Molecular diagnostic approach to determine the degree of photoaging of the skin

by

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining and qualification.

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ABSTRACT

Context: Excessive exposure to ultraviolet radiation (UV) results in the risk of acquiring long-term harmful effects such as photoaging, which is characterised by deep wrinkles, roughness, dyspigmentation and an increased loss in elasticity. As a result, the detection of photoaging at an early stage is crucial to improving morbidity, whilst preventing the advancement of skin cancer.

Aim: The aim of the study was to develop and to validate a diagnostic real-time PCR method in order to establish the gene expression profiles of potential biomarkers in the skin so as to quantify the degree of photoaging: this was conducted by retrieving total RNA from cells adherent to tape strips from sun exposed and non-exposed skin areas.

Materials and methods: Twenty healthy volunteers consisting of seven males and thirteen females aged 25 to 67 years were included in this study. Tape stripping was performed using pre-cut D-Squame® 22 mm adhesive discs. Samples were collected on the right medial thigh area 20 cm above the patella and 2 cm below the lateral canthus of the right eye. Total RNA was extracted and relative standard curve method of gene expression was performed. TGF-β, MMP 9, TNF-α and IL-6 mRNA transcripts were selected as representative cytokines to determine the relative fold-change in sun exposed and non-exposed areas of the skin so as to determine extent of photoaging.

Results: Repeatability and reproducibility was determined by the coefficient of variation (CV) was within an acceptable range. Thirty five percent (n=7) samples displayed down-regulatory effects for TGF-β. Down regulation of MMP 9 was observed within 30% (n=6) of samples, while 15% (n=3) showed marked up regulation. Only two samples showed measurable levels of TNF-α in the assay, of which one showed significant up regulation. Furthermore, we were unable to detect any IL-6 expression in any of the samples prepared.

Conclusion: we have shown that epidermal cytokines can be retrieved from tape stripped samples and can be quantified via real-time PCR. However, the choices of cytokine biomarkers reveal that they are as important as the concentration of starting material. In this study cytokines such as IL-6 is not as informative in determining the extent of photoaging without high doses of ultraviolet radiation before sample collection as opposed to the other explored cytokines.

Key words: real-time PCR, photoaging, cytokines

UITREKSEL

Konteks: Oormatige blootstelling aan ultraviolet (UV) bestraling kan tot ‘n risiko van skadelike en lantermynse nagevolge lei wat gekenmerk word deur foto-veroudering. Dit sluit in diep plooie, growwe vel en ‘n toenemende verlies in elastisiteit. Die ontdekking van foto-veroudering op ‘n vroeë stadium is van kardinale belang vir die verbetering van morbiditeit en die voorkoming van velkanker bevordering.

Doelstelling: Die doel van hierdie studie was om ‘n diagnostiese polimerase kettings reaksie (PKR) metode te ontwikkel om geen uitdrukkings profiele van potensiële bio-merkers te vestig in die vel, om so die graad van foto-veroudering in areas van vel wat blootgestel word aan die son en beskermde van die son te bepaal deur totale RNS te versamel van kleeflintskyfies.

Materiale en metodes: Twintig gesonde vrywilligers (sewe mans en dertien vroue), tussen die ouderdom van 25 en 67 jaar, was ingesluit in hiedie studie. Vel monsters was versamel deur gebruik te maak van D-squame® 22 mm kleeflintskyfies 20 cm bokant die patella van die regterkanste mediale heup en 2 cm onder die regter oog. Totale RNS was geisoleer en die relatiewe vlak van geen uitdrukking was bepaal deur gebruik te maak van die kurwe model. Die boodskapper ribonukleiosier transkripsies van die sitokiene TGF-β, MMP 9, TNF-α en IL-6 was gekies as verteenwoordigers van foto-veroudering om die relatiewe verandering van foto-veroudering in die vel te bepaal.

Resultate: Validering metodes was aanvaarbaar. ‘n Afwaarts reguleringseffek in TGF-β en MMP 9 merker uitdrukking is gevind in vyf en dertig persent (n=7) en dertig persent (n=6) van monsters, onderskuidelik. In vyftien persent (n=3) van monsters is ‘n opwaarts reguleringseffek in die laasgenoemde gevind. Slegs twee monsters het meetbare vlakke van TNF-α getoon in die eksperiment, waarvan slegs een ‘n noemenswaardige opwaartse regulering getoon het. IL-6 uitdrukking is nie gevind in enige van die monsters.

Gevolgtrekkings: Hierdie studie het bepaal dat sitokiene van die vel geisoleer van kleeflint monsters en gekwantifiseer deer relatiewe PKR uitdrukking bepaal kan word. Die keuse van bio-merkers is egter net so belangrik as konsentrasie bepaling van die monsters. Die IL-6 sitokien, in vergelyking met ander, is slegs informaliet tydens hoë ultraviolet bestraling aan die vel blootgestel is.

Sleutelwoorde: relatiewe PKR, foto-veroudering, sitokiene

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LIST OF ABBREVIATIONS

5-prime	**5'**
3-prime	**3'**
Base pair	**bp**
Centimeter	**cm**
Carbon dioxide	**CO_2**
Coefficient of variation	**CV**
Complementary deoxyribonucleic acid	**cDNA**
Degrees Celsius	**°C**
2'-deoxy-nucleotide-5'-triphosphate	**dNTP**
Efficiency	**E**
Ethylenediaminetetraacetic acid	**EDTA**
Genomic deoxyribonucleic acid	**gDNA**
Glyceraldehyde 3-phosphate dehydrogenase	**GAPDH**
Interleukin 6	**IL-6**
Kilogram	**kg**
Lipopolysaccharide	**LPS**
Matrix metalloproteinase 9	**MMP 9**
Messenger ribonucleic acid	**mRNA**
Microlitre	**µl**
Micromolar	**µM**
Millilitre	**ml**
Millimoles per litre	**mM**
Millimeter	**mm**
Minute	**min**
Meter square	**m^2**
Nanogram	**ng**
Nanometer	**nm**
Normality	**N**
Percentage	**%**
Peripheral blood mononuclear cell	**PBMC**
Polymerase chain reaction	**PCR**
Picomole	**pmol**

Quantification cycle	**Cq**
Revolutions per minute	**rpm**
Ribonucleic acid	**RNA**
Roswell Park Memorial Institute	**RPMI**
Seconds	**sec**
Transforming growth factor-beta	**TGF-β**
Melting temperature	**Tm**
No enzyme control	**NEC**
No template control	**NTC**
Texas	**Tx**
Tumor necrosis factor-alpha	**TNF-α**
United States of America	**USA**
Ultraviolet	**UV**
Ultraviolet A	**UVA**
Ultraviolet B	**UVB**
Ultraviolet C	**UVC**
Units	**U**
Universal Probe library	**UPL**
World Health Organisation	**WHO**
Volts	**V**

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Excessive exposure to ultraviolet radiation (UV) results in the risk of acquiring long-term harmful effects such as photoaging, which is characterised by deep wrinkles, roughness, dyspigmentation and an increased loss in elasticity [1]. As a result, the detection of photoaging at an early stage is crucial to improving morbidity, whilst preventing the advancement of skin cancer [2].

This chapter will briefly review the mechanism of photoaging in humans and discuss the ongoing research and challenges faced in the field. Furthermore, this chapter will address the rationale, aim and specific objectives of this study.

1.2 Sunlight: friend or enemy?

Since before the dawn of man, sunlight has been a necessity for life on earth. Many have worshipped it in recognition of its life giving powers [3]. Its benefits as a form of treatment for both mental and skin disorders was discovered and utilized with great achievement [4]. However, prolonged exposure to sunlight may produce harmful effects such as photoaging and induce susceptibility to skin cancer [5].

1.3 Ultraviolet radiation and its effects on human skin

Sunlight is made up of a continuous spectrum of electromagnetic radiation with differing wavelengths as seen in Figure 1.1. This spectrum is sub divided into ultraviolet which consists of wavelengths ranging from ultraviolet C (200–280 nm), ultraviolet B (280–320 nm) to ultraviolet A (320–400 nm) radiation [6].

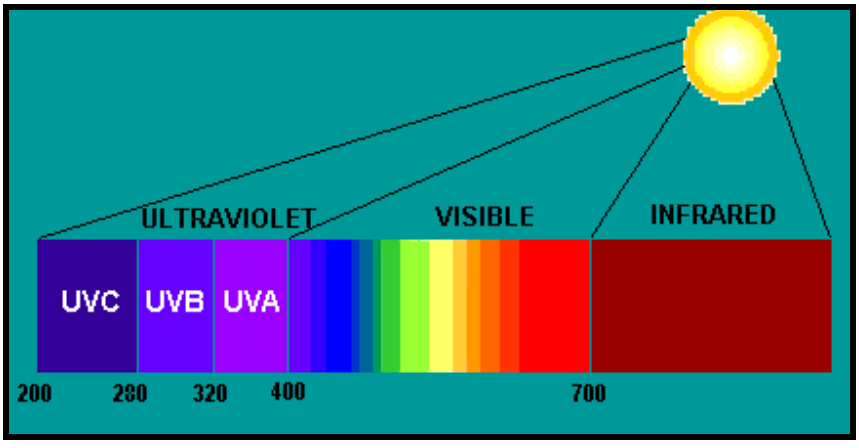

Figure 1.1: Diagrammatic representation of the electromagnetic spectrum. Adapted from [7].

UVC radiation is nearly entirely blocked by the earth's stratospheric ozone layer, but can be sourced from suntan beds and germicidal lamps which may result in a burning of the skin [8]. UVB is mainly responsible for the generation of tanning, burning and carcinogenesis [6][8]. UVA however, contributes for most of the ultraviolet radiation reaching the earth's surface. It possesses the ability to penetrate deeply into the dermis resulting in tanning, burning and photoaging [6][8].

Therefore, according to the abovementioned, excessive exposure to ultraviolet radiation induces a series of adverse effects that contribute to burden of disease [5].

1.3.1 Risk factors influencing susceptibility to ultraviolet radiation

An individuals' susceptibility to ultraviolet radiation is dependant on a number of factors. One such factor is the ozone layer. With each passing day, the ozone layer becomes more depleted, increasing the magnitude of harmful rays that reaches the earth's surface [4].

Furthermore, skin pigmentation is also an important risk factor, because it determines the degree by which UV radiation will affect the skin [9]. Therefore, individuals with fair skin, light coloured hair, have blue eyes and freckles (Fitzpatrick phototype I) are more at risk of UV damage as opposed to those with darker complexions. These individuals should also take into account additional factors such as seasonal differences, latitude, altitude and humidity when traveling to different parts of the world [9][10].

1.3.1.1 Fitzpatrick skin phototype classification

In 1975, Dr Thomas B. Fitzpatrick formulated a skin phototype classification system [9] that is even in this day and age recognised as a valuable tool within the dermatological environment [11]. It is based on a questionnaire to determine an individual's genetic constitution, reaction to sun exposure and tanning habits [9]. The response is thereafter calculated and categorised in an ordinal scale [9]. With the help of this classification system, an individual can be assigned to a specific phototype to suit their profile [11].

1.4 Photoaging

1.4.1 Chronological skin aging versus photoaging

Chronological skin aging, also known as intrinsic aging, is an involuntary process which results in visible changes in the skin's structure and function over time [5][12]. Chronologically aged skin appears thin, dry and lax with fine wrinkles [5]. In contrast, photoaging is brought about by chronic exposure to ultraviolet radiation resulting in premature skin aging. The skin displays distinct clinical features characterised by deep wrinkles, roughness, dyspigmentation and an increased loss in elasticity [1].

The distinctive characteristics between photoaging and chronological skin aging are clearly visible, as seen in Figure 1.2.

