Aus der Hochschulklinik für
Dermatologie, Venerologie und Allergologie,
Immunologisches Zentrum, Städtisches Klinikum Dessau

DISSERTATION

Ageing-related molecular changes of the skin on the protein level – An immunohistochemichal study

Alterungsbedingte molekulare Veränderungen der Haut an der Proteinebene – Eine immunhistochemische Studie

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Rana Elewa

aus Kairo, Ägypten

Datum der Promotion: 05.03.2021

Table of Contents

List of Abbreviations

CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor-1
CRHR2	Corticotropin-releasing hormone receptor-2
CRHBP	Corticotropin-releasing hormone-binding protein
PPAR-α	Peroxisome proliferator-activated receptor alpha
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPAR-δ	Peroxisome proliferator-activated receptor delta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-17	Interleukin-17
TLR-4	Toll-like receptor 4
NF-kB	Nuclear factor kappa beta
TNF-α	Tumor necrosis factor alpha
LPS	Lipopolysaccharides

List of Figures

List of Tables

Ageing-related molecular changes of the skin on the protein level – An immunohistochemical study

Abstract

Introduction: Our planet hosts an ageing community. Ageing is a complex process that results in dysfunction or a decline of function of many systems. Skin ageing is the most apparent form of ageing and could reflect ageing of inner organs. Being a mirror of the body, skin is a major target of ageing research.

Methodology: Immunohistochemistry was used to evaluate molecular changes at the protein level for some of the molecules involved in different possible mechanisms of the ageing process, namely the innate immunity signalling, tumorigenesis, inflammation and the stress response regulation systems. Skin samples used in this study were obtained from the skin of 42 healthy individuals of different ages. The expression of the investigated molecules (corticotropin-releasing hormone, corticotropin-releasing hormone receptors 1 and 2, corticotropin-releasing hormone-binding protein, peroxisome proliferator-activated receptors-alpha, gamma and delta, Toll-like receptor 4, interleukins 6 and 8) was localized in the skin using immunohistochemistry and evaluated using a semiquantitative evaluation method.

Results: The molecules studied were expressed in almost all skin compartments and exhibited significant ageing-associated changes in epithelial tissues, mostly in the sebaceous glands, the sweat glands and the epidermis. Ageing-associated up-regulation was detected for corticotropin-releasing hormone-binding protein, corticotropin-releasing hormone receptor 1 and peroxisome proliferator-activated receptor delta, interleukin 6 and IL-8. Moreover, ageing-associated down regulation was detected for Toll-like receptor 4 and peroxisome proliferator-activated receptor alpha.

Conclusion: This study could explain a part of the physiology of the ageing process. The results obtained confirmed the presence of independent neuroendocrine system, innate immunity and lipid metabolism systems in the skin. They also indicated

an interaction between different pathways of ageing, namely defective stress and down-regulated innate immunity responses and activation of the tumorigenesis pathway, especially apparent in the sebaceous glands.

Alterungsbedingte molekulare Veränderungen der Haut an der Proteinebene – Eine immunhistochemische Studie

Abstract

Einleitung: Unser Planet beherbergt eine alternde Gesellschaft. Alterung ist ein komplexer Prozess, der den Niedergang der Funktion oder eine Dysfunktion vieler Systeme beinhaltet. Die Hautalterung ist die offensichtlichste Form der Alterung und könnte die Alterung der inneren Organe widerspiegeln. Als Spiegel des Körpers ist die Haut ein wichtiges Ziel für die Altersforschung.

Methodik: Immunhistochemische Untersuchung sollten die molekularen Veränderungen einiger Moleküle der verschiedenen möglichen Alterungsprozessmechanismen auf Proteinebene bewerten. Solche Mechanismen sind nämlich die angeborenen Signalwege der Immunität, die Tumorentstehung, Entzündungen und die Regelsysteme der Stressreaktion. Die Hautproben dieser Arbeit wurden von 42 gesunden Personen unterschiedlichen Alters gewonnen. Die Expression der untersuchten Moleküle (Corticotropin freisetzendes Hormon, Corticotropin freisetzendes Hormon-Rezeptoren 1 und 2, Corticotropin freisetzendes-Hormon-Bindungsprotein, Peroxisom-Proliferator-Aktivierte Rezeptoren-alpha, gamma und delta, Toll-like Rezeptor 4, Interleukin 6 und 8) wurden in der Haut mit einer semiquantitativen Bewertungsmethode lokalisiert und bewertet.

Ergebnisse: Die untersuchten Moleküle wurden in fast alle Hautkompartimente nachgewiesen. Eine signifikante alterungsbedingte Änderung der Expression in epithelialen Geweben, insbesondere in den Talgdrüsen, Schweißdrüsen und der Epidermis wurde bemerkt. Eine alterungsbedingte Steigerung der Expression wurde für

Corticotropin freisetzendes-Hormon-Bindungsprotein, Corticotropin freisetzendes Hormon-Rezeptor 1 und Peroxisom Proliferator-aktivierte Rezeptor-delta, Interleukin 6 und Interleukin 8 bemerkt. Darüber hinaus wurde für den Toll-like-Rezeptor 4 und den Peroxisom-Proliferator-Aktivierte Rezeptor-alpha eine alterungsbedingte Herunterregulierung festgestellt.

Schlussfolgerung: Die erhaltenen Ergebnisse zeigen Wechselwirkungen zwischen verschiedenen Alterungswegen, nämlich einer fehlerhaften Stressreaktion und einem abwärtsregulierten Signalwege der angeborenen Immunität und einer Aktivierung der Tumorgenese-Signalwege, besonders deutlich in den Talgdrüsen.

1. Introduction

Ageing is an inevitable physiological process which is influenced by a multiple of heritable and environmental factors. The mechanism of the ageing process is not yet fully elucidated. Ageing results - among others – in dysfunction or a decline of functions of the skin, which exhibits the most apparent ageing-related changes. These changes not only involve morphological changes, such as skin thickness, wrinkle formation, dryness, pigmentation, but also extend to involve functional changes as susceptibility to infections, loss of elasticity, delayed healing and proliferative lesions. As well as changes in the lipid metabolism, sebum composition, extracellular matrix, cytoskeleton proteins, skin stress response reaction and gene expression[1,2]. The use of skin samples for ageing research could overcome the obstacles associated with collecting samples from internal organs.

The study was conducted to detect the changes in expression of different molecules and receptors - on the protein level - during the skin ageing process and to assess their interaction, which could be a supportive investigation towards elucidating the physiology of ageing. The molecules chosen for this study are selected according to their well-known role in inflammation, chronic disease and ageing-associated conditions in humans as well as in other species.

This introduction will shed light on the role of the chosen molecules - (corticotropin-releasing hormone (CRH) system, peroxisome proliferator-activated receptors alpha, gamma and delta (PPAR-α, -γ, -δ), interleukin 6 and 8 (IL-6, IL-8) and the Toll-like receptor (TLR-4) - in different ageing-associated conditions and diseases in humans and other species to justify their selection for the study.

The defective stress response, chronic inflammation, defective immunological response, delayed metabolism and tumourigenesis are events characteristic of ageing[3,4]. The molecules examined in the current study were found in many previous studies to be expressed in the skin. Moreover, they were also found to contribute to the

ageing process of different tissues and species, as they have been shown to be altered in several age-associated conditions[5,6], and in animal ageing models[7-9]. The corticotropin-releasing hormone (CRH) system controls the local stress skin response and is involved in inflammatory skin diseases[10], peroxisome proliferator-activated receptors alpha, gamma and delta (PPAR-α, -γ, -δ), are known to play a critical role in tumourigenesis and metabolism[11], the inflammatory cytokines interleukin 6 and 8 (IL-6, IL-8) and the Toll-like receptor (TLR)-4 are involved in innate immunity and chronic inflammation[12].

Chronic stress - which is mainly controlled through the CRH system molecules - has been accused of being a cornerstone of pathophysiology in several diseases, including those of the skin, causing exacerbation or even initiation of the disease[13]. Interestingly, stress response tends to be less efficient with increasing age, as the CRH system exhibits an age–dependent disturbance of function[14]. Among healthy elderly, an increased cortisol response to inflammatory challenges - a downstream reaction of the CRH system - may be a risk factor for developing Alzheimer's disease, depression[15], diabetes, metabolic syndrome and hypertension[16].

PPARs play an important role in insulin action, lipid metabolism, fat re-distribution and regulation of ageing and longevity[17,18]. PPAR-γ agonists could have a direct anti-ageing effect through increasing the transcription of the Klotho gene, which is an anti-ageing gene[19]. Caloric restriction, which is the most effective intervention known to delay ageing, affects the same physiological functions as PPARs[20]. PPARs were proved to regulate the transcription factor NF-kB, which plays a role in regulating age-related inflammatory process, where it activates transcription of pro inflammatory genes[21]. PPAR-γ and PPAR-α expression were found to be decreased in the brain and spleen of old rodents in comparison to the young ones[8].

Greater susceptibility to infection[22] and poorer response to vaccination[23] - in which TLR-4 plays a major role - are important features of ageing. TLR-4 expression is down-regulated in cervical neoplasia, one of the age-related cancers which could be attributed to the known infective aetiology of the cervical neoplasia, where the defective

anti-human papilloma virus immune response is the main cause of neoplastic transformation[24].

The data involving TLR-4 in ageing animal models showed variable results. The TLR-4 protein surface expression levels were lower in macrophages of aged mice, and their stimulation led to less IL-6 production[7]. Consistent with this, in mouse models of Alzheimer's disease, reduction of TLR-4 led to accumulation of Abeta deposits in neurons, and hence progression of the Alzheimer's disease[25]. Surprisingly contradicting this, the brains of aged mice showed significantly higher TLR-4, associated with higher inflammatory cytokine expression [IL-6 and tumor necrosis factor (TNF)-alpha] in response to lipopolysaccharide (LPS) injection[26]. On the other hand, the pro inflammatory alleles of TLR-4 could be related to successful ageing, due to better resistance to infections[27].

TLR-4 was reported to be involved in Alzheimer's disease progression, as the increased expression of the pro-inflammatory cytokines - IL-6, TNF-alpha and IL-17 - are TLR-4-dependent. Levels of TLR-4 were decreased in inferior parietal cortex tissue specimens from end-stage Alzheimer's disease patients compared to age-matched control subjects, which may indicate that an initial over expression of the TLR-4 in the diseased neurons led to their destruction by the inflammatory process[28].

Numerous studies have shown that levels of several cytokines, and especially IL-6 and TNF-alpha, increase with age even in apparently healthy individuals and in the absence of acute infection[29-32]. This state is further exacerbated in conditions of severe stress such as burns or sepsis[33] and gonadectomy[9].

IL-6 is a non-specific marker for many age-related disease processes[29] such as diabetes[34] atherosclerosis, depression[35], Alzheimer's disease[36], prostate cancer[37], rheumatoid arthritis[38], obstructive sleep apnea, hypertension, increased vulnerability to systemic inflammation, neuronal loss, ageing-related cognitive deficits[39] and reduced muscle strength[40].

Age-related increased vulnerability to systemic inflammation is due in part to augmented IL-6 production by adipose tissue[41]. IL-6 plays a role in progression and development of many tumours[42] including prostate cancer[37] and pancreatic cancer[43]. It also accounts for tumour related constitutional symptoms and cachexia[42]. Together with IL-6, IL-8 may contribute to the inflammatory processes associated with cortical atrophy in the ageing brain as well as the age-related cognitive decline[44]. IL-8 is an important mediator of neuronal death in the age-related Alzheimer's disease[45].

The reported data justifies the choice of the molecules to be examined in the current study.

2. Methodology

2.1 Skin sampling

We performed immunohistochemical staining to examine the expression of the following antigens: CRH, CRHBP, CRHR1, CRHR2, PPAR-α, PPAR-γ, PPAR-δ, IL-6, IL-8 and TLR-4 in skin samples from the face and scalp of 42 healthy individuals aged 18 to 92 years (49.85+/-23.97, males n=26, females n= 16) (Appendix 1).

We performed the sample collection in the period from 01.2009 to 08.2009. The volunteers were recruited from the Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Centre, Dessau, Germany and the Ain Shams Faculty of Medicine University Hospitals, Cairo, Egypt. Written informed consents were received from the volunteers to use the skin samples in experimental research work. We conducted this study according to the ethical standards of the Ethics Committee of the Charité Universitätsmedizin Berlin (# iPF-C 01GN0808) and the Faculty of Medicine Ain Shams University Research Ethics Committee (# FMASU 232/2009).

We excluded individuals suffering from thyroid dysfunction, hyperandrogenism, polycystic ovary, acne, atopic dermatitis, seborrheic dermatitis, or who were pregnant, or breastfeeding. Also, individuals receiving treatment with retinoids, fibrates, PPAR agonists, hormone replacement therapy of all kinds, antihistamines and steroids for the last four weeks before sample collection were excluded. The included individuals had photo-types II, III or IV, and a body mass index < 40. Smokers were excluded.

2.2 Immunohistochemical staining and evaluation

The specimens were fixed in spirit formol 4% fixative for at least 12 hours at 4°C, rinsed in 0.01 phosphate-buffer saline and embedded in paraffin wax. The blocks were sectioned 3 µm thick and mounted on positively charged slides (FLEX IHC Microscope Slides, Dako, code #K8020). Three different specimens were mounted on each slide, to cut down the consumption of the reagents. The specimens on each slide were numbered with a code to ensure blind evaluation. The slides were dried at room

temperature then heated in an oven for 30 minutes at 60°C. Sections from each biopsy were deparaffinized and rehydrated through a graded series of ethanol then rinsed in distilled water. We retrieved the antigens through pre-treatment of the specimens to maximise a specific antigen-antibody interaction. We testes various pre-treatment procedures to detect the most appropriate pre-treatment for antigen retrieval. (steaming in 6.1 or 9 pH buffers, protease pre-treatment with or without boiling).

We used the steaming in pH 6.1 buffer (Dako target retrieval solution code S#1699-lot # 10032884) for staining of IL-6 and TLR-4, and pH 9.0 buffer (Dako target retrieval solution code S#2367-lot # 00052766) for the rest of the antigens. The specimens were left to steam at 99°C for 20 min and then left in the buffer for another 20 min to cool down after boiling, then rinsed with buffer before staining.

The staining of the slides was performed in humidified chambers. All staining and developing system reagents were equilibrated to the room temperature (20-25°C) before use. At the end of each step and before the addition of a second reagent, the slides were thoroughly washed with buffer.

Goat, rabbit or mouse primary antibodies diluted with antibody reducing diluent were used according to manufacturers' instructions. We modified the concentration and incubation time by trial and error (Table 1). We used Biotinylated anti-mouse/ anti-rabbit or rabbit/anti-goat immunoglobulins as secondary antibodies according to the origin of the primary antibodies.

The slides were incubated with streptavidin enzyme conjugate (streptavidin alkaline phosphatase Conjugated, Dako-Cytomation, code # K0674) for 10-30 min according to the adopted protocol for each antibody, rinsed in Dako wash buffer for one min, 2-3 times and the excess was absorbed. As chromogen, we used Fuchsin or permanent red chromogen. Haematoxylin was used as a counterstain. Then the slides were mounted using either standard glass cover mounting or a liquid mounting medium (DAKO Ultramount Aqueous Permanent Mounting Medium Code # 1964) according to availability.

The whole staining procedure was performed three times for each antibody, each individually evaluated by two blinded observers. Negative controls – where the full staining procedure was performed with all staining materials except for the primary antibody – were examined to exclude errors due to non-specific staining, and they exhibited no staining (Appendix 2).

We used a semiquantitative method for the immunohistochemical evaluation. Each skin structure was individually evaluated for the staining intensity. We used a graded scale 0, 1, 2, 3 and 4, where 0 is no stain and 4 is intense staining.

The semiquantitative immunohistochemical evaluation of the slides was repeated twice, each time independently and in a blinded manner and received the same results. A third evaluation performed by an experienced examiner to exclude a subjective evaluation provided similar results.

Table 1**:** Laboratory methods for immunohistochemical staining.
The primary antibodies: manufacturing company, retrieval methods, dilution and incubation periods (*The terms PPAR-β and PPAR-δ correspond to the same molecule, whereas PPAR-β is applied to molecules of animal origin and PPAR-δ to molecules of human origin)

Primary Antibody	Manufacturer	Dilution	Incubation time and temperature	Retrieval procedure
CRH (C-20) Goat polyclonal cat# sc 1759 lot # F1206	Santa Cruz	1:50	30 min at room temperature	Boiling in pH 9 buffer 20 min
CRHBP (c-19) Goat polyclonal cat # sc 1822 lot # D 1105	Santa Cruz	1:50	30 min at room temperature	Boiling in pH 9 buffer 20 min
CRHR1 (c-20) Goat polyclonal cat # sc 1757 lot # 12408	Santa Cruz	1:50	30 min at room temperature	Boiling in pH 9 buffer 20 min
CRHR2 (N-20) Goat polyclonal cat # sc 1826 lot # K2206	Santa Cruz	1:50	30 min at room temperature	Boiling in pH 9 buffer 20 min
PPAR-α (n-19) Goat polyclonal cat # sc 1985 lot# H 1007	Santa Cruz	1:100	1 hr at room temperature	Boiling in pH 9 buffer 20 min

PPAR-β* (H-74) Rabbit polyclonal cat # sc-7197 lot # D1508	Santa Cruz	1:100	24 hr at room temperature	Boiling in pH 9 buffer 20 min
PPAR-γ (n-20) Goat polyclonal cat # sc-1984 lot # F1109	Santa Cruz	1:50	24 hr at room temperature	Boiling in pH 9 buffer 20 min
IL-6 Mouse monoclonal IgG cat# AM06012PU-N lot #14997	Acris	1:200	30 min at room temperature	Boiling in pH 6.1 buffer 20 min
IL-8 Rabbit polyclonal cat #PP1030P1 lot # 0105M018RB	Acris	1.0 μg/ml	2 hrs at room temperature	Boiling in pH 9 buffer 20 min
TLR-4 Mouse monoclonal - Clone 5K155 Cat # LS-B2254	Lifespan biosciences	5 μg/ml	45 min at room temperature	Boiling in pH 6.1 for 20 min

2.3 Statistical analysis

The aim of this study is to examine the changes in the expression (staining degree in a semiquantitative ordinal score from 0 to 4) of the tested molecules in relation to ageing.

Under the supervision of Prof. Zouboulis, the following statistical tests were chosen in 2009-2010.

The two tailed Mann-Whitney U-Test was chosen to compare the data of aged (>70 years) and the young (<30 years) groups. The correlation matrix (Spearman) was chosen to calculate the correlation between age and the staining degree using the whole sample spectrum. The significance level in both tests was chosen to be set at $p<0.05$.

The data was analysed using the online statistical calculator http://vassarstats.net/index.html. Representative Box Plots were drawn using the online program (http://mathwarehouse.com).

The results of this analysis were successfully published in Elewa et al. 2012[89] and Elewa et al. 2015[91]. Based on the same results the author submitted the thesis to the review committee of the Charite-Universitätsmedizin Berlin as a Monography in May 2016.

Based on a request from the review committee to certify the statistical results (November 2016), Prof. Mansmann was approached for advice. He suggested a re-analysis of the data using the following new statistical tests and a new software.

The correlation between age (divided in 4 age groups 18-30, 31-50, 51-70, and 71-100 years) and the staining degree of the examined structures (evaluated in a semiquantitative ordinal score 0,1,2,3 and 4) was calculated using the 'exact version` of Jonckheere-Terpstra and Linear-by-linear association tests, which were specially developed for data with limited number of samples. The tests were performed using the StatXact®11 programme for exact tests for categorial data (http://www.cytel.com/software/statxact).

Due to the use of a semiquantitative score with a confined range of values (0,1,2,3 and 4) and a relatively small number of samples, which revealed a limited amount of information with a high repetition rate, the significance level was chosen to be set at 15% ($p \leq 0.15$).

The data was re-analysed according to the above methods under the supervision of Prof. Mansmann, and the results were approved by him.

The thesis was processed according to the new analysis of the data and re-submitted in July 2018.

According to the new results emerging from the re-analysis of the data, some discrepancies have occurred between the publications[89, 90, 91] and the thesis (Table 2).

Table 2: The discrepancies between the thesis and the publications

Antibody	*Pre-revision results (publications Elewa et al. 2012[89], Makrantonaki et al. 2012[90] and Elewa et al. 2015[91]) and Thesis version May 2016*	*New modified results according to statistical re-analysis of the data. Thesis version July 2018 and current version*
CRH	Up-regulation in sebaceous glands and ducts Down-regulation in sweat glands	No significant changes in the sebaceous gland. Down regulation in sweat glands
CRHBP	Up-regulation in the dermis Down-regulation in the sweat glands and sebaceous glands	Up-regulation in the dermis and sweat glands Down-regulation in the sebaceous glands
CRHR1	up-regulated in the epidermis, hair follicles and sebaceous ducts	up-regulated in the epidermis, hair follicles, sebaceous ducts in addition to: sweat glands, and sebaceous glands
CRHR2	No age-related changes	Up-regulation in the sebaceous ducts
PPAR-α	Down-regulation in the epidermis, hair follicles, sweat glands, sebaceous glands and ducts	Down-regulation in sweat glands and sebaceous glands No significant changes in epidermis and hair follicles
PPAR-δ	Up-regulation in the sebaceous ducts	Up-regulation in the sebaceous ducts and hair follicles
PPAR-γ	Down-regulation in the sebaceous glands	Down-regulation in the sebaceous glands
IL-6	Up-regulation in the dermis Down-regulation in the sebaceous glands and sweat glands	Up-regulation in the dermis and hair follicles Down-regulation in the sebaceous glands
IL-8	Down-regulation in the sweat glands	No significant changes in the sweat glands Up-regulation in epidermis and hair follicles
TLR-4	Down-regulation in epidermis, hair follicles, sweat glands, sebaceous glands and sebaceous ducts	Down-regulation in epidermis, hair follicles, sweat glands, sebaceous glands

The discrepancies mentioned above are, however, of minor clinical importance and do not alleviate the postulations created in the previously mentioned publications[89, 90, 91].

