NOVEL AND ESTABLISHED TECHNIQUES FOR NON-INVASIVE SKIN ASSESSMENT

Observational *in vivo* studies on sensitive skin and skin irritation

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"Considerando in retrospettiva il mio lungo percorso, quello di coetanei e colleghi e delle giovani reclute che si sono affiancate a noi, credo di poter affermare che nella ricerca scientifica, né il grado di intelligenza né la capacità di eseguire e portare a termine il compito intrapreso, siano i fattori essenziali per la riuscita e la soddisfazione personale. Nell'una e nell'altra contano maggiormente la totale dedizione e il chiudere gli occhi davanti alle difficoltà: in tal modo possiamo affrontare problemi che altri, più critici e più acuti, non affronterebbero".

Rita Levi-Montalcini, *Elogio dell'imperfezione* (1987).

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ABBREVIATIONS

AD	Atopic dermatitis
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
CRS	Confocal Raman microspectroscopy
HE	Hematoxylin and eosin
HPA	Hypothalamic–pituitary–adrenal axis
LED	Light emitting diode
NMF	Natural moisturizing factor
NO	Nitric oxide
NSS	Non-sensitive skin
PASI	Psoriasis area and severity Index
RCM	Reflectance confocal microscopy
ROS	Reactive oxygen species
SCORAD	Scoring atopic dermatitis index
SS	Sensitive skin
TAP	Transdermal analyses patch
TEWL	Transepidermal water loss
TRP	Transient receptor potential
UV	Ultraviolet

Chapter 1

General introduction and thesis outline

1.1 STRUCTURE AND FUNCTIONS OF THE SKIN

The skin is the largest organ of the body, extending to an area of approximately 2 m^2 in adults [1]. Anatomically, the skin can be divided in three layers (Figure 1):

- *Epidermis*: a stratified, non-vascularized epithelium, with a thickness of 75-150 μm except on palms and soles where it can be up to 600 μm thick [1]. The epidermis consists primarily of keratinocytes (90-95%) [2], which proliferate as columnar-shaped cells from the innermost, single-cell layer called *stratum basale or germinativum* [1, 3]. Keratinocytes migrate towards the upper part of the epidermis undergoing a process of differentiation, in which they change their morphology and cell content, and express epidermal differentiation proteins (keratins) [1, 3, 4]. In this process, keratinocytes are first part of the *stratum spinosum* (8-10 cell layers), in which they appear as poligonal-shaped cells, and subsequently of the *stratum granulosum* (3–5 cell layers), in which they appear as granule-rich flatter cells where the nucleus and other organelles begin to degenerate [1, 3]. The final product of this process is the *stratum corneum* (15-30 cell layers), composed of non-viable but biochemically active, keratin-filled cells called corneocytes [1]. Corneocytes are eventually shed from the skin surface, a process called desquamation [4]. In normal skin, the journey from the basal layer to the shedding of corneocytes takes approximately 30 days to complete [1].
- *Dermis*: a connective tissue, usually less than 2 mm thick, separated from the epidermis by a basement membrane [1]. The main cell type is the fibroblast [1], which produces the principal structural components of the dermis: collagen fibers, which confer strength and support to the skin, and elastin fibers, which contribute to elasticity and resilience [2]. The dermis houses also appendages (including sweat glands, eccrine glands and pilosebaceous units), blood vessels, and nerves [1].
- *Sub-cutis or hypodermis*: a fatty connective tissue that connects the dermis to underlying skeletal components [1].

The major function of the skin is to form a barrier between the internal milieu and the hostile external environment [5], protecting against physical, chemical and microbial insults, as well as against the loss of water and electrolytes [1, 5]. This barrier function has been termed "la raison d'être of the epidermis" [6].

However, the skin is far beyond a mere physical barrier, since it displays additional features such as immune-competence, psycho-emotion reactivity, ultraviolet (UV) radiation sensing and endocrine functions [2]. For these reasons, the skin has also been termed "a brain on the outside" [1, 2].

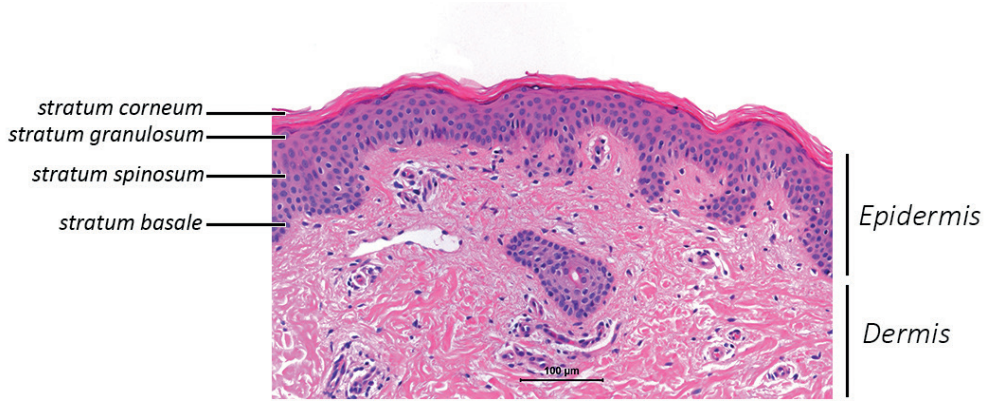

Figure 1. Hematoxylin and eosin staining of normal skin.

1.1.1 Barrier function: *la raison d'être of the epidermis*

The barrier function of the skin localizes primarily to the stratum corneum [2, 5, 7]. Its structure can be described as a "brick and mortar" model, in which corneocytes (bricks) are embedded in a lipid matrix (mortar) [1, 2, 7]. The latter is essential in providing the permeability barrier and is composed of ceramides (50%), cholesterol (25%), and free fatty acids (10-20%), with very little phospholipid [1, 2, 5, 7]. Lipids are delivered in the extracellular spaces of the stratum corneum at the level of the stratum granulosum by organelles contained in keratinocytes, called lamellar bodies [5]. In the matrix, lipids are organized in bilayers (lamellae) between the corneocytes, while within the lamellae they can be packed in three phases, as schematically shown in Figure 2. Different models have been proposed for the organization of lipids in the matrix [8], and evidence is increasing about the involvement of an altered composition as well as organization of lipids in skin diseases characterized by an impaired skin barrier function [4]. The multiple layers of corneocytes contribute to a tough and resilient framework for the lipid matrix [6]. The corneocytes are surrounded by a cornified envelope consisting of a densely cross-linked layer of proteins such as filaggrin, loricrin and involucrin [4]. On the exterior surface of the cornified envelope is a covalently bound layer of lipids, the lipid envelope [6]. The cornified and lipid envelopes minimize the uptake of most substances into the corneocytes and allow the proper formation of the lipid matrix. Indeed, a deficient lipid envelope results in an irregular lipid matrix and in a defective skin permeability function [4]. The corneocytes are filled with keratins and with a mixture of highly hygroscopic substances known as natural moisturizing factor (NMF) [6, 9]. NMF is mainly composed of amminoacids deriving from proteolysis of filaggrin, but it also comprises sweat-derived components [9]. The water-binding property of NMF contributes to the hydration of the stratum corneum, which is necessary for hydrolytic enzymatic processes, required for normal desquamation, to take place [6].

Of note, the barrier function of the skin is not absolute, but it allows a physiological movement of water through the stratum corneum and into the atmoshere [6]. This process is known as transepidermal water loss (TEWL).

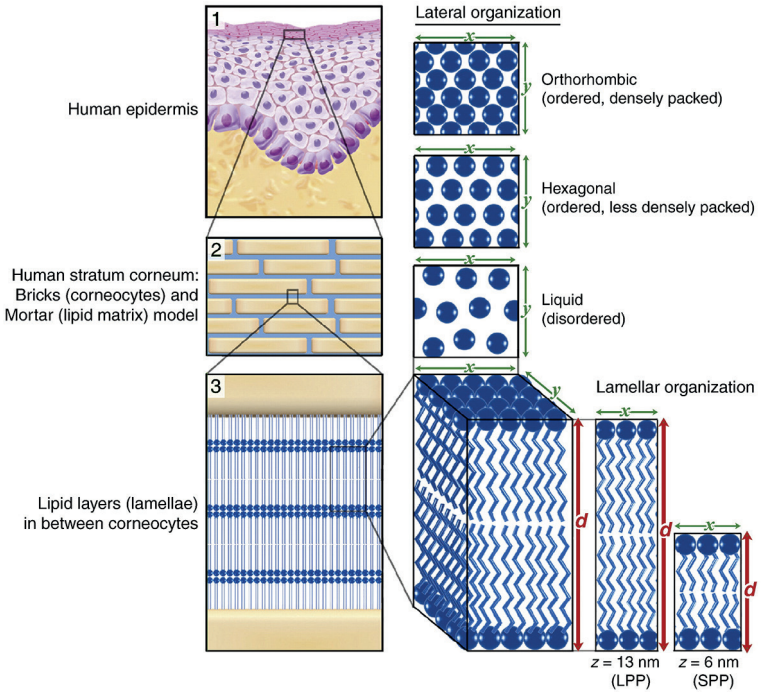

Figure 2. Schematic representation of the skin barrier. **1**) The skin barrier localizes primarily to the stratum corneum, a layer of dead cells (corneocytes) embedded in a lipid matrix. **2**) This structure is also referred to as the "brick" (corneocytes) and "mortar" (lipids) model. **3**) The intercellular lipids are arranged in layers (lamellae), with either a long or short repeat distance (d), referred to as the long periodicity phase (LPP, ~13 nm) and short periodicity phase (SPP, ~6 nm), respectively. The lateral organization is the plane perpendicular to the direction of the lamellar organization. Lipids can be arranged in three ways: a very dense, ordered orthorhombic organization; a less dense, ordered hexagonal organization; a disordered liquid organization. In normal skin, lipids are mainly organized in the orthorhombic phase. Reprinted from "The important role of stratum corneum lipids for the cutaneous barrier function", Biochim Biophys Acta vol. 1841 issue 3, van Smeden J *et al.*, p. 295-313, Copyright (2014), with permission from Elsevier.

1.1.2 Neuro-immune-endocrine function: *a brain on the outside*

The skin is a potent immune-competent tissue [2, 7]. In the last few years it has become clear that practically all cell types residing in and transiting through the skin can exhibit immune functionality [1].

Among the better characterized cellular components are keratinocytes and mast cells [1, 10]. Keratinocytes produce and secrete reactive oxygen species (ROS), antimicrobial peptides (AMPs), cytokines and chemokines [10]. Mediators are released upon keratinocyte activation due to an inflammatory stimulus, but can also be produced under normal conditions in order to maintain tissue homeostasis [10]. Mast cells, normally residing in the dermis, release upon activation a battery of bioactive mediators of both immune and neuroendocrine responses, among which histamine and tryptase [1].

The epidermis can be considered a true sensory tissue [11] and keratinocytes may be at the forefront of the sensory system [12].

It had long been considered that only free nerve terminals in the epidermis played a role in skin surface perception [12]. However, keratinocytes have been shown to express sensory proteins activated by various environmental factors including temperature, mechanical stress, osmotic pressure, and chemical stimuli. These sensory proteins allow transduction of stimuli in intracellular biochemical messages [11]. The transient receptor potential (TRP) and the purinergic families are among the most important sensory proteins [11, 12]. It is therefore speculated that stimuli are sensed by keratinocytes, then processed and transferred to the free nerve terminals [12]. However, the mechanism involved in signal transduction from keratinocytes to free nerve terminals remains to be explored [11, 12].

The skin is capable to act as a neuroendocrine organ in the periphery [1], and has a remarkable stress-sensing capacity [2].

The skin and its appendages have been identified as both a source and target of neuro-transmitters, neuro-hormones and neuro–peptides, thought previously to be the domain of the central nervous system [1, 13]. An example is constituted by the perception of stress: upon the occurrence of psychological stress, the central nervous system activates the hypothalamic–pituitary–adrenal axis (HPA), with subsequent upregulation of key stress hormones believed to have an effect on the skin by triggering or aggravating inflammatory skin diseases. Evidence has shown that a peripheral equivalent of the HPA axis is present in the skin, since, upon environmental stressors such as heat, cold and UV radiation, the same key stress hormones, locally-produced, are released. The skin stress response may be signaled to the brain, where it affects behaviour and leads to an increased vulnerability to additional stress perception [13]. In addition, the skin has receptors for several sex steroid hormones, including oestrogen, androgen and progesterone, and is capable of steroidogenesis [1, 14].

The skin neuro-, immune- and endocrine functions are not disjoint but intermingled through interaction with multiple pro- and anti-inflammatory neuropeptides, cytokines and hormones [14].

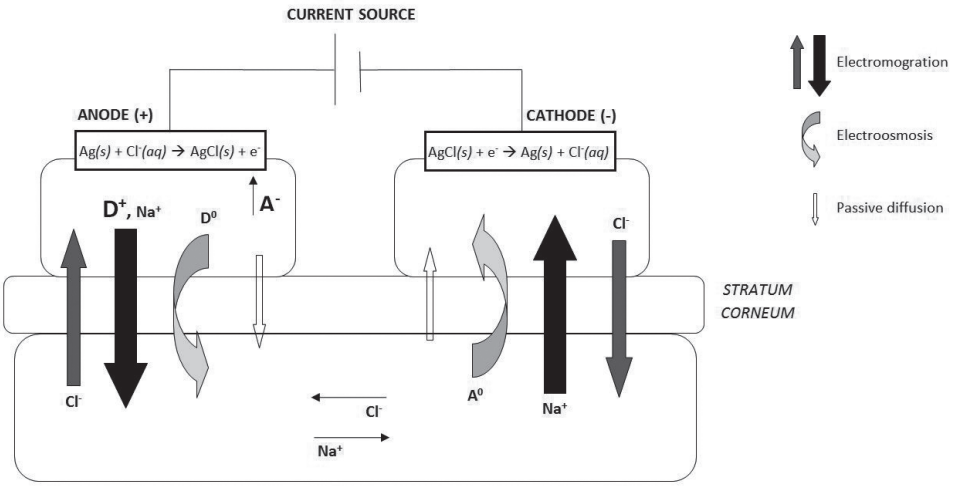

Figure 3. Principle of iontophoresis using an Ag/AgCl electrode system. An iontophoretic system consists of a source of electric current, an electrode containing a reservoir and an indifferent electrode. The formulation containing the ionized molecule of interest (*e.g.* D^+A^-) and saline (NaCl) is placed in the reservoir of the electrode bearing the same charge. Upon application of an eletric current, the ionized molecules are forced into the skin by electrorepulsion (electromigration). To maintain electroneutrality, a ionix flux in the anode-to-cathode direction is set in motion in the skin, which acts as a negatively charged membrane at physiological pH (electroosmosis). This flux, responsible for carrying uncharged molecules (D^0, A^0) and high-molecular-weight cations, is generated primarily by endogenous Na^+ and Cl^-, being saline the principal extracellular electrolyte. Electron fluxes are transformed into ionic fluxes via electrochemical reactions at the electrodes. The electrochemistry occurring at the Ag anode requires the presence of Cl^- ions in the reservoir: this is usually obtained by adding saline or, when available, by using a hydrochloride salt in the formulation. The reduction occurring at the cathode by the arrival of electrons from the power supply releases Cl^- ions; for electroneutrality, this must be compensated for by the arrival of cations from the skin or by the loss of anions. The arrows indicate the contribution of each transport mechanism to the iontophoretic flux: electromigration, electroosmosis, and passive diffusion. When small and hydrophilic ions are used, the efficiency of electromigration is much higher than that of electroosmosis and passive diffusion. Modified from "Iontophoretic drug delivery", Adv Drug Deliv Rev vol. 56 issue 5, Kalia YN *et al.*, p. 619-658, Copyright (2004), with permission from Elsevier, and from "Diagnostic and therapeutic applications of iontophoresis", J Drug Target vol. 17 issue 9, Sieg A *et al.*, p. 690-700, Copyright (2009), with permission from Taylor & Francis Group (www.tandfonline.com).

1.2 *IN VIVO* SKIN MODELS

Given the complexity of skin structure and functions, studying the mechanisms regulating skin homeostasis and underlying skin diseases can be challeging. In order to investigate one specific process or cell type, *in vivo* skin models can be used. These constist in the infliction of a minimally-invasive and standardized challenge to the skin, and in the evaluation of cutaneous growth, differentiation and inflammation at one or several points in time. While *in vivo* models provide a simplification of a complex situation, they allow to retain the interplays and cross-talks among the different cells and mediators in the skin. In this thesis, we focused on two such *in vivo* models: tape stripping and histamine iontophoresis.

1.2.1 Tape stripping

Tape stripping consists in the repetitive application of adhesive tape to remove corneocytes from the stratum corneum. This procedure is used to abrogate, totally or partially, the barrier function of the skin without inducing cytopathic effects on the underlying epidermal keratinocytes [15]. Total abrogation can be assessed clinically with the appearance of a red and homogeneously glistening surface characterizing the viable epidermis. Since its introduction in the 1950s [16], tape stripping has become an established model of acute as well as chronic barrier disruption to investigate homeostatic repair responses [15, 17-19].

Although this method appears simple and easy to perform, several parameters have been shown to influence the outcome, including the type of tape and its rapid or slow removal, the pressure, the duration of pressure application, the anatomical site, and the condition of the skin before stripping [20, 21]. It is therefore important to standardize the procedure and to specify these parameters in order to obtain results which are reproducible and comparable between studies [20, 21].

1.2.2 Histamine iontophoresis

Iontophoresis is a technique which uses a small and defined electric current to facilitate the transport of charged and uncharged molecules across the skin [22]. Charged molecules are driven into the skin under the direct influence of an electric field, a process called electromigration [22-24]. Uncharged molecules are carried by the electroosmotic flow generated by the movement of cations in the anode-to-cathode direction across the skin [22-24]. The contribution of electromigration and electroosmosis to the total iontophoretic flux depends on the physicochemical properties of the molecules being transported [22, 23], the former prevailing for small ions, the second for uncharged molecules and high-molecular-weight cations. Although iontophoresis cannot entirely avoid variability in passive diffusion, the delivery of molecules is less dependent on the condition of the skin [22]. The principle of iontophoresis is described in detail in Figure 3.

Histamine is a well-known pruritogen and vasoactive substance [25]. The delivery of histamine in salt form (*e.g.* histamine dihydrochloride) to the skin via iontophoresis has been extensively performed in the last decades. Histamine iontophoresis is considered to be a quantifiable model for the study of inflammatory skin responses and microcirculation [24, 26], and it has been used to study differences in itch perception and vascular response between subjects with inflammatory skin diseases and controls [27, 28], the effects of treatments to relieve itch [29, 30] and the effects of psychological conditions on itch perception [31, 32].

1.3 INVASIVE AND NON-INVASIVE SKIN ASSESSMENTS IN DERMATOLOGY AND IN COSMETIC SCIENCES

In dermatology, the gold standard to analyse skin physiology and pathology is the histological and immunohistochemical analysis of biopsies. This procedure involves surgical removal of a skin sample following local anesthesia. After undergoing a preparatory processing, the specimens are stained with dyes to label specific cells, cellular components or structures. In histochemistry, for example, labeling is achieved by a chemical reaction between chemicals in the dye and components in the tissue, as in the hematoxylin and eosin (HE) staining (Figure 1). In immunohistochemistry, labeling is obtained by the binding of antibodies in the dye with antigens in the tissue. Examination is carried out by microcopic analysis. While offering unrivalled sensitive and specific analysis of cells and tissue morphology, taking biopsies is invasive and patients may experience discomfort during the procedure.

Another widely used method to analyze skin status in dermatology is visual assessment. Being easily accessible, dermatologists can rely on their visual grading of clinical signs to evaluate the type and severity of skin diseases as well as the efficacy of treatments. This has led to the definition of several scores for the severity of skin diseases based on visual grading of clinical signs such as erythema, dryness and swelling. Examples include the Psoriasis Area and Severity Index (PASI) and the Scoring Atopic Dermatitis Index (SCORAD) [33, 34]. Despite their reliability when performed by trained physicians, justifying their use in clinical trials, visual assessments remain subjective and, most importantly, cannot appreciate the processes unfolding below the surface of the skin.

Over the last four decades, much (cosmetic) research has focused on the development of a third approach to assess skin status, represented by bioengineering techniques [35, 36]. These techniques provide objective and non-invasive biophysical or optical measurements of skin properties. For example, an indirect estimate of the hydration of the stratum corneum can be obtained by measurements of conductance or capacitance on the skin surface [37]. The integrity of the barrier function can be indirectly evaluated by measurement of TEWL, with higher TEWL indicating an impaired skin barrier [37]. Other techniques are based on the interaction of light with various skin structures and on the subsequent measurement

of the exiting photons. Raman microspectroscopy, for instance, exploits inelastic scattering to measure the biochemical composition of the skin [37]. Reflectance confocal microscopy (RCM) uses the different refractive indexes between the cell structures and the surrounding tissue to provide images with morphological information at a resolution comparable to that of conventional light microscopy [38, 39]. Within bioengineering techniques it is possible to include also digital image processing of high-resolution skin photographs, used to enhance, segment and quantify specific features [40]. A selection of bioengineering techniques releveant for this thesis is reported in Table 1, and illustrations are shown in Figure 4. A more exhaustive list of bioengineering techniques can be found in dedicated books [41].

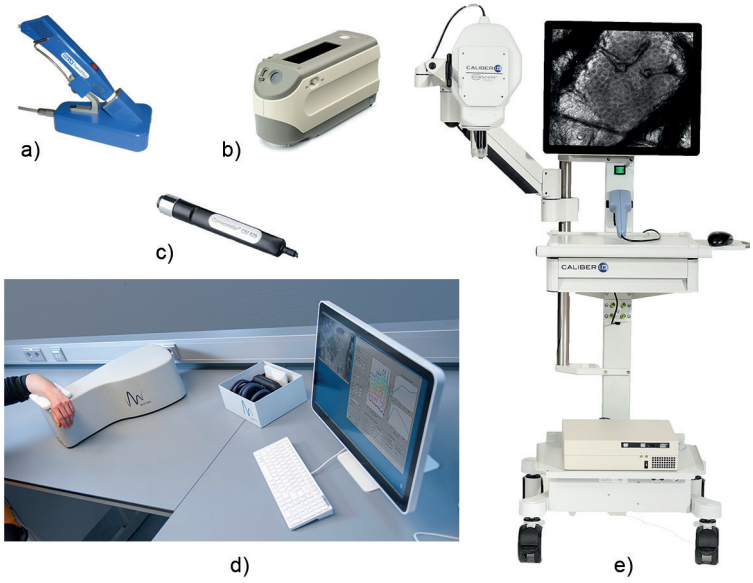

Figure 4. Bioengineering techniques for *in vivo* and non-invasive skin assessments. **a**) Aquaflux AF200 (Biox, UK) for measurement of TEWL; **b**) Spectrophotometer 2600d (Konica Minolta, Japan) for measurement of skin color; **c**) Corneometer CM825 (Courage + Khazaka, Germany) for indirect measurement of stratum corneum hydration; **d**) gen2 Skin Composition Analyzer (RiverD International B.V., the Netherlands) for direct measurement of stratum corneum biochemical composition based on the principle of confocal Raman microspectroscopy; **e**) VivaScope 1500 (Lucid Inc., USA) for imaging of skin morphology based on the principle of reflectance confocal microscopy.

Given their non-invasiveness, bioengineering techniques are invaluable in cosmetic sciences, where the development of new products requires tests on human volunteers [35, 42]. This is even more so since the entry into force of the new European Cosmetics Regulation (1223/2009)

in 2013, which banned animal testing (art. 18) and introduced more stringent requirements for substantiation of claims about the efficacy and effects of cosmetics (art. 20) [35, 42]. On the other hand, bioengineering techniques have not had a great impact in dermatological practice yet [36], with an exception for RCM, on its way towards implementation in clinical practice [43], and dermoscopy, which continues to gain appreciation in general dermatology [44]. The late Professor Albert M. Kligman, one of the greatest exponents of experimental dermatology, was among the first and major advocates of the use of bioengineering techniques in dermatological practice [36]. He warned against relying solely on visual assessment, since even clinically normal-appearing skin could hide abnormal changes, a phenomenon he called "invisible dermatoses" [45]. Unfortunately, bioengineering techniques have their limitations. In addition to their cost, which in the case of top-notch techniques can be extremely high, they can measure only one or a few parameters in the very complex environment which is the skin, and some of them are strongly influenced by external factors such as temperature and humidity [36, 37, 42]. As a consequence, it is frequently necessary to combine more than one method to obtain an overall clinical picture [42] and to follow guidelines to obtain reliable and reproducible measurements [46-50]. Besides careful measurements, attention must be paid to other aspects of the experiment, from the design of the study protocol, to the selection of volunteers and to the analysis and interpretation of the results, as all these aspects might hamper the meaningfulness of the study [35]. Professor Kligman was well aware of these aspects; as he notably remarked, "a fool with a tool is still a fool" [36].

Table 1. Description of bioengineering techniques relevant for this thesis.

	Measurement principle	Output	Measurement depth	Advantages	Limitations	Ref
		BARRIER FUNCTION				
Transepidermal water loss	A measurement chamber is applied on the skin surface; the extremity in contact with the skin is open, the opposite extremity can be open or closed. The chamber contains sensors of relative humidity and temperature. In the open-chamber and closed condenser-chamber methods, the flux of water vapor from the skin surface is calculated from the humidity gradient between the chamber extremities. In the unventilated closed-chamber method, the flux is calculated from the rate of increase of the humidity and temperature readings.	Flux density of water vapor [g/m^2h]	n.a.	Easy-to-use Small-sized probes for measurement in recessed body parts Relatively inexpensive	Indirect measurement Influenced by environment	[50, 51]
Electrical methods	Electrical properties of the SC (mostly capacitance and conductance), dependent on the hydration status, are measured with probes placed in contact with the skin. Capacitance-based instruments apply an oscillating electric field to measure the dielectric constant of the SC. Conductance-based instruments apply an electric currrent to measure the conductance of the SC.	Estimate of the hydration of the SC expressed in abitrary units [a.u.] or conductance [µS]	- 45 µm (capacitance) - 15 µm (conductance)	Easy-to-use Small-sized probes for measurement in recessed body parts Relatively inexpensive	Indirect measurement Influenced by environment	[46, 52]
Confocal Raman microspectroscopy	Monochromatic laser light is focused in the skin. A tiny proportion of photons undergoes inelastic (Raman) scattering with the endogenous molecules, releasing some energy. Of the photons that exit the skin, only the ones coming from the focus region are detected, thanks to the presence of a confocal pinhole. The photons which underwent frequency shifts due to the release of energy to molecules during the interaction are used to obtain (Raman) spectra. The position and intensity of each peak in the spectra are representative of the different molecules and their amounts.	Direct measurement of water, NMF, lipids, carotenoids and exogenously applied compounds + Estimate of SC thickness [µm]	200-500 µm	High spatial and temporal resolution (lateral: 1 µm, axial: 3-5 µm; 1-3 s/spectrum) High biochemical specificity	Expensive Needs trained personnel for interpretation of spectra Bulky set-up not suitable for measurements in recessed body parts	[53-55]

	Measurement principle	Output	Measurement depth	Advantages	Limitations	Ref
		GREASINESS				
Sebumeter©	A probe containing an opaque plastic strip is pressed on the skin for 30 s. As lipids accumulate on its surface, the tape becomes transparent. Transparency is measured by transmitting light through the tape. The light transmission represents the sebum content.	Sebum content on the skin surface expressed in abitrary units [a.u.]	n.a.	Easy-to-use Small-sized probes for measurement in recessed body parts Relatively inexpensive	Influenced by environment	[49]
		SKIN COLOR/ERYTHEMA				
Reflectance spectrophotometry	LED light at two (*e.g. DermaSpectrometer©*) or three (*e.g. Mexameter©*) wavelengths or corresponding to the full visible spectrum (*e.g. Dermacatch©*) is emitted by a probe placed on the skin. The reflected light is detected and used to calculate the light absorbed by hemoglobin and melanin according to predefined formulae.	Erythema index [a.u.] Melanin index [a.u.]	n.a.	Easy-to-use Small-sized probes for measurement in recessed body parts Relatively inexpensive	Influenced by environment No information on extent of erythema or on perceived skin color	[48, 56-59]
Tristimulus colorimetry	LED light corresponding to the full visible spectrum (*e.g. Colorimeter©*) or light from a xenon lamp (e.g. *Chromameter©*) is emitted by a probe placed on the skin. The reflected light is detected and filtered according to the CIE standard observer curves centered in the blue, green and red. L*, a* and b* values are derived from the curves according to predefined formulae.	Color expressed in the L*a*b* CIE color space [a.u.]	n.a.	Easy-to-use Small/medium-sized probes for measurement in recessed body parts Relatively inexpensive	Influenced by environment No information on extent of erythema or on molecular origin of skin color	[48, 56-59]
Diffuse reflectance spectroscopy	Light from a xenon lamp (*e.g. Spectrophotometer CM©)* is emitted by a probe placed on the skin. The L*a*b* values are derived and the addition of a spectrometer allows the measurement of the reflectance spectrum in the 400–700 nm range.	Color expressed in the L*a*b* CIE color space [a.u.] + Reflectance spectrum	n.a.	Easy-to-use Small/medium-sized probes for measurement in recessed body parts	Influenced by environment No information on extent of erythema	[59]

SKIN MORPHOLOGY						
Reflectance confocal microscopy	Monochromatic laser light is focused in the skin. The photons are reflected according to the different refractive indexes of the cells and structures present in the skin. Of the photons that exit the skin, only the ones coming from the focus region are detected, thanks to the presence of a confocal pinhole. The photons which are reflected from the skin are used to obtain images, where skin structures with a higher refractive index appear bright *(e.g.* melanin, keratin) and structures with a lower refractive index appear dark (*e.g.* skin folds).	E*n face* binary images showing skin morphology	200-300 µm	High spatial and temporal resolution (lateral: 0.5-1 µm, axial: 2-5 µm) Real-time imaging Video mode (15-25 frames/s Medium-sized probes for measurement in recessed body parts	Expensive Needs trained personnel for interpretation of images	[38, 39]

a.u.: arbitrary units; CIE: Commission Internationale de l'Éclairage; LED: light emitting diode; n.a.: not applicable; NMF: natural moisturizing factor; SC: stratum corneum.

1.4 SENSITIVE SKIN

Sensitive skin (SS) has gained increasing attention in the cosmetic sciences and dermatology, as demonstrated by the number of publications in scientific journals emerged in the last decades (Figure 5). However, despite extensive research, the understanding of this phenomenon remains elusive. The currently accepted definition of SS is a condition of subjective cutaneous hyper-reactivity to various factors which are not commonly considered irritants [60]. Sensory reactions experienced by subjects characterizing their skin as sensitive include itching, stinging, burning, and pricking; at the same time, objective signs of skin irritation such as redness and dryiness are often absent [60-63]. Factors triggering SS are diverse; they range from contact exposure to chemicals (*e.g.* soaps, parfumes, household cleansers, cosmetics) to environmental insults (*e.g.* wind, cold, heat, UV radiation, temperature changes, pollution) and endogenous insults (*e.g.* stress, hormonal fluctuations) [60, 63, 64]. The frequent lack of objective signs of skin irritation and the heterogeneity in symptoms and triggers are the factors which contributed to the difficulty in fully understanding SS [60-64].

Much of the knowledge on symptoms and triggers of SS has been gained from survey-based epidemiological studies in various industrialized countries (Table 2). In these studies, classification of self-reported SS was mainly made via a four-point scale [65-75], in which the classes, albeit not universally defined, can be distinguished in:

(I) Very sensitive skin, strongly sensitive skin or "I strongly agree with having a sensitive skin";

(II) Sensitive skin, moderately sensitive skin, rather sensitive skin or "I somewhat agree with having a sensitive skin";

(III) Not very sensitive, slightly sensitive, somewhat sensitive skin or "I somewhat disagree with having a sensitive skin";

(IV) Not sensitive at all, not sensitive or "I strongly disagree with having a sensitive skin".

Remarkably, a high prevalence of self-assessed SS across different countries emerged, with approximately 50-60% of women and 30-40% of men declaring to have this condition. For this reason, SS has been indicated as a global challenge [76]. In Table 2 it can be observed that female gender, fair skin phototype, dry skin type, presence of skin diseases and atopic diathesis are most frequently associated with the likelihood of reporting SS. Most studies found that responders claiming their skin as sensitive experience cutaneous reactions to several triggering factors, contrary to responders defining their skin as non-sensitive (NSS). It is recognized that cultural factors and advertisement of products for SS play likely a role in the propensity to report this condition, which could partly explain differences in prevalence among different countries [70, 71]. For example, the lower prevalence found in China was attributed to the low familiarity with or a different interpretation of the term "sensitive skin" [70, 77].

Nevertheless, the overall similarities in prevalence, symptoms and triggers across countries indicate that SS is a true dermatological condition [62].

Besides epidemiological studies, clinical studies have been widely performed with the aim to link self-assessed SS to underlying pathomechanisms. In these studies, quantitative sensory testing methods are generally used to identify subjects with SS [60, 61]. These tests consist in the application of chemicals or other provocative agents to the skin in order to elicit specific cutaneous reactions (*e.g.* itching, stinging, vasodilation); cutaneous reactions are then assessed subjectively by the test subjects and, possibly, objectively by bioengineering techniques [60, 61]. The underlying hypothesis of these methods is that the pathomechanisms of SS might imply an impaired skin barrier function, leading to increased penetration of chemicals, and an increased reactivity of the cutaneous nervous system [60, 63, 64]. To date, however, no quantitative sensory test has emerged to identify subjects with self-assessed SS with sufficient sensitivity and specificity [60, 61, 63]. It has been found that susceptibility to one provocative agent does not predict susceptibility to another [78]; in addition, also subjects describing themselves as having NSS can develop objective signs of skin irritation. As a consequence, evidence from clinical studies has been largely inconclusive, hampering a universal agreement on the definition and pathomechanisms of SS [62]. Authors have emphasized the need to establish more rigorous methodologies for estimating the pathomechanisms of SS [62], and to develop cost-effective, reproducible and minimally invasive metodologies to clarify the correlation between subjective perceptions and objective indications of cutaneous irritation [61].

An attemp in this direction has been recently proposed by Richters and coworkers.

As a first step, the authors performed a systematic review of the available literature on SS [79]. To be included, articles had to contain information about subjective perceptions of cutaneous irritation. The aims of the review were to:

(I) Overview the pathomechanisms possibly involved in SS;
(II) Critically appraise the quality and reliability of diagnostic tests for SS;
(III) Identify key reasons causing ambiguity around the SS phenomenon;
(IV) Formulate recommendations for future research.

The authors found that impaired skin barrier function, sensory hyper-reactivity, vascular responsiveness and atopic predisposition were the pathomechanisms most often associated with SS. They also confirmed that selection by means of sensory testing rarely led to significant differences between subjects with SS and NSS, as measured by established bioengineering techniques. Key reasons causing ambiguity in the understanding of SS were the high heterogeneity in the selection methods of study participants and in the provocations used to elicit skin reactions. In addition, many studies did not consider the possible confounder represented by the concomitant inclusion of healthy subjects and subjects with atopic diathesis or skin

diseases. For future research, the authors proposed that a better selection might be achieved by multifactorial questionnaires spanning a range of provocations of chemical, mechanical and environmental origin, since, in epidemiological studies, several factors have been reported to trigger SS. The combination of clinical, histological and top-notch biophysical measurements was also proposed for a breakthrough in the understanding of the SS phenomenon.

As a second step, Richters and coworkers performed a survey-based epidemiological study on a Dutch sample population to define symptoms and triggers which could discriminate subjects with self-assessed SS from NSS subjects [74]. Triggers of different origin (*i.e.* toiletries, emotions, heat) and both subjective and objective symptoms of skin irritation (*i.e.* discomfort, stinging, dryness and erythema) were found to contribute to the discriminatory capacity of the model. Importantly, the same discriminative factors were found excluding responders with concomitant skin diseases, implying that SS is not defined by concomitant skin diseases only. The authors recommended therefore the exclusion of subjects with concomitant skin diseases from future studies, as symptoms inherent to these diseases overlap with the symptoms of SS and would therefore confound the outcomes.

In the third step, Richters and coworkers implemented the recommendations of the literature review in clinical studies, in which *in vivo* skin models were used to elicit a cutaneous inflammatory response [80-82]. Selection of participants with self-assessed SS and NSS, without concomitant skin diseases, was based on a multifactorial questionnaire designed from the model derived in the survey-based epidemiological study. Differences between the groups could be found at the clinical, immunohistochemical and biophysical level, indicating the discriminative power of the multifactorial questionnaire as a selection tool. These results show that novel insights into the pahtomechanisms of SS could be gained through a rigorous methodology, driven by a thorough knowledge of findings and gaps from previous relevant literature.

As a concluding remark, it is important to address the possible confusion arising when referring to the terms "sensitive skin" and "skin irritation". SS is, in fact, sometimes referred to as "irritable skin" [64], whereas the term "subjective irritation" has been used to describe the occurrence of cutaneous perceptions in the absence of clinical signs of irritation [62]. It is difficult to distinguish the two terms, as experiencing cutaneous perceptions or redness following external insults can be considered as having an irritable skin. However, it is also true that skin irritation can be experienced by subjects not defining their skin as sensitive, and that not every study on skin irritation focuses on SS [83]. For example, *in vivo* skin models can be used to irritate the skin by disruption of the epidermal barrier or by application of a surfactant, in order to study the cutaneous inflammatory processes (chapter 1.2). Studies using surfactants are especially widespread to identify individuals at risk of irritant contact dermatitis in occupational medicine [84]. In the following of this thesis, for clarity purposes, we will refer to "sensitive skin" when this is the self-reported assessment of an individual,

whereas we will refer to "skin irritation" when *in vivo* models are used to elicit cutaneous inflammatory reactions.

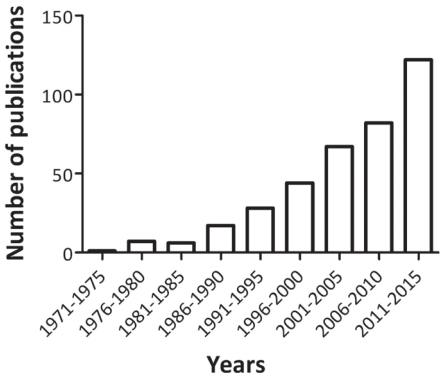

Figure 5. Publications in PubMed in which the term "sensitive skin" appears in the title or abstract.

1.5 PHOTOBIOMODULATION

Light-based therapies are widely used in dermatology and in cosmetic sciences [87]. Photobiomodulation is one such therapy based on the absorbance of non-ionizing light by endogenous photoreceptors to elicit non-thermal, biological reactions through photochemical events [88]. This process is reported to result in beneficial therapeutic outcomes including the alleviation of pain, immunomodulation, and promotion of wound healing and tissue regeneration [88, 89]. However, despite its reported beneficial therapeutic outcomes, two major challenges remain before photobiomodulation can find its way into the mainstream of medicine and dermatology [88-90]. The first challenge concerns the fact that the molecular mechanisms responsible for transducing light into clinical effects have not been established yet. At the present day, several photoreceptors have been discovered in the skin, including cytochtome c oxidase, flavin-containing cryptochromes, and opsins [91-93]. A generally accepted mechanism is that absorption of light by cytochtome c oxidase, an enzime located in the mitochondrial respiratory chain, would trigger the release of mediators, including nitric oxide (NO), adenosine triphosphate (ATP) and ROS, which in turn would activate gene transcription resulting in cellular processes such as proliferation and differentiation [89, 91]. However, the multiplicity of putative photoreceptors and effector pathways suggests that additional mechanisms or a combination thereof likely contribute to mediating light effects [92]. The second challenge pertains to the high number of light parameters which can determine a

Table 2 Survey-based epidemiological studies on the prevalence, symptoms and triggers of sensitive skin.