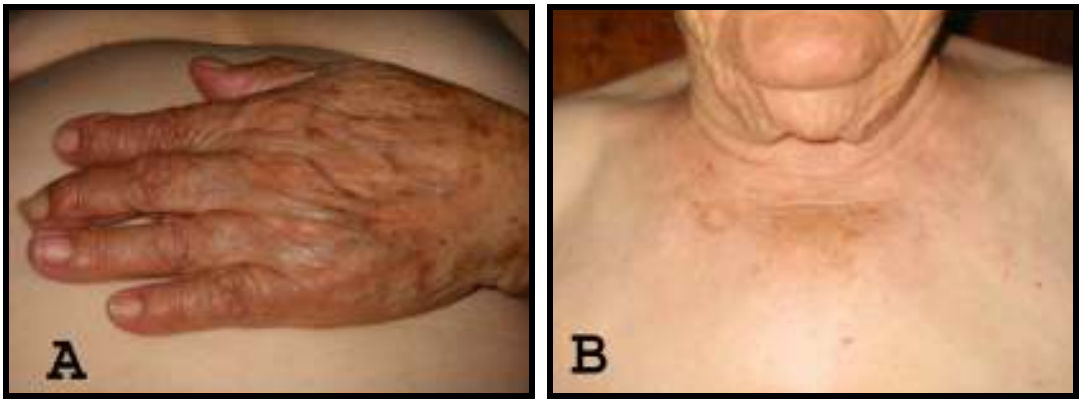

Figure 1.2: Aging of the skin. (A) Contrast between the chronologically aged stomach and photoaged hand of a female. (B) Contrast between the chronologically aged area below neckline and photoaged area above the neckline in same female [13].

1.4.2 Pathophysiology of photoaging

Human skin is continuously subjected to ultraviolet radiation from the sun, since it is in direct contact with the environment [5]. As a result, it undergoes premature aging as a consequence of this cumulative environmental damage, known as photoaging [14].

1.4.2.1 Histological alterations in photoaged skin

The diverse changes in photoaged skin are brought about as a result of various biochemical and morphological changes which affect the dermal and epidermal structural components of the skin [5][15]. Generally, fibroblasts within the photodamaged dermis drastically increase in number. And as a result, become hyperplastic, elongated and collapsed. Furthermore, a marked decrease in type I and III collagen formation is observed as a result of a down-regulation in newly synthesised collagen [16].

The degree of amorphous elastin-containing material, known as solar elastosis, increases but has been shown to correlate to the magnitude of ultraviolet exposure [17].

1.4.2.2 Ultraviolet radiation induced pathophysiological pathways leading to photoaging

Ultraviolet radiation present in sunlight is absorbed in the skin which induces the generation of reactive oxygen species (ROS), resulting in a marked decrease in the body's ability to combat oxidative stress, whilst impeding in collagen metabolism [5][16]. Furthermore, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) transcription factor too is activated by ultraviolet radiation; which is thought to be the initial step in inflammation infiltration. The process enhances the UV response by stimulating the transcription of pro-inflammatory cytokines (interleukin 1 and tumour necrosis factor alpha), as well as a specific increase in matrix metalloproteinase (MMP) expression, causing persistent damage

due to collagen degradation, thus resulting in a collagen deficit and wrinkle formation [5][15]. Furthermore, activation of SMAD 2 and 3 is brought about by the binding of transforming growth factor beta (TGF-β) and activin, which results in the phosphorylation of SMAD 2 and 3 and thereby decreasing collagen synthesis, as seen in Figure 1.3 [5].

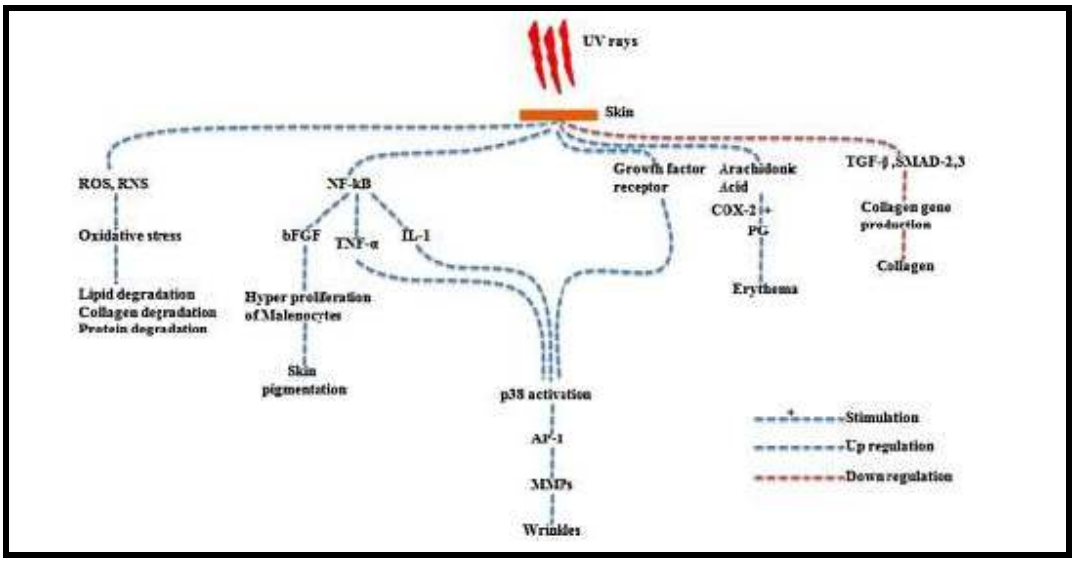

Figure 1.3: Ultraviolet radiation induced pathophysiological pathways leading to photoaging [5].

1.4.3 Importance and strategies for diagnosing photoaging

As outlined above, excessive exposure to ultraviolet radiation has detrimental effects on the structure and function of human skin [5]. The most unfavorable consequence of excessive ultraviolet exposure is skin cancer. This is particularly concerning to individuals located in areas with high ultraviolet radiation, for instance South Africa [18]. In 2011, skin cancer was labelled a notifiable disease since a significant rise in cancer cases were reported within the Cape region. Furthermore, five percent of cancer related deaths today are correlated to the cutaneous melanoma form of skin cancer [18]. For that reason, the need for a safe, reliable and non-invasive method of evaluating the extent of photoaging is needed.

Currently, there are a host of methods available for evaluating photodamage. Clinical examination, immunohistochemistry, skin surface topography and ultrasound are presently available strategies, with histological solar elastosis as the current gold standard [17]. However, according to Baillie [17] practical evidence in terms of sensitivity and specificity is lacking in order to establish which method is best at providing information on photodamage and consequently histological solar elastosis alone cannot be accepted as the gold standard.

With that said, this study proposes a non-invasive method of quantifying biomarkers in the skin so as to determine the extent of photoaging by use of real-time PCR.

Real-time polymerase chain reaction (PCR) is based on a revised approach to the conventional PCR method by relying on real-time monitoring of the amplification process as it occurs [19]. This method has become extensively utilised in many areas of research and diagnostics [20]. The significance of this technique is its sensitivity, specificity and superb ability to detect very low concentrations of starting material [19][21]. Detection chemistries for specific or non-specific amplification have been developed. For the purpose of this study, only hydrolysis probes (Taqman probes) will be referred to [22].

Taqman probes are specific and consists a donor fluorescent moiety at the 5 prime end and an acceptor moiety at the 3 prime end (Figure 1.4). During amplification, the probe becomes degraded by the DNA polymerase which allows for the generation of fluorescence from the donor. This process is repeated in every cycle with exponential synthesis of PCR products [22].

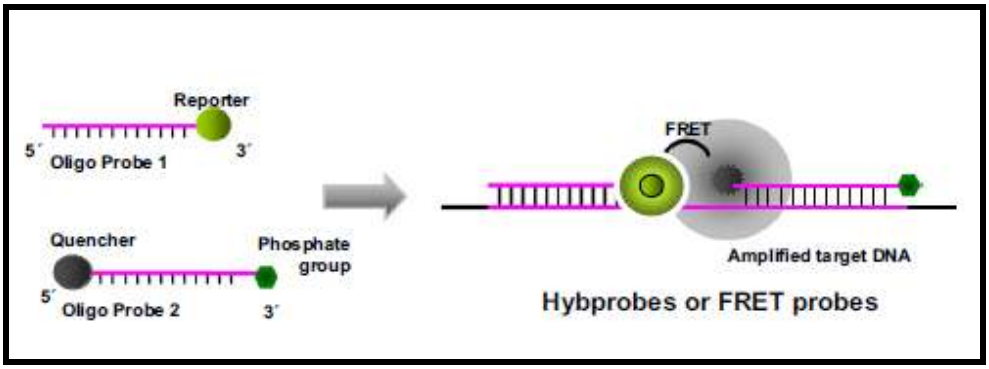

Figure 1.4: Diagrammatic illustration of Taqman probes [22].

1.4.4 Quantification of cytokine messenger ribonucleic acid (mRNA) by real-time PCR and the advantages thereof

Cytokines are proteins that play an important role in the immune system by controlling various processes such as lymphocyte activation, proliferation, differentiation, survival and apoptosis [23].

1.4.4.1 Tumour necrosis factor alpha (TNF-α)

TNF-α is a pleiotropic cytokine. It is involved in processes such as cell growth and differentiation, facilitates immune responses as well as necrotic and apoptotic mechanisms [24].

1.4.4.2 Matrix metalloproteinase 9 (MMP 9)

Matrix metalloproteinase is an enzyme which forms part of the gelatinase family. They are responsible for leukocyte migration during inflammation as well as the turnover and degradation of components within the extracellular matrix [25].

1.4.4.3 Interleukin 6 (IL-6)

Interleukin 6 is a pleiotropic cytokine. It is involved in processes related to inflammation and infection as well as metabolic, regenerative and neural mechanisms [26].

1.4.4.4 Transforming growth factor beta (TGF-β)

Transforming growth factor beta is a pleiotropic cytokine with immunoregulatory properties. In addition, it plays an important role in maintaining pro-inflammatory immune responses [27].

Real-time PCR cytokine mRNA levels allows for the characterization and quantification of cytokine production which is important for understanding the immune response and ultimately assist in understanding disease pathogenesis from a wide range of samples as this method is sensitive and accurate [23][28].
In this study we looked at measuring the gene expression of MMP-9, IL-6, TNF-α and TGF-β, primarily because of the important roles they play in progression to photoaging. According to Shah et al [5] a noticeable increase is observed in MMP-9, TNF- α as well as IL-6, while a decrease is observed in TGF-β.

1.5 Aim

The aim of the study was to develop and validate a real-time PCR method in order to establish the gene expression profiles of potential biomarkers in the skin so as to quantify the degree of photoaging by retrieving total RNA from cells adherent to tape strips applied to sun exposed and non-exposed skin areas.

1.5.1 Specific objectives

1. To design and optimise a real-time PCR method for the detection and quantification of MMP-9, IL-6, TNF-α and TGF-β cytokine transcript levels.
2. To do gene expression analysis of the selected photoaging related genes in sun exposed and non-exposed samples.

1.5.2 Benefits of study

The benefits of this technique (once validated and proven) allows for the ability to apply these validated cytokine expression studies in skin peels to other pathological skin conditions. In addition, in disease states such as psoriasis, where biopsies are the general method of sample collection, the use of tape stripping is quick and patient friendly and allows for resampling at the same site.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Introduction

The structure of this study took on the form of a descriptive research study. The primary goal was to develop and validate a real-time PCR method so as to quantify the degree of photoaging from cells adherent to tape strips from sun exposed and non-exposed areas of the skin.

