wrinkles in a middle-aged to elderly population[18]. This was performed in men and women separately by using digital quantification of wrinkle area measured from facial photographs.

RESULTS

Study population

Between September 2010 and June 2014, a total of 4649 participants visited the in-person examination of the RS, which includes extensive dermatological assessments. After excluding 818 individuals because of non-northwest European origin, poor image quality, make-up, and/or presence of facial hair (e.g., beards), 3831 RS participants with eligible 3D photographs were used to measure facial wrinkle area. The majority were women (N=2229; 58.2%) and the median age was 66.8 (IQR 61.2-71.9) in men and 66.4 (IQR 60.9-71.1) in women (Table 1).

Table 1. Characteristics of 3831 participants of the Rotterdam Study with 3D photographs, for the total study population and stratified by sex

Characteristic[a]	Total study population (N=3831)	Men (N=1602)	Women (N=2229)	P-value men vs. women[j]
Wrinkle area %, median [IQR]	4.0 [2.5 – 6.0]	4.5 [2.9 – 6.3]	3.6 [2.2 – 5.6]	<0.001
Age at photo in years, median [IQR]	66.5 [61.0 – 71.5]	66.8 [61.2 – 71.9]	66.4 [60.9 – 71.1]	0.069
BMI in kg/m^2, mean (SD)	27.6 (4.4)	27.6 (3.7)	27.5 (4.9)	0.360
Skin color				
pale (%)	366 (10)	134 (8)	232 (10)	0.040
white (%)	2912 (76)	1199 (75)	1713 (77)	0.160
white-to-olive (%)	553 (14)	269 (17)	284 (13)	<0.001
Smoking history[b]				
current (%)	707 (19)	339 (21)	368 (17)	<0.001
former (%)	1921 (50)	909 (57)	1012 (45)	<0.001
never (%)	1198 (31)	353 (22)	845 (38)	<0.001
Baldness[c]				
no/mild baldness (%)	2330 (61)	826 (52)	1504 (68)	<0.001
moderate (%)	874 (23)	369 (23)	505 (23)	0.920
extensive (%)	576 (15)	406 (25)	170 (8)	<0.001
Tendency to develop sunburn				
low (%)	2440 (64)	1068 (67)	1372 (62)	0.009
high (%)	1253 (33)	492 (31)	761 (34)	
Lived in sunny country[d]				
no (%)	3483 (91)	1424 (89)	2059 (92)	<0.001
yes (%)	240 (6)	144 (9)	96 (4)	
Sun-protective behavior[e]				
never/almost never (%)	1290 (34)	586 (37)	704 (32)	0.003
often/almost always/always (%)	2433 (64)	983 (61)	1450 (65)	

Table 1. Characteristics of 3831 participants of the Rotterdam Study with 3D photographs, for the total study population and stratified by sex (continued)

Characteristic[a]	Total study population (N=3831)	Men (N=1602)	Women (N=2229)	P-value men vs. women[j]
Spending winter in sunny country				
no or less than 1 month/year (%)	3434 (90)	1430 (89)	2004 (90)	0.460
yes, ≥1 month/year (%)	194 (5)	86 (5)	108 (5)	
missing (%)	203 (5)	86 (5)	117 (5)	
Outdoor work history[f]				
no (%)	1911 (50)	690 (43)	1221 (55)	<0.001
yes (%)	511 (13)	298 (19)	213 (10)	
missing (%)	1409 (37)	614 (38)	795 (36)	
Tanning bed use[g]				
never or less than 10x (%)	1944 (51)	807 (50)	1137 (51)	0.005
more than 10x (%)	325 (9)	108 (7)	217 (10)	
missing (%)	1562 (41)	687 (43)	875 (39)	
Education level[h]				
low (%)	311 (8)	108 (7)	203 (9)	0.009
medium (%)	2388 (62)	906 (57)	1482 (67)	<0.001
high (%)	1090 (29)	570 (36)	520 (23)	<0.001
Alcohol				
median use in glasses/day [IQR]	0.8 [0.1 – 1.8]	1.2 [0.3 – 2.4]	0.5 [0.1 – 1.4]	<0.001
missing (%)	626 (16)	281 (18)	345 (16)	
Dry skin presence				
no (%)	1254 (33)	588 (37)	666 (30)	<0.001
yes (%)	2573 (67)	1014 (63)	1559 (67)	
Testosterone in nmol/l, median [IQR]	na	16.7 [13.1 – 20.6]	na	na
Free androgen index[i], median [IQR]	na	na	1.3 [0.9 – 2.0]	na
missing (%)	na	na	145 (7)	na
Estradiol in pmol/l, median [IQR]	na	na	43.1 [18.4 – 74.6]	na
missing (%)	na	na	122 (6)	na

Abbreviations: BMI, body mass index; na, not applicable; SD, standard deviation.

[a]all variables have missing values <5% unless otherwise specified. Percentages are rounded to integers; [b]cigars, cigarettes, or pipe; [c]based on the Norwood-Hamilton (NH) scale for men and the Ludwig scale for women; None or minimal: NH score 1, 2, 3, 9, 10, 11 and Ludwig scale score none. Moderate: NH score 4, 5, 6, 12 and Ludwig scale score 1. Extensive: NH score 7, 8 and Ludwig scale score 2, 3; [d]history of living in a sunny country >1 year; [e]wearing sunglasses and/or a brimmed hat in the sunshine; [f]worked or been outdoors ≥4 hours daily during at least 25 years; [g]frequency of tanning bed visits in the past 5 years (including facial solarium); [h]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [i]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l); [j]t-test for normally distributed continuous data (BMI); Mann-Whitney U Test for non-normally distributed continuous data (all except BMI); chi^2 test for categorical data.

Wrinkle area measure

The distribution of the facial wrinkle area percentage was skewed towards higher values (Figure 1). The median wrinkle area percentage was higher in men than in women (men: 4.5, IQR 2.9-6.3; women: 3.6, IQR 2.2-5.6), but not for all age groups. Men had a higher wrinkle area than women in the lowest age groups (<65 years old, mean difference in wrinkle area: 1.0, P-value=2.4×10^{-17}; 65-75 years old, difference: 0.6, P-value=6.0×10^{-6}). Women had a higher wrinkle area than men in the highest age group (≥75 years old, mean difference: 0.7, P-value=0.02; Figure 2). We also found similar sex differences after stratifying for the UV variable "outdoor work history" (Supplementary Results, including Supplementary Figure S1).

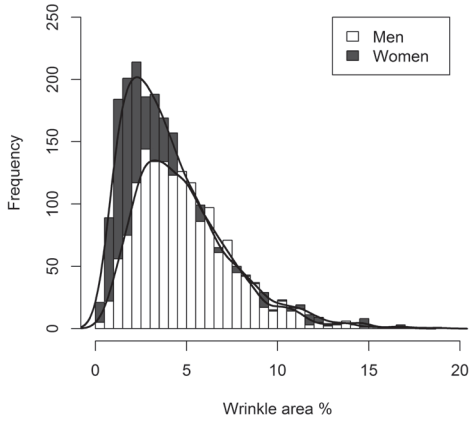

Figure 1. Distribution of the digital wrinkle area percentages split by sex. The distribution of the facial wrinkle area percentage is skewed towards higher values for both sexes. The median wrinkle area percentage was higher in men than in women.

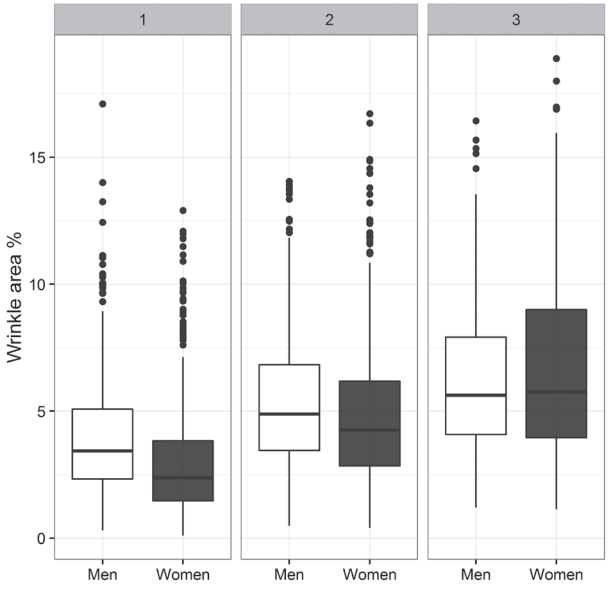

Figure 2. Boxplots of wrinkle area % per age group (1: <65 years old; 2: 65-75 years old; 3: ≥75 years old), split by sex. Men have statistically significant higher wrinkle area than women in the lowest and middle age group. Women have statistically significant higher wrinkle area than men in the highest age group.

Determinants for global facial wrinkle area

Men

We used the R^2 (coefficient of multiple determination) to calculate the percentage of wrinkle variation explained by the regression and the contribution of specific variables to the total R^2 value (see Materials and Methods). In a full model of wrinkle area (all variables included), 22% of the variability (adjusted R^2) of facial wrinkles was explained (Table 2). Age was the most important determinant of wrinkles in the linear regression analysis, as indicated by the R^2 value of 9.0% in 1602 men. Current smoking (15.5%Δ vs. never smoking) and lower BMI (1.7%Δ per unit decrease in BMI) were associated with a higher wrinkle area. Light skin color was associated with a lower wrinkle area (pale vs. white-to-olive colored skin -21.0%Δ; white vs. white-to-olive colored skin -10.3%Δ, Table 2). Testosterone levels showed a positive association with borderline significance (0.5%Δ, 95% confidence interval (CI) -0.00 to 0.94). In addition, the UV variable "spending winter in a sunny country" was borderline statistically significant (10.4%Δ, 95%CI -1.4 to 23.5) and a high susceptibility for sunburn was significantly associated with lower wrinkle area (-5.8%Δ) (Table 2). Age was nonlinearly related to wrinkle area; with increasing age, individuals showed a smaller increase in wrinkle area than younger individuals did (Supplementary Figure S2a, age^2 P-value<0.001).

Women

In 2229 women, the determinants accounted for 37.2% of the variability of facial wrinkles (Table 2). Age showed even stronger effects in wrinkle variation than in men (R^2 21.7%). Current smoking (30.9%Δ vs. never smoking) and lower BMI (1.8%Δ per unit decrease in BMI) were positively associated with wrinkling and a light skin color was inversely associated (pale vs. white-to-olive colored skin -28.5%Δ). In addition, women with a lower education level had more wrinkles compared with those with a high education level (low vs. high 16.5%Δ) and a higher alcohol intake showed a small but significant positive effect (3.9%Δ per daily glass). Extensive female baldness (-16.1%Δ vs. women with no baldness) and a higher free androgen index (FAI) (-5.6% Δ per point) showed protective effects. The nonlinear effects of age were also statistically significant in women (Supplementary Figure S2b, age^2 P-value<0.001).

As shown in Table 2, the differences in the amount of variation in wrinkles explained by the determinants (R^2 differences) between men and women were not only due to additional predictors in women but also due to the magnitude of effects of the variables significant in both groups; in women, R^2 was 0.3% higher for BMI, smoking, and skin color.

Sensitivity analyses

Two UV variables "outdoor work history" and "tanning bed use" had a high percentage of missing values: 37% and 41%, respectively, and were not included in the full model. To better test the influence of UV-exposure, we included these two variables in additional complete case analyses (N=907 men and N=1343 women). In men, outdoor work history was associated with more

Table 2. Sex-stratified multivariable linear regression of global facial wrinkle area among 1602 men and 2229 women in the Rotterdam Study

	Men (N=1602)				Women (N=2229)			
Characteristic	% Δ wrinkle area[a]	95% CI	P-value	Adj. R^2 %	% Δ wrinkle area[a]	95% CI	P-value	Adj. R^2 %
Full model				22.0				37.2
Age				9.0				21.7
Age	11.8	[7.4 – 16.5]	<0.001		17.3	[13.0 – 21.7]	<0.001	
Age^2	-0.06	[-0.10 – -0.02]	<0.001		-0.08	[-0.10 – -0.06]	<0.001	
Resolution variation (batch)	-18.0	[-23.4 – -12.2]	<0.001	1.6	-16.5	[-22.1 – -10.5]	<0.001	0.7
Flash light variation	-0.3	[-0.4 – -0.1]	<0.001	0.8	-0.4	[-0.5 – -0.2]	<0.001	0.8
Lower BMI (per unit)	1.7	[0.9 – 2.4]	<0.001	1.0	1.8	[1.3 – 2.4]	<0.001	1.3
Skin color				0.8				1.1
pale	-21.0	[-29.5 – -11.4]	<0.001		-28.5	[-35.7 – -20.5]	<0.001	
white	-10.3	[-16.4 – -3.8]	0.002		-15.5	[-21.5 – -9.0]	<0.001	
white-to-olive	ref	ref	ref		ref	ref	ref	
Smoking history[b]				1.2				1.5
current	15.5	[6.8 – 24.8]	<0.001		30.9	[21.8 – 40.7]	<0.001	
former	-2.1	[-8.2 – 4.5]	0.529		5.7	[0.2 – 11.4]	0.042	
never	ref	ref	ref		ref	ref	ref	
Baldness[c]								0.4
no/mild baldness	ref	ref	ref		ref	ref	ref	
moderate	-5.9	[-11.9 – 0.5]	0.069		-3.9	[-9.5 – 1.9]	0.183	
extensive	-1.8	[-8.0 – 4.7]	0.577		-16.1	[-23.6 – -7.8]	<0.001	
Tendency to develop sunburn	-5.8	[-11.2 – -0.2]	0.045	0.2	-3.8	[-8.8 – 1.5]	0.159	
Lived in sunny country[d]	4.1	[-4.9 – 13.9]	0.381		-4.9	[-15.4 – 6.9]	0.399	
Sun-protective behavior[e]	0.2	[-5.0 – 5.7]	0.947		-3.8	[-8.6 – 1.4]	0.146	
Spending winter in sunny country	10.4	[-1.4 – 23.5]	0.086		2.9	[-8.0 – 15.1]	0.615	
Education level[f]								0.3
low	2.4	[-7.9 – 14.0]	0.659		16.5	[5.9 – 28.2]	0.002	
medium	2.7	[-2.8 – 8.6]	0.341		7.2	[1.0 – 13.8]	0.023	
high	ref	ref	ref		ref	ref	ref	
Alcohol (per glass/day)	0.4	[-1.2 – 2.0]	0.627		3.9	[1.7 – 6.3]	<0.001	0.4
Dry skin	-1.9	[-7.0 – 3.6]	0.497		0.8	[-4.5 – 6.3]	0.778	
Testosterone (nmol/l)	0.5	[-0.00 – 0.94]	0.050		na	na	na	
Free androgen index[g]	na	na	na		-5.6	[-7.5 – -3.7]	<0.001	1.0
Estradiol (pmol/l)	na	na	na		0.0	[0.0 – 0.0]	0.789	

The adjusted R^2 is shown for the full model and for all statistically significant variables.

Abbreviations: adj. R^2, adjusted R^2; BMI, body mass index; 95% CI, 95% confidence interval; na, not applicable; ref, reference variable.

[a]the regression betas of each determinant are presented as percentage change (%Δ) in wrinkle area (the % increase in the mean value of wrinkle area per unit increase in the independent variables), calculated by the formula: (exp^{β}-

1) * 100%; [b]cigars, cigarettes or pipe; [c]based on the Norwood-Hamilton (NH) scale for men and the Ludwig scale for women; None or minimal: NH score 1, 2, 3, 9, 10, 11 and Ludwig scale score none. Moderate: NH score 4, 5, 6, 12 and Ludwig scale score 1. Extensive: NH score 7, 8 and Ludwig scale score 2, 3; [d]history of living in a sunny country >1 year; [e]wearing sunglasses and/or a brimmed hat in the sunshine; [f]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [g]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l).

wrinkling (18.9%Δ, 95%CI 9.5–29.0), whereas in women, tanning bed frequency of ≥10 vs. <10 in the past five years was associated with more wrinkles (16.5%Δ, 95%CI 6.3–27.6, Supplementary Table S1).

Determinants for site-specific wrinkle area

Besides global facial wrinkles, site-specific wrinkle areas (i.e., crow's feet, forehead wrinkles, and in women upper lip wrinkles) were analyzed. Wrinkling around the mouth was strongly associated with smoking in women (see Supplementary Material and Supplementary Table S2 and S3 for further results) and a higher BMI was associated with greater wrinkling at the crow's feet in women.

DISCUSSION

In this large study of lifestyle and physiological factors for facial wrinkles, known associations between facial wrinkles and lower BMI, sun exposure, and smoking were replicated[4,19]. In addition, we identified protective factors including pale skin color and in women hair loss and a high FAI.

Of all variables examined, age was the largest predictor and explained most of the wrinkle variation ranging from 9.0% in men to 21.7% in women and had a larger effect per year in the middle-aged individuals than in the elderly. Fine wrinkles are regarded as an intrinsic skin aging symptom, whereas coarse wrinkles are considered a typical extrinsic skin aging symptom[20]. Our wrinkling phenotype could not distinguish between these two types of aging. The models used have captured a higher variability for wrinkles in women than in men (R^2 37.2% and 22.0%, respectively). Besides unknown/unmeasured determinants, the main unexplained variation lies in genetics; the heritability of wrinkles is estimated to be 55%[21].

Most skin aging studies have analyzed both sexes together, despite important sex differences that have been described[10,11,14,22,23]. In the sex-stratified analyses, we found that men have more wrinkles than women in the lower age groups, but in the highest age group, this is reversed – confirming earlier research[10]. Men might get more wrinkles earlier in life because of higher occupational sun exposure (e.g., from the construction industry). However, we found that the differences remained significant after adjusting the wrinkling for occupational sun exposure. Alternatively, the difference may be due to the more rapid hormonal changes occurring in postmenopausal women[24], which is supported by a Korean study demonstrating a significant relationship between

early menopause and wrinkle severity[9]. In our study, although estradiol level was not associated with wrinkles, the FAI and presence of female pattern hair loss (FPHL) showed a significant inverse association. As FPHL is, in part, related to sensitivity to testosterone levels, these findings suggest that testosterone plays a significant role in protecting women against wrinkles. Although these are interesting endocrinological findings, wrinkle-reducing strategies are unlikely to incorporate boosting testosterone levels due to undesired side effects (e.g., FPHL) in women; however, identifying the mechanism through which testosterone protects skin from wrinkling in women could help find other analogous routes to reducing wrinkling formation. Obese women have reduced sex hormone binding globulin (SHBG) levels, leading to a higher FAI and thus a relative hyperandrogenic state[25]. In our data, BMI and FAI were both statistically significant in the model together, and stratifying for BMI in women showed a persistent significant association between FAI and wrinkles in all three categories (Supplementary Results). Hence, it is unlikely FAI is only associated with wrinkles due to its relationship to BMI. In men, there is a surprising borderline significant positive effect of testosterone on wrinkling. However, we cannot completely make inferences on this association in men, as total testosterone is an inferior marker to free testosterone[26], which is not available in the RS.

The differences in skin wrinkling across sex were reflected in extent of wrinkles, associated determinants and their effect sizes. Smoking, for example, had a stronger effect in women than in men[6]. Skin color also showed a stronger effect in women. On the contrary, UV variables showed an effect in men, but not in women. Higher education previously showed an association with less wrinkles[7], but we could only confirm this in women. It could be argued that women with higher education put more effort in protecting their skin against aging-related conditions like sun damage, whereas highly educated men do not.

Strikingly, we found that light-skinned northwestern Europeans had less wrinkles than darker skinned ones. In addition, in men, the tendency to develop sunburn – which is often the case in light-skinned individuals – was associated with less wrinkles. This seems contradictive, because people with Fitzpatrick skin types[27] IV and higher have less wrinkles than those with skin types I-III[28]. However, a recent skin aging study confirmed our finding of less wrinkles in light-skinned individuals[29]. An explanation could be that light-skinned individuals are known to avoid the sun because of the UV-sensitive nature of their skin. Yet, our model also included some UV-exposure-related variables that should correct for this effect. Furthermore, light-skinned individuals in this study have already been shown to have more pigmented spots[30], which are also influenced by UV-exposure[31-33]. Alternatively, we argue that different skin types show different phenotypes of skin aging because individuals with lighter skin have atrophic skin changes manifested as fewer wrinkles and more dysplastic changes[34,35].

We found that most variables that were associated with global wrinkles were also significantly associated with specific wrinkle sites (Supplementary Table S3). Of note, smoking showed, in line with a previous study[36], a stronger association with upper lip wrinkles than with any other site or with global wrinkles. This could be the result of the high concentration of smoke around

the mouth, or the increased expression of lip lines while inhaling. Unexpectedly, BMI showed a positive association with crow's feet wrinkles in women and a similar direction but nonsignificant association in men; this finding suggests that the "filler effect" of subcutaneous fat in the crow's feet area is being counteracted by some unknown mechanism – perhaps related to the fat distribution in the face – that needs replication and further investigation.

Limitations of this study include the cross-sectional design, which precludes addressing the temporality of the observed associations. We did not measure wrinkle depth, or distinguish between fine and coarse wrinkles[20] that may have been influenced by different determinants. The assessment of UV-exposure is challenging, and the available UV variables might not have captured cumulative sun exposure accurately. In addition, sex hormones were measured in serum samples on average 5.6 years before photo-collection. This could have influenced the association with wrinkle area although it is not necessarily a limitation, because wrinkles develop over a longer period of time. There was a lack of heterogeneity in the study population – middle-aged to elderly northwestern Europeans with white skin color[37] – which reduces possible residual confounding but limits generalizability. Furthermore, although image analysis techniques were consistently applied to every image, technical variation could influence the extent of wrinkles, although we adjusted for two important technical-related variables in our models.

In conclusion, in the largest wrinkling study to date, facial skin wrinkling differs between men and women regarding the extent of wrinkling, and associated determinants and their effect sizes. This study confirmed known risk factors for facial wrinkles and discovered protectively associated factors such as light skin color in both sexes and hormonal factors such as FPHL and high FAI in women, which could help direct new prevention strategies for skin aging.

MATERIALS AND METHODS

Study population

The Rotterdam Study (RS) is an ongoing prospective population-based cohort study following 14,926 participants aged ≥45 years in Ommoord, a suburb of Rotterdam in the Netherlands, since 1990. Details of the study design and objectives have been described elsewhere[18]. The RS has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sports of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)". All participants provided written informed consent to participate in the study.

Since 2010, skin examinations have been conducted by trained physicians, focusing on the most common skin diseases. In addition, standardized high-resolution digital 3D facial photographs (Premier 3dMDface3-plus UHD, Atlanta, GA, USA) are being collected. Between September 2010 and June 2014, a total of 4649 participants have been photographed and examined at the research center.

Wrinkles

High-resolution standardized full face photographs were obtained in a room without daylight. Details of digital image acquisition and its validation have been described elsewhere[38] and in the Supplementary Material. Enface 2D photographs were exported from the 3D images and masked to isolate the skin areas in the image using semi automated masking (MATLAB, The MathWorks, Inc, Natick, MA, USA, version 2013a). Using MATLAB, wrinkle area was calculated from the 2D facial images, expressed as a percentage of the facial skin area (Figure 3).

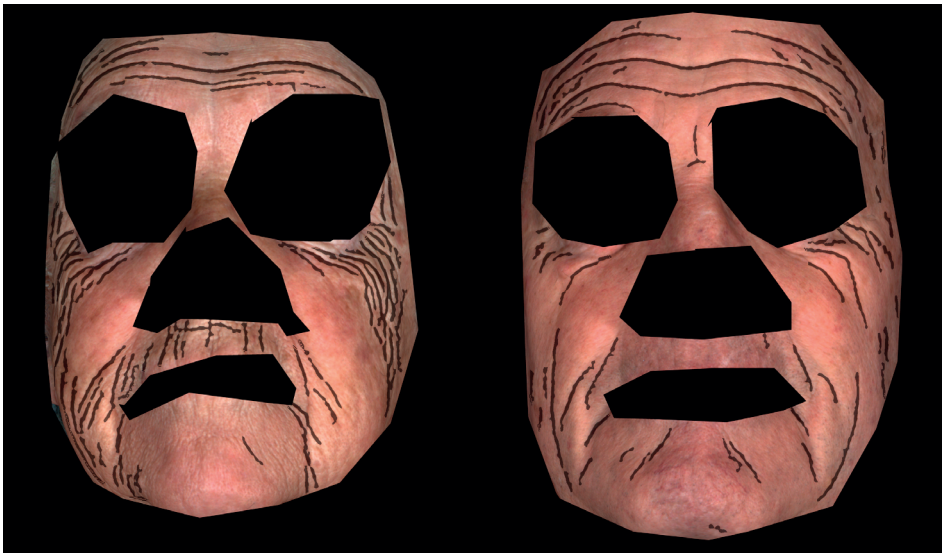

Figure 3. Digital extraction of wrinkles. Non-skin parts (i.e., eyes, nostrils, and mouth) are masked and wrinkle area is measured in units of pixels. Left: female participant; right: male participant.

Besides measuring wrinkles of the whole face, we also measured wrinkle area at three specific sites (crow's feet, forehead wrinkles, and in women upper lip wrinkles) that was readily available for a subset of participants, see Supplementary Methods and Results.

Determinants

Sex and age (in years) at date of photo were retrieved from the database. Information on other physiological and lifestyle factors was collected by interview, physical examination, and blood serum measurements. In the interview, variables on level of education, smoking habit and UV-related questions were collected[18]. Variables collected by physical examination at the research center were BMI (calculated by dividing weight by the squared height), presence of dry skin, baldness, and constitutional skin color assessed at the sun-unexposed skin of the upper body, that is, abdomen and inner upper arm: pale, white, and white-to-olive, as validated by a previous study[37]. Baldness was based on the Norwood-Hamilton scale[39,40] for men and the Ludwig scale[41]

for women. For the analyses, we classified these scales into three categories of male baldness: none or minimal (Norwood-Hamilton score 1, 2, 3, 9, 10, 11), moderate (Norwood-Hamilton score 4, 5, 6, 12), and extensive baldness (Norwood-Hamilton score 7, 8). Female baldness was also classified into three categories, that is, none or minimal (scored as "none"), moderate (Ludwig scale score 1), and extensive baldness (Ludwig scale score 2 and 3). In serum samples, estradiol, testosterone, and sex hormone binding globulin (SHBG) were measured on average 5.6 years before photo-collection. The free androgen index (FAI) was calculated for women, using the formula: (total testosterone / SHBG * 100)[42]. Details of all variables used are described in the Supplementary Methods.

Statistical analysis

We compared characteristics between men and women using chi^2 tests for categorical variables, independent samples t-tests for normally distributed continuous variables and Mann-Whitney U tests for non-normally distributed continuous variables.

For the main association analyses, we excluded variables with >35% missing values, namely the UV variables "outdoor work history" and "tanning bed use". For the other missing values (maximum missing data per variable was 17.5%), we performed multiple imputation based on all available variables shown in Table 1. This was performed using the Multivariate Imputation by Chained Equations (MICE) software package in R (http://www.R-project.org), with an iteration of 20.

To investigate the associations between potential determinants and wrinkle area, we used linear regressions. To correct for technical variation, we included two variables which accounted for possible variations in resolution and flash light in all regression models. For variations in resolution, a variable describing the batch number was used. For flash light variation, the in-person difference between skin lightness in the images and that taken by a spectrophotometer (CM-600d; Konica-Minolta, Osaka, Japan) on the cheek was used, by calculating the residuals of these two lightness variables regressed on each other[30].

The residuals of the linear regression of wrinkle area did not fit a normal distribution. Therefore, wrinkle area was transformed using the natural logarithm (ln). This resulted in an approximately normal distribution of the residuals of the regression. The resulting effect estimates (regression betas) were transformed using the formula: $(\exp^{\beta}-1) * 100\%$ and can be interpreted as the percentage difference (%Δ): the percentage increase in the mean value of wrinkle area per unit increase of a determinant, for example, 3% increase in wrinkle area per 1 year of age.

We found statistically significant interactions between sex and four other variables (age, smoking, skin color, and education – Supplementary Methods, including Supplementary Table S4). Therefore, we stratified by sex in all analyses. Interactions were also tested for all plausible pairs of variables in men and women separately (Supplementary Methods) but none of these were significant (data not shown).

There was a non-linear relationship between age and wrinkle area (Supplementary Figure S2). Therefore, we added the squared term "age^2" to the analyses. Besides age, age^2, and the two technical variables, we added other variables to create a fully adjusted multivariable linear regression analysis. Variables were selected based on known literature and biologically plausible associations. In addition, we investigated the contribution of each variable to the model, by calculating the difference between the adjusted R^2 of the full model with and without each variable of interest (Supplementary Material).

Sensitivity analyses

The two UV variables that represented UV-exposure the best were "outdoor work history" and "tanning bed use". However, these variables had a high percentage of missing values (37% and 41%, respectively) and therefore were not included in the full model. We performed sensitivity analyses including complete cases of these two variables. For incomplete data of the other variables, we performed multiple imputation as mentioned above. Besides the two UV variables, the analyses included the same variables as the main analyses.

All analyses were performed using SPSS for Windows version 21.0 (SPSS, Chicago, IL) and software package R (http://www.R-project.org). A two-sided P-value of <0.05 was considered statistically significant.

ACKNOWLEDGMENTS

The authors are grateful to the study participants, the staff from the Rotterdam Study, and the participating general practitioners and pharmacists. We thank Emmilia Dowlatshahi, Sophie Flohil, Robert van der Leest, Simone van der Velden, Joris Verkouteren and Ella van der Voort for collecting the phenotypes. Additionally, we thank Andreas Wollstein for converting all photographs and Sophie van den Berg for masking and reviewing them. We acknowledge Jaspal Lall for masking the photographs and creating the digital wrinkle measurements and Peter Murray for advice around statistical analyses.

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Digital image acquisition

Details of digital image acquisition have been described elsewhere[1]. In short, high resolution standardized full face photographs were obtained with a Premier 3dMD face3-plus UHD camera (3dMD, Atlanta, GA, USA), in a room without daylight. Participants were asked not to wear any make-up, facial cream, or jewelry. Three 2D photographs (2452×2056 pixels, 14.7MB in BMP format) were taken simultaneously (one upper-frontal and two 45° lateral photos) in uniform surroundings, and by combining these, the 3dMD software rendered a 3D facial image. The machine was calibrated daily to control for camera position and environmental light intensity. Images were obtained under the same conditions, apart from a lighting change halfway through the study, which was accounted for in the analyses. The distance between the subject and the cameras was fixed but slight variation cannot be excluded due to the lack of a face rest. The raw files from the 3DMD system were further processed to regenerate 2D frontal images (1920×1080 pixels) of the whole face.

Wrinkle area global facial wrinkles

Using MATLAB, wrinkle area was calculated from the 2D facial images, expressed as a percentage of the facial skin area. Details of these measurements have recently been published[1]. In short, based on the contrast in color and lightening, the amount of pixels detected as wrinkles divided by the amount of pixels of the whole face resulted in the relative wrinkle area. This was multiplied by 100 to create the facial wrinkle percentage.

Wrinkle area site-specific facial wrinkles

Besides measuring wrinkles of the whole face, we have also measured wrinkles at three specific sites (crow's feet, forehead wrinkles, and in women upper lip wrinkles). The technical wrinkle measurements were the same as for the global facial wrinkles, however these measurements were performed on the original 2D photographs of the left side of the face[1].

Determinants

Information on patient characteristics and lifestyle factors were collected by interview (level of education, smoking and alcohol habits, UV-related questions) and physical examination[2]. Level of education was assessed during the interview, classified into three categories, i.e. low (primary education with or without a higher not completed education), medium (lower secondary education, lower vocational education, and intermediate vocational education), and high (general secondary education, higher vocational education, and university). Smoking habit was transformed into

current, former and never; alcohol habit was shown as glasses per day. In addition, six variables used as proxy for UV-exposure were available from interview data: tendency to develop sunburn (low vs. high), history of more than 25 years of outdoor work (yes vs. no), having wintered in a sunny country between September and May for at least one month during the past 5 years (yes vs. no), having lived in a sunny country for more than 1 year (yes vs. no), sun protective behavior (i.e. wearing sunglasses and/or a brimmed hat in the sun categorized into never/almost never vs. often/almost always/always) and use of tanning beds (fewer vs. more than ten times in the last five years). Body mass index (BMI) was measured at the research center and calculated by dividing weight (in kg) by the squared height (in m).

Other determinants were scored during full body skin examination by trained physicians: presence of dry skin (binary response), skin color (pale, white, and white-to-olive)[3] and baldness based on the Norwood-Hamilton scale[4,5] for men and the Ludwig scale[6] for women. For the analyses, we classified these scales into three categories of male baldness: none or minimal (Norwood-Hamilton score 1, 2, 3, 9, 10, 11), moderate (Norwood-Hamilton score 4, 5, 6, 12), and extensive baldness (Norwood-Hamilton score 7, 8). Female baldness was also classified into three categories, i.e. none or minimal (scored as "none"), moderate (Ludwig scale score 1), and extensive baldness (Ludwig scale score 2 and 3). Furthermore, estradiol (in pmol/l), testosterone (in nmol/l) and sex hormone binding globulin (SHBG, in nmol/l) were measured in serum samples on average 5.6 years before photo-collection. Fasting blood samples were drawn in the morning (≤11 am). Estradiol levels were measured with a radioimmunoassay and SHBG with the Immulite platform (Diagnostics Products Corporation Breda). The corresponding intra- and interassay coefficients of variation with lower limit of detection of the assays were less than 11%, less than 11%, and 18.35 pmol/l for estradiol and less than 4%, less than 5%, and 0.02 nmol/l for SHBG. Serum levels of testosterone were measured with liquid chromatography-tandem mass spectrometry, with a corresponding interassay coefficient of variation of less than 5% and a lower limit of quantification of 0.07 nmol/l. The free androgen index (FAI) was calculated for women, using the formula: (total testosterone / SHBG) * 100 [7].

Statistical analysis

The phenotype (digital global wrinkle area) followed a right-skewed distribution. In order to meet the criteria for performing a linear regression, we ln-transformed the phenotype, resulting in an approximately normal distribution of both the phenotype and the residuals of the regression.

The site-specific wrinkles also followed a right-skewed distribution, but also included a considerable proportion of zeros as value for some photos, indicating there were no wrinkles at that site at all. Therefore, we could not ln-transform the phenotype. To better fit the data for regression, we used rank-based inverse normal transformation, where the mean is set to zero and the standard deviation to one[8].

We investigated statistical interaction between sex and other covariates. We added each potential interaction term to the final model one at a time to investigate its significance, namely:

sex*age, sex*BMI, sex*smoking, sex*skin color, sex*baldness, sex*alcohol, sex*education, and sex*tendency to develop sunburn. Statistical interaction between sex and the following variables was observed after adjusting for other available covariates: smoking (P-value 0.001 and 0.003), age (P-value<0.001), skin color (P-value 0.49 and 0.045) and education (P-value 0.006 and 0.003), Supplementary Table S4. Further analyses were therefore stratified per sex.

We also investigated statistical interaction in men and women separately and found no significant interactions; the following interaction terms were tested: smoking*each UV variable, smoking*BMI, smoking*age, smoking*alcohol, smoking*education, skin color*each UV variable, BMI*education, and BMI*alcohol.

The adjusted R^2 attributable to each variable was calculated by omitting each variable from the final model one by one and calculating the adjusted R^2 of these models. These were subtracted from the adjusted R^2 of the full model. The adjusted R^2 takes into account the number of variables added to the model. The adjusted R^2 of age was calculated for the terms age and age^2 taken together.

Sensitivity analyses

We have investigated the role of FAI in wrinkles for different categories of BMI in women. We created three groups: BMI<25 kg/m^2; BMI 25-30 kg/m^2; BMI>30 kg/m^2. We performed linear regression analyses for the complete cases in these three groups separately, including all variables mentioned in Table 1.

RESULTS

Global facial wrinkles – sensitivity analyses

To investigate whether the sex differences in wrinkle area found within different age groups (Figure 2 main manuscript) were related to a differential UV-exposure (e.g. occupational exposure) we have also performed the analyses correcting for the UV variable “outdoor work history”, by stratifying for this variable (Supplementary Figure S1). Only complete cases of the variable “outdoor work history” were used for this analysis, N=2422.

For the group without occupational UV-exposure, men had significantly more wrinkling in the age group <65 years old (independent samples t-test, P-value 1.4×10^{-9}) and 65-75 years old (P-value 3.7×10^{-3}); women had significantly more wrinkling in the highest age group of ≥75 years old (P-value 1.8×10^{-2}). For the group with occupational UV-exposure, men had significantly more wrinkling in the age group <65 years old (P-value 9.1×10^{-10}). Men also had a trend towards more wrinkling in the age group 65-75 years old, but this was not significant (P-value 0.15). For the highest age group ≥75 years old, women had more wrinkles than men did, but this was also not significant (P-value 0.37). Most probably, the last-mentioned groups were too small (due to stratifying for 3 different variables: sex, age category and occupational exposure) to have enough

power to show a significant effect; in the group with occupational UV-exposure there were 98 men and 73 women aged 65-75 years old and 34 men and 12 women aged ≥75 years old.

To further investigate the relationship between FAI and wrinkles in women, we have stratified women in three groups by BMI category. The association of FAI with wrinkles remained significant in all three groups (multiple linear regression of complete cases, including all variables mentioned in Table 1): -11.6%Δ, 95%CI -17.3 to -5.6 in women with BMI<25 kg/m^2, N=517; -3.9%Δ, 95%CI -6.5 to -1.1 in women with BMI 25-30 kg/m^2, N=633; -6.7%Δ, 95%CI -10.8 to -2.4 in women with BMI>30 kg/m^2, N=392.

Site-specific facial wrinkles

The correlations between crow's feet wrinkles, forehead wrinkles and upper lip wrinkles vs. global facial wrinkles were similar (0.48 – 0.58, Supplementary Table S2). Correlations between different facial sites were not high (0.23 – 0.29), indicating that there might be variation in the effect sizes of the determinants for each different site. As shown in Supplementary Table S3a-c we found that most determinants associated with global wrinkles were also associated with site-specific wrinkles. Age remained significantly associated with all wrinkle sites. BMI remained significantly inversely associated with forehead wrinkles and women's upper lip wrinkles, but was positively associated with crow's feet. Light skin color remained inversely associated with crow's feet in both sexes and with forehead wrinkles in women. Smoking was associated with crow's feet in men and with forehead wrinkles and upper lip wrinkles in women. The association of smoking with upper lip wrinkles was higher than that with global wrinkles or any other wrinkle site. Free androgen index in women remained inversely associated with forehead and upper lip wrinkles.

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table S1. Sex-stratified multivariable linear regression results of global facial wrinkle area: sensitivity analysis, containing all variables from Table 1 for complete cases (907 men and 1343 women in the RS), including the UV variables "outdoor work history" and "tanning bed use"

	Men (N=907)			Women (N=1343)		
Characteristic	% Δ wrinkle area[a]	95% CI	P-value	% Δ wrinkle area[a]	95% CI	P-value
Age	15.8	[7.8 – 24.4]	<0.001	15.9	[9.1 – 23.1]	<0.001
Age^2	-0.1	[-0.15 – -0.03]	0.001	-0.07	[-0.1 – -0.03]	0.002
Resolution variation (batch)	-28.3	[-42.9 – -10.0]	0.004	-20.5	[-32.9 – -5.9]	0.008
Flash light variation	-0.24	[-0.42 – -0.06]	0.008	-0.4	[-0.55 – -0.20]	<0.001
Lower BMI (per unit)	1.5	[0.6 – 2.5]	0.002	2.0	[1.2 – 2.7]	<0.001
Skin color						
pale	-22.8	[-32.9 – -11.2]	<0.001	-29.5	[-38.3 – -19.5]	<0.001
white	-10.4	[-18.8 – -1.2]	0.027	-19.9	[-27.5 – -11.5]	<0.001
white-to-olive	ref	ref	ref	ref	ref	ref
Smoking history[b]						
current	15.6	[4.6 – 27.8]	0.005	33.4	[21.3 – 46.7]	<0.001
former	-2.6	[-10.8 – 6.2]	0.549	5.1	[-2.4 – 13.3]	0.189
never	ref	ref	ref	ref	ref	ref
Baldness[c]						
no/mild baldness	ref	ref	ref	ref	ref	ref
moderate	-3.7	[-12.5 – 6.1]	0.452	-3.6	[-11.5 – 4.9]	0.395
extensive	-0.8	[-9.8 – 9.2]	0.874	-21.8	[-32.6 – -9.1]	0.001
Tendency to develop sunburn	-5.4	[-12.8 – 2.5]	0.175	0.1	[-7.0 – 7.8]	0.973
Lived in sunny country[d]	2.4	[-11.6 – 18.6]	0.749	-3.5	[-16.9 – 12.1]	0.642
Sun-protective behavior[e]	-0.1	[-7.2 – 7.5]	0.978	-6.6	[-13.0 – 0.3]	0.060
Spend winter in sunny country	10.9	[-6.9 – 32.2]	0.246	1.2	[-13.9 – 18.9]	0.890
Outdoor work history[f]	18.9	[9.5 – 29.0]	<0.001	3.5	[-5.6 – 13.5]	0.461
Tanning bed use[g]	6.0	[-5.1 – 18.3]	0.303	16.5	[6.3 – 27.6]	0.001
Education level[h]						
low	-7.4	[-20.1 – 7.3]	0.307	13.6	[0.4 – 28.5]	0.044
medium	-1.5	[-9.0 – 6.6]	0.712	8.5	[0.6 – 17.1]	0.036
high	ref	ref	ref	ref	ref	ref
Alcohol (per glass/day)	0.6	[-1.7 – 2.9]	0.619	3.7	[0.5 – 7.0]	0.025
Dry skin	-3.3	[-10.0 – 3.8]	0.351	-0.7	[-7.2 – 6.3]	0.844
Testosterone (nmol/l)	0.44	[-0.19 – 1.07]	0.172	na	na	na
Free androgen index[i]	na	na	na	-8.2	[-11.0 – -5.2]	<0.001
Estradiol (pmol/l)	na	na	na	0.0	[-0.02 – 0.02]	0.542

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; na, not applicable; ref, reference variable; RS, Rotterdam Study.

[a]the regression betas of each determinant are presented as percentage change (%Δ) in wrinkle area (the % increase in the mean value of wrinkle area per unit increase in the independent variables), calculated by the formula: (exp^{β}-1) * 100%; [b]cigars, cigarettes or pipe; [c]based on the Norwood-Hamilton (NH) scale for men and the Ludwig scale for women; None or minimal: NH score 1, 2, 3, 9, 10, 11 and Ludwig scale score none. Moderate: NH score 4, 5, 6, 12 and Ludwig scale score 1. Extensive: NH score 7, 8 and Ludwig scale score 2, 3; [d]history of living in a sunny country >1 year; [e]wearing sunglasses and/or a brimmed hat in the sunshine; [f]worked or been outdoors ≥ 4 hours daily during at least 25 years; [g]frequency of tanning bed visits in the past 5 years (including facial solarium): more than 10x vs. never or less than 10x; [h]low (primary education); medium (lower secondary education/lower vocational education/ intermediate vocational education); high (general secondary education/higher vocational education/university); [i]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l).

Supplementary Table S2. Spearman's correlation coefficients for global facial wrinkles and site-specific facial wrinkles

	Global wrinkles	Crow's feet wrinkles	Forehead wrinkles
Crow's feet wrinkles	0.48[a]	-	-
Forehead wrinkles	0.58[b]	0.29[d]	-
Upper lip wrinkles	0.52[c]	0.28[e]	0.23[f]

All correlation coefficients have P-values <0.001.

[a]N=3299; [b]N=3261; [c]N=1153 women; [d]N=3261; [e]N=1078 women; [f]N=1068 women.

Supplementary Table S3a. Sex-stratified multivariable linear regression results of crow's feet wrinkle area compared to global facial wrinkle area in the Rotterdam Study

	Global wrinkles Men (N=1602)			Global wrinkles Women (N=2229)			Crow's feet wrinkles Men (N=1370)		Crow's feet wrinkles Women (N=1929)	
Characteristic	% Δ wrinkle area[a]	95% CI	P-value	% Δ wrinkle area[a]	95% CI	P-value	Beta	P-value	Beta	P-value
Age										
age	11.8	[7.4 – 16.5]	<0.001	17.3	[13.0 – 21.7]	<0.001	0.27	<0.001	0.14	<0.001
age^2	-0.06	[-0.10 – -0.02]	<0.001	-0.08	[-0.10 – -0.06]	<0.001	-0.002	<0.001	0.00	<0.001
Batch										
batch 1	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
batch 2	-18.0	[-23.4 – -12.2]	<0.001	-16.5	[-22.1 – -10.5]	<0.001	0.05	0.50	0.04	0.522
batch 3	na	na	na	na	na	na	0.17	0.06	0.09	0.206
Flash light variation	-0.3	[-0.4 – -0.1]	<0.001	-0.4	[-0.5 – -0.2]	<0.001	-0.014	<0.001	0.00	0.016
Lower BMI (per unit)	1.7	[0.9 – 2.4]	<0.001	1.8	[1.3 – 2.4]	<0.001	-0.012	0.11	-0.01	0.004
Skin color										
pale	-21.0	[-29.5 – -11.4]	<0.001	-28.5	[-35.7 – -20.5]	<0.001	-0.20	0.09	-0.22	0.019
white	-10.3	[-16.4 – -3.8]	0.002	-15.5	[-21.5 – -9.0]	<0.001	-0.17	0.02	-0.11	0.105
white-to-olive	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
Smoking history[b]										
current	15.5	[6.8 – 24.8]	<0.001	30.9	[21.8 – 40.7]	<0.001	0.27	<0.001	0.01	0.934
former	-2.1	[-8.2 – 4.5]	0.529	5.7	[0.2 – 11.4]	0.042	0.038	0.58	-0.06	0.235
never	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
Baldness[c]										
no/mild baldness	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
moderate	-5.9	[-11.9 – 0.5]	0.069	-3.9	[-9.5 – 1.9]	0.183	0.11	0.13	0.06	0.239
extensive	-1.8	[-8.0 – 4.7]	0.577	-16.1	[-23.6 – -7.8]	<0.001	0.056	0.41	-0.06	0.511
Tendency to develop sunburn	-5.8	[-11.2 – -0.2]	0.045	-3.8	[-8.8 – 1.5]	0.159	-0.031	0.61	-0.05	0.257

Supplementary Table S3a. Sex-stratified multivariable linear regression results of crow's feet wrinkle area compared to global facial wrinkle area in the Rotterdam Study (continued)

	Global wrinkles Men (N=1602)			Global wrinkles Women (N=2229)			Crow's feet wrinkles Men (N=1370)		Crow's feet wrinkles Women (N=1929)	
Characteristic	% Δ wrinkle area[a]	95% CI	P-value	% Δ wrinkle area[a]	95% CI	P-value	Beta	P-value	Beta	P-value
Lived in sunny country[d]	4.1	[-4.9 – 13.9]	0.381	-4.9	[-15.4 – 6.9]	0.399	0.16	0.10	-0.06	0.532
Sun-protective behavior[e]	0.2	[-5.0 – 5.7]	0.947	-3.8	[-8.6 – 1.4]	0.146	-0.006	0.91	0.01	0.821
Spend winter in sunny country	10.4	[-1.4 – 23.5]	0.086	2.9	[-8.0 – 15.1]	0.615	-0.026	0.83	-0.05	0.646
Education level[f]										
low	2.4	[-7.9 – 14.0]	0.659	16.5	[5.9 – 28.2]	0.002	0.25	0.02	-0.04	0.626
medium	2.7	[-2.8 – 8.6]	0.341	7.2	[1.0 – 13.8]	0.023	0.14	0.02	0.04	0.415
high	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
Alcohol (per glass/day)	0.39	[-1.2 – 2.0]	0.627	3.9	[1.7 – 6.3]	<0.001	0.022	0.22	0.02	0.243
Dry skin	-1.9	[-7.0 – 3.6]	0.497	0.8	[-4.5 – 6.3]	0.778	0.002	0.97	0.01	0.901
Testosterone (nmol/l)	0.47	[-0.00 – 0.94]	0.050	na	na	na	0.0001	0.99	na	na
Free androgen index[g]	na	na	na	-5.6	[-7.5 – -3.7]	<0.001	na	na	-0.02	0.151
Estradiol (pmol/l)	na	na	na	0.0	[0.0 – 0.0]	0.789	na	na	0.00	0.663

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; na, not applicable; ref, reference variable.