For the sake of transparency and scientific accuracy, we communicated the minimally variated results, which emerged from the requested statistical re-analysis of the data, in detail with the editors-in-chief of the three journals, where we the original

results were published. Considering the minor contradiction with the published data, the editors-in-chief of the three journals decided that no corrigendum was required. The communication e-mails are added to the folder of the raw data.

The results in the current thesis version are displayed in context of the new statistical analysis, which are – as previously explained - partially different from the ones in the publications[89, 90, 91].

2.4 Photo documentation

The glass slides of the specimens were evaluated by a semiquantitative evaluation method, as mentioned before. We took pictures only for the aged (> 70 years) and the young groups (< 30 years) to represent the difference in the expression of the molecules in the extremes of age. The pictures were documented as “aged” or “young”.

The photo documentation of the glass slides was performed using an EVOS fL Digital inverted microscope AMG. Since no magnification bar was available, the magnification of each picture was noted manually for each slide according to the microscope lens magnification used at the time of taking the picture. The labels of numbers and antibody names were added to the saved digital photos using power point. The noted magnification power was documented regardless of the zoom-in or crop, which were performed for editing purposes when needed.

A permission is granted from the publishing journals to re-use our own published material and photos in this doctoral thesis.

3. Results

All tested proteins were found to be expressed in almost all skin compartments. The staining was most apparent in the sebaceous glands followed by the sweat glands and hair follicles (Table 3). No gender-related difference in the expression of the tested antigens was observed. A clear variation in protein expression was observed in relation to ageing. The results are displayed according to the statistical re-analysis. (Table 4).

Table 3**:** Localization of the tested antigens in different skin compartments
"x": expressed, "-": not expressed

	Epidermis	Dermis	Hair follicles	Sebaceous glands	Sebaceous ducts	Sweat glands	Blood vessels
CRH	_	_	x	x	x	x	_
CRHBP	_	x	_	x	_	x	_
CRHR1	x	_	x	x	x	x	_
CRHR2	_	_	_	x	x	x	_
PPAR-α	x	_	x	x	x	x	_
PPAR-δ	x	_	x	x	x	x	_
PPAR-γ	x	_	_	x	_	x	_
IL-6	x	x	x	x	x	x	x
IL-8	x	_	x	x	_	x	_
TLR-4	x	_	x	x	x	x	x

Table 4: Ageing-related variation in the intensity of expression of the tested antigens (Jonckheere-Terpstra Test, significant by $p \leq 0.15$)

Antigen	Ageing-related **Up**-regulation	Ageing-related **Down**-regulation
CRH	-	Sweat glands (p=0.109)
CRHBP	Dermis (p=4.082e-005) Sweat glands (p=2.044e-006)	Sebaceous glands (p=0.058)
CRHR1	Epidermis (p=0.0058) Hair follicles (p=0.058) Sweat glands (p=0.107) (Linear-by-linear association Test) Sebaceous ducts (p=0.00143) Sebaceous glands (p=0.082)	-
CRHR2	Sebaceous ducts (p=0,072)	_
PPAR-α	-	Sweat glands (p=0.0028) Sebaceous glands (p=0.133)
PPAR-δ	Hair follicles (p=0.027) Sebaceous ducts (p=0.115, Linear-by-linear Test p=0.068)	-
PPAR-γ	-	Sebaceous glands (p=0.11)
IL-6	Dermis (p=5.01e-005) Hair follicles (0.065)	Sebaceous glands (p= 0.14, linear-by-linear Test p=0.053)
IL-8	Epidermis (p= 0.14) (linear-by linear-test p=0.02) Hair follicles (p=0.021)	-
TLR-4	-	Epidermis (p=0.034) Hair follicles (p=0.091) Sweat glands (p=0.107) Sebaceous glands (p=0.015)

3.1 CRH expression

The epidermis, the dermis and the blood vessels exhibited a negative immunohistochemical staining for CRH, while the hair follicles, sebaceous glands, sebaceous ducts and the sweat glands showed a weak positive staining. The CRH staining of the sebaceous glands was gradually fading from intense staining in the basal undifferentiated cells to weak staining towards differentiated ones (differentiation-dependent). The CRH staining of the sweat glands exhibited an ageing-related down-regulation (Jonckheere-Terpstra-test, significance level 15%, p= 0.109) (Fig. 1 and Tables 5.1-5.3)

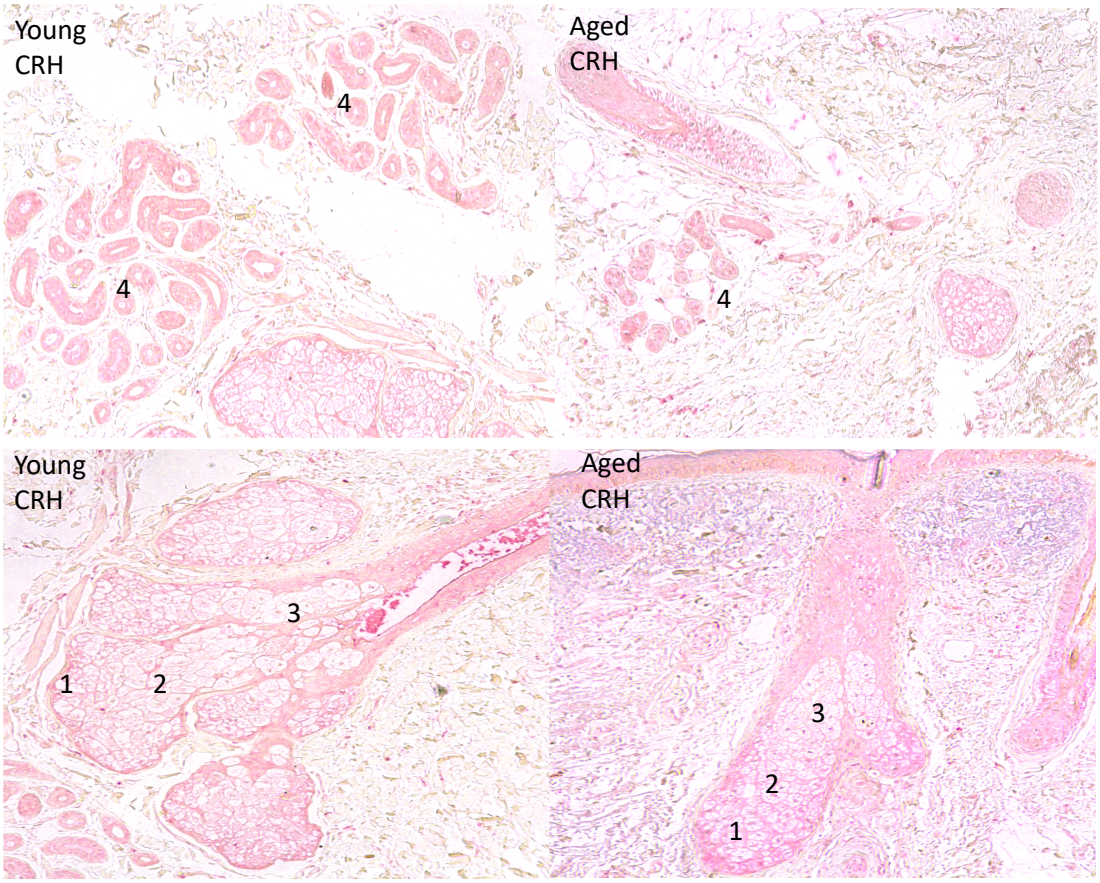


Figure 1: Localization of the immunohistochemical signals of CRH in the skin of the aged and young groups. The intensity of the staining in the sebocytes is differentiation dependent (1,2 and 3). The sweat glands (4) exhibited an ageing-related down-regulation (difficult to capture on photos due to weak staining) A sporadic statistically not significant stronger immune reaction is seen in the sebaceous glands of the aged group. (Original magnification x 100)

Table 5.1-5.3**:** Expression of CRH in the different skin compartments and age groups. An age-related down-regulation was assessed in the sweat glands. Significant p values are typed in ***bold italic***

5.1	CRH in sebaceous glands - Semiquantitative score				
age group	0	1	2	3	4
18-30	0	12	1	0	0
		(92.31 %)	(7.69 %)		
31-50	0	6	2	0	0
		(75.00 %)	(25.00 %)		
51-70	0	8	1	0	0
		(88.89 %)	(11.11 %)		
71-100	0	9	1	0	0
		(90.00 %)	(10.00 %)		
Total	0	35	5	0	0
		(87.50 %)	(12.50 %)		
Jonckheere-Terpstra-Test p= 0.944, Linear-by-linear association test p=1.00					

5.2	CRH in sweat glands - Semiquantitative score				
age group	0	1	2	3	4
18-30	3	10	0	0	0
	(23.08 %)	(76.92 %)			
31-50	2	4	2	0	0
	(25.00 %)	(50.00 %)	(25.00 %)		
51-70	4	4	1	0	0
	(44.44 %)	(44.44 %)	(11.11 %)		
71-100	6	4	0	0	0
	(60.00 %)	(40.00 %)			
Total	15	22	3	0	0
	(37.50 %)	(55.00 %)	(7.50 %)		
Jonckheere-Terpstra-Test ***p= 0.109***, Linear-by-linear association test p=0.123					

5.3	CRH in Hair follicles- Semiquantitative score				
age group	0	1	2	3	4
18-30	5	8	0	0	0
	(38.46 %)	(61.54 %)			
31-50	4	2	2	0	0
	(50.00 %)	(25.00 %)	(25.00 %)		
51-70	6	2	0	0	0
	(75.00 %)	(25.00 %)			
71-100	4	4	2	0	0
	(40.00 %)	(40.00 %)	(20.00 %)		
Total	19	16	4	0	0
	(48.72 %)	(41.03 %)	(10.26 %)		
Jonckheere-Terpstra-Test p=0.962, Linear-by-linear association test p=0.921					

3.2 CRHR1 expression

The CRHR1 exhibited a positive staining in the epidermis, hair follicles, sebaceous ducts, sebaceous glands and sweat glands. Fibroblasts and inflammatory cells were focally positive. The CRHR1 expression in sebaceous glands was differentiation-dependent, being most intense in the basal undifferentiated cells and fading towards maturity. The sebum was strongly stained. The secretory part of the sweat glands showed the strongest positive staining among all skin structures. Like sebum, sweat also showed a strong positive reaction for CRHR1. The epidermis showed relatively stronger staining in the stratum basal and stratum spinosum, gradually fading to negativity towards the stratum corneum. Basement membrane zone and stratum corneum exhibited almost no staining of the CRHR1. The CRHR1 staining of the epidermis, hair follicles, sweat glands, sebaceous ducts and sebaceous glands exhibited an ageing-related up-regulation (Jonckheere-Terpstra-test, significance level 15%, p= 0.005, 0.058, 0.107, 0.001 and 0.082 respectively) (Fig. 2 and Tables 6.1-6.5).

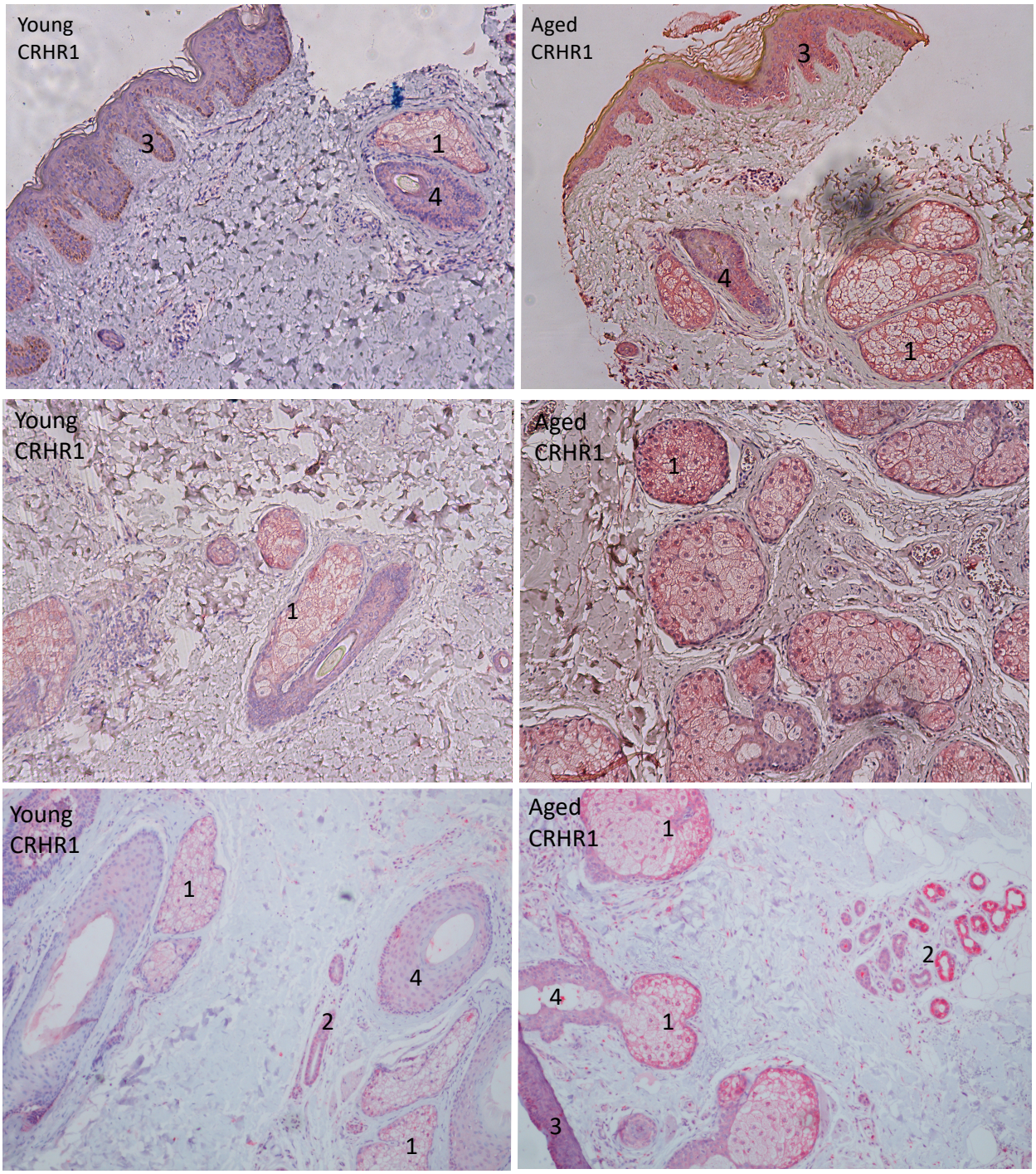


Figure 2**:** Localization of the immunohistochemical signals of CRHR1 in young and aged skin. Ageing-related up-regulation is observed in the sebaceous glands (1), sweat glands (2), epidermis (3) and hair follicles (4). (Original magnification x100). lower panel from Elewa et al. 2012 [(89)]

Table 6.1-6.5**:** Expression of CRHR1 in the different skin compartments and age groups. The CRHR1 exhibited an age-related up-regulation in the epidermis, hair follicles, sweat glands, sebaceous ducts and glands. Significant p values are typed in ***bold italic***

6.1	CRH-R1 Epidermis – Semiquantitative score				
age group	0	1	2	3	4
18-30	8	4	1	0	0
	(61.54 %)	(30.77 %)	(7.69 %)		
31-50	5	3	0	0	0
	(62.50 %)	(37.50 %)			
51-70	0	7	3	0	0
		(70.00 %)	(30.00 %)		
71-100	1	10	0	0	0
	(9.09 %)	(90.91 %)			
Total	14	24	4	0	0
	(33.33 %)	(57.14 %)	(9.52 %)		
Jonckheere-Terpstra-Test ***p=0.005***, Linear-by-linear association test ***p=0.006***					

6.2	CRH-R1 Hair follicles – Semi-quantitative score				
age group	0	1	2	3	4
18-30	8	2	2	1	0
	(61.54 %)	(15.38 %)	(15.38 %)	(7.69 %)	
31-50	3	3	1	1	0
	(37.50 %)	(37.50 %)	(12.50 %)	(12.50 %)	
51-70	0	7	2	0	1
		(70.00 %)	(20.00 %)		(10.00 %)
71-100	1	8	2	0	0
	(9.09 %)	(72.73 %)	(18.18 %)		
Total	12	20	7	2	1
	(28.57 %)	(47.62 %)	(16.67 %)	(4.76 %)	(2.38 %)
Jonckheere-Terpstra-Test ***p=0.058***, Linear-by-linear association test p=0.165					

6.3	CRH-R1 Sebaceous ducts – Semi-quantitative score				
age group	0	1	2	3	4
18-30	8	2	0	0	0
	(80.00 %)	(20.00 %)			
31-50	3	4	0	0	0
	(42.86 %)	(57.14 %)			
51-70	2	3	3	0	0
	(25.00 %)	(37.50 %)	(37.50 %)		
71-100	1	4	2	0	0
	(14.29 %)	(57.14 %)	(28.57 %)		
Total	14	13	5	0	0
	(43.75 %)	(40.63 %)	(15.63 %)		
Jonckheere-Terpstra-Test ***p=0.001,*** Linear-by-linear association test ***p=0.001***					

6.4	CRH-R1 sweat glands- Semiquantitative score				
age group	0	1	2	3	4
18-30	1	2	1	2	7
	(7.69 %)	(15.38 %)	(7.69 %)	(15.38 %)	(53.85 %)
31-50	1	1	1	2	3
	(12.50 %)	(12.50 %)	(12.50 %)	(25.00 %)	(37.50 %)
51-70	0	0	0	5	5
				(50.00 %)	(50.00 %)
71-100	0	0	0	4	5
				(44.44 %)	(55.56 %)
Total	2	3	2	13	20
	(5.00 %)	(7.50 %)	(5.00 %)	(32.50 %)	(50.00 %)
Jonckheere-Terpstra-Test p=0.340, Linear-by-linear association test ***p=0.107***					

6.5	CRH-R1 sebaceous glands - Semiquantitative score				
age group	0	1	2	3	4
18-30	1	2	3	6	1
	(7.69 %)	(15.38 %)	(23.08 %)	(46.15 %)	(7.69 %)
31-50	0	0	2	4	2
			(25.00 %)	(50.00 %)	(25.00 %)
51-70	1	1	0	6	1
	(11.11 %)	(11.11 %)		(66.67 %)	(11.11 %)
71-100	0	0	0	8	1
				(88.89 %)	(11.11 %)
Total	2	3	5	24	5
	(5.13 %)	(7.69 %)	(12.82 %)	(61.54 %)	(12.82 %)
Jonckheere-Terpstra-Test ***p=0.082***, Linear-by-linear association test ***p=0.124***					

3.3 CRHR2 expression

CRHR2 signals were detected in the sebaceous glands, sebaceous ducts and the sweat glands. In contrast the epidermis, dermis, hair follicles and blood vessels exhibited negative CRHR2 staining. There was no evidence of ageing-related changes in the expression of CRHR2 in the different skin compartments, except for the sebaceous ducts which exhibited an ageing-related up-regulation (p= 0,072) (Jonckheere- Terpstra Test, significance level 15%) (Fig. 3 and Tables 7.1-7.3).