2.2 Study population

Twenty healthy volunteers (n=8 Caucasian; n=12 Mixed Ancestry) consisting of seven males and thirteen females aged 25 to 67 years were included in this study and were selected according to the inclusion and exclusion criteria shown in Table 2.1.

The participants included in this study were selected since they form part of South Africa's multi-ethnic population which is representative of all Fitzpatrick skin phototypes [18]. Demographical information regarding the study population is summarised in appendix A.

The study protocol was approved by the Ethics Committee of the Faculty of Health Sciences of Stellenbosch University (Ethics number: N08/08/212). Written consent was obtained from all participants prior to commencement of the study and a skin care and lifestyle questionnaire was completed by all participants subsequent to the inclusion into the study to determine their skin phototype, general demographics and lifestyle patterns. Refer to appendix B and C respectively for an example.

Table 2.1: Inclusion and exclusion criteria for study selection

Inclusion criteria	Exclusion criteria
General good health	Subjects who suffer from skin disorders
Fitzpatrick phototype I - V	Subjects who suffer from any inflammatory diseases
Males and females between the ages of 18 and 70	

2.3 Sample collection

2.3.1 Tape stripping

The skin serves as a suitable portal for evaluating biomarker information, due to the fact that it is easily accessible and provides for much information [29].

Over time several techniques have been created for collecting skin samples to evaluate specific biomarker information however, the majority of these were based on invasive methods of collection. Thus, a quick, yet non-invasive approach in collecting skin samples is required [30].

Tape stripping by definition, is a non-invasive procedure of stratum corneum removal by means of adhesive tape strips (Figure 2.1) [29]. Commonly used in both humans and animals, this technique allows for the investigation of a number of skin disorders, determining drug levels in the skin, as well as the physiology of the stratum corneum [31].

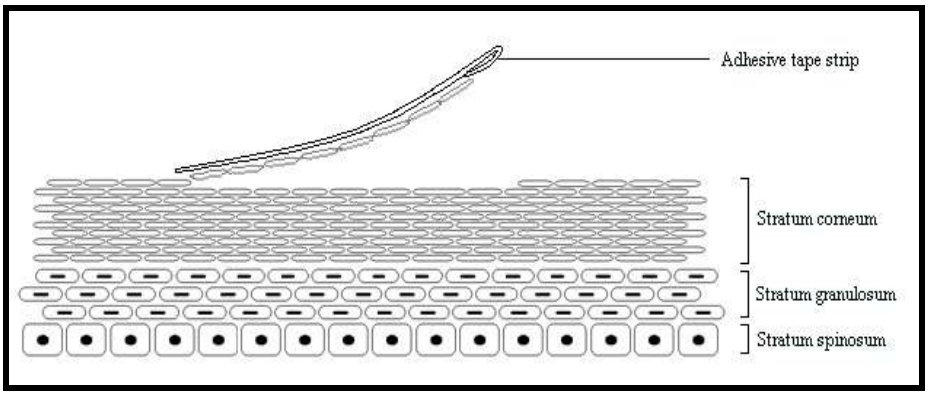

Figure 2.1: Illustration of stratum corneum removal via the tape stripping method. Obtained from [32].

2.3.1.1 Significance of the tape stripping method for retrieving biomarker information

The tape stripping technique is widely used in experimental dermatology [33] and is regarded as an essential advancement that allows one to retrieve and quantify RNA [34] by use of RT-PCR from cells adherent to the adhesive tape. In addition, mRNA's involved in the response to certain disease states, may also be revealed through this technique allowing for the development of various diagnostic opportunities. Therefore tape stripping may be a useful analytical or quantitative tool for the recovery of RNA, as well as being capable of accurately conveying the real time physiology of the skin [34] whilst supplying valuable information concerning the expression of certain cytokine levels in the skin.

Tape stripping was performed using pre-cut D-Squame® 22 mm adhesive discs (CuDerm Corp., Dallas, Texas, USA). Samples were collected on the right medial thigh area 20 cm above the patella and 2 cm below the lateral canthus of the right eye (Figure 2.2). Each area was cleansed with 70% ethanol and shaved to remove vellus hairs prior to the tape stripping procedure. While stretching the area using gloved hands, the adhesive tape was carefully removed from its packaging using sterile forceps and gently placed onto the respective test site. Subsequently, the area was outlined with a marker pen to ensure that the tape strips were consistently applied over the same site. Using the thumb, the test area was rubbed at a constant force with ten circular movements, covering the entire surface area of the tape. The first tape strip from the facial area and the first ten tape strips from the thigh area were discarded to expose the inner layers of the skin. Thereafter, nine successive tape strips were carefully applied to the facial area while twenty four tape

strips were applied to the thigh area and removed with a constant force using sterile forceps. Collected tape strips were individually placed, adhesive side facing inwards, into separate 2 ml microcentrifuge tubes and immediately stored at -80°C for subsequent extraction.

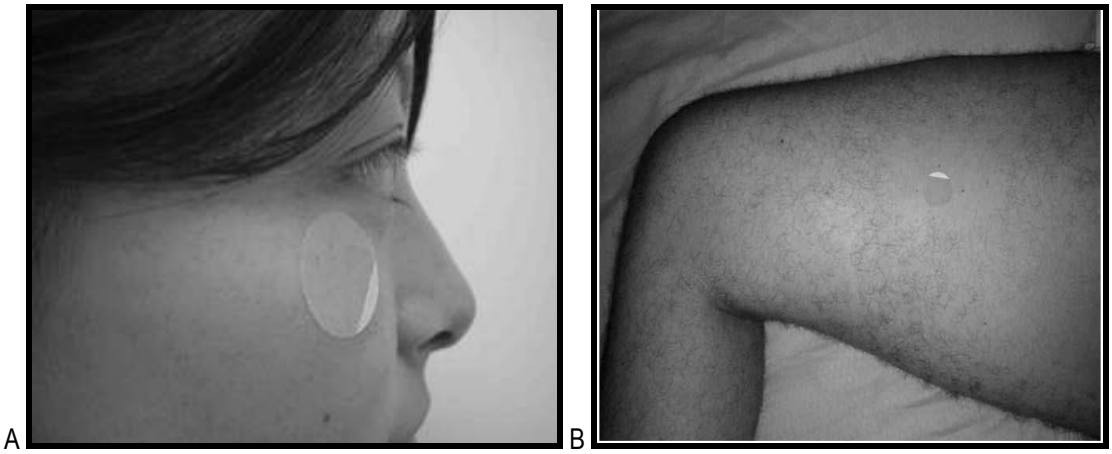

Figure 2.2: An illustration of human stratum corneum collection using the tape stripping method. A) Tape strip collection on the facial area. Obtained from [35]. B) Tape strip collection on the thigh area.

2.4 Sample processing

2.4.1 Total RNA isolation

Nucleic acid extraction is an important technique in molecular biology and is the first step necessary for any downstream application [36].

The RNA extraction protocol used in the current study is based on that reported by Kobaly et al [37] except they used a Braun Mikro Dismembrator and spun the sample at 3000 revolutions per minute (rpm) for 3 min at 20 ºC.

Briefly, 1.5 ml of Buffer RLT (Qiagen, Valencia, California) was added to the first sample tube in the series and vortexed vigorously for 1 min and incubated in a Lab Armor® Chill Bucket (Shel Lab, VWR International LLC) containing Lab Armor® beads for 5 min. Following incubation, the sample tube was centrifuged at 13 000 rpm for 10 min at 4ºC. Thereafter, the RLT buffer was then transferred to the next microcentrifuge tube and the process repeated until all tape strips was processed in the same RLT buffer for each site respectively. Total RNA was extracted using the Rneasy mini kit (Qiagen GmBH, Hilden, Germany), according to manufacturer's instructions. Briefly, the sample was transferred to the upper reservoir of an Rneasy mini spin column placed in a 2 ml collection tube. The column assembly was centrifuged in a microcentrifuge at 10 000 rpm for 15 sec and the flow-through discarded. Subsequently, 700 µl of Buffer RW1 (Qiagen, Valencia, California) was added to the upper reservoir of the spin column and centrifuged at

10 000 rpm for 15 sec and the flow-through discarded. Thereafter, 500 µl of Buffer RPE (Qiagen, Valencia, California) was added to the upper reservoir of the spin column and centrifuged at 10 000 rpm for 15 sec. The flow-through and collection tube was discarded and a further 500 µl of Buffer RPE was added to the upper reservoir of the spin column placed in a new 2 ml collection tube. Subsequently, the tube was centrifuged in a microcentrifuge at 10 000 rpm for 2 min. The collection tube was discarded and the spin column inserted into a sterile 1.5 ml reaction tube. Thirty microlitres of RNase free water was added to elute the RNA and centrifuged at 10 000 rpm for 1 min. The RNA preparation was immediately stored at -80ºC for further analysis. All RNA isolation work was carried out in a Lab Armor® Chill Bucket (Shel Lab, VWR International LLC) containing Lab Armor® beads to reduce the chance of RNA degradation.

2.4.2 RNA cleanup

Irrespective of the method of RNA isolation, an RNA cleanup process has to be implemented to reduce traces of proteins and compounds, such as buffers and enzymes, which could possibly inhibit downstream applications [38].

This study protocol utilised a silica-based spin column technique which allows RNA to bind under high-salt chaotropic buffer conditions ensuring effective RNase inactivation [38].

Total RNA was purified using the RNeasy mini kit (Qiagen GmBH, Hilden, Germany) RNA cleanup protocol, according to manufacturer's instructions. Briefly, the eluted RNA was transferred to a fresh 2 ml microcentrifuge tube and the sample adjusted to a final volume of 100 µl with RNase free water. Thereafter, 350 µl Buffer RLT (Qiagen, Valencia, CA) was added to the RNA sample and mixed well by repeat pipetting. This was followed by the addition of 250 µl of absolute ethanol. The sample was then transferred to the upper reservoir of an Rneasy mini spin column placed in a 2 ml collection tube. The column assembly was centrifuged in a microcentrifuge at 10 000 rpm for 15 sec and the flow-through discarded. Subsequently, 500 µl of Buffer RPE (Qiagen, Valencia, California) was added to the upper reservoir of the spin column and centrifuged at 10 000 rpm for 15 sec. The flow-through and collection tube was discarded and a further 500 µl of Buffer RPE was added to the upper reservoir of the spin column placed in a new 2 ml collection tube. Thereafter, the tube was centrifuged in a microcentrifuge at 10 000 rpm for 2 min. The collection tube was discarded and the spin column inserted into a sterile 1.5 ml reaction tube. Thirty microlitres of RNase free water was added to elute the RNA and centrifuged at 10 000 rpm for 1 min. The RNA preparation was stored at -80ºC for further analysis.

2.4.3 Quantity and quality of extracted total RNA

The concentration of each RNA sample was determined by means of measuring its absorbance at 260 nanometres (nm), using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington, USA). Yields were recorded as nanograms per microlitre (ng/µl). Purity of RNA was evaluated according to the ratios of absorbencies at 260 nm and 280 nm. A ratio of close to 2 was considered to indicate high purity. All readings were determined in duplicate.

2.4.4 Reverse transcription

Reverse Transcription (RT), also known as first strand complementary DNA (cDNA) synthesis, is a technique by which single-stranded RNA is reverse transcribed into complementary DNA [39].

The primer mix used in the cDNA synthesis reaction is made up of a combination of random and oligo-dT primers, to utilise the most out of the number of mRNA molecules that can be analysed from a low sample of RNA [40].

