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Dermal Epidermal Separation for Skin Rejuvenation

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Para os meus avós, tias, pais, irmã e irmão.

Resumo

O aumento da esperança média de vida ao longo das últimas décadas foi acompanhado por uma preocupação crescente com a saúde e estética da pele. Pele fotodanificada e envelhecida é caracterizada pelo aparecimento de características indesejáveis, tais como marcas de pigmentação, atrofia da junção dérmica epidérmica, rugas, perda da radiância, claridade e uniformidade da pele. De forma a contrariar o efeito da exposição solar há uma necessidade crescente de desenvolvimento de métodos e dispositivos que visam o rejuvenescimento da pele. De acordo com o relatório apresentado pela Transparency Market Research [4] sobre o mercado dos dispositivos de tratamento e cuidado de pele é esperado um crescimento de 10% destes dispositivos durante o período de 2012 a 2018.

Rejuvenescimento da pele implica a substituição do tecido foto danificado ou envelhecido por um novo, mais saudável, radiante e uniforme. Desde químicos a fotónicos incluindo sistemas mecânicos, existe uma vasta gama de dispositivos que visam rejuvenescimento de pele. O grau de rejuvenescimento está relacionado com a agressividade do método utilizado. Técnicas suaves como a aplicação de loções e cremes carecem de eficácia, no entanto apresentam poucos efeitos secundários sendo seguras para os utilizadores. Já a maioria dos dispositivos comercialmente disponíveis, como peeling químico, abrasão da epiderme, e *resurfacing* a laser, atuam através da danificação ou remoção de toda a epiderme viável, expondo o corpo humano à acção de agentes químicos, físicos, patogénicos e à radiação ultra violeta (UV). Apesar de eficazes estes métodos apresentam efeitos adversos, nomeadamente o elevado risco de infeção para os pacientes e o longo tempo de recuperação. Por estes motivos é necessário desenvolver uma técnica não invasiva que rejuvenesça eficazmente a pele sem comprometer a saúde do paciente.

Philips Research Eindhoven é uma das maiores organizações de investigação do mundo, localizada no High Tech Campus (HTC) em Eindhoven. O departamento Personal Care and Wellness combina o conhecimento sobre a biologia e morfologia da pele com as mais avançadas técnicas ópticas com o objetivo de inovar e desenvolver produtos com impacte no bem estar da população. O projeto, onde esta tese se insere, visa a geração de propriedade intelectual assim como desenvolver e apresentar a *proof of concept* sobre patentes anteriormente submetidas.

Esta tese teve como principal objetivo desenvolver e construir um dispositivo que permitisse separar a derme da epiderme de forma não invasiva e que possa ser aplicado em pele humana *ex-vivo* e *in-vivo*. Para alcançar este objetivo, um sistema de sucção combinado com método de imagem não invasivo OCT *(do acrónimo inglês Optical Cohenrence Tomography)* foi construído. Este sistema

permitiu pela primeira vez a monitorização em tempo real do processo de separação da derme da epiderme. Devido à capacidade de visualizar em tempo real a separação da derme da epiderme, um modelo que descreve a evolução do processo de separação das duas camadas mais superficiais da pele assim como a acumulação de fluido intersticial foi formulado e descrito. Neste modelo três fases foram definidas e caracterizadas: Latência, Crescimento e Maturação. Foi também possível observar que a separação dérmica epidérmica ocorre segundo dois diferentes processos: 1) formação inicial de uma pequena fenda dérmica epidérmica que progressivamente aumenta de tamanho e 2) formação de várias pequenas separações ao longo de toda a junção dérmica epidérmica que aumentam de dimensão ao longo do tempo e se vão unificando.

Para além de combinado com OCT, o sistema de sucção foi também acoplado a um sistema de aquecimento e a um sistema de Radio-Frequência (RF). A dependência entre o tempo de separação da derme da epiderme e fatores como o diâmetro do prato de sucção aplicado, a pressão e a temperatura da pele foi estabelecida. Os parâmetros óptimos para a separação da derme e epiderme num curto período de tempo foram determinados: um diâmetro entre 1 mm e 1.5 mm, uma pressão de sucção de 600 mmHg e uma temperatura de 40°C. Os resultados em pele *ex-vivo* foram corroborados por um estudo *in-vivo*. Após a optimização dos parâmetros foi possível reduzir o tempo de separação dérmica epidérmica para um sexto do tempo inicial.

De forma a compreender os processos regenerativos que actuam após a separação dérmica épidermica e a avaliar se uma nova e saudável epiderme é formada de forma não invasiva, a resposta regenerativa ao tratamento foi avaliada através de um estudo *in-vivo*. As imagens de OCT obtidas 1 dia e 4 dias após o tratamento revelaram a formação de uma nova e saudável epiderme de baixo da epiderme separada, que age como um escudo biológico protegendo o organismo contra agentes infecciosos. Neste estudo também foi verificado que o tempo de regeneração da epiderme depende da extensão de epiderme separada. Extensões menores requerem um mais curto período de regeneração.

A capacidade da OCT permitir detetar e visualizar características da pele como poros e folículos capilares permitiu a aplicação do tratamento sobre estas. Os resultados obtidos indicam que o processo de separação dérmica epidérmica é facilitado na região folícular e dificultado em poros. Foi ainda testada a aplicação fracionada do tratamento de forma a reduzir o tempo de aplicação e desconforto para os utilizadores.

Apesar de eficiente, OCT é um sistema de monitorização dispendioso e pouco portátil não podendo ser acoplado a um dispositivo comercial de rejuvenescimento de pele. Para colmatar esta necessidade foi testada a possibilidade de usar as propriedade condutivas da pele para detetar a separação da derme da epiderme. Usando um sistema de radio-frequência, a impedância da pele à corrente eléctrica foi monitorizada durante o processo de separação da derme da

epiderme. Uma diminuição da impedância da pele ocorre durante a migração de fluido intersticial para a cavidade dérmica epidérmica.

A principal aplicação visionada para esta técnica assim como os parâmetros determinados é o desenvolvimento de um dispositivo de rejuvenescimento da pele não invasivo. Devido a promover a separação da derme da epiderme em apenas alguns segundos a técnica apresentada nesta tese ganha especial relevância na área médica. Com foco de interesse para dermatologia onde a transplantação epidérmica é umas das técnicas mais utilizadas no tratamento de Vitiligo e também para análises clínicas onde extração do fluido intersticial é utilizado em enúmeros estudos. A redução do tempo de separação é crucial para a diminuação do tempo de tratamento e desconforto para os pacientes.

O próximo passo será um estudo clínico, usando uma significante amostra população de diferentes idades e géneros, de forma a avaliar se o tratamento leva a um rejuvenescimento da pele a curto e longo espaço de tempo. O tratamento deverá ser aplicado no tecido facial e ser acompanhado por um estudo histológico ou de TEM *(do acrónimo inglês Transmission Electron Microscopy)* de forma a estudar o processo de regeneração. A aplicação fracionada do tratamento assim como o efeito de folículos capilares e poros no tempo de separação deverá ser estudada em detalhe.

Em suma, nesta dissertação as principais fases de desenvolvimento de um dispositivo de rejuvenescimento de pele foram levadas a cabo. Desde do design e construção de um protótipo, à otimização do parâmetros terminando num teste clínico e análise dos resultados. O dispositivo construído e descrito nesta tese revelou ser uma técnica promissora, eficiente e segura para o rejuvenescimento de pele e para monitorização em tempo real da cinética da separação dérmica epidérmica.

Palavras Chave: Sucção, Rejuvenescimento, Deteção, Separação Dérmica Epidérmica

Abstract

With the increase of life expectancy over the last decades there is an increasing concern about how to maintain the skin healthy. Skin rejuvenation implies the replacement of the damaged upper layers of the skin with new ones, improving fine lines, radiance and clarity of the skin. The current available techniques for skin rejuvenation including chemical peeling, dermabrasion and laser skin resurfacing, act by removing the upper layer of the skin, the epidermis, which protects the body against physical, chemical, pathogen and UV radiation injuries. The aim of this thesis was to develop and build a non-invasive device which induces dermal epidermal separation and implement this technique to *ex-vivo* and *in-vivo* human skin. For this purpose a suction system integrated with a non-invasive imaging method Optical Coherence Tomography (OCT) was built. This system allowed, for the first time, the real time monitoring of the kinetics of dermal epidermal separation process and to follow the regenerative process non-invasively. The suction device was also combined with a heating and radio-frequency system. A model for the dermal epidermal separation over the time was formulated and described in this thesis. The relation between the dermal epidermal separation time and the diameter of the suction aperture, the suction pressure and the temperature was established. The *ex-vivo* results were validated with *in-vivo* studies. Furthermore it was possible to assess to the feasibility of electric conductivity as dermal epidermal separation detection method. A marked decrease of the skin electric impedance was verified during the migration of interstitial fluid to the dermal epidermal cavity, indicating that electrical impedance can be used as detection method. The experimental set-up and method here in described revealed to be a safe, efficient and promising technique for skin rejuvenation.