Country	n	%females	Body part where SS was assessed	%SS in females	%SS in males	Factors associated with increasing likelihood of SS		Ref
UK (2001)	2316	88.9	n.s.	51.4	38.2	Female gender History of atopic dermatitis, hay fever, asthma	Y Y	[85]
USA (2002)	811	100	face	52	-	Ethnicity	N	[68]
Italy (2005)	2101	88.5	n.s.	56.5	-	Female gender	Y	[86]
France (2006)	8522	59.5	face	61	32	Female gender Younger age	Y Y	[67]
France (2007)	1006 (March) 1001 (July)	-	n.s.	59.4 (March) 71.2 (July)	-	Female gender Summer season Fair phototype Depressive symptoms	Y Y Y N	[72]
France (2008)	400	100	face	85.4	-			[65]
			body	69.8	-			
Europe (2009)	4506	-	n.s.	49.4	37	Female gender Younger age Dermatological disease History of atopic dermatitis Dry skin Oily skin Rural/urban place of living Socio-professional category	Y N Y Y Y Y N N	[71]

USA (2009)	1039	83.6	overall	69.0	64.4	Female gender Younger age Ethnicity	N N N	[66]
			face	78.6	68.1			
			body	60.2	62.0			
			genital area	58.1	44.2	Female gender Younger ag Ethnicity	Y Y Y	
USA (2011)	994	50.2	face	50.9	38.2	Female gender Younger age Fair phototype Ethnicity Dermatological disease Dry skin Combined (dry and oily) skin Rural/urban place of living Socio-professional category	Y N Y N Y Y Y N N	[73]
China (2012)	408	100	overall	23.0	-	Younger age	N	[70]
China (2013)	9154	57.1	n.s.	15.9	8.6	Female gender Younger age Fair phototype Dermatological disease History of atopic dermatitis Dry skin Oily skin Climate place of living	Y Y Y Y Y Y Y Y	[77]

Country	n	%females	Body part where SS was assessed	%SS in females	%SS in males	Factors associated with increasing likelihood of SS		Ref
Japan (2013)	1500	51.8	face	56.0	52.8	Female gender Younger age Dermatological disease History of atopic dermatitis Dry skin Oily skin Combined (dry and oily) skin Socio-professional category	N Y Y Y Y Y Y N	[69]
Russia (2014)	1500	-	face	50.1	25.4	Female gender Younger age Dermatological disease History of atopic dermatitis Rural/urban place of living Socio-professional category	Y Y Y Y Y Y	[75]
Brazil (2014)	1022	-	face	45.7	22.3	Female gender Younger age Dermatological disease History of atopic dermatitis Rural/urban place of living Socio-professional category	Y N Y Y Y N	[75]
the Netherlands (2017)	481	60.8	n.s.	45.3	22.3	Female gender Younger age Fair phototype History of atopic dermatitis, hay fever, asthma Dry skin	Y N Y Y Y	[74]

N: no; n.s.: not specified; SS: sensitive skin; Y: yes.

biological response. Wavelength is the most relevant, as without absorption of the right wavelength by the corresponding photoreceptor, there can be no photochemistry. The delivered dose (J/cm^2), consisting in the product between irradiance (mW/cm^2) and irradiation time (s), plays also a pivotal role. It has been established that cells follow a biphasic dose response, according to which only a narrow window of dose triggers activation, while lower or higher doses lead to no activation or even inhibition, respectively [89, 90]. The dose must also be delivered at the depth at which the photoreceptors are located: attention must be paid to tune the light power at the surface in order to reach a suitable density of photons inside the skin, while ensuring that skin temperature does not increase significantly [94]. This is true especially for shorter wavelengths, as they have a shallower penetration depth in the skin [89]. As a result of this complexity, several inconclusive *in vitro* and *in vivo* studies have been published in literature, often with lack or poor reporting of the rationale for the choice of the optical parameters [92]. A more rigorous approach in selecting the optical parameters, together with the pursuit of fundamental research on the identification of photoreceptors and molecular pathways, is expected to overcome the controversies and ultimately bring phototherapy to the mainstream of therapies [92, 94].

Photobiomodulation with light in the blue spectral range (400-495 nm) is heading toward this direction. Recently, light emitting diodes (LEDs) with well-characterized optical parameters and quasimonochromatic output have been used to irradiate human skin cells in order to distinguish the biological effects of different wavelengths [95, 96]. Blue light at 453 nm emerged for its anti-proliferative and anti-inflammatory effects [95, 97]. Promising results were found also in clinical studies in which blue light at 453 nm was used in the treatment of psoriasis and atopic dermatitis (AD), two common skin diseases characterized by hyper-proliferation of keratinocytes from the basal layer and by an inflammatory infiltrate in the epidermis and dermis [98-100]. The absence of side effects reported in these studies would represent an advantage of blue light compared with UV radiation, whose beneficial effects in the treatment of these dermatoses come at the expense of an increased risk of skin cancer and photoaging [98-100]. Another inflammatory skin disease where beneficial effects of blue light have been widely reported is acne, and expectations are high also for applications in skin rejuvenation and wound healing [94, 101]. Taken together, the current evidence indicates that photobiomodulation with blue light does have biological effects, and might represent a therapeutic paradigm shift from UV irradiation in the treatment of inflammatory dermatoses.

1.6 OBJECTIVES AND THESIS OUTLINE

This thesis focused on the non-invasive, objective and *in vivo* evaluation of skin barrier properties and cutaneous inflammation in healthy volunteers. This evaluation was applied in two research areas: sensitive skin and skin irritation.

Sensitive skin

Rationale

Historically, clinical studies aimed at investigating the pathomechanisms involved in sensitive skin (SS) through linking of subjective perceptions to objective measurements of skin properties by bioengineering techniques have been rather unsuccessful, leading to inclusive results about this condition. In a recent systematic literature review, Richters and coworkers identified impaired skin barrier function, sensory hyper-reactivity and vascular responsiveness as pathomechanisms most often associated with SS [79]. Furthermore, the authors proposed a novel perception-based questionnaire spanning a range of provocations of different origin to select participants with self-assessed SS in clinical studies, and recommended to exclude participants with concomitant atopic diathesis and skin diseases, as these conditions likely represent a confounder [74].

Objective

The *first objective* of this thesis was to investigate three possible pathomechanisms of SS, namely impairment of the skin barrier and enhanced vascular and sensory reactivities, following the recommendations of Richters and coworkers and their multifactorial questionnaire as selection tool.

- Barrier impairment: the possible impairment of the skin barrier function was analyzed, for the first time, using confocal Raman microspectroscopy (CRS), a top-notch technique for the assessment of skin biochemical composition (**chapter 2.2**). Measurements with established biophysical methods were added for comparison. In this respect, a systematic literature review was also conducted in which the advantages and disadvantages of each technique were highlighted, and an association between the skin barrier assessments performed with CRS and with established biophysical methods was sought (**chapter 2.1**).
- Vascular and sensory reactivities: the possible enhanced vascular and sensory reactivities were addressed by histamine iontophoresis, a well-known *in vivo* model eliciting itch and a wheal-and-flare response (**chapter 3.1**). Established biophysical methods, together with digital image processing of skin photographs, were used for the assessment of skin reactions. Immunohistochemistry was additionally employed to provide an in-depth evaluation of the effects of the histamine iontophoresis model on the skin.

As shown in Table 2 in chapter 1.4, epidemiological studies frequently reported a remarkably high percentage of women defining their skin as sensitive. To further characterize SS in women, a cross-sectional digital survey about the perceived influence of fluctuating hormone levels on self-assessed SS was carried out (**chapter 4.1**). Albeit fluctuations in female hormones occurring during the menstrual cycle and menopause have already been suggested among the triggers of SS, data on the prevalence of women perceiving their influence as well as on the associated symptoms and stimuli are lacking. From the same cross-sectional digital survey, risk factors increasing the likelihood of reporting SS were also identified (**chapter 4.2**).

Skin irritation

Rationale

When the skin is perturbed, for example by disruption of the stratum corneum or by penetration of a chemical irritant, a release of molecular markers such as cytokines, chemokines and AMPs occurs, orchestrating the inflammatory response and ultimately leading to the restoration of skin homeostasis. Bioengineering techniques can assess the inflammatory response at the macroscopic level, for example by measuring the extent of barrier disruption by TEWL or the intensity of erythema; however, they do not provide information about which molecular markers are involved in inflammation. As a result, *in vivo* data on the dynamics of molecular markers of cutaneous inflammation are scarce.

Objective

The *second objective* of this thesis was to provide novel insights into the non-invasive and *in vivo* evaluation of skin irritation at the molecular level.

- In the first step, a systematic literature review was conducted to provide an overview of the existing methods to sample in a minimally-invasive fashion molecular markers of inflammation from human skin (**chapter 5.1**).
- In the second step, one of these methods (transdermal analyses patch – TAP) was employed to characterize the dynamics of molecular markers following two *in vivo* models of skin irritation, namely tape stripping and histamine iontophoresis (**chapter 5.2**).
- In the third step, these *in vivo* models of skin irritation and the measurement of molecular makers with TAP were employed in the evaluation of the conditioning effects of photobiomodulation with blue light at 453 nm (**chapter 5.3**). Evidence from *in vitro* and clinical studies suggests that blue light at 453 nm has anti-inflammatory effects in the skin [95, 96, 98-100], whereas *in vivo* data on its biological effects on irritated healthy skin in humans are still lacking.

From these studies, the comparison between the assessment of skin irritation at the molecular level and at the macroscopic level by means of bioengineering techniques was evaluated.

Chapter 6 provides a summary and discussion of the results obtained in this thesis, together with future perspectives offered by bioengineering techniques in dermatology and in cosmetic sciences. **Chapter 7** presents the Dutch summary of the results and discussion, while the last chapter (**chapter 8**) contains a short summary of the findings of this thesis, the research data management, and information about the author.

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Chapter 2

Skin barrier assessment in sensitive skin: established *versus* top-notch approaches

ABBREVIATIONS

AD	Atopic dermatitis
CRS	Confocal Raman microspectroscopy
FLG	Filaggrin
MPE	Maximum permissible exposure
NIR	Near-infrared
NMF	Natural moisturizing factor
OCT	Optical coherence tomography
r	Pearson's correlation coefficient
R^2	Squared correlation coefficient
RCM	Reflectance confocal microscopy
RS	Raman microspectroscopy
SC	Stratum corneum
STROBE	Strengthening the reporting of observational studies in epidemiology
TEWL	Transepidermal water loss
TTP	Tissue tolerable plasma

2.1

Microspectroscopic confocal Raman and macroscopic biophysical measurements in the *in vivo* assessment of the skin barrier: perspective for dermatology and cosmetic sciences

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ABSTRACT

Skin barrier function, confined to the stratum corneum, is traditionally evaluated using established, non-invasive biophysical methods like transepidermal water loss, capacitance, and conductance. However, these methods neither measure skin molecular composition, nor its structure, hindering the actual causes of skin barrier change or impairment. At the same time, confocal Raman microspectroscopy (CRS) can directly measure skin molecular composition and structure and has proven itself to be a powerful technique for biomolecular analysis. The aims of this literature review were to evaluate non-invasive biophysical methods in view of CRS and to outline a direction towards more specific and informative skin measurement methods. We address this by investigating, for the first time, the relation between *in vivo* assessment of the skin barrier using indirect biophysical methods and the actual skin composition and structure as given by CRS and emphasize the high potential of CRS for dermatology and cosmetic sciences. CRS acceptance in these fields will require close collaboration between dermatologists, skin scientists, and spectroscopy experts towards simplifying the technology and creating robust, rapid, easy-to-use, and less expensive CRS applications.

INTRODUCTION

The major function of the skin is to provide a barrier between the body and the environment, protecting the organism from external injurious agents and pathogens and from the loss of water and electrolytes [1]. The barrier function of the skin is fulfilled primarily by the stratum corneum (SC), a thin layer of tightly stacked corneocytes embedded in a lipid matrix [2] The SC is 10-20 µm thick in most body sites except the palms of the hands and the soles of the feet, where it is one order of magnitude thicker [3]. Corneocytes are terminally differentiated keratinocytes containing different types of keratins, water and natural moisturizing factor (NMF), a hygroscopic mixture of amino acids and other components mainly derived from degradation of the epidermal protein filaggrin (FLG) [4]. The lipid matrix is composed of a mixture of ceramides, cholesterol and fatty acids arranged in parallel layers (lamellae) between the corneocytes; within the lamellae, lipids are present in three different lateral organizations, ranging from a very dense to a disordered, liquid phase [2]. Keratinocytes are the major cell type in the epidermis and proliferate from the basal layer, situated at the boundary with the dermis. During their migration towards the skin surface they differentiate to corneocytes, expressing different types of structural proteins, flattening out and losing their nucleus; in addition, keratinocytes deliver lipids to the SC by exocytosis of organelles (lamellar bodies) at the boundary between SC and viable epidermis [1]. The SC is also punctuated by appendages (including hair follicles, sebaceous and sweat glands), which add further structural and molecular elaboration [5]. The skin barrier is thus a complex structure whose integrity depends on the interplay of several factors, including the processes of proliferation and differentiation of keratinocytes, the release of lipids from lamellar bodies, the cohesion of corneocytes and the organization of lipids between them.

The understanding of the skin barrier is of central importance in several fields including dermatology, skin pharmacology, and personal care. In particular, in dermatology, an impairment of the skin barrier is associated with different dermatological diseases and has been suggested in sensitive skin, which nowadays is a widely accepted clinical state [6-8]. In skin pharmacology, new transdermal drug delivery methods have to overcome the skin barrier while maintaining its integrity [9]. In personal care, skin treatments need to be effective yet mild on the SC barrier in order to prevent excessive discomfort and skin irritation. As an example, recent fractional non-ablative skin rejuvenation devices for both professional and home use [10] aim at sparing the skin barrier function to a much higher degree than ablative approaches [11]. It is clear that, in order to understand the mechanisms contributing to SC barrier homeostasis as well as the mechanisms leading to SC impairment, the direct measurement of its molecular components would be required, possibly *in vivo* and non-invasively.

Over the last three decades, SC barrier function and hydration have been extensively evaluated by means of transepidermal water loss (TEWL) and electrical methods like capacitance and conductance, respectively [12, 13]. These established and easy-to-use

biophysical techniques consist of hand-held probes placed in contact with the skin allowing rapid, *in vivo*, and non-invasive measurements. The principle of each technique is schematically shown in Figure 1. The major limitation of TEWL and electrical techniques is that they are indirect methods, not measuring directly the SC molecular composition. In particular, in the case of the electrical techniques, substances or treatments that interact with the keratin-water network of the SC can change the electrical properties of the skin without actually altering the water content [14]. A second limitation is that they are influenced not only by intrinsic factors, such as anatomical site, which are naturally expected to impact the outcome of the measurement, but also by extrinsic factors like ambient temperature and humidity [15]. Moreover, the skin appendages have a biasing effect on measurements through the influence of sweat gland activity, presence of hair follicles and secretion of sebum from the sebaceous gland along the hair shaft. Guidelines have been defined in order to standardize measurement procedures and minimize the influence of these factors [14, 16].

Besides the indirect, probe-based biophysical instruments, optical methods based on light absorption or scattering, like optical coherence tomography (OCT), near-infrared spectroscopy (NIR) and Raman microspectroscopy (RS), have also been reported in the *in vivo* and non-invasive assessment of skin properties [17-19]. Among these, since the pioneering studies of Caspers *et al*. [20-22] in 2000, confocal Raman microspectroscopy (CRS) has emerged for the evaluation of SC barrier function and hydration at high spatial and temporal resolution. CRS allows the direct measurement of the SC molecular composition and distribution by combining the principle of confocal signal acquisition with inelastic (Raman) photon scattering: the signal, coming from a small and spatially defined volume of tissue, can be defined as an "optical sectioning" of the skin. The principle is schematically shown in Figure 2. This methodology has been recognized and widely accepted by scientists working on the frontier of skin research, as well as single cell research, including stem cell characterization [23-25].

It would therefore be of importance to investigate the relation between the composition and distribution of SC molecular components measured by CRS, assumed as the "gold standard" for *in vivo* analysis of molecular composition at high resolution, and the indirect biophysical measurements. For this purpose, we systematically reviewed *in vivo* studies on humans in which both CRS and at least one biophysical parameter between TEWL, capacitance, and conductance were measured.

We are of the opinion that such analysis will help both dermatologists and skin scientists to (i) improve their critical appraise of the values given by indirect biophysical techniques and (ii) understand the advantages offered by CRS in the evaluation of healthy skin and in the diagnosis and treatment of diseased skin.

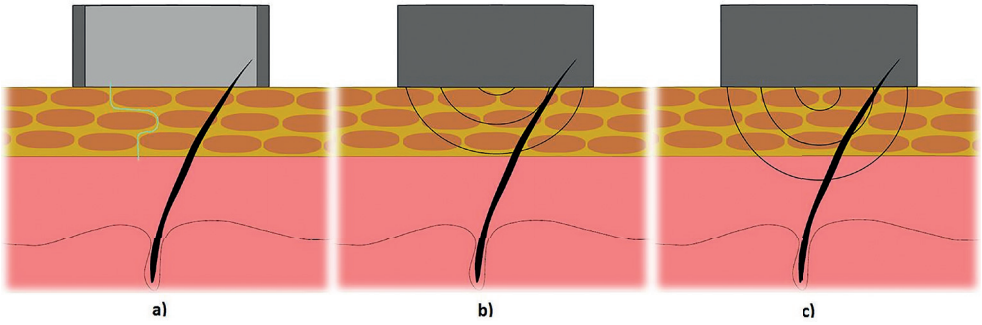

Figure 1. Principle of measurement of indirect biophysical techniques. **a**) Transepidermal water loss (TEWL). TEWL is defined as the flux density of condensed water diffusing from inside the body to the surface. It is measured in grams of water per square meter per hour (g/m^2h). Condensed water flows through a tortuous path via the intercellular lipids in which corneocytes are embedded. The variable measured by all TEWL instruments is actually the flux density of water evaporating from the surface, corresponding to the flux density of condensed water when no other sources of water are present (*e.g.* perspiration, wet skin surface). Instruments feature a chamber equipped with sensors of temperature and humidity; one extremity is open and placed in contact with the skin, while the other extremity can be open or closed. Sensor readings are used to calculate the flux density of water vapour by measurement of the humidity gradient between the extremities of the chamber or by measurement of the linear increase of humidity inside the chamber. **b**) Electrical method based on the conductance principle (Skicon 200EX). The probe head (area: 28 mm^2) features two concentric interdigital electrodes of 75-µm width and 200-µm distance. Upon contact with the skin, an oscillating electric current (frequency: 3.5 MHz) of a few micro Amperes (µA) flows between the electrodes. The conductance of the electric current is measured in micro Siemens (µS) and it is used to indicate stratum corneum hydration. The measurement depth of the instrument is suggested to be very superficial within the stratum corneum. Instrument readings can be influenced by perspiration, sebum and hair. **c**) Electrical method based on the capacitance principle (Corneometer CM825). The probe head (area: 49 mm^2) features a gold grid covered by a glass lamina. An oscillating electric field (frequency: 0.9-1.2 MHz) is developed between the tracks of the grid and penetrates into the skin upon contact with the probe. The capacitance of the system changes according to the moisture content of the superficial skin layers: the changes are then converted in arbitrary units and used to indicate stratum corneum hydration. There is uncertainty over the exact measurement depth of the instrument, with some sources indicating that it might include the upper epidermis. Instrument readings can be influenced by perspiration, sebum and hair.

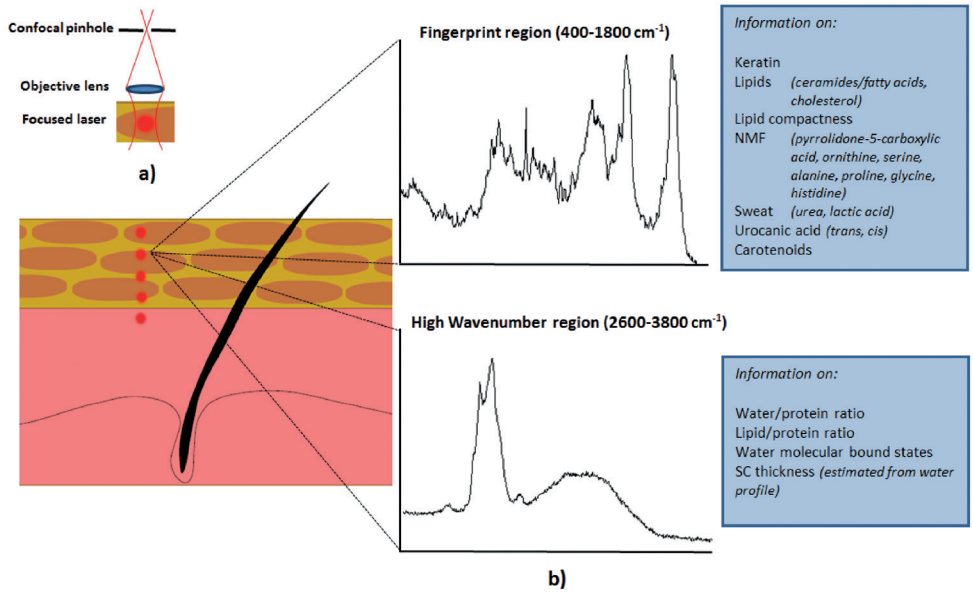

Figure 2. Principle of measurement of confocal Raman microspectroscopy. **a**) Monochromatic laser light is focused in the stratum corneum in a volume of about 5-µm length (axial resolution) and 1-µm width (dimension of the laser spot). Photons interact with the molecules, releasing some of their energy. Of the photons that exit the skin, only the ones coming from the focus region are detected thanks to the presence of a confocal pinhole. **b**) The photons which underwent frequency shifts due to the release of energy to molecules during the interaction are used to obtain Raman spectra. The position and intensity of each peak are representative of the different molecules and their amounts, respectively. Raman spectra can be obtained in a low (fingerprint) as well as a high energetic region: each region contains different information about the molecular composition and structure of the stratum corneum.

METHODS

An extensive literature search was performed in November 2014 in four computerized bibliographical databases: PubMed, EMBASE, Web of Science and the Cochrane Library. Medical subject heading and free text searches embracing the following terms were used: "Raman", "skin", "human", including all possible synonyms. The complete search strategy is shown in supplementary Table S1. The literature search had no date nor language restrictions.

After the initial search, titles and abstracts were reviewed for relevance by two independent reviewers (DF and GRdAS) taking into account the inclusion and exclusion criteria. Inclusion criteria were full text papers in English in which CRS and at least one biophysical measurement were performed *in vivo* on human volunteers. Biophysical measurements

corresponded to TEWL, capacitance, and conductance. Exclusion criteria were review articles and articles published only in abstract form. Once the relevant studies were identified, full publications were retrieved and independently reviewed for inclusion by the two reviewers. The reference lists of the included articles were additionally screened to ensure that all relevant studies were included. Consensus on inclusion was reached by discussion in all phases.

Methodological quality of the included studies was assessed by the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) criteria: each study received, independently by the two reviewers, a score ranging from 0 to 22, which was then expressed as a percentage of the maximum score [26]. Depending of the level of fulfillment of the criteria stated in STROBE, articles were categorized as A (>80%), B (60%-80%) or C (<60%). When scores differed by more than 1.5, consensus was reached by discussion.

RESULTS

Article selection and quality

The article selection process is shown in Figure 3. A total of 33 articles were selected for full-text evaluation. For 15 articles, the inclusion criteria were not applicable following full-text evaluation and were therefore excluded from further analysis. Two articles for which the inclusion criteria applied were excluded because of the impossibility to compare CRS and biophysical measurements given that these measurements were performed in unrelated experiments. Since 15 out of 16 included articles used the confocal Raman spectrometer and data processing method introduced by Caspers *et al.* [21], the reference list of this article was manually screened, in addition to the reference lists of the articles that fulfilled the inclusion criteria. Manual screening resulted in the inclusion of five more articles.

Article quality was, on average, good: four articles were classified as a category C (score <60%), 14 articles were classified as a category B (score 60-80%) and the remaining three articles were classified as a category A (score >80%). Shortcomings were mainly related to no reference to bias and limitations of the study and lack of indication of the number of participants included at each stage.

Results will be discussed for each biophysical technique in the following paragraphs. A detailed summary of each study included in this review can be requested from the first author.

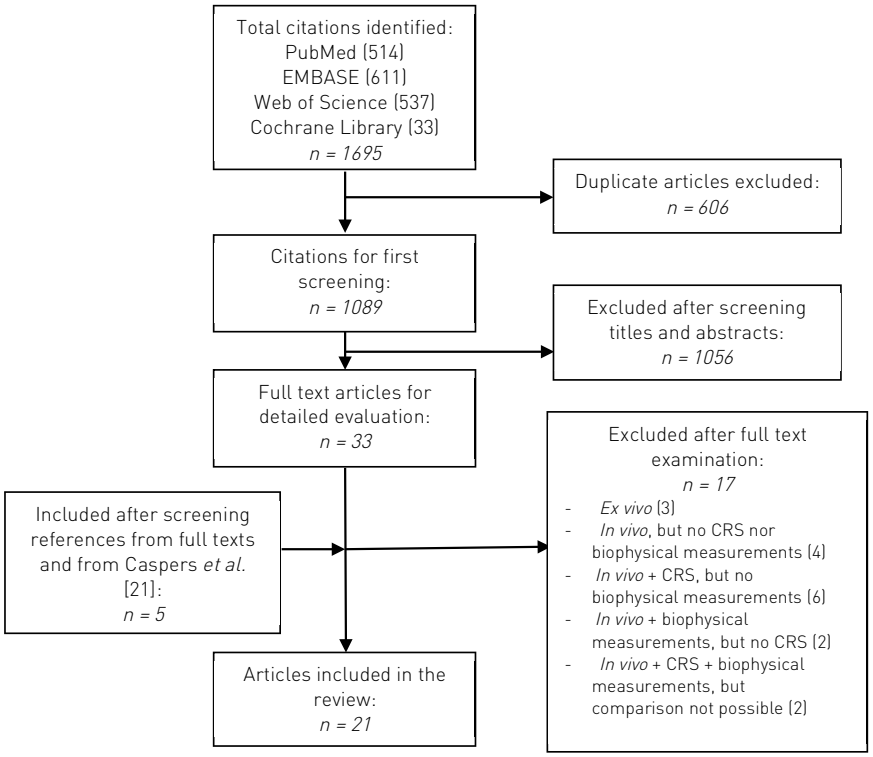

Figure 3. Article selection process.

TEWL

TEWL is widely recognized as an indicator of the SC barrier function and corresponds to the flux density of water diffusing from the inner tissues to the body surface [27]. Under the simplifying assumption of steady-state diffusion in an homogeneous membrane, the flux density can be modeled with the one-dimensional Fick's first law of diffusion [27]:

$$J = D \frac{\Delta c}{\Delta z}$$

where J = flux density ($Kg\ m^{-2}\ s^{-1}$); D = diffusion coefficient ($m^2\ s^{-1}$); Δc = positive concentration difference across the membrane ($Kg\ m^{-3}$); Δz = membrane thickness (m). According to this model, TEWL depends thus on the diffusion coefficient D, on the positive concentration difference across the membrane Δc and on SC thickness. Because the predominant pathway

for water loss through the skin is considered to be the intercellular route, *i.e.* through the lipids in which corneocytes are embedded, major contributors to the diffusion coefficient *D* are the lipids (specifically their amount, relative proportion and lateral organization), as well as the tortuous path created by the stacked packing of corneocytes [28-30].

As to SC thickness, a weak correlation (r = -0.35) with TEWL was found in a study by Bielfeldt *et al.* [31]. SC thickness was estimated indirectly from the water profile, measured on the forearm of healthy volunteers, after fitting with a mathematical model derived from Fick's first law of diffusion. This law was also used to calculate the diffusion coefficient *D*, which correlated strongly with TEWL (r = 0.92). Authors concluded that the physicochemical properties of the SC (like the physical constraint of corneocytes and the chemical constraint of lipids) are mainly responsible for the quality of the barrier in the protection from water loss. Indeed, it is known that the intercellular lipids in the SC play an essential role in preventing loss of water and other electrolytes [1]. Accordingly, in the studies included in this review, CRS measurements of lipids correlated with TEWL. A study by Janssens *et al.* [32] focused on the lipid/protein ratio, measured as the ratio of the integrated signal intensity from 2866 to 2900 cm^{-1} (CH_2 asymmetric stretching of lipids) and from 2910 to 2966 cm^{-1} (CH_3 symmetric stretching of proteins): decreasing values were reported from healthy controls to non-lesional and to lesional forearm skin of atopic dermatitis (AD) patients, with a strong correlation with 1/TEWL (r = 0.86). In agreement with Bielfeldt *et al.* [31], authors concluded that lipid/protein ratio plays a more important role in the impairment of the skin barrier in AD than SC thickness. Janssens *et al.* [33] reported high TEWL in conjunction with impaired lipid composition and organization with techniques other than CRS. The importance of lipids in the skin barrier was demonstrated in another study on AD patients by Simpson *et al.* [34]: TEWL decreased following a four-week application of a moisturizer containing niacinamide and a ceramide precursor, along with increased ceramides levels. Niacinamide, a compound of vitamin B_3 which has been shown to increase the levels of SC ceramides [35], was tested also on healthy skin, resulting in lower increase of TEWL following tape stripping [36]. On the other hand, a study by Boireau-Adamezyk *et al.* [37] showed that the relationship between lipids and TEWL is not straightforward in the facial area, characterized by higher levels of ceramides, cholesterol and lipid/protein ratio, as well as hydration, and yet by poor barrier function indicated by high TEWL. Smaller, immature corneocytes and thinner SC, driven by faster proliferation, have also been reported in literature for facial skin [38, 39]: these factors, leading to a shorter diffusion path length through the corneocytes [40], play most probably the major role for the increase of TEWL. The weak influence of SC thickness on TEWL found by Bielfeldt *et al.* [31] might thus hold primarily for body parts other than the facial area.

Besides lipids and corneocytes, also NMF plays a role in the water-retaining capability of the SC [30]. Following the discovery that mutations in the gene encoding for FLG are a major predisposing factor for AD [41], a number of studies have investigated whether the levels of NMF measured with CRS could be used as markers of FLG genotype. TEWL measurements

were used in these investigations as well. All studies included in this review reported that NMF levels were significantly decreased in carriers compared with non-carriers of FLG mutations. On the other hand, inconsistent results were shown for TEWL, which was significantly increased in AD patients carrying FLG mutations compared to AD patients without FLG mutations when clinically assessed disease severity was higher in the former group [42, 43] and it showed no differences when disease severity was similar between the two groups [33, 44, 45]. In agreement with our findings, the inclusion of different disease severities has been indicated in a recent work as the reason for the discrepancies in TEWL found in past studies [46]. These results suggest that lower NMF levels due to the presence of FLG mutations do not necessarily result in increased barrier impairment, as assessed by TEWL. Inconsistencies between FLG genotype and TEWL have emerged in other studies in literature [47-49]. It has been suggested that increased TEWL in patients with moderate-to-severe AD is independent of FLG genotype [45]; accordingly, the studies of Janssens *et al*. [32, 33] on the impaired skin barrier in AD did not find an effect of FLG genotype either on the lipid/protein ratio or on the composition and organization of lipids.

In terms of SC water content, higher TEWL was found by Nikolovski *et al*. [50] and Boireau-Adamezyk *et al*. [37] in conjunction with high SC water content in infant (3-12 months old) forearm skin and in adult facial skin, respectively. However, increased TEWL was also reported by Fluhr *et al*. [51] in conjunction with decreased SC water content following tissue tolerable plasma (TTP) application, a new approach for topical microbial disinfection of the skin surface employed in wound therapy. Trends of increased TEWL and decreased SC water content were shown also in a study by Mlitz *et al*. [43] comparing mild-to-moderate AD patients to healthy controls [43]. It is thus necessary to emphasize that SC water content and water flux are two separate variables [16] and that increased TEWL may reflect a decreased as well as an increased level of hydration [52].

Electrical methods

Hydration measured with electrical methods is not always representative of the SC water content, as measured with CRS in the studies included in this review. In the same study cited for TEWL, Bielfeldt *et al*. [31] calculated water content in and below the SC by integrating the water profiles from the surface to the SC thickness and from the SC thickness to 20 µm deeper, respectively. The results showed a mild negative correlation with the SC water content and with SC thickness ($r = -0.45$ and $r = -0.49$, respectively), a mild positive correlation with the water content below the SC ($r = 0.55$) and a clear correlation with the SC water gradient ($r = 0.62$) [31]. Another study calculated the water content at 3 µm and at 15 µm through integration of the water profiles, reporting mild squared correlation coefficients with conductance at baseline and following a water patch test (baseline: $R^2 = 0.11$ at 3 µm, $R^2 = 0.31$ at 15 µm; water patch test: $R^2 = 0.36$ at 3 µm, $R^2 = 0.33$ at 15 µm) [53]. Fluhr *et al*. [54] found that capacitance was higher in infant skin (5-7 months old) than in adult skin;

unfortunately, no SC water content was calculated from the water profile for comparison. In a study on skin seasonal changes, Egawa and Tagami [55] reported that capacitance and the sweat components lactic acid and urea increased in summer, while water profiles showed no changes. Of note, the humidity of the experimental room was higher in summer despite the air conditioning; a possible explanation for the higher hydration measured by capacitance could be the presence of sweating, since its influence on electrical methods is known [14]. In another pilot study, Egawa *et al.* [56] reported lower capacitance and conductance and a trend to lower mean amount of water in the upper SC of psoriatic lesional skin compared to non-lesional skin; however, a proper comparison between electrical methods and SC water content is not feasible since the mean water amounts were measured along different depths in lesional and non-lesional skin.

Despite no direct comparison between conductance and capacitance is possible, we found more agreement with the SC water content for the former. Boncheva *et al.* [57] demonstrated that conductance measured across the SC with repetitive tape stripping changed linearly with the water depth profile up to 37 mass percentage (where mass percentage, or mass%, indicates the grams of water per 100 grams of protein), corresponding to a rather superficial depth in the SC. For higher mass%, the relationship of SC water with conductance became exponential, probably due to the different influence of "free" water in the deeper SC with respect to "bound" water closer to the skin surface. In the study by Boireau-Adamezyk *et al.* [37], conductance and SC water content were both higher on the face, but while the former was different between outer and inner arms, the latter showed no differences. It has already been suggested that conductance correlates with the water content of the superficial portion of the SC as well as with that of the whole SC [58]. Accordingly, one *in vitro* study demonstrated that the measurement depth of the conductance instrument covers less than 15 μm, thus reasonably within the SC, while the measurement depth of capacitance is up to 45 μm, corresponding to the viable epidermis; this value is in contrast with the 10-20 μm indicated by the manufacturer [59]. In that study, plastic foils were inserted between the probes and a filter pad saturated with ultrapure distilled water; subsequently, the signal reduction obtained by increasing the number of plastic foils was calculated and used to estimate the measuring depth of each instrument. The correlations calculated by Bielfeldt *et al.* [31] add to the uncertainties as to where in the skin the capacitance signal is coming from and suggest an influence of SC water gradient. In addition, previous studies have stated that capacitance is more sensitive for dry to very dry skin, while conductance is more sensitive for highly hydrated skin [14, 60, 61]. The comparisons described in this review tend to confirm those results. In fact, low capacitance was in agreement with reduced SC water content following TTP application in the study by Fluhr *et al.* [51]. Moreover, in the study by Mlitz *et al.* on mild-to-moderate AD and in several others in literature [49, 62, 63], capacitance correlated with clinical assessment of disease severity in AD, a condition in which dry skin is one of the major hallmarks. Conductance correlated with the SC water content in facial skin, characterized by high hydration [37].

A number of studies investigated the effects of long term use (14 to 27 days) of moisturizers. Crowther *et al.* [35] measured the SC water content through integration of the water profiles from the skin surface to the SC thickness and found a significant increase at two weeks of treatment and at one-week regression of a moisturizer containing glycerol and niacinamide. Two other products containing only glycerol had no significant effects on the SC water content. These results did not correlate with capacitance, which was increased for each moisturizer throughout all time points of the study; authors attributed this outcome to the high dielectric constant of glycerol. Another study did not show full agreement between the two techniques in a one-volunteer experiment [64]. In the study on the moisturizer containing niacinamide and a ceramide precursor, Simpson *et al.* [34] showed that capacitance was increased by 118% with respect to baseline, while only a trend to higher SC water content was found. On the other hand, increased capacitance was in agreement with an improved clinical dryness assessment. These outcomes reveal that values given by capacitance and conductance should be interpreted with caution, as incorrect estimation of the true SC water content might derive from the influence of the electric properties of the products applied rather than from the skin. The performance of capacitance and conductance has been questioned also in terms of the ability to discriminate mild changes in skin moisturization following a comparison with NIR spectroscopy, another optical method to directly measure skin water content [65].

DISCUSSION

In this review we evaluated the outcomes of indirect biophysical measurements in relation to the SC molecular composition and distribution measured by CRS. We found that increased TEWL, indicator of skin barrier impairment, can underlie different amounts of lipids, water and NMF, depending on body site and disease state. The "integral" nature of TEWL measurement (both in the axial and in the lateral dimensions) cannot detect differences at the structural or molecular level if these result in a similar barrier impairment, as found in studies over AD and FLG genotype. It is clear that the advantage of CRS of knowing, *in vivo* and non-invasively, "which" SC components are mostly affected, rather than "if" the SC in its whole is affected, would provide a much more specific and complete understanding of the skin barrier. Applications of CRS to the assessment of the skin barrier could include a more focused diagnosis and rational treatment of skin diseases, a detailed evaluation of the recovery of biochemical components following stimulations of different nature [66], and a deeper insight into the barrier function involvement in sensitive skin. A step towards personalized medicine and personal care would thus be possible.

We confirm that electrical methods are limited by the influence of external factors *(e.g.* sweat) and by uncertainties over the exact measurement depth of the instruments. Their use in the evaluation of moisturizers should always be accompanied by measurements with other

techniques and investigations of different aspects of hydration changes (such as water binding and retention) in order to avoid incorrect conclusions due to the influence of the electric properties of the moisturizer [67]. CRS can help to gain a better understanding of moisturizer mechanism within the skin through the direct measurement of water content, distribution, and SC swelling [35]. In addition, it has recently been demonstrated that CRS can differentiate between different water binding states, including unbound, partially bound and primary bound water [68, 69]. The water content in each binding state has been found to vary differently in function of external stimuli like humidity and mechanical stress and, remarkably, correlations with structural and organizational changes in lipids and proteins were highlighted [68, 70]. This demonstrates the potential of CRS to study the correlation of skin barrier and hydration status in function of stimuli of different nature. It has been reported that nearly all major cosmetic companies have now employed CRS to understand mechanisms of skin hydration and that claims made from the findings of such investigations will continue to emerge [71].