Reverse transcription was performed using the Quantitect® Reverse Transcriptase kit (Promega, USA) following the recommendations of the manufacturer. Total RNA (40 ng) was used to prepare the genomic DNA elimination reaction in a Lab Armor® Chill Bucket (Shel Lab, VWR International LLC) containing Lab Armor® beads which consisted of gDNA Wipeout Buffer [7 X] and sterile nuclease free water to a final volume of 14 µl. The mixture was heated to 42°C for 2 min in a GeneAmp® 9700 standard thermal cycler, and immediately chilled on ice beads. Thereafter, the reverse transcription reaction was prepared with the following component volumes: 1 µl Quantitect Reverse Transcriptase, 4 µl of Quantitect RT Buffer [5 X], 1 µl RT Primer Mix and added to the RNA template. The mixture was incubated at 42°C for 15 min and at 95°C for 3 min to inactivate the reverse transcriptase. The preparation was stored at -20ºC until further analysis.

2.4.5 Real-time PCR

Real-time PCR is based on a revised approach to the conventional PCR method by relying on real-time monitoring of the amplification process as it occurs [41]. It has become a widely used method in the field of research and diagnostics [19].

The significance of this technique is its sensitivity, specificity and superb ability to detect very low concentrations of starting material [19][21]. In addition, this method is quick and allows for quantitative and qualitative results with high sample throughput [42]. And, since no post amplification processing is required, the risk of contamination is drastically minimised [43].

Two methods of real-time quantification are currently available, absolute and relative quantification [21]. For the purpose of this study, only relative quantification via the standard curve method will be referred to.

2.4.5.1 Relative quantification

Briefly, relative quantification determines the changes in expression of the target across various samples relative to a reference gene. The ratio of the concentration of the target to that reference gene is then calculated and expressed as fold-changes in a given experiment [42][44].

Due to experimental variations in the processes prior to real-time, a reference gene is introduced in order to minimize errors and ensure reliable data. However, the reference gene used should be constitutively expressed and should be selected according to the experimental treatment and tissue [40][42][45].

In the present study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the reference gene for relative quantification.

2.4.5.1.1 GAPDH as reference gene

GAPDH forms part of the dehydrogenase enzyme family mainly responsible for glucose metabolism [46].

We selected this reference gene since it proved efficiently as a reference gene within other human skin related studies [47].

2.4.5.1.2 Relative quantification using the standard curve method

The standard curve method requires a cDNA sample to create a dilution series to generate a standard curve. The template for the standard curve can be any cDNA sample, as long as it expresses the target genes.

It is important to note that the samples quantification cycle (Cq) values should fall between the lowest and highest point of the standard curve. These Cq values are used to extrapolate data in terms of the standard curve [48].

2.4.5.1.3 Preparation of standard curves

To create the respective standard curves, cDNA template collected from peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide (LPS) were used to generate standard curves for both the housekeeping and target genes. The standard curves were serially diluted five-fold with PCR grade water to span a concentration range of 160 ng to 0.051 ng. Each serial dilution was performed in triplicate and a negative control comprising of reaction mix and PCR grade water was included in each PCR run. Logarithmic concentrations were plotted against crossing points using the second derivative maximum analysis method incorporated in the LightCycler® quantification software package (Roche).

2.4.5.1.4 Stimulation of PBMCs with LPS

Lipopolysaccharide (LPS) is a mitogen that stimulates immune responses. It interacts with the membrane receptor CD14 to induce the generation of cytokines such as TNF-α and IL-6 [49].

Two archived cryopreserved PBMCs from a healthy donor containing $5x10^6$ cells/ml each, were removed from liquid nitrogen storage and immediately thawed in 37°C waterbath (Memmert) until the cells were almost completely thawed. The cells were then aseptically transferred into a 15 ml conical tube (Fisher Scientific) and topped with Roswell Park Memorial Institute (RPMI) medium (Sigma) and centrifuged at 1800 rpm for 10 min. The supernatant was discarded and the pellet resuspended in approximately 10 ml of RPMI and centrifuged at 1800 rpm for 10 min. Thereafter the supernatant was discarded and the pellet resuspended in approximately 10 ml of RPMI. This was followed by the addition of 1 µl 100 µg/ml of LPS and incubated in a Carbon Dioxide (CO_2) incubator (Thermo Scientific) for 4 hours in an upright position. Following incubation the samples were centrifuged at 1800 rpm for 10 min. The supernatant was discarded and RNA was subsequently extracted using the Rneasy mini kit according to manufacturer's instructions as described in section 2.4.1.

2.4.5.2 Real-time PCR quantification

Real-time PCR assays were performed on a Lightcycler® 1.2 real-time PCR machine (Roche) using the Lightcycler® Taqman® Master kit (Roche Diagnostics GmbH, Germany) in Roche Lightcycler® capillaries (Roche) in a 20 µl reaction mixture volume.

Each reaction contained 10 µl cDNA (diluted in a 1:1 ratio), 1 µl Hydrolysis (TaqMan®) Probe (Roche), 2 µl of each primer and 4 µl of Reaction Mix [5 X] and PCR grade water. The thermal profile for the amplification was as follows: An initial activation step at 95°C for 10 min, followed by 45 cycles of 95°C denaturation for 10 sec, primer annealing at 55°C for 30 sec or 60°C for 30 sec, elongation at 72°C for 4 sec and a final cooling step of 40°C.

2.4.6 Data analysis

Relative quantification of target gene transcripts in comparison to a reference gene transcript was applied by use of the second derivative maximum analysis method incorporated in the LightCycler® 4.1.1 quantification software package (Roche).

2.5 Optimization and validation of real-time PCR method

In order to achieve reliable and reproducible results, optimization and validation of the real-time PCR method is crucial [40].

Two additional tape stripping samples, denoted sample 1 and sample 2, were randomly collected and total RNA retrieved as per protocol described in section 2.4.1. Thereafter, cDNA was synthesised for the validation and optimization of this method, to ensure efficient amplification of the cDNA.

2.5.1 Quality control of sample material

Total RNA preparations were quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington, USA) as described in section 2.4.3 to ascertain the purity and concentration of the sample material.

Sample integrity by use of an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, USA) or denaturing agarose gel, serves as an added measure of sample quality control [50]. However, due to the limited sample volume and concentration this could not be achieved as the former method is expensive and the latter requires approximately 200 ng of total RNA [50].

2.5.2 Primer and probe selection and optimization

In the current study, universal probelibrary (UPL) probes (Roche) were selected and designed using the Probe Finder design software which is based on Primer 3 software to ensure efficient amplification of the target. Since these probes are short hydrolysis probes, approximately 8 to 9 nucleotides in length, minute optimization conditions are required such as slight adjustments to the annealing temperatures.

Primers were synthesized by Inqaba Biotech™ (Pretoria, South Africa) and were designed to span introns to ensure efficient amplification of cDNA from mRNA transcripts and avoid contamination from genomic DNA. Primer sequences and PCR product sizes are outlined in Table 2.2.

Table 2.2: PCR primer and fragment details for PCR

Primer	PCR product size (bp)	Sequences in 5' - 3'	Annealing temperatures	Accession number
TNF-α	123	cagcctcttctccttcctgat gccagagggctgattagaga	55ºC	NM_000594.2
MMP 9	67	gaaccaatctcaccgacagg gccacccgagtgtgtaaccata	55ºC	NM_004994.2
IL-6	85	caggagcccagctatgaact agcaggcaacaccaggag	55ºC	NM_000600.3
TGF-β	73	actactacgccaaggaggtcac tgcttgaacttgtcatagatttcg	55ºC	NM_000660.3
GAPDH	66	agccacatcgctcagacac gcccaatacgaccaaatcc	60ºC	NM_002046.3

5': 5-prime; 3': 3-prime; ºC: degrees Celsius

2.5.2.1 Specificity

A melt-curve analysis was performed to ensure that one distinguished peak is observed, thus indicating that one product is amplified. Amplicons were separated on a 2.0% agarose gel stained with ethidium bromide to confirm expected PCR product size.

2.5.3 Real-time PCR quantification

Real-time PCR assays were performed on a Lightcycler® 1.2 real-time PCR machine (Roche) using the Lightcycler® Taqman® Master kit (Roche Diagnostics GmbH, Germany) in Roche Lightcycler® capillaries (Roche) to monitor assay performance. Master mixes for each primer pair was prepared for individual runs and all pipettes used in this study were calibrated to minimize pipetting errors.

2.5.3.1 Standard curve characteristics

Subsequent to determining optimal primer and probe conditions, standard curves for the respective reference and target genes were generated. The standard curves were serially diluted five-fold with PCR grade water to span a concentration range of 160 ng to 0.051 ng. Parameters in terms of efficiency and linear dynamic range were determined.

To effectively evaluate the amplification efficiency (E) of a standard curve, a minimum of three replicates for every cDNA sample is required, as well as a minimum of five logs of template concentration using the following formula: $E = 10^{(-1/slope)} - 1$ [50][51].

A slope of -3.32 signifies a PCR efficiency of 100%, thus indicating that each target doubles exactly in one PCR cycle. Although slope values between -3.1 and -3.6 are also acceptable [50].

Another important characteristic of a good standard curve is the correlation coefficient (R^2). An R^2 value of >0.99 offers good confidence in correlating two values [50].

2.5.3.2 Assay performance: repeatability and reproducibility

Intra assay variability (repeatability) refers to the closeness of the agreement between repeated samples within the same assay [50]. This was determined by utilising two samples with three replicates each and analysed in a single assay.

Inter assay variability (reproducibility) is a measure of determining results between different assays [50]. This was achieved by use of the same two individual samples as described above, with three replicates each and analysed over two separate assays.

2.5.3.3 Normalisation

As previously pointed out, normalisation to a reference gene is required to compensate for technical differences in the amount of biological material in tested samples [50].

Generally, normalisation against three or more validated reference genes is considered as the most appropriate method for real-time PCR. However, due to budgetary constraints, only one validated reference gene was utilized in this study.

Validated stability data for GAPDH between the different sample conditions can be seen in chapter 3.

2.5.3.4 Controls

A no-template control (NTC) comprising master mix and PCR grade water was included in every run to monitor reagent contamination as well as primer dimer formation.

In addition, a positive control in the form of amplified cDNA was included in every run, to monitor assay variation.

In this study protocol a no-reverse transcriptase control (RT^-) which is used to monitor genomic DNA (gDNA) contamination, was excluded since the primers were designed to span introns and would therefore not amplify gDNA.

CHAPTER THREE

RESULTS

Two tape stripping samples (as referred to in section 2.5), denoted sample 1 and sample 2, were randomly collected and total RNA retrieved as per protocol described in section 2.4.1. Thereafter, cDNA was synthesised for the validation and optimization of this method, to ensure efficient amplification of the cDNA.

3.1 PCR assay performance characteristics

The amplification efficiencies, linear dynamic range as well as the precision of each target and reference gene were determined and are reported below.

3.1.1 Efficiency and linear dynamic range

The mean reaction efficiency of the target and reference gene standard curves was 1.05 with a mean correlation coefficient of 0.99 and displayed a linear dynamic range across the 5-log range. An example of a standard curve generated to represent efficiency and linear dynamic range can be referred to in Figure 3.1. The performance characteristics for the standard curves generated are represented in Table 3.1.

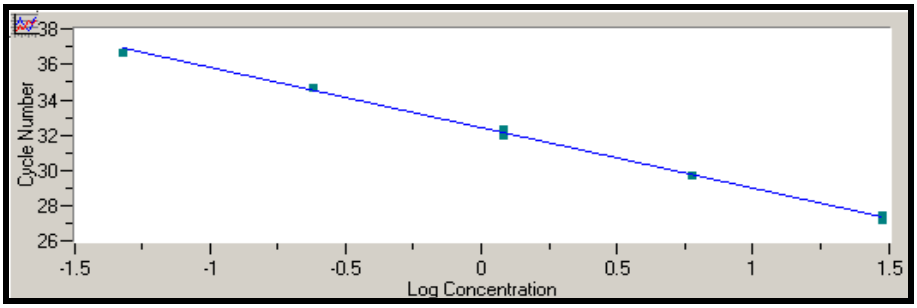

Figure 3.1: The TNF-α standard curve generated to represent efficiency and linear dynamic range.