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Contents

Thesis Overview

An increasing concern about how to maintain the skin healthy emerged in the last decades. Ageing and sun exposition lead to the appearance of undesirable skin features such as pigmentation marks, wrinkles, loss of radiance, clarity and uneven skin tone. For this reason, the demand for skincare devices is growing constantly. According to the report presented by Transparency market Research [4], global skincare devices market is expected to grow at a 10.1% Compounded Annual Growth Rate (CAGR) during the forecast period of 2012 to 2018.

From chemicals to light including mechanical systems, enumerous devices with the purpose of rejuvenate the skin have been developed. The degree of rejuvenation is related with the aggressivity of the method used. Smooth techniques like the application of lotions and creams have lack of efficiency however they present few side effects. On the other hand, commercially available techniques for skin rejuvenation, including chemical peeling, dermabrasion and laser skin resurfacing are efficient but they act by removing the upper layer of the skin, the epidermis, exposing the human body to environmental agents. Accordingly, these techniques present a high risk of infection and a long downtown time. For this reason there is a need of the development of a technique which could rejuvenate the skin without disrupting or damaging the epidermis. This thesis combines the use of a suction device and optical monitoring systems for skin rejuvenation.

Philips Research is one of the leading investigation organizations in the world. This thesis was developed at the department of Personal Care and Wellness at Philips Research, located in the High Tech Campus Eindhoven. This Research group combines the optical insights and techniques with biological knowledge of the skin tissues in order to develop new products through meaningful innovations. This work was part of a project focused on the generation

of Intellectual Property.

This thesis is organized in 6 chapters. Chapter 1 introduces the main concepts about the structure of the skin and the physical principles of the imaging and radio-frequency tools used. In the second chapter the *state of the art* about the factors that influence the blistering time is presented. The materials and methods used to construct the suction system are described in the chapter 3. A model formulated to describe the process of blister evolution over the time is presented in the chapter 4. The main results obtained are reported on the chapter 5. Finally, in chapter 6 the main conclusions, applications and outlook are presented. However in this version only the two first chapters are disclosed.

Objectives

The majority of the skin rejuvenation treatments act by removing or damaging the epidermis, which protects the body against physical, chemical, pathogen and UV radiation injuries. The main goal of this internship was to assess to the feasibility of using the blistering suction pressure technique for non-invasive skin rejuvenation, thus reducing the risk of infection and downtown time.

To achieve this goal it was necessary to develop and build a system which induces dermal epidermal separation that could be applied to *ex-vivo* and *in-vivo* human skin. For this purpose six main objectives were defined:

- To build a suction pressure system which induces blister formation in *ex-vivo* and *in-vivo* human skin.
- To combine a non-invasive imaging technique with the suction pressure system to monitor blister formation in real time.
- To combine a second system to reduce the time of application with a suction pressure system.
- To establish the dependence between blister volume evolution and the suction pressure, the aperture diameter, the temperature of the skin tissue and the morphological effect on the skin.
- To develop a detection method more practical, portable and less expensive than OCT, that could be easily attached to a skin rejuvenation commercial device.
- To assess the process of epidermal regeneration after dermal epidermal separation, through *in-vivo* healing studies.

1 Introduction

1.1 Skin

Skin is the largest organ of the human body and an essential component of the body's life support system. The primary function of the skin is to protect the body against the loss of endogenous substances and against physical, chemical, immune, pathogen and UV radiation injuries. The skin is a sensory organ and the major participant in thermoregulation. It has also an endocrine function, promoting the vitamin D synthesis by conversion of the sunbeams into indispensable Vitamin D. The skin is composed by at least five different cell types such as keratinocytes, melanocytes, Langerhans cells, fibroblasts and endothelial cells [38].

In this chapter an overview about skin structure, ageing effects and skin rejuvenation will be presented.

1.1.1 Struture

The skin consists of two distinct layers. The superficial layer, epidermis, can be repaired from any damage it may suffer from external contacts. The dermis is the deepest layer of the skin and it consists on connective tissue elements. The two layers interact mutually and collaborate during the repair process and remodeling the skin such as in wound healing, see figure 1.1.

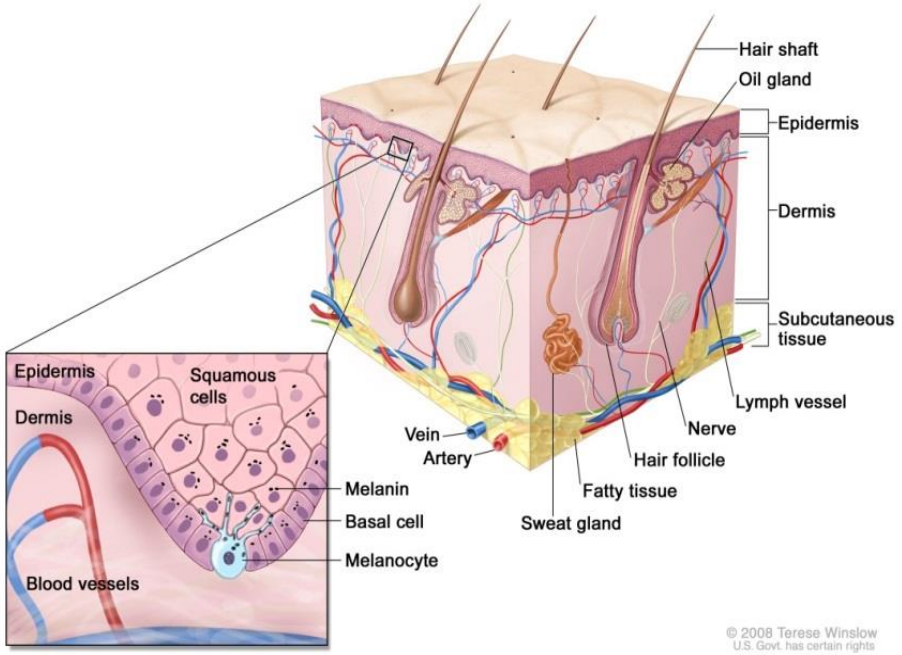

Figure 1.1: Skin overview with different cells, layers and features. An extended figure shows the dermal epidermal junction and melanocyte distribution. Adapted from [3].

The dermis is less cellular than epidermis and it is composed primarily of fibrous and amorphous extracellular matrix. The dermis is highly vascular and includes pilosebaceous units, sweat glands, dermal adipose cells, mast cells, and infiltrating leukocytes. The dermis functions are related with the body protection from mechanical injuries, binding water, temperature regulation and include receptors of sensory stimuli.

1.1.2 Epidermis

The epidermis is a stratified, continually renewing epithelium which exhibits progressive differentiation through basal to superficial direction. It is composed of keratinocytes originated from the basal layer and as they mature they lose their keratin filaments and nuclei to form stratum corneum. The epidermis, which has a thickness between 100 and 150 μm, is divisible into four distinct layers: the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC), see figure 1.2.

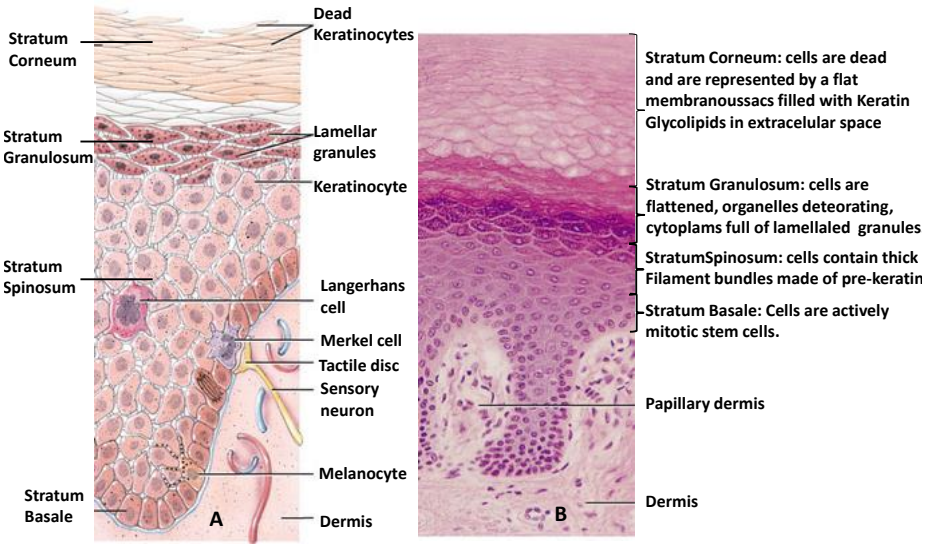

Figure 1.2: A- Schematic representation of epidermal layers and features. B- High resolution histology view of human epidermis. Adapted from [5].