In terms of future perspectives on non-invasive skin measurements, we believe that indirect biophysical techniques will continue to be used due to their capability to provide objective information on healthy and diseased skin in a rapid and relatively inexpensive way. However, the specificity and richness of additional information provided by a new class of measurement like CRS cannot be underestimated. Besides characterizing the SC molecular composition and structure, CRS can track the *in vivo* penetration of actives and drugs within the SC, together with the effect of penetration enhancers, provided the substances under investigation have a Raman signal and the amount applied is sufficient to be detected by currently available devices [72]. Carotenoids, retinol, petrolatum oil and ibuprofen are among the substances successfully measured *in vivo* using CRS [73-76]. Such measurements are obtained in a non-destructive, immediate and labeling-free fashion, representing an advantage compared to other techniques used to track the penetration of substances in the skin, like tape stripping and multiphoton microscopy: the first requires a layer-by-layer removal of SC by adhesive tape followed by extraction and quantification by high performance liquid chromatography, while the second requires, for most procedures, labeling of the substances under investigation with fluorescent dyes, possibly affecting the penetration within the skin [18]. Furthermore, in contrast to infrared spectroscopy, also used to track the penetration of substances in the skin, CRS has a higher spatial resolution and is not affected by interference from water [18]. In addition to the analysis of the skin barrier, RS has proven useful in other dermatological applications. In the oncological field, ample proof-of-concept of Raman-based spectral discrimination between normal and neoplastic skin tissue has been reported: it is suggested that potential *in vivo*, real-time Raman applications could range from cancer diagnosis to tumor-margin detection during surgical resection [77]. Successful attempts at combining RS with imaging methods like OCT and reflectance confocal microscopy (RCM) have been described, allowing concomitant morphological and biochemical characterization of the skin [78-80].

On the other hand, it is important to note that CRS has some inherent limitations. As only one out of 10^7 photons undergoes Raman scattering, the technology requires very

sensitive detectors [18] and can be used for the measurement of molecules present in sufficient concentrations. Moreover, during skin measurements, the detection limit is often hindered by tissue autofluorescence, which gives rise to a high signal background, thereby significantly decreasing the signal-to-noise ratio of the Raman spectra. The effects of tissue autofluorescence are usually minimized with a baseline removal in the spectrum post-processing [21, 22, 69], while fewer approaches focus on reducing autofluorescence prior to the collection of spectra, for example by photobleaching [81, 82]. As any optical measurement, Raman signals undergo attenuation at deeper skin layers, requiring normalization by a skin-derived Raman peak, for example keratin [21], or correction by a mathematical algorithm [83], to allow a reliable definition of the concentration profiles. Further challenges inherent to *in vivo* CRS applications are minimization of body movement, for which an inverted set-up is usually used [21, 22], and of laser heating effects, which have to comply with the maximum permissible exposure (MPE) limits defined internationally [84]. It follows that CRS applications are characterized by high performance requirements in terms of hardware *(e.g.* lasers, detectors, confocal optics) to obtain high quality spectra, as well as by specific software "know-how" to process and extract the information of interest [84] (*e.g.* deconvoluton methods in case of overlapping spectral bands [22, 68]). As a consequence, current state-of-the-art *in vivo* Raman applications are complex, expensive, and relatively bulky, needing trained personnel for their use and not yet allowing measurements on recessed body parts [24, 84, 85]. An approach that would reduce the size, cost, and complexity required for acceptance in mainstream clinical and dermatocosmetic practices would be to develop dedicated Raman devices for specific applications in which, instead of conventional optical components, integrated optics technology is used, the components of which are miniaturized and mass-produced [85]. Such dedicated applications can be developed thanks to the fact that relevant diagnostic information is often contained in a limited number of spectral regions, and that statistical analysis methods could be effectively used to extract this information from the Raman spectra [69, 86]. From the end users point of view (dermatologists and skin scientists), the question is to determine how to include the information provided by the Raman instrumentation in routine clinical or product testing protocols and how it could affect the decision making processes [87]. In order to answer these questions and to widen the use of CRS, we recommend performing clinical trials in which a close collaboration is established between spectroscopists, dermatologists, and skin biology scientists. We hope the endpoint of such collaborations to be robust, rapid, easy-to-use, and less expensive CRS applications allowing a deepening of our understanding of the skin and its barrier function. Meanwhile, we strongly encourage performing clinical investigations to gain further insights into the relationship between indirect biophysical techniques and the skin molecular composition measured by current state-of-the-art CRS applications to create clear rules both for applicability of indirect methods and their limitations. The outcome of such studies will improve the critical appraisal of the measurements performed with indirect, macroscopic biophysical techniques, helping to bridge the gap between the current non-invasive evaluation of the barrier function and future assessments performed by promising techniques like CRS.

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SUPPLEMENTARY MATERIAL

Table S1. Search strategy.

Search Topic	*Search terms: 10-11-2014* **PubMed**	*Search terms: 10-11-2014* **EMBASE**	*Search terms: 10-11-2014* **Cochrane Library**	*Search terms: 10-11-2014* **Web of Science**
Search A:	*Mesh terms:*	*Mesh terms:*	Mesh terms:	*In free text (Topic)*
Raman	#1 Spectrum Analysis, Raman	#1 raman spectrometry/	#1 Spectrum Analysis, Raman (explode all trees)	#1 raman
	In free text:	*In free text:*	*In free text(In title ti or abstract ab or keywords kw):*	
	#2 Raman [tw]	#2 raman.mp	#2 raman:ti, ab, kw	
	#1 OR #2	*#1 OR #2*	*#1 OR #2*	
Search B:	*Mesh terms:*	*Mesh terms:*		*In free text (Topic)*
Skin	#3 Skin OR Dermatology OR Skin Diseases	#3 exp skin/ OR dermatology/ OR skin diseases/		#2 skin OR epidermis OR dermis OR stratum corneum OR dermatology OR cutis OR derma
	In free text: #4 skin [tw] OR epidermis [tw] OR dermis [tw] OR stratum corneum [tw] OR dermatology [tw] OR cutis [tw] OR derma [tw]	*In free text:* #4 (skin OR epidermis OR dermis OR stratum corneum OR dermatology OR derma OR cutis).mp		
	#3 OR #4	*#3 OR #4*		

Search C:	*Mesh terms:*	*Mesh term:*		*In free text (Topic)*
Humans	#5 animals NOT humans	#5 human/		#3 human OR volunteer OR participant OR subject OR patient
		In free text:		
		#6 (human* OR volunteer* OR participant* OR subject* OR patient*).mp		
		#5 OR #6		
Crossed searches:	Search A AND Search B NOT Search C	Search A AND Search B AND Search C	Search A	Search A AND Search B AND Search C

ABBREVIATIONS

AD	Atopic dermatitis
AR	Allergic rhinoconjunctivitis
a.u.	arbitrary units
AUC	Area under the curve
CRS	Confocal Raman microspectroscopy
FLG	Filaggrin
NMF	Natural moisturizing factor
NSS	Non-sensitive skin
SC	Stratum corneum
SS	Sensitive skin
TEWL	Transepidermal water loss
TIS score	Three item severity score
TRP	Transient reception potential

2.2

Sensitive skin: assessment of the skin barrier using confocal Raman microspectroscopy

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ABSTRACT

Background/aims: Sensitive skin (SS), a frequently reported condition in the Western world, has been suggested to be underlined by an impaired skin barrier. The aim of this study was to investigate the skin barrier molecular composition in SS subjects using confocal Raman microspectroscopy (CRS) and to compare it with that of subjects with non-sensitive skin (NSS), atopic dermatitis (AD) and allergic rhinoconjunctivitis (AR), who frequently report SS.

Methods: Subjects with SS (n=29), NSS (n=30), AD (n=11) and AR (n=27) were included. Stratum corneum (SC) thickness, water, ceramides/fatty acids, and natural moisturizing factor (NMF) were measured by CRS along with transepidermal water loss and capacitance on the ventral forearm, thenar, and cheek. Sebum levels were additionally measured on the forearm and cheek.

Results: No differences between SS and NSS subjects were found regarding SC thickness, water and NMF content, yet a trend towards lower ceramides/fatty acids was observed in the cheek. Compared with AD subjects, the SS group showed higher ceramides/fatty acids content in the forearm, whereas no differences emerged with AR subjects. The correlation of macroscopic biophysical techniques and CRS was weak, yet CRS confirmed the well-known lower content of NMF and water, and thinner SC in subjects with filaggrin mutations.

Conclusion: The skin barrier in SS is not impaired in terms of SC thickness, water, NMF and ceramides/fatty acids content. The failure of biophysical techniques to follow alterations in the molecular composition of the skin barrier revealed by CRS emphasizes a strong need in sensitive and specific tools for *in vivo* skin barrier analysis.

INTRODUCTION

The skin protects our internal milieu from the external environment due to its low permeability, which is mainly established by specific properties of the stratum corneum (SC) [1]. The cells of the SC are the result of epidermal differentiation, a gradual maturation process of the basal keratinocytes ascending to the cornified layer of the squamous epithelium, functioning as corneocytes [1-4]. Next to a tough and resilient organization of the flattened corneocytes, the envelope of proteins and lipids surrounding each corneocyte also plays a crucial role in preventing penetration and diffusion of foreign substances through the skin and loss of internal water. The currently accepted model of the SC, although subject to change [5-7], also contains the intercellular lipid lamellae, a product originating from the lamellar bodies of the stratum granulosum, and consisting of ceramides, fatty acids, and cholesterol. Located between the corneocytes, it contributes to the overall skin barrier function [3, 8-12]. Furthermore, water soluble intracorneocyte substances, collectively known as natural moisturizing factor (NMF), contribute to the overall skin barrier function by binding water and limiting water loss from the skin [1]. Impairment of the skin barrier may result in penetration of allergens and pathogens contributing to skin diseases, such as atopic dermatitis (AD), and allergic and irritant contact dermatitis.

Sensitive skin (SS) is a condition characterized by perception of skin discomfort following mild stimuli, frequently without objective signs of skin irritation [13-18]. This skin condition has been shown to be highly prevalent in the Western world but, despite extensive research in the past years, no consensus on its definition and pathomechanisms has been reached [19]. Impaired skin barrier function has been suggested to underlie SS [13, 20, 21], leading to proposals of an association of SS with atopic conditions [22-24]. The assessment of the skin barrier has been traditionally performed by means of indirect macroscopic biophysical techniques such as transepidermal water loss (TEWL). The hydration of the SC, indirectly measured based on capacitance, has been proposed to be lower in facial areas of SS subjects [25] and of subjects perceiving stinging responses following application of lactic acid on the nasolabial folds [20]. However, other studies which used macroscopic biophysical techniques did not find such differences between a SS and a non-sensitive skin (NSS) group [14, 18, 20, 26, 27]. This inconsistency between outcomes could be, on the one hand, due to the fact that subjects were included in tests using different criteria, since a definition of SS is still lacking. It might also be possible that indirect assessments of barrier function and hydration by means of TEWL and capacitance lack sensitivity and specificity if the barrier function impairment in SS is only subtle.

Over recent years, confocal Raman microspectroscopy (CRS) has emerged as a powerful tool for the direct and non-invasive assessment of the molecular components of the skin at high spatial and temporal resolution. CRS is an optical technique based on the principle of inelastic (Raman) scattering: when a monochromatic laser beam is focused in

the skin, incident photons interact with the vibrational levels of the molecules, transferring to them a part of their energy. The exact amount of energy required to excite a vibrational mode is dependent on the masses of the atoms involved in the vibration and on the chemical bonds between them. The resulting Raman spectra are thus molecule-specific and contain detailed information on their type and amounts [28]. In addition, by using the principle of confocal detection, only the light scattered from the laser focus is detected, providing spatially resolved information, which approaches a subcellular resolution [29]. Raman spectroscopy has already established a strong position in non-invasive skin analysis for many applications, ranging from detection of non-melanoma skin cancer [30] to evaluation of skin barrier composition in AD [31, 32]. As CRS is able to detect differences at the molecular level and at high spatial resolution, it could provide a breakthrough in the evaluation of barrier function involvement in SS, which so far is not possible with macroscopic biophysical techniques [33].

The primary objective of this study was to evaluate the SC molecular composition using CRS in subjects with SS and to compare the results with those measured on subjects with NSS, AD, and allergic rhinoconjunctivitis (AR). The underlying hypothesis was that the mechanism of SS could be based on aberrant properties of the SC and could share interface with atopic conditions. The second objective was to perform a comparative study of the penetration kinetics of a solution of glycerol (1%) in water in SS and NSS subjects in order to establish whether faster penetration of topicals due to an impaired skin barrier might occur in SS. Glycerol is a widely used humectant in dermatologic and cosmetic formulations [34]. In this study, it was chosen as a model for substance penetration for its low toxicity, known diffusivity in the SC allowing measurement with CRS, hydrophilic nature, and undisruptive effect on the intercellular lipid lamellae [34]. The penetration of a lipophilic substance might be heavily influenced by its affinity with the lipid compartment of the skin barrier, which is possibly different between SS and NSS. Meanwhile, the lack of a disruptive effect on the intercellular lipid lamellae implies that different penetration kinetics of glycerol could be attributed to the properties of the skin barrier rather than to an active effect of glycerol on the barrier. The knowledge gained should be of high value for clinicians and the cosmetic and pharmaceutical industry involved in developing solutions for individuals with SS.

PATIENTS AND METHODS

Subject selection

The inclusion criteria for this study were an age between 18 and 65 years and skin type II or III (Fitzpatrick scale). Subjects could not use immunosuppressive drugs during the study period. Starting from two days before the test, subjects were advised not to use toiletries (*e.g.* personal care and cosmetic products) in the areas to be investigated and not to excessively expose these areas to sunlight or use artificial tanning methods. Four groups of subjects

were included. Subjects with SS and subjects with NSS were selected based on a perception-based questionnaire, as previously described [21, 35, 36]. Briefly, potential participants filled in questions on self-assessed skin sensitivity and on skin perceptions (*i.e.* discomfort, stinging, redness, dryness) following exogenous and endogenous triggers (*i.e.* toiletries, shaving, heat, cold, clothes, emotions). The severity of reactions to each trigger and the correspondent duration were scored on a visual analogue scale and on an ordinal scale (no reaction, seconds, minutes, less than one hour, hour(s), day(s)), respectively. The scores of each trigger were summed, and the final score was compared to an upper and lower quartile determined in advance by distributing the questionnaire to a large study cohort [37]. If the final score was above the upper quartile and the subject reported SS, he/she was included in the group with SS. If the final score was below the lower quartile and the subject reported NSS, he/she was included in the group with NSS. In addition, subjects in both the SS and NSS groups did not have a specific history of skin diseases or other concomitant diseases, including AD, asthma and AR in particular. The other two groups included in the study were subjects with AR using oral histamine antagonists when experiencing symptoms and without a history of asthma, urticaria, AD or other (skin) diseases; and subjects with moderate to severe AD (three item severity score - TIS - ≥3) currently using no therapy or using solely topical corticosteroids and without a history of asthma, urticaria, AR or other (skin) diseases. Subjects with AR and AD did not fill in the full-length questionnaire, but were asked if they perceived their skin as sensitive or non-sensitive. The study was conducted in accordance with the Declaration of Helsinki principles and was approved by the local ethics committee. Experiments were performed at the dermatology department of the Radboud University Medical Center in Nijmegen, the Netherlands, between January and March 2015.

Instrumentation

Three body sites were measured: (i) the mid-ventral forearm, (ii) the lateral side of the thenar eminence of the arm, and (iii) the malar eminence of the right cheek on the 90-degree angle crosspoint of the ala of the nose and the lateral canthus of the eye. Forearm and thenar measurements were performed in the non-dominant arm (supplementary Figure S1). AD subjects did not have active lesions on these body sites. Subjects were acclimatized for 20 minutes in a temperature- and relative humidity-controlled room (temperature 21 ± 1 °C, relative humidity 50 ± 10%).

In vivo CRS measurements were performed using the gen2-SCA performance model of RiverD International B.V. (Rotterdam, the Netherlands). The device has an axial resolution of 5 µm and is equipped with a 785-nm laser source for measurements in the 400-1800 cm^{-1} spectral region (NMF, ceramides and glycerol) and a 671-nm laser source for measurements in the 2400-4000 cm^{-1} spectral region (water). The laser power complies with the maximum permissible levels for skin as defined by the international laser safety standard (IEC 60285-1:2007; <30mW for 785 nm, and <20mW for 671 nm). In the 400-1800 cm^{-1} region, spectra of

the cheek and forearm were acquired at 4-µm increments in the axial direction up to a depth of 28 µm using a 5-s acquisition time per point, whereas the spectra of the thenar were acquired at 15-µm increments up to a depth of 180 µm using a 5-s acquisition time until 100 µm and a 7-s acquisition time until 180 µm. In the 2400-4000 cm^{-1} region, spectra of the cheek and forearm were acquired at 4-µm increments up to a depth of 40 µm using a 2-s acquisition time per point, whereas spectra of the thenar were acquired at 15-µm increments up to a depth of 180 µm using a 2-s acquisition time until 100 µm and a 5-s acquisition time until 180 µm. Ten measurements per body site were performed in each spectral region.

The macroscopic biophysical techniques employed included: (i) capacitance for the indirect measurement of SC hydration (Corneometer CM825, Courage + Khazaka, Germany); (ii) TEWL for the indirect assessment of skin barrier (Aquaflux AF200, Biox, UK); and (iii) sebumeter for the indirect measurement of sebum level at the skin surface (Sebumeter SM815, Courage + Khazaka, Germany). Three measurements per body site were performed, except for the SC hydration of the cheek which was measured five times. The sebum measurements were performed on the side contra lateral to the one in which CRS was applied and were not performed on the thenar.

Penetration kinetics of glycerol

A solution of glycerol (1%) in demineralised water (200 µl) was applied to the non-dominant volar forearm for one minute. A 13-mm-diameter plastic ring was used to prevent the spreading of the substance over the skin surface. After wiping off the solution with a tissue, CRS measurements in the 400-1800 cm^{-1} spectral region were performed every 60 s during the subsequent 15 minutes using axial steps of 4 µm and up to a depth of 28 µm.

Calculation of parameters from CRS measurements

Concentration profiles of SC molecular components relative to keratin were obtained using the software SkinTools 2.0 (RiverD International B.V.) using a previously reported fitting algorithm [28]. Briefly, the algorithm consists of a least square fitting of the Raman spectra obtained *in vivo* to a library of Raman spectra of SC molecular components obtained *in vitro*, resulting in a set of fit coefficients for the SC constituent spectra. The fit coefficients are subsequently normalized to the fit coefficient of the keratin spectrum in order to compensate for the loss of signal intensity at increasing skin depths [28]. For the measurement of concentration profiles of glycerol, the Raman spectrum of the solution of glycerol (1%) in water was obtained *in vitro* and subsequently added to the library of Raman spectra. Obvious outliers in the concentration profiles (due for example to presence of cosmic rays or high background fluorescence in the corresponding Raman spectra) were removed.

The average thickness of the SC was calculated from the water concentration profiles using an implementation in Matlab (version R2013a, The MathWorks Inc., USA) of the method of Bielfeldt *et al.* [38], in which the SC thickness was defined as the intercept of two straight lines delineating the boundary between the SC and the epidermis. The average water content

in the SC was expressed as area under the curve (AUC) of the water profiles from 4-µm depth to the SC thickness in the cheek and forearm, and from 10-µm depth to the SC thickness in the thenar. The average levels of ceramides/fatty acids (grouped by SkinTools in a single variable, "ceramides") and of NMF between a depth of 4 and 12 µm in the forearm, 4 and 8 µm in the cheek and 10 and 50 µm in the thenar were calculated from the corresponding average concentration profiles. These intervals were chosen in order to avoid influences from skin surface contamination (*e.g.* sebum) and from washout/desquamation effects at the skin surface, as well as to avoid Raman signals from the viable epidermis [39]. The presence of filaggrin (FLG) mutations was established according to the method of O'Regan *et al.* [32], in which the cut-off point of 1.07 arbitrary units (a.u.) used for FLG mutations (either $FLG^{-/-}$ or $FLG^{+/-}$) *versus* no FLG mutation ($FLG^{+/+}$) was derived from the mean NMF level between a depth of 30 and 50 µm measured in the thenar. The presence of exogenous glycerol after topical application was verified by calculating the difference spectrum, obtained by subtraction of the average Raman spectrum where the glycerol signal was not detectable from the average Raman spectrum where glycerol was detected using SkinTools 2.0. If the peaks of the Raman spectrum of glycerol were clearly visible in the difference spectrum, presence of exogenous glycerol was confirmed. If some peaks were missing or were not convincingly above the noise level, the presence of exogenous glycerol was not confirmed.

Statistical analysis

The results are presented as number (percentage) or as median (minimum-maximum). Differences between the groups (SS, NSS, AR and AD) were analyzed using the Mann-Whitney exact test for non-parametric independent values. Correlations were investigated using the Spearman's rho. Statistical analyses were performed with SPSS Statistics version 20 for Windows (IBM SPSS Inc., USA). A p value ≤ 0.05 was considered statistically significant. Missing values were excluded from the analyses. No corrections for multiple comparisons were applied.

RESULTS

Group characteristics

In total, 29 subjects were included in the SS group, 30 in the NSS group, 27 in the AR group and 11 in the AD group. The group characteristics are presented in Table 1. There were no significant differences with respect to age, gender, Fitzpatrick skin type and presence of FLG mutations between the groups. Self-assessed facial and body skin differed between SS and NSS subjects, with the former reporting more frequently dry or combined (concomitant presence of dry and oily parts) skin, and the latter reporting more frequently normal skin ($p=0.007$ and $p=0.000$, respectively). Self-assessed SS was reported by two-thirds of AR subjects and by all AD subjects. The measurements of four subjects in the volar forearm, of one subject

in the cheek and of two subjects in the thenar were excluded, because of artefacts found in the Raman spectra most probably caused by exogenous agents on the skin.

Table 1. Population characteristics.

	NSS	SS	AR	AD
	n (%) or median (range)	*n (%) or median (range)*	*n (%) or median (range)*	*n (%) or median (range)*
Self-assessed sensitive skin	30 (100.0)	29 (100.0)	27 (100.0)	11 (100.0)
SS	0 (0.0)	29 (100.0)	18 (66.7)	11 (100.0)
NSS	30 (100.0)	0 (0.0)	9 (33.3)	0 (0.0)
Gender				
Male	14 (46.7)	11 (37.9)	11 (40.7)	5 (45.5)
Female	16 (53.3)	18 (62.1)	16 (59.3)	6 (54.5)
Age (years)	21.5 (18-28)	21.0 (18-32)	23.0 (19-29)	23.0 (20-27)
Skin type (Fitzpatrick)				
Skin type II	22 (73.3)	21 (72.4)	19 (70.4)	8 (72.7)
Skin type III	8 (26.7)	8 (27.6)	8 (29.6)	3 27.3)
FLG mutations				
FLG-/- or *FLG+/-*	3 (10.0)	3 (10.3)	1 (3.7)	3 (27.3)
FLG+/+	26 (86.7)	25 (86.2)	26 (96.3)	8 (72.7)
missing	1 (3.3)	1 (3.4)	0 (0.0)	0 (0.0)
Facial skin dryness				
Normal	17 (56.7)	6 (20.7)	n.a.	n.a.
Dry	4 (13.3)	9 (31.0)		
Oily	1 (3.3)	2 (6.9)		
Combined (dry and oily)	8 (26.7)	12 (41.4)		
Body skin dryness			n.a.	n.a.
Normal	25 (83.3)	9 (31.0)		
Dry	4 (13.3)	15 (51.7)		
Oily	0 (0.0)	0 (0.0)		
Combined (dry and oily)	1 (3.3)	5 (17.2)		
Questionnaire score	29.5 (0.0-62.0)	157.8 (70.3-287.0)	n.a.	n.a.

SS: sensitive skin; NSS: non-sensitive skin; AR: allergic rhinoconjunctivitis; AD: atopic dermatitis; FLG-/-: homozygous filaggrin mutation; FLG+/-: heterozygous filaggrin mutation; FLG+/+: no filaggrin mutation; n.a.: questions about facial and body skin dryness and the questionnaire to determine skin sensitivity were not asked/distributed to the AR and AD groups.

Macroscopic biophysical measurements

Macroscopic biophysical measurements of the forearm, cheek and thenar are reported in Tables 2-4. In the forearm, SS and AR had lower TEWL compared with NSS and AD (SS *vs.* NSS: *p=0.076*, SS *vs.* AD: *p=0.082*, AR *vs.* NSS: *p=0.038*, AR *vs.* AD: *p=0.044*). Similar findings were found in the thenar (SS *vs.* NSS: *p=0.016*, SS *vs.* AD: *p=0.058*, AR *vs.* NSS: *p=0.050*, AR *vs.* AD: *p=0.071*). No further difference between the groups was found with respect to TEWL measured on the cheek and with respect to SC hydration and sebum level measured on either body site.

Table 2. Measurements in the forearm.

	NSS		SS		AR		AD		SS *vs.* NSS	SS *vs.* AD	SS *vs.* AR	NSS *vs.* AD	NSS *vs.* AR	AD *vs.* AR
	n	median (range)	n	median (range)	n	median (range)	n	median (range)						
Biophysical measurements														
TEWL [g/m²h]	29	13.2 (7.1-21.9)	27	11.4 (5.8-17.7)	26	11.3 (7.0-16.2)	11	13.8 (8.9-28.4)	0.076	0.082	n.s.	n.s.	0.038	0.044
SC hydration [a.u.]	29	31.7 (19.4-55.6)	27	33.9 (16.7-57.7)	26	31.7 (19.4-53.4)	11	31.3 (18.1-39.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Sebum [a.u.]	29	1.0 (0.0-152.0)	27	0.3 (0.0-142.3)	26	1.5 (0.0-138.7)	11	1.0 (0.0-92.3)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CRS parameters														
SC thickness [µm]	29	16.6 (13.5-24.2)	27	16.4 (13.7-23.5)	26	16.9 (14.2-20.3)	11	16.5 (13.6-19.3)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AUC Water 4-SC [a.u.]	29	621.2 (478.6-1010.0)	27	600.5 (503.9-852.3)	26	619.7 (518.2-735.1)	11	582.4 (491.8-719.1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Average NMF 4-12µm [a.u.]	29	1.1 (0.5-1.8)	27	1.0 (0.7-1.8)	26	1.0 (0.6-1.7)	11	0.9 (0.6-1.3)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Average Cer 4-12µm [a.u.]	29	43.8 (27.0-81.5)	27	45.1 (38.8-68.9)	26	44.4 (24.6-58.5)	11	40.9 (36.2-54.1)	n.s.	0.007	n.s.	n.s.	n.s.	n.s.

AD: atopic dermatitis; AR: allergic rhinoconjunctivitis; a.u.: arbitrary units; AUC: area under the curve; Cer: ceramides/fatty acids; CRS: confocal Raman microspectroscopy; NMF: natural moisturizing factor; n.s.: no statistically significant difference; NSS: non-sensitive skin; SC: stratum corneum; SS: sensitive skin; TEWL: transepidermal water loss.

Table 3. Measurements in the cheek.

	NSS		SS		AR		AD		SS *vs.* NSS	SS *vs.* AD	SS *vs.* AR	NSS *vs.* AD	NSS *vs.* AR	AD *vs.* AR
	n	median (range)	n	median (range)	n	median (range)	n	median (range)						
Biophysical measurements														
TEWL [g/m²h]	30	23.8 (12.8-40.8)	28	22.7 (10.2-37.5)	27	23.0 (13.1-40.9)	11	23.2 (15.3-42.7)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SC hydration [a.u.]	30	37.9 (9.0-62.3)	28	30.7 (7.3-54.4)	27	29.8 (16.2-59.0)	11	36.7 (18.5-88.2)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Sebum [a.u.]	30	48.3 (0.3-290.0)	28	50.8 (0.0-209.3)	27	46.7 (0.0-373.0)	11	31.0 (4.3-215.7)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CRS parameters														
SC thickness [µm]	30	14.1 (11.6-17.3)	28	14.0 (10.8-21.5)	27	14.3 (12.0-19.1)	11	13.7 (12.6-15.9)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AUC Water 4-SC [a.u.]	30	516.9 (417.7-639.1)	28	507.5 (393.4-780.4)	27	512.1 (425.5-777.3)	11	482.9 (433.9-623.5)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Average NMF 4-8µm [a.u.]	30	0.9 (0.5-1.3)	28	0.8 (0.5-1.5)	27	0.8 (0.4-1.3)	11	0.8 (0.4-1.0)	n.s.	n.s.	n.s.	0.091	n.s.	n.s.
Average Cer 4-8µm [a.u.]	30	82.2 (47.5-142.9)	28	72.1 (45.1-155.1)	27	72.0 (35.4-143.2)	11	66.3 (39.2-92.0)	0.077	n.s.	n.s.	0.097	n.s.	n.s.

AD: atopic dermatitis; AR: allergic rhinoconjunctivitis; a.u.: arbitrary units; AUC: area under the curve; Cer: ceramides/fatty acids; CRS: confocal Raman microspectroscopy; NMF: natural moisturizing factor; n.s.: no statistically significant difference; NSS: non-sensitive skin; SC: stratum corneum; SS: sensitive skin; TEWL: transepidermal water loss.

Table 4. Measurements in the thenar.

	NSS		SS		AR		AD		SS *vs.* NSS	SS *vs.* AD	SS *vs.* AR	NSS *vs.* AD	NSS *vs.* AR	AD *vs.* AR
	n	median (range)	n	median (range)	n	median (range)	n	median (range)						
Biophysical measurements														
TEWL [g/m²h]	29	39.6 (21.8-80.6)	28	29.9 (19.4-67.8)	27	32.6 (16.5-58.6)	11	39.9 (21.1-60.8)	0.016	0.058	n.s.	n.s.	0.050	0.071
SC hydration [a.u.]	29	32.8 (15.2-82.2)	28	27.0 (9.1-42.5)	27	33.0 (15.3-47.9)	11	29.9 (13.9-56.3)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CRS parameters														
SC thickness [µm]	29	97.6 (57.1-159.1)	28	97.4 (40.1-159.6)	27	111.7 (43.9-149.7)	11	96.7 (41.4-144.2)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AUC Water 10-SC [a.u.]	29	3702.2 (2176.8-6659.5)	28	3741.9 (1401.8-6642.8)	27	4140.7 (1629.3-5609.8)	11	3840.5 (1447.8-5263.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Average NMF 10-50µm [a.u.]	29	1.7 (1.1-2.2)	28	1.8 (1.1-2.6)	27	1.9 (1.1-2.5)	11	1.9 (0.9-2.0)	n.s.	n.s.	n.s.	n.s.	0.076	n.s.

AD: atopic dermatitis; AR: allergic rhinoconjunctivitis; a.u.: arbitrary units; AUC: area under the curve; CRS: confocal Raman microspectroscopy; NMF: natural moisturizing factor; n.s.: no statistically significant difference; NSS: non-sensitive skin; SC: stratum corneum; SS: sensitive skin; TEWL: transepidermal water loss.

Molecular composition measured by CRS

The average concentration profiles of water, NMF, and ceramides in the forearm and cheek for all groups are shown in Figure 1. The SC thickness, SC water content, and average NMF and ceramides levels in the forearm, cheek, and thenar are reported in Tables 2-4. Numerical values of water, NMF and ceramides at each depth and body site are shown in supplementary Tables S1-S3.

No significant differences in SC thickness and SC water content were found between the groups in either body site.

With respect to the average NMF at 4-12 µm measured in the forearm, no significant differences emerged between the groups, yet it is possible to observe a trend towards lower levels in the AD subjects compared to the other subjects (Figure 1). Similarly, in the cheek, AD subjects showed a trend to lower average NMF at 4-8 µm compared to NSS subjects ($p=0.091$). AR subjects tended to have higher average NMF in the thenar at 10-50 µm compared to NSS subjects ($p=0.076$).

With respect to the average ceramides/fatty acids at 4-12 µm measured in the forearm, SS subjects showed higher levels compared to AD subjects ($p=0.007$), whereas no differences emerged between the other groups. In the cheek, SS subjects tended to have lower average ceramides at 4-8 µm compared to NSS subjects, yet these values were again higher than in subjects with AD ($p=0.097$). This trend is more pronounced at the skin surface (Figure 1). Ceramides were not detectable in the thenar.

Presence of FLG mutations

In total, 10 subjects were found to be carriers of FLG mutations (either $FLG^{-/-}$ or $FLG^{+/-}$; Table 1). Differences between subjects with $FLG^{-/-}$ or $FLG^{+/-}$ and subjects with $FLG^{+/+}$ were present in the forearm, with the former having lower NMF levels ($p=0.017$) and a trend towards lower water content and thinner SC ($p=0.071$ and $p=0.058$, respectively). Similarly, in the thenar, subjects with $FLG^{-/-}$ or $FLG^{+/-}$ had lower NMF levels, lower water content and thinner SC ($p=0.000$) compared to subjects with $FLG^{+/+}$. The results found in the cheek are consistent with these findings, albeit no significant differences were present. Numerical results are shown in supplementary Table S4.

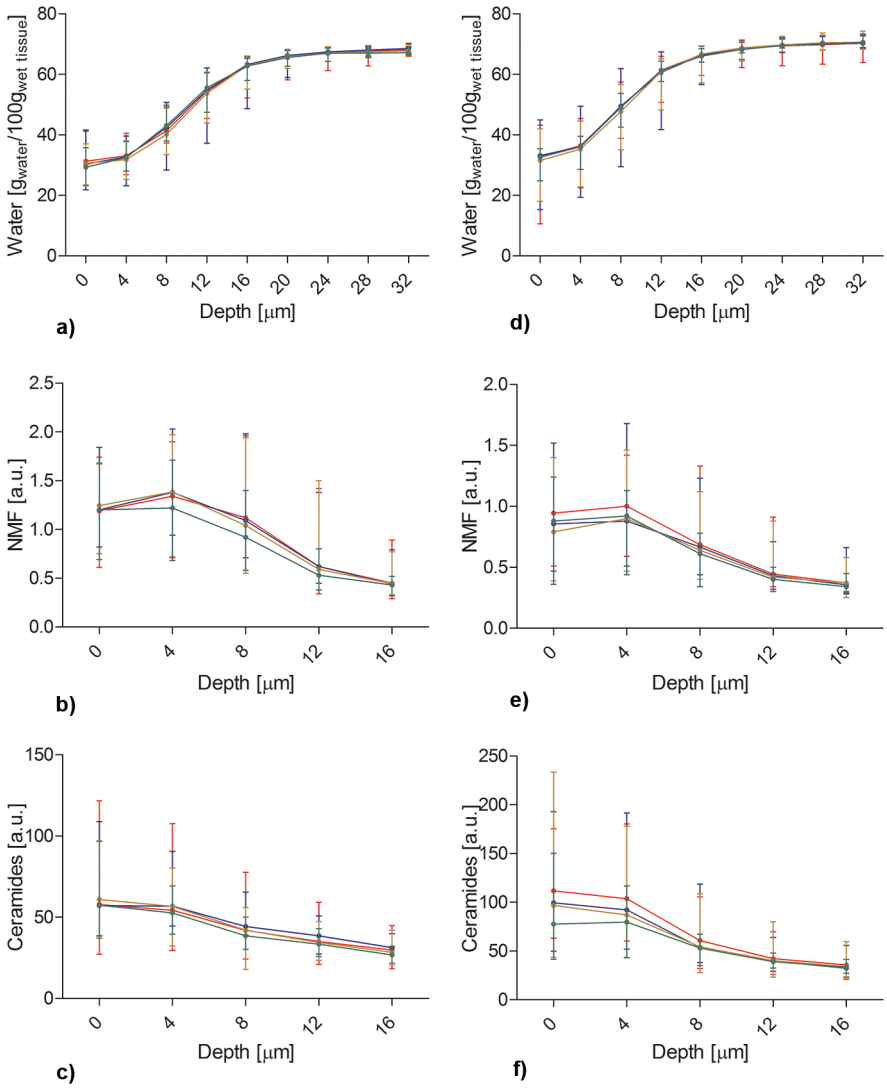

Figure 1. Average concentration profiles of water (**a**, **d**), natural moisturizing factor (**b**, **e**) and ceramides/fatty acids (**c**, **f**) at different depths (µm) measured in the volar forearm (**a**-**c**) and cheek (**d**-**f**). Profiles represent medians (range). Red = non-sensitive skin (NSS), blue = sensitive skin (SS), brown = allergic rhinoconjunctivitis (AR), green = atopic dermatitis (AD).

Correlations between macroscopic biophysical measurements and CRS

A moderate to weak negative correlation was found between TEWL and NMF levels in the forearm (rho = -0.220, *p=0.034*, n=93), in the cheek (rho = -0.408, *p=0.000*, n=96) and in the thenar (rho = -0.208, *p=0.044*, n=95). In the thenar, TEWL correlated weakly with the SC water content (rho = 0.207, *p=0.044*, n=95) and with the SC thickness (rho = 0.202, *p=0.050*, n=95). Of note, the weak correlation with the SC water content remained also when controlling for the corresponding SC thickness. No further correlations were found between TEWL and SC water content, SC thickness or ceramides levels measured in the forearm and cheek.

SC hydration measured with capacitance was not found to correlate with the SC water content in either the forearm, cheek or thenar. In the forearm, on the other hand, capacitance correlated weakly with the water content in the middle part of the SC (4-12 µm) and with the water content up to the upper epidermis (4-32 µm) (rho = 0.222, *p=0.032*, and rho = 0.207, *p=0.046*, n=93, respectively). In the cheek, capacitance correlated weakly with the NMF levels (rho = 0.223, *p=0.029*, n=96).

Penetration kinetics of glycerol

Evidence for the presence of exogenous glycerol was visible only at the skin surface (0-4 µm) and in the first three minutes following application, as verified by the difference spectra shown in Figure 2. Measured levels of glycerol in subjects with NSS and SS are reported in Table 5. NSS subjects showed consistently higher values of glycerol than subjects with SS, reaching statistical significance at one minute after application (*p=0.030*). In both groups, glycerol levels declined slightly from the first to the third minute after application. Baseline levels of endogenous glycerol measured at the skin surface (0-4 µm) were not different between SS and NSS subjects and, in both groups, were significantly lower that the levels measured in the first three minutes after topical glycerol application (*p<0.008*).

Table 5. Levels of glycerol at a depth of 0-4 µm at baseline and after application of a solution of glycerol (1%) in demineralized water on the volar forearm for 1 minute.

	NSS		SS		
	n	median (range)	n	median (range)	
Glycerol content [a.u.]					
Baseline	20	783 (643-1280)	20	816 (656-1008)	n.s.
1 minute	22	1274 (835-2205)	20	1039 (783-1545)	0.030
2 minutes	21	1243 (808-2259)	20	1038 (646-1962)	n.s.
3 minutes	20	1208 (785-2016)	20	994 (672-1925)	n.s.

a.u.: arbitrary units; n.s.: no statistically significant difference; NSS: non-sensitive skin; SS: sensitive skin.

