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).

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REFERENCES

1. Decates T, de Wijs L, Nijsten T, Velthuis P. Numbers on injectable treatments in the Netherlands in 2016. *J Eur Acad Dermatol Venereol*. 2018;32(8):e328-e30.
2. Christensen K, Thinggaard M, McGue M, Rexbye H, Hjelmborg JV, Aviv A, et al. Perceived age as clinically useful biomarker of ageing: cohort study. *BMJ*. 2009;339:b5262.
3. Gupta MA, Gilchrest BA. Psychosocial aspects of aging skin. *Dermatol Clin*. 2005;23(4):643-8.
4. Yaar M, Eller MS, Gilchrest BA. Fifty years of skin aging. *J Investig Dermatol Symp Proc*. 2002;7(1):51-8.
5. Uitto J, Bernstein EF. Molecular mechanisms of cutaneous aging: connective tissue alterations in the dermis. *J Investig Dermatol Symp Proc*. 1998;3(1):41-4.
6. El-Domyati M, Attia S, Saleh F, Brown D, Birk DE, Gasparro F, et al. Intrinsic aging vs. photoaging: a comparative histopathological, immunohistochemical, and ultrastructural study of skin. *Exp Dermatol*. 2002;11(5):398-405.
7. Gilchrest BA. Skin aging and photoaging: an overview. *J Am Acad Dermatol*. 1989;21(3 Pt 2):610-3.
8. Vierkotter A, Ranft U, Kramer U, Sugiri D, Reimann V, Krutmann J. The SCINEXA: a novel, validated score to simultaneously assess and differentiate between intrinsic and extrinsic skin ageing. *J Dermatol Sci*. 2009;53(3):207-11.
9. Yaar M, Gilchrest BA. Ageing and photoageing of keratinocytes and melanocytes. *Clin Exp Dermatol*. 2001;26(7):583-91.
10. Varani J, Warner RL, Gharaee-Kermani M, Phan SH, Kang S, Chung JH, et al. Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J Invest Dermatol*. 2000;114(3):480-6.
11. Rock K, Fischer JW. [Role of the extracellular matrix in extrinsic skin aging] Rolle der extrazellularen Matrix bei der extrinsischen Hautalterung. *Hautarzt*. 2011;62(8):591-7.
12. Gilchrest BA, Stoff JS, Soter NA. Chronologic aging alters the response to ultraviolet-induced inflammation in human skin. *J Invest Dermatol*. 1982;79(1):11-5.
13. Chung JH, Kang S, Varani J, Lin J, Fisher GJ, Voorhees JJ. Decreased extracellular-signal-regulated kinase and increased stress-activated MAP kinase activities in aged human skin in vivo. *J Invest Dermatol*. 2000;115(2):177-82.
14. Bolognia J. Dermatology: Elsevier; 2018. 2752 p.
15. Fisher GJ, Kang S, Varani J, Bata-Csorgo Z, Wan Y, Datta S, et al. Mechanisms of photoaging and chronological skin aging. *Arch Dermatol*. 2002;138(11):1462-70.
16. Kohl E, Steinbauer J, Landthaler M, Szeimies RM. Skin ageing. *J Eur Acad Dermatol Venereol*. 2011;25(8):873-84.
17. Griffiths CE, Wang TS, Hamilton TA, Voorhees JJ, Ellis CN. A photonumeric scale for the assessment of cutaneous photodamage. *Arch Dermatol*. 1992;128(3):347-51.
18. Guinot C, Malvy DJ, Ambroisine L, Latreille J, Mauger E, Tenenhaus M, et al. Relative contribution of intrinsic vs extrinsic factors to skin aging as determined by a validated skin age score. *Arch Dermatol*. 2002;138(11):1454-60.
19. Larnier C, Ortonne JP, Venot A, Faivre B, Beani JC, Thomas P, et al. Evaluation of cutaneous photodamage using a photographic scale. *Br J Dermatol*. 1994;130(2):167-73.
20. Monestier S, Gaudy C, Gouvernet J, Richard MA, Grob JJ. Multiple senile lentigos of the face, a skin ageing pattern resulting from a life excess of intermittent sun exposure in dark-skinned caucasians: a case-control study. *Br J Dermatol*. 2006;154(3):438-44.
21. R B. Skin Aging Atlas - Caucasian Type. Paris: Med Com; 2007.