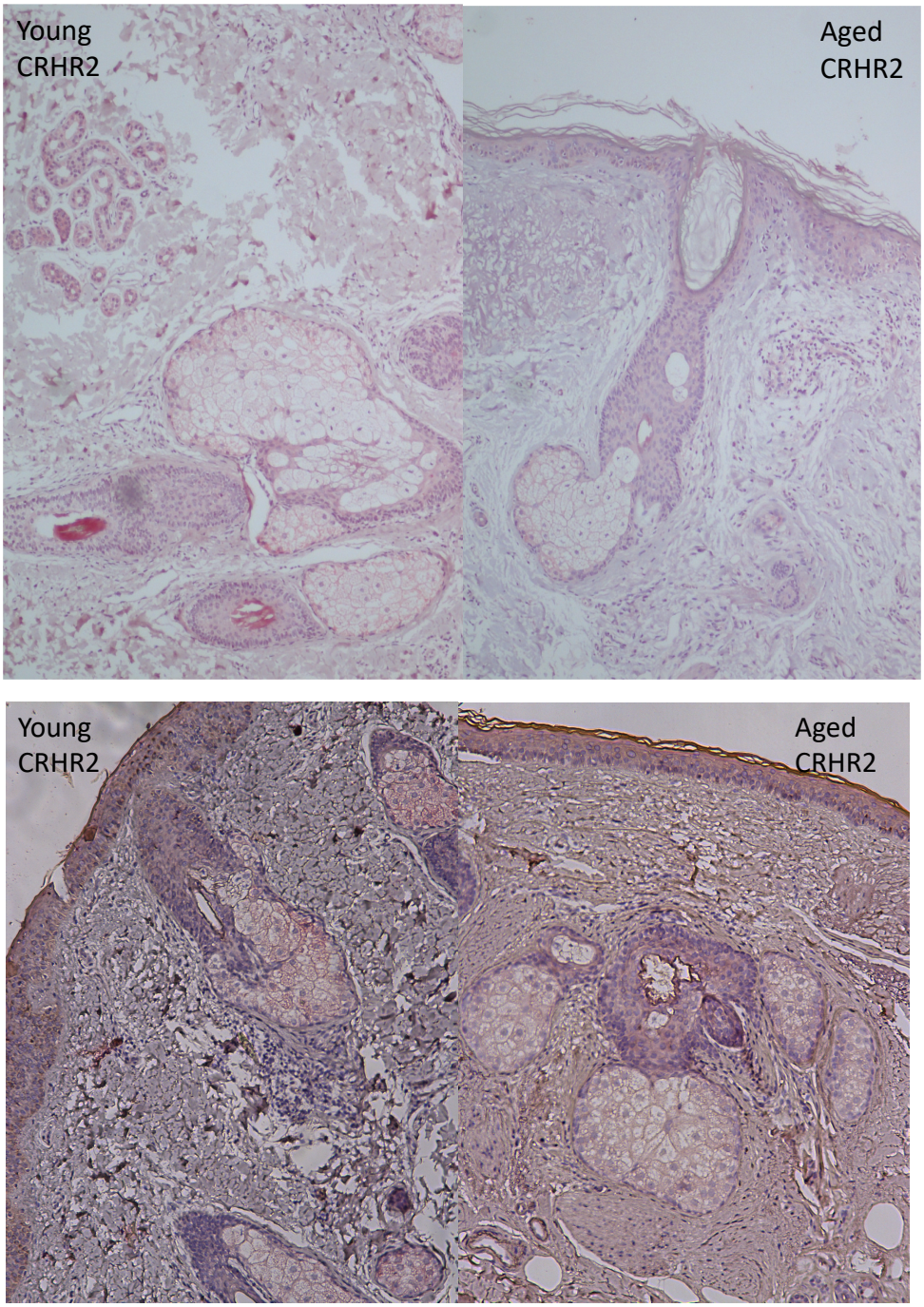


Figure 3**:** Localization of the immunohistochemical signals of CRHR2 in the young and aged skin. There was no ageing-related change in the expression of CRHR2, except for an ageing-related up-regulation in the sebaceous ducts. This change was however difficult to capture on photos due to overall weak stain (Original magnification x100) upper panel from Elewa et al. 2012[89]

Table 7.1-7.3**:** Expression of CRHR2 in the different skin compartments and age groups.

The CRHR2 exhibited age-related up-regulation in the sebaceous ducts. Significant p values are typed in ***bold italic***

7.1	CRH-R2 sweat glands – Semiquantitative score				
age group	0	1	2	3	4
18-30	2	5	5	0	0
	(16%)	(41%)	(41%)		
31-50	1	3	3	1	0
	(12.5%)	(37.5%)	(37.5 %)	(12.5%)	
51-70	5	0	5	0	0
	(50%)		(50%)		
71-100	2	7	2	0	0
	(18.18%)	(63.6%)	(18.18%)		
Total	11	15	15	1	0
	(26.19%)	(35.71%)	(35.71%)	(2.38%)	
Jonckheere-Terpstra-Test p=0.346**,** Linear-by-linear association test p=0.342					

7.2	CRH-R2 sebaceous duct – Semi-quantitative score				
age group	0	1	2	3	4
18-30	9	2	1	0	0
	(75%)	(16.66%)	(8.33%)		
31-50	6	1	0	0	0
	(85.71%)	(14.28%)			
51-70	9	1	0	0	0
	(90%)	(10%)			
71-100	11	0	0	0	0
	(100%)				
Total	35	4	1	0	0
	(87.5%)	(10%)	(2.5%)		
Jonckheere-Terpstra-Test ***p=0.072,*** Linear-by-linear association test ***p=0.078***					

7.3	CRH-R2 Sebaceous glands – Semi-quantitative score				
age group	0	1	2	3	4
18-30	2	6	4	0	0
	(16.66%)	(50%)	(33.33%)		
31-50	2	4	1	1	0
	(25%)	(50%)	(12.5%)	(12.5%)	
51-70	3	4	2	1	0
	(30%)	(40%)	(20%)	(10%)	
71-100	2	5	4	0	0
	(18.18%)	(45.45%)	(36.36%)		
Total	9	19	11	2	0
	(21.95%)	(46.3%)	(26.8%)	(4.87%)	
Jonckheere-Terpstra-Test p=0.97, Linear-by-linear association test p=1					

3.4 CRHBP expression

CRHBP exhibited a strong expression in the sebaceous glands. The staining was differentiation dependent, being strongest in the basal undifferentiated cells and fading towards the mature sebocytes in the centre of the gland. CRHBP exhibited almost a negative staining in the epidermis and hair follicles, whereas the smooth muscles showed a weakly positive stain. The CRHBP staining of the dermis and sweat glands exhibited an ageing-related up-regulation. In contrast, the staining in the sebaceous glands exhibited an ageing–related down-regulation (Jonckheere-Terpstra-test, significance level 15%, p= 0.00001 ,0.00001 and 0.059 respectively) (Figure 4, Tables 8.1-8.3).

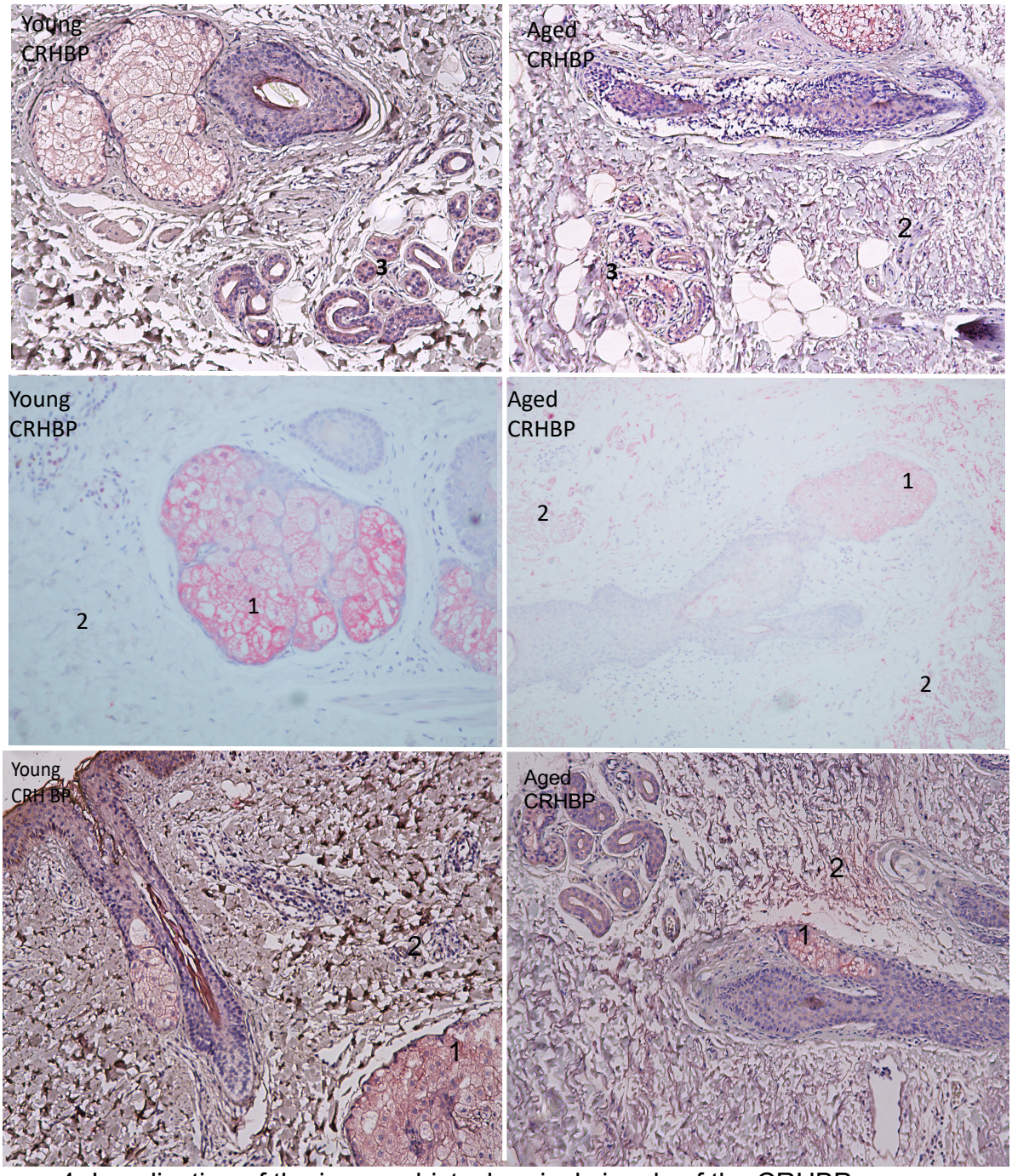


Figure 4: Localization of the immunohistochemical signals of the CRHBP in the skin of the aged and young groups. There was a significant ageing-related down regulation in the sebaceous glands (1) and a significant ageing-related up-regulation in the dermis (2) and sweat glands (3) (Original magnification x100, except middle left picture x200) middle panel from Elewa et al. 2012[89]

Table 8.1-8.3**:** Expression of CRHBP in different skin compartments and age groups. The CRHBP expression exhibited an age-related up-regulation in the dermis and sweat glands, and an age-related down-regulation in the sebaceous glands. Significant p values are Typed in ***bold italic***

8.1	CRHBP dermis - Semiquantitative score				
age group	0	1	2	3	4
18-30	13	0	0	0	0
	(100.00 %)				
31-50	7	1	0	0	0
	(87.50 %)	(12.50 %)			
51-70	6	2	2	0	0
	(60.00 %)	(20.00 %)	(20.00 %)		
71-100	3	6	1	0	1
	(27.27 %)	(54.55 %)	(9.09 %)		(9.09 %)
Total	29	9	3	0	1
	(69.05 %)	(21.43 %)	(7.14 %)		(2.38 %)
Jonckheere-Terpstra-Test ***p=4.082e-005***, Linear-by-linear association test ***p=8.77e00***					

8.2	CRH-BP sweat glands - Semiquantitative score				
age group	0	1	2	3	4
18-30	11	2	0	0	0
	(84.62 %)	(15.38 %)			
31-50	2	4	0	2	0
	(25.00 %)	(50.00 %)		(25.00 %)	
51-70	5	5	0	0	0
	(50.00 %)	(50.00 %)			
71-100	0	1	0	9	0
		(10.00 %)		(90.00 %)	
Total	18	12	0	11	0
	(43.90 %)	(29.27 %)		(26.83 %)	
Jonckheere-Terpstra-Test ***p=2.044e-006***, Linear-by-linear assoc. test ***p=1.8546-006***					

8.3	CRHBP sebaceous glands - Semiquantitative score				
age group	0	1	2	3	4
18-30	1	0	1	7	4
	(7.69 %)		(7.69 %)	(53.85 %)	(30.77 %)
31-50	0	0	1	5	2
			(12.50 %)	(62.50 %)	(25.00 %)
51-70	0	0	4	3	3
			(40.00 %)	(30.00 %)	(30.00 %)
71-100	2	0	2	7	0

	(18.18 %)		(18.18 %)	(63.64 %)	
Total	3	0	8	22	9
	(7.14 %)		(19.05 %)	(52.38 %)	(21.43 %)
Jonckheere-Terpstra-Test ***p=0.058,*** Linear-by-linear association test ***p=0.095***					

3.5 PPAR-α (PPAR-alpha) expression

PPAR-α exhibited a generally weak staining in all skin compartments, namely the hair follicles, sweat glands, ductus seboglandularis and the sebaceous glands. There was evidence of ageing-related down-regulation of the expression of PPAR-α antigen in the sweat and sebaceous glands (Significance level 15% - Jonckheere-Terpstra test, p= 0.002 and 0.13 respectively) (Figure 5, Tables 9.1-9.5).

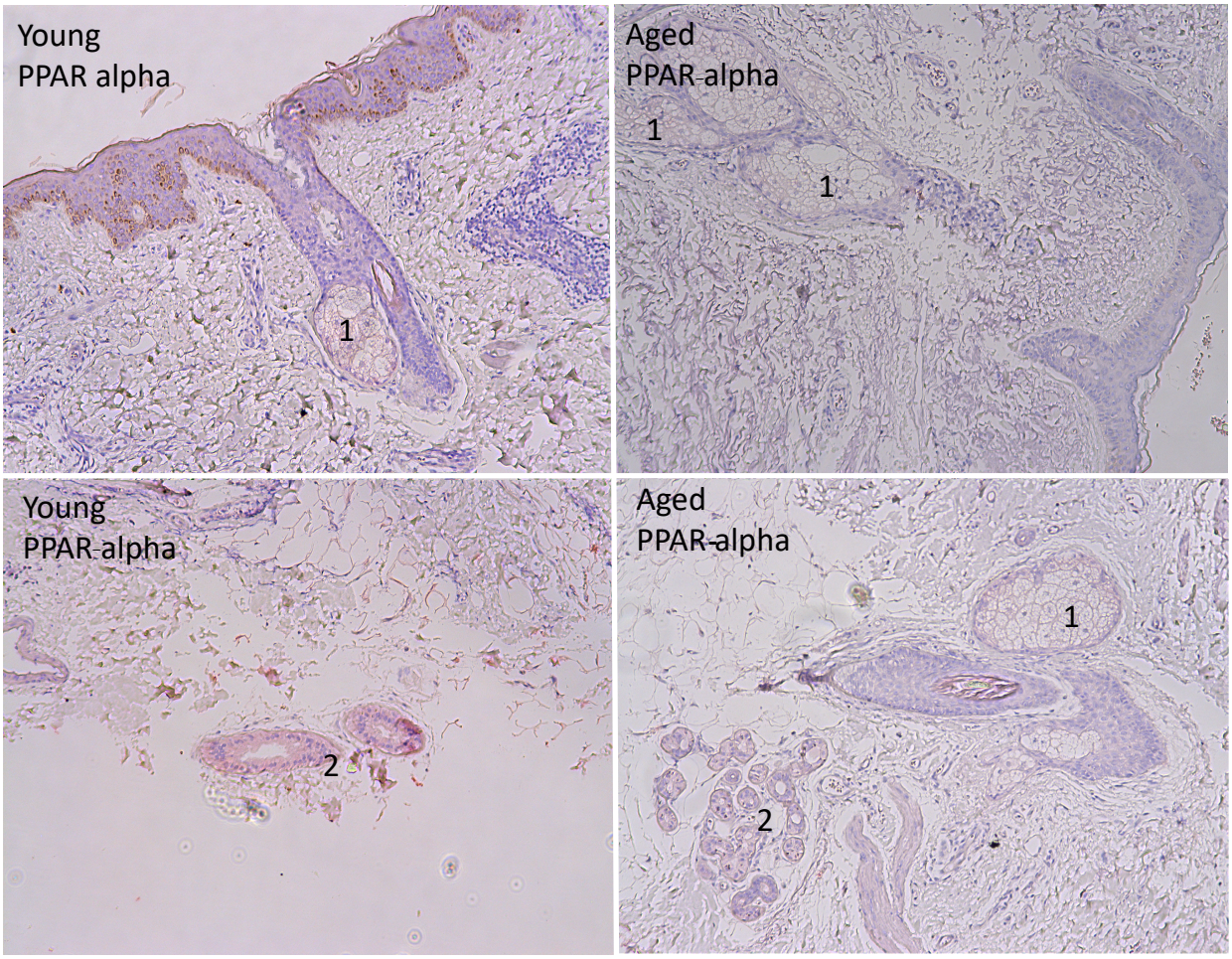


Figure 5**:** Localization of the immunohistochemical signals of PPAR-α **(PPAR-alpha)** in young and aged skin. Ageing-related down-regulation was observed in the sebaceous glands (1) and sweat glands (2). (Original magnification x100).

Table 9 .1-9.5**:** Expression of PPAR-α in the different skin compartments and age groups.

The PPAR-α exhibited an age-related down-regulation in the sebaceous and sweat glands. Significant p values are Typed in ***bold italic***

9.1	PPAR-α epidermis - Semiquantitative score				
age group	0	1	2	3	4
18-30	11	2	0	0	0
	(84.62 %)	(15.38 %)			
31-50	8	0	0	0	0
	(100.00 %)				
51-70	9	1	0	0	0
	(90.00 %)	(10.00 %)			
71-100	11	0	0	0	0
	(100.00 %)				
Total	39	3	0	0	0
	(92.86 %)	(7.14 %)			
Jonckheere-Terpstra-Test p=0.290, Linear-by-linear association test p=0.322					

9.2	PPAR-α hair follicles - Semiquantitative score				
age group	0	1	2	3	4
18-30	11	2	0	0	0
	(84.62 %)	(15.38 %)			
31-50	7	1	0	0	0
	(87.50 %)	(12.50 %)			
51-70	9	1	0	0	0
	(90.00 %)	(10.00 %)			
71-100	11	0	0	0	0
	(100.00 %)				
Total	38	4	0	0	0
	(90.48 %)	(9.52 %)			
Jonckheere-Terpstra-Test p=0.277, Linear-by-linear association test p=0.284					

9.3	PPAR-α sweat glands - Semiquantitative score				
age group	0	1	2	3	4
18-30	5	8	0	0	0
	(38.46 %)	(61.54 %)			
31-50	5	3	0	0	0
	(62.50 %)	(37.50 %)			
51-70	6	4	0	0	0
	(60.00 %)	(40.00 %)			
71-100	11	0	0	0	0
	(100.00 %)				
Total	27	15	0	0	0
	(64.29 %)	(35.71 %)			
Jonckheere-Terpstra-Test ***p=0.002***, Linear-by-linear association test ***p=0.001***					

9.4	PPAR-α sebaceous ducts - Semiquantitative score				
age group	0	1	2	3	4
18-30	11	2	0	0	0
	(84.62 %)	(15.38 %)			
31-50	7	1	0	0	0
	(87.50 %)	(12.50 %)			
51-70	9	1	0	0	0
	(90.00 %)	(10.00 %)			
71-100	11	0	0	0	0
	(100.00 %)				
Total	38	4	0	0	0
	(90.48 %)	(9.52 %)			
Jonckheere-Terpstra-Test p=0.277, Linear-by-linear association test p=0.284					

9.5	PPAR-α sebaceous glands - Semiquantitative score				
age group	0	1	2	3	4
18-30	6	7	0	0	0
	(46.15 %)	(53.85 %)			
31-50	4	4	0	0	0
	(50.00 %)	(50.00 %)			
51-70	4	6	0	0	0
	(40.00 %)	(60.00 %)			
71-100	9	2	0	0	0
	(81.82 %)	(18.18 %)			
Total	23	19	0	0	0
	(54.76 %)	(45.24 %)			
Jonckheere-Terpstra-Test ***p=0.133***, Linear-by-linear association test p=0.155					

3.6 PPAR-δ (PPAR-delta) expression

The sebaceous glands, sebaceous duct and the sweat glands stained positive for PPAR-δ expression, whereas the dermis stained negative. The sebaceous glands having a staining intensity ranging from moderate to highly intense represent the highest PPAR-δ expression among the other skin structures. The staining in the sebaceous glands was differentiation dependent. The basal undifferentiated cells were negative in all samples and the rest of the sebaceous gland showed differentiation-dependent intensity of staining, being maximum in differentiating cells and decreasing in the mature sebocytes. The epidermis showed focal positive PPAR-δ expression, some samples, however, showed homogenous weak staining, the staining was in all cases confined to superficial and mid epidermal layers. There was evidence of ageing-related up-regulation of the expression of PPAR-δ antigen in the hair follicles and sebaceous ducts (Significance level 15% - Jonckheere-Terpstra test, p= 0.02 and 0.11 respectively) (Figure 6, Tables 10.1-10.5).