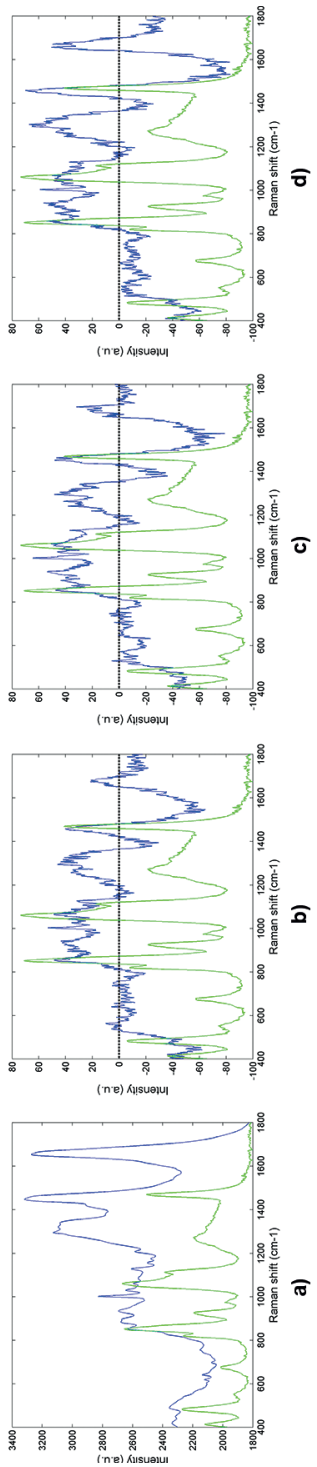

Figure 2. a) The Raman spectrum of normal skin in the fingerprint region (400-1800 cm^{-1}). The difference Raman spectrum obtained by subtraction of the Raman spectra where glycerol was not detectable from the Raman spectra where glycerol was detectable at 1 minute (**b**), 2 minutes (**c**) and 3 minutes (**d**) after topical glycerol application. The Raman spectrum of glycerol (light green) is superimposed for clarity.

DISCUSSION

The main objective of this study was to investigate whether the barrier function is aberrant in SS by directly measuring the molecular composition of the SC by means of CRS. A comparison with the molecular composition of the SC in subjects with NSS, AD and AR was made, together with the indirect assessment of the skin barrier by means of TEWL and capacitance. In line with the literature, also in this study SS subjects reported more frequently dry facial and body skin compared to subjects with NSS. However, we did not find strong evidence to support a hypothesis of an impaired skin barrier in SS in terms of SC water and NMF levels in either the forearm, cheek or thenar. SC thickness was also not different between the groups, which is in line with our previous findings [21]. It is interesting, however, that ceramides/fatty acids showed a trend towards lower levels in the cheek of SS subjects compared to the NSS group, whereas non-significant higher values were found in the forearm. Several studies have suggested the role of an impaired skin barrier function in SS [13, 20, 21] and one in particular demonstrated lower ceramides in the facial skin of subject with SS [27]. This hypothesis concerning a weaker SC barrier is also supported by previous clinical studies performed by our group, in which the same perception-based questionnaire was used to select volunteers with SS and NSS [21, 36]. In those clinical studies, the SC of subjects with SS was shown to be more vulnerable to chemical and mechanical stimuli than the SC of subjects with NSS, suggesting an impaired barrier. Additional mechanisms to those investigated in this study might thus play a major role in the barrier function impairment in SS, including reduced intercorneocyte adhesion, different organization of lipids in the intercellular lamellae, increased inflammatory reactions, and an altered number of mast cells in response to a range of stimuli, as previously reported by our group [21, 35, 36].

In addition to barrier function impairment via alterations in molecular balance of NMF, ceramides and water, an involvement of the family of transient receptor potential (TRP) channels has been hypothesized to play a role in SS [40]. TRP channels are sensory receptors activated by a variety of external stimuli and known to mediate a range of skin sensations including pain, itch and burning feeling. Increased levels or an upregulated sensitivity of these receptors might account for the previously reported increased perception of SS to a variety of stimuli [21, 35, 36]. One could also speculate that the more frequent self-reported perception of skin dryness in SS subjects might be primarily mediated by these receptors, because of the lack of significant differences in SC water content and NMF levels in SS compared to NSS subjects demonstrated here.

The possible association of SS with atopic conditions, as proposed previously [22-24], remains less clear. We did detect lower NMF and water levels and thinner SC in subjects with FLG mutations, which is well-documented in literature [31, 32], albeit indirectly using CRS and in a small population. However, despite frequently reporting SS, in line with previous findings [37], no differences emerged between the AR and NSS and SS subjects, at least with respect

to the inspected parameters. This could be partially due to the overlap in the characteristics of subjects in the groups with respect to skin sensitivity and symptoms, making it difficult to obtain distinct groups since AD and AR subjects frequently report SS [37]. Despite this difficulty, differentiating between subjects with atopy and subjects without atopy is important, since SS cannot be solely explained by having this condition [19]. In fact, the higher ceramides/fatty acids levels found in the forearm of SS subjects compared to AD subjects, together with the observation that FLG mutations do not seem to be reported more frequently in the SS group compared to NSS subjects, do not support the hypothesis of SS being a subclinical form of AD [40]. This is also supported by the lack of association between SS and the dysbiosis of the cutaneous microbiota found in a previous study, in contrast with AD, known to be characterized by overabundance of S*taphylococcus aureus* [40, 41]. Yet, should the TRP receptor family be involved in underlying SS sensations, this could suggest at least one common interface with AD, where these receptors were shown to mediate sensory discomfort and inflammation, at least in murine model [40, 42-44].

In our previous clinical studies we showed a reduced number of mast cells and a reduced number of tapes required to strip off the total SC in the lower back of SS compared to NSS subjects [21, 35, 36]. It could be tempting to speculate that the stronger expression of ceramides in the forearm of SS subjects might be due to a compensating mechanism of the primary defect in activation of the innate immune system, or to an impaired intercorneocyte adhesion [21, 35, 36, 45]. This hypothesis might not be valid for the cheek, which is characterized by a higher number of mast cells compared to other body sites, and thus does not need a compensatory mechanism [46].

Despite the different results on ceramides levels between the forearm and cheek, we are of the opinion that the response of SS is rather universal. Farage *et al.* [47] evaluated whether people claiming to have SS in general also claimed to have SS at specific body sites. Most descriptions of facial and body skin were consistent with the perception of SS in general (60.7% and 68.4% of responders, respectively) or varied by one degree out of four with respect to severity (36.7% and 31.3% of responders, respectively). Our previous findings also support the hypothesis of a generalized SS based on possibly altered immune responses in SS [21].

The second objective of this study was to investigate whether topicals penetrate faster through the skin of subjects with SS compared to subjects with NSS as a result of possible barrier function impairment. We found consistently higher levels of glycerol in the superficial layers of the SC in NSS subjects compared to SS subjects in the first three minutes after application. This might imply a faster spreading of glycerol on the superficial skin of SS because of barrier function impairment. Our results clearly demonstrate that CRS has the potential to detect differences in such assessment provided that a sufficiently long application time is chosen for the exogenous substance to be applied [48].

As a final remark, we confirm the difficulty of finding correspondences between the measurements performed with the macroscopic biophysical methods (TEWL and capacitance)

and the molecular composition of the SC measured with CRS, as demonstrated by the lack of correlations found in this study. In previous studies, the skin barrier function of SS subjects was predominantly analyzed at baseline or after stimulation using these macroscopic biophysical methods and significant differences between SS and NSS subjects could rarely be detected [13, 14, 16, 18, 20, 26, 49, 50]. A range of limitations of these easy-to-use, rapid measurements are known [33], and our study demonstrates that more sensitive and specific tools for the *in vivo* analysis of the skin barrier in general and in SS in particular are needed.

In conclusion, we propose that SS is not a subclinical form of AD and that the skin barrier is not impaired in terms of SC thickness and in terms of water, NMF and ceramides content. Treatments of SS solely based on hydration and ceramides supplementation do not seem to correspond to the identified SC properties, although benefits have been reported. More research efforts should be directed at unraveling the role of the cutaneous nervous system, in particular the involvement of TRP channels, on the onset of subjective perceptions of sensitive and dry skin. Among other mechanisms to be investigated in SS, we would include the role of an altered immune response and intercorneocyte adhesion.

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SUPPLEMENTARY MATERIAL

Table S1. Supplementary table forearm.

	NSS		SS		AR		AD		SS *vs.* NSS	SS *vs.* AD	SS *vs.* AR	NSS *vs.* AD	NSS *vs.* AR	AD *vs.* AR
	n	median (range)	n	median (range)	n	median (range)	n	median (range)						
Water levels [$g_w/100g_t$]														
0	29	31.3 (23.3-41.2)	27	30.4 (21.9-41.6)	26	30.6 (23.2-37.1)	11	29.3 (23.3-35.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	29	33.1 (26.8-40.5)	27	32.7 (23.2-39.7)	26	31.8 (25.3-38.0)	11	32.5 (28.0-37.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	29	41.4 (37.3-49.0)	27	42.4 (28.4-50.8)	26	40.2 (33.5-49.5)	11	43.1 (38.0-49.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
12	29	54.4 (45.4-60.8)	27	54.8 (37.3-62.1)	26	53.8 (43.9-60.7)	11	55.6 (47.5-60.5)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
16	29	62.8 (52.2-65.8)	27	63.2 (48.7-65.9)	26	62.9 (55.1-66.1)	11	62.8 (58.0-65.3)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
20	29	66.0 (58.2-67.9)	27	66.2 (59.0-68.3)	26	65.9 (61.9-68.1)	11	65.6 (62.7-67.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
24	29	66.9 (61.3-68.9)	27	67.4 (64.3-68.8)	26	66.9 (64.3-69.2)	11	67.0 (64.3-68.7)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
28	29	67.7 (62.8-68.9)	27	68.1 (66.0-69.2)	26	67.4 (65.5-69.6)	11	67.1 (65.5-68.6)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
32	29	68.2 (65.9-69.2)	27	68.5 (66.7-70.2)	26	67.8 (66.0-69.8)	11	67.2 (66.2-69.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NMF levels [a.u.]														
0	29	1.2 (0.6-1.7)	27	1.2 (0.8-1.8)	26	1.2 (0.8-1.7)	11	1.2 (0.7-1.7)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	29	1.3 (0.7-1.9)	27	1.4 (0.9-2.0)	26	1.4 (0.7-2.0)	11	1.2 (0.7-1.7)	n.s.	0.095	n.s.	n.s.	n.s.	n.s.
8	29	1.1 (0.6-2.0)	27	1.1 (0.7-2.0)	26	1.0 (0.6-1.9)	11	0.9 (0.6-1.4)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
12	29	0.6 (0.3-1.4)	27	0.6 (0.5-1.4)	26	0.6 (0.4-1.5)	11	0.5 (0.4-0.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
16	29	0.5 (0.3-0.9)	27	0.4 (0.3-0.8)	26	0.4 (0.3-0.8)	11	0.4 (0.3-0.5)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ceramides levels [a.u.]														
0	29	57.8 (27.3-121.7)	27	57.2 (38.5-108.8)	26	61.0 (38.1-97.1)	11	57.5 (37.1-96.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	29	54.3 (29.5-107.5)	27	56.9 (44.6-90.6)	26	56.7 (32.5-80.3)	11	52.7 (39.6-69.4)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	29	41.9 (24.4-77.6)	27	44.3 (38.8-65.4)	26	42.1 (17.9-55.9)	11	38.6 (30.3-50.1)	0.059	0.001	0.065	n.s.	n.s.	n.s.
12	29	35.0 (21.1-59.2)	27	38.6 (27.5-50.8)	26	34.4 (23.5-47.4)	11	33.4 (25.8-43.0)	0.064	0.004	0.060	0.096	n.s.	n.s.
16	29	29.8 (18.4-44.8)	27	31.3 (21.9-39.8)	26	28.5 (20.7-42.1)	11	26.8 (21.8-32.0)	n.s.	0.019	n.s.	0.064	n.s.	n.s.

AD: atopic dermatitis; AR: allergic rhinoconjunctivitis; a.u.: arbitrary units; $g_w/100g_t$: grams of water/100 grams of wet tissue; NMF: natural moisturizing factor; n.s: no statistically significant difference; NSS: non-sensitive skin; SS: sensitive skin.

Table S2. Supplementary table cheek.

	NSS		SS		AR		AD		SS *vs.* NSS	SS *vs.* AD	SS *vs.* AR	NSS *vs.* AD	NSS *vs.* AR	AD *vs.* AR
	n	median (range)	n	median (range)	n	median (range)	n	median (range)						
Water levels [$g_w/100g_t$]														
0	30	32.7 (10.6-43.2)	28	33.2 (15.4-45.0)	27	31.5 (18.0-42.1)	11	32.6 (24.8-35.4)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	30	36.4 (22.5-45.4)	28	36.1 (19.4-49.5)	27	35.3 (22.8-44.6)	11	36.1 (28.6-39.6)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	30	49.2 (38.9-57.4)	28	49.5 (29.5-61.9)	27	47.8 (35.1-56.6)	11	49.1 (42.6-53.7)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
12	30	61.4 (50.8-66.0)	28	60.7 (41.8-67.5)	27	60.9 (48.3-65.1)	11	61.3 (57.7-64.3)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
16	30	66.5 (59.6-69.3)	28	66.4 (56.7-69.3)	27	66.7 (57.2-69.5)	11	66.0 (64.0-68.5)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
20	30	68.6 (62.3-70.6)	28	68.2 (64.9-71.1)	27	68.8 (64.3-71.4)	11	68.3 (67.1-71.1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
24	30	69.5 (62.9-71.8)	28	69.6 (67.2-72.1)	27	69.8 (67.5-72.6)	11	69.5 (68.7-71.7)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
28	30	69.9 (63.4-72.9)	28	70.1 (68.0-72.5)	27	70.5 (68.0-73.7)	11	70.2 (69.4-72.6)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
32	30	70.4 (63.9-72.9)	28	70.3 (68.7-72.7)	27	70.6 (68.3-74.3)	11	70.5 (68.9-73.4)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NMF levels [a.u.]														
0	30	0.9 (0.5-1.4)	28	0.9 (0.5-1.5)	27	0.8 (0.4-1.4)	11	0.9 (0.4-1.2)	n.s.	n.s.	n.s.	n.s.	0.080	n.s.
4	30	1.0 (0.6-1.4)	28	0.9 (0.5-1.7)	27	0.9 (0.5-1.5)	11	0.9 (0.4-1.1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	30	0.7 (0.4-1.3)	28	0.7 (0.4-1.2)	27	0.6 (0.4-1.1)	11	0.6 (0.3-0.8)	n.s.	n.s.	n.s.	0.065	n.s.	n.s.
12	30	0.4 (0.3-0.9)	28	0.4 (0.3-0.7)	27	0.4 (0.3-0.9)	11	0.4 (0.3-0.5)	n.s.	n.s.	n.s.	0.057	n.s.	n.s.
16	30	0.4 (0.3-0.6)	28	0.4 (0.3-0.7)	27	0.4 (0.3-0.6)	11	0.3 (0.3-0.5)	n.s.	n.s.	n.s.	0.080	n.s.	n.s.
Ceramide levels [a.u.]														
0	30	111.7 (63.2-175.4)	28	99.5 (49.9-192.7)	27	96.7 (43.7-233.4)	11	77.6 (41.5-150.3)	n.s.	n.s.	n.s.	n.s.	0.095	n.s.
4	30	103.6 (60.3-180.3)	28	92.2 (52.0-191.6)	27	86.9 (43.0-177.7)	11	79.7 (43.4-116.9)	n.s.	n.s.	n.s.	0.085	n.s.	n.s.
8	30	60.7 (31.9-105.5)	28	53.7 (38.2-118.6)	27	54.2 (28.0-108.7)	11	52.9 (35.1-67.2)	0.086	n.s.	n.s.	0.097	n.s.	n.s.
12	30	42.1 (26.0-69.8)	28	39.8 (29.2-64.0)	27	40.2 (23.2-79.8)	11	39.1 (32.7-48.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
16	30	35.8 (21.0-55.8)	28	32.5 (23.3-55.7)	27	34.1 (21.6-59.8)	11	33.5 (27.3-41.4)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

AD: atopic dermatitis; AR: allergic rhinoconjunctivitis; a.u.: arbitrary units; $g_w/100g_t$: grams of water/100 grams of wet tissue; NMF: natural moisturizing factor; n.s: no statistically significant difference; NSS: non-sensitive skin; SS: sensitive skin.

Table S3. Supplementary table thenar.

	NSS		SS		AR		AD		SS *vs.* NSS	SS *vs.* AD	SS *vs.* AR	NSS *vs.* AD	NSS *vs.* AR	AD *vs.* AR
	n	median (range)	n	median (range)	n	median (range)	n	median (range)						
Water levels [$g_w/100g_t$]														
0	29	28.6 (11.4-38.4)	28	26.5 (13.4-41.6)	27	24.0 (13.5-37.2)	11	20.6 (10.5-37.3)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	29	32.1 (20.7-42.4)	28	31.8 (20.5-40.1)	27	31.0 (21.1-37.7)	11	29.3 (21.1-45.6)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
20	29	34.4 (28.4-40.3)	28	34.9 (29.8-41.8)	27	33.7 (26.6-41.4)	11	33.7 (25.4-43.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
30	29	37.9 (32.6-44.2)	28	36.9 (32.1-51.0)	27	36.5 (31.1-51.4)	11	37.0 (30.0-51.1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
40	29	38.0 (34.2-48.0)	28	38.3 (33.8-60.9)	27	38.1 (32.7-62.0)	11	38.9 (32.0-62.6)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
50	29	39.1 (35.0-57.0)	28	39.9 (35.2-63.7)	27	39.8 (33.3-65.1)	11	39.9 (31.7-65.8)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
60	29	42.5 (35.6-62.0)	28	42.4 (36.6-65.9)	27	41.3 (33.8-67.4)	11	41.5 (33.1-67.1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
70	29	45.6 (36.4-63.5)	28	45.9 (37.6-67.5)	27	43.3 (34.4-69.0)	11	41.7 (34.3-67.9)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
80	29	51.3 (37.3-64.5)	28	52.1 (38.6-68.7)	27	45.5 (34.7-70.0)	11	49.0 (34.6-68.7)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
90	29	58.3 (38.3-65.5)	28	57.5 (39.6-69.3)	27	50.4 (34.3-71.1)	11	55.5 (35.6-70.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
100	29	61.3 (39.4-66.7)	28	61.5 (40.5-69.9)	27	55.7 (34.0-71.8)	11	58.3 (38.2-71.5)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
120	29	65.6 (41.0-67.7)	28	63.6 (42.6-71.9)	27	61.5 37.0-73.1)	11	61.9 (43.8-73.1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
150	29	66.8 (56.1-70.2)	28	65.7 (47.4-74.1)	27	64.6 (45.2-74.6)	11	65.8 (50.4-75.2)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NMF levels [a.u.]														
0	29	1.4 (0.9-3.0)	28	1.5 (1.0-2.6)	27	1.6 (0.9-2.3)	11	1.5 (0.8-2.5)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	29	1.8 (1.2-2.2)	28	1.8 (1.2-2.4)	27	2.0 (1.1-2.5)	11	1.9 (0.9-2.2)	n.s.	n.s.	n.s.	n.s.	0.043	n.s.
20	29	1.8 (1.1-2.2)	28	1.9 (1.3-2.5)	27	2.0 (1.1-2.6)	11	1.9 (0.9-2.3)	n.s.	n.s.	n.s.	n.s.	0.063	n.s.
30	29	1.8 (1.1-2.2)	28	1.9 (1.0-2.6)	27	2.0 (1.1-2.5)	11	1.9 (0.8-2.3)	n.s.	n.s.	n.s.	n.s.	0.069	n.s.
40	29	1.8 (0.7-2.2)	28	1.8 (0.5-2.6)	27	1.9 (1.0-2.56)	11	1.9 (0.5-2.1)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
50	29	1.7 (0.5-2.1)	28	1.7 (0.4-2.4)	27	1.8 (0.8-2.4)	11	1.7 (0.4-2.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

AD: atopic dermatitis; AR: allergic rhinoconjunctivitis; a.u.: arbitrary units; $g_w/100g_t$: grams of water/100 grams of wet tissue; NMF: natural moisturizing factor; n.s.: no statistically significant difference; NSS: non-sensitive skin; SS: sensitive skin.

Table S4. Supplementary table filaggrin mutations.

	n	**FLG$^{+/+}$** median (range)		n	**FLG$^{+/-}$ and FLG$^{-/-}$** median (range)		
CRS parameters Forearm							
SC thickness [µm]	82	16.5	(13.5-24.2)	10	15.7	(14.1-17.9)	0.058
AUC Water 4-SC [a.u.]	82	612.7	(478.6-1010.0)	10	584.4	(505.8-674.9)	0.071
Average NMF 4-12µm [a.u.]	82	1.0	(0.6-1.8)	10	0.9	(0.5-1.2)	0.017
Average Cer 4-12µm [a.u.]	82	44.7	(24.6-81.5)	10	44.4	(33.9-56.0)	1
CRS parameters Cheek							
SC thickness [µm]	84	14.1	(10.8-21.5)	10	13.7	(11.6-14.5)	n.s.
AUC Water 4-SC [a.u.]	84	508.9	(393.4-780.4)	10	491.8	(417.7-562.7)	n.s.
Average NMF 4-8µm [a.u.]	84	0.8	(0.4-1.5)	10	0.7	(0.5-1.2)	n.s.
Average Cer 4-8µm [a.u.]	84	74.7	(35.4-155.1)	10	75.7	(45.1-87.2)	n.s.
CRS parameters Thenar							
SC thickness [µm]	85	101.9	(40.1-159.6)	10	66.9	(41.4-120.2)	0.000
AUC Water 10-SC [a.u.]	85	3917.7	(1401.8-6659.5)	10	2500.0	(1447.8-4279.1)	0.000
Average NMF 10-50µm [a.u.]	85	1.9	(1.1-2.6)	10	0.9	(0.6-1.1)	0.000

a.u.: arbitrary units; AUC: area-under-curve; Cer: ceramides/fatty acids; CRS: confocal Raman microspectrosocpy; FLG$^{-/-}$: homozygous filaggrin mutation; FLG$^{+/-}$: heterozygous filaggrin mutation; FLG$^{+/+}$: no filaggrin mutation; n.s.: no statistically significant difference; NMF: natural moisturizing factor; SC: stratum corneum.

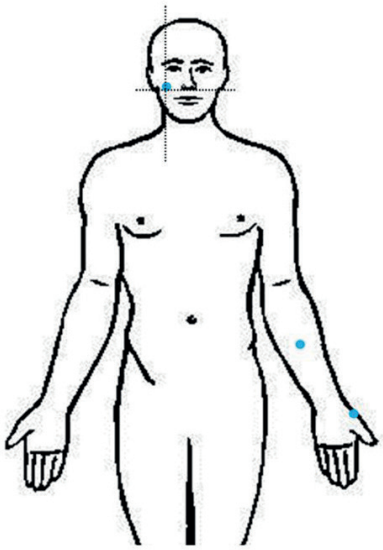

Figure S1. Body locations where the measurements with confocal Raman microspectroscopy (CRS) were performed.

Chapter 3

Vascular and sensory reactivity in sensitive skin

ABBREVIATIONS

a*	CIE spectrum (Commission Internationale de l'Éclairage): a*, b* and L* values
a.u.	arbitrary units
BM	Basal membrane
BSA	Bovine serum albumin
CLAHE	Contrast-limited adaptive histogram equalization
CMOS	Complementary metal-oxide semiconductor
CV	Coefficient of variation
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAPI	Diamidine phenylindole
HE	Hematoxylin and eosin
IFSI	International forum for the study of itch
LED	Light emitting diode
LTB_4	Leukotriene B4
NSS	Non-sensitive skin
PBS	Phosphate buffered saline
ROI	Region of interest
SC	Stratum corneum
SS	Sensitive skin
TEWL	Transepidermal water loss
VAS	Visual analogue scale

3.1

Histamine iontophoresis as *in vivo* model to study human skin inflammation with minimal barrier impairment: pilot study results of application of the model to a sensitive skin panel

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ABSTRACT

Background/aims: Histamine iontophoresis is known to elicit itch and a wheal-and-flare reaction; however, its impact on the skin barrier and underlying compartments has not been thoroughly evaluated yet. The primary objective of this study was to characterize that using immunohistochemistry, biophysical measurements and image analysis. Secondly, to explore whether skin reactions to this model differ in sensitive skin (SS).

Methods: Eighteen healthy subjects, n=9 with SS and n=9 with non-sensitive skin (NSS), were included based on a perception-based questionnaire. Histamine iontophoresis was performed on the buttock and skin reactions evaluated up to 72 hours after stimulation.

Results: The wheal-and-flare peaked at 30 minutes; after 8 hours, no clinical signs were visible. No signs of disruption of the stratum corneum, as well as no increase in the number of Ki67-positive cells emerged, whereas fewer tryptase-positive mast cells and increased epidermal thickness were observed at 1 hour and 72 hours, respectively. SS subjects showed higher perception of itch compared with NSS subjects.

Conclusion: Histamine iontophoresis is a well-standardized *in vivo* model to quantitatively study the early stages of cutaneous inflammation with minimal impact on the skin barrier. In line with previous studies, it highlighted increased sensory perceptions in SS.

INTRODUCTION

The barrier function of the skin is fulfilled primarily by the stratum corneum (SC), the outermost layer of the epidermis [1]. The integrity of the SC as skin barrier depends on the interplay of several factors, including the process of proliferation of keratinocytes from the basal layer, and their gradual differentiation to corneocytes across the epidermis. Upon disruption of the SC, the release of mediators from keratinocytes, including several interleukins and chemokines [2], prompts the onset of an inflammatory response, resulting in a transient infiltrate of immune cells in the dermis, and hyperproliferation and abnormal differentiation in the epidermis [3].

In order to study the cutaneous inflammatory response *in vivo*, human skin models can be used. These models consist in the application of minimally invasive and standardized challenges to the skin, followed by evaluation of skin reactions at one or several points in time. Examples include tape stripping, consisting in repeated application of adhesive tape to mimic disruption of the skin barrier in disease or following skin-material interactions [3, 4], and application of leukotriene B_4 (LTB_4) to mimic psoriasis-like infiltration of immune cells in the epidermis [5]. In this study, we focus on one such *in vivo* model: histamine iontophoresis. Histamine, either exogenously applied or endogenously released from mast cells, is known to elicit a triple response [6, 7]: itch, because of excitation of mechano-insensitive C-fibers [8]; wheal, due to increased capillary permeability; and flare, induced by the release of vasoactive neuropeptides from afferent nerve fibers [7]. Iontophoresis is based on the use of a small and defined electric current to facilitate the transport of ionized molecules across the skin barrier [9, 10]. In addition, albeit it cannot entirely avoid passive diffusion and variability thereof, iontophoresis is less influenced by the condition of the skin at the application site than topically applied molecules on the SC [10]. Histamine iontophoresis has been widely used as experimental itch model both in healthy and diseased state [6, 7, 11-13] and has been described as a quantifiable model for the study of the early stages of cutaneous inflammation [14]. However, to our best knowledge, no study thoroughly evaluated the effect of this model on the epidermal and dermal compartments.

The primary objective of this study was to characterize the effects of histamine iontophoresis on the epidermal and dermal compartments using immunohistochemistry and non-invasive assessments including biophysical methods and image analysis. In this respect, we propose an easy-to-use method to segment the wheal-and-flare reaction using classical image processing techniques. We investigated in particular the impact on the skin barrier, the underlying hypothesis being that histamine iontophoresis exerts its effects primarily on the dermal vasculature and cutaneous sensory system and can thus be used as a model of acute and local skin inflammation with minimal barrier impairment, in contrast with other *in vivo* models such as tape stripping and application of LTB_4.

As a secondary objective, we explored whether skin responses to histamine iontophoresis differ significantly between subjects with sensitive skin (SS) and non-sensitive skin (NSS). With as much as 50-61% women and 30-44% men reporting SS in the industrialized world, and consensus on the definition and pathomechanism remaining elusive despite extensive research [15], SS skin has been identified as a global challenge [16]. However, recent clinical studies based on the selection of a SS and NSS panel according to a novel perception-based questionnaire [17] and on the application of *in vivo* skin models followed by clinical, biophysical and immunohistochemical analysis, have shown that aberrant vascular and inflammatory reactions, accompanied by enhanced sensory reactivity, were the hallmarks of SS [18-20]. We thus hypothesized that the triple response elicited by histamine iontophoresis might differ between SS and NSS subjects. In addition, since histamine has been reported to be normally prevented from entering the skin unless the barrier is compromised [6, 21], topical application of histamine was added to evaluate whether stronger reactions possibly observed in SS subjects might be due to aberrant properties of the SC [15].

PATIENTS AND METHODS

Study participants

Potential participants were recruited via websites and asked to fill in the novel perception-based questionnaire [17], as previously described [18-20]. Briefly, inclusion was based on self-assessed skin sensitivity and on a sensitive skin score obtained by rating skin perceptions (*i.e.* discomfort, stinging, redness, dryness) following exogenous and endogenous triggers (*i.e.* toiletries, shaving, heat, cold, clothes, emotions). The score was compared with upper and lower quartiles previously determined [17]. If the score was above the upper quartile and the subject reported slightly higher or much higher SS compared to others, he/she was included in the group with SS. If the score was below the lower quartile and the subject reported equal of lower SS compared to others, he/she was included in the group with NSS. Additional criteria for inclusion were Fitzpatrick skin type II-III and willingness to give a written informed consent. Pregnant or breastfeeding women and volunteers with a history of skin diseases, atopic or allergic predisposition (*i.e.* atopic dermatitis, asthma, allergic rhinoconjuctivitis), use of immunosuppressive drugs, compromised skin at the experimental sites (volar forearm and lower back) and presence of implanted electrical devices (*e.g.* cardiac pacemakers) were excluded from participation. Volunteers were asked not to apply toiletries on the experimental sites from 24 hours before the experiments and not to sunbathe or use a tanning bed from two weeks before the experiments. The study was performed in a temperature- and humidity-controlled room at the dermatology department of the Radboud University Medical Center in Nijmegen, the Netherlands, between April and November 2015, and was approved by the ethics

committee Regio Arnhem-Nijmegen. Before the start of the study procedures, volunteers were left to acclimatize for 15 minutes with the body sites to be assessed uncovered.

Randomization

All subjects underwent the same study procedures up to 60 minutes after histamine iontophoresis with respect to the non-invasive measurements, whereas they underwent a randomization with respect to the skin biopsies taken at 1, 8, 24 and 72 hours after histamine iontophoresis: namely, two biopsies at two out of the four possible time points were taken from each volunteer. This design was a compromise between getting insights into skin reactions to histamine iontophoresis at the immunohistochemical level and reducing the burden of the study for the volunteers. The choice of the time points was based on previous studies [18-20].

Histamine iontophoresis

Histamine iontophoresis was performed on the buttock (at the level of the upper side of the intergluteal cleft) at 0.4 mA for 2.5 minutes using an iontophoresis sytem (Chattanooga Group, Hixson, TN, USA) and a pair of silver-silver chloride electrodes with an active area of 7.2 cm^2 (Iomed Iogel Iontophoresis Electrode small; Chattanooga Group, Hixson, TN, USA), as previously described [11, 12]. This resulted in a current density of 0.06 mA/cm^2. The active area was filled with 1.5 mL of a solution of 0.5% histamine dihydrochloride (Allergopharma B.V., Zeist, the Netherlands) in 1% hypromellose gel prepared by the local pharmacy. The pH of the solution was 4.5-5.0. Prior to stimulation, the skin areas where the electrodes needed to be applied were gently rubbed with a 5 cm x 5 cm tissue (Cutisoft, BSN medical GmbH, Hamburg, Germany) soaked in 1.5 mL demineralized water in order to remove sebum and other impurities possibly causing localized increased resistance to the ionic flux. Every 15 s during iontophoretic histamine application, subjects were asked to rate their level of perceived itch on a visual analogue scale (VAS) ranging from 0 (no perception) to 10 (strongest possible itch perception), with 3 being the threshold for the willingness to scratch the skin [6]. The average itch score and the time to reach the scratching threshold were calculated. To avoid evaporative cooling, remaining amounts of gel were wiped off immediately after stimulation [14]. The level of perceived itch was asked also at at 5, 30 and 60 minutes after histamine iontophoresis.

Topical application of histamine

Between 30 and 60 minutes after stimulation with histamine iontophoresis, the same amount of histamine (1.5 mL) was applied topically on the contra lateral side of the buttock (10 cm apart) and, subsequently, on the non-dominant mid volar forearm. Histamine was left on the skin for 2.5 minutes and wiped off immediately afterwards. The application area of 2 cm x 3.5 cm was chosen to approximately match the area of the active electrode. Rating of itch perception during topical histamine application and at 5 minutes after wiping off [21] were performed as for histamine iontophoresis.

Non-invasive measurements

Transepidermal water loss (TEWL) (Aquaflux AF200, Biox, UK) and skin redness (a* value) (Spectrophotometer 2600d, Konica Minolta, Japan) were measured at baseline on the buttock and on the non-dominant volar forearm, and at 5, 30 and 60 minutes after histamine iontophoresis. Depending on the randomization performed for skin biopsies, TEWL and a* value were measured on the buttock also at 8, 24 and 72 hours after stimulation. Measurements were repeated three times on adjacent skin areas and averaged.

Skin photographs were taken at 5, 30 and 60 minutes after histamine iontophoresis with a single-lens reflex digital camera (D3200, Nikon, Japan), equipped with a a 32-Light emitting diode (LED) ring flash (PLMRFN, Polaroid, USA) to provide a shadow-free illumination. An algorithm, implemented in Matlab (version R2013a, The MathWorks Inc., USA), was applied to the digital images to segment the wheal-and-flare reaction. Briefly, different combinations of histogram-based contrast enhancement and thresholding were tested and compared to manual erythema segmentation. The best combinations resulted to be the linear histogram-based contrast enhancement followed by thresholding with the Isodata method [22] for the reaction at 5 and 30 minutes, and the non-linear histogram-based contrast ehancement with Isodata thresholding for the reaction at 60 minutes. The acquisition set-up and the algorithm are described in the supplementary material.

Tissue processing and immunohistochemistry

Prior to the biopsy procedure, the skin was cleansed with chlorhexidine 0.5% m/v in alcohol 70% v/v. Punch biopsies (3 mm) were taken under 1% lidocaine chloride. The specific choice of the anesthetic not containing adrenalin as a vasoconstrictor was made not to counteract the vasodilating effect of histamine. One additional biopsy was taken from non-challenged skin, serving as an internal control. In total, three biopsies were taken from each volunteer. All specimens were fixed in 10% formalin for subsequent paraffin embedding and sectioned at 6 µm thickness. Paraffin-embedded specimens were deparaffinized in histosafe (Adamas Instrumenten B.V., the Netherlands) and rehydrated in decreasing concentrations of alcohol (100-50%) and demineralized water. For K16 and filaggrin, xylene (Klinipath B.V., the Netherlands) was used for deparaffinization. Antigen retrieval was achieved by citrate buffer (pH 6.0, 10 min at 100°C) for Ki67, CD1a, CD31, K16 and filaggrin stainings and by EDTA/Tween-20 (10 mM EDTA, + 0.05% Tween-20, pH 8.0, 10 min at 100°C) for the CD3 staining. The tryptase antibody did not require antibody retrieval steps. Endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for Ki67, CD1a, CD31, CD3 and tryptase antibodies. This step was followed by preincubation in 1% bovine serum albumin (BSA) (Sanquin Reagents, the Netherlands) in phosphate buffered saline (PBS) for 15 minutes for Ki67 and tryptase antibodies, 30 minutes for CD1a and CD3 antibodies and 60 minutes for CD31 antibody. For K16 and filaggrin, 5% normal goat serum (Vector Laboratories Inc., USA) in PBS for 15 minutes was used. Incubation with primary antibodies dissolved in 1% BSA was performed overnight at room temperature, except K16 and filaggrin which were incubated for 60 minutes. The

following clones and dilutions were used: Ki67 (1:100, clone MIB-1, Dako), tryptase (1:100.000, clone AA1, Dako), CD1a (1:200, clone 010, Dako), CD3 (1:100, clone F7.2.38, Abcam), CD31 (1:80, clone JC70A, Dako), K16 (1:50, clone LL025, Biotrend), filaggrin (1:100, polyclonal 19058, BioLegend). For all stainings exept K16 and filaggrin, amplification was obtained by EnVision anti-mouse (Dako, Glostrup, Denmark) for 45 minutes, visualization of the antibody with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma-Aldrich, St. Louis, MO) for 10 minutes and counterstaining with Mayer's hematoxylin (Sigma-Aldrich, Saint Louis, USA). Lastly, specimens were dehydrated in increasing concentrations of alcohol (50-100%) and histosafe and mounted using Permount glue (Thermo Fisher Scientific, Waltham, USA). For K16 and filaggrin, visualization of the antibody was obtained with Alexa Fluor 488 conjugate (1:200) and Alexa Fluor 647 conjugate (1:200) (Invitrogen, USA) in 1% BSA. Counterstaining and mounting were obtained with Fluoromont-G with diamidine phenylindole (DAPI, eBioscience, USA).

Semiquantitative assessment of immunohistochemical markers

Specimens were imaged using Axiokop 2 MOT microscope at a magnification of ×200, Axiocam MRc5 digital camera and AxioVision software (version 4.8, Carl Zeiss, Germany). Semiquantitative analysis of immunohistochemical markers was performed using macro colour deconvolution and color thresholding in ImageJ (version 1.49, National Institutes of Health, USA). The thickness of the SC and of the viable epidermis were measured in hematoxylin and eosin (HE)-stained sections by dividing the SC/viable epidermis area by the average length of the basal membrane (BM) (computed as Feret's distance in ImageJ) [18-20]. Ki67-positive cells were expressed as number of cells per mm BM length. CD1a-positive cells were measured as percentage of the viable epidermal surface where a positive signal was detected. The dermis was assessed from the BM down to 350 µm across the specimen. Tryptase-positive cells were expressed as number of cells per mm^2 dermis. CD31- and CD3-positive cells were expressed as percentage of the dermal surface where a positive signal was detected.

Immunofluorescence images of K16 and filaggrin were obtained using Axio Imager M2 microscope at a magnification of ×200, AxioCam 503 Mono digital camera and ZEN 2 pro software (version 5.8, Carl Zeiss, Germany) and qualitatively compared.

Statistical analysis

Results are presented as median (minimum-maximum). To answer the primary objective of the study, differences between values of the same variable measured at baseline and after stimulation with histamine iontophoresis were analyzed with the Wilcoxon sign rank test for non-parametric repeated measures. Of note, for the immunohistochemical markers measured at 1, 8, 24 and 72 hours and for TEWL and a* value measured at 8, 24 and 72 hours following histamine iontophoresis, a paired comparison with baseline values was performed, *i.e.* baseline values from volunteers sampled at other time points were not pooled together. To answer the secondary objective of the study, differences between SS and NSS subjects were

analyzed at baseline and at 5, 30 and 60 minutes after histamine iontophoresis using the Mann-Whitney exact test for non-parametric independent values. In the comparison between SS and NSS subjects, the following variables were considered: TEWL, a* value, wheal-and-flare area, average VAS score for itch during histamine iontophoresis and topical histamine application and time to reach the scratching threshold during histamine iontophoresis. Correlations were done with the Spearman's rho. Statistical analyses were performed with SPSS Statistics version 20 for Windows (IBM SPSS Inc., USA). A p value ≤0.05 was considered statistically significant. Due to the exploratory nature of this study, no corrections for multiple comparisons were applied.