[a]the regression betas of each determinant are presented as percentage change (%Δ) in wrinkle area (the % increase in the mean value of wrinkle area per unit increase in the independent variables), calculated by the formula: $(\exp^{\beta}-1) * 100\%$; [b]cigars, cigarettes or pipe; [c]based on the Norwood-Hamilton (NH) scale for men and the Ludwig scale for women; None or minimal: NH score 1, 2, 3, 9, 10, 11 and Ludwig scale score none. Moderate: NH score 4, 5, 6, 12 and Ludwig scale score 1. Extensive: NH score 7, 8 and Ludwig scale score 2, 3; [d]history of living in a sunny country >1 year; [e]wearing sunglasses and/or a brimmed hat in the sunshine; [f]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [g]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l).

Supplementary Table S3b. Sex-stratified multivariable linear regression results of forehead wrinkle area compared to global facial wrinkle area in the Rotterdam Study

	Global wrinkles Men (N=1602)			Global wrinkles Women (N=2229)			Forehead wrinkles Men (N=1351)		Forehead wrinkles Women (N=1910)	
Characteristic	% Δ wrinkle area[a]	95% CI	P-value	% Δ wrinkle area[a]	95% CI	P-value	Beta	P-value	Beta	P-value
Age										
age	11.8	[7.4 – 16.5]	<0.001	17.3	[13.0 – 21.7]	<0.001	0.01	0.0012	0.03	<0.001
age^2	-0.06	[-0.10 – -0.02]	<0.001	-0.08	[-0.10 – -0.06]	<0.001	na	na	na	na
Batch										
batch 1	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
batch 2	-18.0	[-23.4 – -12.2]	<0.001	-16.5	[-22.1 – -10.5]	<0.001	0.04	0.5787	0.18	0.018
batch 3	na	na	na	na	na	na	0.17	0.0442	0.16	0.076
Flash light variation	-0.3	[-0.4 – -0.1]	<0.001	-0.4	[-0.5 – -0.2]	<0.001	0.00	0.201	0.00	0.165
Lower BMI (per unit)	1.7	[0.9 – 2.4]	<0.001	1.8	[1.3 – 2.4]	<0.001	0.02	0.0104	0.02	0.001
Skin color										
pale	-21.0	[-29.5 – -11.4]	<0.001	-28.5	[-35.7 – -20.5]	<0.001	-0.06	0.5671	-0.41	<0.001
white	-10.3	[-16.4 – -3.8]	0.002	-15.5	[-21.5 – -9.0]	<0.001	-0.05	0.4465	-0.30	<0.001
white-to-olive	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
Smoking history[b]										
current	15.5	[6.8 – 24.8]	<0.001	30.9	[21.8 – 40.7]	<0.001	0.04	0.6026	0.27	<0.001
former	-2.1	[-8.2 – 4.5]	0.529	5.7	[0.2 – 11.4]	0.042	-0.05	0.4061	0.03	0.635
never	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
Baldness[c]										
no/mild baldness	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
moderate	-5.9	[-11.9 – 0.5]	0.069	-3.9	[-9.5 – 1.9]	0.183	-0.03	0.6283	0.06	0.370
extensive	-1.8	[-8.0 – 4.7]	0.577	-16.1	[-23.6 – -7.8]	<0.001	0.07	0.2703	-0.12	0.278
Tendency to develop sunburn	-5.8	[-11.2 – -0.2]	0.045	-3.8	[-8.8 – 1.5]	0.159	-0.10	0.1017	0.01	0.817

Supplementary Table S3b. Sex-stratified multivariable linear regression results of forehead wrinkle area compared to global facial wrinkle area in the Rotterdam Study (continued)

	Global wrinkles Men (N=1602)			Global wrinkles Women (N=2229)			Forehead wrinkles Men (N=1351)		Forehead wrinkles Women (N=1910)	
Characteristic	% Δ wrinkle area[a]	95% CI	P-value	% Δ wrinkle area[a]	95% CI	P-value	Beta	P-value	Beta	P-value
Lived in sunny country[d]	4.1	[-4.9 – 13.9	0.381	-4.9	[-15.4 – 6.9]	0.399	0.19	0.0367	-0.04	0.722
Sun-protective behavior[e]	0.2	[-5.0 – 5.7]	0.947	-3.8	[-8.6 – 1.4]	0.146	-0.02	0.7474	-0.04	0.508
Spend winter in sunny country	10.4	[-1.4 – 23.5]	0.086	2.9	[-8.0 – 15.1]	0.615	0.30	0.0121	-0.11	0.336
Education level[f]										
low	2.4	[-7.9 – 14.0]	0.659	16.5	[5.9 – 28.2]	0.002	0.07	0.5011	0.19	0.066
medium	2.7	[-2.8 – 8.6]	0.341	7.2	[1.0 – 13.8]	0.023	0.03	0.6164	-0.02	0.780
high	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
Alcohol (per glass/day)	0.39	[-1.2 – 2.0]	0.627	3.9	[1.7 – 6.3]	<0.001	-0.02	0.3571	-0.02	0.469
Dry skin	-1.9	[-7.0 – 3.6]	0.497	0.8	[-4.5 – 6.3]	0.778	-0.05	0.3853	-0.01	0.889
Testosterone (nmol/l)	0.47	[-0.00 – 0.94]	0.050	na	na	na	0.00	0.5578	na	na
Free androgen index[g]	na	na	na	-5.6	[-7.5 – -3.7]	<0.001	na	na	-0.06	0.005
Estradiol (pmol/l)	na	na	na	0.0	[0.0 – 0.0]	0.789	na	na	0.00	0.892

Age^2 was not added in the model for forehead wrinkles as it was not statistically significant.

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; na, not applicable; ref, reference variable.

[a]the regression betas of each determinant are presented as percentage change (%Δ) in wrinkle area (the % increase in the mean value of wrinkle area per unit increase in the independent variables), calculated by the formula: $(\exp^{\beta}-1) \cdot 100\%$; [b]cigars, cigarettes or pipe; [c]based on the Norwood-Hamilton (NH) scale for men and the Ludwig scale for women; None or minimal: NH score 1, 2, 3, 9, 10, 11 and Ludwig scale score none. Moderate: NH score 4, 5, 6, 12 and Ludwig scale score 1. Extensive: NH score 7, 8 and Ludwig scale score 2, 3; [d]history of living in a sunny country >1 year; [e]wearing sunglasses and/or a brimmed hat in the sunshine; [f]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [g]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l).

Supplementary Table S3c. Multivariable linear regression results of upper lip wrinkle area in women compared to global facial wrinkle area in women in the Rotterdam Study

	Global wrinkles Women (N=2229)			Upper lip wrinkles Women (N=1153)	
Characteristic	% Δ wrinkle area[a]	95% CI	P-value	Beta	P-value
Age					
age	17.3	[13.0 – 21.7]	<0.001	0.13	0.001
age^2	-0.08	[-0.10 – -0.06]	<0.001	0.00	0.033
Batch					
batch 1	ref	ref	ref	ref	ref
batch 2	-16.5	[-22.1 – -10.5]	<0.001	-0.10	0.178
Flash light variation	-0.4	[-0.5 – -0.2]	<0.001	0.00	0.720
Lower BMI (per unit)	1.8	[1.3 – 2.4]	<0.001	0.02	0.001
Skin color					
pale	-28.5	[-35.7 – -20.5]	<0.001	0.14	0.187
white	-15.5	[-21.5 – -9.0]	<0.001	0.05	0.535
white-to-olive	ref	ref	ref	ref	ref
Smoking history[b]					
current	30.9	[21.8 – 40.7]	<0.001	0.45	<0.001
former	5.7	[0.2 – 11.4]	0.042	0.13	0.019
never	ref	ref	ref	ref	ref
Baldness[c]					
no/mild baldness	ref	ref	ref	ref	ref
moderate	-3.9	[-9.5 – 1.9]	0.183	-0.05	0.385
extensive	-16.1	[-23.6 – -7.8]	<0.001	-0.18	0.066
Tendency to develop sunburn	-3.8	[-8.8 – 1.5]	0.159	0.02	0.768
Lived in sunny country[d]	-4.9	[-15.4 – 6.9]	0.399	-0.10	0.423
Sun-protective behavior[e]	-3.8	[-8.6 – 1.4]	0.146	0.08	0.144
Spend winter in sunny country	2.9	[-8.0 – 15.1]	0.615	-0.18	0.124
Education level[f]					
low	16.5	[5.9 – 28.2]	0.002	0.11	0.289
medium	7.2	[1.0 – 13.8]	0.023	0.19	0.001
high	ref	ref	ref	ref	ref
Alcohol (per glass/day)	3.9	[1.7 – 6.3]	<0.001	-0.02	0.507
Dry skin	0.8	[-4.5 – 6.3]	0.778	0.04	0.395
Free androgen index[g]	-5.6	[-7.5 – -3.7]	<0.001	-0.05	0.035
Estradiol (pmol/l)	0.0	[0.0 – 0.0]	0.789	0.00	0.572

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; ref, reference variable.

[a]the regression betas of each determinant are presented as percentage change (%Δ) in wrinkle area (the % increase in the mean value of wrinkle area per unit increase in the independent variables), calculated by the formula: (exp^{β}-1) * 100%; [b]cigars, cigarettes or pipe; [c]based on the Ludwig scale for women; None or minimal: Ludwig scale score none. Moderate: Ludwig scale score 1. Extensive: Ludwig scale score 2, 3; [d]history of living in a sunny country >1 year; [e]wearing sunglasses and/or a brimmed hat in the sunshine; [f]low (primary education); medium (lower second-

ary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [g]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l).

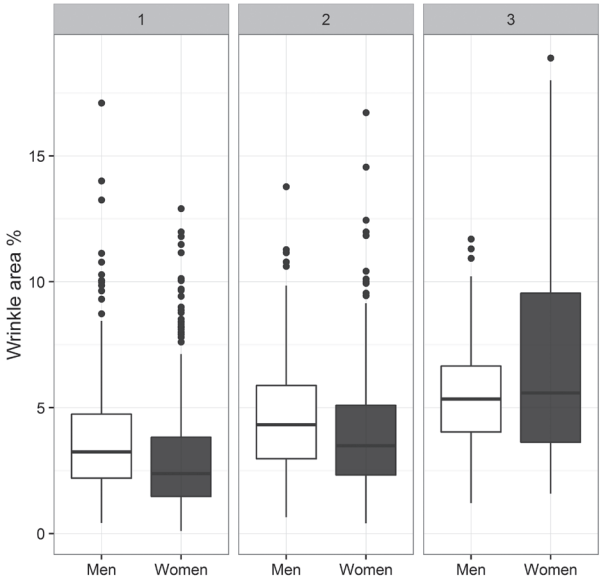
Supplementary Table S4. Betas and P-values for each interaction term, added to the final fully adjusted linear regression model available for both sexes (N=3019; only complete cases used)

Interaction term	Beta	P-value
Sex*age	-2.2×10^{-2}	<0.001
Sex*BMI	-3.6×10^{-4}	0.95
Sex*smoking history[a]		
current	-0.19	3.3×10^{-3}
former	-0.16	1.1×10^{-3}
never	ref	ref
Sex*skin color		
very white	0.18	4.5×10^{-2}
white	0.04	0.49
white-to-olive	ref	ref
Sex*baldness[b]		
no/mild baldness	ref	ref
moderate	-9.0×10^{-2}	7.5×10^{-2}
extensive	6.6×10^{-2}	0.29
Sex*alcohol (per glass/day)	-2.6×10^{-2}	7.6×10^{-2}
Sex*education level[c]		
low	-0.25	2.7×10^{-3}
medium	-0.13	6.3×10^{-3}
high	ref	ref
Sex*tendency to develop sunburn	1.8×10^{-2}	0.68

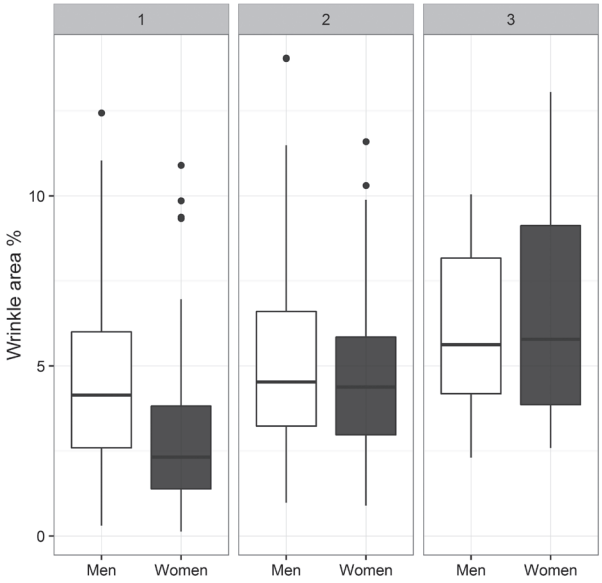
Abbreviation: ref, reference variable.

[a]smoking history: cigars, cigarettes or pipe; [b]baldness: based on the Norwood-Hamilton (NH) scale for men and the Ludwig scale for women; None or minimal: NH score 1, 2, 3, 9, 10, 11 and Ludwig scale score none. Moderate: NH score 4, 5, 6, 12 and Ludwig scale score 1. Extensive: NH score 7, 8 and Ludwig scale score 2, 3; [c]education level: low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university).

A

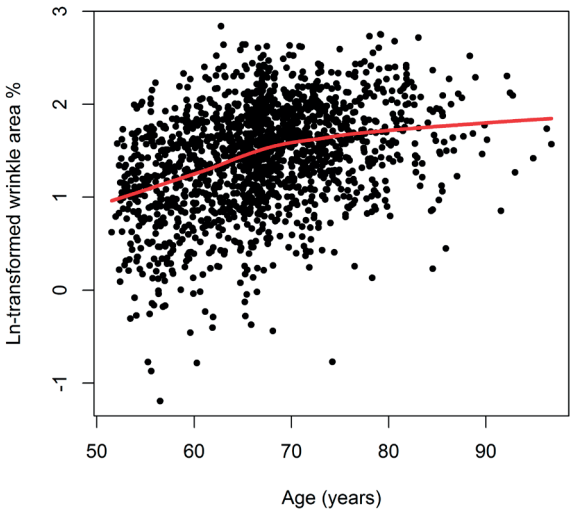


B

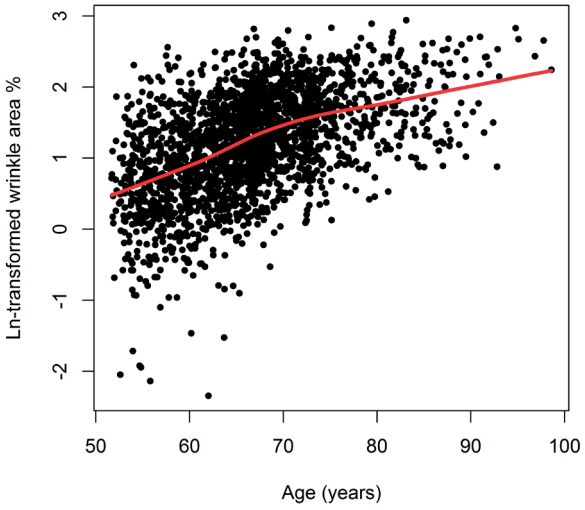


Supplementary Figure S1. Boxplots of wrinkle area percentage per age group (1: <65 years old; 2: 65-75 years old; 3: ≥75 years old), split by sex and stratified by the UV variable "outdoor work". (**A**) 690 men and 1221 women who have not worked/been outdoors for ≥4 hours daily during at least 25 years; (**B**) 298 men and 213 women who have worked/been outdoors for ≥4 hours daily during at least 25 years. In both outdoor groups (A and B), men have a higher wrinkle area than women in the lowest and middle age groups, only in the 65-75 years old group of B this is not statistically significant. In the highest age group (≥75 years) women have a higher wrinkle area than men, which is only statistically significant for group A.

A



B



Supplementary Figure S2. Graphs of ln-transformed wrinkle area percentage by age, for men (**A**, N=1602) and women (**B**, N=2229) separately. The red line represents the regression line of the two variables.

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Chapter 4

Facial wrinkles in Europeans: a genome-wide association study

M.A. Hamer
L.M. Pardo Cortes
L.C. Jacobs
J. Deelen
A.G. Uitterlinden
E. Slagboom
D. van Heemst
H.W. Uh
M. Beekman
M. Kayser
F. Liu
D.A. Gunn
T. Nijsten

J Invest Dermatol. 2018 Aug;138(8):1877-1880

To the Editor,

Wrinkles are among the most notable components of skin aging and are influenced by many different risk factors[1]. Although wrinkle variation has been shown to be a heritable trait, (55%)[2], specific gene variants for wrinkles have not yet been identified. Previous studies have identified the *MC1R* gene as influencing skin photoaging and pigmented spots[3-6], but its role in wrinkling is not clear. In this study, we performed the largest genome-wide association study (GWAS) for global facial wrinkles available to date in 3513 participants from the Rotterdam Study (RS) using a digital wrinkle measure[1] and sought to replicate the most suggestive associations in an independent dataset of 599 participants from the Leiden Longevity Study (LLS).

A detailed description of the methods is presented in the Supplementary Material. The RS is an ongoing Dutch prospective population-based cohort study of 14,926 participants aged 45 years or older[7]. This study includes 3513 northwestern European participants for whom standardized facial photographs and quality-controlled genotype data were available. The RS has been approved by the Medical Ethics Committee of the Erasmus University Medical Center and by the Ministry of Health, Welfare and Sports of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)". All participants provided written informed consent to participate in the study. The LLS is a family-based study[8] that includes 599 participants in this study. In the RS, wrinkle area was digitally quantified as wrinkle area percentage of the face using semi-automated image analysis of high-resolution facial photographs. For wrinkle grading in the LLS, a 9-point photonumeric scale was used[2]. The study protocol was approved by the medical ethics committee of the Leiden University Medical Center, and all participants gave written informed consent. In the RS, DNA from whole blood was extracted following standard protocols, and quality controls were applied on markers and individuals[7]. Imputations were performed with 1000 Genomes (GIANT phase I version 3) as the reference panel[9]. In total, 30,072,738 markers were genotyped/imputed. After quality controls, 9,009,554 autosomal single-nucleotide polymorphisms (SNPs) were available. In the LLS, imputation was performed similarly, and association testing was conducted using QT-assoc[10]. The RS served as discovery dataset. We performed linear regression using an additive model (SNP dosage data)[11] adjusting for age, sex, the first four genetic principal components, and two technical variables. These last two variables correct for possible variations in resolution and flash light of the facial photos[1]. For variations in resolution, a variable describing the batch number was used. For flash light variation, the in-person difference between skin lightness in the images and that taken by a spectrophotometer (CM-600d; Konica-Minolta, Osaka, Japan) on the cheek was used by calculating the residuals of these two lightness variables regressed on each other[4]. We selected all SNPs with P-values $<5\times10^{-6}$ for the replication phase. We also performed a meta-analysis of the RS and LLS together for the top hits, as well as a genome-wide meta-analysis. Several sensitivity analyses (top SNP associations in men and women separately; with different facial wrinkling sites; possible interactions between SNPs and sex, body mass index, and smoking; and a univariate analysis excluding age and sex) and validation of previously published associations between SNPs and skin aging were performed (Supplementary Material).

In the RS, most participants were women (N=2045, 58.2%) and the median age was 66.2 (overall: range 51-98; men: median 66.5, range 51-96; women: median 66.0, range 51-98) years. Men showed a higher average wrinkle area (median facial wrinkle area 4.4%, interquartile range (IQR) 2.9-6.2) than women (3.5%, IQR 2.1-5.5). In the LLS, the mean age was 63.1 years, and 53.8% of participants were women (Supplementary Table S1). The GWAS of global facial wrinkle area in the RS yielded 25 suggestive hits (P-values $<5\times10^{-6}$, Table 1), but none of them were genome-wide significant (Figure 1, Supplementary Figures S1 and S2). The strongest signal was found for an intergenic SNP (rs10476781; P-value 9.5×10^{-8}) on chromosome 5 between the Neuromedin U Receptor 2 (*NMUR2*) and *CTB-1202.1* (long non-coding RNA, *LINC01933*) genes. In the RS, this SNP had a minor allele frequency of 6% and an imputation score of 0.5. The SNP rs10476781 showed moderate linkage disequilibrium (LD, r^2=0.4) with other SNPs on chromosome 5, explaining the moderate imputation score. The effect allele (rs10476781[T], allele frequency 94%) had an effect size of -0.21 (standard error 0.04).

Estimating pairwise LD between all SNPs with suggestive associations (25 SNPs, Table 1) resulted in 11 independent loci (r^2 ≤0.5). There was no LD between rs10476781 and other suggestive SNPs in our dataset (r^2 ≤0.5, Supplementary Table S2 and Supplementary Figure S3). We tested for associations between wrinkles in the LLS replication cohort and the 25 SNPs with suggestive associations. The top SNP, rs10476781, had a nominal P-value of 0.08 in the LLS, and the others could not be replicated (all P-values > 0.2). In a meta-analysis of the two cohorts for the top hits, rs10476781 was genome-wide significant (P-value 2.2×10^{-8}, Table 1). Other suggestive associations (P-values $<=5\times10^{-6}$) from the genome-wide meta-analysis of the two cohorts are presented in Supplementary Table S3 and Supplementary Figure S4. Additional genome-wide meta-analysis of the RS and LLS did not yield any new findings (Supplementary Material, including Supplementary Table S3).

Because of known sex differences in facial wrinkling[1], we also tested for associations between the top SNPs and global wrinkling in a sex-stratified analysis. No genome-wide significant hits or interactions (SNP*sex) were found (Supplementary Table S4).

To our knowledge, this is the largest GWAS of global facial wrinkling conducted thus far, and we found that the rs10476781 SNP was a suggestive hit for global facial wrinkling in the RS (3513 northwestern Europeans) and a significant genome-wide hit in a meta-analysis of the RS and LLS cohorts together (N=4122). However, we cannot exclude that this may be a false positive finding because the imputation score in the RS was moderate, and the SNP has a very low frequency in the general population (minor allele frequency <0.01, and thus it was not included in the latest release of 1000 Genomes). The latter likely explains the moderate imputation quality because rare variants are more difficult to impute. However, it has a higher frequency in Dutch populations (GoNL, a Dutch-specific reference dataset; 2% minor allele frequency, although with low quality[12]), and, among the replicated SNPs in the LLS cohort, this SNP had the lowest P-value. Further confirmation of the association of this SNP with wrinkles is now required.

Table 1. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles in the Rotterdam Study and Leiden Longevity Study and a meta-analysis of these two cohorts[a]

			Discovery cohort: RS (N=3513)						Replication cohort: LLS (N=599)				Meta-analysis: RS & LLS (N=4112)						
SNP	**Chr**	**Position[b]**	**EA**	**OA**	**EAF**	**OAF**	**β (SE)**	**P-value**	**EA**	**EAF**	**β (SE)**	**P-value**	**EA**	**Dir**	**Z[c]**	**P-value**	**I²**	**Cochran's Q**	**Het P-value**
1:3118674:D	1	3118674	D	I	0.12	0.88	0.11 (0.02)	1.8×10^{-6}	I	0.90	0.18 (0.13)	0.18	D	+-	3.90	9.7×10^{-5}	89.40	9.40	0.002
rs11577655	1	3119489	T	C	0.13	0.87	0.11 (0.02)	4.6×10^{-6}	C	0.90	0.17 (0.13)	0.19	T	+-	3.74	1.9×10^{-4}	88.60	8.79	0.003
rs6429657	1	14702354	A	G	0.96	0.04	-0.19 (0.04)	1.6×10^{-6}	G	0.05	0.17 (0.18)	0.35	A	–	-4.79	1.7×10^{-6}	0	0.95	0.33
rs702491	1	54194992	T	C	0.19	0.81	0.09 (0.02)	2.4×10^{-6}	T	0.21	0.09 (0.09)	0.33	T	++	4.74	2.1×10^{-6}	0	0.78	0.38
rs61812508	1	147251772	A	G	0.05	0.95	-0.18 (0.04)	4.3×10^{-6}	G	0.96	0.08 (0.20)	0.69	A	–	-4.40	1.1×10^{-5}	48.20	1.93	0.16
rs11583958	1	147291718	A	T	0.04	0.96	-0.18 (0.04)	3.3×10^{-6}	T	0.96	-0.04 (0.19)	0.84	A	-+	-4.22	2.4×10^{-5}	73.90	3.83	0.05
1:246689691:I	1	246689691	D	I	0.60	0.40	0.07 (0.02)	3.7×10^{-6}	D	0.59	-0.05 (0.07)	0.54	D	+-	4.05	5.2×10^{-5}	81.50	5.42	0.02
rs114667268	2	12433490	T	C	0.01	0.99	-0.49 (0.10)	2.9×10^{-6}	C	0.99	-0.44 (0.65)	0.49	T	-+	-4.07	4.8×10^{-5}	82.90	5.84	0.02
rs7608236	2	180062867	A	G	0.29	0.71	-0.07 (0.02)	4.1×10^{-6}	G	0.72	-0.06 (0.08)	0.43	A	-+	-3.96	7.6×10^{-5}	83.90	6.20	0.01
rs116248825	3	26420135	A	C	0.04	0.96	-0.28 (0.06)	4.1×10^{-6}	C	0.96	0.28 (0.25)	0.27	A	–	-4.68	2.9×10^{-6}	0	0.55	0.46
rs9867656	3	30100084	A	G	0.34	0.66	-0.07 (0.01)	3.7×10^{-6}	A	0.35	-0.06 (0.07)	0.37	A	–	-4.62	3.9×10^{-6}	0	0.89	0.35
rs11711327	3	30101254	A	G	0.66	0.34	0.07 (0.01)	3.1×10^{-6}	G	0.35	-0.06 (0.07)	0.38	A	++	4.65	3.3×10^{-6}	0	0.93	0.34
rs112608607	5	102908739	T	C	0.97	0.03	0.22 (0.05)	3.8×10^{-6}	T	0.97	0.21 (0.22)	0.35	T	++	4.63	3.7×10^{-6}	0	0.83	0.36
rs113322056	5	102913288	A	G	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	A	0.96	0.18 (0.21)	0.41	A	++	4.64	3.4×10^{-6}	3.60	1.04	0.31
rs146551307	5	102915236	T	C	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	T	0.96	0.18 (0.21)	0.42	T	++	4.64	3.5×10^{-6}	3.80	1.04	0.31
5:102915644:D	5	102915644	D	I	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	I	0.96	0.16 (0.21)	0.44	D	–	-4.53	6.0×10^{-6}	6.40	1.07	0.30
rs10476781	**5**	**151763633**	**T**	**C**	**0.94**	**0.06**	**-0.21 (0.04)**	**9.5×10^{-8}**	**T**	**0.94**	**-0.33 (0.19)**	**0.08**	**T**	–	**-5.60**	**2.2×10^{-8}**	**0**	**0.19**	**0.67**
rs72811030	5	179729009	A	G	0.38	0.62	0.07 (0.02)	1.7×10^{-6}	G	0.60	-0.04 (0.08)	0.62	A	++	4.61	4.0×10^{-6}	46.30	1.86	0.17
rs1225927	6	7871037	T	G	0.75	0.25	0.07 (0.02)	3.5×10^{-6}	T	0.75	0.08 (0.08)	0.30	T	++	4.69	2.8×10^{-6}	0	0.67	0.41
9:16847398:D	9	16847398	D	I	0.98	0.02	0.30 (0.07)	4.7×10^{-6}	I	0.02	-0.13 (0.31)	0.68	D	++	4.39	1.1×10^{-5}	46.40	1.86	0.17
rs185291539	10	84338421	A	G	0.98	0.02	0.41 (0.09)	4.8×10^{-6}	A	0.97	0.03 (0.26)	0.90	A	++	4.28	1.9×10^{-5}	62.20	2.64	0.10
rs62047859	16	76826391	A	T	0.03	0.97	0.21 (0.04)	1.0×10^{-6}	T	0.97	-0.26 (0.24)	0.29	A	++	4.92	8.9×10^{-7}	0	0.80	0.37
rs62077967	17	61253263	C	G	0.96	0.04	0.19 (0.04)	4.6×10^{-6}	C	0.96	-0.05 (0.19)	0.81	C	+-	4.15	3.4×10^{-5}	74.10	3.87	0.05
rs72845240	17	61361539	C	G	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	G	0.96	-0.06 (0.19)	0.77	C	-+	-4.12	3.8×10^{-5}	75.50	4.08	0.04
rs189819077	18	34933012	A	G	0.03	0.97	-0.20 (0.04)	1.8×10^{-6}	G	0.97	0.15 (0.23)	0.51	A	–	-4.67	3.0×10^{-6}	32.90	1.49	0.22

Abbreviations: A, adenine; C, cytosine; Chr, chromosome; D, deletion; Dir, direction of the effects; EA, effect allele; EAF, effect allele frequency; G, guanine; GWAS, genome-wide association study; Het P-value, heterogeneity P-value; I, insertion; I^2, heterogeneity I^2; LLS, Leiden Longevity Study; OA, other allele; OAF, other allele frequency; RS, Rotterdam Study; SE, standard error; SNP, single-nucleotide polymorphism; T, thymine. Boldface indicates the top SNP. [a]analyses are adjusted for age, sex, and the first four genetic principal components and in addition, for the RS, for technical variables of the digital measurement; [b]based on GRCh37/hg19; [c]weighted Z-score.

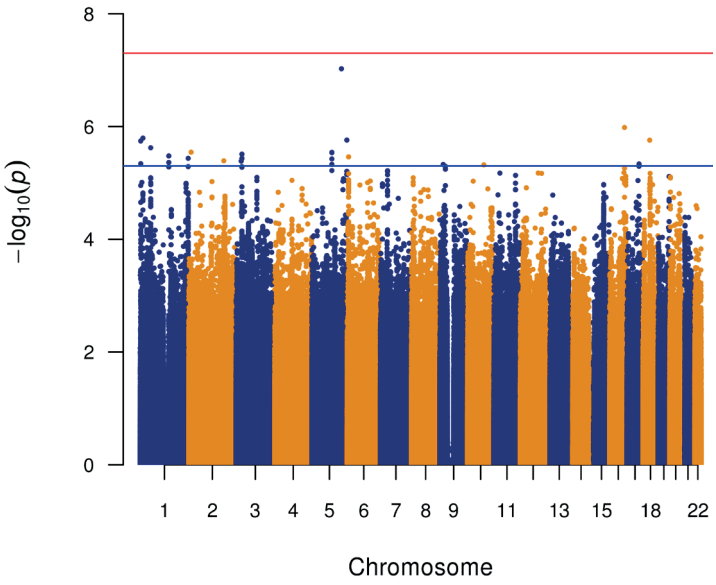

Figure 1. Manhattan plot of the genome-wide associations for wrinkle area in the discovery cohort (Rotterdam Study, N=3513). All SNPs are represented by dots and displayed per chromosome (x-axis); y-axis shows negative $\log_{10}$-transformed P-values.

The *MC1R* gene influences skin aging[3,5,6,13]. However, we did not find any significant association between *MC1R* variants and wrinkles, which suggests that these variants are not influencing facial wrinkle variation as measured in the RS cohort but instead other skin aging phenotypes, such as pigmented age spots[4]. Furthermore, we did not replicate SNPs previously reported as associated with skin aging, except for a nominally significant association between rs12203592 and wrinkles in the LLS. Reasons for the lack of association could be that these SNPs are false positives because of the small sample sizes[14] or because of phenotypic heterogeneity in photoaging versus wrinkling in our study. In addition, genetic heterogeneity could play a role.

We cannot exclude that other SNPs may be associated with wrinkling, because the heritability was 42% in the RS (P-value 4.4×10^{-8}, 95%CI 28% to 61%)[15]. Most likely, the effects of each influencing SNP are too small to be detected with a sample size as used in this study, because we had a 77% power to detect SNPs with moderate effects (Supplementary Material including Supplementary Tables S5-S8). This highlights the importance of increasing sample sizes for future GWASs. Another limitation is that in the replication cohort, only photonumeric grading was available, although there is a high correlation between digital and photonumeric grading (Spearman's rho 0.8-0.9)[16]; hence, we believe that our replication is valid.

In conclusion, we found a genome-wide statistically significant association between the SNP rs10476781 (P-value 2.2×10^{-8}) and global facial wrinkling in a meta-analysis of two independent northwestern European cohorts. This intergenic SNP (628 kilo base pairs downstream of the Neuromedin U Receptor gene) is an interesting candidate but needs further validation.

ACKNOWLEDGMENTS

The authors are grateful to the study participants, the staff from the Rotterdam Study and the Leiden Longevity Study, and the participating general practitioners and pharmacists. Regarding the Rotterdam Study, we thank Sophie Flohil, Emmilia Dowlatshahi, Robert van der Leest, Joris Verkouteren and Ella van der Voort for collecting the phenotypes. Additionally we thank Sophie van den Berg for masking and reviewing all the photographs. We acknowledge Jaspal Lall for masking the photographs and creating the digital wrinkle measurements.

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SUPPLEMENTARY MATERIAL

MATERIAL AND METHODS

Study population

Rotterdam Study (RS)

The RS is an ongoing prospective population-based cohort study of 14,926 participants aged 45 years or older, living in Ommoord, a suburb of Rotterdam, the Netherlands. The RS consists of a major cohort (RS-I, N=7983) and two extensions (RS-II, N=3011 and RS-III, N=3932). Details of the study design have been described elsewhere[1]. The current study includes 3513 participants of northwestern European ancestry (who visited the research center between September 2010 and June 2014), for whom facial photographs and genotype data were available after quality controls. During routine visits at the research center, a full body skin examination was performed by trained physicians, and high-resolution standardized full-face photographs were obtained of participants not wearing make-up, cream, or jewelry, using a Premier 3dMD face3-plus UHD camera (3dMD, Atlanta, GA, USA) in a room without daylight.

Leiden Longevity Study

The LLS has been described in detail previously[2]. This family-based study consists of 1671 offspring of 421 nonagenarian sibling pairs of Dutch descent and their 744 partners[3]. For the current study, there were 599 participants with valid genotype data after quality control and a suitable facial photograph (exclusions were for example due to the presence of beards or poor photo quality). During visits at the Leiden University Medical Center, high-resolution standardized full-face photographs were obtained of participants not wearing make-up, cream, or jewelry, using a Fuji S2 (Tokyo, Japan) camera system. The photos used in this study were collected from November 2006 to April 2008.

Phenotyping

In the RS, wrinkle area was digitally quantified as wrinkle area percentage of the facial skin using semi-automated image analysis of the high-resolution facial photographs. The algorithms, digital rendering, measurement and validation of the outcome measure have been described in detail previously, using a randomly selected subset of photographs of 100 participants[4]. To correct for technical variation, we included two variables in the analyses: the first accounted for differences in resolution between two sets of the images and the second accounted for variations in flash light[5].

For wrinkle grading in the LLS, individuals were graded by two skin aging experts. Skin wrinkling was graded on a 9-point scale by a visual assessment of the number and depth of fine and course wrinkles (i.e. shallow indentations or lines, deep lines, furrows or creases). Fine wrinkles tend to

appear in periorbital and perioral regions and had a small weighting in the 9-point photographic scale; coarse wrinkles had a strong weighting in the scale and tend to appear on the forehead, glabella, chin, nasolabial and periorbital areas and are larger and closer to the eyes and mouth than are fine wrinkles. The mean value from the two graders was used for further analyses[6].

Genotyping and Imputation

In the RS, DNA from whole blood was extracted following standard protocols with details presented elsewhere[1]. In brief, genotyping was carried out using the Infinium II HumanHap 550K Genotyping BeadChip version 3 (Illumina, San Diego, CA, USA) for RS-I (N=6291) and RS-II (N=2157) cohorts while Illumina Human 610 Quad Arrays were used to genotype the RS-III cohort (N=3048). A quality control on markers and individuals was applied to the genotyping[1]. We imputed the RS-I, RS-II and RS-III cohorts separately, with 1000Genomes (GIANT Phase I version 3) as the reference panel[7]. In total, 30,072,738 markers were genotyped and/or imputed. After all quality controls, a total of 9,009,554 autosomal SNPs (minor allele frequency >1%; imputation quality score (r^2_pihat) > 0.3) was available.

In the LLS, genotyping was performed using Illumina Human660W-Quad and OmniExpress BeadChips as described elsewhere[8]. Imputation was performed using IMPUTE with the 1000 Genomes (Phase I version 3) reference panel[7]. Association testing was conducted using QT-assoc[9] to take into account family relations and imputation uncertainty.

Statistical analysis

Stage 1: Discovery phase

In the RS, the wrinkle area distribution was highly right-skewed. Therefore, wrinkle area was transformed using the natural logarithm (ln). We used the three cohorts from the RS as the discovery dataset. The associations between SNPs and (ln-transformed) wrinkles were tested per cohort. We performed linear regression using an additive model using SNP dosage data[10] and adjusting for age, sex, the first four genetic principal components, resolution variability and flash light variability[5]. The significance of the association was tested using the likelihood ratio test with one degree of freedom. The GWAS analyses were implemented in the ProbABEL package[10]. The quality control of the GWAS summary statistics per cohort was performed using the EasyQC software with parameter defaults[11]. Next, a meta-analysis of the three RS cohorts was carried out using the METAL software using an inverse variance approach, allowing for genomic control correction (genomic inflation factors were <1.03, therefore no adjustments were made) and heterogeneity checks[12]. P-values < 5×10^{-8} were considered genome-wide significant.

Stage 2: Replication and joint meta-analysis

We selected all SNPs with P-values <5×10^{-6} from the discovery phase to test for associations between these SNPs and wrinkles in the LLS cohort. The SNPs selected for replication were analyzed in the LLS cohort using linear regression adjusted for age, sex, and familial relations using the

software package QT-assoc[9], which is based on a modified version of the score test. To correct for multiple testing, we calculated the pair-wise linkage disequilibrium (LD; r^2) between the SNPs with suggestive association in plink using genotypes from BestGuess data[13]. The P-values were adjusted by dividing the nominal P-value by the number of independent tests. We identified 11 independent loci ($r^2 \leq 0.5$), leading to a significance threshold of 4.5×10^{-3}. We also performed a meta-analysis of the RS and LLS together for the top hits, using the METAL software mentioned above. P-values < 5×10^{-8} were considered as genome-wide significant using the weighted Z-score method, implemented in METAL[12]. SNP heterogeneity was tested using I^2 and Cochran's Q methods. The top SNPs were annotated to genes using Ensembl (http://browser.1000genomes.org/index.html).

Sensitivity analyses in the RS

Because the top SNP we found in our GWAS has been associated with body weight in other studies[14,15], and given the known associations between BMI and wrinkles[16], we also tested associations between the top SNPs identified in the RS and global wrinkles by additionally adjusting for BMI to determine whether the effects were independent of BMI. We have also added analyses with BMI*SNP as an interaction term for the top SNPs, as well as analyses with smoking*SNP. In addition, we tested for interactions between the suggestive SNPs and sex (by adding a sex*SNP covariate) and performed a sex-stratified analysis to analyze the effects of the suggestive SNPs (P-values $<5 \times 10^{-6}$) separately in men and women, because it is known that wrinkle prevalence is different between sexes[5]. Furthermore, we have also performed a univariate analysis (without age and sex as covariates, but including the two technical variables and the first four genetic principal components). Data on site-specific wrinkling, namely crow's feet, forehead wrinkles and upper lip wrinkles (in women only) was also available[5]. The technical wrinkle measurements were the same as for the global facial wrinkles and area of wrinkles was measured. We investigated whether the suggestive SNPs that were associated with global wrinkling also associate with site-specific wrinkling. Finally, we have also performed a genome-wide meta-analysis, including 4112 subjects from the RS and the LLS.

Validation of previously published associations between SNPs and skin aging

To check the associations of recently found candidate SNPs for skin aging we examined these SNPs in our dataset. The following SNPs, which showed a genome-wide significant association in previous studies, were investigated: rs6975107, rs11863929, rs7616661[17], rs322458[18], rs12203592, rs62543565, rs35063026, rs6059655[19], rs11876749[20], rs185146, rs12203592, rs4268748, rs1805007 and rs1805008[21]. We were unable to determine the effect of rs318125, rs5916727 and rs1578826[17], because we only analyzed autosomal SNPs.

Wrinkle area site-specific facial wrinkles

Besides measuring wrinkles of the whole face, we have also measured wrinkles at three specific sites (crow's feet, forehead wrinkles, and in women upper lip wrinkles[5]). The technical wrinkle measurements were the same as for the global facial wrinkles; however, these measurements were performed on the original 2D photographs of the left-hand side of the face[4].

Statistical analysis

The site-specific facial wrinkles followed a right-skewed distribution, but also included a considerable proportion of zeros as value for some photos, indicating there were no wrinkles at the site at all. Therefore, we could not ln-transform the phenotype. To better fit the data for regression, we used rank-based inverse normal transformation, where the mean is set to zero and the standard deviation to one[22]. We tested for associations between SNPs and site-specific wrinkles using the same methods as for global wrinkles (main manuscript).

RESULTS

Study populations

Rotterdam Study

Between September 2010 and June 2014, a total of 4649 participants visited the in-person examination of the RS, which included extensive dermatological assessments and capturing a facial 3D photo. Of these, 818 were excluded due to poor image quality of the 3D photo, non-northwestern European origin, make-up and/or presence of facial hair (e.g. beards). Another 318 participants were excluded because of missing genetic information. Thus, there were 3513 participants eligible for this study. The majority were women (N=2045, 58.2%) and the median age was 66.2 (range 51-98) years old (Supplementary Table S1). Men showed a significant higher average wrinkle area (median facial wrinkle area 4.4%, IQR 2.9 to 6.2) than women (median facial wrinkle area 3.5%, IQR 2.1 to 5.5).

Leiden Longevity Study

The facial grading in the LLS was performed in 660 participants by visual assessment. Of these, 61 were excluded due to missing genotype data after quality control (N=3) or unsuitable photographs (e.g. presence of beards or poor photo quality), leaving 599 individuals for analysis. The mean age of these individuals was 63.1 years and 53.8% were women (Supplementary Table S1).

Stage 1: Discovery phase

The GWAS of global facial wrinkle area in the RS yielded several suggestive hits (P-values $<5\times10^{-6}$), but none of them were genome-wide significant (Figure 1, Supplementary Figures S1 and S2). In total, 25 SNPs with suggestive associations were identified (Table 1). The strongest signal was

found for the SNP rs10476781 (P-value 9.5×10^{-8}) that maps to an intergenic region on chromosome 5 between the gene Neuromedin U Receptor 2 (*NMUR2*) and *CTB-1202.1*, a gene encoding a long non-coding RNA (*LINC01933*). In the RS, this SNP has a minor allele frequency of 6% and an imputation score of 0.5 (the SNP rs10476781 showed moderate LD – r^2 0.4 – with other SNPs on chromosome 5, explaining the moderate imputation score). The effect allele (rs10476781[T], allele frequency 94%) has a β of -0.21 (SE 0.04). We estimated pairwise LD between the SNPs with suggestive associations and considered 11 SNPs as independent loci ($r^2 \leqq 0.5$). There was no LD between rs10476781 and other suggestive SNPs in our dataset ($r^2 \leqq 0.5$, Supplementary Table S2, Supplementary Figure S3).

Stage 2: Replication and joint meta-analysis

We tested for associations between wrinkles in the LLS replication cohort and the 25 SNPs with suggestive associations. One SNP, rs10476781, had a nominal P-value of 0.08 in the LLS, but the others could not be replicated (all P-values >0.2). In the meta-analysis of the two cohorts for the top hits, we found that rs10476781 showed a genome-wide significant P-value of 2.2×10^{-8} (Table 1).

Power calculation

Using the GWAS/QT Power Detection program[23], we found that with the current sample size of N=3513 and the following parameters: a heritability (effect size) of 0.0084 (based on the R^2 of the most significantly associated SNP); a total of 7,000,000 analyzed SNPs; an LD between a SNP and causative genetic variant of 1; 8 covariates and an explained variance of these covariates of 0.27, the power of our study was 0.77.

Sensitivity analyses in the RS

For the most significant SNPs associated with global wrinkles we performed sensitivity analyses by adding BMI as an additional covariate to the model. This analysis showed similar results as our main analysis (top SNP rs10476781: without BMI, Beta -0.21, P-value 9.5×10^{-8}; with BMI, Beta -0.21, P-value 1.37×10^{-7}), indicating that our findings were independent of BMI. Furthermore, we added BMI*SNP in the analyses for the top SNPs (Supplementary Table S5). For the top SNP rs10476781, the interaction term was not significant. For the 25 top SNPs, the interaction term BMI*SNP was significant for only one SNP (BMI*SNP1:246689691:I, P-value=0.01), however not after Bonferroni correction. Thus, we confirm that the effects of the identified SNPs are not mediated through BMI.