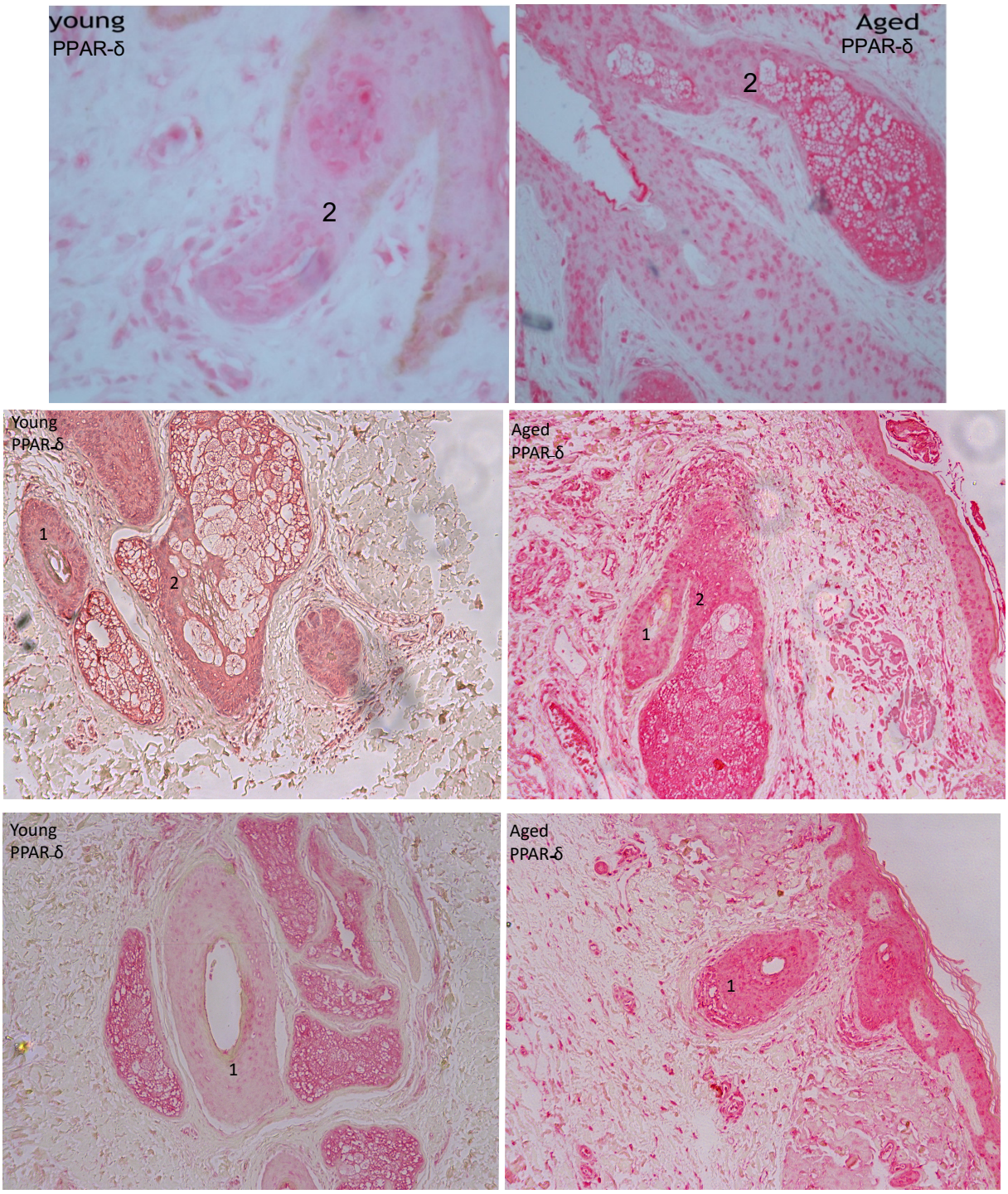


Figure 6: Localization of the immunohistochemical signals of PPAR-δ (PPAR-delta) in young and aged skin. Ageing-related up-regulation was observed in the hair follicles (1) and sebaceous ducts (2). (Original magnification upper left photo x400, upper right photo x200, middle and lower panels x100)

Table 10.1-10.5**:** Expression of PPAR-δ in the different skin compartments and age groups.

PPAR-δ exhibited an ageing-related up-regulation in the hair follicles and sebaceous ducts. Significant p values are typed in ***bold italic.***

10.1	PPAR- δ epidermis - Semiquantitative score				
Age group	0	1	2	3	4
18-30	5	6	2	0	0
	(38.46 %)	(46.15 %)	(15.38 %)		
31 -50	1	7	0	0	0
	(12.50 %)	(87.50 %)			
51 - 70	4	2	0	2	0
	(50.00 %)	(25.00 %)		(25.00 %)	
71 - 100	4	2	1	2	0
	(44.44 %)	(22.22 %)	(11.11 %)	(22.22 %)	
Total	14	17	3	4	0
	(36.84 %)	(44.74 %)	(7.89 %)	(10.53 %)	
Jonckheere-Terpstra-Test p=0.827, Linear-by-linear association test p=0.387					

10.2	PPAR-δ sebaceous gland Semiquantitative score				
Age	0	1	2	3	4
1	0	0	1	4	7
			(8.33 %)	(33.33 %)	(58.33 %)
2	0	0	0	4	3
				(57.14 %)	(42.86 %)
3	0	0	0	5	4
				(55.56 %)	(44.44 %)
4	0	0	0	3	7
				(30.00 %)	(70.00 %)
Total	0	0	1	16	21
			(2.63 %)	(42.11 %)	(55.26 %)
Jonckheere-Terpstra-Test p=0.536, Linear-by-linear association test p=0.4691					

10.3	PPAR-δ sebaceous duct - Semiquantitative score				
Age group	0	1	2	3	4
18-30	4	2	2	0	0
	(50.00 %)	(25.00 %)	(25.00 %)		
31-50	1	5	1	0	0
	(14.29 %)	(71.43 %)	(14.29 %)		
51-70	2	3	3	0	0
	(25.00 %)	(37.50 %)	(37.50 %)		
71-100	3	3	1	3	1
	(27.27 %)	(27.27 %)	(9.09 %)	(27.27 %)	(9.09 %)
Total	10	13	7	3	1
	(29.41 %)	(38.24 %)	(20.59 %)	(8.82 %)	(2.94 %)
Jonckheere-Terpstra-Test ***p=0.115***, Linear-by-linear association test ***p=0.068***					

10.4	PPAR-δ hair follicles - Semiquantitative score				
Age	0	1	2	3	4
1	5	6	0	1	0
	(41.67 %)	(50.00 %)		(8.33 %)	
2	0	6	2	0	0
		(75.00 %)	(25.00 %)		
3	2	4	2	1	0
	(22.22 %)	(44.44 %)	(22.22 %)	(11.11 %)	
4	2	4	0	5	0
	(18.18 %)	(36.36 %)		(45.45 %)	
Total	9	20	4	7	0
	(22.50 %)	(50.00 %)	(10.00 %)	(17.50 %)	
Jonckheere-Terpstra-Test ***p=0.027***, Linear-by-linear association test ***p=0.025***					

10.5	PPAR-δ sweat glands - Semiquantitative score				
Age	0	1	2	3	4
1	2	5	3	1	2
	(15.38 %)	(38.46 %)	(23.08 %)	(7.69 %)	(15.38 %)
2	0	5	0	2	0
		(71.43 %)		(28.57 %)	
3	0	3	1	2	1
		(42.86 %)	(14.29 %)	(28.57 %)	(14.29 %)
4	2	4	1	1	2
	(20.00 %)	(40.00 %)	(10.00 %)	(10.00 %)	(20.00 %)
Total	4	17	5	6	5
	(10.81 %)	(45.95 %)	(13.51 %)	(16.22 %)	(13.51 %)
Jonckheere-Terpstra-Test p=0.891, Linear-by-linear association test p=0.832					

3.7 PPAR-γ (PPAR-gamma) expression

The sebaceous glands, epidermis and sweat glands exhibited positive PPAR-γ staining. The staining of the sebaceous glands was maturation dependent, fading towards the centre of the sebaceous glands where the mature sebocytes are located. There was evidence of age-related down-regulation of the expression of PPAR-γ antigen in the sebaceous glands (Significance level 15% - Jonckheere-Terpstra test, p= 0.11) (Figure 7 and Tables 11.1-11.2).

-

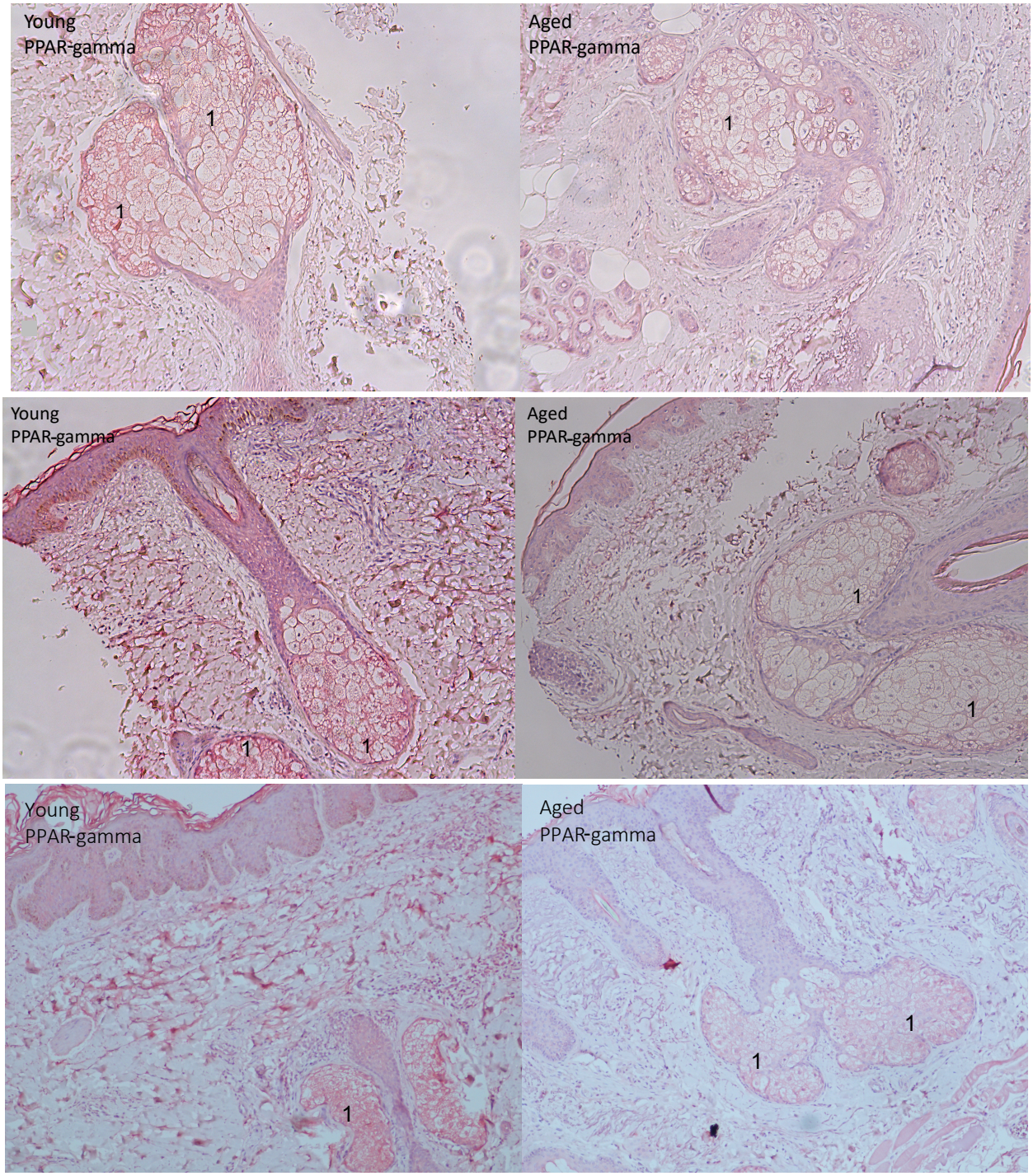


Figure 7: Localization of the immunohistochemical signals of PPAR-γ (PPAR-gamma) in young and aged skin. Ageing-related down-regulation was observed in the sebaceous glands (1) lower panel from Elewa et al 2015[91] (Original magnification x100)

Table 11 .1-11.2**:** Expression of PPAR-γ in the different skin compartments and age groups.
PPAR-γ exhibited an ageing-related down-regulation in the sebaceous glands.
Significant p values are typed in ***bold italic.***

11.1	PPAR-γ sweat gland - Semiquantitative score				
Age group	0	1	2	3	4
18-30	3	7	2	1	0
	(23.08 %)	(53.85 %)	(15.38 %)	(7.69 %)	
31-50	2	2	1	0	0
	(40.00 %)	(40.00 %)	(20.00 %)		
51-70	0	8	0	1	0
		(88.89 %)		(11.11 %)	
71-100	2	6	3	0	0
	(18.18 %)	(54.55 %)	(27.27 %)		
Total	7	23	6	2	0
	(18.42 %)	(60.53 %)	(15.79 %)	(5.26 %)	
Jonckheere-Terpstra-Test p=0.659, Linear-by-linear association test p=0.796					

11.2	PPAR-γ sebaceous gland - Semiquantitative score				
Age group	0	1	2	3	4
18-30	1	3	6	2	0
	(8.33 %)	(25.00 %)	(50.00 %)	(16.67 %)	
31-50	0	0	1	4	0
			(20.00 %)	(80.00 %)	
51-70	0	6	1	1	1
		(66.67 %)	(11.11 %)	(11.11 %)	(11.11 %)
71-100	1	6	4	0	0
	(9.09 %)	(54.55 %)	(36.36 %)		
Total	2	15	12	7	1
	(5.41 %)	(40.54 %)	(32.43 %)	(18.92 %)	(2.70 %)
Jonckheere-Terpstra-Test ***p=0.11***, Linear-by-linear association test ***p=0.131***					

3.8 IL-6 expression

IL-6 exhibited appositive staining in the dermis, epidermis, hair follicles, sweat glands, ductus seboglandularis and the sebaceous glands. The sebaceous glands showed the strongest IL-6 expression among the stained skin structures, IL-6 in the sebaceous glands was differentiation-dependent decreasing towards cell maturation. There was evidence of ageing-related up-regulation of the expression of IL-6 antigen in the dermis and the hair follicles (Significance level 15% - Jonckheere-Terpstra test, p= 0.000001 and 0.06 respectively), while the sebaceous glands showed ageing-related down regulation (p=0.14, Linear-by-linear association test p= 0.05) (Figures 8 and 9, Tables 12.1-12.6).

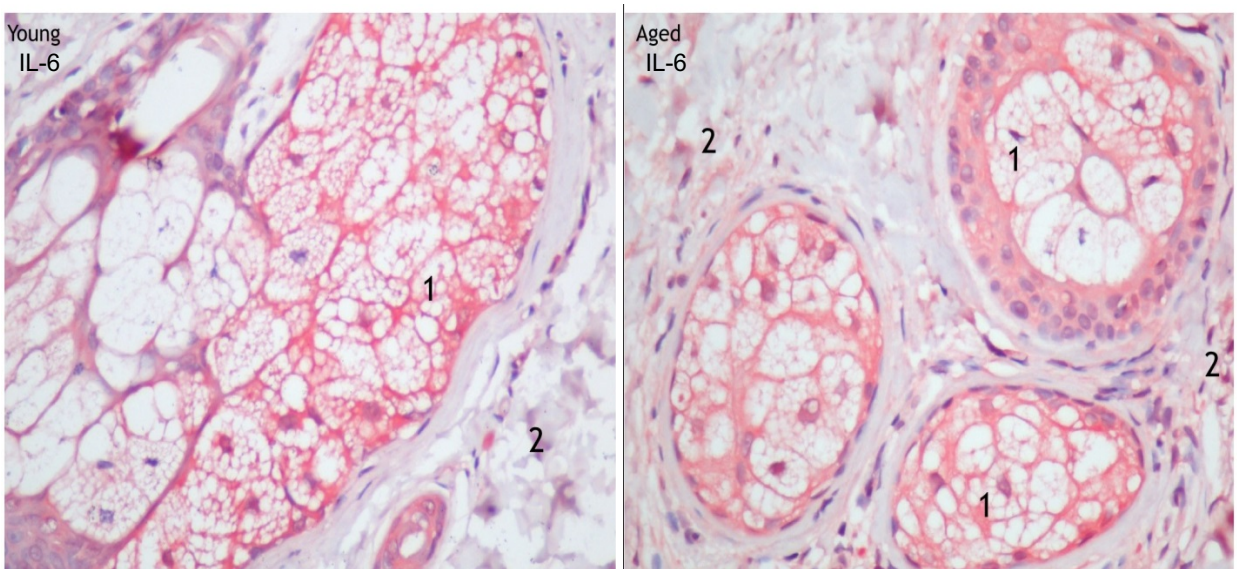


Figure 8**:** Localization of the immunohistochemical signal of IL-6
in young and aged skin. Ageing-related down-regulation in the sebaceous glands (1) and ageing-related up-regulation in the dermal tissue (2) was observed. From Elewa et al. 2015[91]. (Original magnification x400)

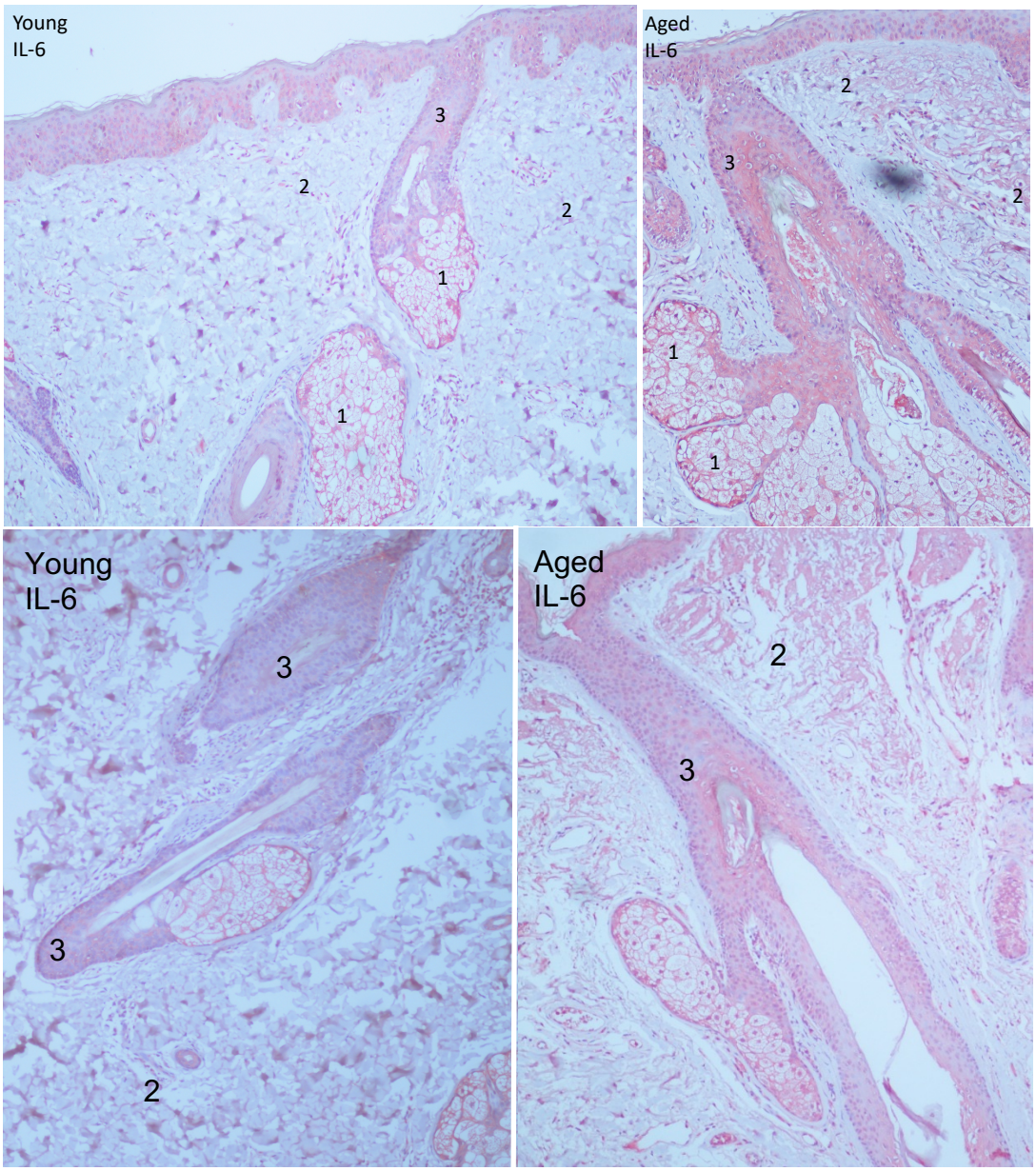


Figure 9: Localization of the immunohistochemical signals of IL-6 in young and aged skin. Ageing-related down-regulation was observed in the sebaceous glands (1) and ageing-related up-regulation in the dermal tissue (2) and the hair follicles (3) (original magnification x100) upper panel from Elewa et al 2015[91]