RESULTS

Study population

A total of 106 potential participants received the questionnaire. Fifty questionnaires were returned and, of these, 32 had to be excluded because of the score being outside the proper range or because of concomitant presence of skin diseases, atopic or allergic predisposition. In total, eighteen subjects were eligible for inclusion in the study, n=9 with SS (one male, eight females) and n=9 with NSS (two males, seven females). In both groups, median age was 21 years old (19-36 in the SS group, 20-32 in the NSS group) and, in both groups, three subjects had Fitzpatrick skin type II and six subjects had Fitzpatrick skin type III. The number of subjects measured at each time point is described in Table 1.

Table 1. Overview of study procedures and measurements including randomization performed for taking skin biopsies at 1, 8, 24 and 72 hours after histamine iontophoresis on the buttock.

		Baseline	During iontophoresis	5min	30min	1h	8h	24h	72h
TEWL	SS	n=9	-	n=9	n=9	n=9	n=4	n=4	n=4
	NSS	n=9	-	n=9	n=9	n=9	n=4	n=4	n=4
a* value	SS	n=9	-	n=9	n=9	n=9	n=4	n=4	n=4
	NSS	n=9	-	n=9	n=9	n=9	n=4	n=4	n=4
VAS score for itch	SS	n=9	n=9	n=9	n=9	n=9	-	-	-
	NSS	n=9	n=9	n=9	n=9	n=9	-	-	-
Skin photograph	SS	-	-	n=9	n=9	n=9	-	-	-
	NSS	-	-	n=9	n=9	n=9	-	-	-
Skin biopsy	SS	n=9*	-	-	-	n=6*	n=4*	n=4	n=4
	NSS	n=9*	-	-	-	n=6*	n=4	n=4	n=4*

NSS: non-sensitive skin; SS: sensitive skin; TEWL: transepidermal water loss; VAS: visual analogue scale
*Biopsies taken from one volunteer with SS at baseline, at 1h and at 8h, as well as biopsies taken from one volunteer with NSS at baseline, at 1h and at 72h could not be evaluated due to damage.

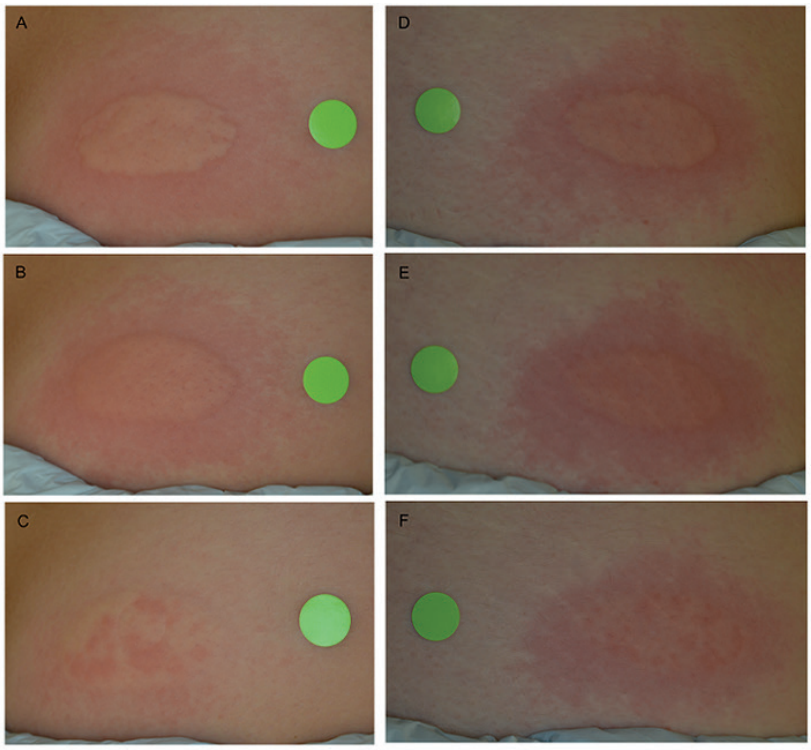

Figure 1. Representative photographs of skin reactions to histamine iontophoresis in two volunteers (**a**,**b**,**c** and **d**,**e**,**f**). **a**,**d**) At 5 minutes, early onset of edema and erythema in the stimulated site and of flare in the surrounding skin were observed. **b**,**e**) At 30 minutes, edema increased while the flare reaction was mostly unchanged. **c**,**f**) At 60 minutes, the edema and the surrounding flare reaction generally decreased, and in most volunteers an irregularly dotted erythema pattern appeared in correspondence of the wheal.

Histamine iontophoresis: clinical assessment and non-invasive measurements

Representative wheal-and-flare reactions at 5, 30 and 60 minutes after histamine iontophoresis are shown in Figure 1. At 8, 24 and 72 hours no wheal or flare were longer visible in skin photographs.

The values of TEWL, a* value and wheal-and-flare area measured at baseline and at 5, 30 and 60 minutes after histamine iontophoresis are shown in Figure 2. In both SS and NSS subjects, TEWL increased by approximately 3.5 g/m^{2}h at 5 minutes and started to recover at 30 minutes, yet at 60 minutes it remained slightly but significantly higher than baseline ($p \leq 0.05$). In both SS and NSS subjects, the a* value increased by approximately 6 arbitrary units (a.u.) at 5 minutes and remained significantly higher than baseline up to 60 minutes ($p \leq 0.05$). Similarly, in both SS and NSS subjects, the wheal-and-flare area at 30 minutes was generally comparable

Table 2. Non-invasive assessments and VAS score for itch measured at baseline and at 5, 30 and 60 minutes after histamine iontophoresis (n=18; n=9 with SS, n=9 with NSS).

	Baseline			5 minutes			30 minutes			60 minutes		
	med	range	p value	med	range	p value	med	range	p value	med	range	p value
TEWL [g/m²h]												
SS	11.5	(8.7 - 16.8)	n.s.	15.2	(11.4-21.5)	n.s.	12.6	(9.8-17.8)	n.s.	12.0	(9.6-16.6)	n.s.
NSS	11.6	(8.3 - 14.7)		15.4	(11.5-18.5)		13.9	(9.5-16.9)		12.8	(9.4-16.0)	
a value [a.u.]*												
SS	4.4	(1.9 - 6.7)	n.s.	10.8	(8.1-13.0)	n.s.	12.1	(9.9-14.3)	n.s.	11.0	(9.5-13.4)	n.s.
NSS	4.0	(3.4 - 5.6)		11.2	(9.1-12.1)		11.3	(11.0-13.9)		8.2	(6.7-13.4)	
Wheal-and-flare area [cm²]												
SS	0.0	(0.0)	n.a.	24.2	(10.8-31.5)	n.s.	24.9	(10.0-34.5)	n.s.	7.9	(4.8-18.7)	n.s.
NSS	0.0	(0.0)		23.8	(16.0-31.1)		20.0	(17.6-27.7)		8.6	(2.8-12.0)	
VAS score for itch [0-10]												
SS	0.0	(0.0)	n.a.	3.0	(1.5-9.5)	n.s.	2.0	(0.0-9.0)	0.004	0.0	(0.0-0.5)	n.s.
NSS	0.0	(0.0)		2.0	(0.0-4.0)		0.0	(0.0-1.0)		0.0	(0.0-0.0)	

a.u.: arbitrary units; med: median; n.a.: not applicable; n.s.: no statistically significant difference; NSS: non-sensitive skin; SS: sensitive skin; TEWL: transepidermal water loss; VAS: visual analogue scale.

p values obtained with Mann-Whitney exact test for non-parametric independent values.

Table 3. Biophysical measurements and (immuno)histochemical markers measured at baseline and at 8, 24 and 72 hours after histamine iontophoresis.

	All (randomized)				SS			NSS		
	n	med	range	p value	n	med	range	n	med	range
Biophysical measurements										
TEWL [g/m²h]										
Baseline 8h	8	9.6	(8.7-14.0)	n.s.	4	9.4	(8.7-11.5)	4	10.3	(8.8-14.0)
8h	8	9.8	(9.3-16.3)		4	9.7	(9.4-10.5)	4	10.4	(9.3-16.3)
Baseline 24h	8	10.9	(8.3-16.8)	n.s.	4	12.3	(10.2-16.8)	4	9.8	(8.3-13.0)
24h	8	10.8	(6.9-15.7)		4	11.9	(9.4-15.7)	4	9.2	(6.9-15.5)
Baseline 72hh	8	12.8	(8.7-16.8)	n.s.	4	12.4	(8.7-16.8)	4	13.5	(11.6-14.7)
72h	8	12.4	(9.2-17.3)		4	12.8	(9.2-17.3)	4	12.2	(10.7-14.9)
a value [a.u.]*										
Baseline 8h	8	4.2	(1.9-6.7)	n.s.	4	5.4	(1.9-6.7)	4	4.0	(3.5-5.3)
8h	8	5.2	(2.2-7.3)		4	6.7	(2.2-7.3)	4	4.5	(3.5-5.2)
Baseline 24h	8	4.6	(3.4-6.6)	0.078	4	4.9	(3.6-6.6)	4	3.9	(3.4-5.6)
24h	8	5.5	(4.0-6.5)		4	5.6	(4.0-6.5)	4	5.1	(4.2-6.1)
Baseline 72h	8	4.2	(1.9-5.6)	0.023	4	4.1	(1.9-4.8)	4	4.7	(4.0-5.6)
72h	8	4.4	(2.3-5.9)		4	4.3	(2.3-5.9)	4	5.0	(3.8-7.2)
Epidermal markers										
SC thickness [µm]										
Baseline 1h	10	19.3	(16.5-32.5)	n.s.	5	21.3	(17.5-29.6)	5	17.3	(16.5-32.5)
1h	10	19.5	(15.0-37.2)		5	20.5	(18.9-28.1)	5	18.6	(15.0-37.2)
Baseline 8h	7	18.2	(15.6-29.6)	n.s.	3	18.5	(18.2-29.6)	4	16.9	(15.6-20.1)
8h	7	18.6	(16.1-27.1)		3	23.2	(21.0-27.1)	4	18.0	(16.1-18.6)
Baseline 24h	8	17.9	(11.3-32.5)	n.s.	4	19.9	(11.3-22.7)	4	16.4	(14.0-32.5)
24h	8	18.3	(11.4-40.3)		4	22.5	(11.4-26.3)	4	16.1	(15.7-40.3)
Baseline 72h	7	18.2	(11.3-20.1)	n.s.	4	17.9	(11.3-18.4)	3	20.1	(14.0-20.1)
72h	7	19.3	(13.7-22.5)		4	17.5	(13.7-21.3)	3	20.4	(16.3-22.5)
Epidermis thickness [µm]										
Baseline 1h	10	64.3	(58.8-78.1)	0.065	5	64.8	(61.3-69.2)	5	63.0	(58.8-78.1)
1h	10	67.5	(61.7-75.5)		5	66.1	(61.7-70.2)	5	69.6	(64.8-75.5)
Baseline 8h	7	67.7	(63.0-80.9)	n.s.	3	67.7	(64.8-69.2)	4	71.1	(63.0-80.9)
8h	7	67.8	(56.2-81.5)		3	67.8	(67.2-76.9)	4	73.4	(56.2-81.5)
Baseline 24h	8	63.9	(58.8-79.4)	0.055	4	65.7	(61.3-79.4)	4	62.7	(58.8-75.5)
24h	8	70.4	(65.7-82.9)		4	71.3	(65.7-72.8)	4	68.8	(66.4-82.9)

	All (randomized)				SS			NSS		
	n	med	range	p value	n	med	range	n	med	range
Baseline 72h	7	69.2	(65.0-80.9)	0.016	4	69.2	(65.7-79.4)	3	75.5	(65.0-80.9)
72h	7	80.3	(71.3-86.3)		4	78.4	(72.1-84.6)	3	80.3	(71.3-86.3)
Ki67 (cells/mm BM)										
Baseline 1h	10	40.8	(23.5-50.0)	n.s.	5	44.0	(23.5-50.0)	5	32.8	(26.0-49.3)
1h	10	30.7	(23.8-52.8)		5	38.6	(26.6-52.8)	5	28.1	(23.8-39.6)
Baseline 8h	7	39.7	(23.5-59.9)	n.s.	3	28.6	(23.5-29.8)	4	48.4	(29.2-59.9)
8h	7	47.8	(28.8-50.1)		3	47.8	(46.0-50.1)	4	44.4	(28.8-49.4)
Baseline 24h	8	46.7	(27.8-50.3)	n.s.	4	47.0	(28.6-50.0)	4	41.0	(27.8-50.3)
24h	8	43.9	(22.4-53.3)		4	43.3	(34.3-53.3)	4	44.7	(22.4-51.5)
Baseline 72h	7	39.7	(26.0-59.9)	n.s.	4	43.1	(37.6-50.0)	3	27.8	(26.0-59.9)
72h	7	39.9	(33.4-85.3)		4	57.1	(33.7-85.3)	3	39.9	(33.4-52.0)
CD1a (% epidermis)										
Baseline 1h	10	4.4	(3.2-6.3)	n.s.	5	3.6	(3.2-5.4)	5	4.5	(4.2-6.3)
1h	10	4.1	(3.2-6.9)		5	3.9	(3.2-6.9)	5	4.2	(4.0-4.7)
Baseline 8h	7	4.4	(3.0-6.6)	n.s.	3	3.6	(3.0-5.7)	4	4.4	(3.9-6.6)
8h	7	4.6	(2.6-7.0)		3	3.6	(3.5-6.1)	4	4.6	(2.6-7.0)
Baseline 24h	8	4.5	(3.2-5.7)	n.s.	4	3.9	(3.2-5.7)	4	4.5	(3.9-4.9)
24h	8	4.7	(2.6-5.5)		4	5.0	(3.1-5.5)	4	3.7	(2.6-5.4)
Baseline 72h	7	4.9	(3.0-6.6)	n.s.	4	4.5	(3.0-5.4)	3	6.3	(4.9-6.6)
72h	7	4.9	(1.9-6.0)		4	4.3	(1.9-4.9)	3	5.0	(5.0-6.0)
Dermal markers										
CD3 (% dermis)										
Baseline 1h	10	0.2	(0.1-0.8)	n.s.	5	0.6	(0.1-0.8)	5	0.2	(0.1-0.2)
1h	10	0.2	(0.1-0.5)		5	0.3	(0.2-0.5)	5	0.2	(0.1-0.5)
Baseline 8h	7	0.3	(0.1-0.5)	n.s.	3	0.4	(0.3-0.5)	4	0.3	(0.1-0.4)
8h	7	0.2	(0.1-0.7)		3	0.2	(0.1-0.3)	4	0.3	(0.2-0.7)
Baseline 24h	8	0.2	(0.1-0.7)	n.s.	4	0.3	(0.1-0.7)	4	0.2	(0.1-0.6)
24h	8	0.4	(0.1-1.0)		4	0.4	(0.3-0.5)	4	0.5	(0.1-1.0)
Baseline 72h	7	0.3	(0.2-0.8)	n.s.	4	0.3	(0.2-0.8)	3	0.4	(0.2-0.6)
72h	7	0.5	(0.2-0.6)		4	0.4	(0.2-0.5)	3	0.5	(0.5-0.6)
CD31 (% dermis)										
Baseline 1h	10	1.0	(0.4-1.9)	n.s.	5	1.2	(0.7-1.9)	5	0.8	(0.4-1.1)
1h	10	1.0	(0.8-1.8)		5	1.1	(0.9-1.8)	5	0.9	(0.8-1.4)
Baseline 8h	7	1.2	(0.4-1.7)	n.s.	3	1.2	(1.2-1.7)	4	1.1	(0.4-1.4)
8h	7	0.8	(0.7-1.4)		3	1.1	(0.8-1.4)	4	0.7	(0.7-0.8)

	All (randomized)				SS			NSS	
	n	med	range	p value	n	med	range	n	med	range
Baseline 24h	8	1.2	(0.5-1.7)	n.s.	4	1.1	(0.7-1.7)	4	1.2	(0.5-1.7)
24h	8	1.0	(0.6-1.4)		4	1.2	(0.8-1.4)	4	0.9	(0.6-1.4)
Baseline 72h	7	1.2	(0.8-1.9)	n.s.	4	1.1	(0.9-1.9)	3	1.2	(0.8-1.7)
72h	7	1.0	(0.6-1.5)		4	1.1	(0.6-1.4)	3	0.8	(0.7-1.5)
Tryptase (cells/mm² dermis)										
Baseline 1h	10	117.0	(79.7-129.4)	0.037	5	114.7	(79.7-129.4)	5	118.4	(85.8-126.1)
1h	10	101.0	(76.0-119.5)		5	104.4	(76.0-112.1)	5	98.0	(79.1-119.5)
Baseline 8h	7	80.6	(61.1-124.5)	n.s.	3	80.6	(79.7-86.3)	4	96.9	(61.1-124.5)
8h	7	76.4	(66.4-99.6)		3	67.2	(66.4-76.4)	4	90.6	(66.9-99.6)
Baseline 24h	8	107.0	(61.1-128.1)	n.s.	4	107.2	(80.6-123.8)	4	112.1	(61.1-128.1)
24h	8	102.0	(70.1-141.7)		4	96.9	(70.1-122.8)	4	114.6	(74.5-141.7)
Baseline 72h	7	105.0	(75.3-129.4)	n.s.	4	109.8	(86.3-129.4)	3	85.8	(75.3-128.1)
72h	7	122.0	(67.3-156.2)		4	129.3	(67.3-147.5)	3	84.0	(71.4-156.2)

BM: basement membrane; med: median; n.s.: no statistically significant difference; NSS: non-sensitive skin; SC: stratum corneum; SS: sensitive skin.
p values obtained with Wilcoxon sign rank test for non-parametric repeated measures.

to the area at 5 minutes, whereas it decreased at 60 minutes. No differences between SS and NSS subjects emerged for either TEWL, a* value or the wheal-and-flare area. There was no correlation between the a* value and the wheal-and-flare area measured at 5 and 30 minutes, whereas a weak yet significant correlation was present at 60 minutes (rho = 0.512, *p=0.030*, n=18). The numerical data are reported in Table 2.

At 8, 24 and 72 hours, considering both groups together, no differences with respect to baseline were present for TEWL, while the a* value was slightly higher at 24 and 72 hours (*p=0.078* and *p=0.023*, respectively). The numerical data are reported in Table 3.

Histamine iontophoresis: VAS score for itch

The average VAS score for itch measured during stimulation and the time necessary to reach the scratching threshold were not different between the groups: average VAS_{SS} = 4.3 (1.6-6.8), average VAS_{NSS} = 4.1 (0.6-6.6), time to $threshold_{SS}$ = 30 s (0 s-120 s), time to $threshold_{NSS}$ = 37.5 s (15 s-75 s). One subject with NSS never perceived VAS scores higher than the scratching threshold.

At 5 minutes after histamine iontophoresis, all subjects except one NSS subject still reported some itch. At 30 minutes, remaining perceptions were reported by two subjects with NSS and eight with SS. At 60 minutes, this was the case only for three subjects with SS. Values are shown in Figure 2 and numerical data are reported in Table 2.

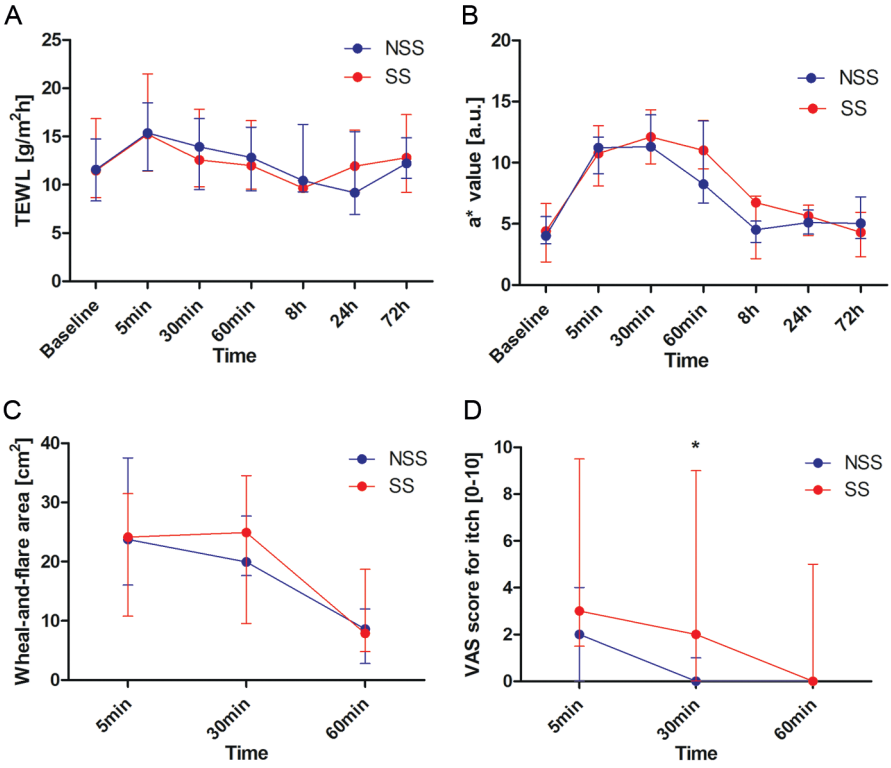

Figure 2. The values of TEWL (**a**), a* value (**b**), wheal-and-flare area (**c**) and average VAS score for itch (**d**) measured at baseline and after histamine iontophoresis in volunteers with sensitive skin (SS, n=9) and non-sensitive skin (NSS, n=9). Values at baseline and up to 60 minutes after histamine iontophoresis were measured in all volunteers (n=18). Values at 8, 24 and 72 hours were measured in a subgroup of volunteers according to the randomization described in the Materials and Methods. Graphs represent median and range (minimum-maximum).

Histamine iontophoresis: histological and immunohistochemical markers

The HE staining revealed no effects of histamine iontophoresis on the SC in terms of change of thickness or presence of parakeratosis. On the other hand, the living epidermis showed a trend to increased thickness at 1 and 24 hours after histamine iontophoresis (*p=0.065* and *p=0.055*, respectively) and was significantly thicker at 72 hours (*p=0.016*). No inflammatory infiltrates in the epidermis or dermis were observed. Representative HE images are shown in Figure 3.

In the epidermal compartment, immunohistochemical stainings neither showed changes in the number of CD1a-positive cells nor in the number of Ki67-positive cells. Of

note, a slight increase of the latter was present only in two SS subjects at 72 hours. In the dermal compartment, no influx of CD3-positive cells or changes in the area covered by CD31-positive cells were visible. A significant decrease in the number of tryptase-positive mast cells was observed at 1 hour after histamine iontophoresis (*p=0.037*). Representative images of the immonohistochemical stainings are shown in Figure 4, whereas the numerical data for each marker are reported in Table 3.

Qualitative assessment of the immunofluorescence staining revealed no presence of increased expression of K16 at any time point, whereas a slight increase in the intensity of the filaggrin signal could be detected at 24 and 72 hours in about half of the volunteers assessed at these time points. Representative images are shown in Figure 5.

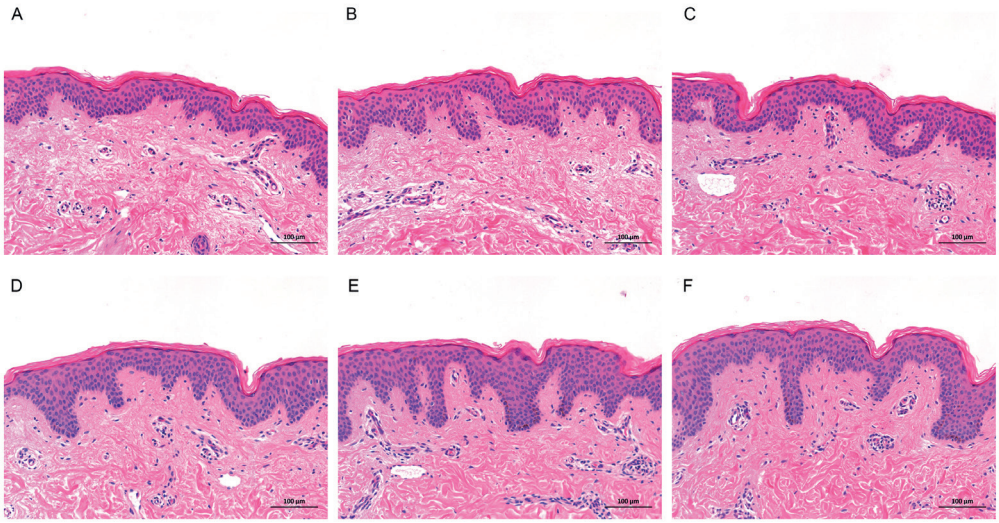

Figure 3. Representative HE photographs of skin reactions to histamine iontophoresis in two volunteers (**a**,**b**,**c**, and **d**,**e**,**f**). **a**,**d**) Control skin. **b**) At 1 hour, spongiosis is present in the epidermis. No changes in the stratum corneum are visible. **c**) At 8 hours, no changes compared to control skin are visible. **e**,**f**) At 24 and 72 hours, a slight increase in the epidermal thickness is visible. No atypia is observed, albeit the higher intercellular spaces in the upper stratum spinosum and in the stratum granulosum indicate spongiosis (more pronounced at 72 hours). No changes in the stratum corneum nor influx of inflammatory infiltrates in either the epidermis and dermis are visible.

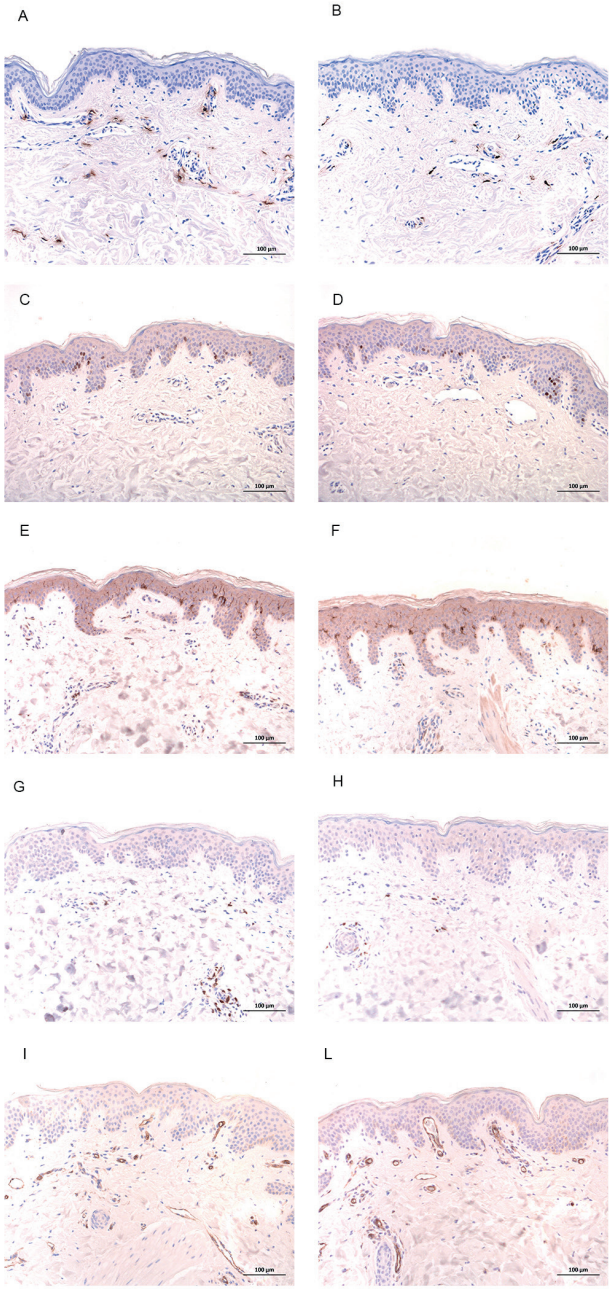

Figure 4. Representative photographs of immunohistochemical data. Tryptase-positive mast cells decreased significantly at 1 hour after histamine iontophoresis (**b**) compared to control skin (**a**). Ki67-positive nuclei, CD1a-positive cells, CD3-positive cells and CD31-positive cells showed no change at 72 hours after histamine iontophoresis (**d**,**f**,**h**,**l**, respectively) compared to control skin (**c**,**e**,**g**,**i**, respectively).

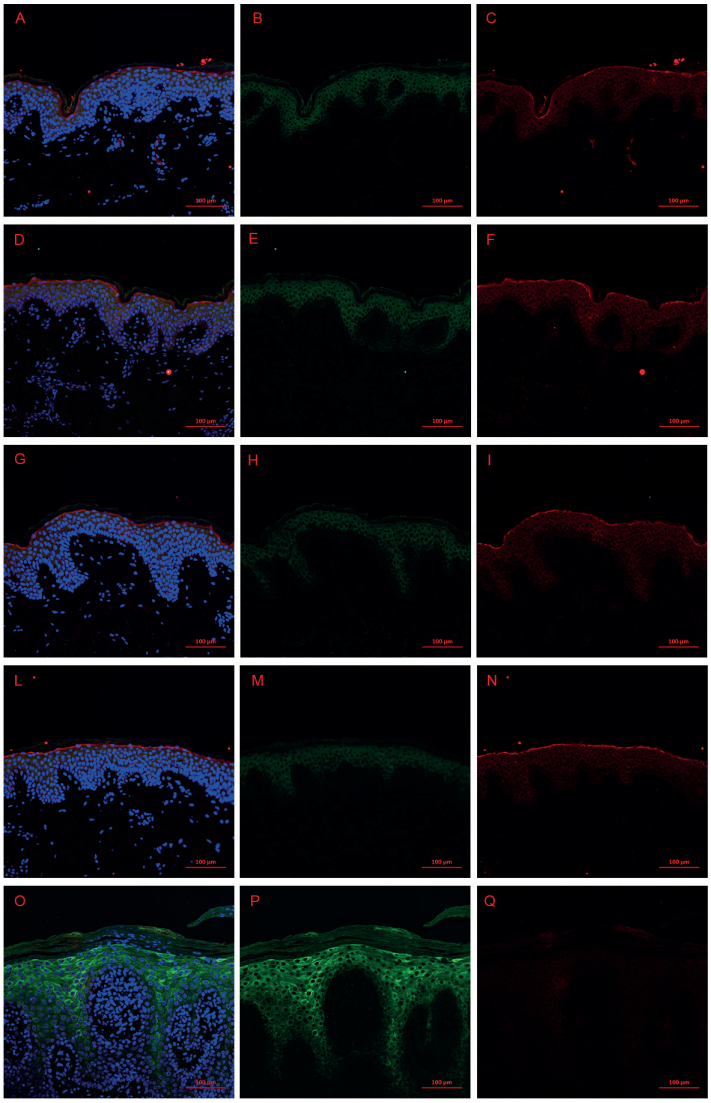

Figure 5. Representative immunofluorescence photographs of expression of K16 (green) and filaggrin (red) in two volunteers (**a**-**f** and **g**-**n**, respectively) at baseline (**a**,**b**,**c** and **g**,**h**,**i**) and at 72 hours after histamine iontophoresis (**d**,**e**,**f** and **l**,**m**,**n**). Diamidine phenylindole was used as counterstaining (blue). In both volunteers, no expression of K16 can be detected at baseline (**b**,**h**) or at 72 hours after histamine iontophoresis (**e**,**m**), whereas a slightly increased intensity in the filaggrin signal is visible at 72 hours (**f**,**n**) compared to control skin (**c**,**i**). The last row (**o**,**p**,**q**) shows an example of psoriatic skin, used as positive control for the increased suprabasal expression of K16 (**p**) and as negative control for the absence of filaggrin expression (**q**) compared to normal skin.

Topical application of histamine

In the buttock, during topical histamine application, five subjects with NSS and four with SS did not perceive any sensation. The remaining volunteers had only transient and subthreshold perceptions, with no differences between SS and NSS subjects except one subject with SS who reported itch values above the scratching threshold: average VAS_{SS} = 0.5 (0.0-4.1), average VAS_{NSS} = 0.0 (0.0-0.8). At 5 minutes, two subjects with SS had remaining perceptions (VAS score of 1 and 5). One subject with NSS and one with SS developed a slight erythema, while edema was not visible in any volunteer.

In the non-domintant mid volar forearm, baseline TEWL values were higher in subjects with SS: TEWLss = 12.0 g/m^{2}h (8.6-16.9), $TEWL_{NSS}$ = 10.6 g/m^{2}h (6.6-14.3) (*p=0.031*), whereas a* values were not different: a* $value_{ss}$ = 6.2 a.u. (3.1-8.3), a* $value_{NSS}$ = 6.2 a.u. (3.4-8.1). During topical histamine application, seven subjects with NSS and four with SS did not perceive any sensation. The remaining volunteers had only transient and subthreshold perceptions, with no differences between SS and NSS subjects except one subject with SS who reported itch values above the scratching threshold: average VAS_{SS} = 0.5 (0.0-4.6), average VAS_{NSS} = 0.0 (0.0-0.5) (*p=0.063*). At 5 minutes, two subjects with SS had remaining perceptions (VAS score of 2). One subject with SS developed a slight erythema, while edema was not visible in any volunteer.

DISCUSSION

The primary objective of this study was to characterize the effects of histamine iontophoresis on the epidermal and dermal compartments combining invasive (immunohistochemistry) and non-invasive assessments.

We confirm that histamine iontophoresis is a quantifiable model for the study of the early stages of cutaneous inflammation [14], as indicated by the fast onset of the wheal-and-flare reaction at 5 minutes. This acute reaction was mirrored by presence of spongiosis in the epidermis (Figure 3), possibly due to the underlying increased capillary permeability, and by the decrease of tryptase-positive mast cells, possibly indicative of degranulation induced by the vasoactive peptides released by histamine [23]. No clinical signs of irritation were visible at time points later than 1 hour. On the other hand, the lack of activation of Langerhans cells in the epidermis and of influx of T-cells in the dermis, evidenced by the unchanged numbers of CD1a- and CD3-positive cells, respectively, confirms that this model (at least with the parametrs chosen in this study) is not suitable for studying key mediators of adaptive immunity activated by sustained inflammation. For studying these mediators other *in vivo* models such as tape stripping and application of LTB_4 would be more appropriate [3-5].

We confirm our hypothesis that histamine iontophoresis causes local and acute cutaneous inflammation with minimal barrier impairment. The minimal barrier impairment was demonstrated by the minor increase and fast recovery of TEWL and by the absence of an

increased proliferative response [24]. Previous works already suggested that the effects of iontophoresis at modest current densities (<0.5 mA/cm^2) on TEWL are largely accounted for by hydration induced throught the contact with the solution to be administered [25], albeit possible disorganization of the lipid bilayers in the SC cannot be ruled out [26]. On the other hand, histology showed that this model had secondary effects in the epidermal compartment, since a slight yet significant increase in epidermal thickness was present at 72 hours. In absence of an increased proliferative response, the thicker epidermis might be partly attributed to an effect on late-differentiation markers like filaggrin. Iontophoresis was previously shown to induce the loss of the epidermal calcium gradient, and in turn the loss of the epidermal calcium gradient has been shown to decrease differentiation-specific markers [27, 28]. In *in vitro* experiments on human keratinocytes it was observed that the addition of histamine reduced the expression of differentiation-specific proteins [29]. The slightly higher expression of filaggrin observed at 24 and 72 hours might thus be a reaction to a transient decrease caused by either iontophoresis or histamine or by the two combined. Another possible explanation might be that the spongiosis elicited by the acute histamine reaction, together with an intact skin barrier limiting free movement of water, could have induced an unbalance in the water transport at the interface between the living epidermis and the SC; hence the higher intercellular spaces visible in the HE images (Figure 3). The increase in epidermal thickness at 24 and 72 hours might in turn be responsible for the change in the a* value measured at these time points.

The secondary objective of this study was to explore whether skin responses to histamine iontophoresis differ significantly between SS and NSS subjects. The only significant difference was a higher VAS score for itch at 30 minutes after stimulation. The occurrence of increased sensory perceptions in SS is in agreement with the results reported by previous clinical studies [18-20], and it has been recognized by the International Forum for the Study of Itch (IFSI), which has recently initiated a special interest group on SS [30]. On the other hand, in contrast to the aberrant vascular reactions in SS reported previously [20], we could not detect differences in skin redness measured by the a* value or in the extent of the wheal-and-flare reaction measured by image analysis. One reason could be due to the low number of volunteers included in each group, preventing to identify subtle differences between SS and NSS subjects. Another reason could be due to insufficient sensitivity and specificity of the measurements used. The a* value is of difficult interpretation in this model, since it is known to be influenced by several factors among which edema [31], blood volume, and vasodilation [32], all variables affected by histamine. To get more insights into the vascular reactions, additional parameters could be evaluated, for example the reflectance spectra from the skin [32], the extent and topographical map of the wheal [33], and the changes in concentration/velocity of erythrocytes [34]. The lack of differences in VAS score for itch during topical histamine application on the buttock and on the volar forearm do not support a major impairment of the skin barrier in SS, contrary to other conditions (*e.g.* atopic dermatitis, dandruff) in which topical histamine was shown to elicit an itchy response [7, 21]. However, the higher baseline TEWL and the trend to

higher VAS score reported by SS subjects during topical histamine application on the volar forearm suggest subtle aberrant properties of the SC, which should be evaluated with more sensitive and specific approaches [35].

In conclusion, we propose that histamine iontophoresis can be used as *in vivo* model to elicit local and acute skin inflammation with minimal impact on the skin barrier, in contrast to tape stripping and topical application of irritants in which the skin barrier is disrupted either mechanically or chemically. In particular, this model could be applied to skin disorders in which aberrant cutaneous mast cells and their mediators play a role; for example, to investigate the still poorly-defined pathomechanisms underlying chronic spontaneous urticaria [36], or to help the diagnosis of different variants of mastocytosis [37]. The effect of the model on mast cells dynamics could be analyzed directly in skin biopsies; otherwise, mast cell degranulation could be indirectly assessed by microdialysis and subsequent histamine measurement [38]. Concomitant biophysical measurements can be added to assess the effects of the model at the (sub)clinical scale. Of note, the status of the barrier function should be assessed before application of the model, to control whether abnormal skin reactions should be related to aberrant barrier properties rather than to a different mast cells dynamics [7]. Should differential responses compared to controls emerge, further clinical trials could employ the histamine iontophoresis model also to monitor disease activity and the response to treatments. With respect to the evaluation of aberrant vascular reactions in SS, future studies using histamine iontophoresis should include a higher number of volunteers and evaluate the wheal-and-flare reaction with sensitive and specific approaches [32-34].