22. Hatzis J. The wrinkle and its measurement--a skin surface Profilometric method. *Micron*. 2004;35(3):201-19.
23. Lemperle G, Holmes RE, Cohen SR, Lemperle SM. A classification of facial wrinkles. *Plast Reconstr Surg*. 2001;108(6):1735-50; discussion 51-2.
24. Jacobi U, Chen M, Frankowski G, Sinkgraven R, Hund M, Rzany B, et al. In vivo determination of skin surface topography using an optical 3D device. *Skin Res Technol*. 2004;10(4):207-14.
25. Luebberding S, Krueger N, Kerscher M. Quantification of age-related facial wrinkles in men and women using a three-dimensional fringe projection method and validated assessment scales. *Dermatol Surg*. 2014;40(1):22-32.
26. Gossage KWW, J.; Velthuizen, R. Segmentation of hyperpigmented spots in human skin using automated cluster analysis. *Proc SPIE*. 2009:7161.
27. Miyamoto K, Takiwaki H, Hillebrand GG, Arase S. Development of a digital imaging system for objective measurement of hyperpigmented spots on the face. *Skin Res Technol*. 2002;8(4):227-35.
28. Stamatas GNB, C..J.; Kollias, N. Hyperspectral image acquisition and analysis of skin. *Proc SPIE*. 2003:4959.
29. Borkan GA, Norris AH. Assessment of biological age using a profile of physical parameters. *J Gerontol*. 1980;35(2):177-84.
30. Bulpitt CJ, Markowe HL, Shipley MJ. Why do some people look older than they should? *Postgrad Med J*. 2001;77(911):578-81.
31. Christensen K, Iachina M, Rexbye H, Tomassini C, Frederiksen H, McGue M, et al. "Looking old for your age": genetics and mortality. *Epidemiology*. 2004;15(2):251-2.
32. Daniell HW. Smoker's wrinkles. A study in the epidemiology of "crow's feet". *Ann Intern Med*. 1971;75(6):873-80.
33. Green AC, Hughes MC, McBride P, Fourtanier A. Factors associated with premature skin aging (photoaging) before the age of 55: a population-based study. *Dermatology*. 2011;222(1):74-80.
34. Ernster VL, Grady D, Miike R, Black D, Selby J, Kerlikowske K. Facial wrinkling in men and women, by smoking status. *Am J Public Health*. 1995;85(1):78-82.
35. Suppa M, Elliott F, Mikeljevic JS, Mukasa Y, Chan M, Leake S, et al. The determinants of periorbital skin ageing in participants of a melanoma case-control study in the U.K. *Br J Dermatol*. 2011;165(5):1011-21.
36. Martires KJ, Fu P, Polster AM, Cooper KD, Baron ED. Factors that affect skin aging: a cohort-based survey on twins. *Arch Dermatol*. 2009;145(12):1375-9.
37. Youn CS, Kwon OS, Won CH, Hwang EJ, Park BJ, Eun HC, et al. Effect of pregnancy and menopause on facial wrinkling in women. *Acta Derm Venereol*. 2003;83(6):419-24.
38. Ezzedine K, Mauger E, Latreille J, Jdid R, Malvy D, Gruber F, et al. Freckles and solar lentigines have different risk factors in Caucasian women. *J Eur Acad Dermatol Venereol*. 2013;27(3):e345-56.
39. Bastiaens M, Hoefnagel J, Westendorp R, Vermeer BJ, Bouwes Bavinck JN. Solar lentigines are strongly related to sun exposure in contrast to ephelides. *Pigment Cell Res*. 2004;17(3):225-9.
40. van Drielen K, Gunn DA, Griffiths CE, Griffiths TW, Ogden S, Noordam R, et al. Markers of health and disease and pigmented spots in a middle-aged population. *Br J Dermatol*. 2015;173(6):1550-2.
41. Isik B, Gurel MS, Erdemir AT, Kesmezacar O. Development of skin aging scale by using dermoscopy. *Skin Res Technol*. 2013;19(2):69-74.
42. Kennedy C, Bastiaens MT, Bajdik CD, Willemze R, Westendorp RG, Bouwes Bavinck JN, et al. Effect of smoking and sun on the aging skin. *J Invest Dermatol*. 2003;120(4):548-54.
43. Jacobs LC, Liu F, Bleyen I, Gunn DA, Hofman A, Klaver CC, et al. Intrinsic and extrinsic risk factors for sagging eyelids. *JAMA Dermatol*. 2014;150(8):836-43.

44. Le Clerc S, Taing L, Ezzedine K, Latreille J, Delaneau O, Labib T, et al. A genome-wide association study in Caucasian women points out a putative role of the STXBP5L gene in facial photoaging. *J Invest Dermatol*. 2013;133(4):929-35.
45. Chang AL, Atzmon G, Bergman A, Brugmann S, Atwood SX, Chang HY, et al. Identification of genes promoting skin youthfulness by genome-wide association study. *J Invest Dermatol*. 2014;134(3):651-7.
46. Elfakir A, Ezzedine K, Latreille J, Ambroisine L, Jdid R, Galan P, et al. Functional MC1R-gene variants are associated with increased risk for severe photoaging of facial skin. *J Invest Dermatol*. 2010;130(4):1107-15.
47. Law MH, Medland SE, Zhu G, Yazar S, Vinuela A, Wallace L, et al. Genome-Wide Association Shows that Pigmentation Genes Play a Role in Skin Aging. *J Invest Dermatol*. 2017;137(9):1887-94.
48. Gunn DA, Rexbye H, Griffiths CE, Murray PG, Fereday A, Catt SD, et al. Why some women look young for their age. *PLoS One*. 2009;4(12):e8021.
49. Vierkotter A, Kramer U, Sugiri D, Morita A, Yamamoto A, Kaneko N, et al. Development of lentigines in German and Japanese women correlates with variants in the SLC45A2 gene. *J Invest Dermatol*. 2012;132(3 Pt 1):733-6.
50. Bastiaens M, ter Huurne J, Gruis N, Bergman W, Westendorp R, Vermeer BJ, et al. The melanocortin-1-receptor gene is the major freckle gene. *Hum Mol Genet*. 2001;10(16):1701-8.
51. Ikram MA, Brusselle GGO, Murad SD, van Duijn CM, Franco OH, Goedegebure A, et al. The Rotterdam Study: 2018 update on objectives, design and main results. *Eur J Epidemiol*. 2017;32(9):807-50.