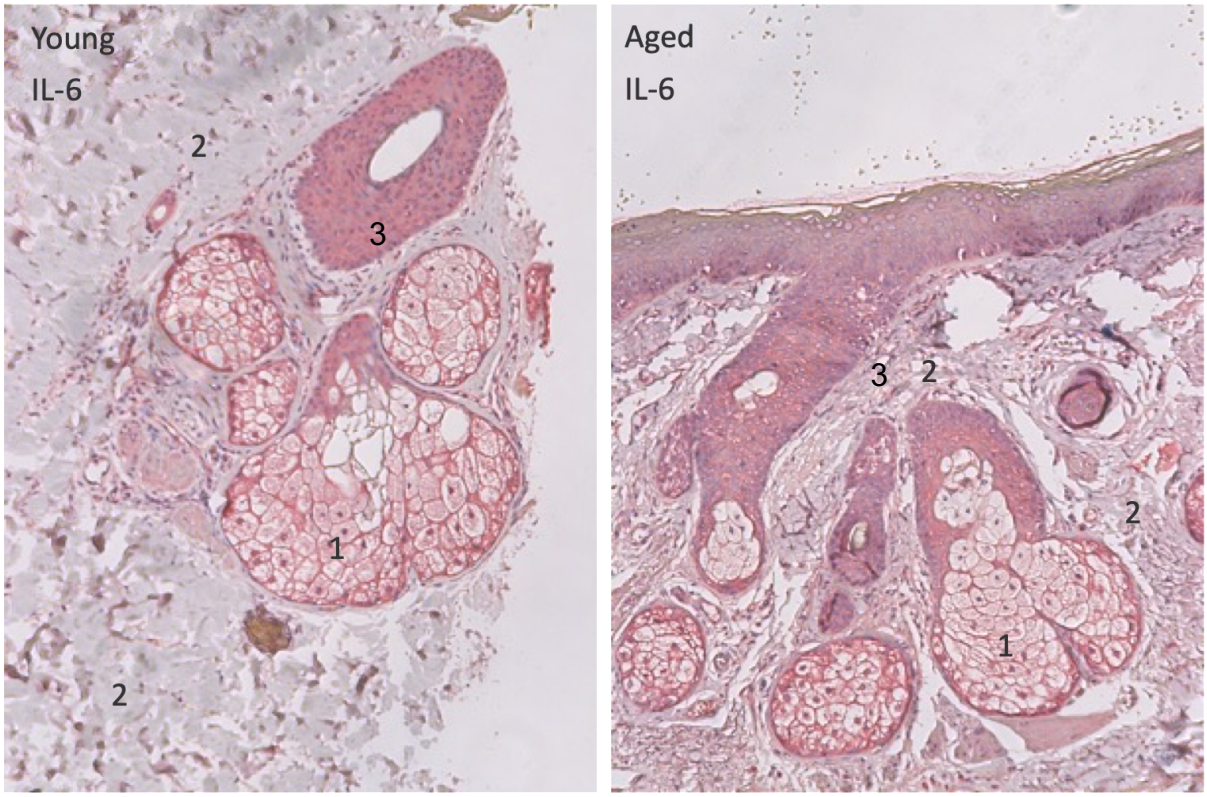


Figure (9): continued

Table 12.1-12.6.**:** Expression of IL-6 in the different skin compartments and age groups. The IL-6 exhibited an age-related down-regulation in the sebaceous glands and an up-regulation in the dermis and hair follicles. Significant p values are typed in ***bold italic.***

12.1	IL 6 epidermis - Semiquantitative score				
Age group	0	1	2	3	4
18-30	2	1	7	2	1
	(15.38 %)	(7.69 %)	(53.85 %)	(15.38 %)	(7.69 %)
31-50	1	2	1	4	0
	(12.50 %)	(25.00 %)	(12.50 %)	(50.00 %)	
51-70	1	0	3	5	1
	(10.00 %)		(30.00 %)	(50.00 %)	(10.00 %)
71-100	2	3	2	4	0
	(18.18 %)	(27.27 %)	(18.18 %)	(36.36 %)	
Total	6	6	13	15	2
	(14.29 %)	(14.29 %)	(30.95 %)	(35.71 %)	(4.76 %)
Jonckheere-Terpstra-Test p=0.902, Linear-by-linear association test p=1.00					

12.2	IL 6 dermis - Semiquantitative score				
Age group	0	1	2	3	4
18-30	13	0	0	0	0
	(100.00 %)				
31-50	6	2	0	0	0
	(75.00 %)	(25.00 %)			
51-70	5	1	3	0	0
	(55.56 %)	(11.11 %)	(33.33 %)		
71-100	4	1	2	4	0
	(36.36 %)	(9.09 %)	(18.18 %)	(36.36 %)	
Total	28	4	5	4	0
	(68.29 %)	(9.76 %)	(12.20 %)	(9.76 %)	
Jonckheere-Terpstra-Test ***p=5.01e-005***, Linear-by-linear association ***p=4.244e-005***					

12.3	IL 6 hair follicles - Semiquantitative score				
Age group	0	1	2	3	4
18-30	1	3	6	2	1
	(7.69 %)	(23.08 %)	(46.15 %)	(15.38 %)	(7.69 %)
31-50	0	0	4	4	0
			(50.00 %)	(50.00 %)	
51-70	0	2	1	6	0
		(22.22 %)	(11.11 %)	(66.67 %)	
71-100	0	2	2	6	1
		(18.18 %)	(18.18 %)	(54.55 %)	(9.09 %)
Total	1	7	13	18	2
	(2.44 %)	(17.07 %)	(31.71 %)	(43.90 %)	(4.88 %)
Jonckheere-Terpstra-Test ***p=0.065***, Linear-by-linear association test ***p=0.112***					

12.4	IL 6 sebaceous ducts - Semiquantitative score				
Age group	0	1	2	3	4
18-30	4	2	4	1	1
	(33.33 %)	(16.67 %)	(33.33 %)	(8.33 %)	(8.33 %)
31-50	3	0	2	3	0
	(37.50 %)		(25.00 %)	(37.50 %)	
51-70	2	1	2	4	0
	(22.22 %)	(11.11 %)	(22.22 %)	(44.44 %)	
71-100	3	1	3	3	1
	(27.27 %)	(9.09 %)	(27.27 %)	(27.27 %)	(9.09 %)
Total	12	4	11	11	0
	(30.00 %)	(10.00 %)	(27.50 %)	(27.50 %)	
Jonckheere-Terpstra-Test p=0.378, Linear-by-linear association test p=0.418					

12.5	IL 6 sebaceous glands - Semiquantitative score				
Age group	0	1	2	3	4
18-30	0	0	0	6	7
				(46.15 %)	(53.85 %)
31-50	0	0	1	2	5
			(12.50 %)	(25.00 %)	(62.50 %)
51-70	0	0	0	4	5
				(44.44 %)	(55.56 %)
71-100	2	0	0	6	3
	(18.18 %)			(54.55 %)	(27.27 %)
Total	2	0	1	18	20
	(4.88 %)		(2.44 %)	(43.90 %)	(48.78 %)
Jonckheere-Terpstra-Test ***p=0.141***, Linear-by-linear association test ***p=0.053***					

12.6	IL- 6 sweat glands - Semiquantitative score				
Age	0	1	2	3	4
18 - 30	0	1	1	7	4
		(7.69 %)	(7.69 %)	(53.85 %)	(30.77 %)
31 – 50	0	0	1	3	3
			(14.29 %)	(42.86 %)	(42.86 %)
51 - 70	1	0	2	4	2
	(11.11 %)		(22.22 %)	(44.44 %)	(22.22 %)
70 - 100	1	0	2	7	1
	(9.09 %)		(18.18 %)	(63.64 %)	(9.09 %)
Total	2	1	6	21	10
	(5.00 %)	(2.50 %)	(15.00 %)	(52.50 %)	(25.00 %)
Jonckheere-Terpstra-Test p=0.179, Linear-by-linear association test p=0.186					

3.9 IL-8 expression

IL-8 was found to be expressed in the sebaceous glands, sweat glands, epidermis and the hair follicles. The IL-8 showed a generally weak staining throughout the whole skin samples and different compartments, the strongest expression however was seen in the sebaceous glands followed by the sweat glands and the hair follicles. The epidermis and the hair follicles exhibited an ageing-related up-regulation. Other skin structures showed no significant ageing-related differences (Significance level 15% - Jonckheere-Terpstra test, p=0.14 and p= 0.01 respectively, Linear-by-linear association test p=0.02 and p=0.012 respectively) (Figure 10 and Tables13.1-13.5).

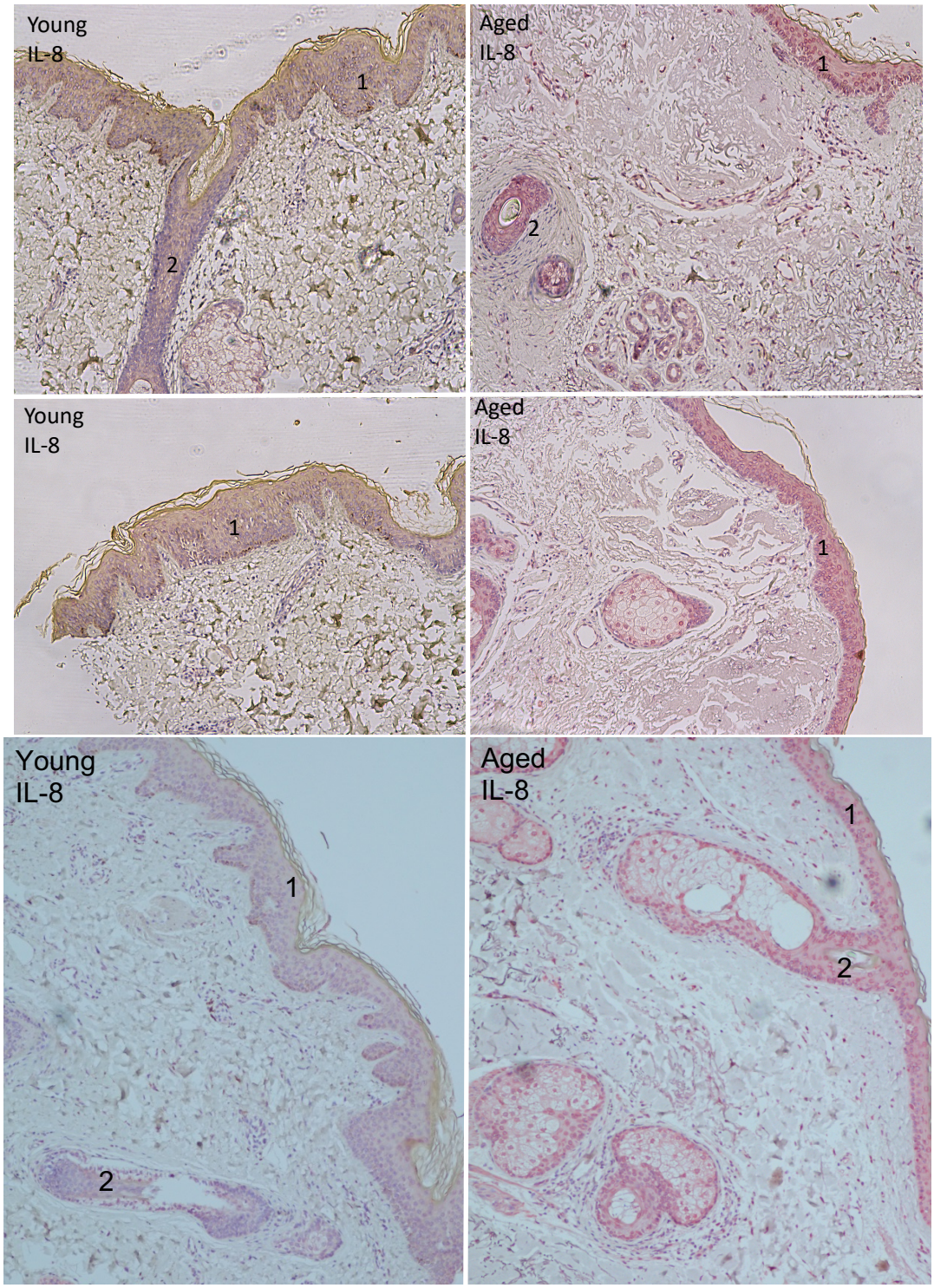


Figure 10 : Localization of the immunohistochemical signals of IL-8 in young and aged skin. IL-8 staining is overall very weak; however, an ageing-related up-regulation was seen in the epidermis (1) and hair follicles (2) (Original magnification x100)

Table 13.1-13.5**:** Expression of IL-8 in the different skin compartments and age groups. It exhibited an age-related up-regulation in the epidermis and hair follicles. Significant p values are typed in ***bold italic.***

13.1	IL-8 sweat glands - Semiquantitative score				
Age group	0	1	2	3	4
18-30	4	6	3	0	0
	(30.77 %)	(46.15 %)	(23.08 %)		
31-50	3	3	2	0	0
	(37.50 %)	(37.50 %)	(25.00 %)		
51-70	4	2	2	1	0
	(44.44 %)	(22.22 %)	(22.22 %)	(11.11 %)	
71-100	6	4	0	0	0
	(60.00 %)	(40.00 %)			
Total	17	15	7	1	0
	(42.50 %)	(37.50 %)	(17.50 %)	(2.50 %)	
Jonckheere-Terpstra-Test p=0.174, Linear-by-linear association test p=0.225					

13.2	IL-8 sebaceous glands - Semiquantitative score				
Age group	0	1	2	3	4
18-30	0	10	2	1	0
		(76.92 %)	(15.38 %)	(7.69 %)	
31-50	1	5	2	0	0
	(12.50 %)	(62.50 %)	(25.00 %)		
51-70	0	10	0	0	0
		(100.00 %)			
71-100	2	6	2	0	0
	(20.00 %)	(60.00 %)	(20.00 %)		
Total	3	31	6	1	0
	(7.32 %)	(75.61 %)	(14.63 %)	(2.44 %)	
Jonckheere-Terpstra-Test p=0.196, Linear-by-linear association test p=0.154					

13.3	IL-8 hair follicles - Semiquantitative score				
Age group	0	1	2	3	4
18-30	8	5	0	0	0
	(61.54 %)	(38.46 %)			
31-50	7	1	0	0	0
	(87.50 %)	(12.50 %)			
51-70	4	4	1	0	0
	(44.44 %)	(44.44 %)	(11.11 %)		
71-100	3	5	3	0	0
	(27.27 %)	(45.45 %)	(27.27 %)		
Total	22	15	4	0	0
	(53.66 %)	(36.59 %)	(9.76 %)		
Jonckheere-Terpstra-Test ***p=0.0219***, Linear-by-linear association test ***p=0.0128***					

13.4	IL-8 epidermis - Semiquantitative score				
Age group	0	1	2	3	4
18-30	10	3	0	0	0
	(76.92%)	(23.07%)			
31-50	7	1	0	0	0
	(87.5%)	(12.5%)			
51-70	7	1	0	0	0
	(87.5%)	(12.5%)			
71-100	6	3	2	0	0
	(54.54%)	(27.27%)	(18.18%)		
Total	30	8	2	0	0
	(75%)	(20%)	(5%)		
Jonckheere-Terpstra-Test ***p=0.14***, Linear-by-linear association test ***p=0.02***					

13.5	IL-8 sebaceous ducts - Semiquantitative score				
Age group	0	1	2	3	4
18-30	10	3	0	0	0
	(76.92%)	(23.07%)			
31-50	8	0	0	0	0
	(100%)				
51-70	8	0	0	0	0
	(100%)				
71-100	6	3	2	0	0
	(54.54%)	(27.27%)	(22.22%)		
Total	32	6	2	0	0
	(80%)	(15%)	(5%)		
Jonckheere-Terpstra-Test p=0.176, Linear-by-linear association test p=0.19					

3.10 TLR-4 expression

TLR-4 was expressed in almost all skin compartments mainly in the hair follicles, secretory portion of sweat glands the sebaceous glands and the sebaceous ducts. The staining in the epidermis was gradually fading towards the superficial epithelial layers and the sebaceous gland staining was differentiation-dependent, decreasing towards cell maturation. There was evidence of ageing-related down-regulation of the expression of TLR-4 antigen in the epidermis, hair follicles, sweat glands and sebaceous glands (Significance level 15% - Jonckheere-Terpstra test, p= 0.03, 0.09, 0.10 and 0.01 respectively) (Figures. 11 and 12, Tables 14.1-14.5).

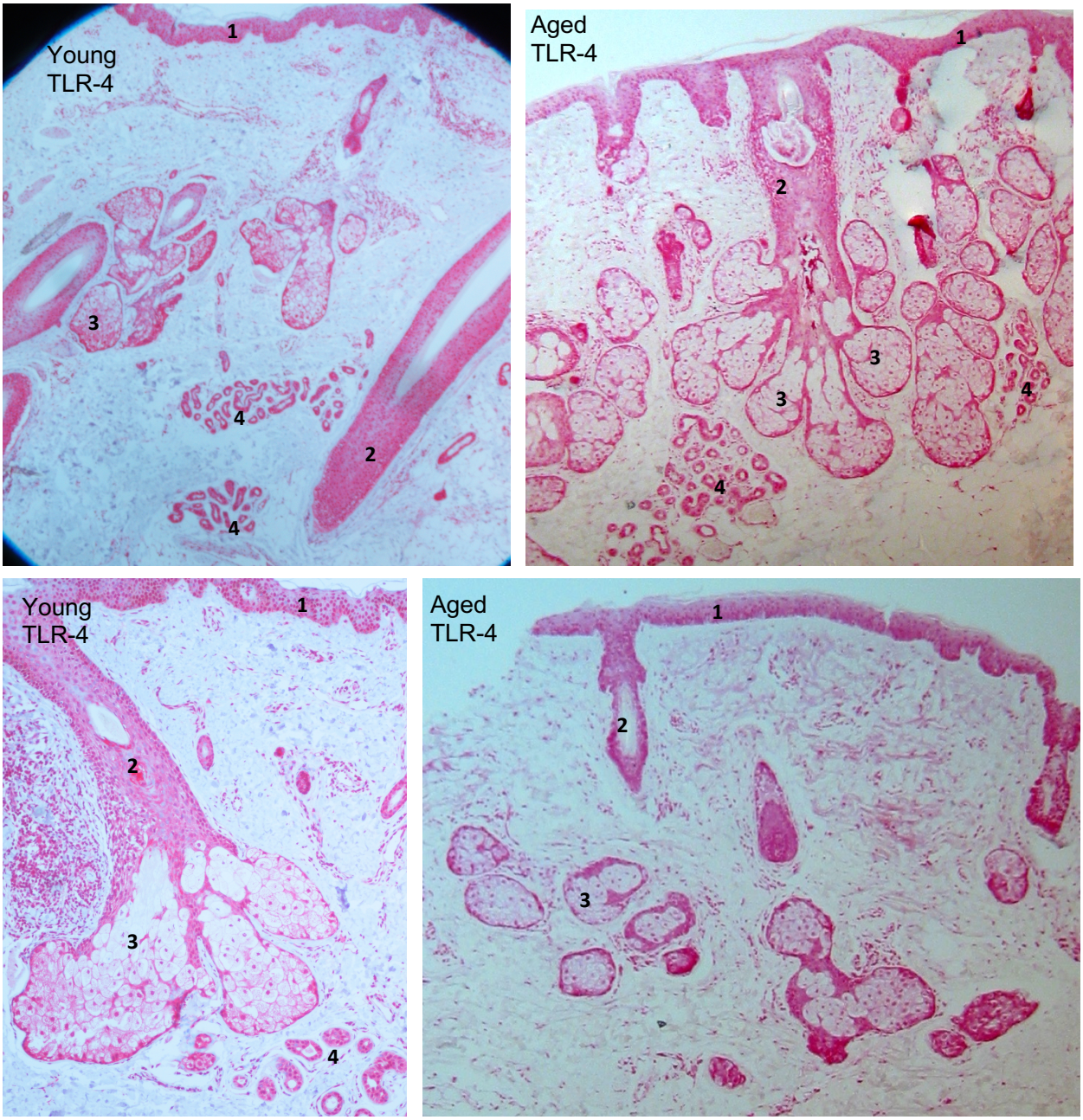


Figure 11: Localization of the immunohistochemical signals of TLR-4 in skin of the young and aged groups. Ageing-related down-regulation of the expression of TLR-4 antigen was shown in the epidermis (1), the hair follicles (2), sebaceous glands (3) and sweat glands (4) (original magnification x40) from Elewa et al 2015[91]