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Acquisition set-up

Skin photographs were acquired using a commercially available single-lens reflex digital camera (D3200, Nikon, Japan), equipped with a complementary metal-oxide semiconductor (CMOS) sensor (23.2 mm x 15.4 mm, 24.2 megapixels) and with a 18-55 mm zoom lens. To provide a shadow-free illumination, a 32-white light LED ring flash (PLMRFN, Polaroid, USA) with a color temperature of 5500 K was mounted on the camera. All experiments were performed under the same ambient light conditions (neon-lighting from the ceiling, curtains closed to avoid daylight). A circular sticker of known area was attached to the skin and used to estimate the area of the wheal-and-flare (in cm^2) by a mathematical proportion between the pixels belonging to the sticker and to the erythema [1]:

$$Area_{erythema} = \frac{(Pixel_{sticker} * Pixel_{erythema})}{Area_{sticker}} \quad (1)$$

Skin photographs were taken while holding manually the camera perpendicular to the skin and the sticker, at a distant sufficient to image the wheal-and-flare reaction and the sticker. The following settings were used to take photographs:

- Manual focus and mode (M) – aperture and shutting speed adjusted to match optimal exposure;
- Focal length 55 mm;
- ISO 200;
- Image quality "FINE" – corresponding to JPEG images with a low (1:4) compression ratio;
- Color space sRGB;
- Ring flash in continuous ("Light") mode;
- Preset manual white balance – obtained by acquiring an image of a white A4-sized sheet in the experimental conditions (ambient lighting and ring flash in continuous mode).

Contrast enhancement: histogram stretching and equalization

The histogram of a grey-level image X represents the number of pixels n_m for each grey level m=0,1,...,L–1 belonging to the image. In histogram equalization, the histogram is flattened across the dynamic range [0, L–1] so that the cumulative histogram, or cumulative

density function, approximates a linear ramp. Each pixel of the contrast-enhanced image *Y* can be expressed with the following formula:

$$Y_i = floor[(L-1) \sum_{m=0}^{X_i} P(X_m)] \quad (2)$$

Where *n* represents the total number of pixels in the image, $P(X_m) = \frac{n_m}{n}$ is the normalized histogram and *floor* [ ] rounds down to the nearest integer.

In histogram stretching, the histogram is stretched between a minimum and maximum grey level, mostly the dynamic range [0, 255]. Each pixel of the contrast-enhanced image Y can be expressed with the following formula:

$$Y_i = 255 * \frac{[X_i - \min(X)]^{\gamma}}{[\max(X) - \min(X)]} \quad (3)$$

Where min(*X*) and max(*X*) represent the maximum and minimum grey level in X, respectively. If $\gamma = 1$ the histogram is linearly stretched across the predefined dynamic range, while if $\gamma > 1$ the histogram is non-linearly stretched with greater emphasis on enhancing contrast of high grey levels.

Thresholding: Otzu's method and Isodata algorithm

Thresholding is the process of reducing a grey-level image into a binary image by selecting a grey level *M* so that all pixels with grey level $m \geq M$ are classified as belonging to the object of interest and all pixels with grey level $m < M$ are classified as belonging to the background. The selection of the most suitable threshold *M* can be improved by increasing the separation between peaks in histogram stretching or equalization. Two widely used approaches for thresholding are the Otzu's method and the Isodata algorithm. In Otzu's method [2], the threshold *M* is chosen by maximizing the variance (distance) between the object and the background. In the Isodata method [3], the threshold *M* is iteratively computed as the average between the average grey level of the background and of the object. The algorithm stops when the absolute difference between the thresholds *M* obtained in iterations *k* and *k* –1 is lower than a predefined value ϑ.

Algorithm for segmentation of histamine-induced wheal-and-flare

The algorithm for erythema segmentation is based on the use of built-in functions implemented in Matlab (version R2013a, The MathWorks Inc., USA). The first step consists in manually choosing a rectangular Region of Interest (ROI) containing the wheal-and-flare reaction. The ROI is converted from the RGB to the CIE Lab color space, in which color is expressed as a three-dimensional quantity defined by an L*-axis (brightness), a*-axis (red-

green chromaticity) and b*-axis (yellow-blue chromaticity) [4, 5]. All subsequent steps are performed on the ROI representing a*, since erythema induces a significant increase in this component making it particularly suited for its evaluation [4-6]. The white point corresponding to the CIE standard illuminant "d55" is used in the conversion between RGB and CIE Lab [6]. As a pre-processing step, contrast-limited adaptive histogram equalization (CLAHE) is applied on small ROI regions (10 x 10 pixels) in order to decrease the occurence of inhomogeneities in the background of the ROI. Subsequently, global contrast enhancement in the range 0-225 and thresholding are performed. Six segmented binary images are obtained by combining the three histogram-based contrast enhancement techniques (histogram equalization and histogram stretching with $\gamma = 1$ and $\gamma = 2$) with the two thresholding methods (Otzu's and Isodata). The aim was to compare these combinations to find the most suitable for segmenting the histamine-induced wheal-and-flare. As a fourth step, post-processing on the binary images is performed: firstly, a median filter of dimension 10 x 10 pixels is applied to eliminate spurious pixels and smoothen the boundary of the segmented wheal-and-flare; secondly, empty regions inside the segmented erythema are "filled", to include pixels belonging to the centrally-developed edema to the total area affected by the histamine reaction. Because this segmentation approach is based only on the pixel intensities and is independent of the shape and configuration of the object of interest, pixels belonging to the borders or to outer regions of the wheal-and-flare could be segmented as well. This issue is solved in the last step by automatically selecting the biggest segmented area in the ROI, which corresponds to the total area affected by the histamine reaction.

In addition to manually select the ROI containing the histamine-induced wheal-and-flare, the user is asked to manually select another ROI containing the sticker. The segmentation of the sticker is performed to calculate the pixels belonging to it in order to apply (1) to the segmented wheal-and-flare. The steps of the algorithm are summarized in Figure S1.

Of note, the application of the algorithm is suitable when the flare surrounds the wheal in the characteristic wheal-and-flare reaction. In our experiments we noticed, however, that when the histamine reaction decreased (usually starting between 30 and 60 minutes after application), the flare decreased in extent and intensity, not necessarily symmetrically around the wheal. In addition, in most volunteers an irregularly dotted erythema pattern appeared in correspondence of the wheal. We propose therefore a variant of the algorithm in which no application of CLAHE in the pre-processing step nor filling and selection of the biggest area in the post-processing step are performed, the reasons being the irregularity in shape and the limited extention of the flare in later time points, not involving background inhomogeneities in skin color. Although the lack of selection of the biggest segmented area may result in the possible inclusion of spurious pixels belonging to outer regions, we noticed that the selection of a small ROI close to the flare could partially overcome this issue resulting in satisfactory results.

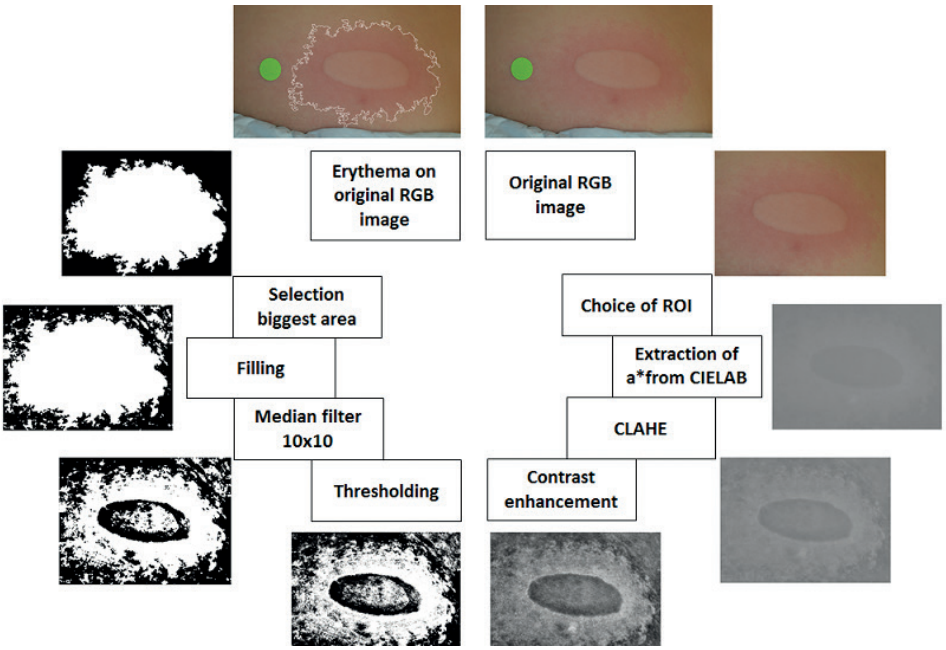

Figure S1. Algorithm for histamine-induced wheal-and-flare segmentation consisting in histogram-based contrast enhancement and thresholding. All steps are implemented in Matlab (version R2013a, The MathWorks Inc., USA).

Data analysis

The algorithm was used to segment the wheal-and-flare reaction at 5 and 30 minutes after histamine iontophoresis. Since in most volunteers a clear decrease in the reaction was visible at 60 minutes after histamine iontophoresis, the variant of the algorithm was applied at this time point.

In order to test the accuracy of the algorithm, the wheal-and-flare reaction on the original ROI was segmented manually by pointing as many points as needed on its border [7]. The area of this polygon was calculated using (1) and used as reference to evaluate the accuracy using a border detection error coefficient, or *XOR* [8]:

$$XOR = \frac{(Pixel_{manual} \oplus Pixel_{erythema})}{Pixel_{manual}}$$

Where $\oplus$ is the exclusive-OR operation giving the pixels for which the semiautomatically and manually segmented areas disagree. To test reproducibility, the algorithm and the manual selection were repeated three times for each image and the coefficient of variation (CV) was computed. Results are presented as mean ± SD.

Also the variant of the algorithm was tested three times on each image and the CV was computed. In this case, no manual segmentation was performed and results are qualitatively presented.

RESULTS

The algorithm was tested on all 18 volunteers at 5 and 30 minutes after histamine iontophoresis. The area, CV and XOR obtained with the six combinations are presented in Table S1.

The method based on histogram equalization ("Histeq") provided satisfactory results but was in general slightly conservative *(i.e.* it tended to segment a smaller area belonging to the wheal-and-flare reaction compared to the manual segmentation). In addition, at 5 minutes and in three volunteers, one to two repetitions of the algorithm provided a poor segmentation (*i.e.* the area belonging to the wheal-and-flare reaction was only partially segmented). At 30 minutes the performance of the algorithm improved, since the poor segmentation occurred in two volunteers and for just one repetition. The thresholding with Otsu's and Isodata methods yielded the same outcome. The method based on linear histogram stretching ("Imadjust$_{\gamma=1}$") provided the best results in terms of accuracy (hence the lowest XOR). However, at 5 minutes after stimulus, two to three repetitions in three volunteers for the Otsu's method and in one volunteer for the Isodata method erroneously segmented a part of the background of the ROI due to strong skin color differences between the sun exposed and non-exposed areas. At 30 minutes, this was the case in the same volunteers but occurred only in one to two repetitions. The method based on non-linear histogram stretching ("Imadjust$_{\gamma=2}$") yielded the worst performance at 5 minutes, in which poor segmentation occurred in five (Otsu's method) and three (Isodata method) volunteers (hence the highest XOR). The method improved at 30 minutes but, especially using Otsu's thresholding, it remained conservative and poor segmentation occurred in two volunteers. Representative segmentation results are shown in Figure S2.

The variant of the algorithm was tested on all 18 volunteers at 60 minutes after histamine iontophoresis. The area and CV obtained with the six combinations are presented in Table S2.

The method based on histogram equalization was the least robust to low-contrast erythema borders and tended to include spurious pixels belonging to the background, whereas the method based on non-linear histogram stretching was the most conservative and the most reproducible as demonstrated by the lowest CV. The method based on linear contrast enhancement had an intermediate behaviour. Thresholding with Otsu's or Isodata methods yielded equal results for the histogram equalization and similar results for the linear histogram stretching approaches, while for the non-linear histogram stretching approach, Otsu's method was more conservative than the Isodata. Representative segmentation results are shown in Figure S3.

Table S1. Results of the algorithm for segmentation of histamine-induced wheal-and-flare at 5 minutes (n=18) and 30 minutes (n=18) after histamine iontophoresis.

Method	**Area** T_{5min} [cm^2]	**CV** T_{5min} [%]	**XOR** T_{5min} [%]	**Area** T_{30min} [cm^2]	**CV** T_{30min} [%]	**XOR** T_{30min} [%]
Manual	23.7 ± 5.7	3.5 ± 1.7	-	22.3 ± 5.1	4.5 ± 1.6	-
Histeq T_{otsu}	21.9 ± 5.5	7.0 ± 6.9	24.1 ± 8.5	20.3 ± 4.4	6.1 ± 3.2	21.3 ± 5.4
Histeq T_{iso}	21.9 ± 5.5	7.0 ± 6.9	24.1 ± 8.5	20.3 ± 4.4	6.1 ± 3.2	21.3 ± 5.4
$Imadjust_{\gamma=1}$ T_{otsu}	25.3 ± 6.9	4.5 ± 3.8	21.6 ± 4.7	23.7 ± 6.4	3.5 ± 3.4	20.2 ± 5.0
$Imadjust_{\gamma=1}$ T_{iso}	24.0 ± 6.3	3.3 ± 3.0	20.8 ± 5.5	22.8 ± 5.9	3.6 ± 2.5	19.2 ± 4.0
$Imadjust_{\gamma=2}$ T_{otsu}	20.1 ± 6.3	5.5 ± 5.2	28.0 ± 11.5	20.8 ± 6.0	5.2 ± 6.2	21.9 ± 7.3
$Imadjust_{\gamma=2}$ T_{iso}	21.1 ± 6.4	4.7 ± 4.1	26.4 ± 10.8	20.6 ± 5.0	4.2 ± 3.5	20.3 ± 5.3

CV: coefficient of variation; Histeq: histogram equalization; Imadjust: histogram stretching; Iso: Isodata method; Otsu: Otsu's method.

Table S2. Results of the variant of the algorithm for segmentation of histamine-induced wheal-and-flare at 60 minutes (n=18) after histamine iontophoresis.

Method	**Area** T_{60min} [cm^2]	**CV** T_{60min} [%]
Histeq T_{otsu}	9.9 ± 4.3	10.4 ± 9.6
Histeq T_{iso}	9.9 ± 4.3	10.4 ± 9.6
$Imadjust_{\gamma=1}$ T_{otsu}	9.0 ± 5.4	8.8 ± 10.1
$Imadjust_{\gamma=1}$ T_{iso}	9.3 ± 4.7	8.3 ± 8.0
$Imadjust_{\gamma=2}$ T_{otsu}	7.1 ± 5.1	4.7 ± 6.3
$Imadjust_{\gamma=2}$ T_{iso}	8.0 ± 4.8	5.0 ± 7.5

CV: coefficient of variation; Histeq: histogram equalization; Imadjust: histogram stretching; Iso: Isodata method; Otsu: Otsu's method.

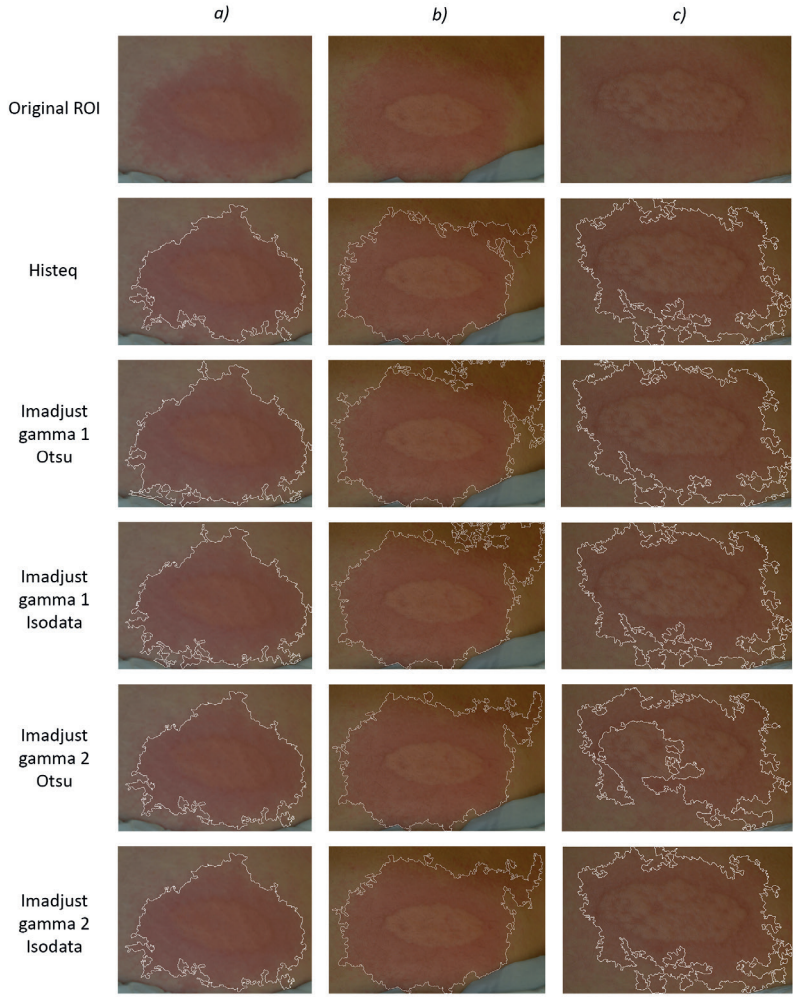

Figure S2. Representative results of the algorithm for histamine-induced wheal-and-flare segmentation obtained in images taken on the buttock of three volunteers (**a**-**c**) after histamine iontophoresis. **a**) Good segmentation results obtained in all six combinations of histogram-based contrast enhancement and thresholding. **b**) The linear histogram stretching methods erroneously selected an area belonging to the background because of strong inhomogeneity in skin color. **c**) Poor segmentation results obtained by the non-linear histogram stretching method with Otsu's thresholding.

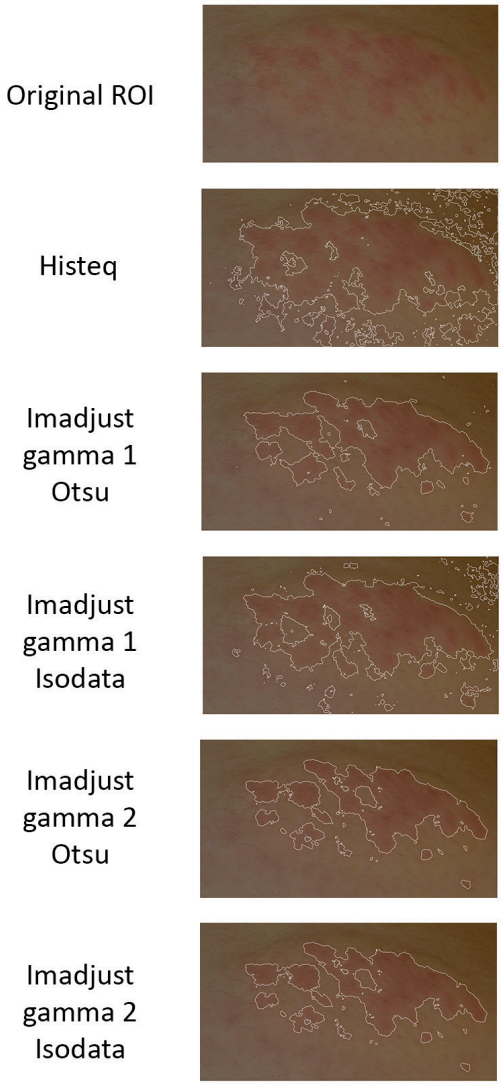

Figure S3. Representative results of the variant of the algorithm for histamine-induced wheal-and-flare segmentation obtained in images taken on the buttock at 60 minutes after histamine iontophoresis. The surrounding flare has faded and there is the appearance of an irregularly dotted erythema pattern in correspondence of the wheal. The non-linear histogram streatching methods show the most conservatory results with least influence of spurious pixels belonging to the background.

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Chapter 4

Role of female hormones and risk factors in sensitive skin

ABBREVIATIONS

BMI	Body mass index
ERs	Estrogen receptors
HRT	Hormone replacement therapy
ICBE	Internal committee biomedical experiments
NSS	Non-sensitive skin
SC	Stratum corneum
SS	Sensitive skin
TEWL	Transepidermal water loss

4.1

Sensitive skin and the influence of female hormone fluctuations: results from a cross-sectional digital survey in the Dutch population

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ABSTRACT

Background: Sensitive skin is a widespread condition, which is most frequently reported by women. Changing hormone levels during the menstrual cycle and menopause have been suggested among the stimuli triggering sensitive skin.

Objectives: To investigate the perceived influence of fluctuating hormone levels on self-assessed sensitive skin, including symptoms and stimuli linked to skin sensitivity, as well as potential changes in facial and body skin and sensitive body parts, depending on hormonal status.

Patients and methods: A digital questionnaire was distributed to a population of women aged 20-65 years old.

Results: A total of 278 women were included in the analysis. About 42% premenopausal women declared a perception of (increased) skin sensitivity just before and during the menstrual cycle, while this was reported by almost 32% of peri- and postmenopausal women following the menopause. The majority of reported symptoms included the presence of bumps/pimples, dryness, itching, and redness, and the majority of reported stimuli were shaving, weather, toiletries, and emotions. No differences emerged regarding characteristics of facial and body skin across different hormonal status. Significant differences in sensitivity of body parts emerged for the face and feet, reported by a larger percentage of premenopausal women and peri- and postmenopausal women, respectively.

Conclusion: The prevalence of the perceived effects of fluctuating hormone levels on self-assessed sensitive skin in women is high. These effects should be taken into consideration in skin testing and dermatological practice, and support the need for selecting personal care routine or treatment during the menstrual cycle and menopause.

INTRODUCTION

As much as 50% to 61% of women in the industrialized world describe their skin as sensitive [1]. Sensitive skin (SS), usually defined as a subjective cutaneous hyper-reactivity to various stimuli [2], has been widely investigated in recent years, but a clear characterization of this condition is still lacking due to the frequent absence of objective signs of skin irritation and the variability of symptoms and severity [3]. Previous work from our group has shown that insights into the pathomechanisms of SS could be gained by selecting volunteers via a perception-based questionnaire, in which a range of eliciting factors of different nature are considered, among which figure toiletries, heat, cold, and emotions [4, 5]. Fluctuations in female hormone levels during the menstrual cycle and menopause have also been suggested among the eliciting factors of SS symptoms in the literature [2, 6]. This hypothesis is supported by the presence of oestrogen receptors (ERs), and, to a lesser extent, progesterone and testosterone receptors, both in the dermis and in the epidermis [7]. Two oestrogen regulatory mechanisms have been described so far, involving a genomic pathway (in which oestrogen binds to intracellular ERs) and a non-genomic pathway (in which oestrogen binds to cell membrane ERs) [8]. Levels of oestrogen and progesterone decline in the late luteal phase, and remain low during the menstrual and early follicular phases [7]. Both hormones fall to low levels also in the menopause, a process leading to the loss of ovarian cyclic activity [7, 8]. Oestrogen decline in menopausal women has been linked to the exacerbation of aging effects, such as skin dryness and wrinkling [8], possibly via influence on dermal collagen content. From observations of menopausal women taking exogenous oestrogen for menopausal symptom relief (hormone replacement therapy – HRT) and those not taking HRT, it was reported that menopausal women on HRT had better lipid coverage of corneocytes in the stratum corneum (SC), better lipid lamellar organization, and improved water-holding capacity in the SC compared to menopausal women who did not take HRT [9-11]. From studies on premenopausal women, a trend towards decreased SC hydration and a weakened skin barrier, measured by transepidermal water loss (TEWL), emerged in correspondence with the perimenstrual phase, in which oestrogen levels are at their lowest [12, 13]. These results suggest that decreased oestrogen levels during menopause and the perimenstrual phase could contribute to the onset or exacerbation of SS through an effect on the skin barrier function. In this respect, the effects of progesterone are more limited and less studied [7]. Furthermore, female hormones could exert an inflammatory and immunological effect via an influence on mast cell behaviour [14], a mechanism which might explain the variability in skin prick tests and allergy patch tests across the menstrual cycle reported in previous studies [15, 16].

The primary objective of this study was therefore to further characterise SS in women by investigating the perceived influence of fluctuating hormone levels during the menstrual cycle and menopause on self-assessed SS. For this purpose, we designed a digital questionnaire to be distributed to a population of women of variable age and hormonal status. Several factors

possibly influencing the effect of hormone levels on SS were evaluated; these included: age, use of exogenous hormones as contraceptives or HRT, self-assessed SS, intensity of menstrual or menopausal symptoms, presence of atopic predisposition or skin diseases, and body mass index (BMI). Questions on stimuli and symptoms which make women aware of the fact that their skin may become (more) sensitive due to the menstrual cycle or menopause were also added. As a second objective, we investigated whether self-assessed characteristics of facial and body skin, as well as sensitive body parts, differ as hormonal status changes.

Given the high prevalence of SS among women, we believe that obtaining deeper insights into the role of fluctuating hormone levels could be of relevance in the evaluation of patients' symptoms in clinical practice, in the assessment of skin responses to dermato-cosmetic treatments, as well as in the selection of personal care routine.

PATIENTS AND METHODS

Participants and recruiting procedure

The digital questionnaire was distributed, in December 2014, to women aged between 20 and 65 years old and registered in the database of a recruiting agency. The database consists of people from several provinces across the Netherlands. A quota of a minimum of 300 respondents was set, *i.e.* respondents could still submit the questionnaire even if the quota had been reached while completing it. General information about the study and the questionnaire was provided. If willing to participate, respondents had to provide informed consent by agreeing to the statement: "I have read the survey information and agree that the information I provide will be used for the stated purpose". To ensure anonymity, participants were not asked personal information. Pregnant and breast-feeding women were asked to refrain from participation. The study was approved by the Internal Committee Biomedical Experiments (ICBE) of Philips Research and by the local ethics committee.

Questionnaire

The questionnaire was created using a web-based survey tool (EFM version 8.1, Verint Systems Inc., USA). Sociodemographic characteristics included age group (20-35 years, 36-45 years, 46-55 years, and 56-65 years), Fitzpatrick skin type, height, and weight. Atopic predisposition was defined as a history of asthma, atopic dermatitis, or hay fever. Hormonal status was defined as: premenopausal (a regular menstrual cycle circa every month), perimenopausal (clear changes in terms of menstrual cycle length and intensity, or no menstrual cycle for up to 12 months), postmenopausal (no menstrual cycle for more than 12 months) [17]. Intensity of perimenstrual and menopausal symptoms could be rated as absent, mild, moderate, or severe. For association analyses, absent and mild responses were categorised as "low" symptom intensities, and moderate and severe responses as "high"

symptom intensities. Self-assessed SS was defined according to the question: "Do you think that your skin is more sensitive with respect to the skin of others?". Women who assessed their skin as much more or slightly more sensitive compared to others, were categorised in the SS group, while women who rated their skin as equally or less sensitive compared to others, were categorised in the non-sensitive skin (NSS) group [4, 5]. The perceived effect of the menstrual cycle on SS was assessed with the question: "Do you notice that your skin becomes sensitive or more sensitive during certain phases of the menstrual cycle?". For association analyses, responses "No" and "Yes, rarely" were categorized as "No or rare effect of menstrual cycle on SS", while responses "Yes, sometimes" and "Yes, often" were categorised as "Effect of menstrual cycle on SS". For these analyses, only premenopausal women were included. The perceived effect of the menopause on SS was assessed with the question: "Do you notice that your skin has become sensitive or more sensitive since the menopause?". For association analyses, responses "No, my skin has become less sensitive since the menopause" and "No, my skin sensitivity has not changed" were categorized as "No increased SS after menopause", while response "Yes" was defined as "Increased SS after menopause". For these analyses, only peri- and postmenopausal women were included. For the analysis of stimuli and symptoms, which make women aware of the fact that their skin may become (more) sensitive due to the menstrual cycle or menopause, only women who reported an effect of the menstrual cycle or menopause on SS were included.

Statistical analysis

Fisher's exact test was used to study associations between categorical variables. Kruskal-Wallis and Mann-Whitney tests were used to evaluate differences between means of BMI. For all comparisons, the significance values (p value) are presented. The level of significance was set at 0.05. Statistical analyses were performed using SPSS Statistics version 20 for Windows (IBM SPSS Inc., USA). No corrections for multiple comparisons were applied.

RESULTS

Characteristics of the study population

A total of 301 women responded to the questionnaire. Women who did not specify their age group or hormonal status were excluded from the analysis (n=23). In total, 278 women were included in this study. Sociodemographic characteristics, presence of atopic predisposition, skin diseases, and self-assessed SS for each hormonal status are shown in Table 1. Skin diseases were less prevalent in perimenopausal women ($p=0.011$), while the BMI was lower in premenopausal women ($p=0.015$). The most frequently reported skin diseases were psoriasis (n=10), contact dermatitis (n=8), and acne (n=7). For atopic predisposition, the majority of women had hay fever (n=76), followed by asthma (n=38) and atopic dermatitis

(n=30). These percentages are in line with the prevalence of skin diseases and atopic conditions in the Netherlands[1]. Of note, when women with skin diseases were excluded from the analysis, the percentage of SS in the perimenopausal group was slightly higher than in the other groups (premenopausal: 45.1%; perimenopausal: 51.0%; and postmenopausal: 42.5%). The difference, however, was not significant (*p=0.643*).

Table 1. Characteristics of the study participants according to hormonal status (n=278).

		Premenopausal		**Perimenopausal**		**Postmenopausal**		
	n	n	%	n	%	n	%	p value
	278	121	*43.5*	55	*19.8*	102	*36.7*	
Age group (years)								n.a.
20 - 35	62	59	*48.8*	3	*5.5*	0	*0.0*	
36 - 45	59	49	*40.5*	9	*16.4*	1	*1.0*	
46 - 55	96	13	*10.7*	41	*74.5*	42	*41.2*	
56 - 65	61	0	*0.0*	2	*3.6*	59	*57.8*	
Body Mass Index (kg/m²)								**0.015**
Mean (±SD)	271	24.85 (± 4.70)		26.09 (± 5.07)		26.70 (± 5.09)		
Skin type (Fitzpatrick scale)								0.674
I-II	98	46	*39.0*	20	*36.4*	32	*31.7*	
III	113	43	*36.4*	24	*43.6*	46	*45.5*	
IV-V-VI	63	29	*24.6*	11	*20.0*	23	*22.8*	
Atopic predisposition								
Yes	98	40	*33.9*	23	*41.8*	35	*36.5*	0.618
Skin disease								
Yes	40	24	*19.8*	2	*3.6*	14	*13.7*	**0.011**
Self-assessed SS								0.921
SS	126	56	*49.1*	26	*51.0*	44	*47.3*	
NSS	132	58	*50.9*	25	*49.0*	49	*52.7*	

n.a.: not applicable (due to expected variation in age during the different hormonal status); NSS: non-sensitive skin; SS: sensitive skin.

1 Data from *Nationaal Kompas Volksgezondheid* (*www.nationaalkompas.nl*).

Perceived effect of menstrual cycle and menopause on self-assessed SS

A total of 121 premenopausal women were included in the analysis of the perceived effect of menstrual cycle on SS, while 157 peri- and postmenopausal women were included in the analysis of the perceived effect of menopause on SS. The results are presented in Figure 1. A perceived effect on SS was reported by premenopausal women before (35.5%) and/or during (28.9%) menstruation. Association analyses are shown in Tables 2 and 3. The majority of premenopausal women with high intensity of perimenstrual symptoms stated that their skin becomes (more) sensitive during certain phases of the menstrual cycle with respect to women with lower symptom intensity ($p=0.002$). Similarly, the majority of peri- and postmenopausal women with high intensity of menopausal symptoms stated that their skin became (more) sensitive following the menopause, compared to women with low symptom intensity ($p=0.018$). In this group, in addition, an association with increased sensitive skin following the menopause was identified for women with self-assessed SS, women with skin diseases, and women currently taking HRT ($p=0.000$, $p=0.042$ and $p=0.001$, respectively).

With respect to symptoms and stimuli that make women aware that their skin may become sensitive or more sensitive due to the menstrual cycle or menopause, for premenopausal women, the most commonly reported symptoms were bumps/pimples (85.2%), dryness (32.8%), and itching (29.5%), while stimuli were shaving (37.7%), the weather (32.8%), emotions (31.1%), and toiletries (29.5%). For peri- and postmenopausal women, the most commonly reported symptoms were dryness (54%), itching (46%), and redness (36%), while stimuli were weather (42%), emotions (38%), and toiletries (38%). The full list of symptoms and stimuli is shown in supplementary Table S1.

Characteristics of facial/body skin and sensitive body parts according to hormonal status

No significant differences in the patterns of facial and body skin were present across different hormonal status. Overall, women reported mostly combined dry and oily skin on the face (47.8%), while normal and dry skin were mostly reported on the body (37.2% and 44.8%, respectively). The characteristics of facial and body skin are reported in supplementary Table S2. The list of sensitive body parts is shown in Table 4. Significant differences were present for the face ($p=0.020$) and feet ($p=0.044$), more frequently reported by premenopausal and perimenopausal women, respectively. Overall, the most frequently reported sensitive body parts were the face (53.6%), legs (27.7%), and hands (27.3%).

Table 2. Relationship between the effect of menstrual cycle on self-assessed sensitive skin and sociodemographic, lifestyle, and health characteristics (n=121).

		Effect of menstrual cycle on SS		
		Yes	No/Rare	
	n	n %	n %	p value
Age group (years)				0.246
20 - 35	58	28 *60.9*	30 *49.2*	
36 - 45	49	18 *39.1*	31 *50.8*	
Use of hormones as contraceptives				0.195
Yes (currently)	58	21 *41.2*	37 *54.4*	
No (in the past or never)	61	30 *58.8*	31 *45.6*	
Self-assessed sensitive skin				0.256
SS	56	27 *56.2*	29 *44.6*	
NSS	57	21 *43.8*	36 *55.4*	
Intensity of MC symptoms				**0.002**
Low (absent or mild)	53	14 *27.5*	39 *56.5*	
High (moderate or severe)	67	37 *72.5*	30 *43.5*	
Atopic predisposition				0.846
Yes	40	18 *35.3*	22 *33.3*	
No	77	33 *64.7*	44 *66.7*	
Skin disease				1
Yes	23	10 *19.6*	13 *18.8*	
No	97	41 *80.4*	56 *81.2*	
Body Mass Index (kg/m²)				0.542
Mean (± SD)	116	24.59 (± 4.54)	25.12 (± 4.83)	

MC: menstrual cycle; NSS: non-sensitive skin; SS: sensitive skin.

Table 3. Relationships between the effect of menopause on self-assessed sensitive skin and sociodemographic, lifestyle, and health characteristics (n=157).

		Increased SS after menopause				
		Yes		No		
	n	n	%	n	%	p value
Age group (years)						0.190
46 - 55	67	29	*63.0*	38	*49.4*	
56 - 65	56	17	*37.0*	39	*50.6*	
Use of hormones as HRT						**0.001**
Yes (currently)	10	9	*18.4*	1	*1.2*	
No (in the past or never)	120	40	*81.6*	80	*98.8*	
Self-assessed sensitive skin						**0.000**
SS	63	34	*72.3*	29	*37.7*	
NSS	61	13	*27.7*	48	*62.3*	
Intensity of M symptoms						**0.018**
Low (absent or mild)	58	15	*30.6*	43	*53.1*	
High (moderate or severe)	72	34	*69.4*	38	*46.9*	
Atopic predisposition						0.442
Yes	44	19	*39.6*	25	*31.6*	
No	83	29	*60.4*	54	*68.4*	
Skin disease						**0.042**
Yes	14	9	*18.0*	5	*6.1*	
No	118	41	*82.0*	77	*93.9*	
Body Mass Index (kg/m²)						0.839
Mean (± SD)	130	26.75 (± 5.61)		26.37 (± 5.08)		

HRT: hormonal replacement therapy; M: menopause; NSS: non-sensitive skin; SS: sensitive skin.

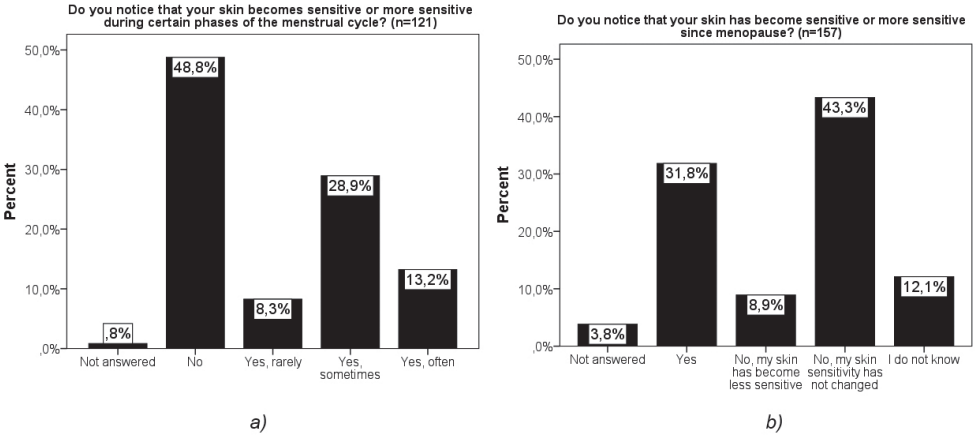

Figure 1. Perceived effects of the menstrual cycle and menopause on self-assessed sensitive skin (SS). **a**) Premenopausal women (*n*=121) answered the question: "Do you notice that your skin becomes sensitive or more sensitive during certain phases of the menstrual cycle?". Women answering "No" (48.8%) and "Yes, rarely" (8.3%) were included in the group "No or rare perceived effect of menstrual cycle on SS", while women answering "Yes, sometimes" (28.9%) and "Yes, often" (13.2%) were included in the group "Perceived effect of menstrual cycle on SS". **b**) Peri- and postmenopausal women (*n*=157) answered the question: "Do you notice that your skin has become sensitive or more sensitive since menopause?". Women answering "No, my skin has become less sensitive" (8.9%) and "No, my skin sensitivity has not changed" (43.3%) were included in the group "No perception of increased SS after menopause", while women answering "Yes" (31.8%) were included in the group "Perception of increased SS after menopause".

Table 4. Sensitive body parts according to hormonal status (n=278). Respondents were allowed to choose multiple body parts.

	Premenopausal		**Perimenopausal**		**Postmenopausal**		
	n	%	n	%	n	%	p value
Equally sensitive	25	*20.7*	10	*18.2*	23	*22.5*	0.841
Face	75	*62.0*	30	*54.5*	44	*43.1*	**0.020**
Hands	28	*23.1*	16	*29.1*	32	*31.4*	0.358
Neck	10	*8.3*	4	*7.3*	5	*4.9*	0.612
Chest	16	*13.2*	14	*25.5*	17	*16.7*	0.136
Back	12	*9.9*	6	*10.9*	9	*8.8*	0.867
Genitals	10	*8.3*	7	*12.7*	13	*12.7*	0.468
Legs	28	*23.1*	15	*27.3*	34	*33.3*	0.244
Feet	14	*11.6*	14	*25.5*	21	*20.6*	**0.044**

DISCUSSION

The primary objective of this cross-sectional study was to investigate the perceived influence of fluctuating hormone levels during the menstrual cycle and menopause on self-assessed SS. A high percentage of premenopausal women reported (stronger) perceptions of symptoms associated with SS just before and during menstruation (42.1%). A lower, but still important, percentage of peri- and postmenopausal women perceived this following menopause (31.8%). Women with a higher intensity of (non-skin-related) perimenstrual and menopausal symptoms were more likely to perceive these effects. The onset or exacerbation of SS due to the menstrual cycle seems to be independent of normal self-assessed skin sensitivity, atopic predisposition, and affection by skin diseases, as demonstrated by the lack of associations found in this study. Atopic predisposition and skin diseases have been included among the risk factors leading to a greater likelihood of reporting SS. It could be speculated that their reported exacerbation during the menstrual cycle [7, 18] has an indirect influence on SS, for example, through a worsening of the skin barrier function or through an alteration of the skin inflammatory mechanisms; however, we could not observe such an effect in our study. In contrast, menopausal women with skin diseases more frequently perceived increased SS following menopause, compared with women without skin diseases. Psoriasis and other autoimmune (skin) diseases are known to occur or be exacerbated during menopause [19, 20], and this could possibly mediate indirect effects on SS. In addition, the lack of a significant difference in prevalence of SS across different hormonal status suggests that the effect of menopause might be perceived primarily by women who had SS already before menopause. Oestrogen depletion during menopause could exacerbate a clinical status already affected by the pathomechanisms involved in SS, while having no major impact on NSS. The association between increased SS following menopause and the use of HRT could be mediated by the intensity of menopausal symptoms, since women taking HRT were also characterised by a higher symptom intensity ($p=0.020$). Given the low number of women taking HRT in this study, its effects on SS should be evaluated in a larger population.