The maintenance of cell number in the epidermis depends upon fine balance between cell birth, proliferation and death, differentiation and apoptosis of keratinocytes. The structure of an individual keratinocyte correlates with its location within the epidermis and its state of differentiation.

While cells produced in the stratum basale move towards the surface, they undergo a sequence of changes which characterize the other strata, until they are shed off the surface of the stratum corneum.

1.1.3 Dermal Epidermal Junction (DEJ)

The DEJ is a specialized basement membrane, which provides adhesion, structural integrity and a dynamic interface between the two distinct skin layers which separates: dermis and epidermis. Through anchoring molecules, the DEJ supports the epidermis and influences the behavior of keratinocytes by modulating cell polarity, proliferation, migration, and differentiation. The DEJ is also important during morphogenesis and development, wound healing and remodeling of the skin [41]. The DEJ also provides the connection between keratin networks within the epithelium to the basolateral surface through the basement membrane and secure the continuous series of connections to the pap-

illary dermis.

The anchoring complex consists of hemidesmosomes, anchoring filaments and anchoring fibrils. Epidermal keratinocytes are secured by hemidesmosomes to DEJ. Hemidesmosomes are located at the plasma membrane of the basal keratinocytes. They anchor the epidermis firmly to the Lamina Densa by connecting with the anchoring filaments, thin threadlike structures with 2-4 nm diameter, they also provide attachment of keratin filaments to the basolateral epidermal surface [12].

1.1.4 Ageing Effects

Ageing of human skin results from intrinsic ageing factors, which is acquired over the years and from extrinsic ageing factors such as cumulative exposure to external influences. The factors related with intrinsic ageing are the decrease of the mitogenic responsiveness and loss of autocrine growth factor production [21]. Factors such as mitochondrial DNA damage, increased Reactive Oxygen Species (ROS), production and telomere shortening cause an accumulation of senescent cells incapable of proliferation.

Photoaged epidermis shows variable: thickness, with alternating areas of severe atrophy and hyperplasia; pigmentation marks, with alternating ephelides, lentigenes, and depigmented areas; degree of structural abnormality for both keratinocytes and melanocytes; and orderliness of keratinocyte maturation. Photoaged epidermis also features melanocytes irregularly dispersed along the basement membrane, and the number of epidermal Langerhans cells are markedly reduced, see figure 1.3.

Both ageing processes, intrinsic and extrinsic, are associated with the loss of tissue compliance, resilience and the formation of wrinkles [41].

Ageing affects the epidermis as well as the dermis. In the epidermis, the tissue changes associated with ageing are: slower turnover of keratinocytes, keratin sloughs more slowly with thickening of the keratin layer, decreased number of melanocytes and less melanin production and uneven melanin pigment distribution. The observed alterations in the dermis are: decreased number of fibroblast which results in a less collagen production that is related to a fragile skin structure, elastin fibers thickened and with less elasticity, decreased of the matrix's quantity and the blood vessels dilation, thinned and weakened walls, prone to rupture.

The major cutaneous changes in aged skin are observed in the DEJ which displays flattening of the rete ridges leading to reduced surface contact between the epidermis and dermis which leads in a reduced exchange of nutrients and metabolites between these two layers [36]. In severely photoaged skin there is a

loss of collagens I, III and VII.

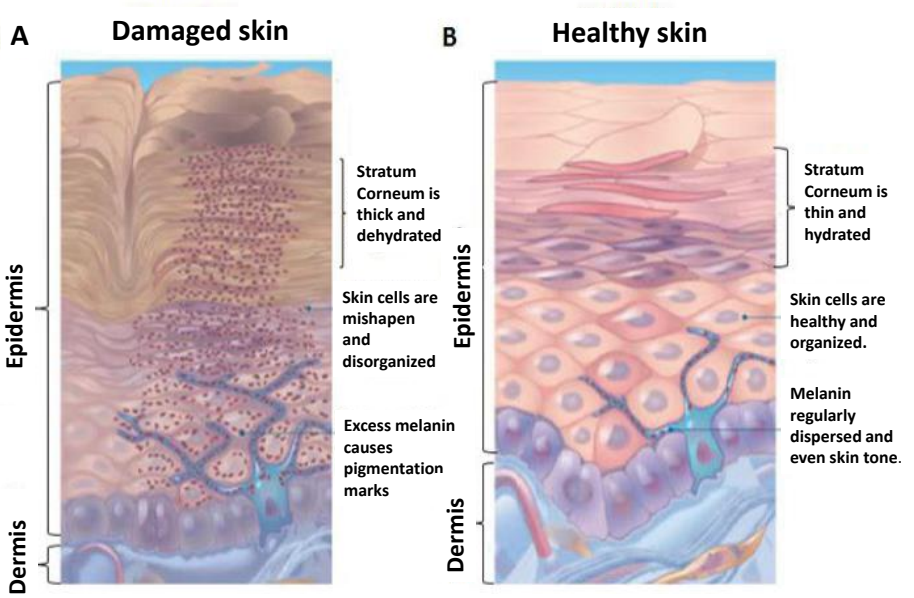

Figure 1.3: Skin diagram of basic epidermal structure illustrating the differences between: A- Aged skin and B- Healthy skin. Adapted from [1].

Mechanical properties of the skin can vary with age in two different ways. The skin elasticity decreases and the relaxation time after strong deformation increases continuously over the time. The progressive loss in the elastic properties of the skin can be explained by a degenerative change in the elastin network in the dermis. However some characteristics such as thickness and extensibility can be constant until 70 years of age and then rapidly change [13][17].

1.1.5 Regeneration

Epidermal regeneration depends on mitosis and migration of keratinocytes from the residual epidermal adnexal structures such as the hair follicle and sweat gland as well from the intact epithelium surrounding the lesion. Epidermal growth factor stimulates growth of keratinocytes and promotes wound healing. The regeneration process is also regulated and controlled by the activation of a specialized stem cell population, located either in the basal epidermis or in the permanent hair follicle called the bulge. Due to the high proliferative potential, lifetime persistence, multipotency, and strategic location they are important for

repopulating the epidermis after injury. They have substantial role in the acute wound-healing response once they rapidly send cells to the epidermis during re-epithelialization.

Epidermal turnover is synonymous with regeneration time and it is defined as the average time taken for the epidermis to replace itself. The turnover time can be subdivided into a proliferative phase, differentiated phase and the passage through the stratum corneum. The total turnover time is the sum of the turnover time of each phase. The time that the normal human epidermis is required to pass the several stages is not clear. It is stated that the skin takes approximately 27 to 28 days to renew it self. However recent studies calculated the turnover time in 39 days [26][36].

1.1.6 Skin Rejuvenation

An increasing interest in the esthetic aspects of the skin has emerged in the last decade. Skin rejuvenation can be defined as the replacement of the damaged tissue with new one, by means of injure or remove the upper layers of the skin in order to stimulate the formation of new collagen, through the generation of a new tissue. Rejuvenation treatments usually aim to resurface the skin, improving fine lines, the radiance and clarity of the skin as well as provide an even skin tone. Also pigmentation marks such as freckles, sun spots and darkened patches that result mainly from sun exposure can be treated by skin rejuvenations techniques. Skin rejuvenation affects collagen and the melanocytes present in the epidermis and in the papillary dermis.