For the top SNP rs10476781, the interaction term smoking*BMI was also not significant. For the top 25 SNPs, the interaction term smoking*SNP was significant for two SNPs and former smoking (former smoking*SNP1:3118674.D, P-value=0.02; former smoking*SNPrs11577655, P-value=0.03, Supplementary Table S5), however not after Bonferroni correction.

Because of differences in facial wrinkling between sexes[5], we also tested for associations between the top SNPs and global wrinkling separately for men and women (i.e. sex-stratified analysis). We found that rs10476781 has a considerably stronger effect in women (Beta -0.27, P-value 1.2×10^{-6}) as compared to men (Beta -0.14, P-value 1.5×10^{-2}). However, the interaction term (sex*SNP) was not significant for any of the suggestive SNPs. For the SNPs with suggestive associations besides the top SNP, the effect sizes were more similar for both sexes and in the same direction (Supplementary Table S4). The univariate analysis – excluding age and sex – (Supplementary Table S8) showed similar results, with a P-value for rs10476781 of 1.3×10^{-7}.

Because global wrinkling only has partial correlation with the site-specific wrinkling (correlations between crow's feet, forehead wrinkles and upper lip wrinkles vs. global wrinkles were 0.48, 0.58 and 0.52, respectively[5]), we also tested for associations between the top SNPs associated with global wrinkling and wrinkling at different facial sites: crow's feet, forehead wrinkles and upper lip wrinkles (women only). Of the 25 tested top SNPs for global facial wrinkling, 22 also associated with the various facial wrinkle sites (Supplementary Table S6).

Results of the genome-wide meta-analysis are shown in Supplementary Table S3 and Supplementary Figure S4. Of note, there are multiple significant associations of SNPs that were only present in the LLS. Because of the small sample size in the LLS, these are likely to be false positives.

Validation of previously published associations between SNPs and skin aging

Finally, we performed a look-up of SNPs that have been previously reported to demonstrate associations with facial aging. The SNPs significantly associated with facial aging in the GWAS of 428 Ashkenazi Jews[17] were not available in the RS cohort, except for rs7616661, which showed a non-significant P-value of 0.19 in our meta-analysis (Supplementary Table S7). The SNP rs322458 significantly associated in the GWAS of 502 French middle-aged women[18] with global skin photoaging (rated using a six-grade ordinal scale) as phenotype[24] had a non-significant P-value of 0.38 in our meta-analysis. The skin color SNPs significantly associated with age pigmented spots in a recent GWAS[19] were all non-significantly associated with wrinkling, as was the case for the SNP associated with sagging eyelids[20] (Supplementary Table S7). One SNP (rs12203592) from the meta-analysis of GWAS of the skin aging phenotype micro-topography score of the back of the hand was significant in the LLS (P-value 0.03), however not in the RS nor in the genome-wide meta-analysis of the two cohorts.

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table S1. Characteristics of participants of the Rotterdam Study (N=3513) used here for discovery and of the Leiden Longevity Study (N=599), used for replication

Characteristic	RS (N=3513)	LLS (N=599)
Wrinkle area % - median [IQR] in RS; mean (SD) in LLS[a]	3.9 [2.4 – 5.9]	4.6 (1.3)
Sex – number of females (%)	2045 (58.2)	322 (53.8)
Age at photo in years - median [IQR] in RS; mean (SD) in LLS[b]	66.2 [60.6 – 71.0]	63.1 (6.7)
BMI in kg/m² - mean (SD)	27.6 (4.4)	25.5 (3.6)[c]

Abbreviations: IQR, interquartile range; LLS, Leiden Longevity Study; RS, Rotterdam Study; SD, standard deviation. [a]in the RS a digital measure was used, which was non-normally distributed; in the LLS a photonumeric scale (0-8) was used, which was normally distributed; [b]in the RS age was non-normally distributed; in the LLS age was normally distributed; [c]based on 594 individuals.

Supplementary Table S2. Pairwise linkage disequilibrium with $r^2 \leq 0.5$ between the top SNPs (P-values $<5\times10^{-6}$) associated with global facial wrinkles in the Rotterdam Study

SNP	Chr	Position[a]	Number of SNPs in LD	Left border position	Right border position	KB span	SNPs in LD
1:3118674:D	1	3118674	1	3118674	3119489	0.816	rs11577655
rs11577655	1	3119489	1	3118674	3119489	0.816	1:3118674:D
rs6429657	1	14702354	0	14702354	14702354	0.001	none
rs702491	1	54194992	0	54194992	54194992	0.001	none
rs61812508	1	147251772	1	147251772	147291718	39.947	rs11583958
rs11583958	1	147291718	1	147251772	147291718	39.947	rs61812508
1:246689691:I	1	246689691	0	246689691	246689691	0.001	none
rs116248825	3	26420135	0	26420135	26420135	0.001	none
rs9867656	3	30100084	1	30100084	30101254	1.171	rs11711327
rs11711327	3	30101254	1	30100084	30101254	1.171	rs9867656
rs112608607	5	102908739	3	102908739	102915644	6.906	rs113322056|rs146551307|5:102915644:D
rs113322056	5	102913288	3	102908739	102915644	6.906	rs112608607|rs146551307|5:102915644:D
rs146551307	5	102915236	3	102908739	102915644	6.906	rs112608607|rs113322056|5:102915644:D
5:102915644:D	5	102915644	3	102908739	102915644	6.906	rs112608607|rs113322056|rs146551307
rs10476781	**5**	**151763633**	**0**	**151763633**	**151763633**	**0.001**	**none**
rs72811030	5	179729009	0	179729009	179729009	0.001	none
rs62077967	17	61253263	1	61253263	61361539	108.277	rs72845240
rs72845240	17	61361539	1	61253263	61361539	108.277	rs62077967

Abbreviations: Chr, chromosome; KB span, kilo base pair span; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism. Boldface indicates the top SNP.
[a]based on GRCh37/hg19.

Supplementary Table S3. Top SNPs (P-values <5×10^{-6}) of the genome-wide meta-analysis[a] for global facial wrinkles in the Rotterdam Study and Leiden Longevity Study

			Meta-analysis (RS & LLS, N=4112)									
SNP	**Chr**	**Position[b]**	**EA**	**OA**	**EAF**	**OAF**	**Dir**	**Z[c]**	**P-value**	**I^2**	**Cochran's Q**	**Het P-value**
rs10476781	**5**	**151763633**	**T**	**C**	**0.94**	**0.06**	**--**	**-5.6**	**2.2×10^{-8}**	**0**	**0.19**	**0.67**
rs11213999	11	111634592	A	C	0.08	0.92	--	-5.04	4.7×10^{-7}	0	0.21	0.65
rs62047859	16	76826391	A	T	0.03	0.97	++	4.92	8.9×10^{-7}	0	0.8	0.37
rs147672305	8	105071112	A	T	0.02	0.98	++	4.91	9.1×10^{-7}	76.9	4.32	0.04
rs78569750	18	34858053	T	G	0.06	0.94	--	-4.88	1.0×10^{-6}	0	0.08	0.78
rs62528382	8	105067863	A	G	0.03	0.97	++	4.83	1.4×10^{-6}	40.8	1.69	0.19
rs6429657	1	14702354	A	G	0.96	0.04	--	-4.79	1.7×10^{-6}	0	0.95	0.33
rs1283106	3	106961498	A	C	0.37	0.63	--	-4.77	1.9×10^{-6}	0	0.01	0.93
rs702491	1	54194992	T	C	0.2	0.8	++	4.74	2.1×10^{-6}	0	0.78	0.38
rs1225927	6	7871037	T	G	0.75	0.25	++	4.69	2.8×10^{-6}	0	0.67	0.41
rs116248825	3	26420135	A	C	0.04	0.96	--	-4.68	2.9×10^{-6}	0	0.55	0.46
rs1299331	3	106965492	A	G	0.62	0.38	++	4.68	2.9×10^{-6}	0	0.13	0.71
rs189819077	18	34933012	A	G	0.03	0.97	--	-4.67	3.0×10^{-6}	32.9	1.49	0.22
rs11711327	3	30101254	A	G	0.65	0.35	++	4.65	3.3×10^{-6}	0	0.93	0.34
rs113322056	5	102913288	A	G	0.96	0.04	++	4.64	3.4×10^{-6}	3.6	1.04	0.31
8:105017098:D	8	105017098	D	I	0.03	0.97	++	4.64	3.4×10^{-6}	22.9	1.3	0.25
rs116873518	9	21005828	C	G	0.02	0.98	--	-4.64	3.4×10^{-6}	0	0.44	0.51
rs1283105	3	106962305	C	G	0.62	0.38	++	4.64	3.5×10^{-6}	0	0.02	0.9
rs146551307	5	102915236	T	C	0.96	0.04	++	4.64	3.5×10^{-6}	3.8	1.04	0.31
rs1150997	12	32070095	A	T	0.72	0.28	--	-4.64	3.5×10^{-6}	0	0.05	0.82
rs184605088	20	24536142	A	C	0.02	0.98	--	-4.64	3.5×10^{-6}	0	0.47	0.49
rs112608607	5	102908739	T	C	0.97	0.03	++	4.63	3.7×10^{-6}	0	0.83	0.36
rs9867656	3	30100084	A	G	0.34	0.66	--	-4.62	3.8×10^{-6}	0	0.89	0.35
10:25869856:D	10	25869856	D	I	0.36	0.64	--	-4.62	3.8×10^{-6}	25.5	1.34	0.25
rs1283108	3	106961285	T	C	0.38	0.62	--	-4.62	3.9×10^{-6}	0	0.01	0.9
rs184880542	8	86293264	T	G	0.02	0.98	++	4.62	3.9×10^{-6}	0	0.2	0.65
rs1283103	3	106962755	A	G	0.62	0.38	++	4.61	4.0×10^{-6}	0	0.04	0.84
rs72811030	5	179729009	A	G	0.38	0.62	++	4.61	4.0×10^{-6}	46.3	1.86	0.17
rs117828793	9	28211487	T	C	0.03	0.97	--	-4.61	4.0×10^{-6}	0	0.12	0.73
rs1283102	3	106962929	A	G	0.38	0.62	--	-4.6	4.3×10^{-6}	0	0.06	0.81
rs382029	6	7870856	A	T	0.27	0.73	--	-4.59	4.3×10^{-6}	0	0.44	0.51
rs1283104	3	106962521	C	G	0.62	0.38	++	4.58	4.7×10^{-6}	0	0.02	0.88

Abbreviations: A, adenine; C, cytosine; Chr, chromosome; D, deletion; Dir, direction of the effects; EA, effect allele; EAF, effect allele frequency; G, guanine; Het P-value, heterogeneity P-value; I^2, heterogeneity I^2; LLS, Leiden Longevity Study; OA, other allele; OAF, other allele frequency; RS, Rotterdam Study; SNP, single-nucleotide polymorphism; T, thymine. Boldface indicates the top SNP.

[a]analyses are adjusted for age, sex, and the first four genetic principal components and in addition, for the RS, for technical variables of the digital measurement. Only SNPs present in both RS and LLS cohorts are displayed; [b]based on GRCh37/hg19; [c]weighted Z-score.

Supplementary Table S4. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles[a] in both sexes in the Rotterdam Study and their effects (β) and P-values tested in men and women separately

			Both sexes (N=3513)							Men (N=1468)		Women (N=2045)	
SNP	Chr	Position[b]	EA	OA	EAF	OAF	β (SE)	P-value	P-value interaction SNP*sex[c]	β (SE)	P-value	β (SE)	P-value
rs10476781	**5**	**151763633**	**T**	**C**	**0.94**	**0.06**	**-0.21 (0.04)**	**9.5×10^{-8}**	**0.12**	**-0.14 (0.06)**	**1.5×10^{-2}**	**-0.27 (0.06)**	**1.2×10^{-6}**
rs185291539	10	84338421	A	G	0.98	0.02	0.41 (0.09)	4.8×10^{-6}	0.48	0.32 (0.13)	1.8×10^{-2}	0.46 (0.12)	1.2×10^{-4}
5:102915644:D	5	102915644	D	I	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	0.92	-0.20 (0.06)	1.2×10^{-3}	-0.18 (0.06)	1.9×10^{-3}
9:16847398:D	9	16847398	D	I	0.98	0.02	0.30 (0.07)	4.7×10^{-6}	0.13	0.16 (0.10)	0.11	0.36 (0.08)	1.5×10^{-5}
rs72845240	17	61361539	C	G	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	6.30×10^{-2}	-0.28 (0.06)	1.1×10^{-6}	-0.14 (0.06)	1.6×10^{-2}
rs11577655	1	3119489	T	C	0.13	0.87	0.11 (0.02)	4.6×10^{-6}	0.69	0.12 (0.04)	5.9×10^{-4}	0.11 (0.03)	6.9×10^{-4}
rs62077967	17	61253263	C	G	0.96	0.04	0.19 (0.04)	4.6×10^{-6}	6.30×10^{-2}	0.28 (0.06)	1.1×10^{-6}	0.14 (0.06)	1.6×10^{-2}
rs61812508	1	147251772	A	G	0.05	0.95	-0.18 (0.04)	4.3×10^{-6}	0.49	-0.17 (0.06)	4.0×10^{-3}	-0.19 (0.05)	2.2×10^{-4}
rs7608236	2	180062867	A	G	0.29	0.71	-0.07 (0.02)	4.1×10^{-6}	0.52	-0.08 (0.02)	2.6×10^{-4}	-0.06 (0.02)	3.6×10^{-3}
rs116248825	3	26420135	A	C	0.04	0.96	-0.28 (0.06)	4.1×10^{-6}	0.42	-0.18 (0.09)	3.2×10^{-2}	-0.34 (0.08)	5.1×10^{-5}
rs112608607	5	102908739	T	C	0.97	0.03	0.22 (0.05)	3.8×10^{-6}	0.98	0.21 (0.07)	1.7×10^{-3}	0.21 (0.07)	1.3×10^{-3}
1:246689691:I	1	246689691	D	I	0.6	0.4	0.07 (0.02)	3.7×10^{-6}	0.6	0.06 (0.02)	9.9×10^{-3}	0.08 (0.02)	9.5×10^{-5}
rs9867656	3	30100084	A	G	0.34	0.66	-0.07 (0.01)	3.7×10^{-6}	0.57	-0.08 (0.02)	2.6×10^{-4}	-0.06 (0.02)	3.6×10^{-3}
rs1225927	6	7871037	T	G	0.75	0.25	0.07 (0.02)	3.5×10^{-6}	0.55	0.07 (0.02)	1.4×10^{-3}	0.07 (0.02)	1.1×10^{-3}
rs11583958	1	147291718	A	T	0.04	0.96	-0.18 (0.04)	3.3×10^{-6}	0.35	-0.16 (0.06)	6.6×10^{-3}	-0.20 (0.05)	9.9×10^{-5}
rs11711327	3	30101254	A	G	0.66	0.34	0.07 (0.01)	3.1×10^{-6}	0.59	0.07 (0.02)	3.1×10^{-4}	0.06 (0.02)	2.8×10^{-3}
rs114667268	2	12433490	T	C	0.01	0.99	-0.49 (0.10)	2.9×10^{-6}	0.26	-0.31 (0.16)	4.9×10^{-2}	-0.58 (0.14)	2.5×10^{-5}
rs113322056	5	102913288	A	G	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	0.96	0.20 (0.06)	1.3×10^{-3}	0.19 (0.06)	1.3×10^{-3}
rs146551307	5	102915236	T	C	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	0.95	0.20 (0.06)	1.3×10^{-3}	0.19 (0.06)	1.4×10^{-3}
rs702491	1	54194992	T	C	0.19	0.81	0.09 (0.02)	2.4×10^{-6}	0.96	0.09 (0.03)	4.6×10^{-4}	0.09 (0.03)	6.3×10^{-4}
1:3118674:D	1	3118674	D	I	0.12	0.88	0.11 (0.02)	1.8×10^{-6}	0.95	0.13 (0.03)	1.4×10^{-4}	0.11 (0.03)	8.4×10^{-4}
rs189819077	18	34933012	A	G	0.03	0.97	-0.20 (0.04)	1.8×10^{-6}	0.76	-0.23 (0.06)	1.1×10^{-4}	-0.19 (0.06)	1.9×10^{-3}

Supplementary Table S4. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles[a] in both sexes in the Rotterdam Study and their effects (β) and P-values tested in men and women separately (continued)

			Both sexes (N=3513)							Men (N=1468)		Women (N=2045)	
SNP	Chr	Position[b]	EA	OA	EAF	OAF	β (SE)	P-value	P-value interaction SNP*sex[c]	β (SE)	P-value	β (SE)	P-value
rs72811030	5	179729009	A	G	0.38	0.62	0.07 (0.02)	1.7×10^{-6}	0.15	0.05 (0.02)	2.7×10^{-2}	0.09 (0.02)	2.3×10^{-5}
rs6429657	1	14702354	A	G	0.96	0.04	-0.19 (0.04)	1.6×10^{-6}	0.88	-0.17 (0.06)	3.4×10^{-3}	-0.23 (0.06)	4.8×10^{-5}
rs62047859	16	76826391	A	T	0.03	0.97	0.21 (0.04)	1.0×10^{-6}	0.93	0.22 (0.06)	3.1×10^{-4}	0.21 (0.06)	4.3×10^{-4}

Abbreviations: A, adenine; C, cytosine; Chr, chromosome; D, deletion; EA, effect allele; EAF, effect allele frequency; G, guanine; GWAS, genome-wide association study; OA, other allele; OAF, other allele frequency; SE, standard error; SNP, single-nucleotide polymorphism; T, thymine. Boldface indicates the top SNP.

[a]analyses are adjusted for age, sex, the first four genetic principal components, and technical variables of the digital measurement; [b]based on GRCh37/hg19; [c]P-value of the interaction term SNP*sex, added as covariate in the linear regression.

Supplementary Table S5. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles[a] in the Rotterdam Study and the P-values of the interaction terms SNP*BMI and SNP*smoking, tested separately in the analyses

			RS (N=3513)								
										P-value interaction SNP*smoking[d]	
SNP	Chr	Position[b]	EA	OA	EAF	OAF	β (SE)	P-value	P-value interaction SNP*BMI[c]	SNP*former smoking	SNP*current smoking
rs10476781	**5**	**151763633**	**T**	**C**	**0.94**	**0.06**	**-0.21 (0.04)**	**9.5×10^{-8}**	**0.25**	**0.64**	**0.50**
rs185291539	10	84338421	A	G	0.98	0.02	0.41 (0.09)	4.8×10^{-6}	0.64	0.76	0.72
5:102915644:D	5	102915644	D	I	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	0.42	0.73	0.80
9:16847398:D	9	16847398	D	I	0.98	0.02	0.30 (0.07)	4.7×10^{-6}	0.65	0.21	0.53
rs72845240	17	61361539	C	G	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	0.54	0.87	0.70
rs11577655	1	3119489	T	C	0.13	0.87	0.11 (0.02)	4.6×10^{-6}	0.76	0.03	0.11
rs62077967	17	61253263	C	G	0.96	0.04	0.19 (0.04)	4.6×10^{-6}	0.53	0.88	0.72
rs61812508	1	147251772	A	G	0.05	0.95	-0.18 (0.04)	4.3×10^{-6}	0.16	0.77	0.24
rs7608236	2	180062867	A	G	0.29	0.71	-0.07 (0.02)	4.1×10^{-6}	0.15	0.23	0.64
rs116248825	3	26420135	A	C	0.04	0.96	-0.28 (0.06)	4.1×10^{-6}	0.09	0.74	0.87
rs112608607	5	102908739	T	C	0.97	0.03	0.22 (0.05)	3.8×10^{-6}	0.31	0.52	0.75
1:246689691:I	1	246689691	D	I	0.6	0.4	0.07 (0.02)	3.7×10^{-6}	0.01	0.77	0.47
rs9867656	3	30100084	A	G	0.34	0.66	-0.07 (0.01)	3.7×10^{-6}	0.99	0.14	0.86
rs1225927	6	7871037	T	G	0.75	0.25	0.07 (0.02)	3.5×10^{-6}	0.6	0.59	0.93
rs11583958	1	147291718	A	T	0.04	0.96	-0.18 (0.04)	3.3×10^{-6}	0.18	0.72	0.20
rs11711327	3	30101254	A	G	0.66	0.34	0.07 (0.01)	3.1×10^{-6}	0.99	0.14	0.86
rs114667268	2	12433490	T	C	0.01	0.99	-0.49 (0.10)	2.9×10^{-6}	0.46	0.66	0.23
rs113322056	5	102913288	A	G	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	0.33	0.83	0.74
rs146551307	5	102915236	T	C	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	0.33	0.83	0.75
rs702491	1	54194992	T	C	0.19	0.81	0.09 (0.02)	2.4×10^{-6}	0.71	0.17	0.10
1:3118674:D	1	3118674	D	I	0.12	0.88	0.11 (0.02)	1.8×10^{-6}	0.73	0.02	0.07
rs189819077	18	34933012	A	G	0.03	0.97	-0.20 (0.04)	1.8×10^{-6}	0.54	0.87	0.70
rs72811030	5	179729009	A	G	0.38	0.62	0.07 (0.02)	1.7×10^{-6}	0.71	0.36	0.31
rs6429657	1	14702354	A	G	0.96	0.04	-0.19 (0.04)	1.6×10^{-6}	0.14	0.93	0.43
rs62047859	16	76826391	A	T	0.03	0.97	0.21 (0.04)	1.0×10^{-6}	0.54	0.17	0.34

Abbreviations: A, adenine; BMI, body mass index; C, cytosine; Chr, chromosome; D, deletion; EA, effect allele; EAF, effect allele frequency; G, guanine; GWAS, genome-wide association study; OA, other allele; OAF, other allele frequency; RS, Rotterdam Study; SE, standard error; SNP, single-nucleotide polymorphism; T, thymine. Boldface indicates the top SNP.

[a]analyses are adjusted for age, sex, the first four genetic principal components, and technical variables of the digital measurement; [b]based on GRCh37/hg19; [c]P-value of the interaction term SNP*BMI, added as covariate in the linear regression; [d]P-value of the interaction term SNP*smoking, added as covariate in the linear regression.

Supplementary Table S6. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles[a] in the Rotterdam Study and their effects (β) and P-values tested in site-specific wrinkles (forehead wrinkles, crow's feet wrinkles, and in women upper lip wrinkles)

						Global facial wrinkles (N=3513)		Forehead wrinkles (N=3499)		Crow's feet wrinkles (N=3544)		Upper lip wrinkles women (N=1136)	
SNP	**Chr**	**Position[b]**	**EA**	**EAF**	**OAF**	**β (SE)**	**P-value**	**β (SE)**	**P-value**	**β (SE)**	**P-value**	**β (SE)**	**P-value**
rs10476781	**5**	**151763633**	**T**	**0.94**	**0.06**	**-0.21 (0.04)**	**9.5×10^{-8}**	**-0.26 (0.07)**	**1.40×10^{-4}**	**-0.13 (0.07)**	**5.2×10^{-2}**	**-0.22 (0.11)**	**5.4×10^{-2}**
rs185291539	10	84338421	A	0.98	0.02	0.41 (0.09)	4.8×10^{-6}	0.25 (0.15)	9.20×10^{-2}	0.25 (0.15)	9.0×10^{-2}	-0.03 (0.25)	0.91
5:102915644:D	5	102915644	D	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	-0.16 (0.07)	2.90×10^{-2}	-0.19 (0.07)	7.2×10^{-3}	-0.18 (0.11)	0.11
9:16847398:D	9	16847398	D	0.98	0.02	0.30 (0.07)	4.7×10^{-6}	0.29 (0.11)	9.40×10^{-3}	-0.08 (0.11)	0.49	0.01 (0.16)	0.94
rs72845240	17	61361539	C	0.04	0.96	-0.19 (0.04)	4.7×10^{-6}	-0.24 (0.07)	5.60×10^{-4}	-0.13 (0.07)	7.0×10^{-2}	-0.14 (0.11)	0.19
rs11577655	1	3119489	T	0.13	0.87	0.11 (0.02)	4.6×10^{-6}	0.09 (0.04)	3.10×10^{-2}	0.13 (0.04)	1.8×10^{-3}	0.26 (0.06)	3.9×10^{-5}
rs62077967	17	61253263	C	0.96	0.04	0.19 (0.04)	4.6×10^{-6}	0.24 (0.07)	5.30×10^{-4}	0.13 (0.07)	7.1×10^{-2}	0.14 (0.11)	0.18
rs61812508	1	147251772	A	0.05	0.95	-0.18 (0.04)	4.3×10^{-6}	-0.14 (0.07)	3.10×10^{-2}	-0.17 (0.07)	1.2×10^{-2}	-0.06 (0.10)	0.55
rs7608236	2	180062867	A	0.29	0.71	-0.07 (0.02)	4.1×10^{-6}	0.08 (0.03)	2.20×10^{-3}	-0.04 (0.03)	8.7×10^{-2}	-0.06 (0.04)	0.17
rs116248825	3	26420135	A	0.04	0.96	-0.28 (0.06)	4.1×10^{-6}	-0.14 (0.10)	0.17	-0.17 (0.10)	9.3×10^{-2}	-0.18 (0.15)	0.22
rs112608607	5	102908739	T	0.97	0.03	0.22 (0.05)	3.8×10^{-6}	0.23 (0.08)	3.60×10^{-3}	0.26 (0.08)	9.7×10^{-4}	0.21 (0.12)	9.1×10^{-2}
1:246689691:I	1	246689691	D	0.6	0.4	0.07 (0.02)	3.7×10^{-6}	0.08 (0.03)	4.10×10^{-3}	-0.001 (0.03)	0.98	0.04 (0.04)	0.28
rs9867656	3	30100084	A	0.34	0.66	-0.07 (0.01)	3.7×10^{-6}	0.04 (0.02)	7.50×10^{-2}	-0.09 (0.02)	2.0×10^{-4}	-0.06 (0.04)	0.11
rs1225927	6	7871037	T	0.75	0.25	0.07 (0.02)	3.5×10^{-6}	0.04 (0.03)	0.19	0.05 (0.03)	6.9×10^{-2}	0.07 (0.04)	0.12
rs11583958	1	147291718	A	0.04	0.96	-0.18 (0.04)	3.3×10^{-6}	-0.14 (0.07)	3.50×10^{-2}	-0.19 (0.07)	4.2×10^{-3}	-0.06 (0.10)	0.57
rs11711327	3	30101254	A	0.66	0.34	0.07 (0.01)	3.1×10^{-6}	0.04 (0.02)	8.70×10^{-2}	0.09 (0.02)	2.3×10^{-4}	0.06 (0.04)	0.11
rs114667268	2	12433490	T	0.01	0.99	-0.49 (0.10)	2.9×10^{-6}	-0.26 (0.15)	8.70×10^{-2}	-0.18 (0.15)	0.22	0.005 (0.22)	0.98
rs113322056	5	102913288	A	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	0.20 (0.07)	6.50×10^{-3}	0.23 (0.07)	1.8×10^{-3}	0.22 (0.11)	5.6×10^{-2}
rs146551307	5	102915236	T	0.96	0.04	0.20 (0.04)	2.9×10^{-6}	0.20 (0.07)	6.60×10^{-3}	0.23 (0.07)	1.7×10^{-3}	0.22 (0.11)	5.6×10^{-2}
rs702491	1	54194992	T	0.19	0.81	0.09 (0.02)	2.4×10^{-6}	0.08 (0.03)	1.10×10^{-2}	0.06 (0.03)	8.4×10^{-2}	0.08 (0.05)	0.11
1:3118674:D	1	3118674	D	0.12	0.88	0.11 (0.02)	1.8×10^{-6}	0.09 (0.04)	2.00×10^{-2}	0.13 (0.04)	1.5×10^{-3}	0.25 (0.06)	5.5×10^{-5}
rs189819077	18	34933012	A	0.03	0.97	-0.20 (0.04)	1.8×10^{-6}	-0.29 (0.07)	4.50×10^{-5}	-0.18 (0.07)	1.3×10^{-2}	0.07 (0.12)	0.54

Supplementary Table S6. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles[a] in the Rotterdam Study and their effects (β) and P-values tested in site-specific wrinkles (forehead wrinkles, crow's feet wrinkles, and in women upper lip wrinkles) (continued)

						Global facial wrinkles (N=3513)		Forehead wrinkles (N=3499)		Crow's feet wrinkles (N=3544)		Upper lip wrinkles women (N=1136)	
SNP	**Chr**	**Position[b]**	**EA**	**EAF**	**OAF**	**β (SE)**	**P-value**	**β (SE)**	**P-value**	**β (SE)**	**P-value**	**β (SE)**	**P-value**
rs72811030	5	179729009	A	0.38	0.62	0.07 (0.02)	1.7×10^{-6}	0.06 (0.03)	2.20×10^{-2}	0.01 (0.03)	0.60	0.09 (0.04)	2.7×10^{-2}
rs6429657	1	14702354	A	0.96	0.04	-0.19 (0.04)	1.6×10^{-6}	-0.10 (0.07)	0.14	-0.06 (0.07)	0.39	-0.002 (0.11)	0.98
rs62047859	16	76826391	A	0.03	0.97	0.21 (0.04)	1.0×10^{-6}	0.24 (0.07)	1.20×10^{-3}	0.15 (0.07)	4.5×10^{-2}	0.18 (0.11)	9.9×10^{-2}

Abbreviations: A, adenine; C, cytosine; Chr, chromosome; D, deletion; EA, effect allele; EAF, effect allele frequency; G, guanine; GWAS, genome-wide association study; OA, other allele; OAF, other allele frequency; SE, standard error; SNP, single-nucleotide polymorphism; T, thymine. Boldface indicates the top SNP.

[a]analyses are adjusted for age, sex, the first four genetic principal components, and technical variables of the digital measurement; [b]based on GRCh37/hg19.

Supplementary Table S7. Previously suggested SNPs significantly associated with facial aging in other recent GWAS and their associations with global facial wrinkles[a] in the Rotterdam Study, the Leiden Longevity Study, and the meta-analysis of both cohorts

					RS (N=3513)				LLS (N=599)				Meta-analysis (RS & LLS, N=4112)			
SNP	Chr	Position[b]	Published P-value	Associated phenotype	EA	EAF	β (SE)	P-value	EA	EAF	β (SE)	P-value	EA	Dir	Z[c]	P-value
rs7616661[d]	3	5965543	4.8×10^{-8}	Photoaging	T	0.98	-0.05 (0.06)	0.37	T	0.98	-0.43 (0.26)	0.09	T	--	-1.47	0.19
rs6975107[d]	7	120380907	4.2×10^{-9}	Photoaging	n.a.	n.a.	n.a.	n.a.	T	0.002	-0.25 (1.57)	0.88	T	?-	-0.16	0.88
rs11863929[d]	16	88304433	1.8×10^{-8}	Photoaging	n.a.	n.a.	n.a.	n.a.	G	0.01	-0.17 (0.58)	0.77	C	?+	0.29	0.77
rs322458[e]	3	120585315	1.5×10^{-8}	Photoaging	T	0.37	-0.01 (0.01)	0.34	C	0.63	0.00 (0.07)	0.97	T	--	-0.89	0.38
rs12203592[f]	6	396321	1.9×10^{-27}	PS	T	0.10	-0.04 (0.02)	0.10	C	0.92	-0.27 (0.12)	0.03	T	-+	-0.67	0.50
rs62543565[f]	9	16901067	1.5×10^{-7}	PS	A	0.60	-0.01 (0.01)	0.70	C	0.41	-0.01 (0.07)	0.94	A	-+	-0.33	0.74
rs35063026[f]	16	89736157	9.4×10^{-15}	PS	T	0.07	-0.01 (0.03)	0.79	C	0.92	-0.10 (0.13)	0.45	T	-+	0.05	0.96
rs6059655[f]	20	32665748	2.6×10^{-9}	PS	A	0.08	-0.00 (0.03)	0.95	A	0.10	0.02 (0.12)	0.86	A	-+	0.01	0.99
rs11876749[g]	18	3942902	1.7×10^{-8}	Sagging eyelids	T	0.52	0.01 (0.01)	0.61	T	0.51	-0.00 (0.07)	0.97	T	+-	0.46	0.65
rs185146[h]	5	33952106	4.1×10^{-9}	MT	T	0.98	-0.04 (0.05)	0.50	C	0.02	-0.44 (0.32)	0.17	T	-+	-0.11	0.92
rs12203592[h]	6	396321	8.8×10^{-13}	MT	T	0.10	-0.04 (0.02)	0.10	C	0.92	-0.27 (0.12)	0.03	T	-+	-0.67	0.50
rs4268748[h]	16	90026512	1.2×10^{-15}	MT	T	0.73	0.00 (0.02)	0.83	T	0.75	-0.12 (0.08)	0.14	T	+-	-0.36	0.72
rs1805007[h]	16	89986117	1.2×10^{-10}	MT	T	0.07	0.00 (0.03)	0.89	C	0.93	-0.19 (0.13)	0.16	T	++	0.66	0.51
rs1805008[h]	16	89986144	1.1×10^{-5}	MT	T	0.10	-0.02 (0.02)	0.49	C	0.91	-0.04 (0.12)	0.71	T	-+	-0.50	0.62

Abbreviations: A, adenine; C, cytosine; Chr, chromosome; Dir, direction of the effects; EA, effect allele; EAF, effect allele frequency; G, guanine; GWAS, genome-wide association study; LLS, Leiden Longevity Study; MT, microtopography score; PS, pigmented spots; RS, Rotterdam Study; SE, standard error; SNP, single-nucleotide polymorphism; T, thymine.

[a]analyses are adjusted for age, sex, and the first four genetic principal components and in addition, for the RS, for technical variables of the digital measurement; [b]based on GRCh37/hg19; [c]weighted Z-score; [d]SNPs found by Chang et al.; [e]SNP found by Le Clerc et al.; [f]SNPs found by Jacobs et al. for pigmented spots; [g]SNP found by Jacobs et al. for sagging eyelids; [h]SNPs found bij Law et al. in a genome-wide meta-analysis for microtopography score of the back of the hand.

Supplementary Table S8. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles in the Rotterdam Study, in a univariate analysis (excluding age and sex)[a]

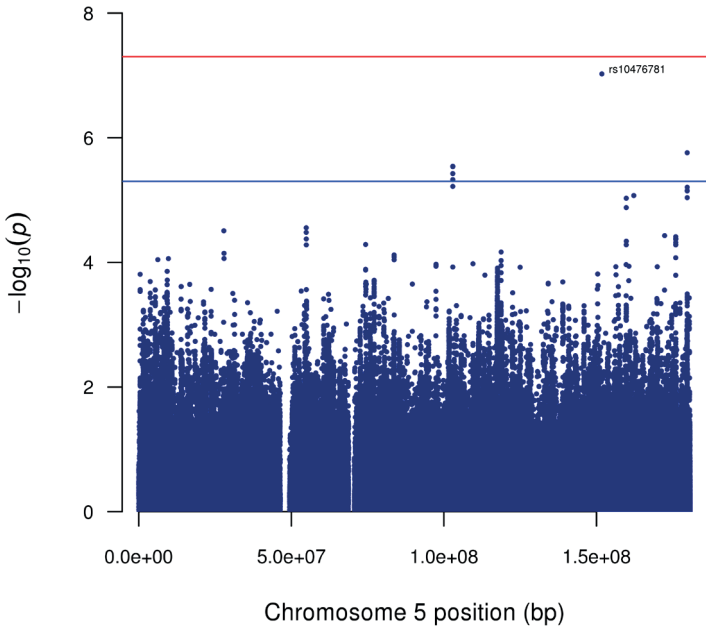
			RS (N=3513)					
SNP	**Chr**	**Position[b]**	**EA**	**OA**	**EAF**	**OAF**	**β (SE)**	**P-value**
rs1857883	3	30052399	A	G	0.54	0.46	0.07 (0.01)	1.2×10^{-7}
rs10476781	**5**	**151763633**	**T**	**C**	**0.94**	**0.06**	**-0.22 (0.04)**	**1.3×10^{-7}**
3:30071057:I	3	30071057	D	I	0.46	0.54	-0.08 (0.01)	1.9×10^{-7}
rs34836863	3	30058003	T	C	0.54	0.46	0.07 (0.01)	2.9×10^{-7}
rs9867656	3	30100084	A	G	0.34	0.66	-0.08 (0.02)	3.4×10^{-7}
rs11711327	3	30101254	A	G	0.66	0.34	0.08 (0.02)	3.6×10^{-7}
rs11129380	3	30072352	T	C	0.45	0.55	-0.07 (0.01)	5.6×10^{-7}
rs11925126	3	30072451	T	C	0.45	0.55	-0.07 (0.01)	5.6×10^{-7}
rs961878	3	30073620	A	G	0.55	0.45	0.07 (0.01)	5.6×10^{-7}
rs1506298	3	30074228	C	G	0.55	0.45	0.07 (0.01)	5.7×10^{-7}
3:30072552:I	3	30072552	D	I	0.45	0.55	-0.07 (0.01)	5.8×10^{-7}
rs6549970	3	30064134	T	C	0.55	0.45	0.07 (0.01)	6.2×10^{-7}
rs1506287	3	30068387	T	G	0.45	0.55	-0.07 (0.01)	6.2×10^{-7}
rs9847686	3	30064795	T	C	0.45	0.55	-0.07 (0.01)	6.6×10^{-7}
rs2371911	3	30065907	A	T	0.55	0.45	0.07 (0.01)	6.6×10^{-7}
rs6795173	3	30055039	A	G	0.57	0.43	-0.07 (0.01)	6.9×10^{-7}
rs61812508	1	147251772	A	G	0.04	0.96	-0.20 (0.04)	7.3×10^{-7}
rs1506285	3	30068158	A	G	0.55	0.45	0.07 (0.01)	7.4×10^{-7}
rs11583958	1	147291718	A	T	0.04	0.96	-0.20 (0.04)	7.8×10^{-7}
rs1506290	3	30069645	T	C	0.55	0.45	0.07 (0.01)	8.0×10^{-7}
3:30058483:D	3	30058483	D	I	0.52	0.48	0.07 (0.01)	8.6×10^{-7}
rs7426945	3	30071380	A	G	0.45	0.55	-0.07 (0.01)	8.6×10^{-7}
rs141920505	3	30095549	A	C	0.35	0.65	-0.07 (0.02)	8.6×10^{-7}
rs72811030	5	179729009	A	G	0.38	0.62	0.08 (0.02)	9.9×10^{-7}
rs61809935	1	147272111	T	C	0.95	0.05	0.20 (0.04)	1.0×10^{-6}
rs7649490	3	30111359	A	G	0.65	0.35	0.07 (0.01)	1.5×10^{-6}
rs6804839	3	30063702	A	G	0.46	0.54	-0.07 (0.01)	1.7×10^{-6}
rs77548552	9	20970280	A	C	0.97	0.03	0.22 (0.05)	1.7×10^{-6}
rs4626055	3	30061866	T	C	0.46	0.54	-0.07 (0.01)	2.2×10^{-6}
rs62047859	16	76826391	A	T	0.03	0.97	0.21 (0.05)	2.4×10^{-6}
rs4426642	3	30074874	A	G	0.44	0.56	-0.07 (0.01)	2.8×10^{-6}
rs55848714	5	179734528	A	C	0.6	0.4	-0.07 (0.01)	3.0×10^{-6}
rs184810693	7	45992257	T	C	0.01	0.99	0.76 (0.16)	3.0×10^{-6}
rs62404922	5	179731466	A	C	0.4	0.6	0.07 (0.01)	3.6×10^{-6}
rs729652	5	179731695	T	C	0.4	0.6	0.07 (0.01)	3.6×10^{-6}
rs62404925	5	179732797	T	C	0.4	0.6	0.07 (0.01)	3.6×10^{-6}
rs12596502	16	83872697	T	G	0.2	0.8	-0.08 (0.02)	3.7×10^{-6}
rs138860040	11	31511241	T	C	0.01	0.99	-0.47 (0.10)	3.8×10^{-6}

Supplementary Table S8. Top SNPs (P-values <5×10^{-6}) of the GWAS for global facial wrinkles in the Rotterdam Study, in a univariate analysis (excluding age and sex)[a] (continued)

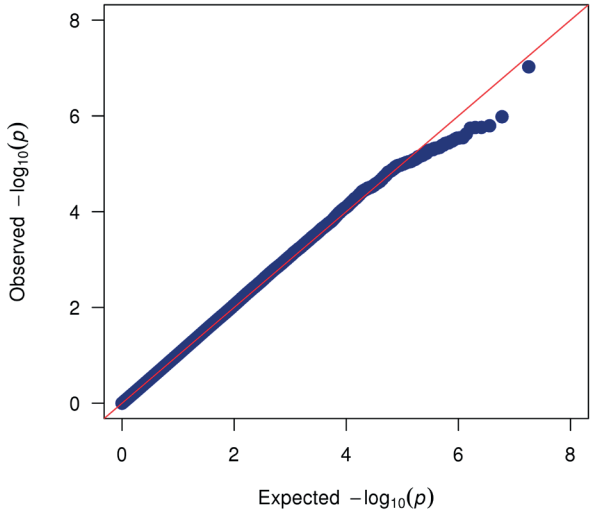
			RS (N=3513)					
SNP	**Chr**	**Position[b]**	**EA**	**OA**	**EAF**	**OAF**	**β (SE)**	**P-value**
rs189819077	18	34933012	A	G	0.03	0.97	-0.20 (0.04)	3.9×10^{-6}
rs76007816	9	20766718	A	G	0.98	0.02	0.25 (0.05)	4.2×10^{-6}
6:105164470:D	6	105164470	D	I	0.38	0.62	-0.07 (0.02)	4.3×10^{-6}
rs12494646	3	30076692	A	G	0.56	0.44	0.07 (0.01)	4.4×10^{-6}
rs2888213	3	30077137	A	G	0.44	0.56	-0.07 (0.01)	4.4×10^{-6}
rs78440239	5	150293381	T	C	0.02	0.98	-0.25 (0.06)	4.5×10^{-6}
rs9837298	3	30066066	A	G	0.52	0.48	0.07 (0.01)	4.6×10^{-6}
5:162220036	5	162220036	A	G	0.01	0.99	-1.20 (0.26)	4.8×10^{-6}
rs34280244	3	30098568	T	C	0.62	0.38	0.07 (0.01)	4.9×10^{-6}
rs118178650	9	20776109	T	C	0.02	0.98	-0.24 (0.05)	4.9×10^{-6}
rs12599838	16	83871039	A	C	0.2	0.8	-0.08 (0.02)	4.9×10^{-6}
rs6549971	3	30064282	A	G	0.48	0.52	-0.06 (0.01)	5.0×10^{-6}
rs62404924	5	179732771	A	G	0.6	0.4	-0.07 (0.01)	5.0×10^{-6}

Abbreviations: A, adenine; C, cytosine; Chr, chromosome; EA, effect allele; EAF, effect allele frequency; G, guanine; GWAS, genome-wide association study; RS, Rotterdam Study; SE, standard error; SNP, single-nucleotide polymorphism; T, thymine. Boldface indicates the top SNP.

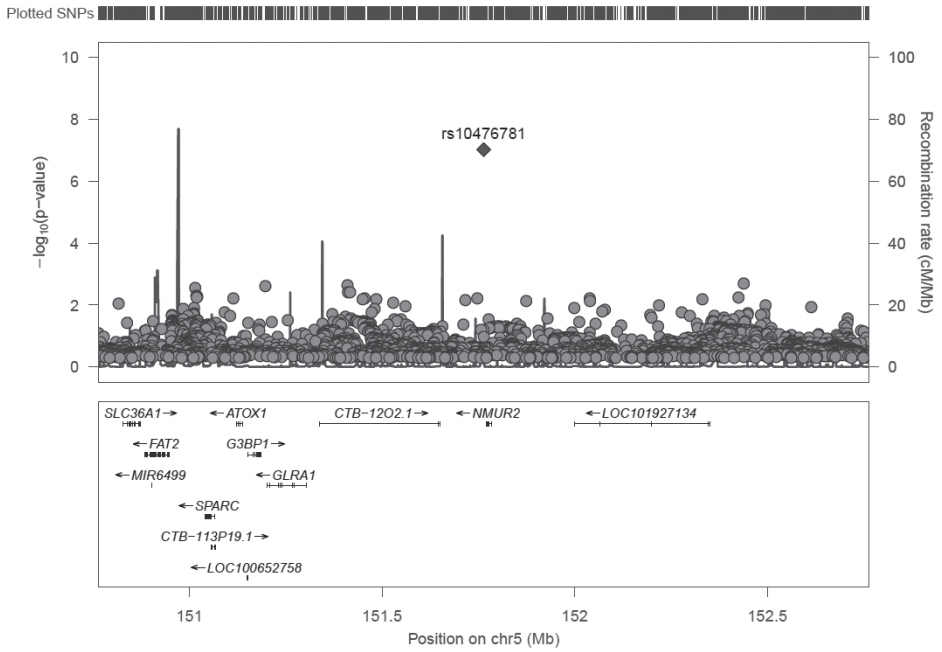
[a]analyses are adjusted for technical variables of the digital measurement and the first four genetic principal components; [b]based on GRCh37/hg19.