As a second objective, we investigated self-assessed characteristics of facial and body skin and sensitive body parts according to hormonal status. No significant differences in skin assessment were identified between different hormonal status for either facial or body skin, despite the fact that oestrogen depletion in menopause has been linked to decreased sebum production and skin hydration [8, 21]. One study showed that the lowest levels of skin surface lipids could be measured in women older than 70 years [22]. Less combined oily and dry or only oily skin, due to decreased sebum secretion, could thus be perceived by women older than the participants included in this study. With respect to skin hydration, a high percentage (54%) of peri- and postmenopausal women reported skin dryness as one of the symptoms responsible for increased SS following the menopause. This result is in line with the effects of oestrogen decline occurring during the menopause [8, 17, 21, 23]. The lack of any difference

in the percentage of dryness of facial or body skin between different hormonal status might be explained by the effect of cold weather at the time when the questionnaire was distributed, prompting an overall higher percentage of women to report dry skin [24]. The lower percentages of peri- and postmenopausal women who reported SS of the face could be related to a lower sensory perception due to age-related decrease in epidermal nerve fibre density [25]. Another mechanism could be the age-related increase in corneocyte size, due to a slower epidermal cell proliferation rate [26], which could improve skin barrier function and lead to decreased skin sensitivity. However, while some studies reported improved skin barrier function in aged facial skin, measured as decreased TEWL, others could not detect differences [22, 27]. The feet, reported by a higher percentage of peri- and postmenopausal women, are described to undergo loss of fat distribution and sensitivity to touch with age, and a cold sensation to the feet was included as a menopausal symptom [28-30]. Whether these factors could have prompted more women to report the feet as a sensitive body part, or whether other skin changes could have played a role, remains to be investigated.

In conclusion, based on the results of our study, we confirm the importance of taking into consideration the fluctuating hormone levels during the menstrual cycle and menopause in the evaluation of patients' symptoms in clinical practice, in the assessment of skin responses to dermato-cosmetic treatments, as well as in the selection of personal care routine [18]. This holds true particularly for women with a higher intensity of perimenstrual and menopausal symptoms, as well as for menopausal women with skin diseases. Subjective assessments of skin dryness during the menstrual cycle and menopause might be linked to quantitative and direct measurement of skin water content by confocal Raman microspectroscopy, a technique for *in vivo* analysis of skin molecular composition [31]. Given the magnitude of influence of oestrogen on female physiology [20], we underline the importance of performing further research in order to unravel the mechanisms by which oestrogen exerts its influence on normal and aging skin, as well as on autoimmune diseases such as psoriasis.

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SUPPLEMENTARY MATERIAL

Table S1. Symptoms and stimuli which make women realize that their skin becomes (more) sensitive during certain phases of the menstrual cycle or in menopause. Respondents were allowed to choose multiple answers.

	MC (n=61)		**M** (n=50)	
Symptoms	n	*%*	n	*%*
Bumps/pimples	52	*85.2*	17	*34.0*
Stinging	2	*3.3*	2	*4.0*
Dryness	20	*32.8*	27	*54.0*
Redness	7	*11.5*	18	*36.0*
Itching	18	*29.5*	23	*46.0*
Burning	7	*11.5*	6	*12.0*
Tightness	6	*9.8*	12	*24.0*
General skin discomfort	5	*8.2*	12	*24.0*
Other	2	*3.3*	4	*8.0*
I do not know	3	*4.9*	2	*4.0*
Stimuli	n	*%*	n	*%*
Weather	20	*32.8*	21	*42.0*
Sun exposure	11	*18.0*	12	*24.0*
Shaving	23	*37.7*	10	*20.0*
Emotions	19	*31.1*	19	*38.0*
Toiletries/cosmetics	18	*29.5*	18	*36.0*
Clothes	5	*8.2*	10	*20.0*
Other	4	*6.6*	5	*10.0*
I do not know	17	*27.9*	9	*18.0*

MC: menstrual cycle; M: menopause.

Definitions of symptoms used in the questionnaire:

Bumps/pimples = bumps or papules with or without white top
Stinging = sensation like having needles prickling the skin, or having alcohol dabbed on a wound
Dryness = the skin is dry and scales
Redness = presence of red spots and stripes, sometimes extended, on the skin
Itching = sensation that causes the desire to scratch
Burning = the skin feels warm, like if a warm object is put on the skin
Tightness = the skin feels tense and less flexible
No description provided for general skin discomfort

Table S2. Characteristics of facial and body skin according to hormonal status (n=278). Respondents were allowed to choose only one skin type.

	Premenopausal		**Perimenopausal**		**Postmenopausal**		
	n	%	n	%	n	%	p value
Facial skin type							0.714
Normal	32	*26.7*	14	*25.9*	32	*31.4*	
Dry	27	*22.5*	7	*13.0*	18	*17.6*	
Oily	7	*5.8*	3	*5.6*	4	*3.9*	
Combined*	54	*45.0*	30	*55.6*	48	*47.1*	
Body skin type							0.160
Normal	39	*32.2*	22	*40.7*	42	*41.2*	
Dry	55	*45.5*	20	*37.0*	49	*48.0*	
Oily	2	*1.7*	0	*0*	0	*0*	
Combined*	25	*20.7*	12	*22.2*	11	*10.8*	

*Combined: concomitant presence of dry and oily parts.

ABBREVIATIONS

AUC	Area under the curve
CI	Confidence interval
OR	Odds ratio
SS	Sensitive skin
NSS	Non-sensitive skin
ROC	Receiver operating characteristic
SC	Stratum corneum

4.2

Risk factors associated with sensitive skin and potential role of lifestyle habits: a cross-sectional study

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ABSTRACT

Sensitive skin (SS) is a widespread condition, but still not completely understood. To identify risk factors that increase the likelihood of SS, 258 women aged between 20 and 65 years old and resident in the Netherlands were surveyed by questionnaire, which included questions on sociodemographic characteristics (age group, Fitzpatrick skin type, hormonal status), health state (atopic predisposition, skin diseases), and lifestyle habits (history of smoking and of sun exposure, frequency of physical exercise). Analysis of the responses confirmed that atopic predisposition, presence of skin diseases and Fitzpatrick skin types I and II are risk factors significantly associated with SS. In addition, as current or past smoking and a history of low sun exposure showed a trend to increase the likelihood of reporting SS, we suggest that the potential role of lifestyle factors in the onset or exacerbation of SS should be investigated further.

In the industrialized world, 50–61% of women and 30–44% of men report having sensitive skin (SS), a condition characterized by the presence of subjective perceptions (*e.g.* stinging, itching, burning) to mild stimuli of different nature [1]. However, clear characterization of SS remains elusive because of the variability of the reported stimuli, symptoms and severity, and the frequent absence of objective signs of skin irritation [1]. An important step towards unraveling the pathomechanisms involved in SS is to perform survey-based studies to evaluate which factors are particularly related to this condition [2, 3]. Knowledge of such risk factors would be instrumental in better understanding these symptoms in clinical practice, and in better selecting individuals who are prone to experience SS and who could be included in clinical studies to evaluate the potential pathomechanisms involved in SS. The objective of this study was therefore to identify risk factors associated with the likelihood of reporting SS. For this purpose, we distributed a digital questionnaire to a sample population of women. Following an exploratory approach, several factors were considered: age, Fitzpatrick skin type, hormonal status, presence of atopic predisposition and presence of skin diseases. In addition, we explored the possible role of lifestyle habits, including smoking, history of sun exposure and frequency of physical exercise. Although an association between SS and fair skin type, atopic predisposition and skin diseases has already emerged in previous studies [2, 3], to our knowledge no survey-based study has as yet evaluated the role of lifestyle habits.

REPORT

Informed consent was obtained from all individual participants included in the study. The recruiting procedure and the design of the questionnaire have been described in detail previously [4]. Briefly, the digital questionnaire was distributed, in December 2014, to women aged between 20 and 65 years old and resident in the Netherlands. In total, 301 women responded to the questionnaire. After exclusion of women who did not specify their age group or hormonal status (n=23), who did not know how to classify the sensitivity of their skin (n=19) or did not answer the question on skin sensitivity (n=1), 258 women were included in the analysis. Of these, the percentage of women with SS was 45.3% (n=126), while the percentage of women with non-sensitive skin (NSS) was 47.4% (n=132). Statistical analyses were performed using SPSS Statistics version 20 for Windows (IBM SPSS Inc., USA) and SAS version 8.2 for Windows (SAS Institute, USA). Significance level was set at p value <0.05. In the first step, univariate logistic regression was used to explore factors related to SS. The crude odds ratios (ORs) for the likelihood of reporting SS using univariate logistic regression are shown in Table 1. Significant factors related to a higher likelihood of reporting SS were presence of atopic predisposition (OR=1.75, 95% CI 1.04–2.95) and presence of skin disease (OR=2.60, 95% CI 1.25–5.42). Significant factors related to a lower likelihood of reporting SS were Fitzpatrick skin type III, IV, V and VI (OR=0.53, 95% CI 0.32–0.90) and history of moderate to high sun

exposure (OR=0.41, 95% CI 0.18–0.91). A positive history of smoking (current or past) increased the risk of SS with respect to having never smoked (*p=0.13*). In the second step, factors with a significance level lower than a predefined cut-off value (*p=0.20*) were included in a multivariate logistic regression model with forward selection procedure. The aim was to identify which factors contributed independently to the risk of SS. The adjusted ORs of the final model are presented in Table 2. Factors significantly (*p=0.001*) associated with the likelihood of reporting SS were presence of skin disease (OR=2.41, 95% CI 1.13–5.14), atopic predisposition (OR=1.77, 95% CI 1.03–3.03) and Fitzpatrick skin type III, IV, V and VI (OR=0.54, 95% CI 0.31–0.92). This indicated that women with skin diseases or atopic predisposition were, respectively, 2.41 and 1.77 times more likely to report SS than women without these conditions, after controlling for other factors in the model. In addition, women with fair skin types (I and II) were 1.85 times (reciprocal of 0.54) more likely to report SS than women with darker skin types (III, IV, V and VI). The model explained 8.16% (pseudo-R^2) of the variance in SS, and its discriminatory power, expressed as area under the curve (AUC) of the receiver operating characteristic curve (ROC), was 63.2%. Our data confirm that atopic predisposition, presence of skin diseases and Fitzpatrick skin types I and II are independent risk factors that increase the likelihood of reporting SS, as reported by previous survey-based studies [2,3]. Of note, current or past smoking and a history of low sun exposure showed a trend to make a significant contribution to the model, although this was not significant (*p=0.06*). As both of these factors increased the risk of SS, this raises the question as to the role that these lifestyle habits might have on SS. Smoking is already associated with the clinical severity of psoriasis [5]. A previous study found that the thickness of the stratum corneum (SC) correlates negatively with years of smoking but not with the smoking status [6], and this might play a role in SS, as a thinner SC could lead to a higher susceptibility to irritants. Controlled, repetitive ultraviolet light exposure improves the barrier function of patients with skin diseases such as atopic dermatitis and psoriasis, and has been shown to decrease the responses to irritants [7]. A history of moderate to high sun exposure might bring similar benefits to SS. Although it is reasonable to believe that skin type and sun exposure are to a certain extent correlated, with people with fairer skin types more likely to avoid sun exposure than those with darker skin types because of the stronger side effects, we believe that the additional independent contribution of sun exposure to SS should be evaluated in further studies. The same holds true for smoking and other lifestyle factors with potential effects on SS, such as dietary habits, urban or rural place of living (with more or less pollution) and stressful (working) environment [8–10].

In conclusion, our study confirms that atopic predisposition, presence of skin diseases and Fitzpatrick skin types I and II are risk factors significantly associated with SS. It also appears that current or past smoking and a history of low sun exposure may also affect SS, and we suggest that the potential role of lifestyle factors in the onset or exacerbation of SS should be investigated further.

Table 1. The (crude) ORs to predict the likelihood of reporting sensitive skin, along with the 95% CI, using univariate logistic regression analysis (n = 258).

	n	OR (95% CI)	p value
Age group			
20-35y	59	1.00 (reference)	0.133
36-45y	57	2.16 (1.03; 4.55)	
46-55y	87	1.33 (0.68; 2.59)	
56-65y	55	0.98 (0.46; 2.06)	
Hormonal status			
Premenopausal	114	1.00 (reference)	0.912
Perimenopausal	51	1.08 (0.56; 2.09)	
Postmenopausal	93	0.93 (0.54; 1.61)	
*Physical exercise**			
Low	35	1.00 (reference)	0.448
Moderate , High	219	0.75 (0.64; 2.71)	
*Fitzpatrick skin type**			
I, II	90	1.00 (reference)	**0.018**
III, IV, V, VI	164	0.53 (0.32; 0.90)	
*Sun exposure**			
Low	31	1.00 (reference)	**0.028**
Moderate, High	223	0.41 (0.18; 0.91)	
*Smoking**			
Never	109	1.00 (reference)	0.129
Currently , In the past	144	1.47 (0.89; 2.43)	
Atopic predisposition#			
No	158	1.00 (reference)	**0.034**
Yes	92	1.75 (1.04; 2.95)	
*Skin disease^*			
No	220	1.00 (reference)	**0.011**
Yes	38	2.60 (1.25; 5.42)	

*Categories were grouped because of similar risk values compared with sensitive skin.

#Defined as history of atopic dermatitis, asthma or hay fever.

^Most often reported skin diseases were psoriasis (n=9), contact dermatitis (n=8) and acne (n=7).

Table 2. The (adjusted) ORs to predict the likelihood of reporting sensitive skin, along with the 95% CI, using multivariate logistic regression analysis with forward selection procedure (n = 258).

	OR	(95% CI)
Fitzpatrick skin type		
I, II	1.00	(reference)
II, IV, V, VI	0.54	(0.31; 0.92)
Atopic predisposition		
No	1.00	(reference)
Yes	1.77	(1.03; 3.03)
Skin disease		
No	1.00	(reference)
Yes	2.41	(1.13; 5.14)

AUC = 63.2%, pseudo R^2 = 8.16%.

LEARNING POINTS

- Sensitive skin (SS) is defined as the presence of heightened sensory perceptions to mild stimuli, often in the absence of objective signs of skin irritation.
- SS is a widespread condition in the industrialized world; however, general consensus on its pathomechanisms is still pending.
- Identifying risk factors that increase the likelihood of reporting SS would be an important step towards a better understanding of this condition in clinical practice and better selection of subjects in clinical studies.
- Fair skin type, presence of skin diseases and atopic predisposition are confirmed to be risk factors that increase the likelihood of reporting SS.
- Past or current smoking and a low history of sun exposure showed a trend to increase the likelihood of reporting SS.
- The effects of these and other lifestyle factors with possible effects on the skin (*e.g.* dietary habits, stress) on the onset or exacerbation of SS should be further investigated.

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Chapter 5

In vivo evaluation of skin irritation at the molecular level

ABBREVIATIONS

AD	Atopic dermatitis
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
IL	Interleukin
PBS	Phosphate buffered saline
SC	Stratum corneum
SLS	Sodium lauryl sulfate
STROBE	Strengthening the reporting of observational studies in epidemiology
TAP	Transdermal analyses patch
TDF	Transdermal fluid
UV	Ultraviolet

5.1

Minimally-invasive sampling of interleukin 1 alpha and interleukin 1 receptor antagonist from the skin: a systematic review of *in vivo* studies in humans

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ABSTRACT

Interleukin-1α (IL-1α) and its receptor antagonist IL-1RA play a pivotal role in skin homeostasis and disease. Although the use of biopsies to sample these cytokines from human skin is widely employed in dermatological practice, knowledge about less invasive, *in vivo* sampling methods is scarce. The aim of this study was to provide an overview of such methods by systematically reviewing studies in Medline, EMBASE, Web of Science and the Cochrane Library using combinations of the terms "IL-1α", IL-1RA", "skin", "human", including all possible synonyms. Quality was assessed using the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) checklist. The search, performed on 14 October 2016, revealed ten different sampling methods, with varying degrees of invasiveness and wide application spectrum, including assessment of both normal and diseased skin, from several body sites. The possibility to sample quantifiable amounts of cytokines from human skin with no or minimal discomfort holds promise for linking clinical outcomes to molecular profiles of skin inflammation.

INTRODUCTION

The primary function of the skin is to provide a physical barrier between the internal milieu and the external environment [1, 2]. In addition, the skin was more recently recognized to have a strong immunological role [3]. Keratinocytes and resident skin cells, such as fibroblasts, mast cells, Langerhans cells and other dermal dendritic cells, have been shown to release a wide variety of mediators, both in the maintenance of tissue homeostasis (immune-surveillance) as well as in case of injury or pathogen invasion [4]. Among the most characterized mediators are interleukins (IL) belonging to the IL-1 family, namely IL-1α, IL-1β and their receptor antagonist IL-1RA [5]. These cytokines are among the first mediators to be released in acute or chronic skin inflammation and are involved in a wide spectrum of (skin) diseases [4, 5]. In this respect, blocking IL-1 activity for therapeutic purposes has entered clinical practice [6]. Of note, unlike IL-1β, IL-1α and IL-1RA have also been shown to be detectable in normal skin [4].

Evidence for the presence of biologically active IL-1 in normal epidermis and stratum corneum (SC) emerged in the mid 1980s by means of bioassays, which measured the ability of IL-1 to augment proliferation of specific cell lines (*e.g.* murine thymocytes in the thymocyte co-stimulation assay) or to stimulate release of collagenase or prostaglandin from human dermal fibroblasts [7, 8]. Bioassays were limited by the impossibility of discriminating between IL-1α and IL-1β as well as between IL-1 and other cytokines [9], prompting some authors to cautiously refer to their findings as IL-1 or IL-1α "like" material [10]. In the late 1980s these obstacles were overcome by the advent of monoclonal antibodies against IL-1α and IL-1β, allowing the two isoforms to be determined and distinguished with high sensitivity and specificity [9]. Since then, enzyme immunoassays (EIA) and enzyme-linked immunosorbent assays (ELISA) have become widely used for the evaluation of soluble analytes in biological samples [11, 12]. The major shortcomings of ELISA are that it allows the measurement of only one analyte at a time in a given sample and that it requires relatively large sample amounts (typically 100 µL) [13]. Building on the principles of ELISA, in the late 1990s multiplex arrays were introduced with the purpose of measuring multiple analytes in the same sample at the same time [13, 14]. An example is constituted by multiplex bead-based assays, in which sets of microscopic color-coded beads (microspheres) coated with capture antibodies for specific analytes are simultaneously used [13, 14]. By flow cytometric analysis, the signal coming from the different bead sets can be distinguished and the binding events between the detection antibodies and the analyte-capture antibody complex on each bead set can be quantified [13, 14]. Comparability analyses between ELISA and multiplex bead-based assays yielded positive results [15].

Given the relevance of IL-1 in skin homeostasis and disease, different sampling methods, followed by the above-mentioned immunoassay quantifications, have been reported to analyze it. While taking skin biopsies remains the most widely used approach in clinical practice, successful attempts using less invasive sampling methods have been reported.

The aim of this study was to provide an overview of such minimally invasive methods by systematically reviewing studies in which IL-1α and/or IL-1RA were sampled from the skin of human volunteers *in vivo*. The choice was restricted to these two mediators because of their constitutive presence both in normal and diseased skin and their clinical relevance [4-6]. For each method, applications as well as advantages and shortcomings were highlighted. We hope that this review will increase awareness of the additional insights offered by analysis of local skin inflammation at the molecular level and will foster research towards implementation of minimally-invasive biomarker analysis in dermatological practice.

METHODS

An extensive literature search was performed on 14 October 2016 in four computerized bibliographical databases: Medline, EMBASE, Web of Science and the Cochrane Library. Medical subject heading and free text searches embracing the following terms were used: "interleukin 1 alpha", "interleukin 1 receptor antagonist", "skin", "human", including all possible abbreviations and synonyms. The complete search strategy is shown in supplementary Table S1. The literature search was limited to publications in English. No date restrictions applied.
The article selection process followed three steps. In step one, a first screening was performed by one reviewer (DF) in which titles and abstracts, and materials and methods when the abstract was not available, were reviewed for relevance, taking into account a list of exclusion criteria. In case of uncertainty or if none of the exclusion criteria were met, in step two a second screening was performed in which the full text was retrieved and inclusion judged by two independent reviewers (DF and PvE) on the basis of a list of inclusion criteria. Consensus on inclusion was reached by discussion. In step three, the reference lists of the eligible studies were additionally screened to ensure that all relevant studies were included. The schematic representation of the article selection process and the list of exclusion and inclusion criteria is shown in supplementary Figure S1.

The methodological quality of the included studies was assessed by the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) criteria: each study received, independently by the two reviewers (DF and PvE), a score ranging from 0 to 22, which was then expressed as a percentage of the maximum score [16]. Depending of the level of fulfillment of the criteria stated in STROBE, articles were categorized as A (>80%), B (60%-80%) or C (<60%). When scores differed by more than 1.5, consensus was reached by discussion.

RESULTS

Article selection and quality

The search in the four bibliographical databases revealed a total of 6598 hits. After exclusion of duplicates and of articles meeting any of the exclusion criteria in the first screening, 535 articles remained for full-text evaluation. For most articles, the exclusion criteria from the first screening applied following full-text evaluation. Four articles for which the inclusion criteria applied were excluded because of the lack of description of the human volunteers from which the skin material was taken or of the method used to obtain the skin material. Manual screening of the reference lists resulted in the inclusion of one more article. In total, 63 articles were included in this review. The number of articles at each step of the selection process is shown in supplementary Figure S2.

Article quality was, on average, good: eight articles were classified as a category C (score <60%), 47 articles were classified as a category B (score 60-80%) and the remaining eight articles were classified as a category A (score >80%). Shortcomings were mainly related to the lack of description of potential confounders and effect modifiers concerning the study participants (*e.g.* age, skin type) or the sampling procedure (*e.g.* season and experimental conditions in which the sampling was performed). In addition, only a few studies specified the number of participants included at each stage.

Each sampling technique is presented in the following paragraphs. An overview of each technique, including collection time, applications, advantages and shortcomings is shown in Table 1. A detailed summary of each study included in this review can be requested from the first author.

Tape stripping

The majority of articles (n=24) sampled IL-1α and/or IL-1RA using tape stripping. This technique was characterized by a high heterogeneity in the sampling procedure. Unfortunately, only a few articles thoroughly described the sampling procedure by specifying the type, area and number of adhesive tapes used, the application time of each tape on the skin, and whether constant pressure was applied, all variables known to influence the procedure [17]. For the quantification of the protein levels of IL-1α and/or IL-1RA, the majority used EIA/ELISA (n=16) [18-33] and the remaining used multiplex arrays [34-41]. Concomitantly to the analysis of IL-1α and/or IL-1RA, nine articles used tape stripping to sample additional cytokines and chemokines [20-23, 34, 36-38, 40]. The additional measurement of the total protein content on the tapes was used as a biomarker of the cohesiveness of the SC [20, 27, 36, 40, 41] .

Among the advantages of tape stripping, the minimal invasiveness and the short duration of the procedure were mentioned [19, 20], suggesting that the cytokine amounts are hardly influenced by the sampling process [20]. Among the drawbacks, the fact that in normal SC only IL-1α and IL-1RA are present in sufficient amounts for routine cytokine determination [19]. In agreement with this, some articles reported that other cytokines and chemokines were not or rarely detected [21-23, 34]. Moreover, a study of de Jongh *et al.* [20] evaluated the distribution of IL-1α and IL-1RA in the SC, showing that, while in normal SC the distribution does not change with depth, after perturbation with chemical substances it changes significantly. The authors concluded that, in the study of irritated or diseased skin, it would be better to sample the whole SC. Observations about the variability of amounts across the SC were also reported in other studies [32, 40].

Skin suction blistering

The second most widespread method to sample IL-1α and/or IL-1RA was based on skin suction blistering (n=15). In this technique, introduced by Kiistala *et al.* [42] in the 1960s, the viable epidermis is separated from the dermis by application of negative pressure, resulting in the generation of artificial blisters. In the articles included in this review, the negative pressure was between 200 and 400 mmHg, the suction cups ranged in size from 5 to 15 mm and the time needed for the formation of the blisters was mostly 2-3 hours. Warming was sometimes added to aid blister formation. For the quantification of the protein levels of IL-1α and/or IL-1RA, the majority used EIA/ELISA (n=10) [43-52] and the remaining used multiplex arrays [53-57]. All studies but two analyzed additional cytokines and chemokines, albeit many reported that not all could be detected [43-45, 51, 52, 54, 55].

The advantage offered by the assessment of the local inflammatory profile in the suction blister fluid [54, 55] was counterbalanced by the suggestion that the local trauma caused by the suction process might induce cytokines release and formation, thereby influencing the measured amounts [46-48, 50]. Other reported issues included the cumbersomeness of the technique [56], the possibility of pain during the procedure [57] and the possibility of blood contamination of the blister [52].

Adsorption by Sebutape

The third most common method consisted in the adsorption of IL-1α and/or IL-1RA from the skin surface by means of a lipophilic tape, Sebutape (CuDerm, Dallas, TX, USA) (n=9). Sebutape, originally developed for the extraction of sebum from the skin, was demonstrated by Perkins *et al.* [58] to be able to recover quantifiable levels of inflammatory proteins from normal skin. All articles used ELISA to quantify the protein levels of IL-1α and/or IL-1RA [58-65], except one which used a multiplex array [66]. In addition to the measurement of IL-1α and/or IL-1RA, four articles sampled additional cytokines and chemokines [58, 63, 64, 66]. However,

authors reported that some of these additional biomarkers were frequently extremely low or not detected.

The minimal-invasiveness of the method was demonstrated by Perkins *et al*. [58] by analyzing the impact of 30 sequential applications of Sebutape on the skin and finding no upregulation of IL-1α nor IL-8, another inflammatory cytokine, up to 24 hours after stimulation. However, the same author warned about the problem of sebum deposition on the tape, which could compete with IL-1α for adsorption. As a consequence, all articles employing this method used short (1-2 minutes) collection times.

Skin chamber technique

Four articles sampled IL-1α and/or IL-1RA using a skin chamber technique. This technique, developed from the skin suction blistering method, was used as *in vivo* model to study the cutaneous inflammatory response in humans and, specifically, the dynamics of leukocyte migration [67]. In this procedure, after removal of the blister roof obtained by application of negative pressure, the denuded dermis ("skin window") is covered by a chamber containing a medium capable to induce a chemotactic response (mostly autologous serum) [67]. All articles included in this review collected the skin chamber fluid between 3 and 24 hours after removal of the blister roofs and, besides IL-1α and/or IL-1RA, used ELISA to analyze additional cytokines and chemokines [68-71].

Microporation

Three articles used microporation to sample IL-1α and/or IL-1RA. In this technique, an infrared laser is used to generate micropores (approximately 100-μm wide) up to the first tens of μm in the epidermis. Transdermal fluid (TDF) is then collected by application of negative pressure similarly to the skin suction blistering technique (around 300 mmHg for 3-4 hours). One article analyzed IL-1α with ELISA and IL-1RA with a multiplex array [72], whereas the others used only multiplex arrays [73, 74]. All studies also assessed additional cytokines and chemokines but reported that some were extremely low or not detected.

While being less invasive than the skin blistering technique, as the impact on the integrity of the tissue is relatively minor [74], concerns arise about the collection efficiency of TDF, since in one out of nine volunteers [72], seven out of 28 volunteers [73] and six out of 16 volunteers [74] collection of TDF failed or was too low. The possible impact of the procedure on the measured amounts of cytokines should be considered and additional data on the reproducibility of the depth of the laser-generated micropores would be needed.

Swabbing

Three articles sampled IL-1α and/or IL-1RA by swabbing the skin with cotton-tipped swabs or cotton buds impregnated with a solution of saline and Triton to lyse cells and extract

proteins. All articles used ELISA to analyze IL-1α and/or IL-1RA together with other cytokines and chemokines [75-77].

Skin surface wash sampling

Two articles sampled IL-1α and IL-1RA by means of skin surface wash. In this technique, proposed by Portugal-Cohen *et al.* [78], a small well is attached to the skin by an adhesive pad and is filled with 0.5-1 mL extraction buffer consisting of phosphate buffered saline (PBS) with pH around 7. After sealing, the well is left on the skin for a 30-minute incubation time. Collected samples were analyzed with ELISA in the study of Portugal-Cohen *et al.* [78] and with multiplex arrays with varying detectability levels in a more recent study [79].

The advantage of the method relies on its minimal invasiveness, whereas its drawback is the relatively long incubation time [80]. Moreover, the extraction buffer is only suitable to extract hydrophilic compounds; lipophilic compounds could be sampled using a more suitable extraction buffer containing surfactants [80].

Of note, a similar version of this method (though defined as a "skin chamber technique") was used by Reilly *et al.* [48] to sample biomarkers from tape-stripped skin. Yields of IL-1α increased by increasing the incubation time and the number of tape strips.

Microdialysis

One study used microdialysis to sample IL-1α and other biomarkers and quantified them by multiplex array [81]. Two linear semi-permeable membranes were inserted into the volar forearm of healthy volunteers at a depth of 0.7 mm for a length of 20 mm. Upon onset of probe perfusion with buffer, extracellular molecules would diffuse through the membranes into the probe lumen to be collected in the exiting fluid (dialysate). Dialysate collection lasted for 30 minutes and was performed at baseline and at several time points following intradermal injection of allergen.

Although almost all biomarkers could be detected following allergen injection, the use of microdialysis for sampling large proteins, such as cytokines, is challenging [82]. In order to increase relative recovery, an in-house membrane with molecular mass cut-off of 3000 kDa was used, much higher than the molecular mass cut-off of 100 kDa usually employed in commercially available membranes for cytokine sampling [82, 83]. This can expose the membranes to ultrafiltration, possibly causing significant loss of dialysate [82, 83]. Other effects potentially hampering the recovery rate of cytokines in microdialysis include low analyte concentration in the extracellular milieu and the adsorption onto the polymeric materials used to construct the membranes and the outlet probe [82, 83]. Finally, commonly to skin suction blistering and microporation, the trauma to the skin due to probe insertion and the possible effect on the measured amounts of biomarkers should be considered.

Scraping

In one article, delicate scraping with a surgical blade was used to obtain extracts of SC from healthy volunteers and scales from plaques of patients with psoriasis [84]. Samples of 10 mg were homogenized in PBS and levels of IL-1RA and other biomarkers were subsequently analyzed with a multiplex array.

Transdermal analyses patch

A novel method to sample IL-1α and IL-1RA from the skin surface was introduced by Orro *et al.* [85]. In this method, a transdermal analyses patch (TAP) is attached to the skin by means of a dermal adhesive plaster. On the side in contact with the skin, the patch contains a circular nitrocellulose micro-array (5 mm in diameter) coated with antibodies for four different protein biomarkers. On the upper side, covering the micro-array, a reservoir allows addition of a standardized amount of buffer (PBS at pH 7.3) for biomarker extraction and ensures close contact between the micro-array and the skin. In the study of Orro *et al.* [85], following optimization of the protein capture and detection protocols, 20 minutes resulted a suitable incubation time for biomarkers extraction. After removal of TAP, biomarkers were analyzed using ELISA.

In the same study, authors compared the biomarkers yield of TAP and of the skin surface wash sampling method, showing higher yield of proteins for the former. They suggested that the higher sensitivity of TAP might be due to higher concentration of proteins in the buffer, or to possible degradation of analytes in skin surface wash. Also Reilly *et al.* [48], using a similar version of skin surface wash, suggested that the reason why some mediators could not be detected could be dilution of the small amount of inflammatory exudates in a large volume of PBS.

Table 1. Overview of the minimally-invasive techniques to sample IL-1α and/or IL-1RA from the skin of human volunteers *in vivo*.

METHOD	n	Collection time	Body site(s) in which collection was performed	Applications	IL-1α and/or IL-1RA detectable?	Other biomarkers detectable?	Advantages	Shortcomings
Tape stripping	24	few minutes	forearm, neck, scalp, cheek, forehead, hand, sole, upper arm, trunk, leg, vulvar area, buttock	healthy skin diseased skin (dandruff, SeD, AD, psoriasis, CICD, acne, senile xerosis)	Yes	Variable	Short collection time Possibility to sample the distribution of biomarkers in the SC	Influence of several collection parameters on the outcome (*e.g.* pressure, type of tape)
Skin suction blistering	15	2-3 hours	forearm, hand, trunk, leg, buttock	healthy skin diseased skin (AD, psoriasis, SSc, PLE, BP, CRPS1)	Yes	Variable	Biomarkers in the blister fluid possibly more representative of the amount in the skin than biomarkers at the surface	Long collection time Cumbersome Possibility of pain/blood contamination Collected biomarkers possibly influenced by skin trauma
Adsorption by Sebutape	9	1-2 minutes	forearm, scalp, cheek, forehead, upper arm, trunk, leg, buttock	healthy skin diseased skin (dandruff, SeD, rosacea, striae distensae, diaper dermatitis)	Yes	Variable	Short collection time	Influence of sebum Only biomarkers at the skin surface are collected
Skin chamber technique	4	3-24 hours	forearm	healthy skin	Yes	Variable	*In vivo* model of cutaneous inflammation	Long collection time Cumbersome Possibility of pain
Microporation	3	3-4 hours	forearm	healthy skin diseased skin (AD)	Yes	Variable	Biomarkers in the transdermal fluid possibly more representative of the amount in the skin than biomarkers at the surface	Long collection time Collected biomarkers possibly influenced by skin trauma More data on the efficiency of transdermal fluid collection and on the depth of micropores needed
Swabbing	3	few seconds	scalp, arm, leg	healthy skin diseased skin (SeD, AD)	Yes	Variable	Short collection time	Only biomarkers at the skin surface are collected
Skin surface wash sampling	2	30 minutes	arm	healthy skin diseased skin (AD, psoriasis)	Yes	Variable	Average collection time	Only biomarkers at the skin surface are collected

Microdialysis	1	1.5 hours	forearm	healthy skin	Yes	Variable	Biomarkers in the dialysate possibly more representative of the amount in the skin than biomarkers at the surface	Long collection time Collected biomarkers possibly influenced by skin trauma Local anesthesia needed
Scraping	1	few minutes	elbow	healthy skin diseased skin (psoriasis)	Yes	Variable	Short collection time	Poor control on the amount of SC removed
Transdermal analyses patch	1	20 minutes	forearm, neck, cheek	healthy skin	Yes	Variable	Average collection time	Only biomarkers at the skin surface are collected

AD: atopic dermatitis; BP: bullous phemphigoid; CICD: chronic irritant contact dermatitis; CRPS1: complex regional pain syndrome type 1; PLE: polymorphic light eruption; SeD: seborrhoeic dermatitis; SC: stratum corneum; SSc: systemic sclerosis.

DISCUSSION

The primary objective of this study was to systematically review minimally-invasive methods whereby the pro-inflammatory cytokine IL-1α and its receptor antagonist IL-1RA were sampled from the skin of human volunteers *in vivo,* and subsequently analyzed at the protein level by means of immunoassays. Ten different methods were found. Common to all methods was the possibility to assess, in addition to IL-1α and/or IL-1RA, other biomarkers including pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines, and various growth factors. Since not every article explicitly mentioned if IL-1α, IL-1RA or the other biomarkers were measurable in all samples, a comparison of the different methods based on the detectability level of the collected biomarkers was not possible. As a general trend, IL-1α and IL-1RA were often or consistently measurable, whereas the other biomarkers were sometimes rarely or not detectable. Possible reasons could be absence in the epidermis, low production or production in different time courses, rapid uptake by target cells, and insufficient sensitivity of the assay [21, 48, 55, 58, 74, 81]. Another frequently reported aspect was the large inter-subject variability in the biomarkers amounts [21, 32, 36, 43, 45-47, 56, 58, 63, 64, 72, 73, 81], for which authors resorted to non-parametric statistical analyses or to log-transformation of the data.

The balance between the expression levels of cytokines of the IL-1 family is decisive in the generation of pro-inflammatory and homeostatic functions [5]. In particular, the ratio between IL-1RA and IL-1α can be used to assess skin inflammation [4]. Compared to healthy volunteers, an increase in the IL-1RA/IL-1α ratio was observed in patients with inflammatory skin diseases including psoriasis, atopic dermatitis (AD), dandruff, seborrhoeic dermatitis and rosacea [32, 36, 63, 64, 75]. This indicates that the increased ratio could be considered a non-specific hallmark of various kinds of inflammation [32, 36, 63], and might reflect an attempt to downregulate the inflammatory response [64]. This is supported by a decrease in the levels of IL-1α and in the IL-1RA/IL-1α ratio observed after topical treatments [18, 25, 27, 32, 35, 36, 39, 75, 77] and by a decreased IL-1α expression found in full-thickness biopsy specimens of psoriatic skin [86]. The ratio might also highlight differential expression of inflammatory markers following different modalities of skin irritation, since an increased ratio compared to normal skin was observed after repeated exposure to sodium lauryl sulfate (SLS) [21, 72] and a lower ratio was reported after single exposure to SLS [58] or after tandem exposure to SLS and another irritant [34]. Interestingly, an increased ratio could also distinguish non-lesional skin in patients with AD, psoriasis and rosacea from skin of healthy volunteers [32, 64], as well as sun-exposed skin on the face compared to unexposed skin [23, 32, 58, 85]. This suggests that inflammatory changes might be present even in normal-appearing skin and supports what Professor Kligman called "invisible dermatoses" [87].

Among the extrinsic factors possibly affecting the recovered amounts of IL-1α and IL-1RA figure season, since a lower IL-1RA/IL-1α ratio was found in summer compared to winter

[41], and ultraviolet (UV) exposure, since release of IL-1α and/or IL-1RA was observed following UV irradiation [23, 43, 56, 73]. These factors indicate that data collection and skin phototype (*e.g.* Fitzpatrick skin type) should be specified, as they might constitute potential confounders. In addition, the presence of IL-1α in sweat [22] and the increased expression of IL-1α at low humidity [88] suggest that the temperature and relative humidity of the experimental room should be standardized, as for measurements with bioengineering techniques [89-91]. Among the intrinsic factors, conflicting results emerged for gender, since differences were found in one study [19] and not in others [28, 35], as well as for age, since an effect was observed in some studies [23, 79] and not in others [31, 34]. In the particular case of infant skin, higher IL-1α levels without concomitant signs of inflammation were found in neonates compared to adults [38] and at one month post-partum compared to birth [62], suggesting that, for this specific age group, IL-1α might be used as a biomarker of skin barrier maturation [62].