Table 3.1: Standard curve performance characteristics for target and reference gene

Gene	Efficiency	Slope	Correlation coefficient (R^2)
GAPDH	1.09	-3.108	0.99
TNF-α	0.99	-3.403	1.00
MMP 9	1.09	-3.153	0.99
IL-6	1.09	-3.102	1.00
TGF-β	0.99	-3.326	0.99

3.1.2 Precision

The mean quantification cycle values among the three experiments designed to evaluate the repeatability and reproducibility (refer to section 2.5.3.2) of the real-time PCR assay are represented in Table 3.2. The mean intra and inter assay variance determined by the coefficient of variation (CV) was within an acceptable range (CV ≤ 30%) and was 0.50%, 0.80%, 0.77% and 1.10% respectively.

Table 3.2: Mean quantification cycle values for precision testing

	Intraday	Interday_1	Interday_2
Sample 1_sun exposed			
Mean Cq	31.80	31.67	31.80
SD	0.31	0.09	0.08
CV (%)	0.97	0.28	0.25
Sample 1_non-exposed			
Mean Cq	28.70	28.25	28.25
SD	0.37	0.18	0.13
CV (%)	1.29	0.64	0.46
Sample 2_sun exposed			
Mean Cq	33.30	32.77	32.79
SD	0.41	0.16	0.19
CV (%)	1.23	0.49	0.58
Sample 2_non-exposed			
Mean Cq	34.40	33.74	33.95
SD	0.48	0.17	0.48
CV (%)	1.40	0.50	1.41

SD: Standard deviation

3.1.3 Stability of reference gene

The reference gene was tested for stability amongst two samples between sun exposed and non-exposed conditions. This was achieved by running the two samples in triplicate in two different assays to determine the mean Cq and SD between the samples. Generally, a reference gene with a low coefficient of variation difference between the control (non-exposed) and experimental sample (sun exposed) is accepted as the most efficient reference gene, see Table 3.3.

Table 3.3: Stability data of the GAPDH reference gene between sun exposed and non-exposed conditions

	Sun exposed	Non exposed
Sample 1		
Mean Cq	31.76	28.40
SD	0.18	0.31
CV (%)	0.57	1.09
Sample 2		
Mean Cq	32.95	34.03
SD	0.36	0.46
CV (%)	1.09	1.35

The next phase of the study involved the quantification of specific biomarkers to evaluate the degree of photoaging recovered from sun exposed and non-exposed areas of the skin.

3.2 Study population characteristics

Twenty subjects were included in the study [65% female (n=13) and 35% male (n=7) with an age range of 25-67. The participants included in this study are characteristic of South Africa's multi-ethnic population which is representative of all Fitzpatrick skin phototypes [18]. Four individuals had Fitzpatrick skin phototype I and II; six had Fitzpatrick skin phototype III and two phototype IV, while four subjects had Fitzpatrick skin phototype V. None of the study subjects displayed any sunburn or skin tanning properties at the time of the study. In addition, six volunteers were current smokers, while thirteen were non-smokers and one a former smoker.

3.3 Total RNA yield

Total RNA was recovered using the tape stripping technique as described in the Materials and methods section. The mean concentration of total RNA recovered from sun exposed sites was 6.48 ng (±5.54) with a range of 2.29-25.10 ng, while the total RNA recovered from non-exposed sites was 5.05 ng (±3.31) with a range of 1.74-14.71 ng, as seen in Figure 3.1.

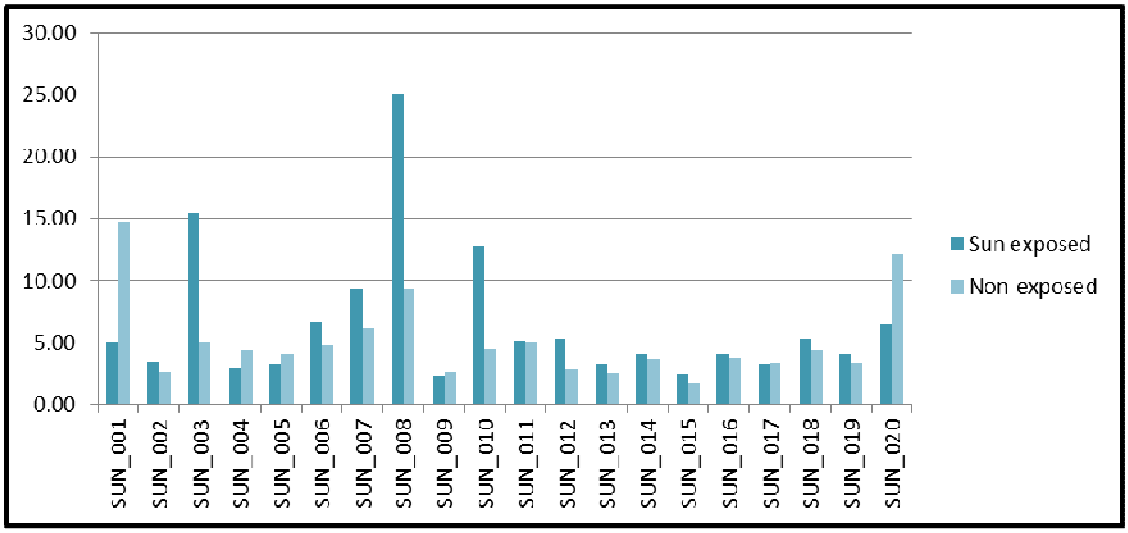

Figure 3.2: Mean total RNA concentrations between sun exposed and non-exposed sites. The y-axis represents the RNA concentrations, while the x-axis represents the sample id's of the study population.

3.4 Relative gene expression fold-change

In this study, TGF-β, MMP 9, TNF-α and IL-6 mRNA transcripts were selected as representative cytokines to determine the relative fold-change in sun exposed and non-exposed areas of the skin so as to determine extent of photoaging.

These markers were normalized to a reference gene (see Table 3.4, 3.5 and 3.6), the GAPDH transcript and compared relative to a calibrator sample using the standard curve method as described in Materials and methods.

Table 3.4: Normalised TGF-β values for sun exposed and non-exposed samples.

Sample ID	Sun exposed	Non-exposed	Sample ID	Sun exposed	Non-exposed
SUN_001	N/R	0.40	SUN_011	0.30	0.09
SUN_002	0.58	0.07	SUN_012	N/R	0.14
SUN_003	0.17	0.04	SUN_013	2.19	0.55
SUN_004	8.49	18.25	SUN_014	0.92	0.99
SUN_005	1.04	0.03	SUN_015	0.22	0.06
SUN_006	0.62	0.50	SUN_016	0.23	0.10
SUN_007	N/R	0.06	SUN_017	1.66	0.35
SUN_008	0.00	0.01	SUN_018	0.05	0.37
SUN_009	3.39	11.44	SUN_019	0.04	0.22
SUN_010	0.68	N/R	SUN_020	0.01	0.15

N/R: not reportable

Table 3.5: Normalised MMP 9 values for sun exposed and non-exposed samples.

Sample ID	Sun exposed	Non-exposed	Sample ID	Sun exposed	Non-exposed
SUN_001	0.06	0.01	SUN_011	0.64	0.48
SUN_002	0.03	N/R	SUN_012	1.14	2.23
SUN_003	0.09	N/R	SUN_013	1.95	0.27
SUN_004	15.98	N/R	SUN_014	0.88	0.25
SUN_005	7.38	0.17	SUN_015	0.36	0.87
SUN_006	0.06	0.54	SUN_016	0.02	0.13
SUN_007	N/R	0.07	SUN_017	2.84	0.03
SUN_008	N/R	N/R	SUN_018	0.04	0.03
SUN_009	1.42	7.46	SUN_019	0.03	0.15
SUN_010	1.40	11.24	SUN_020	1.10	0.02

N/R: not reportable

Table 3.6: Normalised TNF-α values for sun exposed and non-exposed samples.

Sample ID	Sun exposed	Non-exposed	Sample ID	Sun exposed	Non-exposed
SUN_001	N/R	0.00	SUN_011	N/R	N/R
SUN_002	N/R	N/R	SUN_012	N/R	N/R
SUN_003	N/R	N/R	SUN_013	N/R	0.16
SUN_004	1.68	26.85	SUN_014	N/R	N/R
SUN_005	N/R	N/R	SUN_015	N/R	N/R
SUN_006	N/R	N/R	SUN_016	N/R	N/R
SUN_007	N/R	N/R	SUN_017	N/R	N/R
SUN_008	58.80	0.00	SUN_018	N/R	0.16
SUN_009	N/R	N/R	SUN_019	0.14	N/R
SUN_010	N/R	N/R	SUN_020	N/R	N/R

N/R: not reportable

Normalised values for both the sun exposed and non-exposed values were each divided by the normalised calibrator values for each gene. The data illustrating the relative fold-change of the target compared to the calibrator sample can be viewed in Table 3.7, 3.8 and 3.9 respectively.

Table 3.7: TGF-β fold-change ratio in sun exposed and non-exposed samples.

Sample ID	Sun exposed	Non-exposed	Sample ID	Sun exposed	Non-exposed
SUN_001	N/R	0.22	SUN_011	0.37	0.11
SUN_002	0.32	0.04	SUN_012	N/R	0.17
SUN_003	0.09	0.02	SUN_013	2.72	0.68
SUN_004	4.62	9.92	SUN_014	1.14	1.23
SUN_005	0.57	0.01	SUN_015	0.28	0.07
SUN_006	0.34	0.27	SUN_016	0.28	0.13
SUN_007	N/R	0.04	SUN_017	2.06	0.44
SUN_008	0.00	0.01	SUN_018	0.06	0.45
SUN_009	1.84	6.22	SUN_019	0.05	0.28
SUN_010	0.84	N/R	SUN_020	0.01	0.18

N/R: not reportable

Table 3.8: MMP 9 fold-change ratio in sun exposed and non-exposed samples.

Sample ID	Sun exposed	Non-exposed	Sample ID	Sun exposed	Non-exposed
SUN_001	0.00	0.00	SUN_011	0.10	0.07
SUN_002	0.00	N/R	SUN_012	0.17	0.34
SUN_003	0.01	N/R	SUN_013	0.30	0.04
SUN_004	1.19	N/R	SUN_014	0.13	0.04
SUN_005	0.55	0.01	SUN_015	0.06	0.13
SUN_006	0.00	0.04	SUN_016	0.00	0.02
SUN_007	N/R	0.00	SUN_017	0.43	0.00
SUN_008	N/R	N/R	SUN_018	0.01	0.00
SUN_009	0.11	0.56	SUN_019	0.01	0.02
SUN_010	0.21	1.72	SUN_020	324.45	0.00

N/R: not reportable

Table 3.9: TNF-α fold-change ratio in sun exposed and non-exposed samples.