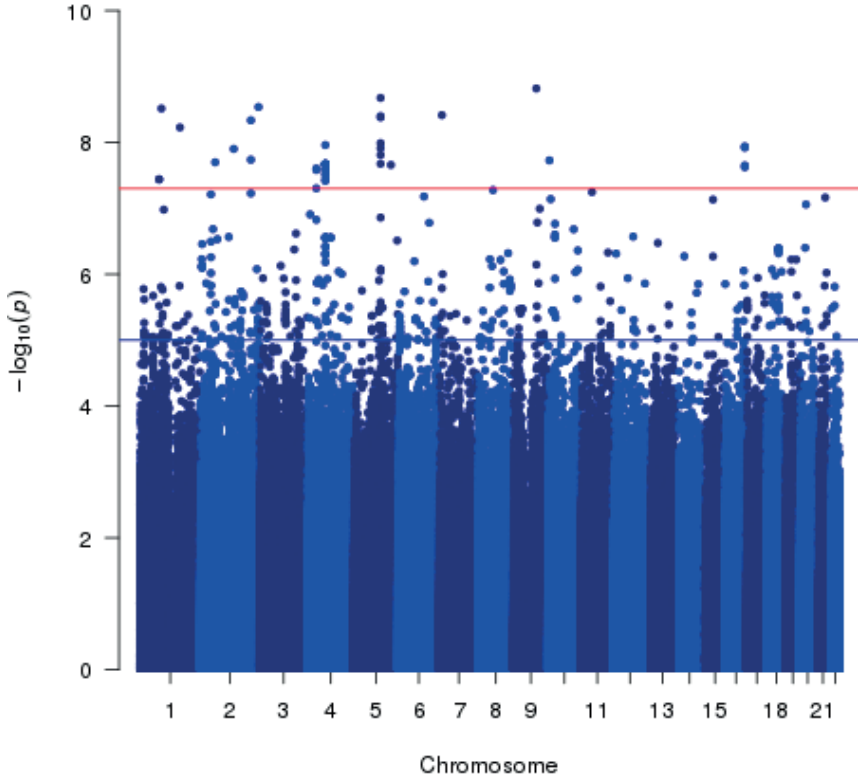
Supplementary Figure S1. Manhattan plot of the genome-wide associations for wrinkle area on chromosome 5 in the discovery cohort (Rotterdam Study, N=3513). All SNPs are represented by dots; x-axis shows the base pairs on chromosome 5; y-axis shows negative $\log_{10}$-transformed P-values.



Supplementary Figure S2. Quantile-quantile (QQ) plot of the expected (x-axis) vs. observed (y-axis) P-values of the genome-wide associations for wrinkle area in the discovery cohort (Rotterdam Study, N=3513). The higher observed than expected P-values are due to the lack of power in our study.



Supplementary Figure S3. LocusZoom[25] plot showing the region on chromosome 5 around the top suggestive SNP rs10476781 associated with wrinkle area in the discovery cohort (Rotterdam Study, N=3513). There is no LD between rs10476781 and other SNPs shown in this region.



Supplementary Figure S4. Manhattan plot of the genome-wide associations for wrinkle area in the meta-analysis of the two cohorts (Rotterdam Study, N=3513 & Leiden Longevity Study, **N=599).** All SNPs are represented by dots and displayed per chromosome (x-axis); y-axis shows negative $\log_{10}$-transformed P-values. There are multiple significant associations of SNPs that were only present in the LLS. Because of the small sample size in the LLS, these are likely to be false positives and therefore not reported as significant hits.

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PART III

OTHER SKIN AGING PHENOTYPES

Chapter 5

A genome-wide association study identifies the skin color genes *IRF4*, *MC1R*, *ASIP*, and *BNC2* influencing facial pigmented spots

L.C. Jacobs
M.A. Hamer
D.A. Gunn
J. Deelen
J.S. Lall
D. van Heemst
H.W. Uh
A. Hofman
A.G. Uitterlinden
C.E.M. Griffiths
M. Beekman
P.E. Slagboom
M. Kayser
F. Liu
T. Nijsten

J Invest Dermatol. 2015 Jul; 135(7):1735-42

ABSTRACT

Facial pigmented spots are a common skin aging feature, but genetic predisposition has yet to be thoroughly investigated. We conducted a genome-wide association study for pigmented spots in 2844 northwestern Europeans from the Rotterdam Study (mean age: 66.9 ±8.0; 47% male). Using semi-automated image analysis of high-resolution digital facial photographs, facial pigmented spots were quantified as the percentage of affected skin area (mean women: 2.0% ±0.9, men: 0.9% ±0.6). We identified genome-wide significant association with pigmented spots at three genetic loci: *IRF4* (rs12203592, P-value 1.8×10^{-27}), *MC1R* (compound heterozygosity score, P-value 2.3×10^{-24}), and *RALY/ASIP* (rs6059655, P-value 1.9×10^{-9}). In addition, after adjustment for the other three top-associated loci, the *BNC2* locus demonstrated significant association (rs62543565, P-value 2.3×10^{-8}). The association signals observed at all four loci were successfully replicated (P-value<0.05) in an independent Dutch cohort (Leiden Longevity Study N=599). Although the four genes have previously been associated with skin color variation and skin cancer risk, all association signals remained highly significant (P-value<2×10^{-8}) when conditioning the association analyses on skin color. We conclude that genetic variations in *IRF4*, *MC1R*, *RALY/ASIP*, and *BNC2* contribute to the acquired amount of facial pigmented spots during aging, through pathways independent of the basal melanin production.

INTRODUCTION

Facial pigmented spots are regarded as a common skin aging feature[1]. In global populations, the demand for products that prevent the appearance of skin aging features has increased markedly. However, to offer substantiated advice and effective treatment, it is prerequisite to understand skin aging etiology.

Pigmented spots (solar lentigines and seborrheic keratoses) are part of the complex skin aging phenotype, which also includes wrinkling, sagging, and telangiectasia, which together have been considered as one skin aging phenotype in previous studies[2,3]. Important known risk factors for skin aging include age, cumulative UV-exposure, and light skin color[4-6]. Candidate gene studies have been performed, where gene variants in the pigmentation genes *SLC45A2* (solute carrier family 45 member 2) in Asians[7] and *MC1R* (melanocortin 1 receptor) in Europeans[8] have been found to be associated with the presence of solar lentigines. However, the genetic predisposition to facial pigmented spots has not been investigated at the genome-wide scale.

To provide insight into which other genes may be involved in the development of pigmented spots during aging, we performed a genome-wide association study (GWAS) in 2844 individuals of northwestern European ancestry from the Rotterdam Study (RS). Facial pigmented spots were quantified from high-resolution digital photographs, using semi-automated image analysis. We then replicated our findings in an independent cohort of 599 Dutch participants of the Leiden Longevity Study (LLS). To clarify whether the genetic associations with pigmented spots were independent of skin color or not, we additionally adjusted the identified associations for skin color.

RESULTS

Discovery GWAS

All 2844 individuals from the discovery RS cohort (mean age: 66.9 ±8.0, 47% men, Table 1) were of northwestern European ancestry. Women were more severely affected with on average 2.0% (±0.9%) of their facial area being covered by pigmented spots (Figure 1), compared to men (0.9% ±0.6%, Table 1). A total of 168 single-nucleotide polymorphisms (SNPs; nine genotyped SNPs, Supplementary Table S1) in three distinct loci showed genome-wide significant association with pigmented spots (P-value<5×10^{-8}, Figure 2, Supplementary Figure S1). All three loci harbor a known skin color gene, namely *IRF4* (6p25), *MC1R* (16q24) and *ASIP* (20q11). The most strongly associated SNP was rs12203592(T) in the 4th intron of *IRF4* (24.9%Δ per allele, P-value 1.9×10^{-27}, Table 2, Supplementary Figure S2A). The association at 16q24 consisted of a large number of SNPs (Supplementary Figure S2B). This locus contains many genes, and the top associating SNP was rs35063026(T) (20.29%Δ, P-value 9.4×10^{-15}) located in exon 3 of *c16orf55/SPATA33*, which is ~250 kb upstream from the skin color gene *MC1R*. The third locus was found at 20q11, where the most strongly associated SNP rs6059655(A) was located in intron 8 of the *RALY* gene (14.6%Δ, P-value

Table 1. Characteristics of 2844 northwestern European participants from the Rotterdam Study

Characteristics	Men (N=1323)	Women (N=1521)
Pigmented spots; mean (SD)	0.9% (0.6)	2.0% (0.9)
Age (years); mean (SD)	67.1 (7.9)	66.8 (8.0)
Skin color; number (%)	1323	1521
very white	100 (7.6)	141 (9.3)
white	1016 (76.8)	1196 (78.6)
white-to-olive	207 (15.6)	184 (12.1)

Abbreviation: SD, standard deviation.
Pigmented spots were measured as affected area per total measured facial area.

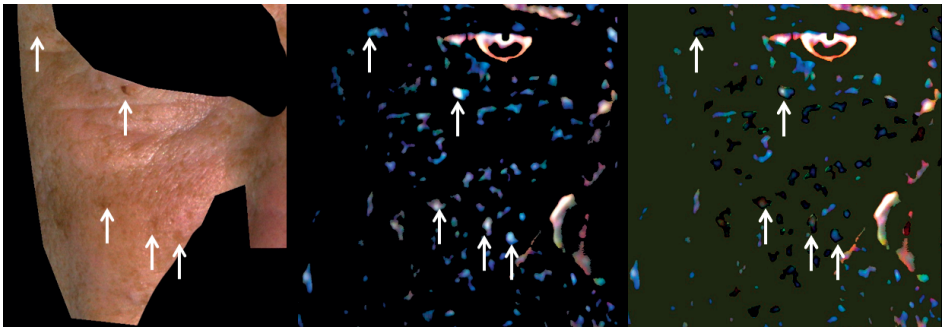

Figure 1. Example image of pigmented spots detection. (**A**) All non-skin areas were masked. The arrows are pointing at pigmented spots. (**B**) This contrasted image targets features approximate in size to pigmented spots, which appear blue to white in color (brown to black in the regular photograph). (**C**) Spots subsequently detected as pigmented spots are shaded.

2.6×10^{-9}, Table 2, Supplementary Figure S2C). *RALY* is located <200 kb upstream from the skin color gene *ASIP.* Linkage disequilibrium between the top-associated SNP in *ASIP* (rs1205312(A), P-value 1.8×10^{-6}) and rs6059655 was substantial (r^2=0.59).

We performed a second genome-wide association analysis for pigmented spots in the RS, conditional on the three most strongly associating SNPs (rs12203592 (*IRF4*), rs35063026 (*MC1R*), and rs6059655 (*RALY/ASIP*)). In this conditional analysis, none of the SNPs at the *IRF4* and *RALY* loci were associated with pigmented spots at genome-wide significance (P-value>0.005). In contrast, at the *MC1R* locus, a large number (N=31) of SNPs still showed genome-wide significant association. Interestingly, one additional locus at 9p22 was identified to be significantly associated with pigmented spots, where rs62543565(C) showed the most significant association (-6.4%Δ, P-value 2.3×10^{-8}). This SNP is located 30 kb upstream from Basonuclin 2 (*BNC2;* Supplementary Figure S2D), which was recently found to be involved in skin coloration[9]. A sex-stratified GWAS could not identify new loci or SNPs with strong sex-specific effects (Supplementary Table S2).

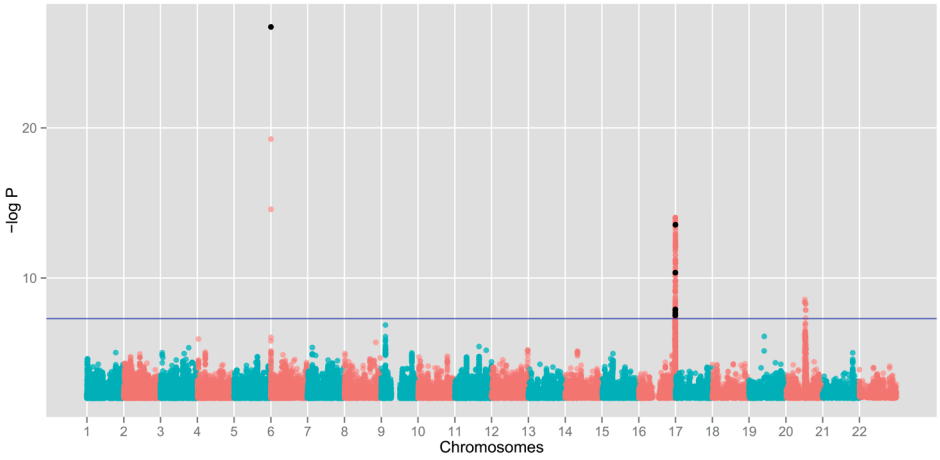

Figure 2. Manhattan plot of the GWAS in 2844 northwestern European individuals from the Rotterdam Study. The observed -$\log_{10}$ P-values (y-axis) of the association between each single-nucleotide polymorphism (SNP) and pigmented spots are shown. All SNPs are represented by dots and displayed per chromosome (x-axis). The horizontal line indicates the genome-wide significant threshold of P-value 5×10^{-8}. Genotyped SNPs passing this threshold are colored black.

Table 2. GWAS in the discovery cohort and the replication results, for pigmented spots in two independent cohorts

				Discovery cohort				Replication cohort			
				RS (N=2844)				LLS (N=599)			
Chromosome	**Gene**	**SNP**	**EA**	**EAF**	**%Δ**	**SE**	**P-value**	**EAF**	**β**	**SE**	**P-value**
6p25	*IRF4*	rs12203592	T	0.09	24.94	2.05	1.9×10^{-27}	0.08	0.44	0.12	4.4×10^{-4}
9p22	*BNC2*	rs62543565	C	0.37	-6.32	1.25	1.5×10^{-7}	0.41	-0.15	0.07	0.033
16q24	*MC1R*	rs35063026	T	0.07	20.29	2.40	9.4×10^{-15}	0.08	0.33	0.13	0.011
20q11	*RALY/ASIP*	rs6059655	A	0.08	14.58	2.31	2.6×10^{-9}	0.10	0.30	0.11	0.009

Abbreviations: %Δ, percentage change in the pigmented spots area, per increase in effect allele; β, the increase in pigmented spots severity category, per increase in effect allele; EA, effect allele, or minor allele; EAF, effect allele frequency; GWAS, genome-wide association study; LLS, Leiden Longevity Study; RS, Rotterdam Study; SE, standard error of the %Δ or β; SNP, single-nucleotide polymorphism.
The most significant signal per locus of the GWAS in the RS with a P-value<5×10^{-7} is shown; the signals were replicated in the LLS.

Replication of findings

A replication study for the 168 top-associated SNPs was conducted in an independent cohort, the LLS, of Dutch ancestry (Supplementary Table S1). This study consisted of 599 individuals (mean age: 63.1 ±6.7, 46% men), with facial pigmented spots graded in severity categories (ranging from 2 to 8, mean: 4.4 ±1.2). Although the pigmented spots phenotype of this replication cohort was assessed differently (categorical) compared with the discovery cohort (percentage of affected area), both methods reflect the severity of facial pigmented spots. The four top SNPs from the discovery GWAS were all successfully replicated in the LLS (P<0.05, Table 2). These included rs12203592(T) (*IRF4*, beta 0.44, P-value 4.4×10^{-4}), rs35063026(T) (*MC1R*, beta 0.33, P-value 0.011), rs6059655(A)

(*RALY/ASIP*, beta 0.30, P-value 0.009) and rs62543565(C) (*BNC2* (non-conditional analysis), beta -0.15, P-value 0.033).

MC1R compound heterozygosity

The association pattern at chr16q24 was consistent with previous GWAS findings of skin color[10-12], where multiple SNPs in a large region around *MC1R* demonstrate independent association (Supplementary Figure S2B). There are six SNPs within *MC1R* that are frequent in Europeans (MAF>1%) and associate with skin color together in a compound heterozygous manner[13]. Compound heterozygosity implies that if both homologous chromosomes carry one effect allele but in different SNPs, the effect is similar to that of a homozygous allele. In the RS, a compound heterozygosity score (CHS) was calculated from the haplotypes of the six independent skin color-associated *MC1R* SNPs (Supplementary Table S3). The CHS was more significantly associated with pigmented spots (14.0%Δ, P-value 1.6×10^{-24}) than the top-associated SNP in this region, demonstrating that compound heterozygosity also has a role in pigmented spot development. In addition, when the GWAS was adjusted for the CHS, no more genome-wide significant SNPs on chr16q24 could be detected; this implies that these six *MC1R* skin color SNPs together explained a large part of the *MC1R* association with pigmented spots.

Skin color-adjusted analyses

Because all four identified loci are known to be involved in skin color, we performed additional skin color-adjusted and -stratified analyses in the RS. Adjustment for skin color showed that *IRF4* and *BNC2* SNPs hardly reduced in association effect size. *MC1R* (CHS) and *RALY/ASIP* (rs6059655) slightly reduced in effect size (~8.5% lower %Δ), but were still genome-wide significant (Table 3).

Stratification for the three skin color categories showed that the effect sizes (%Δ) in the very white and the white skin color subgroups were similar for all four top SNPs (Table 3). The SNPs in *RALY/ASIP* (rs6059655) and *BNC2* (rs62543565) did not reach significance in the very white subgroup, likely because of the small sample size (N=241). The effect sizes were stronger in the white-to-olive skin color subgroup, although only rs12203592 (*IRF4*) showed a significant interaction with skin color (P-value 0.04).

In addition, we investigated whether other well-known pigmentation genes associated with pigmented spots, which we might not have picked up with the GWAS, due to smaller effect sizes (Table 4). However, none of the eight additional pigmentation genes we selected showed significant association with pigmented spots (P>0.005). The total variance of the pigmented spots phenotype explained by age, sex, skin color, and the pigmentation genes combined was very high (r^2=40.3%, Table 4), with sex (r^2=30.4%) and age (r^2=3.5%) being the strongest predictors. The *IRF4* SNP rs12203592 explained the largest proportion of the phenotypic variance (r^2=2.3%) of the four top SNPs combined (r^2=5.6%).

The four genes that associated with pigmented spots risk here, also showed association with perceived skin color in a previous investigation in the RS[9]. The most striking difference between

the association with skin color and pigmented spots, in terms of significance, was observed for *HERC2*, where rs12913832 showed a highly significant association with skin color (P-value 1.5×10^{-109})[9], but not with pigmented spots (P-value 0.49, Table 4).

Table 3. Skin color-adjusted and -stratified analysis in 2844 northwestern European individuals from the Rotterdam Study

		IRF4 rs12203592		***MC1R*** CHS		***RALY/ASIP*** rs6059655		***BNC2*** rs62543565	
Analysis	**N**	**%Δ**	**P-value**	**%Δ**	**P-value**	**%Δ**	**P-value**	**%Δ**	**P-value**
SC-adjusted	2844	24.12	2.3×10^{-26}	12.85	3.5×10^{-21}	13.32	3.7×10^{-8}	-6.20	2.0×10^{-7}
SC-stratified									
very white	241	21.85	2.8×10^{-5}	11.88	2.9×10^{-3}	8.91	0.179	-5.43	0.141
white	2212	21.54	4.5×10^{-17}	11.70	3.7×10^{-15}	13.25	7.7×10^{-7}	-5.38	6.7×10^{-5}
white-to-olive	391	46.37	6.3×10^{-10}	19.80	9.5×10^{-7}	19.10	0.028	-12.09	3.1×10^{-4}

Abbreviations: %Δ, percentage change in the pigmented spots area, per increase in effect allele; CHS, compound heterozygosity score; SC, skin color; SC adjusted, regression analysis additionally adjusted for skin color; SC stratified, regression analysis per skin color stratum.

Table 4. Multivariable analysis of pigmentation genes and pigmented spots in 2844 northwestern European individuals from the Rotterdam Study

Factor	**SNP**	**EA**	**EAF**	**%Δ**	**SE**	**P-value**	**r^2 (%)**
Age (years)				1.50	0.11	1.1×10^{-42}	3.54
Female sex				86.91	1.69	1.0×10^{-245}	30.39
Light skin color				9.39	1.87	1.2×10^{-7}	0.57
SLC45A2	rs16891982	C	0.03	-6.83	3.78	0.056	0.06
IRF4	rs12203592	T	0.09	24.43	2.03	5.4×10^{-27}	2.26
TYRP1	rs1408799	T	0.31	1.74	1.28	0.175	0.03
BNC2	rs62543565	C	0.37	-6.54	1.21	2.3×10^{-8}	0.58
TPCN2	rs35264875	T	0.17	-1.58	1.57	0.308	0.02
TYR	rs1393350	A	0.23	2.71	1.41	0.057	0.06
KITLG	rs12821256	C	0.13	0.83	1.76	0.637	0.004
SLC24A4	rs12896399	G	0.49	-0.15	1.19	0.900	0.0003
OCA2	rs1800407	T	0.05	-4.50	3.04	0.125	0.04
HERC2	rs12913832	A	0.22	1.07	1.57	0.494	0.01
MC1R	CHS	-	-	13.42	1.27	6.6×10^{-23}	2.02
RALY/ASIP	rs6059655	A	0.08	13.45	2.26	1.9×10^{-8}	0.74
Total							**40.32**

Abbreviations: %Δ, percentage change in the pigmented spots area, per increase in effect allele; EA, effect allele; EAF, effect allele frequency; r^2, percentage variance in pigmented spots area, explained by the predictor; SE, standard error of the %Δ; SNP, single-nucleotide polymorphism.
Multivariable linear regression analysis. Age, sex, skin color, the four top SNPs from the GWAS, and the top SNPs of eight known pigmentation genes were tested. For *MC1R*, the CHS was used (compound heterozygosity score).

DISCUSSION

We detected SNPs in and around the genes *IRF4*, *MC1R*, *ASIP*, and *BNC2* that demonstrated genome-wide significant associations with facial pigmented spots, and all were successfully replicated in a second independent cohort. Furthermore, our data demonstrate that the associations of *IRF4*, *MC1R*, *ASIP*, and *BNC2* with facial pigmented spots were at least partially independent of skin color.

The four identified genes are known to be associated with visible skin traits in Europeans, including pigmentation variation (eye, hair, and skin color)[9,10,12], freckling[12,14], tanning response[15], and different types of skin cancer (basal cell carcinoma, squamous cell carcinoma, and melanoma)[16-19]. However, not all skin color-associated genes have an additional effect on the development of pigmented spots, such as *HERC2*. Previously, GWAS on skin sagging and global photoaging did not identify any skin color genes being involved[20,21], but we now demonstrate that skin color genes clearly have a role in the appearance of a specific feature of skin aging.

The SNP rs12203592 in *IRF4* (interferon regulatory factor 4) showed the strongest association with pigmented spots, explaining more than two percent of the phenotypic variance. Gene variants in *IRF4* are also associated with related phenotypes, namely skin color, freckling, and all skin cancer types[10,14,22]. Similarly, the compound *MC1R* haplotype was strongly associated with pigmented spots, explaining two percent of the variation. Many SNPs located close to *MC1R* showed association and, after adjusting for the *MC1R* CHS, no more SNPs were genome-wide significantly associated. All common variants in *MC1R* are associated with hair and skin color, freckling, and skin cancer types[10,11,23,24], showing that *MC1R* is pleiotropic in nature. The rs6059655 SNP in *RALY* (heterogeneous nuclear ribonucleoprotein) is located close to the skin color gene *ASIP* (agouti signaling protein). In previous studies, many variants around *ASIP* showed association with skin color-related phenotypes such as freckling, sun sensitivity and skin cancer[11,25]. Hence, the *RALY* SNP could affect *ASIP* expression via a long-range regulation, or it is in LD with another SNP closer to *ASIP*, which is affecting *ASIP* expression[26]. Finally, rs62543565 close to *BNC2* (basonuclin 2) was genome-wide significantly associated with pigmented spots after adjusting for the other three top-associated SNPs. Variants in *BNC2* are associated with skin color[9], and with freckling[14], but not yet found to be associated with skin cancer. This is a relatively new skin color gene and the function of *BNC2* in pigmentation needs to be further investigated in future studies. The four pigmentation genes together explain a non-trivial portion of 5.6% of the phenotypic variance, which is large compared with typical human complex traits – e.g., for adult body height ~2000 SNPs together could explain about 21% of the phenotypic variance[27].

The gene variant associations with pigmented spots were found to be independent of skin color, similar to what is found for gene variants that are associated with different types of skin cancer[24,28,29]. Pigmented spots and skin cancer share cumulative UV-exposure as a major risk factor, and facial pigmented spots have also shown to be a risk factor for skin cancer[30,31]. In addition, in the rare recessively inherited disease xeroderma pigmentosum, all affected individuals suffer

from many solar lentigines and skin (pre-)malignancies from a young age onward because of a defect in DNA repair mechanisms[23]. Therefore, it could be hypothesized that a less effective repair of UV-induced DNA damage explains the skin color independent effects of skin color genes in pigmented spots and skin cancer. In support of this, *MC1R* loss of function alleles have been associated with a higher level of UV-induced DNA damage in melanocytes[32,33], which is independent of the total melanin content[34]. Possibly, the melanocytes react to DNA damage by locally boosting melanin production to provide a subsequent UV protection. However, the specific role of these genes in the development of pigmented spots histology remains elusive.

DNA variants at all four loci, in particular *IRF4*, showed a stronger effect in darker colored individuals compared with white skinned individuals in a skin color-stratified analysis. Such an effect has been shown before for *MC1R* and melanoma[35]. Possibly, individuals with a darker skin color are less likely to avoid the sun as they will burn less easily, which aggravates the effect by cumulative UV-exposure. A second hypothesis is that the gene variant effects in the lighter skin color groups are ameliorated by other gene variants prevalent in these groups. This is supported by the observation that light skin color is still significantly associated with pigmented spots after adjustment for the top SNPs found here.

In women, we found a much higher prevalence of facial pigmented spots with 30% of the pigmented spot variance explained by sex, which could not be explained by genetic differences in our study. Previous studies are inconclusive about sex differences; some found a higher risk in women[4] and others in men[36]. Although we cannot rule out that our computer-aided phenotyping method used here was biased for pigmented spot detection in female compared to male skin, the same sex difference was also present in the LLS expert grading data, which were manually graded by experts[37]. Possible explanations are that higher levels of estrogen and progesterone may increase the risk of developing pigmented spots[5], women may exhibit a different lifestyle, or epigenetic regulation mechanisms may differ among the sexes.

To our knowledge, digitally quantified pigmented spots to identify risk factors are previously unreported. Photonumeric scales have been used to assess pigmented spot severity[36,38], but the advantage of digital quantification is a more objective and a more sensitive approach. However, a possible disadvantage is the inability to differentiate between the different facial pigmented lesions. We aimed to measure solar lentigines as a skin aging characteristic but simultaneously measured seborrheic keratosis (brown warty lesions in elderly). It is unlikely that other types of pigmented facial spots (melanocytic nevi, freckles, and melasma[5,39,40]) have biased our measure because they are more common in young individuals, and we additionally excluded all heavily freckled individuals. Therefore, our pigmented spots phenotype consists of solar lentigines and (a minority of) seborrheic keratosis. Because these two are often assumed to reflect the same phenotype (histologically they show clear overlapping features[41]), the elucidated genes likely influence both, but this should be confirmed in future research.

Conclusion

DNA variants in *IRF4*, *MC1R*, *ASIP*, and *BNC2* are significantly associated with facial pigmented spots independently of age, sex, and skin color. Future studies should investigate the biological function of these genes in the skin and, in particular, how they could be influencing pigmented spot development independently of basal melanin production.

MATERIALS & METHODS

Study populations

Rotterdam Study

The RS is a population-based prospective study of unrelated elderly subjects (>45 years of age) consisting of an initial cohort and two extensions[42]. The present study includes 2844 participants of northwestern European ancestry, for whom facial photographs and genotype data were available, after quality control. During routine visits at the research center, a full-body skin examination was performed by trained physicians and high-resolution standardized full-face photographs were obtained of participants not wearing make-up, cream, or jewelry, using a premier 3dMD face3-plus UHD (3dMD, Atlanta, GA, USA). The photos used in this study were collected from September 2010 to July 2013. The medical ethics committee of the Erasmus MC University Medical Center approved the study protocol, and all participants provided written informed consent.

Leiden Longevity Study

The LLS has been described in detail previously[43]. This family-based study consists of 1671 offspring of 421 nonagenarian sibling pairs of Dutch descent and their 744 partners. The current study includes 599 participants with facial pigmented spot grades and genotype data available after quality control. During routine visits at the Leiden research center, high-resolution standardized full-face photographs were obtained of participants not wearing make-up, cream, or jewelry, using a Fuji S2 (Tokyo, Japan) camera system. The photos used in this study were collected from November 2006 to April 2008. The study protocol was approved by the medical ethics committee of the Leiden University Medical Center, and all participants gave written informed consent.

Phenotyping

In the RS, pigmented spot presence was digitally quantified using semi-automated image analysis of high-resolution facial frontal photographs. The algorithms, digital rendering, measurement, and validation of the outcome measure have been described in detail using a randomly selected subset of images of 100 participants[44]. In short, the analysis detects areas that are dark brown, i.e., hyperpigmented relative to the surrounding skin with a roundish shape, present on the forehead, cheeks, and nose (Figure 1). It subsequently calculates the percentage of skin area detected as hyperpigmented spots. To test the image analysis accuracy, two independent physi-

cians manually graded the 100 photographs using a 5-point photonumeric scale. There was a high correlation between the average of the two manual grades and the values from the image analysis (Spearman's rho correlation coefficient 0.69)[44]. Furthermore, all 2844 photos were visually controlled for the type of hyperpigmentation, which should be solar lentigines or seborrheic keratoses. Therefore, individuals with freckles (N=23), facial contusion (N=1), facial scars with hyperpigmentation (N=1), and post inflammatory hyperpigmentation (N=1) were excluded. During the full-body skin examination, constitutional skin color was assessed at sun-protected skin sites (trunk, upper legs)[45]. The skin color was graded into three levels: very white (9%), white (78%), and white-to-olive (14%) (Table 1).

In the LLS, severity of pigmented spots was manually graded using a 9-point photonumeric scale, taking area, intensity of color, and uniformity of distribution into account[46]. Grading was performed independently by two skin aging experts using frontal digital photographs, as described previously[37,47].

Genotyping

In the RS, genotyping was carried out separately in the initial cohort and the two extension cohorts using the Infinium II HumanHap 550K and 660K Genotyping BeadChip version 3 (Illumina, San Diego, CA, USA). Collection and purification of DNA have been described previously[48]. All genotyped SNPs (N=537,405) were imputed using the MACH software[49] based on the 1000G Phase I Integrated Release Version 3 (released in March 2012) reference panel[50] separately for the three cohorts. Genotyping and quality control have been described in detail previously[51]. After quality control, the current study included a total of 6,846,125 autosomal SNPs (MAF>0.03, imputation Rsq>0.3, SNP call rate>0.97, HWE>1×10^{-4}) and 2844 individuals (individual call rate >0.95, pairwise identity by descent (IBD) sharing <0.25 (--genome option in PLINK), excluding x-mismatches and outliers from MDS analysis). We additionally conducted a GWAS using a more stringent IBD sharing threshold (IBD<0.1, N=2501). The results are identical in terms of the loci showing significant association with pigmented spots and the effect sizes (Supplementary Table S2, Supplementary Figure S3), which shows that including individuals with an IBD sharing <0.25 does not affect the reliability of GWAS results.

The LLS offspring and partners were genotyped using Illumina Infinium HD Human660W-Quad BeadChips and Illumina OmniExpress and imputation was performed using IMPUTE with the 1000G Phase I Integrated Release Version 3 (released in March 2012) reference panel. Family relations and imputation uncertainty were taken into account in the analysis by specialized software, QT-assoc[52].

Statistical analysis

In the RS, the phenotype (area of pigmented spots) showed a highly right-skewed distribution. We thus log transformed the phenotype, resulting in an approximately normal distribution of both the phenotype and the regression residuals. Because effect estimates (regression betas) of log

transformed outcome variables are not directly interpretable, we represent all regression betas as the percentage change (%Δ) – i.e., the percentage increase of the mean value of the dependent variable (in our case pigmented spots area) per unit increase of the independent variables (such as one year of age or carrying one additional minor allele), calculated as (exp (beta) - 1) * 100.

All analyses in the RS were adjusted for age, sex, the first four genetic principal components, and for variance between participants in flashlight illumination of the skin (Supplementary Methods). In the discovery GWAS (RS), association with autosomal SNPs was tested using linear regression assuming an additive allele effect. The inflation factor lambda was close to 1.0 (λ=1.02) and not further considered. A conditional GWAS, adjusted for the top SNP per locus, was performed. We also conducted GWAS separately in men (N=1323) and in women (N=1521). All GWAS analyses were conducted using PLINK [53].

A total of 167 SNPs in three loci with P-values<5×10^{-8} from the GWAS in the RS, plus the top SNP (at *BNC2*) from the conditional GWAS, were selected for replication analysis in the LLS. SNPs selected for replication were analyzed using linear regression, adjusting for age, sex and familial relations using the software package QT-assoc[52], which is based on a modified version of the score test. P-values<0.05 were considered as a significant replication.

The CHS of *MC1R* was calculated based on the haplotypes of six known and independent skin color SNPs in *MC1R* (rs1805005, rs2228479, rs1805007, rs1805008, rs885479, and rs1805009)[26], which were present in the RS. The haplotypes were calculated with statistical software R (www.R-project.org), package "haplo.stats". To calculate the *MC1R* CHS, we added up the number of variant type haplotypes per individual (Supplementary Table S3). A variant type haplotype carries at least one effect allele. The CHS is therefore coded as 0, 1, or 2 and comparable to a SNP in linear regression analysis.

Additional skin color-adjusted analyses were conducted in the RS. A skin color-adjusted and a skin color-stratified analysis were conducted for the top SNPs per locus in relation to pigmented spots. Furthermore, known pigmentation genes were tested for association with pigmented spots. Selection of the pigmentation genes was based on significant association with hair, eye, or skin color in previous GWAS studies[10-12,14,15,19] and included the reported top-associated SNP at each of the gene loci: *MC1R*, *HERC2*, *OCA2*, *ASIP*, *TYR*, *TYRP1*, *IRF4*, *SLC45A2*, *SLC24A4*, *TPCN2*, *KITLG*, and *BNC2*, unless a different SNP was associated with pigmented spots in this study. Association was tested in a multivariable analysis, including these 12 pigmentation SNPs, age, sex, and skin color (to test their independent effects, significance threshold P-value<0.005) and calculated the explained variance of pigmented spots (r^2). All statistical analyses were conducted using statistical software R.

ACKNOWLEDGMENTS

The authors are grateful to the study participants, the staff from the Rotterdam Study and the participating general practitioners and pharmacists. We thank Sophie Flohil, Emmilia Dowlatshahi, Robert van der Leest, Joris Verkouteren, Ella van der Voort and Shmaila Talib for collecting the phenotypes. Additionally we thank Sophie van den Berg for masking and reviewing all the photographs and Kirk Gossage for helping design and construct the pigmented spot image analysis method. We also thank Pascal Arp, Mila Jhamai, Marijn Verkerk, Lizbeth Herrera and Marjolein Peters for their help in creating the GWAS database, and Karol Estrada and Maksim V. Struchalin for their support in creation and analysis of imputed data.

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Chapter 6

Epidemiology and determinants of facial telangiectasia: a cross-sectional study

M.A. Hamer*
S. Mekić*
C. Wigmann
D.A. Gunn
M. Kayser
L.C. Jacobs
T. Schikowski
T. Nijsten
L.M. Pardo Cortes

**Shared first authorship*

J Eur Acad Dermatol Venereol. 2020 Apr; 34(4):821-826

ABSTRACT

Background: Telangiectasia or red veins are one of the prominent features of facial skin aging. To date, there are few studies investigating the determinants of telangiectasia.

Objectives: We investigated lifestyle and physiological factors associated with facial telangiectasia in a large prospective Dutch cohort study.

Methods: Telangiectasia were quantified digitally from standardized facial photographs of 2842 northwestern European participants (56.8% female, median age 66.9) from the Rotterdam Study, collected in 2010-2013. Effect estimates from multivariable linear regressions are presented as the percentage difference in the mean value of telangiectasia area per unit increase of a determinant (%Δ) with corresponding 95% CI.

Results: Significant determinants were older age (1.7%Δ per year, 95%CI 1.4 to 2.0), female sex (18.3%Δ, 95%CI 13.2 to 23.6), smoking (current versus never 38.4%Δ, 95%CI 30.3 to 47.0; former versus never 11.6%Δ, 95%CI 6.6 to 16.9), a high susceptibility to sunburn (10.2%Δ, 95%CI 5.4 to 15.3), and light skin color (pale versus white-to-olive 31.4%Δ, 95%CI 19.7 to 44.1; white vs. white-to-olive 9.2%Δ, 95%CI 2.8 to 16.0).

Conclusions: In this large cohort study, we confirmed known and described new determinants of facial telangiectasia.

INTRODUCTION

Facial telangiectasia are a feature of skin aging, alongside wrinkling, pigmented spots, and sagging. Most skin aging studies have focused on aging as a compound phenotype, predominantly using manual photonumeric scales[1-3]. This makes it difficult to make inference on the role of lifestyle and physiological factors associated with specific features such as telangiectasia, if they have varying influence on different skin aging features.

In line with this, recent skin aging research into pigmented spots, wrinkles, and sagging eyelids showed differences in genetic background as well as different environmental risk factors per subtype[4-6]. This highlights the need for separate analysis of risk factors for telangiectasia.

To date, few studies have specifically focused on telangiectasia. In one cross-sectional study of 1400 subjects (aged 20-54 years), telangiectasia were associated with increasing age, male sex, fair skin, smoking, and mainly outdoor occupations[7]. Smoking has repeatedly been associated with telangiectasia[8,9], but little is known about other lifestyle and physiological factors associated with red veins in the middle-aged to elderly.

In the Rotterdam Study, a large population-based cohort study, we investigated multiple lifestyle and physiological factors associated with facial telangiectasia in 2842 northwestern European elderly, using multiple linear regression.

METHODS

Study design, setting and participants

The Rotterdam Study (RS) is an ongoing prospective population-based cohort study of middle-aged to elderly (≥45 years of age) inhabitants of Ommoord, a suburb of Rotterdam in the Netherlands[10]. Since 2010, skin examinations have been conducted by trained physicians, focusing on the most common skin diseases. In addition, standardized high-resolution digital facial photographs (Premier 3dMDface3-plus UHD, Atlanta, GA, USA) are collected of participants not wearing make-up, cream, or jewelry. The present study aimed to include all participants who visited the dermatological screening at the research center between September 2010 and July 2013. For this study, a cross-sectional design was applied where data were measured at a single moment. The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus University Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports[10]. All participants provided written informed consent to participate in the study.

Telangiectasia assessment

The presence of telangiectasia was digitally quantified using semi-automated image analysis of high-resolution facial frontal photographs. The algorithms, digital rendering, measurement,

and validation with numerical grading have been described in detail previously[11]. In short, the analysis detects areas that are colored red to purple and linear or branch-like in shape (Figure 1). It subsequently calculates the percentage of skin area detected as telangiectasia.

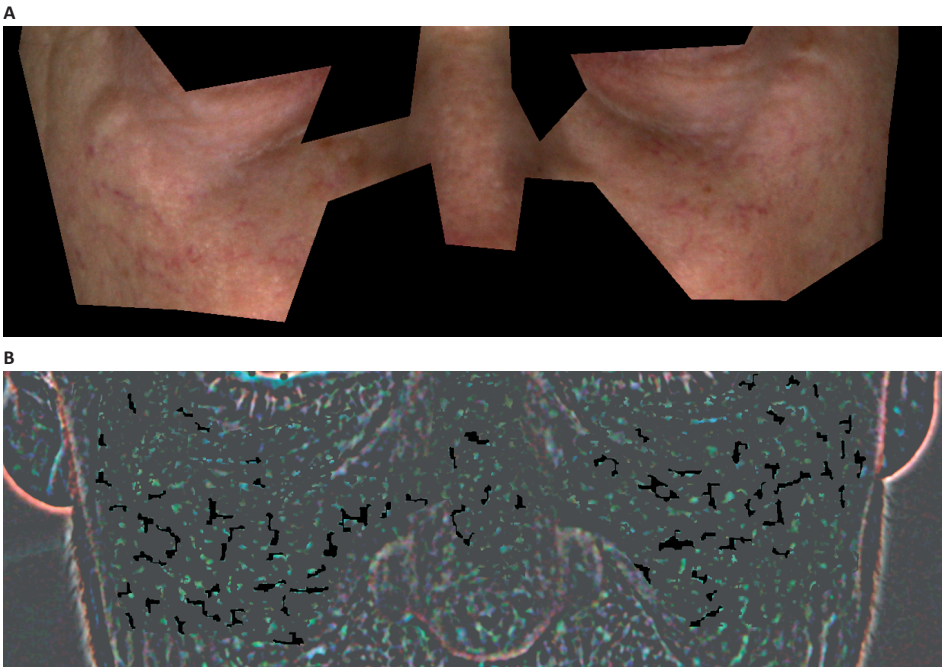

Figure 1. (**A**) Example of masked image. (**B**) Example of image analysis technique; the black structures are picked up as telangiectasia.

Determinants

Variables were selected based on known literature and biologically plausible associations. Level of education, smoking habit, alcohol consumption, and UV-related questions were collected through interview[12]. Variables collected by physical examination were body mass index (BMI), presence of dry skin, validated constitutional skin color assessment at sun-protected sites (pale, white, and white-to-olive)[13], rosacea (graded as centrofacial redness and red papules), and baldness. We used the Norwood-Hamilton scale[14,15] for baldness in men and the Ludwig scale[16] for baldness in women and classified these into none to minimal, moderate, and extensive baldness. Serum estradiol, testosterone and sex hormone binding globulin (SHBG) were measured on average 5.6 years before photograph collection. For women, the free androgen index (FAI) was calculated: (total testosterone / SHBG * 100)[17]. Details of all variables have been previously published[4].

Statistical analysis

We excluded variables with >35% missing values, namely the UV variables "outdoor work history" and "frequency of tanning bed visits". For the other missing values (maximum per variable: 16.4%), we performed multiple imputation based on all available variables shown in Table 1, with 20 iterations. To investigate the associations between lifestyle and physiological factors and telangiectasia, we used multivariable linear regressions, where all these variables are adjusted for one another in one model. Additionally, we adjusted for two technical variables in all analyses: one which accounted for possible variations in resolutions and another which accounted for variation in flash light[11,13]. Interaction terms for age, sex, smoking, and UV variables were tested. They were not significant or did not change the betas significantly and hence not added to our model.

Because the residuals of the linear regression of telangiectasia area did not fit a normal distribution, we transformed the outcome using the natural logarithm (ln), resulting in an approximately normal distribution of the regression residuals. To interpret the effect estimates (regression betas), we transformed the betas back, using the formula: $(\exp^{\beta}-1) * 100\%$. This outcome is interpreted as the percentage change (%Δ): the percentage increase in the mean value of telangiectasia area per unit increase of the independent variable, e.g. 3% increase in telangiectasia area per 1 year of age. There was no statistical interaction between sex and other variables (data not shown); therefore, all analyses were performed for men and women together. FAI, estradiol, and testosterone, hence, were excluded from this analysis. All analyses were performed using SPSS for Windows version 21.0 (SPSS, Chicago, IL) and software package R. A two-sided P-value of <0.05 was considered statistically significant.

Sensitivity and additional analyses

The missing UV variables ("outdoor work history" and "tanning bed use"), which were missing for ≥35% of the participants, were analyzed for association with telangiectasia in an exploratory complete-case analysis. Rosacea could have falsely been detected as telangiectasia, although people with rosacea do not necessarily show telangiectasia. In the RS cohort, we manually graded 54 individuals as having rosacea. To show their relationship, we calculated the correlation coefficient between rosacea and telangiectasia area. Lastly, we also retrieved data on telangiectasia from another cohort, the German SALIA cohort of elderly women. Because this cohort was a lot smaller, contained only women, and was different in terms of telangiectasia assessment and studied determinants, information on methods and results of these analyses is presented separately in the Supplementary Material. In an attempt to make a comparison between the RS and the SALIA cohort, we performed a linear regression analysis in RS women only, also including the variables FAI and estradiol.

Table 1. Characteristics of 2842 participants of the Rotterdam Study with telangiectasia measurements

Characteristic	Men N=1321	Women N=1521
Telangiectasia % - median [IQR]	0.77 [0.49 – 1.21]	0.96 [0.62 – 1.41]
Age at photo in years - median [IQR]	66.80 [61.3 – 72.0]	66.39 [61.0 – 71.3]
BMI in kg/m^2 - mean (SD)	27.70 (3.70)	27.56 (4.76)
Skin color		
pale (%)	100 (7.57)	141 (9.27)
white (%)	1014 (76.76)	1196 (78.63)
white-to-olive (%)	207 (15.67)	184 (12.10)
Baldness[a]		
no/mild baldness (%)	656 (49.66)	1013 (66.60)
moderate (%)	299 (22.63)	365 (24.00)
extensive (%)	365 (27.63)	111 (7.30)
Tendency to develop sunburn		
low (%)	870 (65.86)	921 (60.55)
high (%)	414 (31.34)	528 (34.71)
Outdoor work history		
no (%)	536 (40.58)	717 (47.14)
yes (%)	244 (18.47)	140 (9.20)
missing (%)	541 (40.95)	664 (43.66)
History of living in a sunny country >1 year		
no (%)	1178 (89.17)	1399 (91.98)
yes (%)	118 (8.93)	67 (4.40)
Sun-protective behavior[b]		
never/almost never (%)	482 (36.49)	485 (31.89)
often/almost always/always (%)	814 (61.62)	980 (64.43)
Tanning bed use		
never or less than 10x (%)	631 (47.77)	717 (47.14)
more than 10x (%)	74 (5.60)	140 (9.20)
missing (%)	616 (46.63)	664 (43.66)
Spend winter in sunny country		
no or less than 1 month (%)	1169 (88.49)	1366 (89.81)
yes, ≥1 month/yr (%)	61 (4.62)	69 (4.54)
missing (%)	91 (6.89)	86 (5.52)
Smoking history[c]		
current (%)	275 (19.45)	241 (15.84)
former (%)	766 (57.99)	695 (45.69)
never (%)	280 (21.20)	583 (38.33)
Education level[d]		
low (%)	91 (6.89)	139 (9.14)
medium (%)	745 (56.40)	1021 (67.13)
high (%)	469 (35.50)	349 (22.95)

Table 1. Characteristics of 2842 participants of the Rotterdam Study with telangiectasia measurements (continued)

Characteristic	Men N=1321	Women N=1521
Alcohol		
median use in glasses/day [IQR]	1.24 [0.31 – 2.42]	0.45 [0.05 – 1.40]
missing (%)	242 (18.32)	225 (14.79)
Dry skin		
No (%)	444 (33.61)	388 (25.51)
Yes (%)	877 (66.39)	1132 (74.42)
Testosterone in nmol/l - median [IQR]	16.58 [13.09 – 20.48]	na
Free androgen index[e] - median [IQR]	na	1.34 [0.89 – 1.93]
missing (%)		76 (5.00)
Estradiol in pmol/l - median [IQR]	na	39.72 [18.35 – 73.09]

Abbreviatons: BMI, body mass index; IQR, interquartile range; na, not applicable; SD, standard deviation.
[a]based on the Norwood-Hamilton (NH) scale for men and the Ludwig scale for women; None or minimal: NH score 1, 2, 3, 9, 10, 11 and Ludwig scale score none. Moderate: NH score 4, 5, 6, 12 and Ludwig scale score 1. Extensive: NH score 7, 8 and Ludwig scale score 2, 3; [b]wearing sunglasses and/or a brimmed hat in the sunshine; [c]cigars, cigarettes or pipe; [d]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [e]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l).