Invasive rejuvenation treatments include chemical peeling, dermabrasion and laser skin resurfacing. These treatments do not treat just a specific area but the entire skin treatment area is injured. On the other hand, skin tightening requires tissue reduction, which can be the total area of the skin or just one layer of skin, it is usually achieved by remodeling the tissue . Skin tightening is related with the regeneration of collagen fibers of the dermis and subsequent remodeling of skin tissue. It aims to reduce the skin laxity, attenuate folds and wrinkles and to provide a firmer aspect to the skin. The main skin characteristics which can be treated by tightening procedures are: static wrinkles, deep fold into the skin that is always present and does not change in appearance with facial movements or expressions and scars that result of acne or injury to the skin. Scars can be discolored or have a wavy appearance to the skin.

There are several techniques for tightening skin, such as laser therapy and face lift. However they can be extremely invasive, for example filling material below the wrinkle to flatten the fold, or to modulate the muscular movement by either activating muscles that pull the tissue opposite to the folds for a smoothing effect or immobilizing the muscles that move the skin to form the fold. Other disadvantage of these techniques is the fact that the neuromodulators can di-

minish the capacity to create facial expressions. Futhermore, it is believed that epidermal rejuvenation will reduce the skin pigmentation spots and an evenness skin tone will be obtained.

1.2 Blisters

1.2.1 Formation

Under normal circumstances, the molecular interactions of the structural proteins of the epidermis, DEJ, and dermis sustain the scaffolding of the skin. Blisters can occur by protease degradation of the structural protein or by alteration of the protein-protein interaction or adhesive function of the molecules. According to Bork et al. [11] a blister formation is composed of three processes which can occur successively and simultaneously: 1) loss of structure, due to a diminished cohesion of epidermal cells or a weakened DEJ; 2) discontinuity, which is related with cleft formation between the keratinocytes or in different levels of the junction zone and 3) fluid accumulation, movement of fluid into the space created by the antecedent damage.

There are three compartments in the skin where blisters might arise: the epidermis, dermal epidermal junction (DEJ), and the dermis. Based on the location of fluid accumulation blisters can be classified as intraepidermal, dermal epidermal or dermal [15], see figure 1.4.

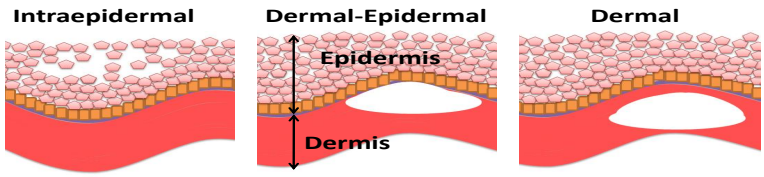

Figure 1.4: Schematic representation of the different types of blisters. Intraepidermal blisters can be subcorneal or occur within the stratum spinosum. Dermal Epidermal blisters occur between the stratum basale of the epidermis and the dermis basement membrane. Dermal blisters occur between the dermis basement membrane and the papillary dermis.

Intraepidermal and dermal blisters are usually related with specific pathologies. In case of an intraepidermal blister the hemidesmosomes are well preserved and the intraepidermal clefts contain the remnants of the degenerate cells. It is

well accepted that the dermal epidermal junction is the natural cleavage plane of the skin: it is virtually devoid of fibrillar structures and is the site of a 'viscous bond' between dermis and epidermis which can be easily disturbed by thermal, mechanical, osmotic and chemical factors. For this reason this introduction is mainly focused on dermal epidermal blisters.

Regarding mechanically induced blisters, suction blisters are typically located at the dermal epidermal junction, however friction blisters are located intraepidermally, resulting from the necrosis of spinous cells [50].

Blisters are formed as a result of a breakdown of tissue integrity and fluid accumulation. It occurs when one or more of the skin's structural components responsible for the functional connection are weakened or destroyed by a variety of mechanisms. The main mechanisms which lead to blister formation are: virus invasion of the epidermal cell, infection of the epidermis with fungi and bacteria, sensitization of the skin due to application of chemicals, vascular occlusion, thiol-binding agents such as mustard gas and physical injury which could be related with friction, pressure, heat, cold and ionizing radiation. There are also blister-forming diseases as: necrobiosis lipoidicadiabetirum, lichen planus, systemic lupus erythematosus and mastocytosis [46].

When the epidermal layer of the skin is traumatized, an acute accumulation of extracellular fluid develops between the epidermal and dermal layers of the skin. Secondary inflammation then occurs as part of the healing process. If the epidermal layer opens, the secondary inflammation also may be associated with infection and as a result, may develop purulent fluid with an infiltration of white blood cells. Moreover, the actual exposure of the dermal layer of skin often results in pain once the nerve endings are more exposed [50].

1.2.2 Physical Mechanisms Involved on Blister Formation: Suction Pressure

The suction blister technique was first described in 1964 as a method of generating skin blisters. This technique separates the epidermal basal layer from the basement membrane of the dermis, due to the disruption of the DEJ. The dermal epidermal separation can be understood as a process of viscous slip, it is supposed that a highly viscous resistance promotes the adherence of the epidermis to the dermis [9].

In Vivo blistering can be produced by the application of a suction pressure to the skin surface, which will lead to the separation between the dermal and epidermal layers resulting in the formation of superficial blisters. DEJ separation occurs by detachment of hemidesmosomes from the basement membrane. The anchoring filaments are lifted with the hemidesmosomes. The basement membrane itself and the region of the anchoring fibrils underneath remained

unchanged throughout suction blister formation.

The stronger the suction forces or its duration, the higher are the damages on the skin. Beerens et al. [8] showed that the product of suction pressure (P) and blistering time (t_b) is constant, which suggest a viscous nature of DEJ. A second study showed that the adherence decreased exponentially with the increase of skin temperature. After some time of suction, an hydrostatic pressure gradient is generated over the stratum corneum. Since this layer represents the skin water barrier, the hydrostatic pressure promotes the accumulation of fluid under the layer, until tonofibrils become stretched.

In the past years there has been an increasing interest about the use of suction blistering devices to epidermal grafting. The viable epidermal layer is removed without disrupting the dermis which can be transferred to a distant recipient site. As the dermis of the donor site is left undamaged there is no scarring although variable loss of pigmentation may occur. The repigmentation obtained by blister grafting is permanent. This technique is mainly used to transfer melanocytes to depigmented skin in patients with vitiligo [25].

Hyperpigmentation and hypopigmentation are related to the basement membrane and with the over or sub-activity of melanocytes caused by external and/or internal factors. The hypothesis that will be tested in this work, is that the dermal epidermal separation will lead to the formation of a new epidermis which will contain healthy melanocytes regularly dispersed along the basement membrane.

1.2.3 The healing of a Blister

Following the process of tissue injury, a dynamic cascade of wound repair process is initiated to restore skin integrity. Wound healing in skin is a dynamic and interactive biological phenomenon, which involves three main phases: 1) inflammation and exudation; 2) tissue regeneration which is characterized by proliferation and migration of cells to the wound and 3) tissue remodeling, in this stage the epidermis is restored and scarred if deeper layers were involved during the tissue destruction [18].

The suction blister is a model of epidermal regeneration *in-vivo*, while the dermis remains uninjured. The active process of wound healing begins with an inflammatory reaction, including local vasodilation and fluid extravasation into the extracellular space. The epithelialization process follows angiogenesis and begins with migration of adjacent epidermal keratinocytes from the wound edges and from dermal appendages into the wound as well as from hair follicles. Epidermal proliferation takes place at the wound edge and covers the defective area by migration. This migration is enabled by alterations in the cytoskeleton of keratinocytes and by changes on the integrin expression. The keratinocytes

become flat and elongated [31] [48].

It is stated that intact blisters usually heal faster due to the fact that after the blister fluid got absorbed by the body or drained during the course of conservative treatment, blister roof acts as a natural occlusive dressing, protects the underlying wound and provides a moist environment. Blister skin that collapsed to the surface of skin serves the dual purpose of expediting wound healing and enhancing quality of healing. Studies also indicate that opening of blisters increases pain, converts a closed wound into an open wound and increase the potential for wound infection. Some of these studies are based on the measurement of water evaporation and blood flow in suction-created blister wounds. However, Leivo et al. [32] showed that re-epithelialization process was considerably slower in intact blisters than in roofless open wounds, which is probably related with the fact that in intact blisters contact inhibition can occur by the pressure of the blister fluid, difference in protease or cytokine expression or in interstitial fluid calcium concentration and accumulation of inhibitory compounds into the blister cavity.