Sample ID	Sun exposed	Non-exposed	Sample ID	Sun exposed	Non-exposed
SUN_001	N/R	0.00	SUN_011	N/R	N/R
SUN_002	N/R	N/R	SUN_012	N/R	N/R
SUN_003	N/R	N/R	SUN_013	N/R	N/R
SUN_004	0.60	9.59	SUN_014	N/R	N/R
SUN_005	N/R	N/R	SUN_015	N/R	N/R
SUN_006	N/R	N/R	SUN_016	N/R	N/R
SUN_007	N/R	N/R	SUN_017	N/R	N/R
SUN_008	20.99	0.00	SUN_018	N/R	0.06
SUN_009	N/R	N/R	SUN_019	0.05	N/R
SUN_010	N/R	N/R	SUN_020	N/R	N/R

N/R: not reportable

Normalised sun exposed ratios were divided by the normalised non-exposed ratios to determine the fold-change of exposed versus non-exposed. Samples with an expression ratio above one for a specific gene, depicts up-regulation, while less than one illustrates down-regulation as seen in Table 3.10. Mean fold-change ratios are illustrated in Table 3.11.

Table 3.10: Sun exposed versus non-exposed fold-change expression ratios

Sample ID	TGF-β	MMP 9	TNF-α	Sample ID	TGF-β	MMP 9	TNF-α
SUN_001	N/R	5.85	N/R	SUN_011	3.32	1.34	N/R
SUN_002	8.43	N/R	N/R	SUN_012	N/R	0.51	N/R
SUN_003	4.55	N/R	N/R	SUN_013	4.01	7.19	N/R
SUN_004	0.47	N/R	0.06	SUN_014	0.93	3.50	N/R
SUN_005	41.29	42.36	N/R	SUN_015	3.84	0.42	N/R
SUN_006	1.29	0.10	N/R	SUN_016	2.23	0.19	N/R
SUN_007	N/R	N/R	N/R	SUN_017	4.69	94.66	N/R
SUN_008	0.10	N/R	13189	SUN_018	0.14	1.44	N/R
SUN_009	0.30	0.19	N/R	SUN_019	0.17	0.23	N/R
SUN_010	N/R	0.12	N/R	SUN_020	0.07	70.61	N/R

N/R: not reportable

Table 3.11: Mean fold-change ratios of sun exposed versus non-exposed

	TGF-β	MMP 9	TNF-α
Sun exposed			
Mean fold-change ratio	4.74	15.25	6594.53
SD	10.03	29.71	9325.99
	TGF-β	**MMP 9**	**TNF-α**
Non-exposed			
Mean fold-change ratio	1.08	0.19	2.43
SD	2.56	0.43	4.27

The data shows variation between samples for the respective genes as depicted by the standard deviation values.

It has been reported that TGF-β is generally down-regulated upon exposure to ultraviolet radiation [5]. In this study, only 35% (n=7) samples displayed similar down-regulatory effects.

Down regulation of MMP 9 was observed within 30% (n=6) of samples, while 15% (n=3) showed marked up regulation, indicative of MMP 9 in response to ultraviolet radiation [5].

Only two samples showed measurable levels of TNF-α in the assay, of which one showed significant up regulation.

Furthermore, we were unable to detect any IL-6 expression in any of the samples prepared: No expression data is available since no amplification of samples above the fluorescent background was observed.

Additionally, we conducted a small pilot study to evaluate the gene expression of specific growth factors subsequent to repetitive microneedling to determine extent of photoaging. This study was initiated so as to observe in what manner the validated technique would perform clinically by means of a local anti-aging expert wanting to make use of the method in his clinical practice. The data from this small pilot study is presented in Appendix D.

CHAPTER FOUR

DISCUSSION

Human skin is continuously subjected to ultraviolet radiation from the sun, which consequently over time results in photoaging [5]. Therefore, the detection of photoaging at an early stage is crucial to improving morbidity, whilst preventing the advancement of skin cancer [2].

Because the skin is an easily accessible organ whilst possessing a high metabolic activity, it enables investigators to evaluate the physiology as well as provide a means to monitor disease states and outline a therapeutic strategy [30][34].

Therefore, the purpose of the study was to develop and validate a real-time PCR probe-based method for quantifying biomarkers to monitor the degree of photoaging from total RNA recovered from sun exposed and non-exposed areas.

The first phase of the study was to develop, optimise and validate a real-time PCR technique based on the standard curve method using the Lightcycler® 1.2 real-time PCR machine (Roche). The advantage of utilising this system is its speed, since an optimised protocol takes approximately 40 min. The disadvantage however, is the inability to perform multiplexing. This meant that the target and reference genes had to be amplified individually. For that reason, all pipettes used in the study were regularly calibrated and checked to reduce pipetting errors as well as normalising the expression of target genes to a reference gene to avoid any bias [52].

Standard curves for both the reference and target genes were generated. Performance characteristics for each curve were determined based on reaction efficiency, correlation coefficient, linear dynamic range, as well as the slope of the standard curve. We found that all the above mentioned criteria were in optimal range. Furthermore, the assay also proved to be highly repeatable and reproducible with CV percentages within an acceptable range.

In the next phase of the study we demonstrated that total RNA can be recovered from cells adherent to tape strips. The tape stripping technique has shown to be a quick, non-invasive procedure of stratum corneum removal by means of adhesive tape strips [30].

Despite the fact that the mean concentrations of the total RNA recovered from both sun exposed and non-exposed areas were comparable, the actual concentrations were quite low, even when optimal extraction volumes were achieved. I feel that because of these low total RNA concentrations, it was quite challenging to include technical replicates within the real-time PCR reaction. This however, could have been overcome by possibly utilising a RNA pre-amplification kit, which magnifies low quantities of RNA by linearly amplifying the original RNA transcript [53]. With that said, as a consequence of budgetary constraints this option was not exploited.

In essence, despite the low quantities, we still were able to make use of the total RNA for the expression of cytokine biomarkers for evaluating the extent of photoaging in the skin.

Subsequent to optimization and validation of the real-time PCR method, the investigation of cytokine biomarkers TGF-β, MMP 9, TNF-α and IL-6 mRNA expression was determined to study the possible involvement of these cytokines in the pathogenesis of photoaging. Normalisation to a reference gene was required to compensate for technical differences in the amount of biological material in tested samples [54]. Generally, normalisation against three or more validated reference genes is considered as the most appropriate method for real-time PCR. However, due to budgetary constraints, only one validated reference gene was utilized in this study.

We validated the GAPDH reference gene transcript by comparing quantification cycles, standard deviation as well as coefficient of variation between two sun exposed and non-exposed samples. We found that the GAPDH transcript used in this study was quite stable. According to Ma et al. [54], lower CV percentage is an indication of stability of the reference gene, which is indicative of our results. Furthermore, we selected this reference gene since it proved efficiently as a reference gene within another human skin related study [55].

Only two samples showed measurable levels of TNF-α in the assay. No outliers could be calculated since you require more than two data points to determine an outlier. No amplification above fluorescence levels for IL-6 was observed. Normally these cytokines are expressed at very low levels [54] and therefore, a sample that has low expression when amplified, usually has a Cq value close to the limit of detection. In such an event it is advised to automatically increase the template; however, with the limited amount of sample this could not have been possible. Secondly, one would check the PCR optimisation to ensure that there are no discrepancies that could possibly affect the outcome of the genes and add controls to ensure the assay is running optimally. In this case, we have proved that our method is acceptable and that the controls are amplifying successfully.

In addition, since this study only involves healthy individuals no significant levels of expression can truly be determined between the sun exposed versus non-exposed fold-changes. However, any ratio above one represents upregulation, so one would therefore assume that a 2:1 ratio would imply signicant expression.

According to a former study, the levels of IL-6 *in vivo* were very low or undetected without the presence of ultraviolet radiation. However, excessive quantities of IL-6 were detected after one, three and twelve hours post UV radiation in plasma samples [56].

Furthermore, since none of the study participants displayed any sun-tanning properties or exposed themselves to excessive ultraviolet radiation prior or during the study, it can be presumed that IL-6 is not an informative gene without significant exposure to ultraviolet radiation.

The mRNAs selected in the study have shown that they are directly involved in the pathogenesis of photoaging [5]. However, our results demonstrate variation between samples for the respective genes as depicted by the standard deviation values. This variation I feel was contributed by the low concentrations of starting material recovered from tape stripping as well as low level cytokine mRNA expression, such as for TNF-α and IL-6.

The advantage of the study is that we were able to show that a real-time PCR method is repeatable and reproducible based on the validated information. The limitation of the study is no pre-amplification kit was utilised. The use of this kit would have allowed us to observe the detection of low expressed genes. This method could be applied in areas where a significant increase in expression would be observed, no need for pre-amplification kit, especially if study is limited to a budget.

In conclusion, we have shown that epidermal cytokines can be retrieved from tape stripped samples and can be quantified via real-time PCR. However, the choices of cytokine biomarkers reveal that they are as important as the quantity of sample recovered. In this study cytokines such as IL-6 is not as informative in determining the extent of photoaging without high doses of ultraviolet radiation before sample collection as opposed to the other explored cytokines.

CHAPTER FIVE

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Appendix A: Demographical information regarding the study population

Sample ID	Sex	Age	Fitzpatrick phototype	Skin care regime	Type of sun protection, if any	Smoking status
SUN_001	Female	43	Type I	Logica skin care treatment	Sunscreen (SPF 30)	Non-smoker
SUN_002	Female	31	Type II	Olay face wash	None	Non-smoker
SUN_003	Female	31	Type V	Avon firming lotion	None	Non-smoker
SUN_004	Male	30	Type II	Bio-oil	None	Non-smoker
SUN_005	Female	36	Type II	Yardley skin care treatment	Sunscreen (SPF 15)	Non-smoker
SUN_006	Male	38	Type I	None	Hat	Non-smoker
SUN_007	Female	27	Type II	Pond's skin care treatment	None	Former
SUN_008	Male	32	Type IV	None	None	Non-smoker
SUN_009	Female	43	Type I	Loreal day and night cream	Sunscreen (SPF 30)	Current
SUN_010	Male	30	Type I	None	None	Current
SUN_011	Female	52	Type III	None	None	Current
SUN_012	Male	57	Type IV	None	None	Current
SUN_013	Female	29	Type III	None	None	Current
SUN_014	Male	30	Type III	None	None	Non-smoker
SUN_015	Female	60	Type III	Elizabeth Arden skin care treatment	None	Non-smoker
SUN_016	Male	67	Type III	None	None	Non-smoker
SUN_017	Female	25	Type V	None	None	Non-smoker
SUN_018	Female	44	Type V	None	None	Current
SUN_019	Female	55	Type V	None	None	Non-smoker
SUN_020	Female	30	Type III	Annique skin care treatment	Sunscreen (SPF 30)	Non-smoker

Skin care treatment: face wash, toner and moisturiser

Appendix B: Participant information leaflet and consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF RESEARCH PROJECT

Molecular diagnostic approach to determine the degree of photoaging of the skin

REFERENCE NUMBER: N08/08/212

PRINCIPAL INVESTIGATOR: Mrs Stephany Wilcox

ADDRESS:
Synexa Life Sciences (Pty) Ltd
4 Kunene Circle
Omuramba Business Park
Montague Gardens
7441

AND:
Discipline of Medical Microbiology: Immunology unit
Department of Pathology
Faculty of Health Sciences
Stellenbosch University

CONTACT PERSON: Mrs Stephany Wilcox

CONTACT NUMBER: 021 938 4032

Dear Sir/ Madam

You are being invited to take part in a research project. Please take some time to read the information presented since this will explain the details of this project. Please ask any questions about any part of this project that you do not understand. It is very important that you are fully satisfied with the details provided and that you clearly understand what this research entails and how you could be involved. Also, your participation is voluntary and you are under no obligation to participate. You are free to withdraw at any point during the study.

This study has been approved by the **Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the **International Declaration of Helsinki**, South African Guidelines for Good Clinical Practice and the **Medical Research Council (MRC) Ethical Guidelines for Research**.

Appendix B: Participant information leaflet and consent form

What is the research project about?