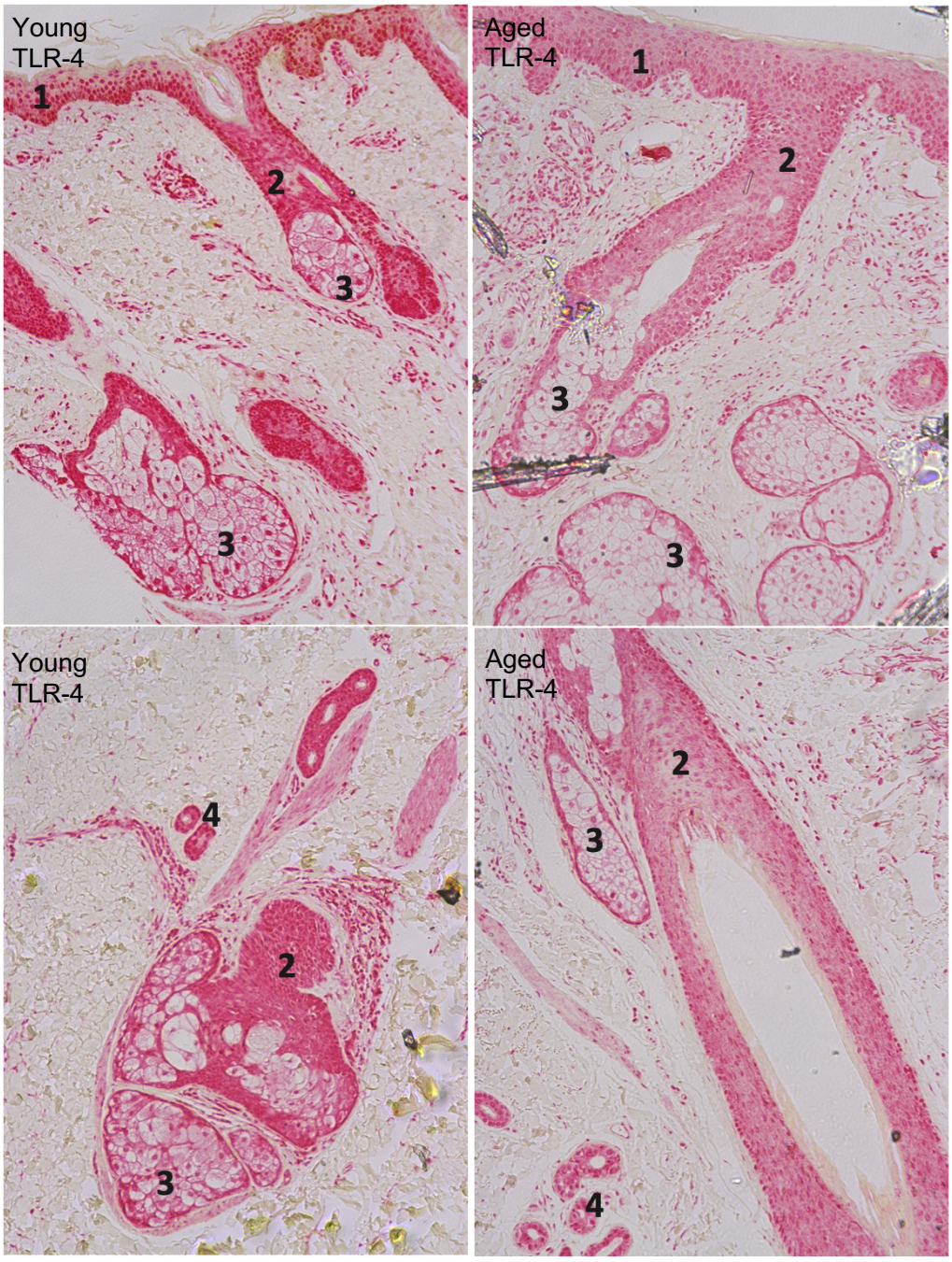


Figure 12: Localization of the immunohistochemical signals of TLR-4 in skin of the young and aged groups. Ageing-related down-regulation of the expression of TLR-4 antigen was shown the epidermis (1), hair follicles (2), sebaceous glands (3) and sweat glands (4) (original magnification x100)

Table 14.1-14.5**:** Expression of TLR-4 in the different skin compartments and age groups. TLR-4 exhibited an ageing-related down-regulation in almost all skin compartments. Significant p values are typed in ***bold italic.***

14.1	TLR-4 – Epidermis - Semiquantitative score				
Age group	0	1	2	3	4
18-30	0	0	1	7	5
			(7.69 %)	(53.85 %)	(38.46 %)
31-50	0	0	3	5	0
			(37.50 %)	(62.50 %)	
51-70	0	1	3	3	3
		(10.00 %)	(30.00 %)	(30.00 %)	(30.00 %)
71-100	0	0	4	7	0
			(36.36 %)	(63.64 %)	
Total	0	1	11	22	8
		(2.38 %)	(26.19 %)	(52.38 %)	(19.05 %)
Jonckheere-Terpstra-Test ***p=0.034***, Linear-by-linear association test ***p=0.04***					

14.2	TLR 4 – Hair follicle - Semiquantitative Score				
Age group	0	1	2	3	4
18-30	0	0	1	4	6
			(9.09 %)	(36.36 %)	(54.55 %)
31-50	0	0	0	4	4
				(50.00 %)	(50.00 %)
51-70	0	0	2	3	5
			(20.00 %)	(30.00 %)	(50.00 %)
71-100	0	0	0	10	1
				(90.91 %)	(9.09 %)
Total	0	0	3	21	16
			(7.50 %)	(52.50 %)	(40.00 %)
Jonckheere-Terpstra-Test p=0.152, Linear-by-linear association test ***p=0.09***					
14.3	TLR-4 - sweat glands - Semiquantitative score				
Age group	0	1	2	3	4
18-30	0	0	1	4	8
			(7.69 %)	(30.77 %)	(61.54 %)
31-50	0	0	1	5	2
			(12.50 %)	(62.50 %)	(25.00 %)
51-70	0	0	1	3	5
			(11.11 %)	(33.33 %)	(55.56 %)
71-100	0	0	0	9	1
				(90.00 %)	(10.00 %)
Total	0	0	3	21	16
			(7.50 %)	(52.50 %)	(40.00 %)
Jonckheere-Terpstra-Test ***p=0.107***, Linear-by-linear association test p=0.194					

14.4	TLR-4 - sebaceous duct - Semiquantitative score				
Age group	0	1	2	3	4
18-30	2	0	1	4	3
	(20.00 %)		(10.00 %)	(40.00 %)	(30.00 %)
31-50	0	0	0	3	5
				(37.50 %)	(62.50 %)
51-70	0	0	2	3	3
			(25.00 %)	(37.50 %)	(37.50 %)
71-100	0	0	0	10	0
				(100.00 %)	
Total	2	0	3	20	11
	(5.56 %)		(8.33 %)	(55.56 %)	(30.56 %)
Jonckheere-Terpstra-Test p=0.578, Linear-by-linear association test p=0.614					

14.5	TLR-4 sebaceous glands - Semiquantitative score				
Age group	0	1	2	3	4
18-30	0	2	1	5	4
		(16.67 %)	(8.33 %)	(41.67 %)	(33.33 %)
31-50	0	2	3	2	1
		(25.00 %)	(37.50 %)	(25.00 %)	(12.50 %)
51-70	0	3	1	2	4
		(30.00 %)	(10.00 %)	(20.00 %)	(40.00 %)
71-100	0	7	1	2	0
		(70.00 %)	(10.00 %)	(20.00 %)	
Total	0	14	6	11	9
		(35.00 %)	(15.00 %)	(27.50 %)	(22.50 %)
Jonckheere-Terpstra-Test ***p=0.0154***, Linear-by-linear association test ***p=0.0178***					

4. Discussion

This study is an attempt to elucidate a part of the physiology of the ageing process. This discussion will be carried out in the context of the statistically re-analysed results.

The process of ageing could be grouped in different possible pathways, which can give a clue to the complex mechanisms of skin senescence. In this work, I examined the expression of molecules involved in the following pillar pathways of ageing: the stress response and the neuroendocrine regulation, innate immunity reaction, chronic inflammation and tumorigenesis.

The results of this study could track some changes occurring along the different pillars of ageing and a concept of their possible interaction could be postulated.

The CRH system is responsible for the neuroendocrine regulation of the skin and the stress response reaction. It also influences directly the synthesis and quality of skin lipids, which vary enormously during the different stages of life[10,46]. The CRH system can affect cell proliferation and differentiation, chronic inflammation and age-related diseases[47,48]. The skin has a CRH system of its own, this was detected in previous studies on the protein and mRNA; moreover, CRH was found to be synthesised in the skin in the sebaceous gland, the so-called "brain" of the skin[49,50,51,52].

In this study, all CRH system components were found to be expressed almost all skin compartments, primarily concentrated in the sebaceous and sweat glands. Looking at the distribution of the CRH system components in the aged skin, we see that the sebaceous glands -lacking the buffering binding protein- fall under an unopposed function of the CRH, which in turn functions on highly expressed effector receptor (CRHR1). The hair follicles and the epidermis - having a highly expressed CRHR1- also fall under a heightened CRH function. This makes almost the whole components of the aged skin suffer a chronic stress-like condition. This is consistent with the drastic ageing-related changes in skin activity and function.

The CRH system was proved to play a role in skin disease known to be mediated by psychological stress as vitiligo[53] alopecia areata[54] and cutaneous lupus erythematosus[55].

The exaggerated stress response and overactivity of the CRH system in the aged skin corresponds to the previously detected heightened systemic hypothalamic-pituitary-adrenal (HPA) stress response[15]. However, in the sweat glands- a highly innervated compartment of the skin, - the CRH system ageing-related changes could represent one of the stages occurring in the ageing brain with Alzheimer's disease, as it was found that the CRH protects against amyloidosis in neuronal cultures[56].

Ideally, the CRH system is activated in cases of stress to combat possible dangers and attacks from the environment. In this study, however, I found that CRH system in the aged skin is continuously activated- in a way similar to what happens in the central nervous system, where ageing is associated with a chronic stress like condition where the stimulated HPA axis fails to be promptly arrested[57]. This continuous activated or alert status leaves the aged skin in a chronic stress-like situation with all its damaging consequences.

In the present study, some of the molecules involved in the innate immune regulation were examined. TLRs recognize both pathogen-associated molecular patterns and molecules that are released from damaged tissue. TLR activation regulates the expression of proinflammatory cytokines and chemokines - such as IL-6 and IL-8 - that are required for the innate immunity response, the activation of adaptive immunity. And the clearance and attenuation of viral infections in the respiratory tract epithelium and central nervous system in humans and mice[58].

Extreme IL-8 activation promotes the survival and replication of the respiratory syncytial virus and cytomegalovirus by promoting neutrophil recruitment, leading to pathologic consequences resulting from extreme inflammatory tissue damage[59]. Here we see the inflammation as a double-sided weapon, where the protection can turn into

damage. In the present study, we see IL-8 highly expressed in the aged epidermis and hair follicles.

IL-6, which in the current study showed a strong expression in sebaceous glands, seems to have a great control on the sebaceous gland function as it induces sebaceous lipogenesis and its inhibition directly reduces it[60,61]. The reduced levels of IL-6 in the aged sebaceous glands detected in the present study may explain the reduced lipogenesis known in aged skin and confirms the reduced response of epithelial compartments to inflammatory signals.

In contrast to the sebaceous glands, IL-6 exhibited ageing-dependent up-regulation in the dermis and the hair follicles, reflecting the molecular inflammatory state of ageing which has been detected in many other studies, where IL-6 is shown to be elevated in the aged healthy individuals[29-32] and associated with increased risk of ageing-related conditions, such as atherosclerosis, diabetes, obesity, obstructive sleep apnoea and hypertension as well as Alzheimer's disease[62,63].

TLR-4 in the current study exhibited an ageing-related down regulation in almost all skin compartments. This could explain the greater susceptibility to infections[22], poor response to vaccination[23] and the suboptimum delayed hypersensitivity reaction, which are known features of ageing.

Interestingly, the brain of aged mice expressed significantly higher TLR-4 levels, associated with higher inflammatory cytokine expression (IL-6 and TNF-α) in response to lipopolysccharide (LPS) injection[26]. At the end-stage Alzheimer's disease, however, the brain exhibits a down-regulation of TLR-4[28] which could be a late stage of the disease after the neurons expressing high levels of TLR-4 have been destroyed by the inflammatory process. In analogy to the previous, the ageing-related reduced expression of TLR-4 and IL-6 detected in the current study, especially in sebaceous glands, may indicate an almost end stage of skin ageing.

On the other hand, other studies have shown that high TLR-4 expression in age is associated with tumour cell proliferation[64] which could - in part - be explained by TLR-

4 polymorphisms, in which proinflammatory alleles of TLR-4 are related to unsuccessful ageing[27].

It has been shown in several studies, that the PPAR-α and PPAR-γ are known to have antitumorigenic properties. PPAR-α was found to inhibit tumour cell proliferation and tubule genesis in animal models[11] and PPAR-γ activation leads to decreased cancer cell proliferation in several malignancies[65]. A reduced PPAR-γ expression has been associated with poor tumour differentiation, higher cell proliferation and higher aneuploidy[66]. PPAR-α and PPAR-γ activators could protect against UVB-induced tissue damage and eventually carcinogenesis by reducing metalloproteinases and reactive oxygen species production and suppressing pro-inflammatory cytokines[67,68]. PPAR-α and PPAR-γ could be used as predicting markers for possible tumour development and later on as cancer treatment marker[69]. Moreover, they are known inhibitors of NF-kB signalling and its expression products[70,71]

In agreement with the previous data, PPAR-α and PPAR-γ in the present study exhibited ageing-related down-regulation, most significantly in the sebaceous and sweat glands, which could explain the tendency for ageing-related tumour development in the skin and indicates increased susceptibility to photo-ageing with time[67].

PPAR-δ, which was found in our study to be intensively expressed in the aged hair follicles and sebaceous ducts, was also found to be up-regulated in malignant and premalignant epithelial skin tumours[72] and it was found to directly stimulate the expression of Src, which is an oncogene promoting the development of UVB-induced skin cancer [73]. This could explain the high incidence of epithelial tumours in old age. In contrary to the above data, PPAR-δ expression and activation was found to reduce tumorigeneity in human skin carcinoma cells in vitro[74]. Also, in melanoma, the PPAR delta has a controversial role[75].

This work presents the ageing-related protein changes in mixed skin ageing pattern in both intrinsic and extrinsic ageing. The hormonal variations most probably do not play a role in the changes detected in this study, at least in the sebaceous glands. In

a study on human sebocyte culture in conditions resembling the hormonal ageing conditions[76], the genes coding for CRH, CRHBP, PPAR-γ, PPAR-δ, IL-6 and IL-8 showed no age-related changes, while the CRHR1 and PPAR-α were down regulated and TLR-4 was up regulated in a clear contrast to my study. This leaves us with the conclusion that the changes detected in this study are a result of extrinsic or mixed rather than hormonal ageing.

Reflected through the results of the current study we could visualise the molecular status of each of the compartments of the aged skin and the interaction between the involved molecules. This visualisation is diagrammatically represented in Figure 13.

Aged Epidermis: in addition to the chronic stress like status seen in the up-regulation of the effector receptor CRHR1, the epidermis shows an exaggerated IL-8 expression. Both together simulating the state of chemically or physically stressed skin (through either tape stripping[77] or chemical sensitizers[78]).

Aged dermis: The aged skin is well-known to be associated with reduced healing potential[79]. The ageing dermis in this study exhibits an exaggerated IL-6 expression, simulating the response of normal dermis to danger or physical trauma[80]. The CRH effect of reducing the healing capability of fibroblasts[81], was however not exhibited in this study, as the dermis was partially protected by the buffering effect of the overexpressed CRHBP.

Aged hair follicles in the current study suffer a chronic stress-like condition through the up-regulated expression of the CRHR1 effector receptor, and also a high IL-6 and IL-8 expression. This could contribute to the ageing-associated hair loss, slower hair growth[82] and hair greying[83]. The ageing-related up-regulated expression of the PPAR-δ seen in this study is essential for the normal development of hair[84], which could be seen as a protective mechanism against the elevated inflammatory cytokines. PPAR-δ however, as mentioned before, has a very controversial role weather in promoting or inhibiting skin carcinogenesis[85,73,74].

Aged sebocytes in this study suffered a chronic stress-like condition presented in lack of the buffering power of the CRHBP and the up-regulated expression of the effector receptor CRHR1. It is noteworthy that IL-6 - together with other cytokines - is responsible for the up-regulation of PPAR-α levels in human sebocytes[60]. In the present study, both the IL-6 and PPAR- α were down-regulated in the sebocytes of aged skin. We can see that the lack of IL-6 did not only make the sebaceous glands prone to infections, but also contributed to the down-regulation of PPAR-α, which in turn caused the sebaceous gland to become more prone to tumorigenesis. Furthermore, the age-dependent down-regulation of PPAR-γ in the sebaceous glands may explain the occurring of the ageing-related skin dryness[86] due to decreased sebum production, sebaceous gland hyperplasia and the development of the rare sebaceous carcinoma[86]. The aged sebocytes also lack the wound healing role, immune protective effect and chemotaxis, which are normally partially provided by the TLR-4 after being stimulated with LPS[87].

Aged sweat glands have a clearly disturbed stress response mechanism represented in elevated CRHBP and CRHR1 expression levels and reduced CRH expression level. This could be one of the mechanisms contributing to the defective response to the stress of heat, where the elderly individuals exhibited a decremented sweating sensitivity and a lower sweating rate, leading to less efficient thermoregulation[88]. Like the aged sebocytes, the aged sweat glands also exhibited a reduced PPAR-α and TLR-4 expression, with all their consequences.

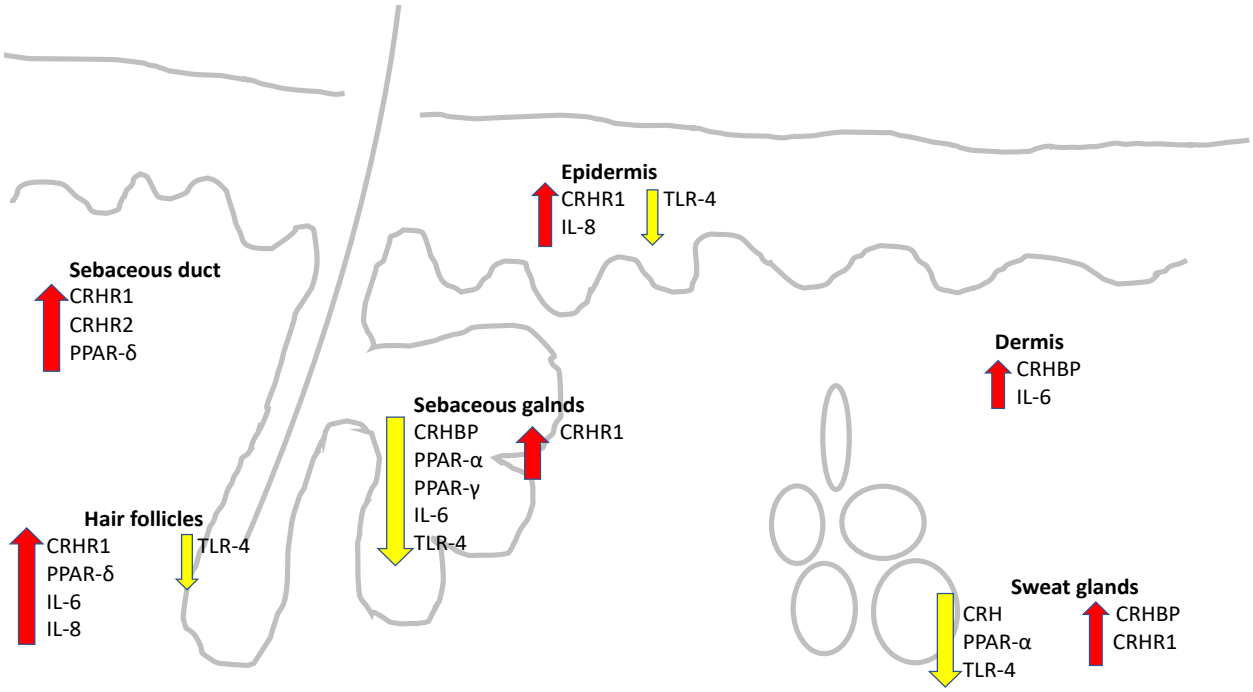


Figure 13: Diagrammatic representation of the molecular status of aged skin.
Red arrow pointing up: ageing-related up-regulation, yellow arrow pointing down: ageing-related down-regulation

In conclusion, the ageing process could be considered as a loss of the accurate balance between a minimum level of inflammation and stress response alertness which are needed for protection, and an overshooting level of inflammation and continuous stress-like condition which cause tissue damage and consequently support the ageing process, tumour development and enhance the susceptibility to infections. IL-6 and IL-8 – being down-regulated in some ageing skin compartments – could reflect an end stage ageing process where the inflammatory cytokines, when once in an overshooting level which damaged the skin, are finally reduced to a level that leaves the skin in an infection- and tumour-prone condition.

The ageing-dependent molecular skin changes detected in our study could partly explain the tumorigenesis and defective stress and immune responses of the ageing process. An interaction between the molecules involved in the study along the complicated ageing pathways was apparent[89,90,91]. (Figure 14) is an attempt to diagrammatically represent this interaction.