Suction Blister Healing

Suction blister formation occurs by successive detachment of hemidesmosomes from the basement membrane. After a partial separation of the epidermis from the dermis, a rapid regeneration of the dermal epidermal junction takes place. This regenerative process consists of two steps: realignment of basal cells to the basement membrane accompanied by autophacytosis of detached hemidesmosomes; and formation of new hemidesmosomes [9]. Detached basal cells occupy the empty spaces at the basement membrane by means of pseudopod formation and phagocytosis of the detached hemidesmosomes, these process takes about 2 hours for a 1 cm diameter blister, after the epidermal-dermal separation to be completed.

Studies with interrupted suction revealed a process of rapid repair of DEJ. Beerens et al. [8] showed that after using a suction device during half of the blistering time, an interval of 2 hours was sufficient for a complete repair of DEJ, the connection regained its initial strength. Half an hour after the disruption of the dermal epidermal junction, fine filaments of hemidesmosomes of basal cells were found in contact with the basement membrane. A complete lining-up of keratinocytes with the basement membrane was also found, while autophagic vacuoles containing detached hemidesmosomes, lay at about 0.2 μm from the basement membrane.

1.3 Detection Methods for Dermal Epidermal Separation

1.3.1 Optical Coherence Tomography (OCT)

OCT is based on a technique called low-coherence interferometry, where the light reflected or backscattered from inside the tissue is measured by correlating it with light that has travelled a known reference path. OCT imaging is performed using a fiber-optic Michelson interferometer with a low-coherence length light source [20]. OCT uses light sources with wavelengths in the near infrared. The light is coupled to a single-mode fiber optic interferometer and divided into a reference beam and a probe beam, represented respectively by A and B on figure 1.5. The reference signal is reflected from a scanning mirror system. The light in the sample arm is focused onto the superficial skin layers, backscattered and recombined again with the reflected reference signal.

The skin surface represents the location of maximum intensity. Interference occurs only if the path length of both beams matches within the short coherence length of the light source. OCT image contrast results from a combination of absorption and scattering.

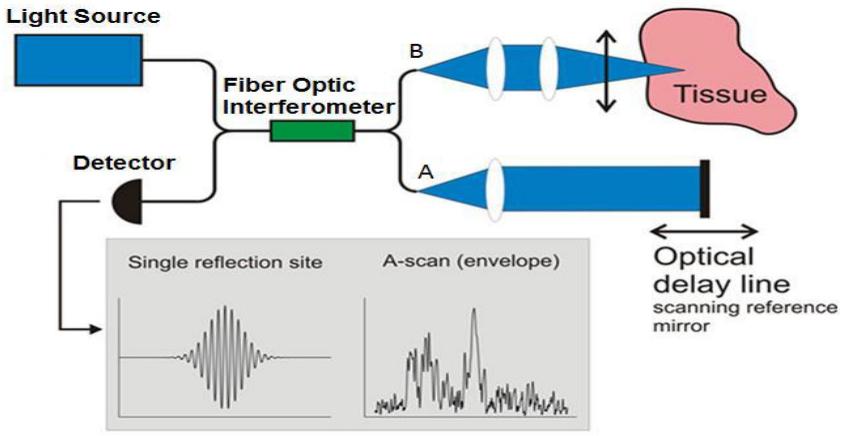

Figure 1.5: Schematic representation of OCT operation system [2].

The interference signal gives information about the path length distribution of the sample beam due to optical inhomogeneities of the tissue. OCT measures echo delays and the intensity of backreflected infrared light from internal tissue structures. The reflectivity of the skin leads to a signal with a intense bright band on the surface, which is also considered the entrance signal. Light propagation in tissue differs from that in air due to differences in refraction

indexes. The skin refractive index is about 1.4 [54]. The skin surface represents the location of maximum intensity.

As observed in previous studies, the border between the stratum corneum and living epidermis is usually distinct, whereas the dermal epidermal border is frequently blurred. The dermis shows intense signals with some lower reflecting regions, corresponding to hair follicles and sebaceous glands, figure 1.6. Hairs on the skin surface cause signal shadows. The blood vessels appear as longish structures [54].

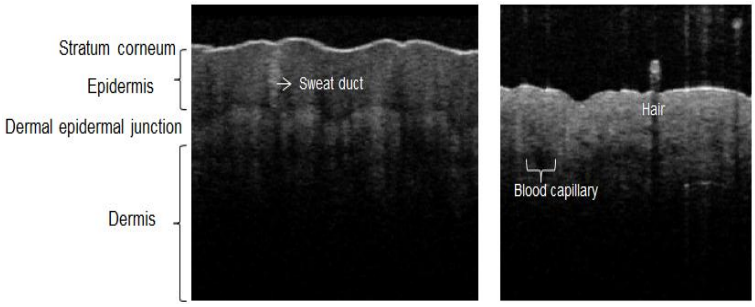

Figure 1.6: OCT images of skin features: Sweat Duct (Left) and Hair (right).

By lateral scanning, OCT provides two-dimensional cross-sectional images of the skin. The axial resolution depends on the coherence length of the light source, whereas the lateral resolution is given by the focal spot size and the scan step. The detection depth depends on the wavelength, the scattering and the attenuation of the light inside the tissue and varies from 1 to 1.6 mm in skin, which is sufficient for investigation of the stratum corneum, the living epidermis, and the upper parts of the dermis.

1.3.2 Reflectance Confocal Microscopy (RCM)

Confocal scanning microscopy is based on the imaging of thin sections at high resolution and contrast without physically dissecting the tissue. Small volumes of tissue are sampled, producing images with microscopic resolution at depths up to several hundred micrometers within tissue. This optical sectioning capability of confocal microscopy enables cellular structures to be imaged without taking biopsies from the human body. A thin plane or section can be optically or non-invasively imaged within a scattering medium with high resolution and contrast [43]. Currently are being developed miniaturized objective optics which enable confocal imaging of internal organs for in situ detection of pathology [47].

A confocal microscope uses a point source of light, generally a focused laser beam, to illuminate a point within the sample. First the laser light reflects off a

dichroic mirror, which reflects the wavelength and passes light longer than that wavelength. From there, the laser hits two mirrors that scan the laser across the sample. The sample emits light that gets descanned by the same mirrors that are used to scan the laser light. The emitted light passes through the dichroic and is focused into the pinhole, see figure 1.7. The pinhole aperture in a screen that allows only the light emitting from the desired focal spot to pass through. The light that passes through the pinhole is measured by a detector, for example a photomultiplier tube. The light source, illuminated spot and detector aperture lie in optically conjugate focal planes. In order to block any light that is not contributed from the back-scatter of the focused spot, the size of the detector aperture should match to the size of the illuminated spot. By scanning the focused spot over the sample, the detector only receives light from the thin plane at the focus. Light from out-of-focus planes is rejected by the pinhole or spatially filtered by the detector aperture. The optical sectioning is created as long as the sample is optically transparent or translucent. Consequently the confocal microscope can create non-invasive images of thin sections, within turbid, scattering media without having to cut the sample physically into thin slices [53].

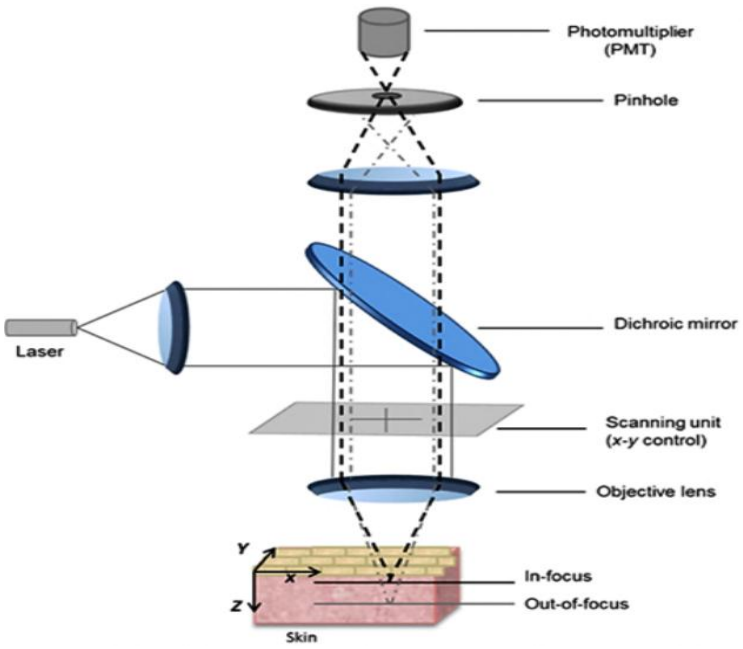

Figure 1.7: Schematic diagram of the principle of confocal laser scanning microscopy [44].