The aim of the study is to determine which factors are responsible for the aging process by looking at various biological parameters that have been activated or deactivated in your skin using a non-invasive stripping technique of the skin with adhesive tape (so-called skin peel).

Who will conduct the research project?

The project will be conducted at **Synexa Life Sciences Laboratories** and at **Tygerberg Hospital** by **Mrs. Stephany Wilcox** and will be supervised by **Prof Patrick Bouic**.

Why have you been invited to participate?

As a healthy individual, you are suitable to take part in this study: by taking part, we may be able to better provide you with information about the amount of aging that your skin displays.

What will your responsibilities be?

You will be asked to give consent to take part in the study. A skin peel will be done to determine the significance in change in photoaging.

Will you benefit from taking part in this research?

You will be provided with information which is specific to your skin. This study will be the first time that the amount of aging can be shown in the skin and hopefully lead to better products to stop the process in other patients.

Are there any risks involved in your taking part in this research?

The procedure will be handled in a sterile manner in order to prevent any allergic reactions.

What happens if you do not agree to take part?

If you do not agree to take part in the study your consent will not be needed and you will not forfeit the advice by not taking part.

Is there any access to your medical records?

Once the skin peel is taken, the sample is given a project number: for the rest of the study, only this code is used. Information concerning your name, age, date of birth and contact details would be required; however, the information will be kept strictly confidential.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

The procedure will be handled in a sterile manner in order to prevent any allergic reactions, but in the case of an infection your medical practitioner will provide the necessary medical care.

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid to take part in the study. Also, you will not incur any costs for the laboratory analysis of the skin peel.

Further information related to the study:

- You can contact **Prof Patrick Bouic (Synexa Life Sciences)** at tel: **021 933 9580** if you have any further queries or encounter any problems.
- You can contact the **Committee for Human Research** at **021 938 9207** if you have any concerns or complaints that have not been adequately addressed by the investigator.
- **You will receive a copy of this information and consent form for your own records.**

Appendix B: Participant information leaflet and consent form

Declaration by participant

By signing below, I ……..…… agree to take part in a research study entitled:

Molecular diagnostic approach to determine the degree of photoaging of the skin

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I may request that my results be withdrawn from the collective data if I choose to withdraw.
- I may be asked to leave the study before it has finished, if the researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ..... on (*date*) …….…….. 2013.

.....	.....
Signature of participant	Signature of witness

Appendix B: Participant information leaflet and consent form

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above.
- I did/did not use an interpreter. (*If a interpreter is used then the interpreter must sign the declaration form)*

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2013.

…… ……

Signature of investigator Signature of witness

Appendix B: Participant information leaflet and consent form

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) …….. to explain the information in this document to (*name of participant*) ……..……..
  using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……..…….. 2013.

…… ……

Signature of interpreter Signature of witness

Appendix C: Patient skin care and lifestyle questionnaire

PATIENT SKIN CARE AND LIFESTYLE QUESTIONNAIRE

By completing this skin care and lifestyle questionnaire, you will be assisting in efficiently evaluating your skin care needs. All information will be kept strictly confidential.

Last Name……..

First Name……...

Date of Birth……..….

Age…….

Sex……..

Skin Type Test

Skin phototype classification is founded on subjects' recollection of their response to natural sunlight in terms of whether, and to what degree, they tan and develope erythema and afterward, their response is then categorised in an ordinal scale which ranges from vary fair (skin type I) to very dark (skin type V) [1].

Please answer the following questions pertaining to the main factors that influence skin type and add up the total scores for each section.

Genetic Disposition

What colour are your eyes?	
Light blue, gray or green	0
Blue, gray or green	1
Blue	2
Dark brown	3
Brownish black	4

What colour is your skin (non exposed)?	
Reddish	0
Very pale	1
Pale with beige tint	2
Light brown	3
Dark brown	4

What is the natural colour of your hair?	
Sandy red	0
Blond	1
Chestnut/Dark blond	2
Dark brown	3
Black	4

Do you have freckles on non exposed areas?	
Many	0
Several	1
Few	2
Incidental	3
None	4

Obtained from [1]

Appendix C: Patient skin care and lifestyle questionnaire

Tanning Habits

When last did you expose your body to sun/artificial lamp?	
More than 3 months ago	0
2 - 3 months ago	1
1 - 2 months ago	2
less than a month ago	3
less than 2 weeks ago	4

Obtained from [1]

Reaction to Sun Exposure

What happens when you stay too long in sun?	
Painful redness, blistering, peeling	0
Blistering, followed by peeling	1
Burns sometimes followed by peeling	2
Rare burns	3
Never had burns	4

How deeply do you tan?	
Not at all, or very little	0
Lightly	1
Moderately	2
Deeply	3
Very deeply	4

To what degree do you turn brown?	
Hardly, not at all	0
Light colour tan	1
Reasonable tan	2
Tan very easy	3
Turn brown quickly	4

How does your face react to the sun?	
Very sensitive	0
Sensitive	1
Normal	2
Very resistant	3
Never had a problem	4

Obtained from [1]

Total score……..

Skin type score
0 to 7
8 to 16
17 to 25
26 to 30
over 30

Fitzpatrick skin type
I - always burns, never tans
II - usually burns, then tans
III - may burn, tans well
IV - very rarely burns, tans well, brown skin
V - very rarely burns, tans well, very dark skin

Obtained from [1]

When outdoors, what precautionary measures do you take to protect yourself from the sun?

……

……

Appendix C: Patient skin care and lifestyle questionnaire

……

……

Lifestyle

Have you made use of any medication, oral or topical, within the last 6 months?

If yes, please specify……..

Do you follow a skin care regime or use any speciality products?

If yes, please specify……..

Smoking status: Non-smoker [ ] Former [ ] Current [ ]

Total cigarettes smoked per day……

- **Non-smoker** – defined as someone who has not smoked approximately 100 cigarettes in his/her life.
- **Former** – characterised as someone who has last smoked approximately 6 months ago.
- **Current** – defined as someone who has one or more cigarettes on a daily basis.

Signature……

Date of collection……..

Tape strip number…….

Reference

[1] Sachdera S. Fitzpatrick skin typing: Applications in dermatology. *Indian Journal of Dermatology, Venereology and Nephrology* 2009;**75**:93-96.

Appendix D: Percutaneous assessment of growth factors subsequent to skin needling to determine extent of photoaging

Percutaneous assessment of growth factors subsequent to skin needling to determine extent of photoaging

Stephany Wilcox[1] and Prof Patrick JD Bouic [1,2]

Medical Microbiology and Immunology Unit, Department of pathology, University of Stellenbosch[1] and Synexa Life Sciences, South Africa[2]

Abstract

Chronic exposure to ultraviolet radiation results in distinct characteristics and undesirable changes in the skin. Consequently, countless individuals are seeking therapeutic remedies to reverse these changes to maintain a youthful appearance.

The purpose of this pilot study was to evaluate the gene expression of specific growth factors subsequent to repetitive microneedling to determine extent of photoaging. This study was initiated by a local anti-aging expert wanting to make use of the method in his clinical practice.

Five volunteers were included in the study. Response to treatment was assessed by comparing pre- and post-treatment by means of relative real-time PCR.

Abbreviations: PCR, polymerase chain reaction; RNA, ribonucleic acid;

1. Introduction

Chronic exposure to ultraviolet radiation results in distinct characteristics and undesirable changes in the skin [1]. Consequently, countless individuals are seeking therapeutic remedies to reverse these changes to maintain a youthful appearance [2].

Therefore, the need for safe, non-invasive and cost-effective alternative ways to revitalise the skin has significantly increased [3].

Skin needling, also known as microneedling [3], is a technique by which microneedles attached to a rolling barrel [2] is repetitively penetrated into the skin so as to induce collagen and elastin production. It is believed that the needling procedure breaks down the old collagen while promoting the synthesis of new collagen below the epidermal layer. In doing so, the skin appears tightened and lifted, while improving the signs of photoaging, scars and stretch marks [4][5].

It is advisable that the skin undergoes a preparation process, which involves the addition of a topical formulation of vitamin A, C and E. Vitamin A, as a retinol, plays an important role in the restoration of the skin as well as assist in collagen production. Vitamin C too provides a basis for collagen production and in the instance of skin needling, where a significant increase in collagen is produced, and an even greater need for vitamin C is required [5].

Appendix D: Percutaneous assessment of growth factors subsequent to skin needling to determine extent of photoaging

Although this technique seems relatively new, it has been utilised for many years as in the art of tattooing, but lacking pigment. Clinicians believe that this method will not only provide facial rejuvenation, but can be applied to the entire body [2].

The aim of the study was to quantify growth factors by use of relative real-time PCR subsequent to microneedling from cells adherent to tape strips.

2. Materials and methods

2.1 Study population

Five healthy volunteers consisting of three males (36 ± 7.00) and two females (44.5 ± 16.26) were included in this pilot study based on the inclusion and exclusion criteria shown below. Demographical information regarding the study population is summarised in Table 1.

Written consent was obtained from the participants prior to commencement of the study and a skin care and lifestyle questionnaire was completed subsequent to the inclusion into the study to determine their skin phototype, general demographics and lifestyle patterns.

Table 1: Demographical information of study population

Subject ID	Sex	Age	Fitzpatrick phototype
Subject_001	female	56	type II
Subject_002	female	33	type IV
Subject_003	male	43	type III
Subject_004	male	36	type III
Subject_005	male	29	type IV

2.1.1 Inclusion and exclusion criteria

Males and females aged eighteen and above and experiencing good health were included in the study. Participants suffering from renal or hepatic impairment, or who have undergone procedures which require anaesthesia during the progression of the trial, as well as pregnant or lactating females were excluded from participation in the study.

2.2 Study design

All participants received a topical application of Environ® Vitamin A, C and E enhanced body oil (Environ Skin Care (Pty) Ltd, Cape Town, South Africa) throughout the progression of the study. Furthermore,

participants were subjected to repetitive facial needling treatments with a microneedling instrument (Environ® Roll-CIT™ 1 mm; Environ Skin Care (Pty) Ltd, Cape Town, South Africa) to inflict micro-wounds in the skin. The needling treatments were scheduled at weekly intervals for two weeks and every alternate day thereafter for one week. Therefore each subject had seven visits in total. Tape stripping was performed every 18-24 hours after each needling treatment.

2.3 Preparation of the skin and microneedling

The skin was routinely prepared by using a topical application of Environ® Vitamin A, C and E enhanced body oil (Environ Skin Care (Pty) Ltd, Cape Town, South Africa) before and after every microneedling treatment.

Skin needling was performed using a microneedling instrument (Environ® Roll-CIT™ 1 mm; Environ Skin Care (Pty) Ltd, Cape Town, South Africa) by rolling the tool over the entire area of the face in right, left, horizontal and vertical directions to ensure that the entire surface area was covered.

2.4 Sample collection

2.4.1 Tape stripping

Tape stripping was performed using pre-cut D-Squame® 22 mm adhesive discs (CuDerm Corp., Dallas, Texas, USA). Samples were collected 2 cm below the lateral canthus of the right eye. The first tape strip was discarded to expose the inner layers of the skin. Thereafter, nine successive tape strips were carefully applied to the facial area and removed with a constant force using sterile forceps. Collected tape strips were individually placed, adhesive side facing inwards, into separate 2 ml microcentrifuge tubes and immediately stored at -80°C for subsequent extraction.