RESULTS

Study population

Between September 2010 and July 2013, a total of 3831 participants visited the dermatological examination of the RS. We excluded individuals due to non-northwestern European origin, poor image quality, and make-up, leaving 2842 participants with eligible 3D photographs used to measure facial telangiectasia area. There were slightly more women than men (N=1521; 53.5%), and the median age was 66.6 years old (Table 1). The median telangiectasia area was higher in women than in men (men: 0.77%, IQR 0.49 to 1.21; women: 0.96%, IQR 0.62 to 1.41).

Determinants for facial telangiectasia area

With higher age, telangiectasia area increased 1.7% per year (95%CI 1.4 to 2.0). Women had 18.3% (95%CI 13.2 to 23.6) more telangiectasia than men, and the lighter the skin color, the higher the risk for more red veins was. Having a white skin color associated with a 9.2% (95%CI 2.8 to 16.0) larger telangiectasia area and having a pale skin color with 31.4% more red veins, compared to white-to-olive skinned participants. Interestingly, not only did current smokers have 38.4% (95%CI 30.3 to 47.0) more telangiectasia than non-smokers, but former smokers also had 11.6% (95%CI 6.6 to 16.9) more telangiectasia than non-smokers. Finally, participants with a tendency to develop sunburn also showed a 10.2% (95%CI 5.4 to 15.3) larger telangiectasia area than those not susceptible to sunburn (Table 2).

Table 2. Multivariable linear regression of facial telangiectasia: determinants of facial telangiectasia among 2842 participants of the Rotterdam Study

Determinant	%Δ telangiectasia area[a]	95% CI	P-value
Sex			
male	ref	ref	ref
female	**18.3**	**[13.2 – 23.6]**	**<0.001**
Age (per year)	**1.7**	**[1.4 – 2.0]**	**<0.001**
BMI (per point)	0.2	[-0.2 – 0.7]	0.405
Skin color			
white-to- olive	ref	ref	ref
white	**9.2**	**[2.8 – 16.0]**	**0.004**
pale	**31.4**	**[19.7 – 44.1]**	**<0.001**
Baldness			
no/mild baldness	ref	ref	ref
moderate	1.7	[-3.1 – 6.8]	0.500
extensive	$-1.1*10^{-2}$	[-5.7 – 11.0]	0.997
Tendency to develop sunburn	**10.2**	**[5.4 – 15.3]**	**<0.001**
History of living in a sunny country	0.5	[-7.3 – 8.9]	0.905
Sun-protective behavior[b]	-0.6	[-4.8 – 3.7]	0.772
Spending winter in sunny country	-8.1	[-16.4 – 0.9]	0.076
Smoking history[c]			
never	ref	ref	ref
former	**11.6**	**[6.6 – 16.9]**	**<0.001**
current	**38.4**	**[30.3 – 47.0]**	**<0.001**
Education level[d]			
low	ref	ref	ref
medium	4.31	[-3.23 – 12.4]	0.270
high	2.26	[-5.76 – 11.0]	0.592
Alcohol (per glass per day)	-0.8	[-2.2 – 0.7]	0.291
Dry skin			
no	ref	ref	ref
yes	-1.5	[-5.8 – 3.1]	0.519
Batch[e]	**32.26**	**[23.4 – 41.7]**	**<0.001**
Residual[f]	**2.27**	**[2.0 – 2.5]**	**<0.001**

Abbreviations: 95% CI, 95% conference interval; BMI, body mass index; ref, reference variable. Boldface indicates statistically significant determinants.

[a] %Δ: the percentage change in telangiectasia area (the % increase in the mean value of telangiectasia area per unit increase of the independent variable, calculated by the formula: $(\exp^{\beta}-1) \cdot 100\%$. E.g. 1.7% increase in telangiectasia area per 1 year of age; [b]wearing sunglasses and/or a brimmed hat in the sunshine; [c]cigars, cigarettes or pipe; [d]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [e]technical variable which accounts for possible changes in resolution; [f]technical variable which accounts for possible changes in flash light variability. R^2 total model: 0.354.

Sensitivity and additional analyses

In a complete case analysis including the two additional UV variables "outdoor work history" and "tanning bed use", both were not significantly associated (Supplementary Table S1). Additionally, the effect estimates of the significant determinants remained similar to the previous analysis, indicating there was no meaningful association between these two UV variables and telangiectasia. However, the variable "spending winter in a sunny country" showed a negative association (-20.8%, 95%CI -31.4 to -8.5) instead of no association in the previous analysis. Spearman's rho correlation coefficient between rosacea and telangiectasia area was 0.04, indicating no correlation between the two conditions. In the SALIA cohort, age, light skin color type, and smoking were significantly associated determinants (Supplementary Material & Supplementary Tables S3 and S4).

When analyzing RS women only (N=1521), we found similar results to those in the analysis of men and women together. The only meaningful difference was that BMI was associated with more telangiectasia area in women (0.6%Δ per 1 point BMI increase, 95%CI 0.1 to 1.2) (Supplementary Table S2).

DISCUSSION

In this large cross-sectional study, the most important variables associated with facial telangiectasia were light skin color type and smoking. Increasing age was also significantly associated with more facial red veins, although with a smaller effect size. Additionally, we found that female sex and tendency to develop sunburn were significant determinants for telangiectasia in the RS. We replicated our associations in a smaller cohort of women from European ancestry, showing the relevance of our findings. Although the cohort was smaller and only assessed telangiectasia in women with a different assessment, we demonstrated that main determinants were indeed associated with telangiectasia.

Smoking was the most important determinant for telangiectasia with the largest effect size. Current smokers had more than a third extra telangiectasia compared with non-smokers. This is not surprising, as we know that smoking is one of the most important lifestyle factors inducing premature skin aging[9,18-20]. It might even be the most important risk factor for telangiectasia since it is repeatedly replicated in all telangiectasia studies. Even former smoking had a significant effect in our cohort. The underlying mechanism on how smoking could lead to more red veins is not yet known; however, smoking induces DNA damage, elastosis, and more atrophy of the skin, which could make red veins more visible[21]. Smoking has also been associated with dilated venules in other human organs such as the retina[22]. Alternatively, smoking causes vasoconstriction of the small vessels which leads to a chronic hypoxemic state in the skin[23]. This could result in proliferation of new red veins, visible as more telangiectasia.

Pale skin color type was associated with more telangiectasia, as previously reported[7]. Similar to smoking, the underlying mechanism is not yet elucidated, but we hypothesize that UV-induced DNA damage will play a role, as in other types of skin aging. Alternatively, telangiectasia might be more visible on lighter skin.

Female sex was associated with more telangiectasia, which was opposite to what has been previously reported[7]. This could be explained in part by the higher average age in the RS population compared to the age of the participants in the previous report. Men tend to show signs of skin wrinkling earlier in life, with women showing similar wrinkling prevalence as men later on in life[4]. Hence, men could also develop telangiectasia earlier in life. Additionally, male skin is 10-20% thicker than female skin and therefore might be less susceptible to thinning and showing red veins[24].

Light skin color type and current smoking were also significant determinants for telangiectasia in the SALIA cohort. Unexpectedly, in this relatively small cohort, older age was associated with less telangiectasia. However, the age range of the replication cohort is much smaller than in the RS and lies within the ages in which the RS also showed a decline in telangiectasia (Supplementary Figures S1 and S2). This phenomenon has not been described earlier, which indicates it is probably a coincidental finding. However, unknown confounders might also have a part in this. In the sensitivity analysis in the women of the RS, we found that increasing BMI associates with more telangiectasia but this was not found in the SALIA cohort. A higher BMI has previously been linked with fewer facial wrinkles, which probably has to do with the filler effect of facial fat[4]. Research into skin circulation showed that with increasing BMI, oxygenation in skin increased[25]. Furthermore, dermal microvascular dysfunction is common in diabetes patients who often have a higher BMI than healthy subjects[26]. However, how BMI exactly associates with telangiectasia remains to be fully understood.

The results of this study confirm the hypothesis that the different features of skin aging have different determinants. Age and sun exposure are the exception and are important risk factors for all skin aging phenotypes (i.e., wrinkling, pigmented spots, and telangiectasia). However, skin color, for example, is different. Pale skinned individuals are more at risk for having telangiectasia and pigmented spots while they have less wrinkles[4,5]. Smoking is the major lifestyle risk factor for wrinkling and telangiectasia, and although it can cause smokers' melanosis in the oral cavity[27], it has not been proven to stimulate facial pigmented spots. This clustering of specific risk factors could be of use in the risk stratification and personalized approach of skin aging prevention strategies.

There are several limitations of this study. Firstly, the cross-sectional nature of the associations prevents from determining causal inferences. Secondly, we used a digital method to measure telangiectasia where most previously performed studies used photonumeric grading. However, validation of our digital method[11] has shown that there is a moderate to good correlation between digital and photonumeric measurement of telangiectasia (Spearman's rho 0.60 in women and 0.75 in men), which suggests this will not have a large effect in our conclusions. Also, we found

only one of our UV variables to be associated with telangiectasia. This illustrates that the quality of our used questions for sun exposure was suboptimal and that it remains a difficult variable to capture by questionnaire. Furthermore, besides in telangiectatic aging, facial erythema and telangiectasia are also often associated with the erythematotelangiectatic subtype of rosacea (ETR, besides the other three types of rosacea: papulopustular, phymatous and ocular). It is therefore important to recognize the differences between ETR and telangiectatic aging[28]. However, in our data, the number of rosacea patients was low and rosacea correlated poorly with telangiectasia. Looking more carefully into these rosacea cases, there was a substantial proportion with the papulopustular subtype and telangiectasia were poorly picked up in the ETR group. The latter is a limitation of our image analysis technique where it seems to pick up telangiectasia less well in an erythematous environment, probably due to lack of contrast. Lastly, our findings hold for a predominantly northwestern European population. It is not clear to which extent these can be extrapolated to other populations.

In conclusion, this large study confirmed some of the earlier found risk factors for telangiectasia such as pale skin and smoking which are similar in men and women, while identifying potential new associations such as BMI. These results support the evidence that different skin colors show varying prevalence of specific skin aging features. The correlated factors of telangiectasia can help future studies to unravel causal versus consequence determinants as more insight into etiology of telangiectasia is gained, and longitudinal or experimental studies are added to this field of research.

ACKNOWLEDGMENTS

Rotterdam study: The authors are grateful to the study participants, the staff from the Rotterdam Study, and the participating general practitioners and pharmacists. We thank Emmilia Dowlatshahi, Sophie Flohil, Robert van der Leest, Simone van der Velden, Joris Verkouteren and Ella van der Voort for collecting the phenotypes. Additionally, we thank Sophie van den Berg for masking and reviewing all the photographs. We acknowledge Jaspal Lall for masking the photographs and creating the digital telangiectasia measurements.

SALIA Study: We thank all study members and staff involved in data collections in each cohort and also the respective funding bodies for SALIA. Study directorate: R. Dolgner; U. Krämer, U. Ranft, T. Schikowski, A. Vierkötter. Scientific Team Baseline: A.W. Schlipköter, M.S. Islam; A. Brockhaus, H. Idel, R. Stiller-Winkler, W. Hadnagy, T. Eikmann. Scientific Team Follow-up: D. Sugiri, A. Hüls, B. Pesch, A. Hartwig, H. Käfferlein, V. Harth, T. Brüning, T. Weiss. Study Nurses: G. Seitner-Sorge, V. Jäger, G. Petczelies, I. Podolski, T. Hering, M. Goseberg. Administrative Team: B. Schulten, S. Stolz. During the last decades many scientists, study nurses and laboratories were involved in conducting the study. We are most grateful for all the women from the Ruhr area and Borken who participated in the study over decades and the local health Departments for organizing the study.

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SUPPLEMENTARY MATERIAL (SALIA COHORT)

METHODS

Study population

The Study on the influence of Air pollution on Lung function, Inflammation and Ageing (SALIA) is a cohort study including middle-aged women from the urban Ruhr area (Dortmund, Duisburg, Essen, Gelsenkirchen and Herne) and two rural northern counties (Southern Münsterland) in West Germany. The baseline investigation started in 1985, when the women were about 55 years of age. Men were not recruited because of the high occupational exposure of many men in this area, where coal mining and steel industry constituted the predominant sources of income in the time period before the baseline examination[1]. The replication analysis is based on data from the clinical follow-up examination (2007–2010), in which 834 women participated. All participants gave written informed consent. The Medical Ethics Committee of the University of Bochum approved the follow-up examination[2].

Telangiectasia assessment

Severity of telangiectasia was manually graded using a photonumeric 0-5 scale, as part of the SCINEXA™ method[3].

Determinants

BMI was assessed by physical examination. Information on skin color type (based on the Fitzpatrick scale[4]), household education level (highest level of education of the participants and their partners combined) and lifestyle (use of sun protection cream and sunbeds, holidays in sunny regions, smoking and alcohol consumption) was collected via interview.

Statistical analysis

In SALIA, we investigated the influence of lifestyle and physiological factors on telangiectasia using a multivariable linear regression model including age, BMI, skin type, use of sun protection cream and tanning beds, holidays in sun rich regions, smoking history, education level and alcohol consumption as independent variables. Information on these variables and on telangiectasia were available for 784 women and we included only these complete cases. The analysis was performed in R. A two-sided P-value of <0.05 was considered statistically significant.

RESULTS

Between May 2007 and March 2010, 834 women were screened on telangiectasia. A number of 50 women were excluded due to missing data, leaving 784 women included in the final analysis. The women were slightly older than in the RS with a mean age of 73.5 years (Supplementary Table S3). The mean value of the telangiectasia was 2.1.

The age range in the SALIA cohort was smaller (66-79 years) than the age range in the RS (51-98 years) and showed a decrease in telangiectasia with increasing age, whereas the RS showed an overall increase in telangiectasia with increasing age. However, when zooming in on the age range of 60-75 years in the RS, a decrease in telangiectasia was seen, similar to the SALIA cohort in the comparable age range (Supplementary Figures S1 and S2). Light skin color type (skin type I/II vs. III/IV: ß=0.44 [95%CI 0.22 to 0.66]) and smoking (current vs. never smoking: ß=0.66 [95%CI 0.002 to 1.33]) were replicated as potential determinants. Women using sun-cream protection showed less telangiectasia (ß=-0.21 [95%CI -0.45 to 0.02]). Age was associated with less telangiectasia (ß=-0.09 [95%CI -0.12 to -0.05], as opposed to the findings in the RS (Supplementary Table S4).

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table S1. Sensitivity analysis complete cases Rotterdam Study (N=1146)

Determinant	%Δ telangiectasia area[a]	95% CI	P-value
Sex			
male	ref	ref	ref
female	**19.0**	**[11.1 – 27.4]**	**0.008**
Age (per year)	**1.3**	**[0.7 – 2.0]**	**<0.001**
BMI (per point)	0.4	[-0.3 – 1.1]	0.243
Skin color			
white-to-olive	ref	ref	ref
white	8.8	[-0.6 – 19.1]	0.068
pale	**27.7**	**[12.6 – 44.9]**	**<0.001**
Baldness			
no/mild baldness	ref	ref	ref
moderate	-2.2	[-9.6 – 5.9]	0.588
extensive	2.9	[-6.9 – 13.8]	0.570
Tendency to develop sunburn	**14.0**	**[6.4 – 22.2]**	**<0.001**
Outdoor work history	2.0	[-5.6 – 10.3]	0.610
History of living in a sunny country	2.3	[-10.2 – 16.5]	0.733
Tanning bed use >10 times	-3.0	[-11.4 – 6.2]	0.508
Sun-protective behavior[b]	2.0	[-4.4 – 8.9]	0.550
Spending winter in sunny country	**-20.8**	**[-31.4 – -8.5]**	**0.002**
Smoking history[c]			
never	ref	ref	ref
former	**10.4**	**[2.9 – 18.3]**	**0.006**
current	**36.8**	**[25.2 – 49.5]**	**<0.001**
Education level[d]			
low	ref	ref	ref
medium	-2.8	[-13.2 – 8.9]	0.627
high	-5.3	[-16.1 – 7.0]	0.384
Alcohol (per glass per day)	4.0×10^{-4}	[-2.0 – 2.0]	0.999
Dry skin			
no	ref	ref	ref
yes	-1.2	[-7.2 – 5.3]	0.714
Batch[e]	**28.2**	**[16.5 – 41.0]**	**0.004**
Residual[f]	**2.2**	**[1.8 – 2.5]**	**<0.001**

Abbreviations: 95% CI, 95% conference interval; BMI, body mass index; ref, reference variable. Boldface indicates statistically significant determinants.

[a] %Δ: the percentage change in telangiectasia area (the % increase in the mean value of telangiectasia area per unit increase of the independent variable, calculated by the formula: $(\exp^{\beta}-1) * 100\%$. E.g. 1.7% increase in telangiectasia area per 1 year of age; [b]wearing sunglasses and/or a brimmed hat in the sunshine; [c]cigars, cigarettes or pipe; [d]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [e]technical variable which accounts for possible changes in resolution; [f]technical variable which accounts for possible changes in flash light variability.

Supplementary Table S2. Sensitivity analysis women Rotterdam Study (N=1521)

Determinant	% Δ telangiectasia area[a]	95% CI	P-value
Age (per year)	**1.5**	**[1.1 – 1.8]**	**<0.001**
BMI (per point)	**0.6**	**[0.1 – 1.2]**	**0.034**
Skin color			
white-to-olive	ref	ref	ref
white	**10.2**	**[1.3 – 20.0]**	**0.024**
pale	**31.4**	**[16.2 – 48.7]**	**<0.001**
Baldness			
no/mild baldness	ref	ref	ref
moderate	2.4	[-3.8 – 9.1]	0.453
extensive	-3.3	[-12.5 – 7.0]	0.521
Tendency to develop sunburn	**7.8**	**[1.7 – 14.3]**	**0.012**
History of living in a sunny country	5.4	[-7.3 – 19.8]	0.424
Sun-protective behavior[b]	1.9	[-3.8 – 7.9]	0.524
Spending winter in sunny country	-7.7	[-18.7 – 4.8]	0.218
Smoking history[c]			
never	ref	ref	ref
former	**7.8**	**[1.7 – 14.3]**	**0.011**
current	**45.0**	**[33.7 – 57.3]**	**<0.001**
Education level[d]			
low	ref	ref	ref
medium	4.06	[-5.2 – 14.2]	0.401
high	1.7	[-8.4 – 13.0]	0.748
Alcohol (per glass per day)	0.9	[-1.5 – 3.3]	0.477
Dry skin			
no	ref	ref	ref
yes	-2.7	[-8.5 – 3.5]	0.388
Free androgen index[e]	0.8	[-1.3 – 2.8]	0.464
Estradiol (per pmol/l)	3.0×10^{-3}	[-0.01 – 0.02]	0.682
Batch[f]	**25.6**	**[14.5 – 37.9]**	**<0.001**
Residual[g]	**2.0**	**[1.7 – 2.4]**	**<0.001**

Abbreviations: 95% CI, 95% conference interval; BMI, body mass index; ref, reference variable. Boldface indicates statistically significant determinants.

[a] %Δ: the percentage change in telangiectasia area (the % increase in the mean value of telangiectasia area per unit increase of the independent variable, calculated by the formula: $(\exp^{\beta}-1) * 100\%$. E.g. 1.7% increase in telangiectasia area per 1 year of age; [b]wearing sunglasses and/or a brimmed hat in the sunshine; [c]cigars, cigarettes or pipe; [d]low (primary education); medium (lower secondary education/lower vocational education/intermediate vocational education); high (general secondary education/higher vocational education/university); [e]free androgen index (calculated as total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l); [f]technical variable which accounts for possible changes in resolution; [g]technical variable which accounts for possible changes in flash light variability.

Supplementary Table S3. Characteristics of 784 female participants of the SALIA cohort with telangiectasia measurements

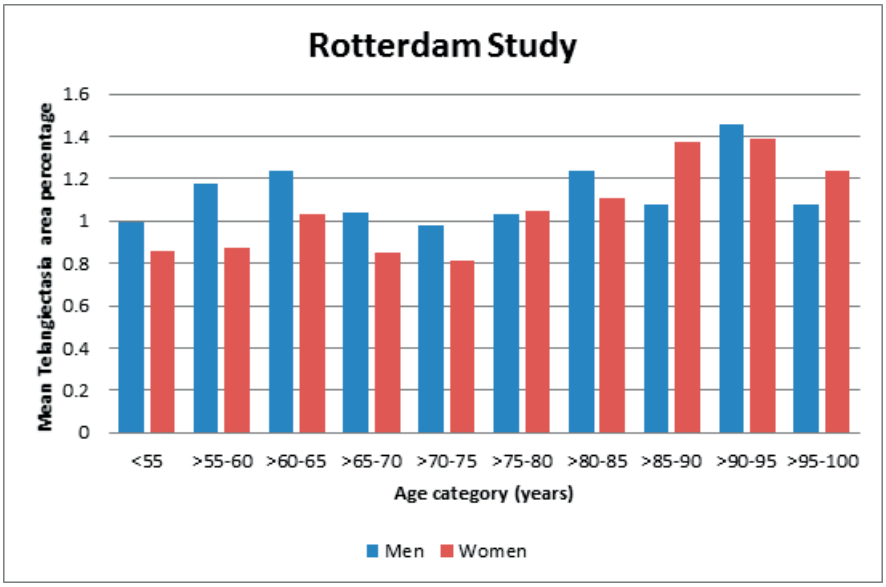
Characteristic	Women N=784
Telangiectasia score - mean (SD)	2.1 (1.5)
Age at photo in years - mean (SD)	73.5 (3.0)
BMI in kg/m^2 - mean (SD)	27.3 (4.5)
Skin color	
I/II (%)	437 (55.7)
III/IV (%)	347 (44.3)
Regular use of sun protection cream	
no (%)	309 (39.4)
yes (%)	475 (60.6)
Tanning bed use	
never (%)	644 (82.1)
ever (%)	140 (17.9)
Holidays in sunrich regions in weeks per year – mean (SD)	1.4 (2.6)
Smoking history	
current (%)	21 (2.7)
former (%)	138 (17.6)
never (%)	625 (79.7)
Education level	
low: <10yrs education (%)	139 (17.7)
medium: 10yrs education (%)	385 (49.1)
high: >10yrs education (%)	260 (33.2)
Alcohol	
never (%)	130 (16.6)
ever (%)	654 (83.4)

Abbreviations: BMI, body mass index; SD, standard deviation.

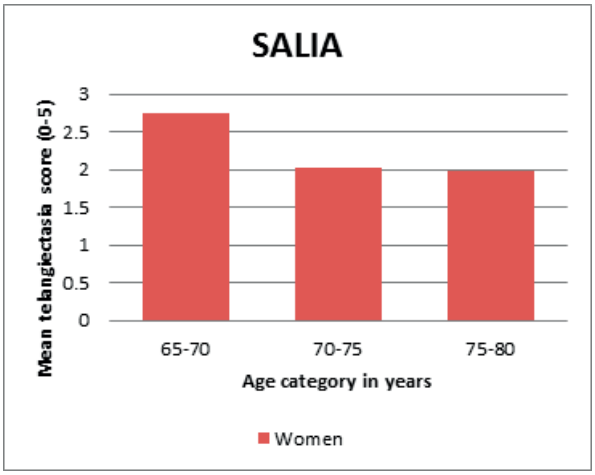
Supplementary Table S4. Multivariable linear regression of facial telangiectasia: determinants of facial telangiectasia among 784 women of the SALIA cohort

Determinant	ß	95% CI	P-value
Age (per year)	**-0.09**	**[-0.12 – -0.05]**	**<0.001**
BMI (per point)	0.005	[-0.02 – 0.03]	0.714
Skin type (Fitzpatrick)			
III/IV	ref	ref	ref
I/II	**0.44**	**[0.22 – 0.66]**	**<0.001**
Regular use of sun protection cream			
no	ref	ref	ref
yes	-0.21	[-0.45 – 0.02]	0.074
Tanning bed use			
never	ref	ref	ref
ever	0.03	[-0.26 – 0.33]	0.827
Holidays in sunrich regions in weeks (per year)	-0.01	[-0.05 – 0.04]	0.797
Smoking history			
never	ref	ref	ref
former	0.20	[-0.09 – 0.48]	0.174
current	**0.66**	**[0.002 – 1.33]**	**0.049**
Education level			
low	ref	ref	ref
medium	-0.01	[-0.30 – 0.29]	0.958
high	-0.06	[-0.38 – 0.26]	0.701
Alcohol consumption			
never	ref	ref	ref
ever	0.003	[-0.29 – 0.29]	0.983

Abbreviations: BMI, body mass index; SD, ref, reference variable. Boldface indicates statistically significant determinants.



Supplementary Figure S1. Distribution of telangiectasia per age category in the Rotterdam Study.



Supplementary Figure S2. Distribution of telangiectasia per age category in the SALIA cohort.

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Chapter 7

The *MC1R* gene and youthful looks

F. Liu
M.A. Hamer
J. Deelen
J.S. Lall
L.C. Jacobs
D. van Heemst
P.G. Murray
A. Wollstein
A.J.M. de Craen
H.W. Uh
C. Zeng
A. Hofman
A.G. Uitterlinden
J.J. Houwing-Duistermaat
L.M. Pardo Cortes
M. Beekman
P.E. Slagboom
T. Nijsten
M. Kayser
D.A. Gunn

Curr Biol. 2016 May 9;26(9):1213-20

ABSTRACT

Looking young for one's age has been a desire since time immemorial. This desire is attributable to the belief that appearance reflects health and fecundity. Indeed, perceived age predicts survival[1] and associates with molecular markers of aging such as telomere length[2]. Understanding the underlying molecular biology of perceived age is vital for identifying new aging therapies among other purposes, but studies are lacking thus far. As a first attempt, we performed genome-wide association studies (GWASs) of perceived facial age and wrinkling estimated from digital facial images by analyzing over eight million single-nucleotide polymorphisms (SNPs) in 2693 elderly northwestern Europeans from the Rotterdam Study. The strongest genetic associations with perceived facial age were found for multiple SNPs in the *MC1R* gene (P-value $<1\times10^{-7}$). This effect was enhanced for a compound heterozygosity marker constructed from four pre-selected functional *MC1R* SNPs (P-value=2.69×10^{-12}), which was replicated in 599 Dutch Europeans from the Leiden Longevity Study (P-value=0.042) and in 1173 Europeans of the TwinsUK Study (P-value=3×10^{-3}). Individuals carrying the homozygote *MC1R* risk haplotype looked on average up to two years older than non-carriers. This association was independent of age, sex, skin color, and sun-damage (wrinkling, pigmented spots) and persisted through different sun-exposure levels. Hence, a role for *MC1R* in youthful looks independent of its known melanin synthesis function is suggested. Our study uncovers the first genetic evidence explaining why some people look older for their age and provides new leads for further investigating the biological basis of how old or young people look.

RESULTS

The discovery cohort included 2693 northwestern European subjects from the Rotterdam Study[3] (Table S1). As expected, perceived facial age (termed perceived age from now on) was strongly correlated with chronological age of the subjects (r^2 44%, P-value <10^{-300}). However, women tended to look slightly older (by 1.53 years on average) and men slightly younger (by -1.49 years on average) for their respective chronological age (Figure S1A). On average, the percentage of facial skin covered by wrinkling was estimated as 1.27% (SD 0.66%, Table S1). Facial wrinkling was strongly correlated with perceived age, as measured by the residuals of regressing perceived age on chronological age, in women (r^2 35%, P-value=9.5×10^{-138}) as well as in men (r^2 21%, P-value=3.1×10^{-65}) (Figure S1B). The effect of wrinkling and non-wrinkling components on facial aging is illustrated using averaged faces of women who, although being of the same chronological age, looked younger or older either influenced by (Figures 1A and 1B) or irrespective of (Figures 1C and 1D) facial wrinkling. Facial pigmented spots showed a weaker correlation with perceived age in women (r^2 1.0%, P-value=0.001) and in men (r^2=0.8%, P-value=0.002) (Figure S1C). Most subjects were not sunbed users and had white as opposed to pale skin color or white-to-olive skin color (Table S1).

Genome-wide association studies on perceived age and wrinkles in the Rotterdam Study

In the discovery GWAS using 2693 samples from the Rotterdam Study, we searched for SNPs that associated with perceived age, wrinkling, and the non-wrinkling component of perceived age (i.e., adjusted for wrinkles). Although genome-wide significant associations for perceived age (Table S2) and wrinkling were not observed (Table S3), multiple SNPs at the *MC1R* gene locus on chromosome 16 showed borderline genome-wide significant association with perceived age after adjustment for age, sex, and wrinkles (Tables 1 and S2; Figure 2, S2A, and S2B).

We then constructed a collapsed compound heterozygosity marker (herein termed *MC1R* compound marker) based on a haplotype analysis of four *MC1R* DNA variants, rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H), which were selected a priori because of previous knowledge that they (1) are missense loss-of-function variants[4], (2) are causing phenotypes such as red hair color and pale skin in a compound heterozygote manner[4,5], and (3) are involved in age-related skin phenotypes such as pigmented spots[6]. These four missense *MC1R* DNA variants were collapsed into three possible haplotypes, WT/WT, WT/R, and R/R, where R is the risk haplotype consisting of at least one risk allele from any of the four *MC1R* variants and the WT is the wild-type haplotype consisting of none of the risk alleles (Supplemental Information). This *MC1R* compound marker demonstrated a genome-wide significant association with perceived age after adjustment for age, sex, and wrinkles (P-value=2.69×10^{-12}, Table 1; Figure 2). On average, the homozygote *MC1R* risk haplotype carriers (R/R) looked almost two years older (1.81 years) than the non-carriers (WT/WT); the heterozygote carriers (R/WT) looked almost one year older (0.87 years) than the non-carriers (WT/WT) (Table 2), with a slightly larger effect size in men compared to women (Figure S2C).

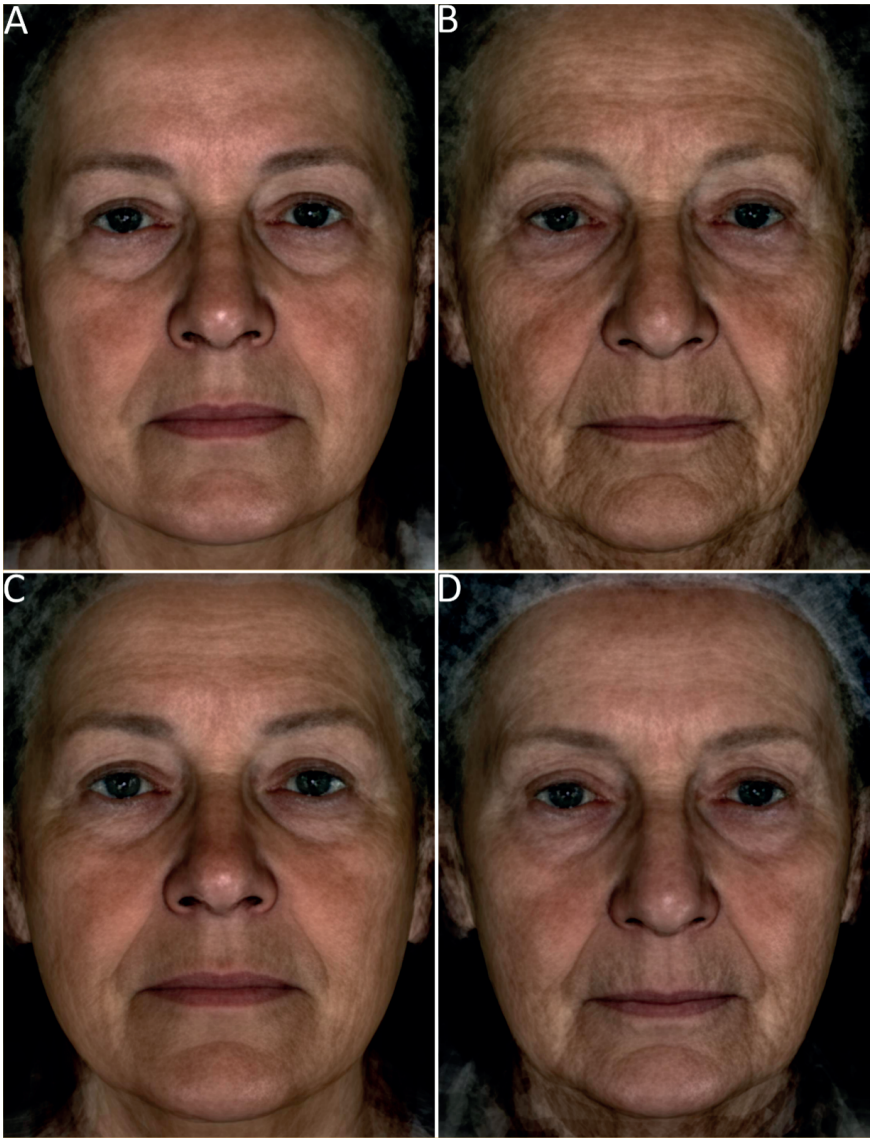

Figure 1. Illustration of the effect of wrinkling and non-wrinkling components on perceived facial age. (**A**–**D**) Facial averages of northwestern European women who looked young or old for their chronological age without (**A** and **B**) and with (**C** and **D**) adjustment for the effect of wrinkles. Enface average image of 22 women (mean chronological age 70) who looked young for their chronological age (mean perceived age 59) (**A**) and 22 women (mean chronological age 70) who look old for their chronological age (mean perceived age 80) (**B**); differences in face shape changes (e.g., lip size, jawline sag, nasolabial fold) and wrinkles (average percent of skin covered by wrinkles was 2% for A and 10% for B) are evident. Enface average image of 20 women (mean chronological age 69) who looked young for their chronological age (average perceived age after adjusting for wrinkles was 60) (**C**) and 20 women (mean chronological age 69) who looked old for their chronological age (mean perceived age after adjusting for wrinkles was 78) (**D**); differences in face shape changes and skin color are evident. The mean total skin area covered by wrinkles for (**C**) and (**D**) was the same (5%). See Figure S1 for correlations of perceived age with chronological age and age-related sub-phenotypes such as wrinkles and pigmented spots in the Rotterdam Study discovery cohort.

Table 1. SNPs associated with perceived facial age from a GWAS in the discovery cohort (Rotterdam Study), their association in the first replication cohort (Leiden Longevity Study), and in a meta-analysis

					Discovery cohort (N=2693)				First replication cohort (N=599)				Meta-analysis (N=3292)		
Gene	**CHR**	**MBP**	**SNP**	**EA**	**EAF**	**β**	**SE**	**P-value**	**EAF**	**β**	**SE**	**P-value**	**β**	**SE**	**P-value**
CALN1	7	71.4	rs10259553	C	0.26	-0.64	0.13	9.4×10^{-7}	0.25	-0.06	0.22	0.796	-0.49	0.11	1.2×10^{-5}
CORO2A	9	100.9	rs35480968	G	0.33	-0.61	0.12	3.9×10^{-7}	0.33	0.09	0.22	0.668	-0.44	0.10	2.2×10^{-5}
MC1R	16	89.8	rs34265416	A	0.09	0.98	0.19	5.1×10^{-7}	0.10	0.43	0.35	0.214	0.85	0.17	5.5×10^{-7}
MC1R	16	89.8	rs4785704	G	0.10	1.00	0.19	2.6×10^{-7}	0.10	0.46	0.36	0.200	0.88	0.17	2.6×10^{-7}
MC1R	16	89.8	rs34714188	A	0.07	1.10	0.22	5.1×10^{-7}	0.08	0.63	0.38	0.098	0.98	0.19	2.0×10^{-7}
MC1R	16	89.8	rs12924124	T	0.07	1.10	0.22	5.1×10^{-7}	0.07	0.66	0.38	0.084	0.99	0.19	1.7×10^{-7}
MC1R	16	89.8	rs35026726	T	0.07	1.10	0.22	5.1×10^{-7}	0.07	0.66	0.38	0.084	0.99	0.19	1.7×10^{-7}
MC1R	16	89.8	rs12931267	G	0.07	1.09	0.22	5.7×10^{-7}	0.07	0.66	0.38	0.084	0.98	0.19	2.0×10^{-7}
MC1R	16	89.8	rs75570604	C	0.07	1.11	0.22	3.5×10^{-7}	0.07	0.68	0.39	0.079	1.01	0.19	1.1×10^{-7}
MC1R	16	89.9	MERGED_DEL_2_86235	D	0.07	1.14	0.22	1.9×10^{-7}	0.07	0.69	0.39	0.077	1.03	0.19	5.8×10^{-8}
MC1R	16	89.9	16:89913406:D	D	0.07	1.15	0.23	3.8×10^{-7}	0.06	0.96	0.46	0.036	1.11	0.20	3.9×10^{-8}
MC1R	16	90.0	Compound	R	0.26	0.93	0.13	2.7×10^{-12}	0.28	0.61	0.30	0.042	0.88	0.12	1.7×10^{-13}
MC1R	16	90.0	rs1805007	T	0.07	1.09	0.22	9.2×10^{-7}	0.07	0.80	0.40	0.046	1.02	0.19	1.3×10^{-7}
MC1R	16	90.1	rs112556696	G	0.06	1.18	0.24	9.5×10^{-7}	0.05	0.55	0.56	0.321	1.08	0.22	9.1×10^{-7}

Abbreviations: β (beta), increase in perceived age per increase in effect allele; CHR, chromosome; Compound, a collapsed compound heterozygosity marker based on a haplotype analysis of four pre-selected *MC1R*-coding DNA variants rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H); EA, effect allele; EAF, effect allele frequency; MBP, mega base pair position of the SNPs according to GRCh37.p13; SE, standard error of the β.

All SNPs with perceived age association P-value $<1\times10^{-6}$ in the Rotterdam Study GWAS are shown.

All analyses were adjusted for age, sex, and wrinkles.

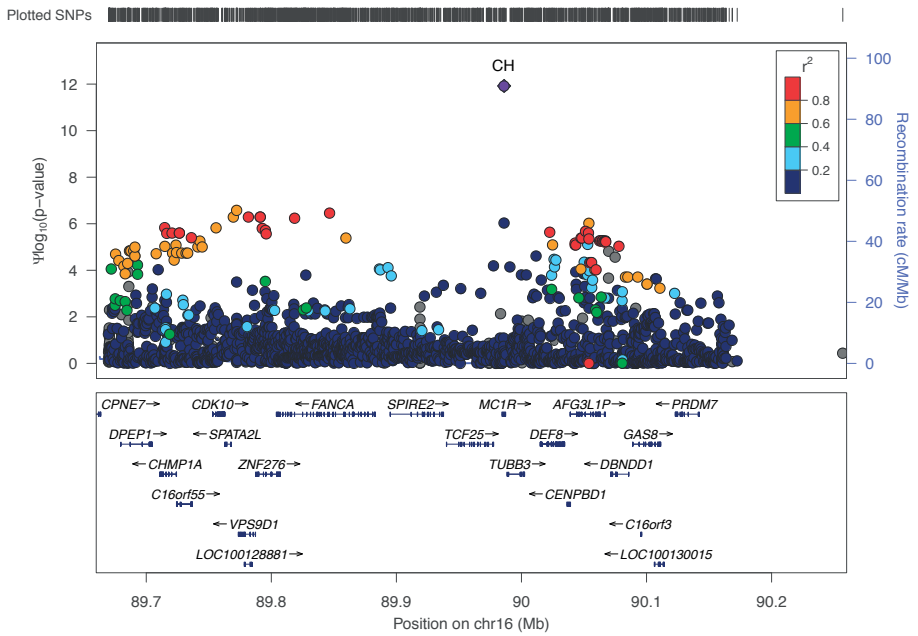

Figure 2. Regional Manhattan plot of the *MC1R* gene locus with perceived facial age in the Rotterdam Study discovery cohort. The physical positions of the SNPs used in the GWAS (using hg19) are plotted against the -$\log_{10}$P-values (left-hand axis) for their association with perceived age after adjustment for age, sex, and wrinkling in the Rotterdam Study (N=2693). The genomic region from 89.66 to 90.26 Mb on chromosome 16 is displayed along the x-axis. The association signal for the *MC1R* compound marker was superimposed onto the plot using the same physical position as rs1805007. Linkage disequilibrium (LD) r^2 values between all SNPs and rs1805007 are scaled by redness, and known genes are aligned below. See Figure S2 for genome-wide Manhattan and Q-Q plots and for the perceived age effect of the *MC1R* compound marker in the Rotterdam Study discovery cohort.

Replication analyses in the Leiden Longevity Study and the TwinsUK Study

To replicate our findings, we used the Leiden Longevity Study[7] with perceived age and wrinkle grading from facial photographs and genetic data of 599 Dutch European subjects (Table S1 and Supplemental Information). This analysis successfully confirmed the perceived age association (also after adjusting for age, sex, and wrinkles) of SNPs within or close to *MC1R* (e.g., rs1805007(T), β=0.80, P-value=0.046) but no other loci (Table 1). One of the *MC1R* variants (chr16:89913406:D) became genome-wide significant (P-value=3.85×10^{-8}) when combining the test statistics from both cohorts using a meta-analysis (Table 1). The *MC1R* compound marker in the Leiden Longevity Study (Table 2) also replicated with nominal significance in this sample (P-value=0.042, Table 1) and demonstrated a genome-wide significant association with perceived age in the combined analysis (P-value=1.68×10^{-13}).

To further confirm that the *MC1R* compound marker association with perceived age in the Rotterdam Study was genuine and the replication in the Leiden Longevity Study was not a false-positive finding, e.g., due to multiple testing, we performed a second replication analysis of the *MC1R* compound marker in 1173 European subjects (99% female) of the TwinsUK Study[8].

Table 2. Frequencies of the *MC1R* compound marker haplotypes and their associated mean perceived facial ages in the discovery cohort (Rotterdam Study), the first replication cohort (Leiden Longevity Study), and the second replication cohort (TwinsUK Study)

	Discovery cohort (N=2693)				First replication cohort (N=599)				Second replication cohort (N=1173)			
MC1R haplotype[a]	N	%	Perceived age[b]	SE	N	%	Perceived age[b]	SE	N	%	Perceived age[b]	SE
WT/WT	1426	52.95	65.29	0.08	317	52.92	62.99	0.01	674	65.76	59.54	0.10
WT/R	1119	41.55	66.16	0.09	240	40.06	63.41	0.01	310	30.24	60.01	0.15
R/R	148	5.5	67.10	0.25	42	7.01	63.99	0.09	41	4.00	61.07	0.43

Abbreviations: SE, standard error of the perceived age estimate in years; R, risk haplotype; WT, wild-type haplotype. [a]The *MC1R* compound marker haplotypes were constructed from four pre-selected *MC1R*-coding DNA variants rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H), except in the second replication cohort TwinsUK Study where only rs1805007and rs1805008 were available (see Supplemental Information); [b]mean perceived age in years after adjusting for age, sex, and wrinkles.

Although the two rarest of the four *MC1R* SNPs (rs1805005 and rs1805009) were unavailable in the TwinsUK dataset used (Table 2; Supplemental Information), the *MC1R* compound marker constructed from the two available and more common SNPs (rs1805007 and rs1805008) demonstrated statistically significant association with perceived age after adjusting for age, sex, and wrinkles (P-value=3.6×10^{-3}). Moreover, the effect size seen in the TwinsUK Study (β=0.60 per risk haplotype) was almost identical to that found in the Leiden Longevity Study (β =0.61).

Testing the genetic effects of additional sub-phenotypes of perceived age

MC1R SNPs have previously been associated with variation in skin color[9,10] and pigmented spots[6]. In a skin color stratified analysis, the *MC1R* compound marker association with perceived age persisted though different skin color groups with weakening effect sizes (β=0.95 in pale, β=0.81 in white, β=0.80 in white-to-olive, Table S4). Furthermore, a candidate gene analysis of eight SNPs from eight pigmentation genes selected from a recent skin color GWAS[10] revealed nominally significant association (P-value<0.05) with perceived age in the Rotterdam Study for SNPs in four genes, i.e., *IRF4*, *RALY/ASIP*, *SLC45A2*, and *TYR*, in addition to the *MC1R* compound marker (Table S5). The significance levels all remained when skin color was additionally adjusted for (Table S5), and *TYR* rs1393350 remained nominally significant (P-value=0.04) after Bonferroni correction.

A multivariable regression analysis of perceived age was performed to test the independent effects of genetic factors and sub-phenotypes on perceived age (Table S6). In this analysis, the *MC1R* compound marker association with perceived age remained genome-wide significant, and *TYR* rs1393350 (P-value=6.8×10^{-3}) and *SLC45A2* rs183671 (P-value=0.02) showed nominally significant association with perceived age (Table S6). Including sunbed usage as a covariate in the multivariable analysis had little impact on the effect of *MC1R* in the model (β remained the same at 0.74, and P-value slightly changed from 2.1×10^{-8} to 2.3×10^{-8}), as also shown in a sunbed-use stratified analysis, where the *MC1R* effect was slightly attenuated in frequent sunbed users (Table

S4). Adjusting for sun-exposure in the Leiden Longevity Study (i.e., mainly, often, or rarely in the sun in the summer) had little effect on the *MC1R* association (β changed from 0.61 from 0.66, and P-value decreased from 0.042 to 0.031), and in the stratified analysis, *MC1R* SNPs also showed an attenuated effect in the high exposure group (Table S4).

DISCUSSION

There have been no studies to date investigating the genetic basis of perceived age, despite its links to health[1] and the evidence of a large additive genetic component to perceived age variation[11]. In the present study, we detected in Dutch Europeans a significant association between DNA variants in the *MC1R* gene and perceived age, after removing the influence of age, sex, and wrinkles, which successfully replicated in two independent European samples from the Netherlands and the UK. The observed *MC1R* perceived age associations were independent of skin color and pigmented spots, indicating other facial features were responsible for the associations. In addition, we found little evidence that sun exposure was the main route through which *MC1R* gene variants were associating with perceived age.