In the healthy skin the strata granulosum and spinosum present a honeycombed pattern, formed by keratinocytes. They can be identified as 10 to 20 μm polygonal and polyhedral cells with dark nuclei and bright edges, due to

occasional melanin granules and intercellular connections (structures with high refractive index). Cells of the stratum spinosum and stratum granulosum form a cohesive honeycomb pattern with bright granular cytoplasm and dark oval to round nuclei in the center, see figure 1.8.

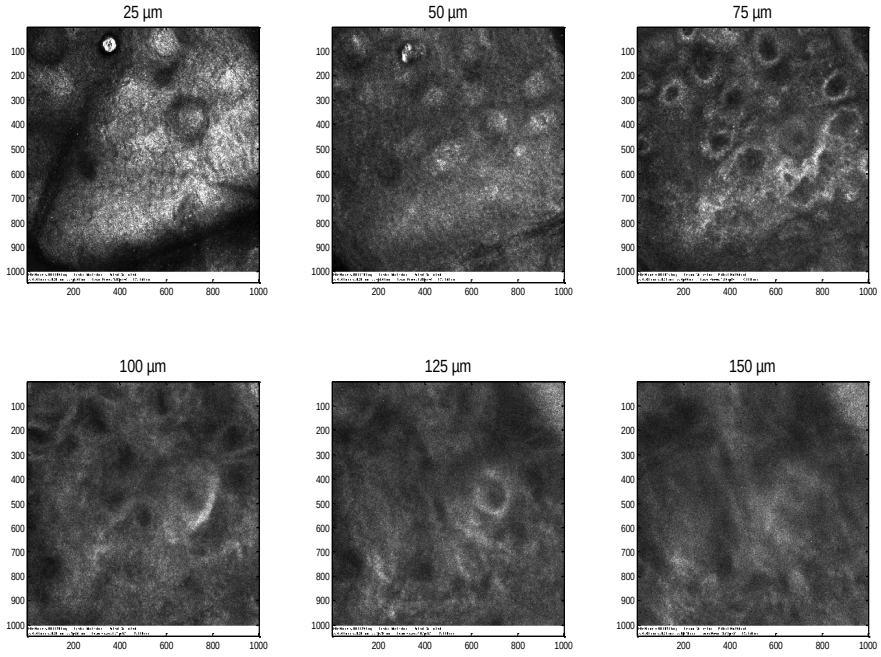

Figure 1.8: RCM images of human hand at different depths, and with a field of view of 0.5 mm x 0.5 mm. The images were obtained using a RCM beam with 658 nm wavelength.

Usually the stratum basale appear at a depth of approximately 50 μm to 100 μm below the stratum corneum and it is characterized by the presence of melanocytes, extremely refractive cells that appear bright in the RCM images due to the high refractive index of melanin. Typically basal cells, diameter of about 7 to 12 μm, appear as bright, round or oval shapes is RCM images. Basal cells form a cobblestone pattern.

1.3.3 Electrical Impedance

According to an electric point of view skin can be characterized as a parallel RC circuit composed of a resistor, R_s in series with the parallel combination of a resistor R_{sc} and a capacitor C_{sc}. Where R_s represents the resistance associated with deeper tissues. R_{sc} and C_{sc} represent the properties of the stratum corneum, as presented on the scheme of figure 1.9.

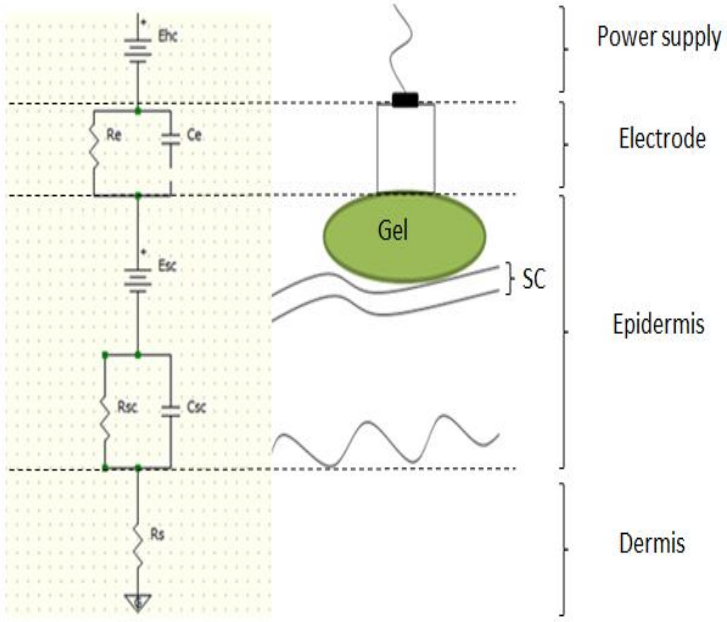

Figure 1.9: Electrical model representing the skin, gel and the electrode.

The electrical connection between an electrode and the skin was modeled by Meziane at al. [39]. The gel establishes the contact between the electrode and the skin. The potential difference *Esc* is related with the semi-permeability of the stratum corneum to the ions flow, which can be expressed by the Nernst Equation.

The fluid secreted by sweat glands contains ions with a different concentration of the extracellular fluid. This produces an electric potential between the lumen of the sweat duct and the dermis and subcutaneous layers.

Electrical impedance is a measure of the materials opposition to the flow of alternating electric currents of various frequencies. Impedance at low frequencies, in the range of 1Hz to 1MHz, is related to the electrical properties of the extracellular environments, whereas impedance at high frequencies is related with the electrical properties of the intra and extracellular environments and the capacitive properties of the cell membranes.

The epidermis plays the most important role in the electrode-skin interface, once the skin impedance is greatly associated with the stratum corneum; if this layer was abraded the impedance will be strongly reduced. In order to make

the skin surface more uniform, a thin layer of Tripolar®RF gel is applied to the stratum corneum. The presence of the gel allows a better penetration of the current into the pores of the stratum corneum, which becomes more conductive.

The radiofrequency system used in this thesis operates under a frequency of 1 MHz. This frequency allow: to deposit the power on the dermis, to measure impedance without concerning the capacitive properties of the skin and to study the migration of the interstitial fluid to the blister cavity, extracellular space.

The skin impedance is influenced by several factors such as: temperature, pressure of the electrode, cutaneous region, skin moisturization and viscosity, electric field mafnitude and gel used. Stronger electric field, high temperatures, moisturized skin facilitate the ion mobility and thus decrease the impedance on tissue impedance. According with Tregear at al.[51] the impedance measured, at low-frequencies on the skin surface it is influenced by the way in which the contact is made between the electrode and the skin surface. Also for low-frequency biopotentials, the resistance is the major source of the observed impedance changes. Based on this, we tested the hypothesis: blisters present lower impedance that the untreated skin. We supposed that the swelling of the stratum corneum and the presence of interstitial fluid inside the blister reduces the impedance. It is expected that the transmission of the radiofrequency through the tissue will be promoted by the fluid-enriched interface.

2

Background of the Study

2.1 Blister Formation and the Bistering Time

In the previous chapter the biophysical mechanisms of blister formation were described, in this chapter an overview about the blistering time dependence with the aperture diameter, suction pressure and temperature will be disclosed.

It is expected that, during the first moments of suction application a skin dome will be formed (figure 2.1 B). It is hypothesized that skin rejuvenation effects do not require a complete blister formation . If the treatment stops at stage C, formation of several smaller separated blisters, the epidermis will be damaged and detached enough to mechanisms of formation of new epidermis being activated. The fluid content of the blisters will be absorbed, new hemidesmosomes will be formed and they will attach the recently formed epidermis to the uninjured dermis. The new DEJ formed will present a wavy pattern (figure 2.1 E). However if the blisters formed at stage C were too small, then the dermis and epidermis can attach again without formation of new epidermis.

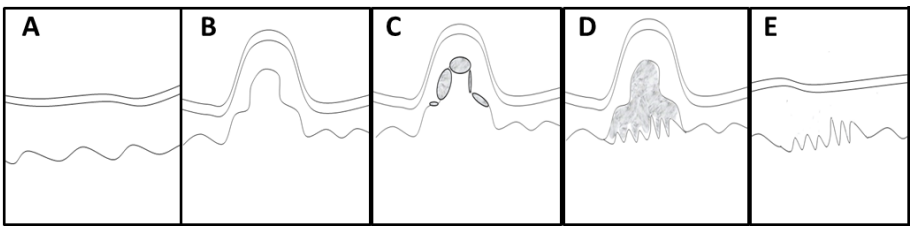

Figure 2.1: Schematic representation of the hypothesis: A- Untreated Skin; B- Beginning of the application of the suction pressure device; C-Formation of blisters with fluid content; D-Full blister formed by coalescence of smaller blisters and E- Rejuvenated Skin.