2.5 Sample processing

2.5.1 RNA isolation

Briefly, 1.5 ml of Buffer RLT (Qiagen®, Valencia, California) was added to the first sample tube in the series and vortexed vigorously for 1 min and incubated in a Lab Armor® Chill Bucket (Shel Lab, VWR International LLC) containing Lab Armor® beads for 5 min. Following incubation, the sample tube was centrifuged at 13 000 rpm for 10 min at 4ºC. Thereafter, the RLT buffer was then transferred to the next microcentrifuge tube and the process repeated until all tape strips was processed in the same RLT buffer. Total RNA was extracted using the RNeasy mini kit (Qiagen® GmBH, Hilden, Germany), according to manufacturer's instructions.

2.5.2 RNA cleanup

Total RNA was purified using the RNeasy mini kit (Qiagen GmBH, Hilden, Germany) RNA cleanup protocol, according to manufacturer's instructions.

2.5.3 Reverse transcription

Reverse transcription was performed using the Quantitect® Reverse Transcriptase kit (Promega, USA) following the recommendations of the manufacturer. Total RNA (40 ng) was used to prepare the genomic DNA elimination reaction in a Lab Armor® Chill Bucket (Shel Lab, VWR International LLC) containing Lab Armor® beads which consisted of gDNA Wipeout Buffer [7 X] and sterile nuclease free water to a final volume of 14 µl. The mixture was heated to 42°C for 2 min in a GeneAmp® 9700 standard thermal cycler, and immediately chilled on ice beads. Thereafter, the reverse transcription reaction was prepared with the following component volumes: 1 µl Quantitect Reverse Transcriptase, 4 µl of Quantitect RT Buffer [5 X], 1 µl RT Primer Mix and added to the RNA template. The mixture was incubated at 42°C for 15 min and at 95°C for 3 min to inactivate the reverse transcriptase. The preparation was stored at -20ºC until further analysis.

2.5.4 Real time PCR quantification

Real-time PCR assays were performed on a Lightcycler® 1.2 real-time PCR machine (Roche) using the Lightcycler® Taqman® Master kit (Roche Diagnostics GmbH, Germany) in Roche Lightcycler® capillaries (Roche) in a 20 µl reaction mixture volume.

In the present study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the reference gene for relative quantification.

Each reaction contained 10 µl cDNA (diluted in a 1:1 ratio), 1 µl Hydrolysis (TaqMan®) Probe (Roche), 2 µl of each primer and 4 µl of Reaction Mix [5 X] and PCR grade water. The thermal profile for the amplification was as follows: An initial activation step at 95°C for 10 min, followed by 45 cycles of 95°C denaturation for 10 sec, primer annealing at 55°C for 30 sec or 60°C for 30 sec, elongation at 72°C for 4 sec and a final cooling step of 40°C.

2.5.6 Data analysis

Gene expression fold-change of target gene transcripts in comparison to a reference gene transcript was applied by use of the second derivative maximum analysis method incorporated in the LightCycler® 4.1.1 quantification software package (Roche) using the standard curve method.

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3. Results

3.1 Total RNA yields

Total RNA was recovered by means of the tape stripping method as described in the Materials and methods section. The mean concentration of RNA recovered from each individual is shown in Table 2.

Table 2: Summary of total RNA recovered from tape stripping

Subject ID	Mean Concentration (ng)	Standard Deviation
Subject_001	8.11	4.58
Subject_002	9.64	6.37
Subject_003	8.10	3.27
Subject_004	10.14	9.52
Subject_005	10.64	5.10

ng: nanograms

3.2 Relative gene expression fold-change

In this study, TGF-β, MMP 9, TNF-α and IL-6 mRNA transcripts were selected as representative cytokines to determine the relative fold-change in microneedling treatment over various time points (treated) and a baseline (untreated) so as to determine extent of photoaging. These markers were normalized to a reference gene, the GAPDH transcript and compared relative to a calibrator sample using the standard curve method as described in Materials and methods.

Normalised values for both the treated and untreated were each divided by the normalised calibrator values for each gene. The data illustrating the relative fold-change can be viewed in Table 3.

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Table 3: Relative fold-change of treated and untreated samples relative to calibrator

Subject_001	IL-6	MMP 9	TGF-β	TNF-α
Baseline	9.19E+04	8.07E-02	N/R	N/R
Visit 1	N/R	2.14E-01	3.92E+00	N/R
Visit 2	N/R	6.17E-03	2.65E-01	1.65E-03
Visit 3	1.45E-03	1.01E-02	2.13E-01	6.58E-04
Visit 4	N/R	1.35E-03	1.60E-03	N/R
Visit 5	1.21E-04	1.02E-03	1.45E-01	3.94E-04
Visit 6	N/R	6.50E-03	1.44E-01	9.50E-06
Subject_002	**IL-6**	**MMP 9**	**TGF-β**	**TNF-α**
Baseline	N/R	5.19E-04	9.98E-02	3.05E-04
Visit 1	1.41E-04	4.83E-04	2.36E-02	N/R
Visit 2	8.40E-04	1.25E-03	4.25E-02	2.00E+06
Visit 3	N/R	9.34E-03	1.89E-01	1.97E+02
Visit 4	1.90E-04	N/R	2.54E-02	N/R
Visit 5	2.39E-03	3.90E-03	8.99E-02	N/R
Visit 6	1.67E-03	2.41E-02	5.13E-02	N/R
Subject_003	**IL-6**	**MMP 9**	**TGF-β**	**TNF-α**
Baseline	N/R	N/R	N/R	N/R
Visit 1	N/R	3.75E-03	2.53E-01	5.75E-04
Visit 2	2.76E-04	2.95E-03	8.95E-02	N/R
Visit 3	1.10E-02	N/R	N/R	N/R
Visit 4	1.35E-04	3.73E-03	2.42E-01	8.59E-02
Visit 5	3.00E+00	3.77E-02	1.01E-01	5.63E-01
Visit 6	9.97E-02	1.88E-02	6.79E-01	9.01E-04

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Table 3: continued

Subject_004	IL-6	MMP 9	TGF-β	TNF-α
Baseline	N/R	N/R	1.81E-03	1.08E+07
Visit 1	4.08E-04	2.28E-02	3.86E-01	1.27E-02
Visit 2	4.56E-03	1.02E-03	2.10E-03	N/R
Visit 3	N/R	4.55E-04	1.41E-03	4.19E-05
Visit 4	3.81E-05	N/R	2.04E-02	N/R
Visit 5	2.11E-04	5.75E-02	5.82E-01	3.33E-03
Visit 6	N/R	1.68E-03	6.62E-03	3.07E-05
Subject_005	**IL-6**	**MMP 9**	**TGF-β**	**TNF-α**
Baseline	N/R	1.77E-02	5.92E-02	4.84E-05
Visit 1	6.30E-06	1.22E-02	1.90E-01	2.08E-05
Visit 2	N/R	1.94E-02	5.52E-02	1.11E-03
Visit 3	6.91E-05	5.72E-03	8.98E-02	1.04E-03
Visit 4	N/R	N/R	6.31E-03	N/R
Visit 5	1.56E-06	4.49E+00	1.20E-01	5.29E-04
Visit 6	N/R	1.72E-03	7.18E-03	N/R

N/R: not reportable

The samples indicated in yellow displays an outlier in the amplification process, since these were significantly up-regulated. Furthermore, those samples demonstrating a positive exponent denote up-regulation, while those with a negative exponent denote down-regulation.

Normalised treated ratios were divided by the normalised untreated ratios to determine the fold-change of treated versus untreated. Samples with an expression ratio above one for a specific gene, depicts up-regulation, while less than one illustrates down-regulation as seen in Table 4.

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Table 4: Relative fold-change of treated versus untreated

Sample ID	Visit	MMP 9	TGF-b	TNF-a
Subject_001	Visit 1	2.66	N/R	N/R
	Visit 2	0.08	N/R	N/R
	Visit 3	0.12	N/R	N/R
	Visit 4	0.02	N/R	N/R
	Visit 5	0.01	N/R	N/R
	Visit 6	0.08	N/R	N/R
Subject_002	Visit 1	0.93	0.24	N/R
	Visit 2	2.41	0.43	6562947707.74
	Visit 3	18.00	1.89	645785.19
	Visit 4	N/R	0.25	N/R
	Visit 5	7.52	0.90	N/R
	Visit 6	46.36	0.51	N/R
Subject_003	Visit 1	N/R	N/R	N/R
	Visit 2	N/R	N/R	N/R
	Visit 3	N/R	N/R	N/R
	Visit 4	N/R	N/R	N/R
	Visit 5	N/R	N/R	N/R
	Visit 6	N/R	N/R	N/R
Subject_004	Visit 1	N/R	213.43	0.00
	Visit 2	N/R	1.16	N/R
	Visit 3	N/R	0.78	0.00
	Visit 4	N/R	11.30	N/R
	Visit 5	N/R	321.88	0.00
	Visit 6	N/R	3.66	0.00
Subject_005	Visit 1	0.69	3.21	0.43
	Visit 2	1.09	0.93	22.97
	Visit 3	0.32	1.52	21.52
	Visit 4	N/R	0.11	N/R
	Visit 5	253.73	2.03	10.94
	Visit 6	0.10	0.12	N/R

N/R: not reportable

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The samples indicated in yellow displays an outlier in the amplification process, since these were significantly up-regulated. Furthermore, samples above one are up-regulated, while those less than one are down-regulated. We were unable to calculate fold-change in treated versus untreated for IL-6, since no amplification of samples above the fluorescent background was observed in the baseline samples.

No trend in fold-change for growth factors over the various time points was observed.

Down regulation of MMP 9 was observed within 30% (n=9) of samples, while 23% (n=7) showed marked up regulation, indicative of MMP 9 in response to ultraviolet radiation [6].

It has been reported that TGF-β is generally down-regulated upon exposure to ultraviolet radiation [6]. In this study, only 30% (n=9) samples displayed similar down-regulatory effects.

Five samples (16.6%) showed down regulated fold-change of TNF-α, while 10% (n=3) showed up regulation.

4. Discussion

The purpose of the study to demonstrate the quantification of growth factors by use of relative real-time PCR subsequent to microneedling from cells adherent to tape strips.

No trend is observed between relative ratios between pre treatment and post treatment, so the needling did not significantly increase expression in these individuals. However, due to low sample quantity and low expression, changes in expression could possibly have been observed if this was otherwise.Only ten samples showed measurable levels of TNF-α in the assay, while no amplification above fluorescence levels for IL-6 was observed in the relative fold-change for treated versus untreated. Normally these cytokines are expressed at very low levels [7] and therefore, a sample that has low expression when amplified, usually has a Cq value close to the limit of detection. In such an event it is advised to automatically increase the template; however, with the limited amount of sample this could not have been possible. Secondly, one would check the PCR optimisation to ensure that there are no discrepancies that could possibly affect the outcome of the genes and add controls to ensure the assay is running optimally. In this case, we have proved that our method is acceptable and that the controls are amplifying successfully.

The mRNAs selected in the study have shown that they are directly involved in the pathogenesis of photoaging [5]. However, our results demonstrate variation between samples for the respective genes as depicted by the expression values. This variation I feel was contributed by the low quantities of starting material recovered from tape stripping as well as low level cytokine mRNA expression, such as for TNF-α and IL-6.

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The advantage of the study is that we were able to show that a real-time PCR method is repeatable and reproducible based on the validated information. The limitation of the study is no pre-amplification kit was utilised. The use of this kit would have allowed us to observe the detection of low expressed genes. This method could be applied in areas where a significant increase in expression would be observed, no need for pre-amplification kit, especially if study is limited to a budget.

In conclusion, we have shown that epidermal cytokines can be retrieved from tape stripped samples and can be quantified via real-time PCR. However, the choices of cytokine biomarkers reveal that they are as important as the quantity of sample recovered. In this study cytokines such as IL-6 is not as informative in determining the extent of photoaging without high doses of ultraviolet radiation before sample collection as opposed to the other explored cytokines.

5. References

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