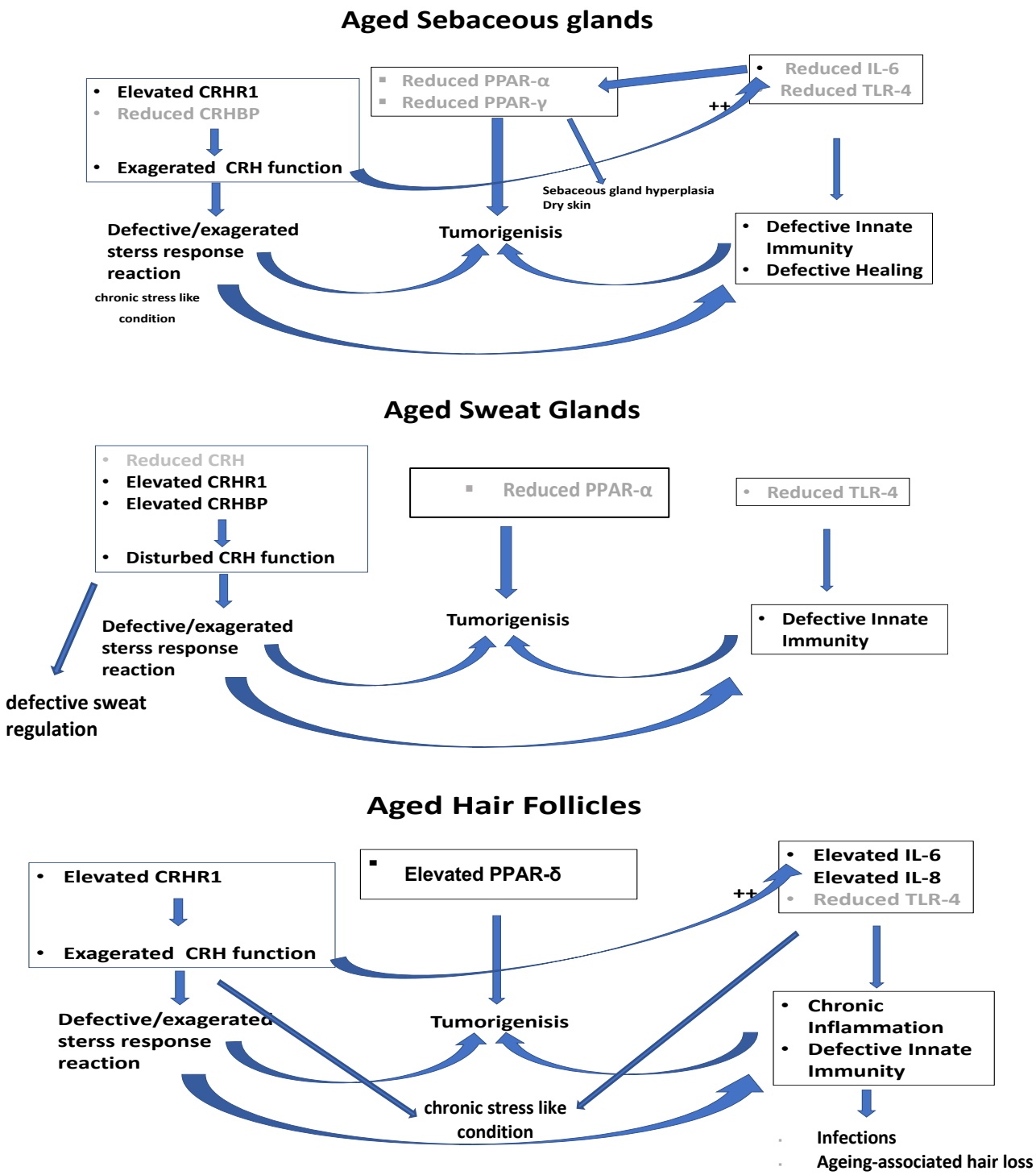


Figure 14: Diagrammatic representation of the interaction between ageing-associated pathways in different skin compartments. Adapted from Elewa et al 2015 [91]

References

1- Makrantonaki E, Zouboulis C. The skin as a mirror of the ageing process in the human organism –State of the art and results of the ageing research in the German National Genome Research Network 2 (NGFN-2). Experimental Gerontology 2007; 42: 879–886

2- Callaghan T.M and Wilhelm K.P. A review of ageing and an examination of clinical methods in the assessment of ageing skin. Part 2: Clinical perspectives and clinical methods in the evaluation of ageing skin. International Journal of Cosmetic Science 2008; 30: 323–332

3- Chung HY, Lee EK, Choi YJ, Kim JM, Kim DH, Zou Y, Kim CH, Lee J, Kim HS, Kim ND, Jung JH, Yu BP. Molecular inflammation as an underlying mechanism of the ageing process and age-related diseases. J Dent Res 2011; 90(7):830-40.

4- Zouboulis CC, Makrantonaki E . Clinical aspects and molecular diagnostics of skin ageing. Clin Dermatol 2011 ;29(1):3-14.

5- Yang HC, Deleuze S, Zuo Y, Potthoff SA, Ma LJ, Fogo AB. The PPAR gamma agonist pioglitazone ameliorates aging-related progressive renal injury. J Am Soc Nephrol Nov 2009 ;20(11):2380-8.

6- Baune BT, Ponath G, Rothermundt M, Roesler A, Berger K. Association between cytokines and cerebral MRI changes in the ageing brain. J Geriatr Psychiatry Neurol 2009; Mar;22(1):23-34.

7- Renshaw M, Rockwell J, Engleman C. Cutting edge: Impaired Toll like receptor expression and function in ageing. J Immunol 2002; 169: 4697–4701.

8- Gelinas D.S., McLaurin J. PPAR-alpha expression inversely correlates with inflammatory cytokines IL-1beta and TNF-alpha in ageing rats. Neurochem. Res 2005 ; 30, 1369–1375.

9- Kireev RA, Tresguerres AC, Garcia C, Borras C, Ariznavarreta C, Vara E, Vina J, Tresguerres JA. Hormonal regulation of pro-inflammatory and lipid peroxidation processes in liver of old ovariectomized female rats. Biogerontology 2010; Apr;11(2):229-43.

10- Ganceviciene R, Graziene V, Fimmel S, Zouboulis CC. Involvement of the corticotropin-releasing hormone system in the pathogenesis of acne vulgaris. Br J Dermatol 2009; 160:345-52.

11- Pozzi A, Popescu V, Yang S, Mei S, Shi M, Puolitaival SM, Caprioli RM, Capdevila JH. The anti-tumorigenic properties of peroxisomal proliferator-activated receptor alpha are arachidonic acid epoxygenase-mediated. J Biol Chem 2010; Apr 23;285(17):12840-50.

12- Ospelt C, Gay S. TLRs and chronic inflammation. Int J Biochem Cell Biol 2010; Apr;42(4):495-505.

13- Bove M, Carnevali L, Cicero AF, Grandi E, Gaddoni M, Noera G, Gaddi AV. Psychosocial factors and metabolic parameters: is there any association in elderly people? The Massa Lombarda Project. Ageing Ment Health 2010; 14:801-6.

14- Cai A, Wise PM. Age-related changes in the diurnal rhythm of CRH gene expression in the paraventricular nuclei. Am J Physiol 1996; 270:238-43.

15- Raadsheer FC, van Heerikhuize JJ, Lucassen PJ, Hoogendijk WJ, Tilders FJ, Swaab DF. Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. Am J Psychiatry.1995;152(9):1372-6.

16- Szczepanska-Sadowska E, Cudnoch-Jedrzejewska A, Ufnal M, Zera T. Brain and cardiovascular diseases: common neurogenic background of cardiovascular, metabolic and inflammatory diseases. J Physiol Pharmacol. 2010; 61(5):509-21.

17- Nunn AV, Bell J, Barter P. The integration of lipid-sensing and anti-inflammatory effects: how the PPARs play a role in metabolic balance. Nucl Recept. 2007; May 25;5(1):11-17.

18- Cha DR, Han JY, Su DM, Zhang Y, Fan X, Breyer MD, Guan Y. Peroxisome proliferator-activated receptor-alpha deficiency protects aged mice from insulin resistance induced by high-fat diet. Am J Nephrol. 2007 ;27(5):479-82.

19- Zhang H, Li Y, Fan Y, Wu J, Zhao B, Guan Y, Chien S, Wang N.Klotho is a target gene of PPAR-gamma. Kidney Int. 2008; Sep;74(6):732-9.

20- Michal M., Masternak and Andrzej Bartke. PPARs in Calorie Restricted and Genetically Long-Lived mice. PPAR Research 2007; Article ID: 28436, 7 pages.

21- Chung H.Y., Sung B., Jung K.J., Zou Y., Yu B.P. The molecular inflammatory process in ageing Antioxid. Redox Signal.2006 ;8: 572–581.

22- Gomez CR, Nomellini V, Faunce DE, Kovacs EJ. Innate immunity and ageing Exp Gerontol 2008; 43:718–728.

23- Gross PA, Hermogenes AW, Sacks HS, Lau J, Levandowski RA. The efficacy of influenza vaccine in elderly persons. Ann Int Med 1995; 123:518-27.

24- Yu L, Wang L, Li M, Zhong J, Wang Z, Chen S. Expression of toll-like receptor 4 is down-regulated during progression of cervical neoplasia. Cancer Immunol Immunother. 2010 Jul;59(7):1021-8.

25- Jin JJ, Kim HD, Maxwell JA, Li L, Fukuchi K.Toll-like receptor 4-dependent up-regulation of cytokines in a transgenic mouse model of Alzheimer's disease. J Neuroinflammation. 2008; 29; 15:23

26- Huang Y, Henry CJ, Dantzer R, Johnson RW, Godbout JP.Exaggerated sickness behaviour and brain pro inflammatory cytokine expression in aged mice in response to intracerebro-ventricular lipopolysaccharide. Neurobiol Ageing 2008; 29(11):1744-53.

27- Balistreri CR, Candore G, Lio D, Colonna-Romano G, Di Lorenzo G, Mansueto P, Rini G, Mansueto S, Cillari E, Franceschi C, Caruso C. Role of TLR4 receptor polymorphisms in Boutonneuse fever. Int J Immunopathol Pharmacol. 2005; 18(4):655-60.

28- Tang SC, Lathia JD, Selvaraj PK, Jo DG, Mughal MR, Cheng A, Siler DA, Markesbery WR, Arumugam TV, Mattson MP. Toll-like receptor-4 mediates neuronal apoptosis induced by amyloid beta-peptide and the membrane lipid peroxidation product 4-hydroxynonenal. Exp Neurol. 2008; Sep;213(1):114-21.

29- Singh T, Newman AB. Inflammatory markers in population studies of ageing. Ageing Res Rev. 2011 ;10(3):319-29

30- Wei J, Xu H, Davies JL, Hemmings GP. Increase of plasma IL-6 concentration with age in healthy subjects. Life Sci. 1992; 51(25):1953–1956.

31- Ershler WB, Sun WH. The role of interleukin-6 in certain age-related diseases. Drugs Ageing 1994; 5(5):358–365.

32- Ferrucci L, Corsi A, Lauretani F, Bandinelli S, Bartali B. The origins of age-related pro inflammatory state. Blood. 2005; 105(6): 2294–2299.

33- Wang L, Quan J, Johnston WE, Maass DL, Horton JW, Thomas JA, Tao W. Age-dependent differences of interleukin-6 activity in cardiac function after burn complicated by sepsis. Burns 2010; ;36(2):232-8.

34- Kristiansen OP, Mandrup-Poulsen T. Interleukin-6 and diabetes: the good, the bad, or the indifferent? Diabetes 2005; 54 Suppl 2: S114–24.

35- Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctot KL. A meta-analysis of cytokines in major depression. Biological Psychiatry 2010; 67 (5): 446–457.

36- Swardfager W, Lanctôt K, Rothenburg L, Wong A, Cappell J, Herrmann N. A meta-analysis of cytokines in Alzheimer's disease. Biological Psychiatry 2010; 68 (10): 930–941.

37- Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET. Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Rev. 2001; 12 (1): 33–40

38- Nishimoto N. Interleukin-6 in rheumatoid arthritis. Curr Opin Rheumatol 2006; 18 (3): 277–81.

39- Dugan LL, Ali SS, Shekhtman G, Roberts AJ, Lucero J, Quick KL, Behrens MM. IL-6 mediated degeneration of forebrain GABAergic interneurons and cognitive impairment in aged mice through activation of neuronal NADPH oxidase.PLoS One. 2009; 4(5):e5518.

40- Oliveira DM, Narciso FM, Santos ML, Pereira DS, Coelho FM, Dias JM, Pereira LS. Muscle strength but not functional capacity is associated with plasma interleukin-6 levels of community-dwelling elderly women. Braz J Med Biol Res.2008 ;41(12):1148-53.

41- Starr ME, Evers BM, Saito H.Age-associated increase in cytokine production during systemic inflammation: adipose tissue as a major source of IL-6. J Gerontol A Biol Sci Med Sci. 2009 ;64(7):723-30.

42- Weidle UH, Klostermann S, Eggle D, Krüger A. Interleukin 6/interleukin 6 receptor interaction and its role as a therapeutic target for treatment of cachexia and cancer. Cancer Genomics Proteomics. 2010 Nov-Dec;7(6):287-302.

43- Holmer R, Goumas FA, Waetzig GH, Rose-John S, Kalthoff H. Interleukin-6: a villain in the drama of pancreatic cancer development and progression. Hepatobiliary Pancreat Dis Int. 2014 Aug;13(4):371-80.

44- Baune BT, Ponath G, Golledge J, Varga G, Arolt V, Rothermundt M, Berger K. Association between IL-8 cytokine and cognitive performance in an elderly general population--the MEMO-Study. Neurobiol Ageing 2008 ; 29(6): 937-944.

45- Thirumangalakudi L, Yin L, Rao HV, Grammas P. IL-8 induces expression of matrix metalloproteinases, cell cycle and pro-apoptotic proteins, and cell death in cultured neurons. J Alzheimers Dis 2007 ;11(3):305-11.

46- Zouboulis CC, Boschnakow A. Chronological ageing and photo-ageing of the human sebaceous gland. Clin Exp Der- matol 2001;26:600–607.

47- Krause K, Schnitger A, Fimmel S, Glass E, Zouboulis CC. Corticotropin-releasing hormone skin signalling is receptor- mediated and is predominant in the sebaceous glands. Horm Metab Res 2007;39:166–170

48- Ziegler CG, Krug AW, Zouboulis CC, Bornstein SR. Corti- cotropin releasing hormone and its function in the skin. Horm Metab Res 2007;39:106–109

49- Slominski A, Wortsman J, Luger T, Paus R, Solomon S. Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol Rev 2000;80:979–1020.

50- Slominski A, Wortsman J, Pisarchik A, Zbytek B, Linton EA, Mazurkiewicz JE, Wei ET. Cutaneous expression of corti- cotropin-releasing hormone (CRH), urocortin, and CRH re- receptors. FASEB J 2001;15:1678–1693.

51- Slominski A, Pisarchik A, Tobin DJ, Mazurkiewicz JE, Wortsman J. Differential Expression of a cutaneous cortico- tropin-releasing hormone system. Endocrinology 2004;145: 941–950.

52- Zouboulis CC, Seltmann H, Hiroi N, Chen W, Young M, Oeff M, Scherbaum WA, Orfanos CE, McCann SM, Born- stein SR. Corticotropin-releasing hormone: An autocrine hormone that promotes lipogenesis in human sebocytes. Proc Natl Acad Sci USA 2002;99:7148–7153.

53- Shaker OG, Eltahlawi SM, Tawfic SO, Eltawdy AM, Bedair NI.Corticotropin-releasing hormone (CRH) and CRH receptor 1 gene expression in vitiligo. Clin Exp Dermatol. 2016 Oct;41(7):734-40.

54- Katsarou-Katsari A, Singh LK, Theoharides TC. Alopecia areata and affected skin CRH receptor upregulation induced by acute emotional stress. Dermatology. 2001;203(2):157-61.

55- Schmitz MK, Botte DA, Sotto MN, Borba EF, Bonfa E, de Mello SB. Increased corticotropin-releasing hormone (CRH) expression in cutaneous lupus lesions. Lupus. 2015 Jul;24(8):854-61. doi: 10.1177/0961203315569335. Epub 2015 Jan 28.

56- Pedersen WA, McCullers D, Culmsee C, Haughey NJ, Her- man JP, Mattson MP. Corticotropin-releasing hormone protects neurons against insults relevant to the pathogenesis of Alzheimer's disease. Neurobiol Dis 2001;8:492–503

57- Aguilera G. HPA axis responsiveness to stress: implications for healthy aging. Exp Gerontol. 2011 Feb-Mar;46(2-3):90-5.

58- Peng BH, Borisevich V, Popov VL, Zacks MA, Estes DM, Campbell GA, Paessler S. Production of IL-8, IL-17, IFN-gamma and IP-10 in human astrocytes correlates with alphavirus attenuation.Vet Microbiol. 2013 May 3;163(3-4):223-34.

59- Stoppelenburg AJ1, Salimi V, Hennus M, Plantinga M, Huis in 't Veld R, Walk J, Meerding J, Coenjaerts F, Bont L, Boes M.Local IL-17A potentiates early neutrophil recruitment to the respiratory tract during severe RSV infection. PLoS One. 2013; Oct 23;8(10):e78461.

60- Alestas T, Ganceviciene R, Fimmel S, Müller-Decker K, Zouboulis CC. Enzymes involved in the biosynthesis of leukotriene B4 and prostaglandin E2 are active in sebaceous glands. J Mol Med 2006; 84: 75-87.

61- Zouboulis CC , Angres S, Seltmann H.Regulation of stearoyl-coenzyme A desaturase and fatty acid delta-6 desaturase-2 expression by linoleic acid and arachidonic acid in human sebocytes leads to enhancement of proinflammatory activity but does not affect lipogenesis.Br J Dermatol. 2011 Aug;165(2):269-7

62- Scheller J, Ohnesorge N, Rose-John S. Interleukin-6 trans-signalling in chronic inflammation and cancer. Scand J Immunol 2006; 63: 321-329.

63- van Exel E, Eikelenboom P, Comijs H, Frölich M, Smit JH, Stek ML, Scheltens P,Eefsting JE, Westendorp RG.Vascular factors and markers of inflammation in offspring with a parental history of late-onset Alzheimer disease. Arch Gen Psychiatry 2009; 66: 1263-1270.

64- Huang B, Zhao J, Unkeless JC, Feng ZH, Xiong H. TLR signaling by tumor and immune cells: a double-edged sword. Oncogene 2008; 27: 218-224.

65- Harris G, Ghazallah RA, Nascene D, Wuertz B, Ondrey FG. PPAR activation and decreased proliferation in oral carcinoma cells with 4-HPR. Otolaryngol Head Neck Surg 2005; 133:695-701.

66- Espadinha C, Pinto AE, Leite V. Underexpression of PPARgamma is associated with aneuploidy and lower differentiation of thyroid tumours of follicular origin. Oncol Rep 2009; 22: 907-913.

67- Kim JK, Mun S, Kim MB, Sa BK, Hwang JK. 5,7-Dimethoxyflavone, an activator of PPARα/γ inhibits UVB-induced MMP expression in human skin fibroblast cells. Exp Dermatol 2012; 21: 211-216.

68- Park MH, Park JY, Lee HJ et al. The novel PPAR α/γ dual agonist MHY 966 modulates UVB-induced skin inflammation by inhibiting NF-κB activity. PLoS One 2013; 8: e76820.
69- Vamecq J, Colet JM, Vanden Eynde JJ, Briand G, Porchet N, Rocchi S. PPARs: Interference with Warburg' effect and clinical anticancer trials. PPAR Res 2012: 304760.

70- Poynter ME, Daynes RA. Peroxisome proliferator-activated receptor alpha activation modulates cellular redox status, represses nuclear factor-kappa B signaling, and reduces inflammatory cytokine production in aging. J Biol Chem 1998; 273: 32833-32841.

71- Chung JH, Seo AY, Chung SW et al. Molecular mechanism of PPAR in the regulation of age-related inflammation. Ageing Res Rev 2008; 7: 126-136.

72- Nijsten T, Geluyckens E, Colpaert C, Lambert J..Peroxisome proliferator-activated receptors in squamous cell carcinoma and its precursors. J Cutan Pathol. 2005 May;32(5):340-7

73- Montagner A, Delgado MB, Tallichet-Blanc C, Chan JS, Sng MK, Mottaz H, Degueurce G, Lippi Y, Moret C, Baruchet M, Antsiferova M, Werner S, Hohl D, Saati TA, Farmer PJ, Tan NS, Michalik L, Wahli W. Src is activated by the nuclear receptor peroxisome proliferator-activated receptor β/δ in ultraviolet radiation-induced skin cancer. EMBO Mol Med. 2014 Jan;6(1):80-98.

74- Borland MG, Kehres EM, Lee C, Wagner AL, Shannon BE, Albrecht PP, Zhu B, Gonzalez FJ, Peters JM. Inhibition of tumorigenesis by peroxisome proliferator-activated receptor (PPAR)-dependent cell cycle blocks in human skin carcinoma cells. Toxicology. 2018 Jul 1;404-405:25-32.

75- Peters JM, Gonzalez FJ, Müller R. Establishing the Role of PPARβ/δ in Carcinogenesis. Trends Endocrinol Metab. 2015 Nov;26(11):595-607.

76- Makrantonaki E, Adjaye J, Herwig R, Brink TC, Groth D, Hultschig C, Lehrach H, Zouboulis CC. Age-specific hormonal decline is accompanied by transcriptional changes in human sebocytes in vitro. Aging Cell 2006;5:331–344.

77- Koppes SA, Brans R, Ljubojevic Hadzavdic S, Frings-Dresen MH, Rustemeyer T, Kezic S. Stratum Corneum Tape Stripping: Monitoring of Inflammatory Mediators in Atopic Dermatitis Patients Using Topical Therapy. Int Arch Allergy Immunol.2016;170(3):187-93.

78- Frankart A, Coquette A, Schroeder KR, Poumay Y. Studies of cell signaling in a reconstructed human epidermis exposed to sensitizers: IL-8 synthesis and release depend on EGFR activation. Arch Dermatol Res. 2012 May;304(4):289-303.