2.2 Factors that Influence the Blistering Time

2.2.1 Aperture Diameter

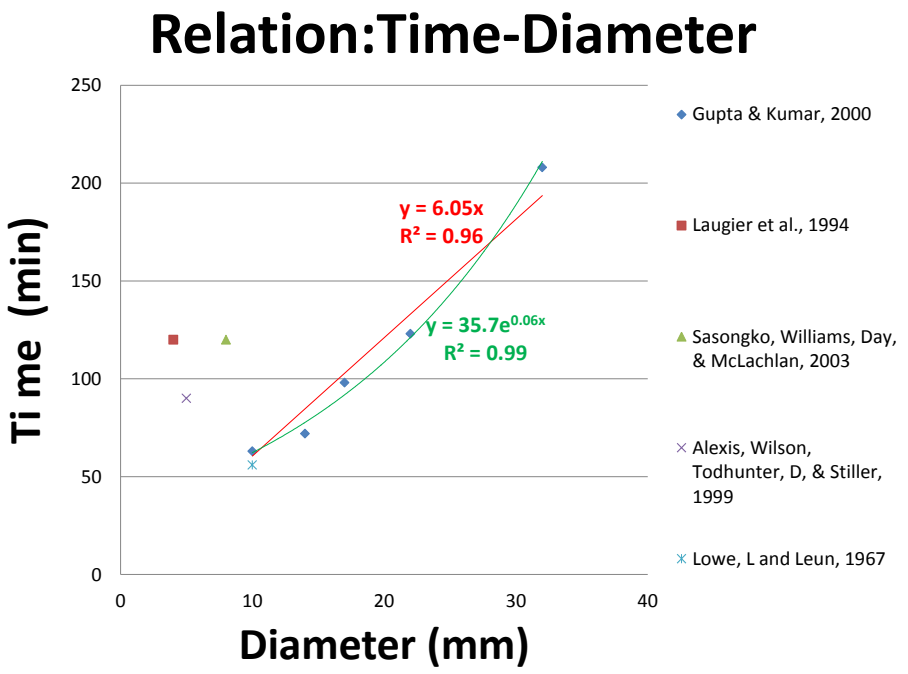

Figure 2.2: Relationship between suction blister induction time and the diameter of the blister, obtained for a constant pressure of 300 mmHg. The temperature of the skin surface during the experiments it was not reported. The red line represents the fit presented by Gupta et al [22], the green curve represents the fit that we think that decribes more accurately the diameter blistering time relation.

The relationship between aperture diameter and the blistering time was obtained by summarizing the results of five studies. The graphic, figure 2.2, shows a direct proportionality relationship between the suction blister induction time and the diameter of the blister (red regression line). However for diameters smaller than 1.4 cm, the slope is attenuated, being not evident what is the relationship between the suction blister induction time and the diameter of the blister for these range of diameters [22]. An exponential relation between the blistering time and the diameter of the suction aperture ($R^2 = 0.99$) also shows best fit to the data than a linear regression ($R^2 = 0.96$). A linear fit suggests that for a very small diameter the blistering time will approach zero, which is not possible due to the necessity of a minimum time interval for biological processes take place. Also for very small diameteres, there are more strecthing forces applied to the skin area, difficulting the skin dome formation and blister-

ing. An exponential fit seems to be more appropriate once it takes into account that there is a minimum time interval since the application of suction until blistering. Based on the studies of [34],[45] and [6] , which used diameters below 1 cm, it is not possible to confirm either the exponential or linear relation between the blistering time and the blister diameter.

Leun et al [34] stated that within the range of few centimeters, (0.5 to 2 cm) the diameter of the suction orifice does not appreciably influence blistering time. Also previous studies which used a small suction diameter (3 mm) showed no significant difference in blistering time regarding orifices within a range between 3-20 mm. It is possible that different authors have used different methods and the blister formation may have occurred in dissimilar conditions, regarding temperature, moisture and body site. These factors can influence drastically the blistering time, they will be described later in this chapter. Also the thickness and resiliency of the skin region selected can affect the blistering time.

Small suction orifices present the advantage of providing a good contact with the skin. Only a slight distention of the skin, when the suction pressure is being applied, is verified [42]. Moreover Kiistala et al. [28] stated that the regeneration process of a suction blister depends on its size. Blisters with small diameters, less than one millimeter, tend to disappear within some minutes. On the other hand, larger blisters require a few days to one week to heal.

In order to understand how the blistering time is related with small aperture diameters it is necessary to reproduce previous cited studies maintaining constant environmental conditions such as temperature, humidity and body site. Futhermore it will be necessary to accomplish and study blister formation for diameters smaller than 2 mm.

2.2.2 Suction Pressure

Blistering time is highly reduced with the increasing of the suction pressure applied.

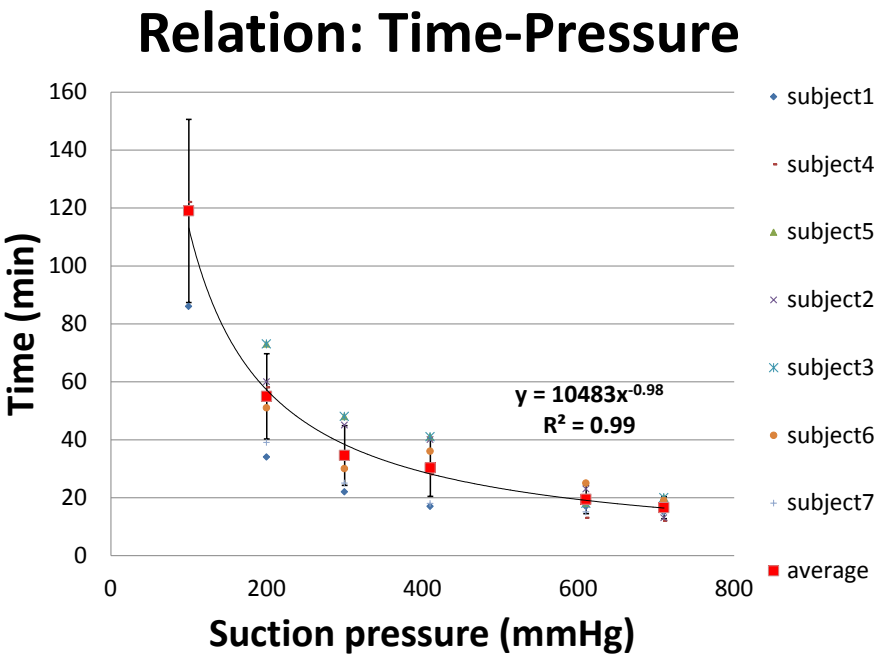

Figure 2.3: Representation of the relation between the blistering time and the suction pressure obtained for 7 subjects with a suction aperture of 1 cm. The temperature of the skin surface during the experiments it was not reported.[37]

The results of Lowe at al. [37] (figure 2.3) showed a power fit between blistering time and suction pressure. As the power coefficient β, calculated based on the average of the subjects is approximately -1, which describes a straight line under a slope of -45°, it is possible to approximate within a certain limit of error the relation between suction pressure and blistering time to the expression: [37].

$$A = P * t_b \tag{2.1}$$

Where $\boldsymbol{p}$ is the suction pressure, t_b the blistering time and $\boldsymbol{A}$ a constant. Each experimental test gives a value for a. The mean of the observational values for human male lower abdominal skin, showed on figure 2.3, is

$$A = 10483(mmHg.min) \tag{2.2}$$

The constant, $\boldsymbol{A}$ can be expressed in units of viscosity and may be interpreted as the skin resistance against the blister formation forces [37]. Previous studies concluded that for the formation of blisters with large diameters (>1 cm), low values of suction pressure (200 to 300 mmHg) favor blistering, while high pressures usually results in bruising and failure of blister formation. To induce blister with a diameter smaller than 1 cm, higher pressures are required [22]. However the implementation of high suction pressures, above 680 mmHg, can cause infiltration of inflammatory cell of the tissue, immediately after blistering and during the healing period [40].