The *MC1R* gene encodes the melanocortin 1 receptor, which is a key regulator of melanogenesis, and controls the ratio of pheomelanin to eumelanin synthesis. A diminished *MC1R* activity, as caused by multiple loss-of-function polymorphisms in *MC1R*, produces the yellow to reddish pheomelanin, which has a weaker UV shielding capacity than that of the brown to black eumelanin[12]. However, multiple studies have shown that loss-of-function *MC1R* variants significantly associate with age spots, actinic keratosis, and various types of skin cancers in a skin-color-independent and/or UV-exposure-independent manner[6,13-18], and in the present study, we showed that *MC1R* variants associated with perceived age after skin color and sun exposure adjustments. These observations are in line with previous findings from functional studies suggesting a pleotropic role for *MC1R* in inflammation[19] and nucleotide excision repair[20], as well as in fibroblasts during wound healing and tissue repair[21], and are consistent with the previously demonstrated UV-independent carcinogenesis mechanism of *MC1R* via oxidative damage[22].

Small-scale GWASs on photoaging[23] and a skin age score[24] have been performed previously; these two studies each identified different genes, and none were *MC1R*. A direct comparison with the present study is difficult, as both previous studies used very different skin aging phenotypes compared to perceived age used here as well as smaller sample sizes (<503 subjects). The *MC1R* association with perceived age we found here and replicated in two independent cohorts, and these DNA variants having been significantly associated with other skin aging-related phenotypes in recent studies (e.g., pigmented spots[6]) also independently of skin color, together provide confidence that our findings are non-spurious. In addition, a previous candidate gene study in 530 middle-aged French women reported associations between variants in *MC1R* and severe facial photoaging[25]. However, a key feature of the photoaging measure was facial wrinkles, whereas

we found that the *MC1R* variants mainly explained the non-wrinkling components of perceived age. Our data therefore highlight that further studies are needed to identify the specific cellular pathway (e.g., DNA repair) and facial feature (e.g., skin sag) responsible for the link between *MC1R* variants and facial aging.

The discovery set of this study uses a relatively small sample size compared to current GWAS standards, which minimized the statistical power to detect genetic effects smaller in size than the observed *MC1R* compound marker effect of almost two years. The GWAS quantile-quantile (QQ) plot (Figure S2B) indeed shows many SNPs with a lower P-value than expected, albeit to only a small degree. This is in line with many SNPs having small effects on perceived age, which is not surprising giving the wide variety of facial features that influence age perception, i.e., it is a very complex phenotype. Much larger sample sizes are now required to reveal additional gene variant effects on perceived age as well as their effects in younger and non-European populations.

Appearance and age prediction from DNA with the aim to find unknown perpetrators, who in principle cannot be identified via conventional DNA profiling, has gained enormous interest in the forensic genetics field over the last few years[26, 27]. Given that the *MC1R* compound marker explained only a small proportion of the perceived age variation, a more complete list of genetic loci involved in perceived age is required to accurately predict perceived age (given chronological age is available or can be reliably estimated from molecular biomarkers thereof), such as in forensic applications. In support of this, we found SNPs in several other skin color genes associated in the expected direction with perceived age in a multivariable model.

Finally, as *MC1R* correlates with advanced facial aging, it provides clues to mechanisms of biological aging beyond cosmetic and forensic interests. Indeed, it is notable that the 2-year effect of the *MC1R* DNA variants on perceived age observed here is similar to the effect of smoking reported previously in the Leiden Longevity Study[28], indicating that *MC1R* variants can have a considerable impact on facial appearance over many years.

In conclusion, this study is the first to identify genetic variants significantly associated with perceived age. We provide evidence that, of eight million tested, DNA variants in the *MC1R* gene had the strongest association with perceived age in subjects of European ancestry, and a *MC1R* compound marker was genome-wide significant independently of age, sex, skin color, sun-exposure, wrinkles, and pigmented spots. Follow-up work on how the MC1R protein is affecting facial aging, for example, through non-UV pro-oxidant pheomelanin effects[22] or fibroblast function[21], is now required. Moreover, as this study demonstrates that a GWAS of perceived facial age is indeed feasible, future studies using large consortia GWASs should be performed to identify additional genetic loci that associate with perceived facial age. Expectedly, this will provide further insights into the biological pathways that underlie variation in facial aging and eventually on the utility of genotype-based prediction of perceived age alongside chronological age estimation from molecular biomarkers.

EXPERIMENTAL PROCEDURES

Each study was approved by the research ethics committees of the contributing institutions, and all participants provided written informed consent.

Rotterdam Study

The Rotterdam Study is a prospective cohort study ongoing since 1990 in the city of Rotterdam in The Netherlands[3]. Perceived age, i.e., how old the subjects looked, was assessed from front and side facial images from the 3dMD system by on average 27 assessors per image (totaling ~73,000 assessments) using a previously used[28] and validated method ([29] and Supplemental Information). Pigmented spots and wrinkles were measured quantitatively from the frontal images using image analysis algorithms (Matlab 2013b) as previously described and validated ([30] and Supplemental Information). Sunbed use (i.e., never, <10 times, 10-50 times, >50 times) was assessed through questionnaires. Skin color was graded as pale, white, or white-to-olive skinned based on a full body examination whilst subjects were in a state of undress[31]. To merge photographs together for comparisons of facial appearance, facial images were combined together as previously detailed[11,32] using face shape, color, and texture information. Genotyping, imputation, and quality control procedures are described in detail elsewhere ([3] and Supplemental Information).

Leiden Longevity Study

The Leiden Longevity Study has been described in detail elsewhere[7,33,34]. Perceived age was assessed from front and side facial images by on average 60 assessors (totaling ~36,000 assessments) and wrinkles graded into nine photonumeric grades, both as previously reported[35]. Summer sun exposure (mainly in the sun, often in the sun, and rarely in the sun) was captured through questionnaires[28]. Genotyping was performed using Illumina Human660W-Quad and OmniExpress BeadChips as described elsewhere[34]. Association testing was conducted using QTassoc[36].

TwinsUK Study

The UK Adult Twin Registry (TwinsUK Study) is described elsewhere[8]. Perceived age was graded from 3dMD photos by four assessors per image, and wrinkles were graded according to the above-described photonumeric grading by five assessors (Supplemental Information). *MC1R* SNP data from TwinsUK were ascertained from the imputed genome-wide SNP dataset described elsewhere[8].

Statistical analyses

Genetic association was tested per SNP in the GWAS using a linear model assuming an additive allele effect, always including sex, chronological age, and the top four genetic principal components as covariates using PLINK[37]. Wrinkles, skin color, sunbed use, and summer sun exposure were adjusted for where appropriate. The *MC1R* compound marker analysis in each of the three co-

horts is detailed in Supplemental Information. We conducted a stepwise multivariable regression analysis to investigate the independent effects of all phenotypes and factors as performed using R version 3.2.0 (http://www.r-project.org/); see Supplemental Information for further details.

ACKNOWLEDGMENTS

We thank two anonymous reviewers for their comments, which helped improving the manuscript. Access to TwinsUK facial images and genotype data was kindly provided by the Department of Twin Research and Genetic Epidemiology at King's College London, which the authors highly appreciate. The authors are grateful to the study participants and staff from the Rotterdam Study, the Leiden Longevity Study, and the TwinsUK Study. We thank Sophie Flohil, Emmilia Dowlatshahi, Robert van der Leest, Joris Verkouteren, Ella van der Voort, and Shmaila Talib for help in phenotype collection in the Rotterdam Study. Additionally, we thank Sophie van den Berg for masking and reviewing the Rotterdam Study photographs. We would like to thank Professor Christopher Griffiths, Dr Tamara W. Griffiths, Sharon Catt and Dr Stephanie Ogden for the wrinkle grading; Cyrena Tomlin and Corrie Groenendijk for their work generating the perceived ages; and Professor David Perrett for the use of Pyschomorph for facial averaging.

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Chapter 8

No causal association between 25-hydroxyvitamin D and features of skin aging: evidence from a bidirectional Mendelian randomization study

M.A. Hamer*
R. Noordam*
L.M. Pardo Cortes
T. van der Nat
J.C. Kiefte-de Jong
M. Kayser
P.E. Slagboom
A. Uitterlinden
M.C. Zillikens
M. Beekman
T. Nijsten
D. van Heemst
D.A. Gunn

**Shared first authorship*

J Invest Dermatol. 2017 Nov;137(11):2291-2297

ABSTRACT

Data from in-vitro experiments suggest that vitamin D reduces the rate of skin aging, whereas population studies suggest the opposite, most likely due to confounding by UV-exposure. We investigated whether there are causal associations between 25-hydroxyvitamin D concentrations and features of skin aging in a bidirectional Mendelian randomization study. In the Rotterdam Study (N=3831; 58.2% women, median age 66.5 years) and Leiden Longevity Study (N=661; 50.5% women, median age 63.1 years), facial skin aging features (perceived age, wrinkling, pigmented spots) were assessed either manually or digitally. Associations between 25-hydroxyvitamin D and skin aging features were tested by multivariable linear regression. Mendelian randomization analyses were performed using single-nucleotide polymorphisms identified from previous genome-wide association studies. After meta-analysis of the two cohorts, we observed that higher serum 25-hydroxyvitamin D was associated with a higher perceived age (P-value=3.6×10^{-7}), more skin wrinkling (P-value=2.6×10^{-16}), but not with more pigmented spots (P-value=0.30). In contrast, a genetically determined 25-hydroxyvitamin D concentration was not associated with any skin aging feature (P-values>0.05). Furthermore, a genetically determined higher degree of pigmented spots was not associated with higher 25-hydroxyvitamin D (P-values>0.05). Our study did not indicate that associations between 25-hydroxyvitamin D and features of skin aging are causal.

INTRODUCTION

A higher perceived age – estimated age based on facial appearance – is associated with an increased risk of morbidity and mortality[1], making it a useful marker in aging research. In addition to well-described extrinsic factors, such as smoking and UV-exposure[1-3], a higher perceived age also has an intrinsic component[4-6]. It has previously been shown that high serum concentrations of glucose and cortisol were associated with a higher perceived age[7,8], whereas a high concentration of insulin-like growth factor-1 (IGF-1) was associated with a lower perceived age mainly through skin wrinkling[6,9]. Besides skin wrinkling, facial pigmented spots are also an important component of skin aging.

Although sun-exposure contributes to premature skin aging[1,2], it is essential for vitamin D synthesis in the skin[10] and vitamin D is essential for musculoskeletal health. Moreover, in clinical practice, low serum concentrations of 25-hydroxyvitamin D, or vitamin D deficiency, is a broadly accepted marker for general health status, and has been associated with multiple extraskeletal age-related diseases (e.g., type 2 diabetes mellitus and cardiovascular disease), and mortality[11-16].

Different in-vitro studies have shown that physiological concentrations of 1,25-hydroxyvitamin D, the active vitamin D metabolite, protect the skin against factors that promote skin aging, including cellular damage induced by UVB irradiation. Vitamin D has been demonstrated to influence keratinocyte proliferation[17] and differentiation[18] with the response dependent on vitamin D concentrations and culture conditions[17,19]. Although the bioavailable levels of vitamin D in human skin are unknown, a higher serum concentration of 25-hydroxyvitamin D was associated with a higher number of facial pigmented spots in the Leiden Longevity Study[20]. However, the nature of these studies is observational, and causality cannot be ascertained due to influences of, for example, residual confounding by sunlight.

Causality can be inferred between a certain exposure and outcome using Mendelian randomization studies[21,22]. With such analyses, genetic polymorphisms that are strongly related to the exposure are investigated in relation to the outcome, in the absence of confounding. Therefore, we aimed to investigate whether associations between serum 25-hydroxyvitamin D and features of skin aging are causal using a bidirectional Mendelian randomization study.

RESULTS

Characteristics of the study populations

A maximum of 3831 participants from the Rotterdam Study (median [IQR] age: 66.5 [61.0 to 71.5] years) and 661 participants from the Leiden Longevity Study (median [IQR] age: 63.1 [58.9 to 67.5] years) were included in the present study (Table 1). Compared with participants from the Leiden Longevity Study, participants from the Rotterdam Study were more frequently women (58.2% versus 50.4%), smokers (18.5% versus 13.8%), and had a lower 25-hydroxyvitamin D concentra-

tion (median: 61.0 nmol/l vs. 68.3 nmol/l). In addition, in line with the higher mean chronological age, participants from the Rotterdam Study had a higher mean perceived age (mean: 65.9 years vs. 59.4 years).

Table 1. Characteristics of the study populations

	Rotterdam Study (N=3831)	Leiden Longevity Study (N=661)
General		
Chronological age in years, median [IQR]	66.5 [61.0 – 71.5]	63.1 [58.9 – 67.5]
Females, N (%)	2229 (58.2)	334 (50.4)
Body mass index in kg/m^2, mean (SD)	27.6 (4.4)	26.6 (4.0)
Current smoking, N (%)	707 (18.5)	91 (13.8)
Skin aging features		
Perceived age in years, mean (SD)	65.9 (7.6)[a]	59.4 (7.6)
Degree of skin wrinkling, median [IQR][b]	3.9 [2.5 – 6.0]	4.5 [3.5 – 5.5]
Degree of pigmented spots, median [IQR][b]	1.3 [0.9 – 2.1][c]	4.5 [3.5 – 5.0]
Serum measurements		
25-hydroxyvitamin D in nmol/l, median [IQR]	61.0 [42.7 – 82.3]	68.3 [54.0 – 139.2]
Serum taken in winter season, N (%)	930 (24.3)	160 (24.2)
Serum taken in spring season, N (%)	1107 (28.9)	214 (32.4)
Serum taken in summer season, N (%)	863 (22.5)	135 (20.4)
Serum taken in autumn season, N (%)	850 (22.2)	152 (23.0)

Abbreviations: IQR, interquartile range; N, number of participants; SD, standard deviation.
[a]assessed in 2679 individuals; [b]for the Rotterdam Study, measured digitally as area (wrinkles or pigmented spots) as a percentage of the total facial area. For the Leiden Longevity Study, wrinkle score and pigmented spots were measured manually by 2 expert dermatologists using a photonumeric scale ranging from 1 to 9; [c]assessed in 2843 individuals.

Observational associations between 25-hydroxyvitamin D concentration and skin aging features

After meta-analyzing the results of the Rotterdam Study and the Leiden Longevity Study (Table 2), a higher 25-hydroxyvitamin D concentration was associated with a higher perceived age (β=0.149 SD per 1 ln[25-hydroxyvitamin D]; SE=0.029; P-value=3.58×10^{-7}). However, this association disappeared after additional adjustment for the degree of skin wrinkling (β=0.020 SD per 1 ln[25-hydroxyvitamin D]; SE=0.022; P-value=0.36). In line with this, a higher 25-hydroxyvitamin D concentration was associated with a higher degree of skin wrinkling (β=0.250 SD per 1 ln[25-hydroxyvitamin D]; SE=0.030; P-value 2.61×10^{-16}). In contrast, a higher 25-hydroxyvitamin D was only associated with a higher degree of pigmented spots in the Leiden Longevity Study, and not in the Rotterdam Study. After meta-analysis, a higher 25-hydroxyvitamin D was not associated with a higher degree of pigmented spots (β=-0.033 SD per 1 ln[25-hydroxyvitamin D]; SE=0.031;

P-value=0.30). These results were similar when we additionally adjusted for UV-exposure, physical activity, and dietary vitamin D and any vitamin D supplementation in the Rotterdam Study (Supplementary Table S1), despite that we observed strong associations between these factors and 25-hydroxyvitamin D level (Supplementary Table S2).

Table 2. Association between serum 25-hydroxyvitamin D and features of skin aging

	Rotterdam Study (N=3831)		Leiden Longevity Study (N=661)		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
Perceived age	0.128 (0.030)[b]	1.8×10^{-5}	0.516 (0.127)	5.4×10^{-5}	0.149 (0.029)	3.6×10^{-7}
Perceived age, adjusted[a]	0.006 (0.023)[b]	7.8×10^{-1}	0.225 (0.087)	9.8×10^{-3}	0.020 (0.022)	3.6×10^{-1}
Degree of skin wrinkling	0.241 (0.031)	2.2×10^{-14}	0.507 (0.169)	2.7×10^{-3}	0.250 (0.030)	2.6×10^{-16}
Degree of pigmented spots	-0.050 (0.032)[c]	1.2×10^{-1}	0.571 (0.189)	2.6×10^{-3}	-0.033 (0.031)	3.0×10^{-1}

Abbreviation: SE, standard error.

Effect estimates presented as the increase in standardized outcome per 1 ln-transformed unit increase in 25-hydroxyvitamin D serum concentration. Analyses adjusted for chronological age, sex, season, current smoking status and body mass index. The results of the digitally measured wrinkles and pigmented spots in the Rotterdam Study were additionally adjusted for technical variables.

[a]analyses additionally adjusted for the degree of facial skin wrinkling. Effect estimates of the meta-analysis obtained using fixed-effect models; [b]analysis based on 2679 individuals from the Rotterdam Study; [c]analysis based on 2843 individuals from the Rotterdam Study.

Mendelian randomization analyses between 25-hydroxyvitamin D concentration and skin aging features

We calculated, per participant, a weighted genetic score for 25-hydroxyvitamin D concentration based on the SNPs that were identified in a genome-wide association study (GWAS) on 25-hydroxyvitamin D concentration (notably, rs2282679 [*GC*], rs3829251 [*NADSYN1*], and rs2060793 [*CYP2R1*][23]). Based on the observational effect estimates, we had an 82% and 84% power to detect significant (α=0.05) associations between the 25-hydroxyvitamin D genetic risk score (GRS) and perceived age and degree of skin wrinkling, respectively.

After meta-analysis, all three selected 25-hydroxyvitamin D genotypes were associated with 25-hydroxyvitamin D concentration (Supplementary Table S3). In line with this, the calculated weighted genetic score for higher 25-hydroxyvitamin D concentration was associated with a higher 25-hydroxyvitamin D concentration in our study populations and meta-analysis (β=0.24 units ln[25-hydroxyvitamin D] increase per 1 unit increase in genetic score; SE=0.01; P-value=2.23×10^{-64}).

After meta-analyzing the observed estimates of the Rotterdam Study and the Leiden Longevity Study (Table 3), a higher genetically determined 25-hydroxyvitamin D concentration was not associated with (i) a higher perceived age (β=0.030 SD per 1 genetically-determined ln[25-hydroxyvitamin D]; SE=0.023; P-value=0.18); (ii) a higher perceived age additionally adjusted for skin wrinkling (β=0.017 SD per 1 genetically determined ln[25-hydroxyvitamin D]; SE=0.016; P-

value=0.28); (iii) a higher degree of skin wrinkling (β=0.000 SD per 1 genetically determined ln[25-hydroxyvitamin D]; SE=0.028; P-value=1.00); (iv) a higher degree of pigmented spots (β=0.055 SD per 1 genetically-determined ln[25-hydroxyvitamin D]; SE=0.030; P-value=0.07).

Table 3. Association between 25-hydroxyvitamin D genetic risk score and skin aging features

	Rotterdam Study (N=3831)		Leiden Longevity Study (N=661)		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
Perceived age	-0.017 (0.045)[b]	0.69	0.046 (0.026)	0.08	0.030 (0.023)	0.18
Perceived age, adjusted[a]	-0.003 (0.034)[b]	0.93	0.023 (0.018)	0.19	0.017 (0.016)	0.28
Degree of skin wrinkling	-0.064 (0.048)	0.18	0.034 (0.035)	0.32	0.000 (0.028)	1.00
Degree of pigmented spots	0.004 (0.051)[c]	0.93	0.084 (0.038)	0.03	0.055 (0.030)	0.07

Abbreviation: SE, standard error.
Effect estimates presented as the increase in the standardized outcomes per 1 unit increase in the genetic risk score. Analyses adjusted for age and sex. Effect estimates of the meta-analysis obtained using fixed-effect models.
[a]analyses additionally adjusted for the degree of facial skin wrinkling; [b]analysis based on 2679 individuals from the Rotterdam Study; [c]analysis based on 2843 individuals from the Rotterdam Study.

Mendelian randomization analyses between pigmented spots and 25-hydroxyvitamin D concentration

We found no evidence after meta-analyzing the results of the Rotterdam Study and Leiden Longevity Study that any of the genotypes for pigmented spots or perceived age (*MC1R* gene only) or the genetic risk score for pigmented spots was associated with a higher 25-hydroxyvitamin D concentration (Table 4; e.g., β=0.146 ln[25-hydroxyvitamin D in nmol/l] per 1 unit increase in pigmented spots GRS; SE=0.089; P-value=0.10).

Table 4. Mendelian randomization analyses for pigmented spots and 25-hydroxyvitamin D concentration

	Rotterdam Study (N=2843)		Leiden Longevity Study (N=661)		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
IRF4 gene, rs12203592	0.017 (0.014)	0.20	0.056 (0.023)	0.01	0.028 (0.012)	0.02
MC1R gene, rs35063026	-0.007 (0.017)	0.67	-0.014 (0.023)	0.54	-0.009 (0.014)	0.49
ASIP gene, rs6059655	0.000 (0.015)	1.00	0.020 (0.020)	0.30	0.007 (0.012)	0.55
Pigmented spots GRS	0.079 (0.106)	0.45	0.306 (0.163)	0.06	0.146 (0.089)	0.10

Abbreviations: *ASIP*, agouti signaling protein; GRS, genetic risk score; *IRF4*, interferon regulatory factor 4; *MC1R*, melanocortin 1 receptor; SE, standard error.
Effect estimates presented as the increase in the standardized outcomes per one unit increase in the genetic risk score. Analyses adjusted for age and sex. Effect estimates of the meta-analysis obtained using fixed-effect models.

DISCUSSION

We found evidence that a higher serum 25-hydroxyvitamin D concentration was associated with a higher perceived age and a higher degree of skin wrinkling. However, we found no evidence that a higher genetically determined 25-hydroxyvitamin D was associated with any of the studied skin aging features, nor was there evidence that a higher genetically determined degree of pigmented spots was associated with a higher 25-hydroxyvitamin D concentration. These results suggest that the association between 25-hydroxyvitamin D and skin aging features is not likely causal.

In several observational studies, a low 25-hydroxyvitamin D[11-15] and a higher perceived age[1] are associated with an increased risk of morbidity and mortality[16]. Therefore, low 25-hydroxyvitamin D might associate with a higher perceived age. However, participants with high 25-hydroxyvitamin D concentrations likely have a higher frequency of outdoor activities (e.g., physical activity, sun bathing), better dietary quality, and lower fat mass[24]. As UVB exposure by sunlight is a predominant factor of 25-hydroxyvitamin D[25] production and contributes to skin aging, a higher 25-hydroxyvitamin D concentration might be associated with a higher perceived age. Indeed, a higher 25-hydroxyvitamin D concentration was associated with a higher perceived age in our study populations. However, the attenuation of this association by the adjustment for skin wrinkling suggests that 25-hydroxyvitamin D only associates with certain aspects of skin aging. In addition, although the Leiden Longevity Study described an association between high 25-hydroxyvitamin D and the degree of pigmented spots in an earlier publication[20], this association was not observed in the Rotterdam Study. There is no clear reason for this difference, as different methodologies (image analysis vs. photonumeric grading) show large similarities[26].

We did not find evidence of an association between higher genetically determined 25-hydroxyvitamin D levels and features of facial skin aging. Our findings suggest that the observations in the previously published in-vitro experiments[17-19] might not have in-vivo relevance. This could be because most in-vitro studies demonstrate beneficial effects of the most potent vitamin D metabolite (1,25-hydroxyvitamin D) at very high physiological levels ($\geq$100nmol/l) compared with no vitamin D[17-19]. In contrast, most participants in the present study had a 25-hydroxyvitamin D concentration between 40 and 140 nmol/l; hence, the biological effects in this range will likely be lower. However, bioavailable levels of 25-hydroxyvitamin D and 1,25-hydroxyvitamin D in skin need to be ascertained to determine the relevance of the in-vitro studies to in-vivo conditions.

There was no significant association between the genetic score for pigmented spots and vitamin D levels. However, there was a borderline significant association between a SNP in the *IRF4* gene and 25-hydroxyvitamin D concentration, replicating a similar finding in a different cohort[27]. This finding warrants follow-up particularly because many of the pigmented spot genes are also linked to melanin levels in skin, which protects skin from UVB radiation effects, the key determinant of vitamin D production in skin.

The observational associations between 25-hydroxyvitamin D concentration and a higher perceived age and a higher degree of skin wrinkling could be the result of residual confounding

or reverse causality. However, SNPs in the *MC1R* gene associate with a higher perceived age, but were unrelated to 25-hydroxyvitamin D concentration in our study population. This suggests that reverse causality is not at play here. We believe that the most likely explanation for the association between 25-hydroxyvitamin D concentration and features of skin aging is residual confounding, probably due to UVB radiation exposure.

The present study has a number of limitations. First, the assessment of the degree of skin wrinkling and pigmented spots was different in the Rotterdam Study and the Leiden Longevity Study, which might have caused increased disparity in the data. The differences in perceived age between the two cohorts (despite having a similar chronological age) might originate from slight methodological differences as well as differences in lifestyle factors and medical history. However, we used the data on comparable scales (Z-scores) and there is large agreement between digital and manual assessment of skin wrinkling and pigmented spots[26]. The present study populations only comprised individuals from European ancestry, and our study findings might therefore not necessarily be generalizable to populations of different ancestry backgrounds. In addition, regarding the observational associations found between 25-hydroxyvitamin D, the available UV variables used might not have captured cumulative sun exposure accurately. However, this would not affect the Mendelian randomization analyses. Furthermore, although we have validated the GRS for 25-hydrxoyvitamin D against 25-hydroxyvitamin D levels in our study populations, we cannot completely rule out that the lack of evidence for an association between the GRS and features of skin aging is the result of a lack of power for the GRS to detect 25-hydroxyvitamin D effects in skin. Lastly, the facial photographs of the Rotterdam Study and the Leiden Longevity Study were taken at a later moment in time than the blood drawing for 25-hydroxyvitamin D assessment, which could have weakened any observational links.

In summary, we did not find evidence that the previously described beneficial in-vitro effects of vitamin D on cellular processes are detectable at a population level. The observational associations in our study between 25-hydroxyvitamin D and features of skin aging are, most likely, predominately due to residual confounding.

MATERIALS AND METHODS

Study setting

The present study was conducted using data from the population-based Rotterdam Study and the Leiden Longevity Study. The Rotterdam Study is an ongoing prospective population-based cohort study following 14,926 inhabitants aged ≥45 years in Ommoord, a suburb of Rotterdam in the Netherlands since 1990. Participants were examined at baseline at the study center and invited every 4-5 years for follow-up visits at the study center. Details of the study design and objectives have been described elsewhere[28]. The Leiden Longevity Study recruited a total of 421 families containing long-lived Caucasian siblings[29]. Families were only included when at least two long-

lived siblings were still alive and met the age criteria upon study inclusion (≥ 89 years for men; ≥ 91 years for women). Here, the study was conducted in the offspring of the long-lived individuals with the partners of the offspring as controls. A more detailed description of the recruitment strategy of the study participants has been published elsewhere[30].

Both studies were approved by local Medical Ethics Committees and all included participants provided written informed consent.

Serum measurements

In the Rotterdam Study, fasted blood samples were collected between 1997 and 1999, 2000 and 2001, and 2006 and 2009, for each participant only once. Serum 25-hydroxyvitamin D concentrations were measured using electrochemiluminescence immunoassay (COBAS; Roche Diagnostics, Mannheim, Germany).

In the Leiden Longevity Study, nonfasted blood samples were collected between 2002 and 2006. Plasma 25-hydroxyvitamin D levels were measured with monoclonal antibodies using a standardized protocol with electrochemiluminescence immunoassays on a fully automated Cobas e411 analyzer (Roche Diagnostics, Almere, the Netherlands). As part of the standard protocol, standardization was performed to make the measures comparable to assays using polyclonal antibodies.

Skin aging features

In the Rotterdam Study, standardized high-resolution digital three-dimensional (3D) facial photographs (Premier 3dMDface3-plus UHD, Atlanta, GA, USA) are being collected since 2010. Enface and side 2D photographs were exported from the 3D images. The current study included 3831 participants of northwestern European ancestry, who have been photographed and examined at the research center from September 2010 until June 2014. Perceived age was assessed from front and side facial exported 2D images by on average 27 assessors per image using a previously established[31] and validated[32] method. Pigmented spots and wrinkles were measured quantitatively from frontal 2D images using image analysis algorithms (Matlab 2013b) as previously described and validated[26]. Visual inspection of the image analyses measurements[33] highlighted that the measurement mainly detected solar lentigines and very few nevi. Individuals with freckles (N=23), facial contusion (N=1), facial scars with hyperpigmentation (N=1), and postinflammatory hyperpigmentation (N=1) were excluded.

In the Leiden Longevity Study, the method to determine a person's perceived age has been described and validated previously[1,5,32]. From all participants, without make-up or hairstyling product, we took one facial photograph from the front and one at 45˚. Photographs, with hair and clothing concealed, were assessed to determine the average perceived age by 60 independent assessors. Skin wrinkling grade was determined on a nine-point scale by visual assessment of front-on, whole-face photographs[2]. Pigmented spots were graded by visual assessment of light, patchy, mottled hyperpigmentation, actinic lentigines, seborrheic keratosis, and solar freckling[5];

nevi were excluded from the grading. The grade on a nine-point photographic scale was determined using quantitative and qualitative criteria such as the area/density of pigmentation, color intensity, and uniformity of distribution[5,34].

Covariables

Chronological age was determined on the day the facial photographs were taken. Weight and height were determined by research nurses at the study center. Body mass index was calculated by dividing weight (in kilograms) by the squared height (in meters). Smoking status was determined using a home questionnaire. Season was determined at the moment blood was drawn for measuring 25-hydroxyvitamin D. For the digital measurements in the Rotterdam Study, analyses were additionally adjusted for two technical variables. For both wrinkles and pigmented spots, flashlight variance was taken into account: the within-person difference between skin lightness in the images and that assessed by a spectrophotometer (CM-600d; Konica-Minolta, Osaka, Japan) on the cheek. In addition, for wrinkles, a difference in resolution between two sets of the images was taken into account using a variable described as batch[26]. In a random subpopulation of the Rotterdam Study, six variables were available as proxy for UV-exposure based on interview data: tendency to develop sunburn (low vs. high), history of working or being outdoors ≥ 4 hours daily during at least 25 years (yes vs. no), having wintered in a sunny country between September and May for at least one month during the past 5 years (yes vs. no), having lived in a sunny country for more than 1 year (yes vs. no), sun protective behavior (i.e. wearing sunglasses and/or a brimmed hat in the sun categorized into never/almost never vs. often/almost always/always), and frequency of tanning bed visits including facial solarium (fewer vs. more than 10 times in the past 5 years). Vitamin D from dietary intake (measured in ug/day) was calculated using data collected by a food-frequency questionnaire (FFQ)[35]. The Dutch Food Composition Table of 2006 and 2011[36] was used to transform the data into daily macronutrient intake and total energy intake (kcal/day). Physical activity (measured in METhours/week) was assessed using the LASA Physical Activity Questionnaire (LAPAQ)[37]. Participants were categorized as vitamin D supplement users if they used vitamin D or multivitamin supplements at least once a week.

Genotyping

For the Rotterdam Study, DNA from whole blood was extracted and genotyped following standard protocols[28]. In brief, genotyping was carried out using the Infinium II HumanHap 550K Genotyping BeadChip version 3 (Illumina, San Diego, CA, USA) for the largest part of the cohort and Illumina Human 610 Quad Arrays for the rest of the cohort. Genome-wide genotype data was imputed using 1000-Genomes (GIANT Phase I version 3) as the reference panel[38], using a two-step procedure imputation algorithm implemented in the program MACH-Minimac with default parameters[39]. For the Leiden Longevity Study, genotyping was conducted with the Illumina Human 660W-Quad and OmniExpress BeadChips (Illumina, San Diego, CA, USA). Individuals were excluded from

further investigation if they had a mismatch in sex or familial relatedness based on genotype and phenotype.

We extracted three genetic variants as instrumental variables for 25-hydroxyvitamin D: rs2282679 (*GC*), rs3829251 (*NADSYN1*), and rs2060793 (*CYP2R1*)[23], and extracted three genetic variants as instrumental variables for pigmented spots: rs12203592 (*IRF4*), rs35063026 (*MC1R*), rs6059655 (*ASIP*)[33]. The *MC1R* gene has also been associated with perceived age[40].

Based on the effect sizes observed in the genome-wide association studies, we calculated a weighted GRS for the abovementioned determinants. GRS for 25-hydroxyvitamin D: rs2282679-C*0.38 + rs3829251-C*0.18 + rs2060793-A*0.25 [23]. GRS for facial pigmented spots: rs12203592-T*0.097 + rs35063026-T*0.080 + rs6059655-A*0.059 [33].

Statistical analyses

Characteristics of the study populations are presented as means (standard deviations) for normally distributed determinants, medians (interquartile range) for non-normally distributed determinants and frequencies (percentages) for categorical determinants, separately for the Rotterdam Study and Leiden Longevity Study.

As methodologies for determining the skin aging features differed between the Rotterdam Study and the Leiden Longevity, study outcomes were standardized to obtain a standard normal distribution. Analyses were done separately for the two cohorts, and subsequently meta-analyzed using fixed-effect meta-analysis, as part of the rmeta package in R (http://www.R-project.org). For the analyses in the Rotterdam Study, multiple imputation was performed, using the Multiple Imputation by Chained Equations (MICE) package in R, with an iteration of 20 (maximum missing data per variable was 6%). We used linear regression analyses, adjusted for age, sex, BMI, current smoking, and season to obtain the observational effect estimates for the association between 25-hydroxyvitamin D concentrations and the skin aging features. On the basis of the observational effect estimates in our total study population and considering an α of 0.05, we calculated the statistical power for the Mendelian randomization analysis on the skin aging features using a publically available power calculator (http:/cnsgenomics.com/shiny/mRnd/). Associations between 25-hydroxyvitamin D genotypes and the GRS for 25-hydroxyvitamin D were adjusted for age and sex. We also performed the Mendelian randomization analyses for the genetic instruments for pigmented spots and perceived age (*MC1R* only) with linear regression analyses, adjusted for age and sex.

All analyses for wrinkles and pigmented spots in the Rotterdam Study were additionally adjusted for the two technical variables mentioned above. Two-sided P-values below 0.05 were considered statistically significant.

ACKNOWLEDGMENTS

Rotterdam Study: The generation and management of GWAS genotype data for the Rotterdam Study (RS I, RS II, RS III) was executed by the Human Genotyping Facility of the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands. We thank Pascal Arp, Mila Jhamai, Marijn Verkerk, Lizbeth Herrera and Marjolein Peters, MSc, and Carolina Medina-Gomez, MSc, for their help in creating the GWAS database, and Karol Estrada, PhD, Yurii Aulchenko, PhD, and Carolina Medina-Gomez, MSc, for the creation and analysis of imputed data. The authors are grateful to the study participants, the staff from the Rotterdam Study and the participating general practitioners and pharmacists. We thank Emmilia Dowlatshahi, Sophie Flohil, Leonie Jacobs, Robert van der Leest, Simone van der Velden, Joris Verkouteren and Ella van der Voort for collecting the phenotypes. Additionally, we thank Andreas Wollstein for converting all photographs and Sophie van den Berg for masking and reviewing them. We acknowledge Jaspal Lall for masking the photographs and creating the digital wrinkle measurements.

Leiden Longevity Study: We would like to thank all participants, the secretary staff, Meriam H.G.F. van der Star and Ellen H.M. Bemer-Oorschot for their contribution to this study. We would also like to thank Christopher Griffiths and Tamara Griffiths for the pigmented spots and wrinkle score measurements.

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SUPPLEMENTARY TABLES

Supplementary Table S1. Sensitivity analyses in the Rotterdam Study, using only complete cases for all variables

	Model 1		Model 2	
	β (SE)	P-value	β (SE)	P-value
Perceived age (N = 1153)	0.15 (0.04)	5.8×10^{-4}	0.15 (0.05)	9.6×10^{-4}
Perceived age, adjusted (N = 1153)[a]	0.01 (0.03)	0.75	0.02 (0.04)	0.49
Degree of skin wrinkling (N = 1487)	0.29 (0.05)	1.6×10^{-8}	0.24 (0.05)	3.8×10^{-6}
Degree of pigmented spots (N = 1003)	-0.08 (0.06)	0.18	-0.03 (0.06)	0.63

Abbreviation: SE, standard error.
Effect estimates presented as the increase in standardized outcome per 1 ln-transformed unit increase in 25-hydroxyvitamin D serum concentration.
Model 1: Analyses adjusted for age and sex, smoking status, season, body mass index and technical variables.
Model 2: Analyses additionally adjusted for UV variables, physical activity, energy intake, vitamin D derived from diet and from supplement use.
[a]adjusted for wrinkles.

Supplementary Table S2. Association between UV- and vitamin D-related covariables and 25-hydroxyvitamin D in the Rotterdam Study, using only complete cases for all variables

		Rotterdam Study (N=1487)	
		β (SE)	P-value
UV-related variables	Tendency to develop sunburn[a]	-0.09 (0.02)	7.4×10^{-5}
	Lived in a sunny country[b]	-0.07 (0.05)	0.20
	Sun-protective behavior[c]	0.03 (0.02)	0.25
	Spending winter in a sunny country[d]	0.15 (0.05)	3.6×10^{-3}
	Outdoor work history[e]	4.8×10^{-3} (0.03)	0.86
	Tanning bed use[f]	0.24 (0.03)	7.3×10^{-14}
	Physical activity[g]	1.3×10^{-3} (2.4×10^{-4})	2.3×10^{-7}
Vitamin D-related variables	Vitamin D from diet[h]	0.02 (5.9×10^{-3})	3.4×10^{-3}
	Vitamin D from supplement use[i]	0.06 (0.02)	7.9×10^{-3}

Abbreviation: SE, standard error.
Effect estimates presented as the increase in ln-transformed 25-hydroxyvitamin D serum concentration, e.g. effect estimate of -0.09 meaning the ln-transformed 25-hydroxyvitamin D serum concentration is decreased by 0.09 when tendency to develop sunburn is high. Analyses additionally adjusted for age, sex, smoking status, season, body mass index and energy intake.
[a]tendency to develop sunburn (high vs. low); [b]history of living in a sunny country >1 year (yes vs. no); [c]wearing sunglasses and/or a brimmed hat in the sunshine (often/always vs. never/almost never); [d]having wintered in a sunny country between September and May for at least one month during the past 5 years (yes vs. no); [e]worked or been outdoors ≥4 hours daily during at least 25 years (yes vs. no); [f]frequency of tanning bed visits in the past 5 years, including facial solarium (more than 10x vs. never or less than 10x); [g]physical activity measured in Metabolic Equivalent of Task (MET)hours/week; [h]vitamin D from dietary intake (ug/day); [i]vitamin D or multivitamin supplement use at least once a week (yes vs. no).

Supplementary Table S3. Association between genetic variants for 25-hydroxyvitamin D and 25-hydroxyvitamin D

	Rotterdam Study		Leiden Longevity Study		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
GC gene, rs2282679	0.135 (0.008)	8.2×10^{-57}	0.073 (0.013)	2.8×10^{-8}	0.118 (0.007)	4.0×10^{-67}
NADSYN1 gene, rs3829251	0.037 (0.010)	3.4×10^{-4}	0.015 (0.016)	0.33	0.031 (0.008)	1.4×10^{-4}
CYP2R1, rs2060793	0.039 (0.008)	6.7×10^{-7}	0.025 (0.011)	2.7×10^{-2}	0.034 (0.006)	1.3×10^{-7}
25-hydroxyvitamin D GRS	0.279 (0.017)	1.1×10^{-58}	0.152 (0.026)	5.7×10^{-9}	0.241 (0.014)	2.3×10^{-64}

Abbreviations: *CYP2R1*, cytochrome P450 family 2 subfamily R member 1; *GC*, GC vitamin D binding protein; GRS, genetic risk score; *NADSYN1*, nicotinamide adenine dinucleotide synthetase 1; SE, standard error.
Analyses adjusted for age, sex, and season. Effect estimates of the meta-analysis obtained using fixed-effect models. Effect estimates of the individual genetic instruments presented as the additive effect of the effect allele on log(25-hydroxyvitamin D) serum concentration. Effect estimates of the genetic risk score presented as the increase in ln(25-hydroxyvitamin D) serum concentration per 1 point increase in the weighted score.

Chapter 9

Principal component analysis of seven skin aging features identifies three main types of skin aging

L.M. Pardo Cortes
M.A. Hamer
F. Liu
P. Velthuis
M. Kayser
D.A. Gunn
T. Nijsten

Br J Dermatol. 2019 Sep 13 [Epub ahead of print]

ABSTRACT

Background: The underlying phenotypic correlations between wrinkles, pigmented spots (PS), telangiectasia and other related facial aging sub-phenotypes are not well understood.

Objectives: To analyze the underlying phenotypic correlation structure between seven features for facial aging: global wrinkling, perceived age (PA), Griffiths photodamage grading, PS, telangiectasia, actinic keratosis (AK) and keratinocyte cancer (KC).

Methods: This was a cross-sectional study. Facial photographs and a full-body skin examination were used. We used principal-component analysis (PCA) to derive principal components (PCs) of common variation between the features. We performed multivariable linear regressions between age, sex, body mass index, smoking, and UV-exposure and the PC scores derived from PCA. We also tested the association between the main PC scores and 140 single-nucleotide polymorphisms (SNPs) previously associated with skin aging phenotypes.

Results: We analyzed data from 1790 individuals with complete data on seven features of skin aging. Three main PCs explained 73% of the total variance of the aging phenotypes: a hypertrophic/wrinkling component (PC1: global wrinkling, PA and Griffiths grading), an atrophic/skin color component (PC2: PS and telangiectasia) and a cancerous component (PC3: AK and KC). The associations between lifestyle and host factors differed per PC. The strength of SNP associations also differed per component with the most SNP associations found with the atrophic component [e.g. the *IRF4* SNP (rs12203592); P-value = 1.84×10^{-22}].

Conclusions: Using a hypothesis-free approach, we identified three major underlying phenotypes associated with extrinsic aging. Associations between determinants for skin aging differed in magnitude and direction per component.

INTRODUCTION

Facial aging occurs alongside the progressive decline of functionality of all organs in the body that occurs over time. Facial aging has been partitioned into intrinsic and extrinsic features, each with overlapping and distinctive histological hallmarks[1]. Histologically, intrinsic aging is characterized by a loss of cells and extracellular matrix components from the dermal and epidermal layers[1]. Extrinsic aging is mostly due to chronic ultraviolet radiation (UVR) exposure and is characterized by deposits of abnormal amorphous proteins of abnormal elastin in the papillary dermis (solar elastosis) and inflammatory changes that lead to a thickened epidermis[2].

The alterations due to both intrinsic and extrinsic factors in the skin result in notable skin aging phenotypes (to varying degrees), namely: wrinkles (fine and coarse), pigmentation changes, telangiectasia and skin sagging. However, the latter is arguably mainly due to subcutaneous changes in the musculature and bone structure. When facial skin is assessed in the clinic, two main clinical types have been described: hypertrophic and atrophic aging[3]. Hypertrophic-type skin presents as leathery inelastic skin with coarse wrinkles and pigmentation changes; it mainly affects individuals with darker skin (i.e. Fitzpatrick skin type III). The atrophic variant is characterized by telangiectasia, hypertrophic sebaceous glands, and absence of wrinkles; it is more frequent in fair-skinned individuals[3]. In addition, people with the atrophic type have an increased risk for skin malignancies[4]. The above clinical definition assess mainly photoaging (damage due to UVR), although they also involve features of intrinsic skin aging such as fine wrinkling and atrophy[3]. From the above classification it is not clear how wrinkles, pigmented spots (PS), telangiectasia and keratinocyte growths are related to each other. Moreover, it is unclear which patients are at risk of these distinct phenotypes with respect to their exposure, lifestyle and genetics. In addition, the clinical assessment is difficult to quantify as it is based on the experience of the clinician doing the assessments.

Epidemiological studies have shown that in addition to UVR (sun exposure or tanning beds), smoking, body mass index (BMI)[5], hormones[5] and pollutants[6,7] are risk factors for facial aging. The role of genetic variation in skin aging is less well studied. Several genome-wide association studies (GWAS) have been published using different facial aging phenotypes[8,9], but only very few single-nucleotide polymorphisms (SNPs) have been identified and replicated to date. A possible explanation could be that the phenotypes used in these studies are too heterogeneous for the sample sizes of the GWAS used to date[10]. In addition, studies have rarely used the same phenotype to identify SNP associations; skin aging GWAS include perceived age (PA), wrinkles, loss of fine skin patterning[11] and scores of photoaging manually graded using facial photographs (e.g. Griffiths grading)[12]. However, it is not clear how these different phenotypes correlate to each other, making it difficult to compare results across studies.

For observational studies, where different determinants are investigated for associations with facial aging, it is important to have phenotypes that are relatively easy to measure in large series of individuals. Phenotypes derived from digital photographs are a good alternative because of

their objectivity and easy implementation for epidemiological and genetic studies of skin aging. As part of the dermatological investigations conducted in a population-based study (the Rotterdam Study)[13], fully standardized three-dimensional (3D) photographs of the face have been taken of participants to assess skin aging. So far, we have investigated the association between lifestyle factors and/or genetic variants to several skin aging-related features including PS[14], PA[15], global wrinkling[5], actinic keratosis (AK)[16] and keratinocyte cancer (KC)[17]. Interestingly, several determinants previously associated with skin aging showed contrasting effects across specific skin aging features (e.g. a pale skin color was associated with more PS but fewer wrinkles)[5]. We hypothesize that a better understanding of the phenotypic correlation structure between these different phenotypes will help to clarify such unexpected findings.

Here, we estimated the amount of shared variation between skin aging-related phenotypes (PA, Griffiths grading, AK and KC) using principal-component analysis (PCA)[18]. PCA can be used to investigate the underlying variance-covariance structure of multiple features across participants[18]. Using PCA we identified main components of common variation, and used these as dependent variables to test for the direction and magnitude of effects of both lifestyle factors and genetic variants previously associated with skin aging.

MATERIALS AND METHODS

Study population

The Rotterdam Study is an ongoing prospective population-based cohort study following 14,926 participants aged ≥ 45 years in Ommoord, a district of Rotterdam in the Netherlands, since 1990. Details of the study design and objectives have been described elsewhere[13]. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sports of the Netherlands, implementing the 'Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)'. All participants provided written informed consent to participate in the study.