79- Ashcroft GS, Mills SJ, Ashworth JJ. Ageing and wound healing. Biogerontology. 2002;3(6):337-45. Review.
80- Sjögren F, Anderson C. Sterile trauma to normal human dermis invariably induces IL1beta, IL6 and IL8 in an innate response to "danger". Acta Derm Venereol. 2009;89(5):459-65.

81- Rassouli O, Liapakis G, Lazaridis I, Sakellaris G, Gkountelias K, Gravanis A, Margioris AN, Karalis KP, Venihaki M. A novel role of peripheralcorticotropin-releasing hormone (CRH) on dermal fibroblasts. PLoS One.2011;6(7):e21654.

82- Yu M, Kissling S, Freyschmidt-Paul P, Hoffmann R, Shapiro J, McElwee KJ. Interleukin-6 cytokine family member oncostatin M is a hair-follicle-expressed factor with hair growth inhibitory properties. Exp Dermatol. 2008 Jan;17(1):12-9.

83- Dasanu CA, Mitsis D, Alexandrescu DT. Hair repigmentation associated with the use of lenalidomide: graying may not be an irreversible process! J Oncol Pharm Pract. 2013 Jun;19(2):165-9.

84- Di-Poï N, Ng CY, Tan NS, Yang Z, Hemmings BA, Desvergne B, Michalik L, Wahli W. Epithelium-mesenchyme interactions control the activity of peroxisome proliferator-activated receptor beta/delta during hair follicle development. Mol Cell Biol. 2005 Mar;25(5):1696-712.

85- Peters JM, Gonzalez FJ, Müller R. Establishing the Role of PPARβ/δ in Carcinogenesis. Trends Endocrinol Metab. 2015 Nov;26(11):595-607.

86- Dozsa A, Dezso B, Toth BI, Bacsi A, Poliska S, Camera E, Picardo M, Zouboulis CC, Bíró T, Schmitz G, Liebisch G, Rühl R, Remenyik E, Nagy L. PPARγ-mediated andarachidonic acid-dependent signaling is involved in differentiation and lipidproduction of human sebocytes. J Invest Dermatol. 2014 Apr;134(4):910-920.

87- Törőcsik D, Kovács D, Póliska S, Szentkereszty-Kovács Z, Lovászi M, Hegyi K, Szegedi A, Zouboulis CC, Ståhle M. Genome wide analysis of TLR1/2- and TLR4-activated SZ95 sebocytes reveals a complex immune-competence and identifies serum amyloid A as a marker for activated sebaceous glands. PLoS One. 2018 Jun 21;13(6):e0198323.

88- Inbar O, Morris N, Epstein Y, Gass G. Comparison of thermoregulatory responsesto exercise in dry heat among prepubertal boys, young adults and older males. ExpPhysiol. 2004 Nov;89(6):691-700.

89- Elewa R, Abdallah M, Youssef N, Zouboulis CC, Aging-related changes in cutaneous corticotropin-releasing hormone system reflect a defective neuroendocrine-stress response in aging. Rejuvenation Res 2012 Aug;15(4):366-73.

90- Makrantonaki E, Brink TC, Zampeli V, Elewa R, Mlody B, Hossini AM, Hermes B, Krause U, Knolle J, Abdallah M, Adjaye J, Zouboulis CC. Identification of biomarkers of human skin ageing in both genders. Wnt signalling - a label of skinageing? PLoS One. 2012;7(11): e50393.

91- Elewa RM, Abdallah M, Zouboulis CC. Aging-associated skin changes in innate immunity markers reflect a complex interaction of aging mechanisms in the sebaceous gland. The journal of dermatology 2015 May;42(5):467-76.

Eidesstattliche Versicherung

„Ich, Rana, Elewa versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: [Ageing-related molecular changes of the skin on the protein level – An immunohistochemichal study]
[Alterungsbedingte molekulare Veränderungen der Haut an der Proteinebene – Eine immunhistochemische Studie] selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -www.icmje.org) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Betreuer, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Städtisches Klinikum Dessau
Akadem. Lehrkrankenhaus d. Martin-Luther-Universität Halle-Wittenberg

Städtisches Klinikum Dessau • Auenweg 38 • 06847 Dessau-Rosslau

An die Charité Universitätsmedizin Berlin
z. Hd. Prof. Dr. med. Jörg-Wilhelm Oestmann
Vorsitzenden der Promotionskommission
Campus Virchow Klinikum der Charité
Augustenburger Platz 1
13353 Berlin

Klinik für Dermatologie, Venerologie und Allergologie, Immunologisches Zentrum

Chefarzt: Prof. Dr. med. Prof. h.c. Dr. h.c. Christos C. Zouboulis
Chefarztsekretariat: (03 40) 5 01-40 00
(03 40) 5 01-40 25
Stationssekretariat: (03 40) 5 01-40 59
(03 40) 5 01-40 65
ramona.rückert@klinikum-dessau.de

Dessau, 19. April 2016

Oberärzte
Dr. med. Dietrich Trebing, Ltd. OA -40 58
Dr. med. Andreas Altenburg -40 00
Dr. med. Martina Brunner -40 13
Dr. med. Peggy Seele -40 64
Dr. med. Thomas Wild (Chirurg) -40 26

Ambulanz (03 40) 5 01-40 10
(30 40) 5 01-40 22
OP-Bereich (03 40) 5 01-40 19

Chefarzt-Sprechstunde
Montag ab 11.00 Uhr
Donnerstag ab 08.00 Uhr

Station (03 40) 5 01-40 50
Allergologie (03 40) 5 01-40 57
Balneophotochemotherapie (03 40) 5 01-48 48

HKZ HAUTKREBS ZENTRUM DESSAU-ANHALT
Leiter: OA Dr. Dietrich Trebing
Sekretariat (03 40) 5 01-40 59
britt.binus@klinikum-dessau.de

ZKSD
Zentrum für Klinische Studien Dessau
Leiter: Prof. Dr. Prof. h.c. Dr.h.c. C.C. Zouboulis
Sekretariat (03 40) 5 01-40 04
sabine.schmalle@klinikum-dessau.de

Kliniklaboratorien (03 40) 5 01-40 20
Allergologisches Labor (03 40) 5 01-40 18
Histologisches Labor (03 40) 5 01-40 16
Immunologisches Labor (03 40) 5 01-40 17
Mykologisches Labor (03 40) 5 01-40 14

EURL ECVAM
European Union Reference Laboratory for Alternatives to Animal Testing

Forschungslaboratorien
(03 40) 5 01-40 15

Anteilserklärung an etwaigen erfolgten Publikationen

Sehr geehrter Herr Kollege Oestmann,

Frau Rana Elewa hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1:
Authoren: Elewa RM, Abdallah M, Youssef N, Zouboulis CC
Titel: Aging-related changes in cutaneous corticotropin-releasing hormone system reflect a defective neuroendocrine-stress response in aging
Zeitschrift: Rejuvenation Research
Erscheinungsjahr: 2012
Beitrag im Einzelnen:
Sammlung der Hautproben, Modifikation und Anpassung der Methodik, histochemische und immunhistochemische Färbung der Präparate, blinde Beurteilung der gefärbten Präparate, Fotografieren der Präparate, statistische Analyse, Diskussion der Ergebnisse, Schreiben und Korrektur des Manuskriptes

Städtisches Klinikum Dessau
Akadem. Lehrkrankenhaus d. Martin-Luther-Universität Halle-Wittenberg

Publikation 2:
Authoren: Makrantonaki E, Brink TC, Zampeli V, Elewa RM, Mlody B, Hossini AM, Hermes B, Krause U, Knolle J, Abdallah M, Adjaye J, Zouboulis CC
Titel: Identification of biomarkers of human skin ageing in both genders. Wnt signalling - a label of skin ageing?
Zeitschrift: PLoS One
Erscheinungsjahr: 2012
Beitrag im Einzelnen:
Sammlung der Hautproben, immunhistochemische Färbung der Präparate mit PPAR-delta-, Wnt-, und Frizz-Antikörpern, blinde Beurteilung der gefärbten Präparate, Fotografieren der Präparate, Teilschreiben des Manuskriptes

Publikation 3:
Authoren: Elewa R, Abdallah M, Zouboulis CC
Titel: Ageing-associated skin changes in innate immunity markers reflect a complex interaction of aging mechanisms in the sebaceous gland
Zeitschrift: The Journal of Dermatology
Erscheinungsjahr: 2015
Beitrag im Einzelnen:
Sammlung der Hautproben, Modifikation und Anpassung der Methodik, histochemische und immunhistochemische Färbung der Präparate, blinde Beurteilung der gefärbten Präparate, Fotografieren der Präparate, statistische Analyse, Diskussion der Ergebnisse, Schreiben und Korrektur des Manuskriptes

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers

Dessau, den 19. April 2016

Prof. Dr. med. habil. Prof. h.c. Dr. h.c. Christos C. Zouboulis
Chefarzt
Klinik für Dermatologie, Venerologie und Allergologie / Immunologisches Zentrum
Städtisches Klinikum Dessau
Auenweg 38 • 06847 Dessau-Roßlau

Unterschrift der Doktorandin

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publication list

1. Elewa R, Altenburg A, Zouboulis CC. Recalcitrant severe erosive cutaneous lichen planus treated with extracorporeal photopheresis monotherapy. Br J Dermatol. 2011 Aug;165(2):441-3.
2. Ragab N, Abdallah M, El-Gohary E, Elewa R. Stress and serum TNF-alpha levels may predict disease outcome in patients with pemphigus: a preliminary study. Cutis. 2011 Apr;87(4):189-94.
3. Elewa RM, Abdallah M, Youssef N, Zouboulis CC, Aging-related changes in cutaneous corticotropin-releasing hormone system reflect a defective neuroendocrine-stress response in aging. Rejuvenation Res. 2012 Aug;15(4):366-73.
4. Elewa R and Zouboulis CC. Vitamin A and the skin.in: Nutrition and Skin-Lessons for Anti-Aging, Beauty and Healthy skin, A. Pappas (ed.) 1st Ed. New York: Springer, 2012, pp 7-24.
5. Makrantonaki E, Brink TC, Zampeli V, Elewa RM, Mlody B, Hossini AM, Hermes B, Krause U, Knolle J, Abdallah M, Adjaye J, Zouboulis CC. Identification of biomarkers of human skin ageing in both genders. Wnt signalling - a label of skinageing? PLoS One. 2012;7(11): e50393.
6. Elewa R, Makrantonaki E, Zouboulis CC. Neuropeptides and skin aging. Horm Mol Biol Clin Investig. 2013 Dec;16(1):29-33.
7. Elewa R, Zouboulis CC, Review: Molecular mechanisms of action of topical anti-aging compounds, Journal of the Egyptian women's dermatologic society, May 2014 - Volume 11 - Issue 2
8. Elewa R M, Abdallah MA, Zouboulis CC. Age-associated skin changes in innate immunity markers reflect a complex interaction between aging mechanisms in the sebaceous gland. J Dermatol. 2015 May;42(5):467-76.

9. Abdel-Naser MB, Liakou AI, Elewa R, Hippe S, Knolle J, Zouboulis CC. Increased Activity and Number of Epidermal Melanocytes in Lesional Psoriatic Skin. Dermatology. 2016;232(4):425-30. Epub 2016 Aug 6.
10. Zouboulis CC, Elewa R, Ottaviani M, Fluhr J, Picardo M, Bernois A, Heusèle C, Camera E. Age influences the skin reaction pattern to mechanical stress and its repair level through skin care products. Mech Ageing Dev. 2018 Mar; 170: 98-105. doi: 10.1016 / j.mad.2017.11.011. Epub 2017 22. November.
11. Zouboulis CC, Ganceviciene R, Liakou AI, Theodoridis A, Elewa R, Makrantonaki E. Aesthetic aspects of skin aging, prevention, and local treatment. Clin Dermatol. 2019 Jul - Aug;37(4):365-372. doi: 10.1016/j.clindermatol.2019.04.002. Epub 2019 Apr 26. PubMed PMID: 31345325.

Certificate for approval of the statistical methods- Prof. Dr. rer. nat. Ulrich Mansmann

LMU LUDWIG-MAXIMILIANS UNIVERSITÄT MÜNCHEN

MEDIZINISCHE FAKULTÄT
INSTITUT FÜR MEDIZINISCHE INFORMATIONSVERARBEITUNG, BIOMETRIE UND EPIDEMIOLOGIE
LEHRSTUHL BIOMETRIE UND BIOINFORMATIK

IBE · Marchioninistraße 15 · 81377 München

Direktor: Prof. Dr. Ulrich Mansmann

Marchioninistraße 15
D-81377 München

Telefon: 089 / 4400-74491
Telefax: 089 / 4400-77491

http://www.ibe.med.uni-muenchen.de/index.htmlulrich.mansmann@lmu.de

München, 11.06.2018

To whom it may concern

Statistisches Begleitschreiben zur Promotion von Frau Rana Elewa

Sehr geehrte Damen und Herrn,

hiermit bestätige ich, dass Frau Rana Elewa von mir hinsichtlich der statistischen Auswertung zu ihrem Promotionsvorhaben beraten wurde. Weiterhin bestätige ich, die Umsetzung der Beratungsinhalte in der vorgelegten Arbeit geprüft zu haben.

Frau Elewa erfasst semiquantitative die Färbung verschiedener Antikörper in verschiedenen Zelltypen. Hier handelt es sich um ordinale Variablen. Die zentrale Fragestellung betraf eine Untersuchung der Korrelation der ordinalen Färbungsbewertungen mit dem Alter, das ebenfalls in Altersgruppen eingeteilt eine ordinale Variable darstellt.

Frau Elewa hat in ihrer Arbeit die entsprechenden Kreuztabellen (Alter versus Färbung) mit den vorhandenen Rohdaten dargestellt. Somit kann jeder Leser versuchen, die Ergebnisse selbst nachzurechnen.

Um die Korrelation zwischen zwei ordinalen Variablen zu prüfen werden typischerweise folgende Tests eingesetzt: Der „linear-by-linear association test“ oder der Jonckheere-Terpstra-Test. Beide Tests überprüfen, ob ein monotoner Zusammenhang zwischen den eingebrachten Variablen vorliegt. Beide Tests lassen sich auch auf nxm Tafeln anwenden um das monotone Verhalten der einen Variablen (mit m ordinalen Ausprägungen) zu prüfen wenn die zweite Variable (mit n ordinalen Ausprägungen) zunimmt.

Erschwerend kam bei der Analyse hinzu, dass Frau Elewa nur über eine übersichtliche Anzahl von Proben verfügt. Damit können statistische Testverfahren, die theoretisch auf der Annahme einer Asymptotik beruhen (Annahme einer sehr großen Stichprobenanzahl) nicht verwendet werden. Um dieses Problem zu lösen wurden von Statistikern sogenannte „exakte Versionen“ der Testverfahren entwickelt, die auch auf kleinen Stichproben verlässlich arbeiten. Frau Elewa hat sich dieser exakten Versionen des „linear-by-linear association test“ und des Jonckheere-Terpstra-Tests bedient. Diese werden in der Software StatXact zur Verfügung gestellt.

Ich hatte Frau Elewa auch geraten aufgrund der geringen Fallzahl und des Pilotcharakters Ihrer Studie den Wert 0.15 als Signifikanzniveau zu verwenden. Damit erhöht sich die Rate falsch positiver Ergebnisse, andererseits wird aber die falsch negativ Rate verringert. Die Verringerung der falsch negativen Rate ist im Falle einer Pilotstudie extrem wichtig. Einer Pilotstudie werden weitere Validierungsschritte folgen. Für den nun anstehenden Validierungsprozess sollen alle relevanten Kandidaten zur Verfügung stehen. Falsch positive Kandidaten werden im Laufe des Validierungsprozesses eliminiert werden. Die richtig positiven dann - gegeben eine ausreichende Power - sichtbar werden. Frau Elewa hat nur die Pilotstudie durchgeführt, die Validierung der Ergebnisse müssen weitere Studien leisten.

Ich hoffe, dass sich damit die statistische Analysestrategie von Frau Elewa verdeutlicht und von den Reviewern der Arbeit als angemessen erachtet wird.

Mit freundlichen Grüßen

Prof. Dr. rer. nat. Ulrich Mansmann

Acknowledgement

First and last of all, all praises to God.

I am truly grateful to Univ.-Prof. Dr. med. Prof. honoraire Dr. h.c. Christos C. Zouboulis, Chair, Departments of Dermatology, Venerology, Allergology and Immunology, Dessau Medical Center, Brandenburg Medical School Theodor Fontane, Dessau, Germany for his close supervision, sincere help, valuable suggestions and continuous encouragement throughout the entire work.

I am deeply thankful to Prof. Dr. rer. nat. Ulrich Mansmann director of the Institute for Medical Information Processing, Biometry and Epidemiology (IBE), Faculty of Medicine, Ludwig Maximilian University, Munich, Germany for his sincere help in revising and optimizing the statistical analysis of the results.

I would like to express my deepest gratitude to my son, my parents and friends for their continuous support throughout this work.

Appendixes

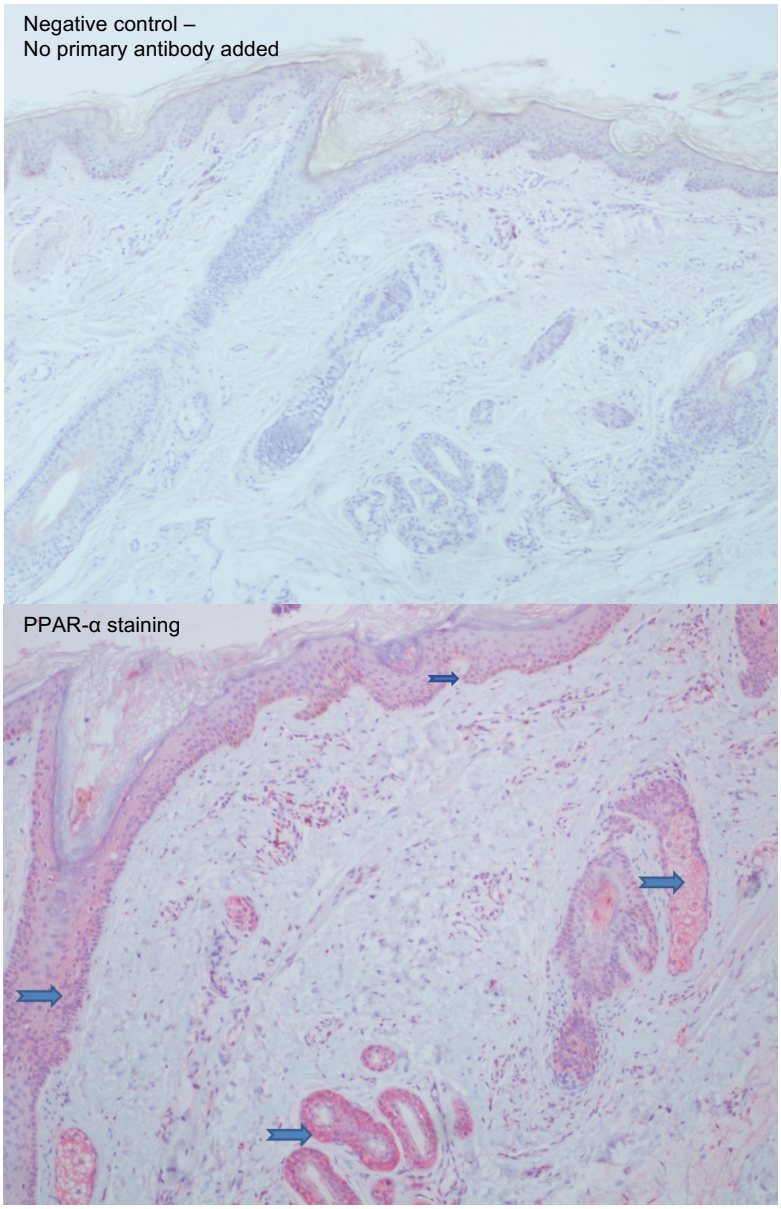
Appendix -1-

Table representing the population of the study, showing the gender and age.
(m = male, w= female, age in years)

Volunteer Number	gender	age
1	w	18
2	m	19
3	m	20
4	m	21
5	m	22
6	m	22
7	m	23
8	m	24
9	w	25
10	m	26
11	w	29
12	m	30
13	w	30
14	w	31
15	m	35
16	w	36
17	w	38
18	m	39
19	m	41
20	w	44
21	w	45

Volunteer number	gender	age
22	w	51
23	w	52
24	m	54
25	m	54
26	m	55
27	m	58
28	w	60
29	m	69
30	m	70
31	w	70
32	m	74
33	w	75
34	m	76
35	m	77
36	m	83
37	m	83
38	w	84
39	m	85
40	m	87
41	w	87
42	m	92

Appendix -2



An example for a negative control slide (upper panel) – where the full staining procedure was performed with all staining materials except for the primary antibody. The upper panel shows no staining except for the haematoxylin counterstain apparent in the crisp blue colour of the nuclei. Compared to another cut of the same specimen in which the PPAR-α primary antibody was added (lower panel). The lower panel shows the red stain in the epidermis, hair follicles, sebaceous glands and sweat glands representing the PPAR-α immunohistochemichal staining. (marked with blue arrows). (lower panel from Elewa et al. 2015[91]) (Original magnification x40)