According to the model of dermal epidermal adhesion, anchoring fibrils are assumed to have the property of extending at a steady rate under a constant stress and tend to break if the viscous slip has caused sufficient elongation. The dermal epidermal separation will occur only when the suction pressure applied exceeds a critical value of elongation. This critical value varies from one cell to cell. Below that critical value, no blistering will occur even during a long exposure time [37].

2.2.3 Skin Temperature

Blistering time decreases rapidly and continuously with the increase of temperature. Which sustains the hypothesis that adherence is an exponential function of temperature. The relation between blistering time and temperature can be approximated to be an exponential function, described by [33]:

$$t_b = C * e^{-\beta T} \tag{2.3}$$

Where C is a constant determined by the suction pressure and the adherence of the skin. β characterizes the effect of temperature on blistering time and T represents the temperature value.

According Leun et al.[33] these relationship can be simplified and it can be assumed that the adherence of the DEJ is directly proportional to the temperature.

$$\frac{t_b\,(T)}{t_b\,(T_0)} = \beta * \Delta T \tag{2.4}$$

In DEJ separation by suction, blistering time may be shortened significantly by increasing the skin temperature as high as reasonably accepted. Application of moist heat to the surrounding skin during the blistering process can reduce substantially the blistering time. The optimal temperature in the suction area is about 40°C to 50°C [23]. In excised skin, the epidermis could be easily removed

from the dermis if the skin was heated at 50°C. However if temperatures of 50°C to 51°C were applied, for a sufficiently long time it could result to epidermis necrosis [7]. Previous studies showed that at 50°C the blistering time was so short that epidermis and dermis can be separated at this temperature without burning.

According Peachey et al.[42]. indirect heating or cooling, such as warm baths, where the blister formation system is not being applied, do not affect the speed of blistering, once it was demonstrated that alterations in skin blood flow related with vasodilation and vasoconstriction do not significantly affect the speed of blister formation.

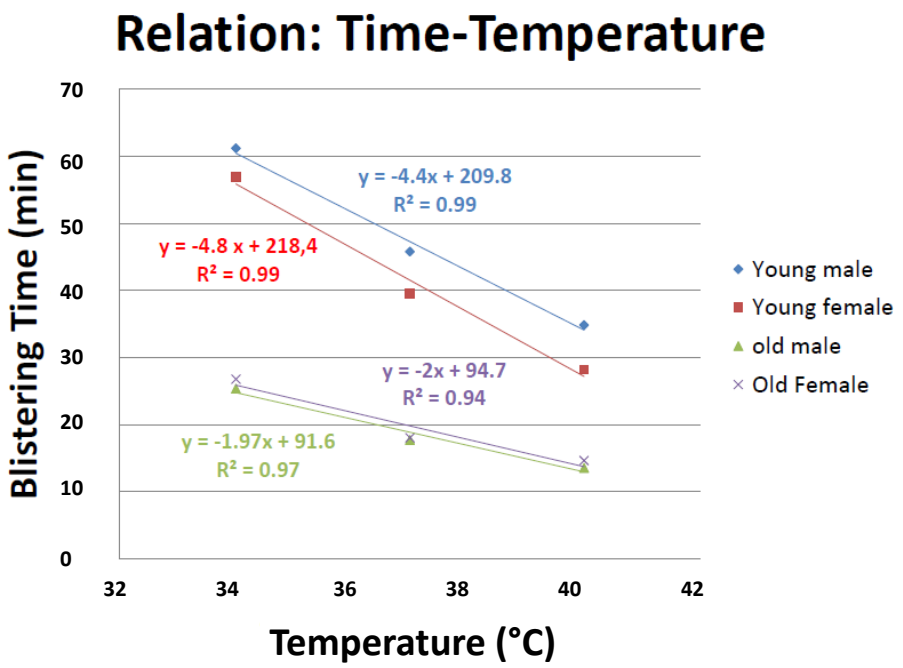

Figure 2.4: Relation between the blistering time and the temperature. During the experiment a 3 mm suction aperture was used.

The time needed to create a blister is correlated with age and gender of the subjects, as is represented in the figure 2.4. This study presents a clear difference, in the speed of blister formation between elderly and young persons. For the same pressure and temperature, the blistering time is longer for young persons than in old persons. The difference between the gender of the subjects is more evident in young persons, where it is necessary to apply a certain pressure during a longer time to male subjects in order to obtain the same results as female subjects. Regarding elderly persons the differences related with the

gender are insignificant.

Table 2.1: Influence of the temperature on blistering times.

Subject Group	$t_b(T)$ **(min)**	$t_b(T_0)$ **(min)**	$\Delta\mathbf{T}$	β (%)	$\frac{t_b(T_0)}{t_b(T)}$
Young Male	16	61	10	2.7	4
Young Female	11	57	10	2.0	5
Old Male	5	25	10	2.0	5
Old female	6	27	10	2.1	5

$t_b(T)$ is the blistering time after an increase of the skin temperature by $\mathbf{\Delta T}$ and $t_b(T_0)$ is the blistering time at skin temperature.

These results based on the data, from the report of Peachey et al.[42], are also in accordance with the conclusions obtained by Leun et al.[33] which stated that an increase of skin temperature by 10 degrees decreases blistering time by approximately a factor of 4, see table 2.1. An even stronger influence of temperature $\frac{t_{b,T}}{t_{b,(T+10)}} = 6.5$ was obtained by Kiistala et al.[29].

2.2.4 Other Factors that Influence Blistering

The blistering time can be influenced by other factors such as age and gender when variables as suction pressure, skin site, aperture diameter and skin temperature are standard. In general, blister formation is shorter in elderly persons than in young adults, and slightly faster in young females than in young males [42]. Age is an important factor in determining the rate of blister formation, once with the increasing age of the subject, the thickness of the skin is reduced and the dermal epidermal junction weakens which results in easier and faster separation of the DEJ by means of suction in older individuals.

The time required for blister formation is affected not only by the suction pressure and temperature but also by variations between subjects and different parts of the body [29]. Regarding the variations from one area of the body to another, it is estimated to be required approximately twice as long to produce a blister on the surface of the lower leg as on the surface of the lower lateral abdomen under the same conditions of pressure and temperature. However, according to the data provided from Tidman et al. [49] and Kakasheva-Mazenkovska et al. [27] there is no relation between the blistering in different body parts and the thickness of the epidermis.

Also some diseases can influence the blister formation, such as epidermolysis bullosa dystrophica recessive, pemphigus vulgaris, pemphigus erythematosus

and dermatitis herpetiformis. The patients with these diseases develop blisters with lower pressures than healthy skin [34]. Insulin-dependent diabetics need approximately half of the time that a healthy subject to generate a blister [10].

2.2.5 Key Learnings

In the previous subchapters it was described the blister formation dependence on four parameters: pressure, time, area and temperature. The product between pressure and time is constant, as described by the equation 2.1. In previous studies it was clear that higher the temperature lower the blistering time. Due to the ambiguity of the data related with the relation between the blistering time and diameter with respect to small blister diameters, in this thesis it is assumed that for diameters lower than 1 cm, the suction orifice does not appreciably influences blistering time.

Parameters To Use

It is believed that skin rejuvenation effects do not require a complete blister formation, but only the separation of dermis and epidermis. According to Beerens et al.[8] separation of the epidermis to dermis is a gradual process that involves progressive detachment of hemidesmosomes and basal cells from basement membrane. Considering t_b as the time required for a complete blister formation. At $t = \frac{1}{2}t_b$ basal cells begin to detach from the basement membrane and at $t = \frac{3}{4}t_b$ basal cells are completely separated from the basement membrane. It is possible to estimate that the dermal epidermal separation will occur between $t = \frac{1}{2}t_b$ and $t = \frac{3}{4}t_b$. Based on this assumption, it is feasible to assume that a time interval between 250 and 374 seconds will be sufficient to generate epidermal dermal separation in a skin heated at 40 degrees.

Attending the previously cited relations between the parameters and the blistering time, it will be necessary in average 3 minutes and 20 seconds for a complete blister formation, considering skin temperature of 44 degrees. A more realistic approach will be heat the skin until 40 degrees, in this case the blistering time will be approximately 8 minutes and 20 seconds.

The device comprises several orifices with diameters between 0.5 mm and 1.5 mm. A suction pressure of 600 mmHg should be applied through a suction system. The temperature of the area being treated should be between 38 and 40 degrees. A predefined treatment time should be in the range 250 and 374 seconds.

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