Facial skin aging features

Standardized high-resolution digital 3D facial photographs (Premier 3dMDface3-plus UHD, Atlanta, GA, U.S.A.) are being collected as part of dermatological examinations carried out in the Rotterdam Study. Between September 2010 and June 2014, a total of 4649 participants were photographed and examined at the research centre. Using frontal and 3D photographs, three main phenotypes were derived with semi-quantitative image analysis of frontal images, namely: PS, telangiectasia, and global wrinkling. A detailed description of PS and wrinkling is presented elsewhere[19]. In short, two-dimensional images were generated from the 3D photographs using Blender v.2.7 (http://www.blender.org). Next, the photographs were masked to isolate skin areas of interest using semi-automated masking (MATLAB, The MathWorks, Inc, Natick, MA, USA, ver-

sion 2013a)[19]. PS were digitally quantified as areas that are dark brown relative to the surrounding skin, with a roundish shape, present on the forehead, cheeks and nose[19]. Further, the percentage of skin area detected as hyperpigmented spots was calculated. Telangiectasia were quantified as areas that are colored red to purple and linear or branch-like in shape. Global wrinkling was quantified as a percentage of the masked facial skin area. These phenotypes have been validated against clinical assessment of the same photographs[19].

In addition, PA was included in the analysis because it has been used recently as a phenotype to investigate lifestyle factors and genes for skin appearance[8,15]. Independent observers who were blinded to chronological age assessed the same photos for PA using an ordinal scale of 10 age groups (with an average number of 27 assessments per image/participant and a minimum of 20)[15]. PA was calculated as the best linear unbiased predictor of the mean estimated age of the person in the image from a Proc-mixed model (SAS 9.3; SAS, Cary, NC, U.S.A.) with the viewing order as a fixed effect and assessor and image as random effects. For comparison, we also scored wrinkles by grading the facial photographs using the photonumerical grading scale from Griffiths *et al*[12]. We used nine ordinal levels from the 3D photos by four independent raters. Before phenotyping, a total of 100 photos (50% females) were randomly selected and openly discussed to reach a consensus between all raters. The concordance between the raters was evaluated using Pearson correlation coefficients and Cohen's kappa's coefficient. The average score of the four raters was considered as a quantitative phenotype.

Furthermore, the number of total AKs and KCs were also included. These were generated based on full-body examination.[20] AK was defined as rough red scaling lesions, not fitting another diagnosis and categorized into four levels, namely: 0, 1-3, 4-9 and ≥ 10.[16] KCs were assessed as a binary outcome [1: presence of KC: basal cell carcinoma (BCC) or squamous cell carcinoma (SCC); 0: absence]. In total, we analyzed seven features of skin aging.

Statistical analysis

Principal component analysis

Characteristics of the three main phenotypes (e.g. global wrinkles, PS and telangiectasia) and the associated variables (Griffiths grading, PA, AK and KC) are presented as means (SD) or medians (interquartile range) for non-normally distributed determinants and frequencies (percentages) for categorical determinants.

To investigate the underlying correlation structure of the seven features we used PCA as an exploratory analysis (details of the procedure and analysis to assess whether our data were suitable for PCA are presented in File S1, see Supporting Information). In short, PCA was carried out using the matrix correlations on complete cases and using varimax rotation, where residual correlation between latent components is not assumed. The loading factors (principal components, PCs) with eigenvalues higher than 1[21] were selected. The above analysis was performed using SPSS for Windows version 21.0 (SPSS; Chicago, IL, U.S.A.).

Multivariable linear regressions on demographic and lifestyle factors

Scores were derived from the main PCs and used as dependent variables to test for associations with main lifestyle factors, using multivariable linear regressions to calculate adjusted beta coefficients and 95% confidence intervals. Based on previous results from the Rotterdam Study[5], the following variables were tested: chronological age, sex, smoking status (never, former, current), skin color (pale, white and white-to-olive), BMI and four variables used as proxy to assess UVR exposure. Details on how the variables were defined are presented in File S1 (see Supporting Information).

Multivariable linear regressions on single-nucleotide polymorphisms involved in skin aging

To test for associations between previously identified genetic variants and PC scores we searched the literature for SNPs with genome-wide associations (P-value ≤ 5×10^{-8}) (Table S1, see Supporting Information) in at least two different studies with different skin aging phenotypes[14,15] and related phenotypes including AK[16], skin color[22], hair color[23] and eye color[14].

Next, we extracted the SNP allele scores (derived from imputation/genotypes) from an existing SNP GWAS dataset available from the Rotterdam Study [a description of how the genotypes were generated is presented in File S1 (see Supporting Information) and the references therein]. Further, we performed a linkage disequilibrium analysis to remove SNPs that were highly correlated with each other (see File S1, Fig. S1 and Table S2, Supporting Information). Lastly, we tested for per-SNP association with the three PCA-derived scores using a multivariable linear regression on complete cases. We adjusted this analysis for age, sex and four covariates used to correct for possible population stratification. The threshold for significance of the SNP associations was defined by dividing the nominal P-value by the total number of SNPs that show low correlations (P-value = 0.05/140; File S1, see Supporting Information). These analyses were performed using the R statistical package, v.5.1.3 (http://www.R-project.org).

RESULTS

Principal component analysis

In total, there were 1793 participants with complete data on the seven features. The mean distribution and standard error of the features we analyzed are presented in Table S3 (see Supporting Information). Table 1 presents Pearson's correlations derived from the PCA between global wrinkles, PS, telangiectasia and related features (PA, Griffiths grading, AK and KCs). Global wrinkling was poorly correlated with PS with a negative estimate of -0.20 and there was no correlation between global wrinkling and telangiectasia. Likewise, the correlation between PS and telangiectasia was only 0.27. On the other hand, strong correlations were found between global wrinkles and PA (0.65), and global wrinkles and Griffiths grading (0.74). Although AK has previ-

Table 1. Principal component analysis: pairwise correlations between seven facial skin phenotypes and skin cancer

	Global wrinkling	Pigmented spots	Telangiectasia	Perceived age	Griffiths grading	Actinic keratosis	Keratinocyte cancers
Global wrinkling	1.00						
Pigmented spots	**-0.20****	1.00					
Telangiectasia	**-0.24****	**0.27****	1.00				
Perceived age	**0.65****	**0.15****	0.01[a]	1.00			
Griffiths grading	**0.74****	**0.08****	**-0.06****	**0.75****	1.00		
Actinic keratosis	**0.15****	**0.04****	0.02[a]	**0.21****	**0.13****	1.00	
Keratinocyte cancers	**0.05****	**0.06***	-0.02[a]	**0.13****	**0.05****	**0.26****	1.00

*correlation is significant at the 0.05 level (one-tailed); **correlation is significant at the 0.01 level (one-tailed); [a]non-significant correlations.

ously been considered to be a marker of UVR-induced skin aging, it correlated poorly with the other skin features with correlations of 0.21 between AK and PA, and 0.26 between AK and KC.

Table 2 presents the main PC components derived from the PCA. The first three components had eigenvalues ≥ 1 and accounted for 73% of the variance. The first component (hypertrophic component; PC1) was strongly correlated with the wrinkling-related assessments (global wrinkles, Griffiths grading and PA) with correlations higher than 0.80 (Table 3). The second component (atrophic component; PC2) correlated with PS and telangiectasia, accounting for almost 82% of the variation of PS and 77% of telangiectasia. Global wrinkling was negatively correlated with PC2 (-0.32). AK and KC, on the other hand, were grouped together in a separate component (cancerous component; PC3), with correlations of 0.77 between AK and PC3 and 0.81 between KC and PC3.

Table 2. Principal component analysis. Total variance shown by components

	Initial eigenvalues		
Component	Total	% of variance	Cumulative %
1	2.52[a]	35.98	35.98
2	1.40[a]	20.04	56.02
3	1.16[a]	16.54	72.56
4	0.74	10.51	83.07
5	0.72	10.31	93.38
6	0.26	3.68	97.06
7	0.21	2.94	100.00

Kaiser–Meyer–Olkin measure of sampling adequacy: 0.663. [a]Three PCs account for 73% variation.

Table 3. Rotated components. Correlations between the seven phenotypes and the three main principal components

	Principal components		
	1	2	3
Global wrinkling	0.87	-0.32	0.05
Pigmented spots	0.07	0.82	0.05
Telangiectasia	-0.1	0.77	-0.02
Perceived age	0.89	0.15	0.15
Griffiths grading	0.93	0.04	0.02
Actinic keratosis	0.14	-0.004	0.77
Keratinocyte cancers	0	0.04	0.81

Multivariable regression analysis on lifestyle factors and single-nucleotide polymorphism variants

Using the PCA scores generated from the three main components (PC1, PC2 and PC3) as dependent variables, we tested for their association with a range of determinants (Table 4). Effect sizes and direction of effects were different for the determinants across the three PCs. For example, age had the largest effect on the wrinkling component (PC1). Light skin color was positively associated with PC2 and weakly associated with PC3, but negatively associated with PC1, although the latter was not significant. Current smoking was positively associated with PC1 and PC2 and negatively associated with PC3. BMI was negatively associated only with PC1, although the effect was rather small. Being female was positively associated with PC2 and PC3, and negatively with PC1. Of the four UVR-related variables available in the Rotterdam Study, sun-protective behavior was significantly associated with PC1 and PC3, and propensity to sunburn was positively associated with PC2 and PC3. Although the direction and effect of the associations cannot be directly extrapolated as these are statistical constructs, it does show that the associations differ per component.

We also conducted a candidate gene analysis on 140 SNPs from 75 genes and 51 intergenic regions (Table S4, see Supporting Information) with the three PCs. These SNPs have been associated previously with skin aging-related features in previous GWAS (see references in File S1, Supporting Information). A total of 17 SNPs showed significant association with at least one skin PC after multiple testing correction (Table S4, see Supporting Information). Some of these associations were stronger for the PCs than those from individual skin features (e.g. rs2233173, beside the *melan-A* gene, was significant for PC2 but only nominally significant for PS), although for other SNP associations the opposite was true (e.g. rs12203592; Table S5, see Supporting Information). There were stronger effect sizes for PC2 when compared with telangiectasia and PS, although this was not the case for all features (Fig. S2, see Supporting Information). Overall, PC2 showed the most significant associations (Fig. 1; Table S4, see Supporting Information), of which rs478882, a hair color-associated SNP, was the top hit (P-value = 3.52×10^{-14}). This SNP was also nominally significant with PC3. Other significant associations included the rs12203592 SNP (*IRF4* gene), which has previously been associated with skin color, AK and skin cancer. There were clusters of associations that were similar between PC2 and PC3 components, but not PC1, as well as associations that were specific to each PC (Fig. 1; Table S4, see Supporting Information). PC1, where global wrinkling, PA and Griffiths grading were clustered, had the weakest genetic associations. The most significant association found for this component was for rs12350739, an intergenic SNP that has been assigned to the *BNC2* gene in previous GWAS of skin color. The genes associated with the SNPs are listed in Table S4 (see Supporting Information).

Table 4. Statistical associations between main lifestyle risk factors and the scores derived from principal component analysis (PCA)

	PC1			PC2			PC3		
	Effect size (SE)	95% CI	P-value	Effect size (SE)	95% CI	P-value	Effect size (SE)	95% CI	P-value
Age	**0.08 (0.003)**	**[0.08 – 0.09]**	**$<2.0 \times 10^{-16}$**	0.001 (0.003)	[-0.004 – 0.01]	0.62	**0.04 (0.003)**	**[0.03 – 0.05]**	**$<2 \times 10^{-16}$**
Sex									
female	**-0.24 (0.04)**	**[-0.32 – -0.17]**	**7.1×10^{-10}**	**0.97 (0.04)**	**[0.89 - 1.05]**	**$<2.0 \times 10^{-16}$**	**0.24 (0.05)**	**[0.14 – 0.33]**	**4.7×10^{-7}**
male	ref	ref	ref	ref	ref	ref	ref	ref	ref
Skin color									
pale	-0.14 (0.09)	[-0.31 – 0.03]	0.10	**0.86 (0.09)**	**[0.69 – 1.04]**	**$<2.0 \times 10^{-16}$**	**0.22 (0.10)**	**[0.03 – 0.42]**	**2.7×10^{-2}**
white	-0.07 (0.06)	[-0.18 – 0.05]	0.24	**0.25 (0.06)**	**[0.13 – 0.37]**	**2.5×10^{-5}**	0.10 (0.07)	[-0.04 – 0.23]	0.15
white-to-olive	ref	ref	ref	ref	ref	ref	ref	ref	ref
BMI	**-0.03 (0.00)**	**[-0.04 – -0.02]**	**8.9×10^{-13}**	0.00 (0.00)	[-0.01 – 0.01]	0.89	0.00 (0.01)	[-0.01 – 0.01]	0.73
Smoking[a]									
current	**0.44 (0.06)**	**[0.33 – 0.56]**	**2.6×10^{-14}**	**0.22 (0.06)**	**[0.10 – 0.33]**	**2.9×10^{-4}**	**-0.16 (0.07)**	**[-0.30 – -0.03]**	**1.7×10^{-2}**
former	0.08 (0.04)	[-0.01 – 0.16]	9.4×10^{-2}	**0.13 (0.05)**	**[0.04 – 0.22]**	**4.0×10^{-3}**	-0.09 (0.05)	[-0.20 – 0.01]	7.8×10^{-2}
never	ref	ref	ref	ref	ref	ref	ref	ref	ref
Lived in a sunny country[b]	-0.04 (0.08)	[-0.20 – 0.12]	0.62	0.03 (0.08)	[-0.13 – 0.19]	0.73	0.15 (0.09)	[-0.034 – 0.34]	0.11
Sunburn easily[c]	-0.03 (0.04)	[-0.12 – 0.05]	0.45	**0.29 (0.04)**	**[0.20 – 0.37]**	**1.8×10^{-10}**	**0.22 (0.05)**	**[0.12 – 0.32]**	**2.4×10^{-5}**
Winter in a sunny country[d]	0.13 (0.09)	[-0.05 – 0.32]	0.16	-0.02 (0.10)	[-0.21 – 0.17]	0.81	0.17 (0.11)	[-0.05 – 0.39]	0.14
Sun-protective behavior[e]	**-0.09 (0.04)**	**[-0.17 – -0.01]**	**3.3×10^{-2}**	0.02 (0.04)	[-0.06 – 0.10]	0.66	**0.12 (0.05)**	**[0.020 – 0.21]**	**1.8×10^{-2}**

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; PC, principal component; PC1, PC2 and PC3 refer to the PC scores computed from the PCA and were used here as dependent variables; SE, standard error. Boldface indicaties significant associations (P-value<0.05).

[a]cigars, cigarettes or pipe; [b]history of living in a sunny country > 1 year (reference group: no); [c]tendency to develop sunburn (reference group: low); [d]spending winter in sunny country (reference group: no or less than 1 month per year); [e]wearing sunglasses and/or a brimmed hat in the sunshine (reference group: never/almost never).

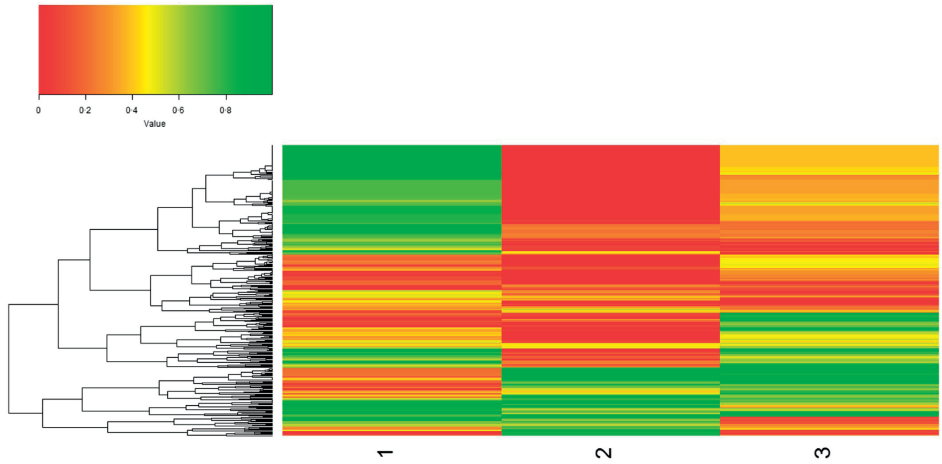

Figure 1. Heatmap of the correlation of associations between 140 single-nucleotide polymorphisms (SNPs) and the three principal components. The dendrogram on the left corresponds to clusters of SNPs with similar associations across the three components. The colors represent the strength of the associations (P-values) with red color for the most significant associations and green for the less significant associations.

DISCUSSION

The principal component analysis of seven skin features identified three main components of extrinsic skin aging that explained 73% of the total variance. Although it is difficult to give a biological label to these components, because these are statistical constructs, it could be argued that PC1 is related to hypertrophic skin aging[3], while PC2 corresponds to the atrophic component[3]. In contrast with earlier studies based on clinical assessments only, our analysis separated a cancerous component (PC3) from the other two PCs. This suggests that hypertrophic skin aging and atrophic skin aging occur via partly different pathophysiological mechanisms than those producing keratinocyte growths, in contrast to previous clinical-based studies[4]. The analysis presented is more robust compared with previous clinical classifications of photoaging as the latter are subjective and operator dependent and therefore more prone to bias, while our classification was based on a data-driven and hypothesis-free approach in a large population sample which is more likely to provide an objective differentiation between skin aging phenotypes.

The associations between the main lifestyle factors for skin aging and the PCs differed in magnitude and the direction of effects. Light skin color was positively associated with the atrophic component of skin aging (PC2) and weakly associated with the cancerous factor (PC3), but inversely associated with the hypertrophic factor (PC1), although it was not significant. This is more likely due to a reduced sample size because in our previous publication on the epidemiology of wrinkles, which included over 3000 participants, the association was significant[5]. Current smoking was positively associated with both hypertrophic and atrophic skin aging, but negatively associated with the cancer-related component (PC3). All these associations were significant (Table 4).

Although smoking is positively associated with SCC, the protective effect of smoking on KC has recently been shown for cutaneous BCC[24] and is in line with our results, as most of the KCs in our study were BCCs (80%).

The differential strength and direction of known determinants on PCs highlight the importance of understanding the different aspects of skin aging in general. This has clinical implications as, for example, individuals with a predominant hypertrophic component will benefit the most from interventions to modify lifestyle factors, while this may be less relevant for individuals with an atrophic component (except for UVR exposure, as these individuals will be more susceptible due to their genetic background as shown in Fig. 1). Furthermore, the fact that the cancerous component is separated from the atrophic one highlights the importance of screening for skin cancer even if individuals do not show strong signs of skin aging. The three components could help in the interpretation of results of other assessments of epidemiological studies on facial aging. For example, in a recent publication by Law *et al*.[11], the phenotype "microtopography scores" was used as an assessment for loss of fine skin patterning, also a feature of skin aging. In that article, SNPs associated with *IRF4* and *MC1R* genes, which are skin color genes, were significantly associated. The microtopography phenotype is in our view an atrophy-related phenotype, which explains the associations with skin color genes. Thus, in light of our analysis, it may be easier to understand results from other studies using our PCA-derived components than with individual's phenotypes.

To understand whether genetic correlations mirrored the phenotypic analysis, we calculated pairwise genetic correlations (the extent of shared variance between two traits due to genetic effects; see Table S6, Supporting Information) between the seven features and found higher correlation between phenotypes within the hypertrophic component, mostly driven by the correlations with wrinkling. The difference could be due to genetic correlations being less prone to residual confounding than the phenotypic ones. We also found a borderline significant negative correlation between PS and global wrinkling (-0.52; SE 0.27; P-value = 0.03; Table S6, see Supporting Information). The latter suggests that although some genetic variants may be associated with both traits, they could have opposite effects and, therefore, would cancel each other out if the phenotypes were combined together. However, our sample size is probably too modest for a robust estimation of genetic correlations and therefore larger samples may be needed to validate the findings. The per-SNP associations we found with atrophic skin aging (PC2) showed that the genetic variation of PS is largely determined by pigmentation genes, while these have very little influence on wrinkles, supporting our early GWAS findings on wrinkles.[9] Our study cautions against combining different features of skin aging into a single score without a previous understanding of the phenotype and genotypic correlations, as this can dilute the effects of individual genes and/or risk factors. Even combining moderately correlated phenotypes can decrease the power of genetic identification, as illustrated by the *IRF4* SNP (rs12203592), which had a weaker association with PC2 than for PS. This may be related to the fact that genetic associations are more susceptible to phenotypic heterogeneity than non-genetic association analysis and therefore other approaches for gene finding may be needed.

This study has several limitations. Firstly, the PCA is a statistical method that uses assumptions – such as there is little error in the measurement of the variables – that may not hold true in practice. In addition, we used a method (varimax approach) that assumes that there are no residual correlations between the main PCs. Residual correlations between the main PCs may still be significant due to, for example, shared determinants (e.g. UVR exposure, skin color). Although there are multivariable techniques that account for residual correlations between components, this would make the interpretation of the PCs more difficult. Furthermore, UVR-related variables used in this paper are more related to individual behavior towards sun exposure than actual UVR exposure. This limits the conclusions we can make for the association of these variables with the PCs. This issue is not limited to this study but is a known problem when measuring environmental exposure with questionnaire-based instruments in epidemiological research. This needs to be improved in the future. Lastly, although the PCA approach can be considered to be unbiased, the interpretation of what the PCs mean can be subject to debate. Here, we interpreted the PCs in the context of known literature on different clinical phenotypes and taking into account which original phenotypes were accounted for by the PCs. Because this is an exploratory PCA and not a confirmatory one, the interpretation of the PCA is largely research dependent. For example, it could be argued that PC2 is more a pigmentation component than an atrophic one because of the strong association of this component with pigmentation-related SNPs. We tested this by including skin color, eye color and hair color in the PCA and they formed a separate component (data not shown). However, with the added variables the new component explained less total phenotypic variance (four PCs, 66%; correlation between PS and PC4 was 44%) and did not improve the fitting of the data. Therefore, we did not include this component as a major skin aging component.

To conclude, using an unbiased approach in a large sample, we found three underlying phenotypes for photoaging, two of which matched the previously recognized clinical subtypes of photoaging, namely hypertrophic and atrophic skin aging. The cancerous component of skin aging adds to the complexity of the latter with a partial genetic overlap additionally found between PS and skin cancer, which can now be explored in further depth.

ACKNOWLEDGMENTS

The authors are grateful to the study participants, the staff from the Rotterdam Study, and the participating general practitioners and pharmacists. We thank Sophie Flohil, Emmilia Dowlatshahi, Robert van der Leest, Joris Verkouteren and Ella van der Voort for collecting the phenotypes. Additionally, we thank Sophie van den Berg for masking and reviewing all the photographs. We acknowledge Jaspal Lall for masking the photographs and helping create the digital wrinkle measurements.

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Chapter 10

General discussion

IMPACT OF SKIN AGING RESEARCH

From cosmetic relevance towards healthy skin aging

Facial skin aging has a large impact on the general population, as it is directly visible and influences the perception of a person's wellbeing. The cosmetic relevance is clear (as described in **Chapter 1**), but there is much more to it. Increasing life expectancy and the resulting aging of the population have made skin aging a topic that is of growing concern. With older age, prevalence of dry skin rises; it affects 60% of the middle-aged and elderly population[1]. This gives rise to higher risk of skin diseases such as late onset atopic dermatitis and pruritus. A better understanding of skin aging will help to unravel aging in general and can be of importance for promoting healthy aging. The skin is the most feasible organ to investigate using non-invasive approaches, making it an ideal target to understand aging as it may even be seen as a mirror of the internal organs. Skin aging could therefore act as a robust biomarker of aging in general and even of survival[2-4], which makes it valuable in clinical decision-making. Furthermore, global disease prevention strategies might benefit from emphasizing that certain risk factors or protective measures are also linked to facial aging. An example of this is the finding that certain dietary habits are also associated with facial wrinkling[5]. Creating a tailored approach to prevention of (skin) aging and related diseases is an important ambition. In doing so, many different phenotypes can be used. Finding the most suitable and standardized one is important to be able to replicate findings and reach the above-mentioned goals.

A MULTI-FACTORIAL PHENOTYPE

Confirming known and discovering new determinants

As described in the general introduction (**Chapter 1**), facial skin aging is a complex phenotype. There are distinctive characteristics between intrinsic and extrinsic aging, but in practice it is difficult to separate these two in UV-exposed areas such as the face. The combined effects of both intrinsic and extrinsic facial aging result in a wide range of observable physical characteristics, which can be divided into four major phenotypes: wrinkles, pigmented spots (PS), telangiectasia and sagging. Perceived age (PA, the estimated age of a person based on facial appearance) is composed of all these aspects. To date there is no gold standard approach to investigate skin aging; a wide range of assessments are used, mostly regarding skin aging as a compound phenotype, rather than specifying a certain component. In the past, two main phenotypes for photoaging have been described based on photoaging characteristics: hypertrophic aging (leathery inelastic skin with coarse wrinkles) and atrophic aging (telangiectasia, hypertrophic sebaceous glands and the striking absence of wrinkles) as depicted in Figure 1[6,7]. There are indications that individuals with atrophic-type skin aging have an increased risk of keratinocytic skin cancer (KC)[8]. Given the complexity of facial skin aging and the need of homogeneous phenotypes to increase the

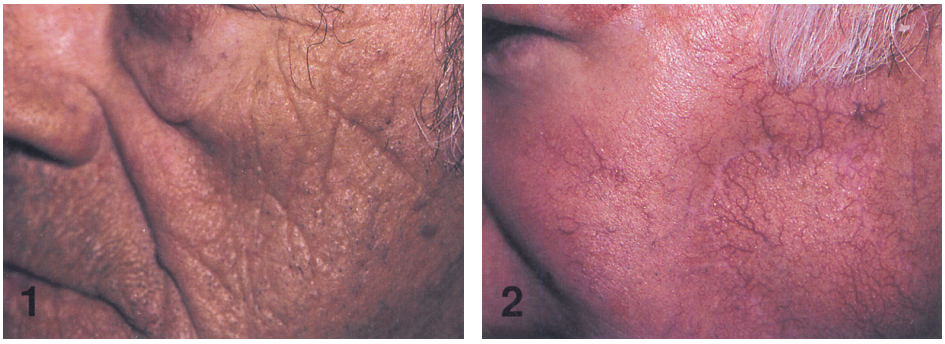

Figure 1. Hypertrophic (**1**) and atrophic (**2**) skin aging, adapted from Calderone et al[6]
1: Coarse wrinkles; inelastic and leathery appearance.
2: Smooth skin without wrinkles; prominent telangiectasia and hypertrophic sebaceous glands.

power to identify (genetic) determinants, in this thesis we studied different skin aging aspects separately, allowing to discover different determinants associated with each one of them. This is shown in several chapters of this thesis: **Chapter 3** and **4** (wrinkles), **Chapter 5** (PS), **Chapter 6** (telangiectasia), and **Chapter 7** (PA). In this thesis we mainly used continuous digital skin aging measures, quantified using image analysis techniques. They correlated well with the different manual grades (**Chapter 2**) and provide an objective, consistent and fast method to produce powerful continuous variables. We have not added sagging as separate feature as it is arguably mainly due to subcutaneous changes in the musculature and bone structure rather than to cutaneous changes.

We replicated known determinants, but also encountered surprising associations. Age was the strongest determinant for wrinkles (**Chapter 3**). It was non-linearly related to wrinkle area; with increasing age, older individuals showed a smaller increase in wrinkle area than younger individuals did. Interestingly, the effect of age was smaller for PS and telangiectasia. As age has more influence on wrinkles than on the other phenotypes, perhaps it is a more suitable proxy for chronological age.

One of the most striking differences in determinants per phenotype was found for skin color. Light skin color was associated with less wrinkles (**Chapter 3**), but with more pigmented spots (**Chapter 5**) and more telangiectasia (**Chapter 6**). The negative association of light skin color with wrinkles seems contradictive at first, because people with Fitzpatrick skin types IV and higher have less wrinkles than those with skin types I-III[9,10]. The possible explanation that very light-skinned individuals are known to avoid the sun because of the ultraviolet (UV) sensitive nature of their skin does not suffice because our model also included UV-exposure related variables which should correct for this effect. Furthermore, pigmented spots and telangiectasia are also influenced by UV-exposure[11-14]. This strengthens the hypothesis that the association between skin color and the skin aging features are independent of UV-exposure and confirms the concept that different skin types age in a different manner. Fair-skinned individuals tend to show more atrophic

signs of aging (e.g. PS and telangiectasia), whereas darker-skinned individuals show more hypertrophic signs of aging (e.g. wrinkles).

We discovered important sex differences for wrinkles, stressing the importance of a sex-tailored approach regarding skin aging management (**Chapter 3**). Men are first at developing wrinkles (<75 years old: wrinkle area is higher in men), but women catch up with them at a higher age (≥75 years old: wrinkle area is higher in women). This difference could be due to hormonal changes in postmenopausal women, as early menopause has been associated with more wrinkles[15]. Women with premature ovarian insufficiency are shown to have an unfavorable cardiovascular risk profile[16], although perhaps the cardiovascular risk is related to the association with their increased free androgen index (FAI; total testosterone in nmol/l divided by sex hormone binding globulin in nmol/l)[17]. In contrast, endogenous estrogen exposure in women was found to be harmful for all-cause mortality[18]. In this thesis, however, we found no association between estradiol levels and wrinkles. Nonetheless, we did find an inverse association of FAI and wrinkles in women. Moreover, female pattern hair loss was also inversely associated with wrinkles. These findings suggest that testosterone could play a role in protecting women against wrinkles. However, this does not coincide with the abovementioned association of an increased FAI with an unfavorable cardiovascular risk profile, when assuming skin aging might reflect internal aging. Surprisingly, we have not found a significant association of testosterone and wrinkles in men. This could be explained by the fact that we have used an inferior measurement; total testosterone is less suitable than bioavailable testosterone in men. Interestingly, the effect sizes of the determinants also differ per sex. The effect sizes for skin color and smoking are larger in women than in men (**Chapter 3**). Furthermore, higher educational level is associated with less wrinkles in women, but not in men.

UV-exposure has well-known negative effects, but it is also essential for vitamin D synthesis in the skin[19], which is vital for our health. In the past decades, vitamin D deficiency has been seen as a marker for health status, as it has been associated with cardiovascular disease as well as with mortality[20-23], although recent studies debate this[24,25]. We found that higher serum 25-hydroxyvitamin D was associated with more skin wrinkling and a higher PA (**Chapter 8**). In order to discover whether these associations were causal, we performed Mendelian Randomization (MR).

Genetically determined 25-hydroxyvitamin D (i.e. the genetic variants that are associated with serum 25-hydroxyvitamin D) was not associated with wrinkling, PS or PA, suggesting that these associations are not likely causal. The observational associations between serum 25-hydroxyvitamin D and the two skin aging phenotypes are most probably due to residual confounding (e.g. UV-exposure) or reverse causality. These findings confirm the need for more MR studies to investigate causality.

Understanding the underlying correlation structure of skin aging

The abovementioned differences between the distinct skin aging features prompted us to investigate the underlying variance of these phenotypes using principal component analysis (PCA)[26], as

described in **Chapter 9**. Besides wrinkles, PS, telangiectasia and PA, we also used data on actinic keratosis (AK) and keratinocytic cancer (KC), the most common skin (pre)malignancies[27,28]. The PCA identified three main components of skin aging that explained 73% of the total variance (Figure 2). It can be argued that principal component 1 (PC1) is related to the hypertrophic skin aging component, while PC2 corresponds to the atrophic component. This analysis also revealed a third component (PC3), which represents a cancerous component. It suggests that hypertrophic aging, atrophic aging, and the cancerous component occur via partly different pathophysiological mechanisms. This PCA confirms what has been described earlier, but is more robust because it is data-driven and hypothesis-free, making it an objective measure (as opposed to the subjective clinical measurement done by clinicians). As expected, the associations between the main lifestyle and physiological determinants and the three components of skin aging differed in magnitude and direction of effects and confirmed the distinctions found in earlier analyses. This highlights the importance of understanding their underlying mechanisms. It also has clinical implications; it can be of use in risk stratification and personalized approach of skin aging prevention strategies. Furthermore, the finding that the cancerous component is separated from the skin aging components stresses the relevance of skin cancer screening even in the absence of obvious signs of skin aging.

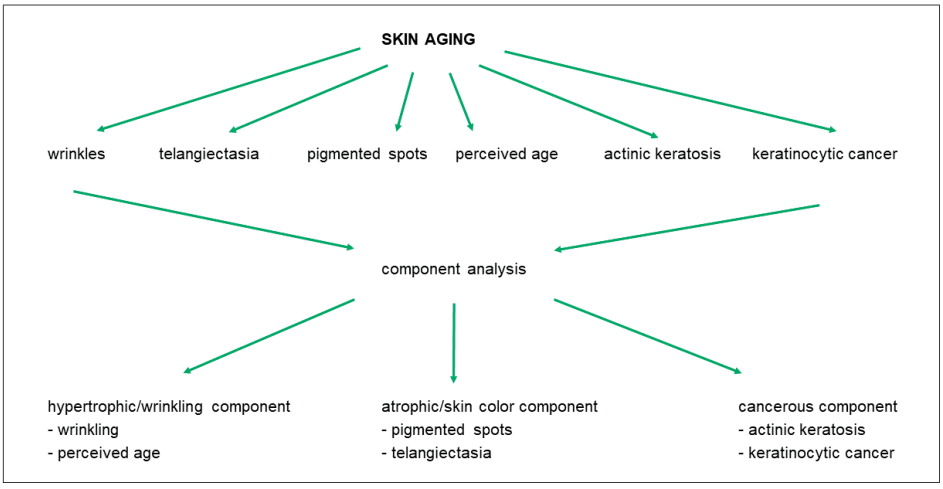

Figure 2. The accordion model. Skin aging is a complex phenotype, consisting of six different features, which can be re-grouped into three main components: hypertrophic, atrophic, and cancerous.

Genetic epidemiology – usual suspects *MC1R* and *IRF4* not related to wrinkles

Results from previous genetic association studies have been conflicting. In the general introduction (**Chapter 1**), we discussed suggestive single-nucleotide polymorphisms (SNPs) from previous skin aging genome-wide association studies (GWAS) and candidate gene studies, of which *MC1R* and *IRF4* have the strongest effects. In this thesis, we found that these and other pigmentation

variants were associated with the phenotypes PS and PA. For PS we found associations at four genetic loci (**Chapter 5**): *IRF4* (rs12203592, $P=1.8\times10^{-27}$), *MC1R* (compound heterozygosity score, $P=2.3\times10^{-24}$), *RALY/ASIP* (rs6059655, $P=1.9\times10^{-9}$), and *BNC2* (rs62543565, $P=2.3\times10^{-8}$). These four genes are associated with skin color variation and skin cancer risk, but remained highly significant ($P<2\times10^{-8}$) after adjustment for skin color. Therefore, these genes seem to contribute to PS through pathways independent of the basal melanin production as they may have pleiotropic effects.

The *MC1R* gene is also associated with PA ($P=2.7\times10^{-12}$, **Chapter 7**). Individuals carrying the homozygote *MC1R* risk haplotype looked on average up to 2 years older than non-carriers did. A diminished *MC1R* activity can cause a weaker UV shielding capacity and cause more photodamage[29]. The effect was, however, found to be independent of UV variables. This is in line with findings that *MC1R* has pleiotropic effects, perhaps influencing aging via different pathways, for example via inflammation[30]. Of importance, the association of the *MC1R* gene with PA was only found when PA was corrected for wrinkles. Therefore, it can be hypothesized that this corrected PA represents the "atrophic" perceived age, including PS and telangiectasia, as well as sagging, which is not quantified in this thesis but also plays a role in PA.

In line with this, *MC1R* was not associated with wrinkles (**Chapter 4**). We only found a suggestive hit for the SNP rs10476781 ($P=2.2\times10^{-8}$), an intergenic variant 628 KB downstream of the Neuromedin U Receptor gene. In contrast to the other phenotypes, the classical skin pigmentation genes did not play a role in global wrinkling. This is somehow unexpected since we found a clear association between skin color and wrinkles. Perhaps other yet to be discovered skin color genes influence wrinkles. The remarkable differences between wrinkles and PS/PA imply different genetic backgrounds for these skin aging components. The genetic architecture of wrinkles seems more complex than that of PS and PA.

Missing heritability

The estimated heritability using all SNP data[31] was highest for wrinkles, namely 46%. For PS it was 28% and for PA 33% (**Chapter 9**). However, in our GWAS the top hit accounted for <1% of wrinkling variation (**Chapter 4**). The remaining genetic variation for wrinkles remains unknown. There are several hypotheses for these gaps[32]. A large part of missing heritability is likely explained by variants with effects too small to be detected. Most probably, there are many different genes affecting wrinkles, but all with small effect sizes. There seems to be too much genotypic and phenotypic heterogeneity. In other words, the well-known but unfortunate power problem for genetic studies. Increasing the sample size can solve this problem. In addition, non-tagged SNPs could be of importance. Previously, GWAS on skin aging mainly took into account common variants with a frequency of >0.01. However, also rare variants may have substantial effects[33]. Focusing on rare SNPs can offer important additional information. Finally, a part of the missing heritability could simply be due to artificial factors, such as imprecise phenotypic measurement, genotyping or imputation errors.

FUTURE PERSPECTIVES – FILLING GAPS OF MISSING HERITABILITY AND BEYOND

In order to discover the beforementioned missing heritability, higher sample sizes are required, for which collaborations are important. With future collaborations in mind, it is important to be able to use a standardized phenotype. Which phenotype is most suitable is under discussion, but several factors are important: it should be easy to retrieve, non-invasive, objective and of high quality. The use of photographs seems the most feasible, as these can be stored for later use and can be replicated by several graders or digital methods. In this thesis, perceived age was assessed by human grading. This was however quite time-consuming; this could possibly be replaced by artificial intelligence in the future, as this is a fast evolving field[34]. Nevertheless, perhaps there is no such thing as "the perfect phenotype" for research in skin aging. It may be more appropriate to select the phenotype based on the underlying question. Combining different assessments for skin aging may obscure identification of genes if variants have different effects on the phenotypes (as shown for wrinkles and PS). On the other hand, PCA-like approaches may help to understand the outcomes from previous studies or be of use for clinical advice. Furthermore, the phenotype PA could perhaps help to identify parameters for healthy aging.

In this thesis, we showed that for certain phenotypes such as wrinkles, large samples will be needed to identify new genes associated with facial skin aging. In GWAS, both common DNA variants and rare DNA variants can be targeted, depending on the underlying genetic architecture of the phenotype. For both, large sample sizes are needed. In future studies, there should be special attention for rare DNA variants. GWAS based on whole-genome sequencing permit the full frequency spectrum of variants to be studied, including rare variants that are difficult to capture using SNP arrays and imputation. This could help solve the missing heritability problem. Performing GWAS will hopefully allow to identify new pathways for skin aging. Another approach is to look into already identified pathways or determinants to reduce the burden of multiple testing associated with testing millions of markers. For example, by using MR we can confirm associations that have been seen in epidemiological studies. In **Chapter 3,** we showed that body mass index (BMI) was negatively associated with wrinkles. It is unclear if this is simply due to the "filling effect" or if there is more to it. In preliminary analyses, we performed MR to understand whether this association was causal. The results showed that some BMI-SNPs were associated with wrinkle variation (data unpublished).

Other major factors are gene-gene and gene-environment interactions. In this thesis, we only evaluated SNP associations one at a time (main effects). However, it is very likely that there are gene-gene interactions and gene-environment interactions for skin aging features, e.g. polymorphisms in repair genes that affect skin aging features via UV-exposure. Therefore, in the future it is important to also take these interactions into account (Figure 3). However, several other challenges are then likely to arise[35] and the importance of environmental exposures is not always straightforward. Also, it requires large sample sizes, only reachable by generating consortia.

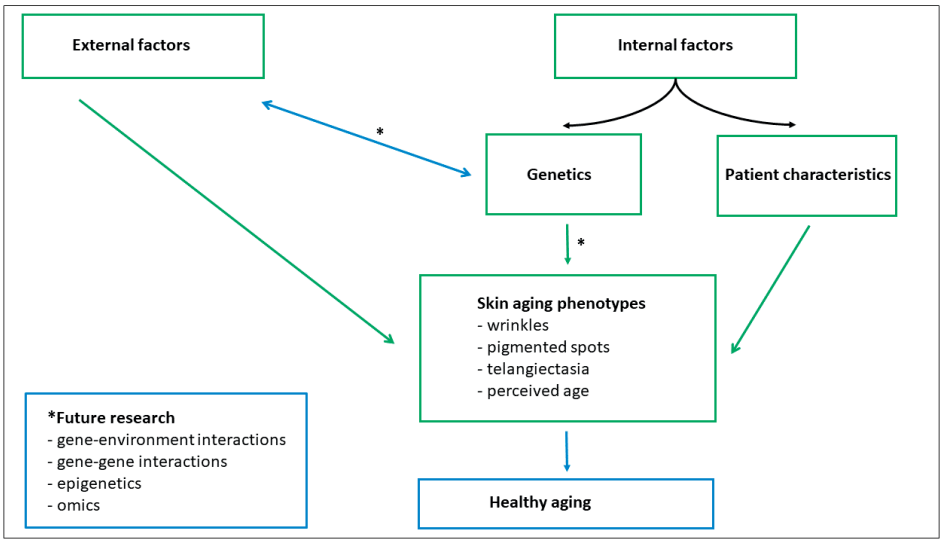

Figure 3. An overview of associations investigated in this thesis (depicted in green) and future possibilities (depicted in blue). In this thesis, we have investigated external factors (lifestyle and environmental exposures) and internal factors (patient characteristics and genetics) related to the skin aging phenotypes. This has offered valuable information, although much is still unexplained. This can perhaps be solved by focusing future research on gene-environment interactions, gene-gene interactions, epigenetics and omics. Furthermore, the interesting link towards healthy aging can be made.

Epigenetics is described by modifications that regulate gene activity and/or expression by methylation, but do not directly change the DNA sequence[36]. Epigenetic modifications are highly dynamic and display great tissue specificity. To what extent epigenetic modifications contribute to skin aging is unknown. Examples of epigenetic studies are next-generation sequencing (especially to search in detail for rare/unknown variation in a specific genetic area) and epigenome-wide association studies (EWAS) researching DNA-methylation profiles. The concept of epigenetic drift describes changes in methylation that occur with age across various tissues, including skin. Several studies show contradictory results of either hypo- or hypermethylation related to skin aging[37,38]. The strong effects of smoking in methylation make it likely that smoking may play an important role in skin aging via epigenetics.

"Omics", high-throughput analyses including genomics, epigenomics, transcriptomics, proteomics and metabolomics, are widely applied in aging research[39]. For skin aging, however, this field has not been extensively researched. Most proteomics research is done on photodamaged skin[40], but there are also examples of comparisons between UV-exposed and UV-unexposed sites, revealing differences in relative protein abundance[41,42]. At the UV-exposed sites, age-altered proteins were associated with conferring structure, energy and metabolism. At the UV-unexposed site, proteins associated with gene expression, free-radical scavenging, protein synthesis and protein degradation were most frequently altered[41], giving rise to inferences on intrinsic and extrinsic aging.

Needless to say, it is first and foremost important that our GWAS findings are replicated in independent cohorts. The relevance of these gene variants can then be confirmed by functional follow-up studies, e.g. through gene expression.

Further missing links may be found in other research fields, as the microbiome. Investigation of dietary habits has revealed interesting findings[43-49]. Most recently discovered, a red meat and snack-dominant dietary pattern was associated with more facial wrinkles in women, whereas a fruit-dominant dietary pattern was associated with fewer wrinkles[5]. Although not much is known on microbiome and skin aging, it could be an interesting new field to discover.

CONCLUDING REMARKS

In this thesis, I discovered lifestyle and genetic associations for different skin aging features, using validated digital measurements. I identified valuable determinants and gene variants for several features and created robust evidence for the hypertrophic and atrophic components. However, this is only the start of understanding skin aging, as it seems much more complex; the relationships are not simply linear, but involve multiple complicated pathways, still to be discovered. This and future research on associations with other aging organ systems will contribute to a better understanding of (healthy) aging.

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Chapter 11

Summary / Samenvatting

SUMMARY

Chapter 1 gives a general introduction to this thesis. Facial skin aging has a large impact on the general population. Besides the clear cosmetic relevance, it reflects a person's general health and emotional well-being and it is associated with mortality. Skin aging is a compound phenotype, which can be assessed in many different ways. It is often divided in intrinsic (or innate) and extrinsic (or acquired) aging. There are distinctive characteristics between the two, but in practice it is difficult to separate them in UV-exposed areas such as the face. Their combined effects result in the compound phenotype facial skin aging, which can be divided into four major phenotypes: wrinkles, pigmented spots, telangiectasia and sagging. Another approach to investigate skin aging is by the phenotype perceived age (the estimated age of a person based on facial appearance). Skin aging is affected by multiple lifestyle and physiological factors. Given the complexity of facial aging, we decided to investigate determinants for different features of skin aging instead of focusing on the compound phenotype. In doing so, we aim to validate our digital measurement method used to quantify the different facial skin aging phenotypes. Furthermore, we aim to define main epidemiological determinants and genetic factors for the several skin aging features. In addition, we aim to clarify the relationship between vitamin D and skin aging. Lastly, we aim to understand the underlying correlation structure of skin aging. We performed the abovementioned epidemiological and genetic studies using data from the Rotterdam Study (RS), a large ongoing population-based cohort study in Ommoord (a suburb of Rotterdam in the Netherlands) with mainly northwestern European participants aged ≥40 years.

In **Chapter 2,** we validated a new digital image analysis technique to measure severity of different skin aging features including wrinkling, pigmented spots and telangiectasia. Within the RS, standardized three-dimensional facial photographs were taken. Two physicians independently graded 150 photographs for severity of wrinkles (full face, forehead, crow's feet, nasolabial fold, and upper lip), pigmented spots and telangiectasia, using photonumeric grading scales. Additionally, the affected percent of skin was digitally quantified. Sex-specific system optimization (N=50) and blinded validation using the photonumeric grades (N=100) were performed for each skin aging feature. The inter-rater reliability of the manual grading was good to excellent in all skin aging features (intra-class correlation coefficient between 0.65 and 0.93). A comparison of the digital and the manual approach showed excellent correlations between most features (Spearman's rho correlation coefficients between 0.52 and 0.89, except male upper lip wrinkling (ρ=0.30)). This shows that digitally quantified skin aging feature measures are comparable with the traditional manual measures, and therefore seem useful in future studies.

In **Chapter 3**, we investigated sex-specific determinants for facial wrinkles using the digitally quantified measures. We included standardized facial photographs of 3831 northwestern European participants of the RS (51-98 years, 58% female). Effect estimates from multivariable linear

regressions are presented as the percentage difference in the mean value of wrinkle area per unit increase of a determinant (%Δ). Wrinkle area was higher in men (median 4.5%, interquartile range: 2.9-6.3) than in women (3.6%, interquartile range: 2.2-5.6). Age was the strongest determinant; current smoking (men: 15.5%Δ; women: 30.9%Δ) and lower BMI (men: 1.7%Δ; women: 1.8%Δ) were also statistically significantly associated with increased wrinkling. Pale skin color showed a protective effect (men: -21.0%Δ; women: -28.5%Δ) and, in men, sunburn tendency was associated with less wrinkling. In women, low educational levels and alcohol use were associated with more wrinkling, whereas female pattern hair loss and a higher free androgen index were associated with less wrinkling. Thus, we validated known and identified additional determinants for wrinkling.

In **Chapter 4**, we searched for genetic variants associated with facial wrinkles using the digitally quantified measures. We performed a genome-wide association study in 3513 northwestern European participants of the RS (51-98 years). Replication analyses were performed in an independent Dutch cohort (Leiden Longevity Study, N=599), making use of photonumerically graded photographs. For the most significant single-nucleotide polymorphisms (SNPs) we performed sensitivity analyses to determine the effect of BMI, sex and site-specific wrinkling (crow's feet, forehead and in women upper lip wrinkles). None of the SNPs showed a genome-wide significant effect in the RS cohort, nor in the sensitivity analyses. However, one SNP, rs10476781, showed a genome-wide significant association with global facial wrinkling in the meta-analysis of the two cohorts ($P=2.19\times10^{-8}$). This intergenic SNP is located between the Neuromedin U Receptor 2 *(NMUR2)* gene and a non-protein coding RNA gene (*LINC01933*). *NMUR2* is expressed in the brain, which may suggest nervous system effects on facial wrinkling. Further studies are needed to validate the findings and understand their biological relevance.

In **Chapter 5**, we searched for genetic variants associated with pigmented spots using the digitally quantified measure. We performed a genome-wide association study in 2844 northwestern European participants of the RS. Age, female sex and pale skin color were associated with more pigmented spots. The percentage of affected facial skin area (mean 1.5% ± 0.9) was significantly associated with three genetic loci: *IRF4* (rs12203592, $P=1.8\times10^{-27}$), *MC1R* (compound heterozygosity score, $P=2.3\times10^{-24}$), and *RALY/ASIP* (rs6059655, $P=1.9\times10^{-9}$). Additionally, after adjustment for the other three top associated loci the *BNC2* locus demonstrated significant association (rs62543565, $P=2.3\times10^{-8}$). The association signals observed at all four loci were successfully replicated ($P<0.05$) in an independent Dutch cohort (Leiden Longevity Study, N=599). Although the four genes have previously been associated with skin color variation and skin cancer risk, all associations remained highly significant ($P<2\times10^{-8}$) when conditioning the association analyses on skin color. We conclude that genetic variation in *IRF4*, *MC1R*, *RALY/ASIP* and *BNC2* contribute to the acquired amount of facial pigmented spots during aging, through pathways independent of the basal melanin synthesis.

In **Chapter 6**, we investigated lifestyle and physiological factors associated with facial telangiectasia using the digitally quantified measure. We included standardized facial photographs of 2842 northwestern European participants of the RS (51-98 years, 57% female). Effect estimates from multivariable linear regressions are presented as the percentage difference in the mean value of telangiectasia area per unit increase of a determinant (%Δ). Significant determinants were older age (1.7%Δ per year), female sex (18.3%Δ), smoking (current versus never 38.4%Δ; former versus never 11.6%Δ), a high susceptibility to sunburn (10.2%Δ) and light skin color (pale vs. white-to-olive 31.4%Δ; white vs. white-to-olive 9.2%Δ). In this large cohort study, we confirmed known and described new determinants of facial telangiectasia.

In **Chapter 7**, we searched for genetic variants associated with facial perceived age, estimated from standardized facial photographs of 2693 northwestern European participants of the RS (51-87 years). We performed a genome-wide association study of perceived age, analyzing over eight million SNPs. The strongest genetic associations with perceived age were found for multiple SNPs in the *MC1R* gene ($P<1\times10^{-7}$). This effect was enhanced for a compound heterozygosity marker constructed from four pre-selected functional *MC1R* SNPs ($P=2.69\times10^{-12}$), which was replicated in an independent Dutch cohort (Leiden Longevity Study, N=599; P=0.042) and among 1173 Europeans of the TwinsUK Study ($P=3\times10^{-3}$). Individuals carrying the homozygote MC1R risk haplotype looked on average up to two years older than non-carriers. This association was independent of age, sex, skin color, wrinkling and pigmented spots. Furthermore, it persisted through different sun-exposure levels. Hence, a role for *MC1R* in youthful looks independent of its known melanin synthesis function is suggested. Our study uncovers the first genetic evidence explaining why some people look older for their age and provides new leads for further investigating the biological basis of this.

In **Chapter 8**, we investigated whether there are causal associations between 25-hydroxyvitamin D concentrations and features of skin aging in a bidirectional Mendelian randomization study. In the RS (N=3831; 58.2% women, median age 66.5 years) and Leiden Longevity Study (N=661; 50.5% women, median age 63.1 years), facial skin aging features (wrinkles, pigmented spots, perceived age) were assessed either manually or digitally using standardized facial photographs. Associations between 25-hydroxyvitamin D and skin aging features were tested by multivariable linear regression. Mendelian randomization analyses were performed using SNPs identified from previous genome-wide association studies. After meta-analysis of the two cohorts, we observed that higher serum 25-hydroxyvitamin D was associated with a higher perceived age ($P=3.6\times10^{-7}$), more skin wrinkling ($P=2.6\times10^{-16}$), but not with more pigmented spots (P=0.30). In contrast, a genetically determined 25-hydroxyvitamin D concentration was not associated with any skin aging feature (P>0.05). Furthermore, a genetically determined higher degree of pigmented spots was not associated with higher 25-hydroxyvitamin D (P>0.05). Our study did not indicate that associations between 25-hydroxyvitamin D and features of skin aging are causal.

In **Chapter 9**, we analyzed the underlying phenotypic correlation structure between seven features for facial aging: global wrinkles, perceived age (PA), Griffiths' manual photodamage grading, pigmented spots, telangiectasia, actinic keratosis (AK), and keratinocytic cancer (KC). Data on AK and KC were obtained via a full-body skin examination and the other features via standardized facial photographs of 1790 northwestern European participants of the RS with complete data on all of these features. We used principal component analysis (PCA) to derive principal components (PCs) of common variation between the features. We performed multivariable linear regressions between age, sex, BMI, smoking, and UV-exposure and the PC scores derived from PCA. We also tested the association between the main PC scores and 140 SNPs previously associated with skin aging phenotypes. Three main PCs explained 73% of the total variance of the aging phenotypes: a hypertrophic/wrinkling component (PC1: global wrinkling, PA and Griffiths' grading), an atrophic/skin color component (PC2: pigmented spots and telangiectasia) and a cancerous component (PC3: AK and KC). The associations between lifestyle and physiological determinants differed per PC. The strength of SNP associations also differed per component with the most SNP associations found with the atrophic component (e.g. the *IRF4* SNP (rs12203592); P-value=1.84×10^{-22}). Thus, using a hypothesis-free approach, we identified three major underlying phenotypes associated with skin aging. Associations between determinants for skin aging differed in magnitude and direction per component.

In **Chapter 10**, a general overview of the main findings is provided. The thesis shows that skin aging is a complex phenotype. Four different features of skin aging were analyzed, namely wrinkles, pigmented spots, telangiectasia, and perceived age. In doing so, mainly continuous digital measurements were used, as this is a feasible approach and offers objective and consistent outcomes. I have replicated known and identified additional lifestyle and physiological determinants for the different skin aging features. The varying associations of light skin color with the different skin aging features (less wrinkles, more pigmented spots and more telangiectasia) strengthen the concept that different skin types age in a different manner. Fair-skinned individuals tend to show more atrophic signs of aging (e.g. PS and telangiectasia), whereas darker-skinned individuals show more hypertrophic signs of aging (e.g. wrinkles). This is also confirmed by the principal component analysis. With the genome-wide association studies for wrinkles, pigmented spots and perceived age, associated gene variants were identified, with varying results per feature. Skin color genes were associated with pigmented spots and perceived age, but surprisingly not with wrinkles. This emphasizes the hypothesis that different skin aging features have different genetic architectures; that of wrinkles seems more complex. Furthermore, I presented a view on future skin aging research.

SAMENVATTING

Hoofdstuk 1 geeft een algemene inleiding op dit proefschrift. Huidveroudering in het gelaat heeft een grote invloed op de samenleving. Naast de duidelijke cosmetische relevantie, weerspiegelt het de algemene gezondheid van een persoon alsook de emotionele gesteldheid. Bovendien is het geassocieerd met mortaliteit. Huidveroudering is een samengesteld fenotype, dat op verschillende manieren kan worden geëvalueerd. Vaak wordt het gecategoriseerd in intrinsieke (aangeboren) en extrinsieke (verworven) veroudering. Er zijn kenmerkende verschillen tussen deze twee, maar in de praktijk is het moeilijk om ze van elkaar te onderscheiden in een UV-blootgesteld gebied zoals het gelaat. Het samengestelde fenotype huidveroudering kan worden ingedeeld in vier hoofdkenmerken: rimpels, pigmentvlekken, teleangiëctasieën en 'verzakking'. Een andere invalshoek om huidveroudering te benaderen is door het fenotype geschatte leeftijd (waargenomen leeftijd van iemand gebaseerd op uiterlijke kenmerken in het gelaat). Huidveroudering wordt beïnvloed door vele leefstijl en fysiologische determinanten. Vanwege de complexiteit van huidveroudering hebben wij besloten om niet het samengestelde fenotype, maar de afzonderlijke hoofdkenmerken te analyseren. Hiertoe gebruiken we digitale meetmethoden die we in dit proefschrift streven te valideren. Verder beogen we de belangrijke epidemiologische en genetische determinanten van de afzonderlijke huidverouderingskenmerken te definiëren. Tevens onderzoeken we de relatie tussen vitamine D en huidveroudering. Tot slot geven we de onderlinge verhoudingen tussen de verschillende huidverouderingskenmerken weer. Bovengenoemde vraagstukken hebben we beantwoord middels epidemiologische en genetische studies binnen de Rotterdam Studie (RS). Dit is een groot prospectief bevolkingsonderzoek in de Rotterdamse wijk Ommoord, met deelnemers van veelal Noordwest-Europese afkomst van 40 jaar en ouder.

In **Hoofdstuk 2** valideren we een nieuwe digitale beeldanalyse techniek, die de ernst van verschillende huidverouderingskenmerken kan meten, namelijk rimpels, pigmentvlekken en teleangiëctasieën. In de RS werden gestandaardiseerde driedimensionale foto's van het gelaat gemaakt. Twee artsen scoorden onafhankelijk 150 foto's op ernst van rimpels (hele gelaat, voorhoofd, kraaienpootjes, neuslippenplooi en bovenlip), pigmentvlekken en teleangiëctasieën, handmatig met behulp van foto-numerieke schalen. Vervolgens werd het aangedane percentage huid digitaal gekwantificeerd. Geslachtsspecifieke optimalisatie van het systeem (N=50) en geblindeerde validatie met behulp van de foto-numerieke schalen (N=100) werd uitgevoerd voor elk huidverouderingskenmerk. De betrouwbaarheid tussen verschillende scoorders ('inter-rater reliability') van de handmatige scores was voor alle huidverouderingskenmerken goed tot uitstekend (intraklasse correlatiecoëfficiënt tussen 0,65 en 0,93). De vergelijking tussen de digitale en handmatige methode liet een uitstekende correlatie zien voor de meeste kenmerken ('Spearman's rho' correlatie coëfficiënten tussen 0,52 en 0,89; behalve voor de bovenlip rimpels in mannen (ρ=0,30)). Dit laat zien dat digitaal gekwantificeerde huidverouderingsmaten overeenkomen met traditionele handmatige maten en daarom bruikbaar lijken voor toekomstig onderzoek.

In **Hoofdstuk 3** onderzoeken wij geslachtsspecifieke determinanten voor rimpels in het gelaat, gebruikmakend van de digitaal gekwantificeerde uitkomstmaten. We includeerden gestandaardiseerde foto's van het gelaat van 3831 Noordwest-Europese deelnemers van de RS (51-98 jaar, 58% vrouw). De effectmaten van de multivariabele lineaire regressie worden gepresenteerd als het percentage verschil in gemiddeld rimpel oppervlak (%Δ) per eenheid toe- of afname van een determinant; voor BMI bij mannen betreft dit -1,7%Δ. Dit houdt in dat per BMI punt hoger een man 1,7% minder rimpel oppervlak in het gelaat heeft. Rimpel oppervlak was overigens hoger bij mannen (mediaan 4,5%, interkwartielafstand [2,9-6,3]) dan bij vrouwen (mediaan 3,6%, interkwartielafstand [2,2-5,6]). Leeftijd was de sterkste determinant. Roken (mannen: 15,5%Δ; vrouwen: 30,9%Δ) en lager BMI (mannen: 1,7%Δ; vrouwen: 1,8%Δ) waren ook statistisch significant geassocieerd met toegenomen rimpels. Lichte huidskleur toonde een beschermend effect (mannen: -21,0%Δ; vrouwen: -28,5%Δ) en bij mannen was zonovergevoeligheid geassocieerd met minder rimpels. In de groep vrouwen waren laag opleidingsniveau en alcoholgebruik geassocieerd met meer rimpels, terwijl haarverlies volgens het vrouwelijk patroon en een hogere hoeveelheid androgenen (gemeten als vrije androgenen index) geassocieerd waren met minder rimpels. Concluderend hebben we in deze studie bekende determinanten voor rimpels bevestigd en nieuwe determinanten beschreven.

In **Hoofdstuk 4** zijn we op zoek gegaan naar genetische varianten geassocieerd met rimpels in het gelaat, gebruikmakend van de digitaal gekwantificeerde uitkomstmaten. We verrichtten een genoomwijde associatie studie (GWAS, 'genome-wide association study') in 3513 Noordwest-Europese deelnemers van de RS (51-98 jaar). Replicatie analyses werden uitgevoerd in een onafhankelijk Nederlands cohort (Leiden LangLeven Studie; LLS, N=599), gebruikmakend van handmatig foto-numeriek gescoorde foto's. Voor de meest significante enkel nucleotide polymorfismen (SNP's, 'single-nucleotide polymorphisms'; varianten in het DNA) hebben we sensitiviteitsanalyses verricht om het effect van BMI, geslacht en locatie van rimpels (kraaienpootjes en rimpels t.p.v. van voorhoofd en in vrouwen bovenlip) te bepalen. Geen van de SNP's toonden een genoom-wijd significant effect in het RS cohort, noch in de sensitiviteitsanalyses. Desondanks toonde in een meta-analyse van de twee cohorten één SNP (rs10476781) een genoom-wijd significante associatie met rimpels in het gehele gelaat ($P=2{,}19\times10^{-8}$). Deze SNP ligt tussen 2 genen in: het Neuromedin U Receptor 2 (*NMUR2*) gen en een non-eiwit-coderend RNA gen (*LINC01933*). *NMUR2* wordt tot expressie gebracht in het brein, wat suggereert dat het zenuwstelsel een rol zou kunnen spelen bij rimpels in het gelaat. Meer studies zijn echter nodig om deze bevindingen te valideren en de biologische relevantie te kunnen doorgronden.

In **Hoofdstuk 5** zijn we op zoek gegaan naar genetische varianten geassocieerd met pigmentvlekken, gebruikmakend van de digitaal gekwantificeerde uitkomstmaat. We verrichtten een GWAS in 2844 Noordwest-Europese deelnemers van de RS. Leeftijd, vrouwelijk geslacht en lichte huidskleur waren alle geassocieerd met meer pigmentvlekken. Het percentage aangedaan ge-

laatsoppervlakte (gemiddeld 1,5% ± 0,9) was significant geassocieerd met 3 genetische loci: *IRF4* (rs12203592, $P=1,8\times10^{-27}$), *MC1R* (samengestelde heterozygositeits score, $P=2,3\times10^{-24}$) en *RALY/ASIP* (rs6059655, $P=1,9\times10^{-9}$). Bovendien werd na aanpassing voor deze top 3 geassocieerde loci een associatie met het *BNC2* locus ontdekt (rs62543565, $P=2,3\times10^{-8}$). De associaties van deze vier loci werden succesvol gerepliceerd ($P<0,05$) in een onafhankelijk Nederlands cohort (LLS, N=599). Hoewel de vier genen eerder een associatie met huidskleur en huidkanker risico hebben vertoond, bleven alle associaties sterk significant ($P<2\times10^{-8}$) na aanpassing voor huidskleur. Wij concluderen dat de genetische varianten in *IRF4*, *MC1R*, *RALY/ASIP* en *BNC2* bijdragen aan de verworven hoeveelheid pigmentvlekken in het gelaat gedurende veroudering, via mechanismen onafhankelijk van de basale melanine aanmaak.

In **Hoofdstuk 6** onderzoeken wij leefstijl en fysiologische determinanten geassocieerd met teleangiëctasieën in het gelaat, gebruikmakend van de digitaal gekwantificeerde uitkomstmaat. We includeerden gestandaardiseerde foto's van het gelaat van 2842 Noordwest-Europese deelnemers van de RS (51-98 jaar, 57% vrouw). De effectmaten van de multivariabele lineaire regressie worden gepresenteerd als het percentage verschil in gemiddeld teleangiëctasieën oppervlak (%Δ) per eenheid toe- of afname van een determinant. Significante determinanten waren leeftijd, (1.7%Δ per jaar), vrouwelijk geslacht (18.3%Δ), roken (huidig t.o.v. nooit 38.4%Δ; vroeger t.o.v. nooit 11.6%Δ), zonovergevoeligheid (10.2%Δ) en lichte huidskleur (zeer licht t.o.v. olijfkleurig 31.4%Δ; licht t.o.v. olijfkleurig 9.2%Δ). In dit grote cohort onderzoek hebben we bekende determinanten voor teleangiëctasieën in het gelaat bevestigd en nieuwe determinanten beschreven.

In **Hoofdstuk 7** zijn we op zoek gegaan naar genetische varianten geassocieerd met geschatte leeftijd. Deze schatting werd gebaseerd op uiterlijke kenmerken in het gelaat op gestandaardiseerde foto's van 2693 Noordwest-Europese deelnemers van de RS (51-87 jaar). We verrichtten een GWAS van geschatte leeftijd, waarbij we meer dan acht miljoen SNP's analyseerden. De sterkste genetische associaties met geschatte leeftijd werden gevonden voor meerdere SNP's in het *MC1R* gen ($P<1\times10^{-7}$). Dit effect was versterkt voor een samengestelde heterozygositeits score, gecreëerd m.b.v. vier voorgeselecteerde functionele *MC1R* SNP's ($P=2,69\times10^{-12}$). Dit werd gerepliceerd in een onafhankelijk Nederlands cohort (LLS, N=599, P=0,042) en in 1173 Europese deelnemers van de 'TwinsUK' studie ($P=3\times10^{-3}$). Individuen met een homozygoot *MC1R* risico haplotype werden gemiddeld 2 jaar ouder geschat dan individuen die dit haplotype niet hadden. Deze associatie was onafhankelijk van leeftijd, geslacht, huidskleur, rimpels en pigmentvlekken. Het effect bleef significant onafhankelijk van verschillende maten van zon-expositie. Dit suggereert een rol voor *MC1R* in een jeugdig uiterlijk, onafhankelijk van zijn bekende functie in basale melanine aanmaak. Deze studie toont het eerste genetische bewijs dat verklaart waarom sommige mensen er ouder uitzien voor hun leeftijd. Verder biedt het aanknopingspunten voor het ontrafelen van de biologische basis van dit fenomeen.

In **Hoofdstuk 8** onderzoeken wij of er een causaal verband is tussen 25-hydroxyvitamine D en de verschillende huidverouderingskenmerken in een bidirectionele Mendeliaanse randomisatie studie. In de RS (N=3831; 58,2% vrouw, mediane leeftijd 66,5 jaar) en LLS (N=661; 50,5% vrouw, mediane leeftijd 63,1 jaar) werden huidverouderingskenmerken (rimpels, pigmentvlekken, geschatte leeftijd) digitaal dan wel handmatig gescoord o.b.v. gestandaardiseerde foto's van het gelaat. Associaties tussen 25-hydroxyvitamine D en huidverouderingskenmerken werden getest middels multivariabele lineaire regressie. Mendeliaanse randomisatie analyses werden uitgevoerd met SNP's die geïdentificeerd zijn uit eerdere genoomwijde associatie studies. Na meta-analyse van de twee cohorten zagen wij dat een hoger serum 25-hydroxyvitamine D geassocieerd was met een hogere geschatte leeftijd ($P=3,6\times10^{-7}$) en meer rimpels ($P=2,6\times10^{-16}$), maar niet met meer pigmentvlekken (P=0,30). Een genetisch vastgestelde 25-hydroxyvitamine D concentratie was daarentegen niet geassocieerd met de huidverouderingskenmerken (P>0,05). Voorts was een genetisch vastgestelde hogere mate van pigmentvlekken niet geassocieerd met een hogere 25-hydroxyvitamine D concentratie (P>0,05). Derhalve toonde onze studie niet aan dat de associaties tussen 25-hydroxyvitamine D en de huidverouderingskenmerken causaal waren.

In **Hoofdstuk 9** analyseren we de onderliggende fenotypische correlatie tussen zeven huidverouderingskenmerken: rimpels in het gehele gelaat, geschatte leeftijd, Griffiths' handmatige foto-numerieke huidverouderingsmaat, pigmentvlekken, teleangiëctasieën, actinische keratosen (AK) en keratinocyten huidkanker (KC, basaalcel- en plaveiselcelcarcinoom). Data over AK en KC werden verkregen middels volledig huidonderzoek en de andere kenmerken via gestandaardiseerde foto's van het gelaat. Wij onderzochten 1790 Noordwest-Europese deelnemers van de RS met complete onderzoeksgegevens. We verrichtten een hoofdcomponentenanalyse (PCA, 'principal component analysis') om de hoofdcomponenten (PC's, 'principal components') van de gezamenlijke variantie van de huidverouderingskenmerken te verkrijgen. We verrichtten tevens multivariabele lineaire regressie voor de verkregen PC scores en leeftijd, geslacht, BMI, roken en UV-blootstelling. We onderzochten ook de associaties tussen de hoofd PC scores en 140 SNP's die eerder geassocieerd werden met huidverouderingsfenotypes. Drie hoofd PC's verklaarden 73% van de totale variantie van de huidverouderingsfenotypes: een hypertrofische/rimpel component (PC1: rimpels in het gehele gelaat, geschatte leeftijd, Griffiths' handmatige foto-numerieke huidverouderingsmaat), een atrofische/huidskleur component (PC2: pigmentvlekken en teleangiëctasieën) en een cutane (pre-)maligniteit component (PC3: AK en KC). De associaties tussen leefstijl en fysiologische determinanten verschilden per PC. De sterkte van de SNP associaties verschilden ook per PC; de meeste SNP associaties werden gevonden in de atrofische component (bijv. *IRF4* SNP (rs12203592); $P=1,84\times10^{-22}$). Concluderend identificeerden wij middels een hypothese-vrije benadering drie onderliggende basis fenotypes geassocieerd met huidveroudering. Associaties met de determinanten verschilden zoals verwacht in richting en sterkte per component.

Hoofdstuk 10 geeft een algemeen overzicht weer van de belangrijkste bevindingen. Dit proefschrift laat zien dat huidveroudering een complex fenotype is. Er zijn vier verschillende huidverouderingskenmerken onderzocht, namelijk rimpels, pigmentvlekken, teleangiëctasieën en geschatte leeftijd. Daarbij zijn er voornamelijk continue digitale huidverouderingsmaten gebruikt aangezien dit objectieve en consistente uitkomsten biedt. Voor de verschillende huidverouderingskenmerken zijn bekende leefstijl en fysiologische determinanten bevestigd en nieuwe determinanten beschreven. De variërende associaties van lichte huidskleur met de verschillende huidverouderingskenmerken (minder rimpels, meer pigmentvlekken en meer teleangiëctasieën) bekrachtigen het concept dat verschillende huidtypes op verschillende manieren verouderen. Individuen met een lichte huid hebben de neiging om meer atrofische huidverouderingskenmerken te ontwikkelen (bijv. pigmentvlekken en teleangiëctasieën), terwijl individuen met een donkerdere huid meer hypertrofische kenmerken vertonen (bijv. rimpels). Dit wordt ook bevestigd door de hoofdcomponenten analyse. Met de GWAS voor rimpels, pigmentvlekken en geschatte leeftijd zijn er genetische varianten getoond met verschillende uitkomsten per kenmerk. Huidskleurgenen zijn geassocieerd met pigmentvlekken en geschatte leeftijd, maar verrassend genoeg niet met rimpels. Dit benadrukt de hypothese dat de verschillende huidverouderingskenmerken een verschillende genetische opmaak hebben; daarbij lijkt die van rimpels complexer. Voorts heb ik een visie op toekomstig huidverouderingsonderzoek gepresenteerd.

Chapter 12

Appendices

Abbreviations

List of co-authors

List of publications

PhD portfolio

Curriculum Vitae

Dankwoord

ABBREVIATIONS

%Δ	percentage change
AK	actinic keratosis
BMI	body mass index
EWAS	epigenome-wide association study
FAI	free androgen index
FPHL	female pattern hair loss
GWAS	genome-wide association study
GRS	genetic risk score
ICC	intraclass correlation coefficient
IQR	interquartile range
KC	keratinocyte cancer
MAF	minor allele frequency
MC1R	melanocortin 1 receptor
MMPs	matrix metalloproteinases
MR	Mendelian randomization
IBD	identity-by-descent
LD	linkage disequilibrium
LLS	Leiden Longevity Study
ρ	Spearman's correlation coefficient
PA	perceived age
PC	principal component
PCA	principal component analysis
PS	pigmented spots
ROS	reactive oxygen species
RS	Rotterdam Study
SD	standard deviation
SE	standard error
SHBG	sex hormone binding globulin
SNP	single-nucleotide polymorphism

LIST OF CO-AUTHORS

Marian Beekman
Department of Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands

A.J. de Craen
Department of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, The Netherlands

Joris Deelen
Department of Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands

Kirk W. Gossage
Formerly Unilever R&D, Trumbull, CT, United States

Christopher E. Griffiths
Dermatology Centre, Salford Royal Hospital, Manchester Academic Health Science Centre, The University of Manchester, Manchester, United Kingdom

David A. Gunn
Unilever Research and Development, Colworth Science Park, Sharnbrook, Bedfordshire, United Kingdom

Diana van Heemst
Department of Internal Medicine, Section of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, the Netherlands

Albert Hofman
Department of Epidemiology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

Loes M. Hollestein
Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

J.J. Houwing-Duistermaat
Formerly Department of Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands

M.A. Ikram
Department of Epidemiology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

Leonie C. Jacobs
Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

Manfred Kayser
Department of Genetic Identification, Erasmus MC University Medical Center Rotterdam, Rotterdam, Netherlands

J.C. Kiefte – de Jong
Department of Epidemiology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

Jaspal S. Lall
Unilever Research and Development, Colworth Science Park, Sharnbrook, Bedfordshire, United Kingdom

J.S. Laven
Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynaecology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

Fan Liu
Department of Genetic Identification, Erasmus MC University Medical Center Rotterdam, Rotterdam, Netherlands

S. Mekíc
Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

P.G. Murray
Formerly Unilever Research and Development, Colworth Science Park, Sharnbrook, Bedfordshire, United Kingdom

T. van der Nat
Department of Internal Medicine, Section of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, the Netherlands

Tamar E.C. Nijsten
Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

R. Noordam
Department of Internal Medicine, Section of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, the Netherlands

Luba M. Pardo Cortes
Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

Alastair R. Rae
Tessella, Abingdon Science Park, Abingdon, United Kingdom

T. Schikowski
IUF – Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany

Eline P. Slagboom
Department of Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands

Hae-Won Uh
Formerly Department of Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands

André G. Uitterlinden
Departments of Epidemiology and Internal Medicine, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

Peter Velthuis
Department of Dermatology, Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands

C. Wigmann
IUF – Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany

Andreas Wollstein
Section of Evolutionary Biology, Department of Biology II, University of Munich, Planegg-Martinsried, Germany

C. Zeng
Key Laboratory of Genomic and Precision Medicine, Beijing Institute of Genomics, University of Chinese Academy of Sciences, Beijing, China

M.C. Zillikens
Department of Internal Medicine, Erasmus MC University Medical Center Rotterdam, Rotterdam, the Netherlands

LIST OF PUBLICATIONS

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Jacobs LC, **Hamer MA**, Gunn DA, Deelen J, Lall JS, van Heemst D, Uh HW, Hofman A, Uitterlinden AG, Griffiths CEM, Beekman M, Slagboom PE, Kayser M, Liu F, Nijsten T. A Genome-Wide Association Study Identifies the Skin Color Genes IRF4, MC1R, ASIP, and BNC2 Influencing Facial Pigmented Spots. *J Invest Dermatol. 2015 Jul;135(7):1735-1742.*

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PHD PORTFOLIO

Name PhD student:	M.A. Hamer
Erasmus MC Department:	Dermatology
Research schools:	NIHES / MolMed
PhD period:	2012 – 2020
Promotor:	Prof. T.E.C. Nijsten
Copromotor:	Dr. L.M. Pardo Cortes

Activity	Year	Workload
General courses		
"Regelgeving en organisatie" (BROK), Erasmus MC	2013	1.0 ECTS
Integrity in Research	2014	0.3 ECTS
DOO-course Hospital Management	2017	1.0 ECTS
DOO-course Teach the Teacher	2018	1.0 ECTS
DOO-course Communication	2020	1.0 ECTS
Specific courses / workshops / seminars		
Biostatistics (NIHES)	2012	1.0 ECTS
Principles of Research in Medicine and Epidemiology (NIHES)	2012	0.7 ECTS
Classical Methods for Data-analysis (NIHES)	2012	0.7 ECTS
Principles of Genetic Epidemiology (NIHES)	2012	0.7 ECTS
Dermoscopy training	2012	5 hours
Introduction to SPSS (MolMed)	2013	1.0 ECTS
Genome-Wide Association Analysis (NIHES)	2013	1.4 ECTS
Logistic Regression (NIHES)	2013	1.0 ECTS
Introduction to R (MolMed)	2014	1.4 ECTS
Short course Biomedical English writing (MolMed)	2014	2 ECTS
Presentation techniques	2014	3 hours
Media contact for researchers	2015	2.5 hours
Attendance of (inter)national conferences / symposia		
6th International Dermato-Epidemiology Association (IDEA) conference, Malmö, Sweden	2012	1.0 ECTS
1st PhD weekend Dermatology Erasmus MC, Maastricht, the Netherlands	2012	1.0 ECTS
3rd Netherlands Consortium for Healthy Aging (NCHA) outreach meeting, den Haag, the Netherlands	2013	1.0 ECTS
14th Annual Scientific Meeting NVED, Lunteren, the Netherlands	2013	1.0 ECTS
AAV "Wetenschapsmiddag", Erasmus MC, Rotterdam	2013	5 hours
International Investigative Dermatology (IID) conference, Edinburgh, UK	2013	1.0 ECTS
4th Netherlands Consortium for Healthy Aging (NCHA) outreach meeting, Den Haag, the Netherlands	2013	1.0 ECTS

Activity	Year	Workload
23rd European Academy for Dermatology and Venerology (EADV) Conference, Amsterdam, Netherlands	2014	1.0 ECTS
2nd PhD weekend Dermatology Erasmus MC, Maastricht, the Netherlands	2014	1.0 ECTS
15th Annual Scientific Meeting NVED, Lunteren, Netherlands	2014	1.0 ECTS
Spa III: "Oncologie in de parel van de Ardennen", Spa, Belgium	2014	1.0 ECTS
3rd PhD weekend Dermatology Erasmus MC, Wassenaar, the Netherlands	2015	1.0 ECTS
AAV "Wetenschapsmiddag", Erasmus MC, Rotterdam	2015	5 hours
NVDV Scientific Meeting, Rotterdam, the Netherlands	2015	8 hours
17th International Master Course on Aging Skin (IMCAS) Congress, Paris, France	2015	1.0 ECTS
13th Aesthetic & Anti-aging Medicine World Congress (AMWC), Monte Carlo, Monaco	2015	1.0 ECTS
24th European Academy for Dermatology and Venerology (EADV) Conference, Copenhagen, Denmark	2015	1.0 ECTS
4th PhD weekend Dermatology Erasmus MC, Antwerpen, Belgium	2016	1.0 ECTS
1st European Dermato-Epidemiology Network (EDEN) Forum, Madrid, Spain	2017	1.0 ECTS
5th World Congress of Dermoscopy, Thessaloniki, Greece	2018	1.0 ECTS
28th European Academy for Dermatology and Venerology (EADV) Conference, Madrid, Spain	2019	1.0 ECTS
Oral presentations		
Skin Aging in the Rotterdam Study; 2020 Epidemiology meeting Erasmus MC, Rotterdam	2013	1.0 ECTS
Skin Aging; Skintermezzo, Erasmus MC, Rotterdam	2013	1.0 ECTS
Risk Factors for Facial Skin Aging; 23rd European Academy for Dermatology and Venerology (EADV) Conference, Amsterdam, Netherlands	2014	1.0 ECTS
Biostatistics; 2nd PhD weekend Dermatology Erasmus MC, Maastricht, the Netherlands	2014	1.0 ECTS
Huidvergrijzing in Rotterdam; NVDV Scientific Meeting, Rotterdam, the Netherlands	2015	1.0 ECTS
Onderzoek en de media; 4th PhD weekend Dermatology Erasmus MC, Antwerpen, Belgium	2016	1.0 ECTS
Vitamin D and Features of Skin Aging; 1st European Dermato-Epidemiology Network (EDEN) Forum, Madrid, Spain	2017	1.0 ECTS
Poster presentations		
Risk Factors for Facial Wrinkles in Europeans; Poster healthy aging conference	2013	1.0 ECTS
Epidemiology of Facial Wrinkles in Europeans; 15th Annual Scientific Meeting NVED, Lunteren, the Netherlands	2014	1.0 ECTS
Facial Aging; 13th Aesthetic & Anti-aging Medicine World Congress (AMWC), Monte Carlo, Monaco	2015	1.0 ECTS
Teaching		
Principal Component Analysis; "methodenuur"	2013	8 hours
The dilemma of the UV questions; research meeting	2014	8 hours
Outliers; research meeting	2015	8 hours
Mendelian randomization; research meeting	2016	8 hours
EADV fostering course on Clinical Research and Epidemiology, Erasmus MC, Rotterdam	2014	4 hours
EADV fostering course on Clinical Research and Epidemiology, Erasmus MC, Rotterdam	2016	4 hours
Pre-intern lessons for medical interns (e.g. acne, inflammatory diseases)	2018	1.0 ECTS

Activity	Year	Workload
Other		
PhD Day Erasmus MC, the Netherlands	2012	5 hours
PhD Day Erasmus MC, the Netherlands	2013	5 hours
National PhD day, the Hague, the Netherlands	2014	8 hours
"Methodenuur" Dermatologie Erasmus MC	2012-2016	50 hours
2020 meetings Epidemiology, Erasmus MC	2012-2013	10 hours
Organizing committee 2nd PhD weekend Dermatology Erasmus MC, Maastricht, the Netherlands	2014	1.0 ECTS
Organizing the start-up of Erasmus MC Aesthetics	2016	2.0 ECTS

CURRICULUM VITAE

Merel Aline Hamer is geboren op 15 mei 1984 te Dordrecht. Ze is opgegroeid in afwisselend Alblasserdam, Hong Kong, Taipei en Manilla. Zij behaalde in 2002 haar VWO-diploma cum laude aan Scholengemeenschap de Lage Waard in Papendrecht. Hierna is zij begonnen met de studie geneeskunde aan de Erasmus Universiteit Rotterdam. In het 2e jaar van de studie geneeskunde heeft zij een onderzoeksstage gedaan aan het Karolinska Institutet te Stockholm, Zweden. Haar afstudeeronderzoek rondde ze in 2007 af op de afdeling Nefrologie van het Erasmus MC Rotterdam. Tijdens haar co-schappen werd al snel haar interesse voor de Dermatologie gewekt; ter verdieping verrichtte zij in 2009 een keuze-coschap Dermatologie in het Sint Franciscus Gasthuis te Rotterdam. Om een brede basis als arts te vormen en vanwege de vele raakvlakken met de Dermatologie, deed ze ervaring op als ANIOS op de afdeling Interne Geneeskunde van het Sint Franciscus Gasthuis van 2009-2011. In 2011-2012 was ze een half jaar werkzaam als ANIOS Dermatologie in het Amphia Ziekenhuis te Breda. Per juni 2012 startte zij op de afdeling Dermatologie van het Erasmus MC met haar huidige promotieonderzoek. Daarnaast heeft zij in 2016 met veel plezier een actieve rol gespeeld bij het opzetten van Erasmus MC Aesthetics, het opleidings- en kenniscentrum op het gebied van cosmetische Dermatologie. Sinds 1 januari 2017 is zij in opleiding tot dermatoloog in het Erasmus MC Rotterdam.

DANKWOORD

Onvoorstelbaar, het is af! Ik had dit niet voor elkaar kunnen krijgen zonder steun van een heleboel mensen… dit laatste hoofdstuk is dan ook voor hen.

Mijn promotor, prof. dr. Nijsten. Beste Tamar, dank voor deze kans die je me hebt gegeven. En het vertrouwen dat je in me hebt gehouden. Ik zal ons pittige maar eerlijke gesprek eind 2019 niet snel vergeten. Je weet precies hoe je iemand kunt motiveren, maar ook je eigenzinnige helikopter view blijft me verbazen. Jouw kamer binnenkomen met tientallen vragen betekent naar buiten wandelen met concrete antwoorden in verhelderende schetsen. De kracht van een goede promotor! Daarbij onthield je ondanks je drukke schema ook mijn belangrijke life events, dat vind ik bijzonder.

Dear Luba, thank you for being my co-supervisor and helping me out with all the complicated genetic analyses. And for motivating me for that last step before finishing it all off. I remember our car trip to Antwerpen a few years ago, where our conversations moved further than just research and statistics. It was good to have you by my side.

Beste prof. dr. van der Lei, prof. dr. Kayser en prof. dr. Prens, dank voor de bereidheid om plaats te nemen in mijn leescommissie en dank voor de genomen moeite voor het doornemen van mijn proefschrift. Prof. dr. van der Lei, ik was aangenaam verrast door de doortastendheid van het telefoontje dat ik van u ontving tijdens mijn spreekuur over het versturen van mijn proefschrift. U had destijds al een vooruitziende blik m.b.t. COVID-19, maar gelukkig kan mijn verdediging in deze aangepaste setting toch doorgang vinden. Prof. dr. Prens, beste Errol, ik bewonder de rust die je uitstraalt, niets is je teveel en nooit verlies je je “cool”. Dat in combinatie met je creatieve klinische visie maakt tot een bijzondere supervisor van deze AIOS Dermatologie. Prof. dr. Kayser, dear Manfred, many thanks for your comments on my thesis, which showed you have really taken the time to go over it. I look back on many successful collaborations, even attracting the media’s attention at times…

Beste leden van de grote commissie, prof. dr. Ikram, prof. dr. Uitterlinden, dr. Gunn, dr. Velthuis; dank voor de bereidheid om deel uit te maken van mijn grote commissie en voor het met mij van gedachten wisselen tijdens de verdediging. Dear Dave, not sure how your Dutch is coming along so also a few words in English here for you… You are one of the main reasons I was able to do my PhD. You have played a vital role and have pushed all my papers to a higher level. I will never forget my visit to Unilever in England; you made me feel welcome and I much enjoyed your English sense of humor. Beste Peter, we hebben nauw samengewerkt tijdens het opzetten van Erasmus MC Aesthetics en ik ben bevoorrecht dat ik de cosmetische dermatologie van jou heb mogen

leren. Ik waardeer je geduld, rust, indrukwekkende ervaring en buitengewone vriendelijkheid: je straalt plezier uit in het delen van kennis en daarmee ben je voor mij de ultieme leermeester.

Alle coauteurs en anderen die aan de manuscripten hebben meegewerkt, dank voor jullie samenwerking. Ook wil ik alle deelnemers van de Rotterdam Studie en andere cohorten bedanken. Daarbij wil ik graag m'n waardering uitspreken voor alle medewerkers van het ERGO centrum: jullie maakten het altijd een fijne plek om te komen werken.

Mijn paranimfen Leonie en Marisa, wat een fijn gevoel dat jullie "achter" mij staan tijdens mijn promotie. Lieve Leo, mijn voorganger, mijn oud-collega, maar bovenal mijn vriendin. We hebben veel meegemaakt de afgelopen jaren en altijd kon ik op je bouwen. Ook tijdens de afronding wist ik je vaak te vinden, maar niets was je te veel. Wat een geluk heb ik toch met jou! Lieve M, onze vriendschap is op de werkvloer ontstaan toen wij als Jansen & Jansen door het ziekenhuis raasden. Ik bewonder je eindeloze toewijding en loyaliteit; je hebt een hart van goud en het staat je goed, paradijsvogel!

Lieve oud-kamergenoten: *GK-026* was lang een begrip op de Dermatologie. Het was een bijzondere tijd, we zagen elkaar soms vaker dan het thuisfront en hebben lief en leed met elkaar gedeeld. Robert, Emmilia, Leonie, Joris, Martijn, Hilke, Wendy, Joan, Eline en Kirtie: dank voor die onvergetelijke jaren. Sophie, jij hebt mij destijds geïntroduceerd bij Tamar en dat waardeer ik nog altijd.

Lieve (oud-)AIOS, ANIOS en onderzoekers, wat hebben we toch een leuke groep. Het werk voelt daarmee als een echte team effort; dank voor de flexibiliteit in de afronding van mijn promotie. Maar ook buiten werktijd zien we elkaar graag – ik heb al vele vrijdagmiddagborrels en (ski-) weekenden mogen meemaken met jullie gezelligerds! Lieve Kaar en Juut, m'n fijne mede-sjaarsen; inmiddels zijn we al een stuk verder in de opleiding, hebben we met z'n drieën de mannenkamer succesvol geïnfiltreerd en zijn we omgedoopt tot derma-blondes…

Ook de huidige onderzoekers wil ik graag extra bedanken, met name ook voor het openstellen van kamer NA-501 afgelopen januari waarin ik me weer even onderzoeker waande: dank voor jullie gastvrijheid! Loes, als post-doc ben je een belangrijke steunpilaar van de onderzoeksafdeling en een ster in epidemiologie – dank voor het delen van je kennis. Selma, ik had me geen betere opvolgster kunnen wensen; jouw discipline en doorzettingsvermogen zijn bewonderenswaardig.

Beste stafleden van de afdelingen Dermatologie van het Erasmus MC en het Albert Schweitzer Ziekenhuis: dank voor jullie inspirerende begeleiding in mijn opleidingstraject. In het bijzonder wil ik ook de ondersteuning bedanken voor alle hulp rondom de spreekuren en patiëntenzorg. Dr. van

Montfrans, beste Bibi, als opleider heb jij er mede voor gezorgd dat de olievlek van m'n promotie in een mooi vat kon worden gegoten.

Marry, ik waardeer je creativiteit en je directheid; terug te vinden in mijn interieur en inmiddels ook in de mooie kaft van dit proefschrift, samen met je kleindochter ontworpen.

Lieve vriendinnen! Vroeger zagen we elkaar elke zondag op het hockeyveld en tegenwoordig regelmatig bij etentjes, weekendjes weg of verre reizen: An, Fiep, Jos, Juud, Kel, Kiek, Liek, Miek, Mies, Nien, San: de mooie momenten met jullie zijn me heel veel waard. Ils en Roos, wij drieën kennen elkaar al sinds de peuterspeelzaal en zijn elk een andere weg ingeslagen. Toch vinden we gelukkig altijd nog ergens de tijd om elkaar weer even bij te praten. Claire en Marloes, m'n honingbijstraat-huisgenoten, jullie vormden de perfecte thuisbasis van m'n studententijd. An, jouw Zweedse levenslessen brengen me wijsheid, eller hur?! Sil, wat een geluk dat je ooit mijn bovenbuurvrouw was; je Italiaanse spontaniteit, stoere kijk op het leven en je oprechte lach maken je tot een uniek mens. Lies, je bent een voorbeeld voor mij hoe je ondanks de drukte van werk en gezin alles zo goed kunt managen en toch ook tijd voor anderen hebt. Lotte, jouw eerlijke en wijze woorden geven me rust in alle hectiek. Tanne, jij bent voor mij als "thuis"; een grenzeloos waardevolle constante factor in mijn leven bij wie ik altijd aan mag schuiven.

Lieve pap en mam, de onvoorwaardelijke liefde die ik van jullie voel is onbeschrijfelijk. Jullie zijn er altijd. Zorgeloos opgroeien aan de andere kant van de wereld heeft me gevormd tot wie ik nu ben en daar ben ik jullie zó dankbaar voor. Thuiskomen voelt nog altijd even vertrouwd en ik hou zielsveel van jullie.

Simon en Charlotte – onze band reikt verder dan iemand zich ooit kan voorstellen. Wij zijn een absolute drie-eenheid. Simon, voor altijd ben jij mijn grote broer. Trots ben ik op wat jij allemaal hebt bereikt en waar je voor staat. Samen met Roelie kun je de hele wereld aan. Lot, mijn kleine zusje maar vooral mijn grote vriendin. Wij zijn twee handen op één buik, compleet op elkaar ingespeeld en hoeven elkaar niets uit te leggen. Ik vertrouw je blindelings. Luc haalt het beste in jou naar boven en ik ben blij dat onze jaarlijkse ski-roadtrips inmiddels traditie worden. Kleine grote schatten Gijs, Floor en Florian: jullie geven ongelofelijk veel liefde en ik rijd graag naar den Haag voor jullie dikke knuffels.

Lieve Pieter Jan. Wat ben ik blij dat ik jou heb leren kennen! Jij bent zowel m'n *instant gratification monkey* als de reden dat dit proefschrift nu is afgerond. Ik geniet van jouw aanwezigheid in m'n buurt en kijk uit naar onze toekomstige avonturen... Tot hier en nu samen verder!