The limitation of this study is that the restriction of the search strategy to IL-1α and IL-1RA could have excluded minimally-invasive methods used to sample other biomarkers. The addition of relevant cytokines and chemokines such as IL-1β, IL-6 and IL-8 [4] would have strengthened and, possibly, broadened the overview of minimally-invasive sampling methods; on the other hand, it would have also complicated the search strategy and prolonged the article selection process. Despite this obvious limitation, we believe that the relevance of the chosen biomarkers and the systematic article selection process maintain the validity of the overview of minimally-invasive methods presented in this review.

In conclusion, this review showed that several methods are available to collect quantifiable amounts of IL-1α, IL-1RA and other biomarkers from the skin, causing no or minimal discomfort. This is relevant on both practical and ethical grounds. Whereas it might be possible to argue that the levels of biomarkers measured in the fluid extracted by skin suction blistering, microporation and microdialysis are more representative of the overall levels present in the skin, the long collection time and the relative invasiveness make them less practical for use in routine testing. Tape stripping, adsorption with Sebutape, swabbing, skin surface wash sampling, scraping and TAP, characterized by shorter collection time and lower invasiveness, would be more fit for that purpose. However, it needs to be realized that these methods sample biomarkers at the skin surface, and that surface levels do not necessarily correspond to the ones in deeper skin layers. Independently of the method used, assessing the individual cytokine profile locally in the skin would bring additional insights than assessing it only systemically in blood [54, 55, 69, 81]. We strongly encourage performing clinical investigations to gain further insights into the relationship between clinical responses and biomarkers at the molecular level. This will hopefully lead to implementation these minimally-invasive methods in clinical practice to target and monitor therapies, predict disease progression and response to treatment.

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SUPPLEMENTARY MATERIAL

Table S1. Search strategy.

Search Topic	*Search terms: 14-10-2016* ***MEDLINE***	*Search terms: 14-10-2016* ***EMBASE***	*Search terms: 14-10-2016* ***Cochrane Library***	*Search terms: 14-10-2016* ***Web of Science***
Search A:	Mesh terms:	Mesh terms:	Mesh terms:	
Interleukin 1α	#1 exp Interleukin 1alpha/	#1 exp Interleukin 1alpha/	#1 Interleukin-1 alpha (explode all trees)	
Interleukin 1RA	#2 exp Interleukin 1 Receptor Antagonist Protein/	#2 exp Interleukin 1 Receptor Antagonist Protein/	#2 Interleukin 1 Receptor Antagonist Protein (explode all trees)	
	In free text (In title .ti or abstract .ab or keywords .kf):	*In free text (In title .ti or abstract .ab or keywords .kw):*	*In free text(In title ti or abstract ab or keywords kw):*	*In free text (Topic)*
	#3	#3	#3	
	((interleukin? adj2 1 alpha) OR (interleukin? adj2 1alpha) OR (interleukin? adj2 1 a) OR (interleukin? adj2 1a) OR (interleukin? adj2 1 receptor antagonist) OR (interleukin? adj2 1receptor antagonist) OR (interleukin? adj2 1 RA) OR (interleukin? adj2 1RA) OR (IL? 1 alpha) OR (IL? 1alpha) OR (IL? 1 a) OR (IL? 1a) OR (IL? 1 receptor antagonist) OR (IL? 1receptor antagonist) OR (IL? 1 RA) OR (IL? 1RA)).ti,ab	((interleukin? adj2 1 alpha) OR (interleukin? adj2 1alpha) OR (interleukin? adj2 1 a) OR (interleukin? adj2 1a) OR (interleukin? adj2 1 receptor antagonist) OR (interleukin? adj2 1receptor antagonist) OR (interleukin? adj2 1 RA) OR (interleukin? adj2 1RA) OR (IL? 1 alpha) OR (IL? 1alpha) OR (IL? 1 a) OR (IL? 1a) OR (IL? 1 receptor antagonist) OR (IL? 1receptor antagonist) OR (IL? 1 RA) OR (IL? 1RA)).ti,ab	((interleukin* near/2 ("1 alpha")) OR (interleukin* near/2 1alpha) OR (interleukin* near/2 ("1 a")) OR (interleukin* near/2 1a) OR (interleukin* near/2 ("1 receptor antagonist")) OR (interleukin* near/2 ("1receptor antagonist")) OR (interleukin* near/2 ("1 RA")) OR (interleukin* near/2 1RA) OR (IL* next "1 alpha") OR (IL* next 1alpha) OR (IL* next "1 a") OR (IL* next 1a) OR (IL* next "1 receptor antagonist") OR (IL* next "1receptor antagonist") OR (IL* next "1 RA") OR (IL* next 1RA)):ti,ab,kw	(interleukin$ near/2 ("1 alpha")) OR (interleukin$ near/2 1alpha) OR (interleukin$ near/2 ("1 a")) OR (interleukin$ near/2 1a) OR (interleukin$ near/2 ("1 receptor antagonist")) OR (interleukin$ near/2 ("1receptor antagonist")) OR (interleukin$ near/2 ("1 RA")) OR (interleukin$ near/2 1RA) OR ("IL 1 alpha") OR ("IL 1alpha") OR ("IL 1 a") OR ("IL 1a") OR ("IL 1 receptor antagonist") OR ("IL 1receptor antagonist") OR ("IL 1 RA") OR ("IL 1RA")
	#4 3.kf	#4 3.kw		
	#1 OR #2 OR #3 OR #4	*#1 OR #2 OR #3 OR #4*	*#1 OR #2 OR #3*	

Search B: **Skin**	*Mesh terms:* #1 exp Skin/ #2 exp Dermatology/ #3 exp Skin disease/ *In free text (In title .ti or abstract .ab or keywords .kf):* #4 ((skin) OR (epidermis) OR (dermis) OR (stratum corneum) OR (dermatology) OR (cutis) OR (derma)).ti,ab #5 4.kf *#1 OR #2 OR #3 OR #4 OR #5*	*Mesh terms:* #1 exp Skin/ #2 exp Dermatology/ #3 exp Skin disease/ *In free text (In title .ti or abstract ab or keywords .kw):* #4 ((skin) OR (epidermis) OR (dermis) OR (stratum corneum) OR (dermatology) OR (cutis) OR (derma)).ti,ab #5 4.kw *#1 OR #2 OR #3 OR #4 OR #5*	*Mesh term:* #1 Skin (explode all trees) #2 Dermatology (explode all trees) #3 Skin Diseases (explode all trees) *In free text(In title ti or abstract ab or keywords kw):* #4 ((skin) OR (epidermis) OR (dermis) OR (stratum corneum) OR (dermatology) OR (cutis) OR (derma)):ti,ab,kw *#1 OR #2 OR #3 #4*	*In free text (Topic)* skin OR epidermis OR dermis OR stratum corneum OR dermatology OR cutis OR derma
Search C: **Humans**	*Mesh terms:* #1 exp Human/ *In free text (In title .ti or abstract .ab or keywords .kf):* #2 ((human*) OR (volunteer*) OR (participant*) OR (subject*) OR (patient*)).ti,ab #3 2.kf *#1 OR #2 OR #3*	*Mesh term:* #1 exp Human/ *In free text (In title .ti or abstract .ab or keywords .kw):* #2 ((human*) OR (volunteer*) OR (participant*) OR (subject*) OR (patient*)).ti,ab #3 2.kw *#1 OR #2 OR #3*	*Mesh term:* #1 Humans (explode all trees) *In free text(In title ti or abstract ab or keywords kw):* #2 ((human*) OR (volunteer*) OR (participant*) OR (subject*) OR (patient*)):ti,ab,kw *#1 OR #2*	*In free text (Topic)* human* OR volunteer* OR participant* OR subject* OR patient*
Crossed searches:	Search A AND Search B AND Search C *Limits: English*	Search A AND Search B AND Search C *Limits: English*	Search A AND Search B AND Search C *No limits*	Search A AND Search B AND Search C *Limits: English*

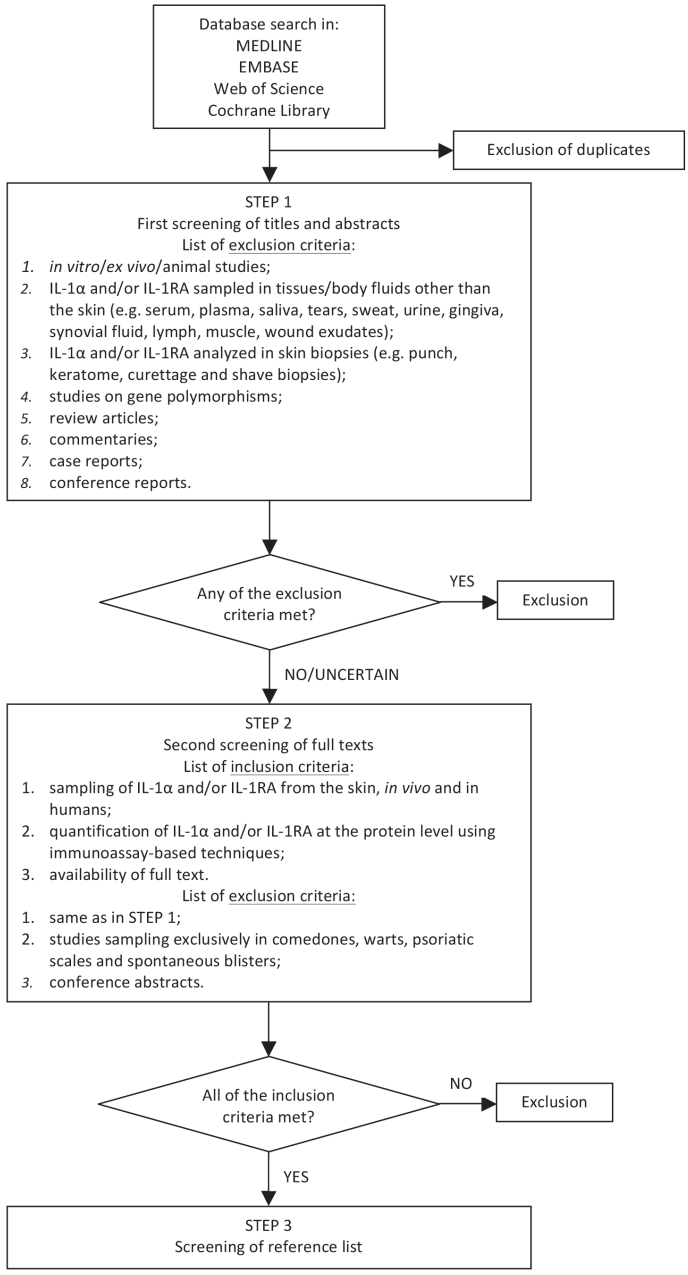

Figure S1. Article selection process

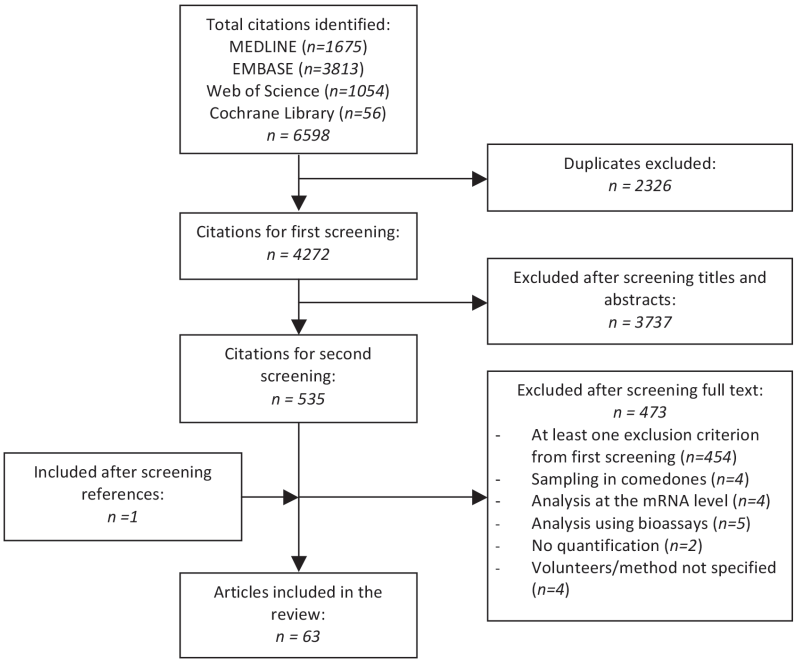

Figure S2. Results of the article selection process.

ABBREVIATIONS

a*	CIE spectrum (Commission Internationale de l'Éclairage): a*, b* and L* values
CCL	(C-C motif) ligand
CRP	C-reactive protein
CXCL	(C-X-C motif) ligand
ELISA	Enzyme-linked immunosorbent assay
hBD	Human beta defensin
IL	Interleukin
PBS	Phosphate buffered saline
RCM	Reflectance confocal microscopy
SC	Stratum corneum
TAP	Transdermal analyses patch
TEWL	Transepidermal water loss
TL-1A	TNF-like ligand 1A
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor

5.2

Measurement of skin surface biomakers by Transdermal Analyses Patch following different *in vivo* models of irritation: a pilot study

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ABSTRACT

Background/aims: FibroTx transdermal analyses patch (TAP) is a novel technology for non-invasive measurements of protein biomarkers on the skin surface, *in vivo*. The aim of this study was to explore the potential of TAP in detecting skin surface biomarkers following mild perturbations, *in vivo*, using two experimental models: tape stripping, mimicking acute barrier disruption, and histamine iontophoresis, mimicking acute and local inflammation at minimal skin barrier insult.

Methods: Tape stripping and histamine iontophoresis were performed in two separate experiments on the volar forearm of healthy volunteers (n=27 and n=10, respectively). Biomarker levels were assessed with TAP at baseline and up to 72 hours after stimulation. Functional (transepidermal water loss – TEWL – and a* value) and morphological (reflectance confocal microscopy – RCM) assessments were added in the tape stripping and histamine iontophoresis experiments, respectively.

Results: Cytokines IL-1α and IL-1RA and the antimicrobial peptide hBD-1 showed distinct dynamics, despite substantial inter-individual variation in levels, with an increase following tape stripping and a decrease following histamine iontophoresis. These dynamics could be related to the assessments made by TEWL and RCM. In the tape stripping experiment, additional biomarkers could be detected.

Conclusion: TAP measurements, especially IL-1α, IL-1RA and hBD-1, from the skin surface were sensitive enough for monitoring dynamic changes in the skin in the two models of skin perturbation. We conclude that TAP holds promise for non-invasively unraveling the dynamics of processes related to skin perturbation and repair.

INTRODUCTION

The barrier function of the skin is mainly fulfilled by the stratum corneum (SC), consisting of a compact structure of dead, flattened cornified cells (corneocytes) embedded in a lipid matrix [1]. Corneocytes derive from differentiation of keratinocytes in the underlying epidermis, a process initiated at the stratum basale, the boundary between the epidermis and dermis. Upon disruption of the skin barrier, a cascade of events occurs, leading to the onset of an inflammatory response that triggers the repairing mechanisms that ultimately restore the barrier integrity. Present evidence shows that this process involves different cytokines and intercellular interactions [2]. The release of a pre-formed pool of interleukin (IL)-1α, which is constitutively present in keratinocytes and the SC, is considered to be the first step in the inflammatory cascade [3]. IL-1α stimulates keratinocytes and fibroblasts to produce early pro-inflammatory cytokines and chemokines, *e.g.* IL-1β, IL-6, IL-8, and tumour necrosis factor (TNF)-α [3]. One of the effects of these secondary pro-inflammatory cytokines and chemokines is to stimulate and attract a variety of immune cells, *e.g.* T- and B-cells, neutrophils, and immature dendritic cells [2]. To counteract these inflammatory processes, keratinocytes also release anti-inflammatory mediators, such as IL-1 receptor antagonist (IL-1RA), which is also constitutively present in keratinocytes and the SC and functions by blocking the IL-1 receptors [3, 4]. The balance between IL-1RA and IL-1α plays an important role in the regulation of the inflammatory response in skin and in skin homeostasis in general [2, 5]. In addition to cytokines and chemokines, keratinocytes release lamellar bodies in the upper epidermis upon disruption of the skin barrier [6-8]. These organelles release lipids and anti-microbial peptides (*e.g.* human beta defensin (hBD)-1 and hBD-2) in the extracellular space, thus forming a chemical barrier against invasion of pathogens during the repairing process [8]. Several studies have shown co-regulation between processes regulating skin permeability and microbial defense during skin repair, indicating that these distinct barrier functions of the skin are inter-dependent rather than independent [6, 7].

Given their essential role in orchestrating skin defense and repair, several *in vivo* approaches have been developed to sample proteins that regulate these processes. Among the invasive methods figure skin punch biopsies, in which both inflammatory mediators and antimicrobial peptides can be detected [9-11]. Minimally invasive methods are based on harvesting material from the superficial layers of the skin; examples include removal of SC by sequential application of adhesive tape (tape stripping) [3-5, 12-14], extraction of interstitial fluid by microporation of the SC and epidermis followed by vacuum application [15, 16], and adsorption of skin surface mediators by a lipophilic tape (Sebutape, CuDerm, Dallas, TX, USA) [17-19]. A non-invasive method based on skin surface wash sampling of mediators secreted from the skin has also been described [20, 21]. Despite their undeniable value for skin research, these methods affect skin and/or have technical restrictions which limit their potential for measuring subtle changes in skin at different time-points over a condensed period of time.

Recently, a novel and non-invasive technique based on enzyme-linked immunosorbent assay (ELISA) has been developed for simultaneous measurements of proteins directly from the skin surface [22]. The so-called transdermal analyses patch (TAP) is applied directly to the skin surface, where it captures proteins of interest by immune-recognition. With this method, IL-1α, IL-1RA, and hBD-1, skin mediators that are constitutively present in the skin [3, 7], could be detected on healthy, intact skin [22]. However, it has as yet not been addressed whether TAP can measure qualitative and/or quantitative changes of skin regulatory proteins during, for instance, skin irritation, inflammation, infections, skin ageing or skin repair.

The aim of this study was to explore whether TAP is able to detect dynamic changes in skin responding to irritants *in vivo* by measuring, on the skin surface, a panel of proteins expressed in the skin at various time-points. For this, two well-established *in vivo* models of skin irritation were employed on healthy volunteers. In a first experiment, tape stripping was used as a model of acute barrier disruption. In addition to TAP measurements, transepidermal water loss (TEWL) and redness (a* value) were measured in order to characterize the irritant reaction by traditional biophysical methods. In a second experiment, histamine iontophoresis was used as a model of acute and local skin inflammation with minimal barrier impairment. Morphological changes induced by the irritant reaction were characterized by reflectance confocal microscopy (RCM). In both experiments, levels of biomarkers were measured at baseline and at different time points following skin irritation. In addition to IL-1α, IL-1RA and hBD-1, TAPs for detection of several other constituent and inducible mediators were included.

PATIENTS AND METHODS

Study participants

Twenty-seven (25 females, 2 males) healthy volunteers were included in the first study, carried out between September and November 2015, and 10 (7 females, 3 males) healthy volunteers were included in the second study, carried out between May and July 2016. Mean age was 27 (18-62) years old in the first study and 34.6 (19-62) years old in the second study. Criteria for inclusion were Fitzpatrick skin type I-II-III and willingness to give a written informed consent. Pregnant or breastfeeding women and volunteers with a history of skin diseases, atopic or allergic predisposition (*i.e.* atopic dermatitis, asthma, allergic rhinoconjuctivitis), use of immunosuppressive drugs and compromised skin at the experimental sites (volar forearms) were excluded from participation. For the second study, presence of implanted electrical devices (*e.g.* cardiac pacemakers) was an additional exclusion criterium. In both studies, volunteers were asked not to apply toiletries (*e.g.* shower gels, moisturizers) on their forearms from 24 hours before the experiments and not to sunbathe or use a tanning bed from two weeks before the experiments. To comply with the guidelines of biophysical measurements, the first experiment was performed under controlled temperature and relative humidity conditions (20-

22 °C, 40-60% relative humidity). Both studies were performed at the dermatology department of the Radboud University Medical Center in Nijmegen, the Netherlands, and were approved by the ethics committee Regio Arnhem-Nijmegen.

Detection of skin surface biomarkers by TAP

Four different TAPs were used, each containing a micro-array coated with antibodies for the measurement of protein biomarkers: TAP1, containing antibodies for IL-1α, IL-1RA, hBD-1, and chemokine (C-X-C motif) ligand 2 (CXCL-2); TAP2, containing antibodies for IL-8 (or CXCL-8), TNF-α, thymic stromal lymphopoietin (TSLP), and vascular endothelial growth factor-A (VEGF-A); TAP3, containing antibodies for CXCL-3, TNF-like ligand 1A (TL-1A), hBD-2, and hBD-4; TAP4, containing antibodies for IL-4, C-reactive protein (CRP), CXCL-1, and chemokine (C-C motif) ligand 2 (CCL-2). Each TAP was attached to the skin via the provided adhesive bandage and, after addition of a standardized amount of buffer on the reservoir covering the micro-array (phosphate buffered saline – PBS – at pH 7.3), it remained in contact with the skin for 20 minutes; during this time, the antibodies printed on the micro-array captured biomarkers through immune-recognition. After removal, TAPs were stored at 4 °C until analysis using spot-ELISA, as previously described [22].

Study 1: tape stripping and biophysical measurements

Tape stripping was performed on four sites (Site 1, Site 2, Site 3 and Site 4) on the non-dominant mid volar forearm using a metal oblong plate with an oval aperture (13 mm x 22 mm, 2.9 cm^2) covered by adhesive tape (6890 PVC Tape, Scotch, 3M, Maplewood, MN, USA) to standardize extension of the skin and the velocity and angle of removal [23]. Tape application, performed by the same investigator, was stopped when the skin became homogeneously refulgent and the tape did not stick anymore, indicative of the removal of the whole SC. The number of strips necessary to remove the SC was counted in each site and averaged.

Volunteers were divided in four groups and in each group the same type of TAP (either TAP1, TAP2, TAP3, or TAP4) and study protocol (summarized schematically in supplementary Figure S1) were applied. Immediately after tape stripping (0-20 minutes), TAP was applied on Site 1. At 20-40 minutes after tape stripping, TAP was applied on Site 1 and Site 2; at 24 hours, on Site 2 and Site 3; at 72 hours, on Site 2, Site 3 and Site 4. The rationale for the application of TAP on different sites was to evaluate whether the previous application affected the successive measurement. The overall dynamics of biomarkers levels was obtained by taking the measurement performed on the site in which TAP was applied for the first time (*i.e.* 0-20 minutes: Site 1; 20-40 minutes: Site 2; 24 hours: Site 3; 72 hours: Site 4). In addition, TEWL (Aquaflux AF200, Biox, UK) and a* value (Spectrophotometer 2600d, Konica Minolta, Japan) were measured on Site 4 at 10 minutes, 30 minutes, 24 hours and 72 hours after tape stripping, in order to exclude possible effects of previous TAP application. Three and two measurements,

respectively, were performed and the average value was calculated. Baseline measurements with TAP, TEWL and a* value were performed on the contra lateral mid volar forearm.

Study 2: histamine iontophoresis and RCM measurement

Histamine iontophoresis was performed on the non-dominant mid volar forearm using 1.5 mL of a solution of 0.5% histamine dihydrochloride (Allergopharma B.V., Zeist, the Netherlands) in 1% hypromellose gel prepared by the local pharmacy. Stimulation was performed at 0.4 mA for 2.5 minutes using a iontophoresis sytem (Chattanooga Group, Hixson, TN, USA) and a couple of silver-silver chloride electrodes with an active area of 7.2 cm^2 (Iomed Iogel Iontophoresis Electrode Small; Chattanooga Group, Hixson, TN, USA). Prior to stimulation, the skin areas where the electrodes needed to be applied were gently rubbed with a 5 cm x 5 cm tissue (Cutisoft, BSN medical GmbH, Hamburg, Germany) soaked in 1.5 mL demineralized water in order to remove sebum and other impurities.

All four TAP types were applied on the area stimulated by histamine iontophoresis at the following time points: immediately after stimulation, at 1 hour after stimulation, and at 72 hours after stimulation. In addition, imaging with RCM (VivaScope 1500 system, Lucid Inc., USA) was performed at 30 minutes, 90 minutes, and 72 hours according to a standardized protocol. Two horizontal maps of 4 mm x 4 mm (Vivablock) were made at the level of the SC/granulosum and at the stratum spinosum, while two vertical mappings (Vivastack) were performed by capturing a series of images of 0.5 mm x 0.5 mm starting from the skin surface up to 150 µm in depth with steps of 3 µm. Of note, for the RCM measurement at 30 minutes, only two Vivastacks were taken, in order to minimize the contact time of the device with the skin, possibly influencing the subsequent measurement at 1 hour with TAP. The thicknesses of the SC and living epidermis were measured as previously described [24] by two observers (DF and MP), and averaged. Baseline measurements with TAP and RCM were performed on the contra lateral mid volar forearm.

Statistical analysis

Results are presented as median (minimum-maximum). Differences in the measurement of the same biomarker between different sites in study 1, as well as differences in the measurement of the same biomarker between baseline and post stimulus time points in study 1 and 2, were evaluated with the Wilcoxon signed rank test for matched pairs. Statistical analyses were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software Inc., USA). A p value ≤ 0.05 was considered statistically significant. Missing values were excluded from the analyses. Due to the exploratory nature of this study, no corrections for multiple comparisons were applied.

RESULTS

Study 1: levels of biomarkers following tape stripping

A total of n=7 volunteers per TAP type were included, with exception for the TAP2 group in which n=6 volunteers were included. No differences with respect to age, gender, Fitzpatrick skin type and mean number of strips needed to remove the SC (n=25.7, range 14.3-40.3) were present between the four groups.

We were able to detect 15 out of 16 biomarkers. In two subjects, TAP3 biomarkers at 0-20 minutes and 20-40 minutes after tape stripping could not be measured, possibly because of a damage to the patches. The results of the 7 most consistently measurable biomarkers are shown in Figure 1. The levels of biomarkers showed large inter-individual differences. However, despite this variation, some showed a clear upregulation following stimulation: IL-1RA, IL-1α, and hBD-1. The median level of IL-1RA increased 4.6- to 6.9-fold in the first 24 hours after tape stripping, while at 72 hours it was back at baseline (0.9-fold increase). The median level of IL-1α showed a similar trend, albeit the increase was less pronounced in the first 24 hours (2.7- to 3.5-fold increase) and at 72 hours it was lower than baseline (0.3-fold increase). The median level of the ratio between IL-1RA and IL-1α was higher at 24 and 72 hours when compared to baseline (1.8 at 24 hours and 0.9 at 72 hours *versus* 0.5 at baseline), albeit the differences were not significant (supplementary Figure S2). The median level of hBD-1 increased 2- to 3.2-fold following tape stripping and, differently from IL-1RA and IL-1α, was still higher than baseline at 72 hours (1.9-fold increase). The median level of hBD-2 seemed to show a delayed upregulation similar to hBD-1, with the highest increase at 24 hours (2.2-fold), but at 72 hours it was back at baseline (0.9-fold increase). Other biomarkers, despite being measurable, either did not show a clear trend toward upregulation (CXCL-3 and CRP), or were too close to the detection limit of the assay (hBD-4), to be able to define their dynamics following tape stripping.

When a comparison between different sites was made for the previously mentioned biomarkers, differences were found for IL-1RA, IL-1α and hBD-1: all showed decreased levels on Site 1 compared to Site 2 at 20-40 minutes after tape stripping. For IL-1α, levels were also lower on Site 2 compared to Site 3 at 24 hours after tape stripping. At other time points, and for the other biomarkers (hBD-2, CXCL-3, CRP and hBD-4), no differences emerged between different sites (supplementary Figure S3).

Eight mediators were rarely detected, preventing an assessment of the dynamics after tape stripping: IL-8, TNF-α, TSLP, VEGF, TL-1A, CXCL-1, CCL-2 and IL-4. CXCL-2 was never detected in any of the samples.

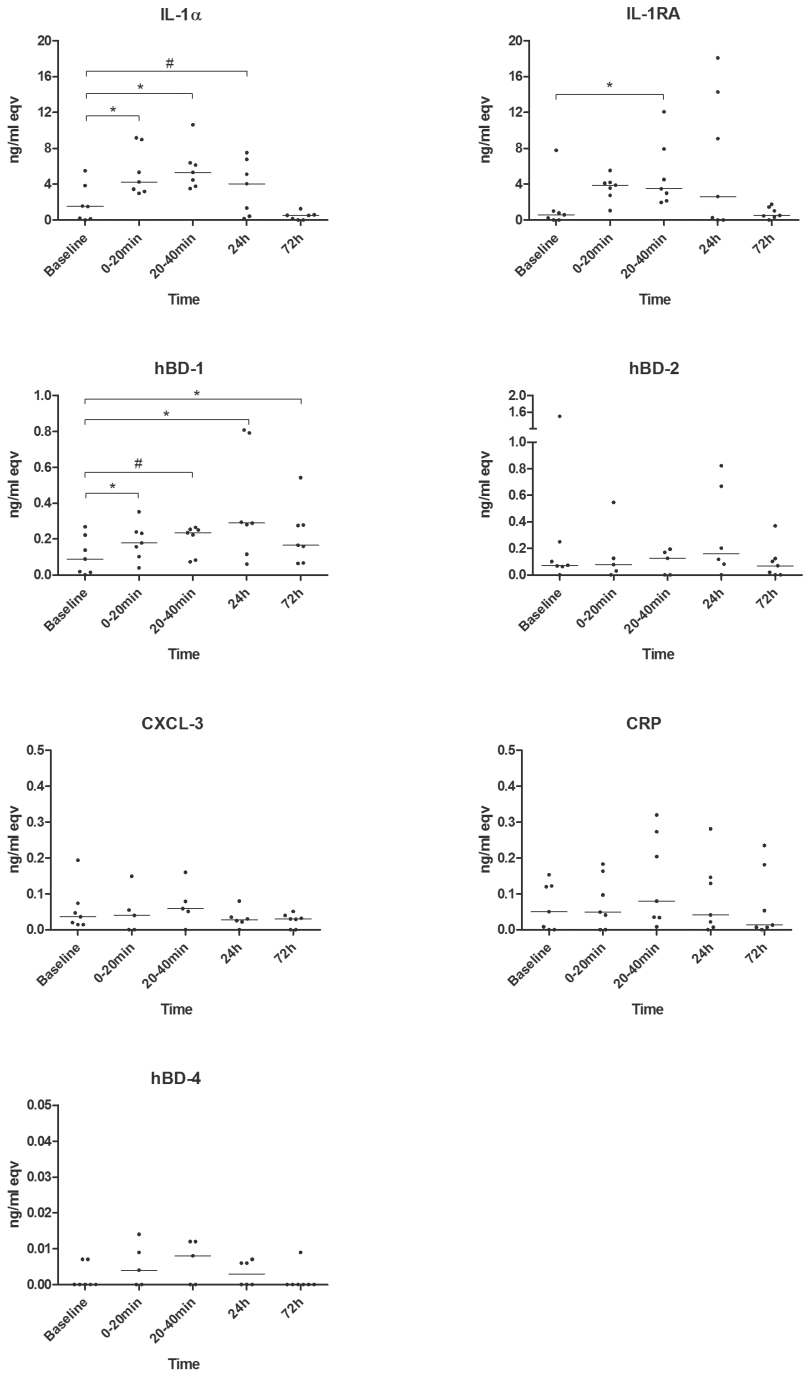

Figure 1. Results of the 7 most consistently detectable biomarkers measured at baseline and at several time points after tape stripping (n=7/biomarker; horizontal bar represents median). * = p ≤0.05; # = 0.05<p< 0.08.

Study 1: biophysical measurements

TEWL and a* value measured at baseline and after tape stripping are shown in Figure 2. Both measurements increased significantly immediately after tape stripping; at 24 hours they started to recover, but were still higher than baseline after 72 hours (*p<0.001*).

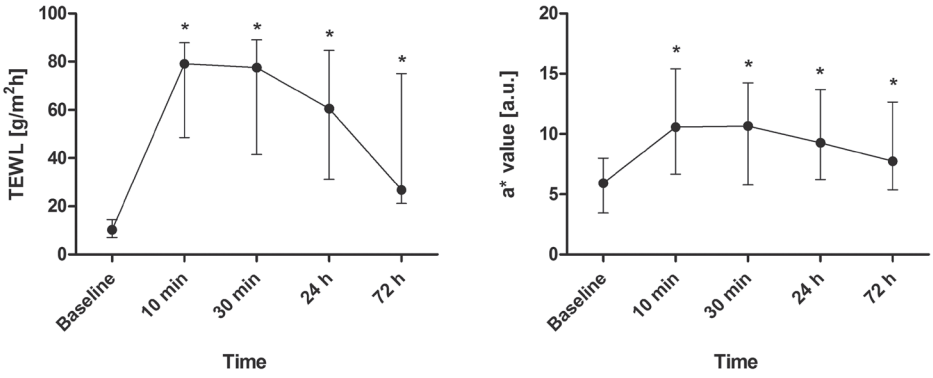

Figure 2. TEWL and a*value measured at baseline and at several time points after tape stripping (n=27; median and range). * = p ≤0.05.

Study 2: levels of biomarkers following histamine iontophoresis

No differences with respect to age and Fitzpatrick skin type were present between the volunteers included in this study and the volunteers included in each group of the tape stripping study.

We were able to detect 6 out of 16 biomarkers. Of these, IL-1RA, IL-1α, and hBD-1 were the most consistently measurable and their dynamics after histamine iontophoresis is shown in Figure 3. The median levels of IL-1RA and of hBD-1 had a similar dynamics, as both decreased in the first 80 minutes after histamine iontophoresis (1.6- and 2.2-fold decrease for IL-1RA, and 2.4- and 4.1-fold decrease for hBD-1), and both were back at baseline at 72 hours (1.1- and 1.4-fold decrease for IL-1RA and hBD-1, respectively). Compared to baseline, the median level of IL-1α increased slighlty immediately after histamine iontophoresis (1.6-fold increase), then decreased at 60 minutes after stimulation (1.7-fold decrease). At 72 hours, the median level of IL-1α was back at baseline (1.2-fold increase). The median level of the ratio between IL-1RA and IL-1α was lower immediately after histamine iontophoresis compared to baseline (0.3 at 20 minutes *versus* 0.4 at baseline), while no differences were present at later time points (supplementary Figure S2).

CXCL-2, CXCL-3 and TNF-α were rarely detected, preventing an assessment of their dynamics after histamine iontophoresis. All other biomarkers were never detected in any of the samples.

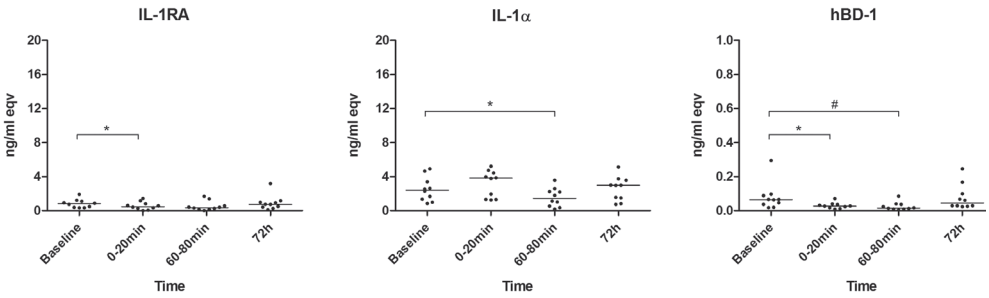

Figure 3. Results of the 3 most consistently detectable biomarkers measured at baseline and at several time points after histamine iontophoresis (n=10/biomarker; horizontal bar represents median).
* = p ≤0.05; # = 0.05<p<0.08.

Study 2: SC and epidermal thickness measured with RCM

SC and living epidermis thicknesses measured at baseline and after histamine iontophoresis are shown in Figure 4. The thickness of the SC increased markedly at 30 minutes after stimulation (*p=0.006*) and, despite a recovery, was still higher than baseline at 90 minutes (*p=0.036*). No significant changes in the thickness of the living epidermis were detected. Of note, in most volunteers an increase in the reflectance of the SC was visible at 30 and 90 minutes, often accompanied by a decrease in the contrast of the first nucleated cells at the boundary between the SC and stratum granulosum, as shown in Figure 5.

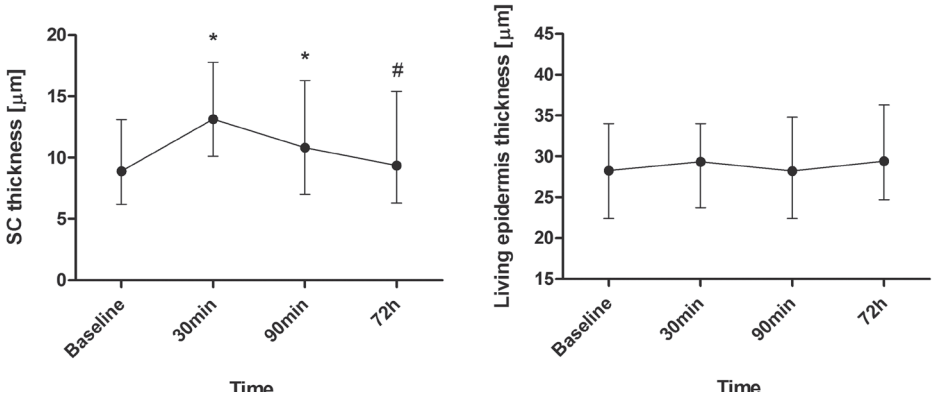

Figure 4. Stratum corneum (SC) and living epidermis thicknesses measured by reflectance confocal microscopy (RCM) at baseline and at several time points after histamine iontophoresis (n=10; median and range). * = p ≤0.05; # = 0.05<p<0.08.

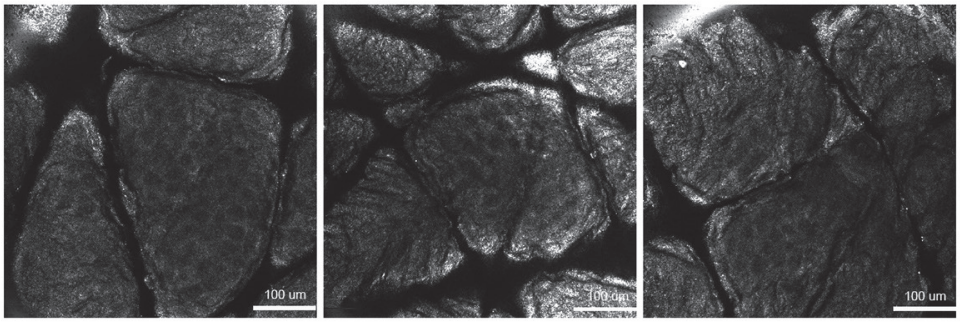

Figure 5. Representative RCM images at the boundary between the stratum corneum (SC) and stratum granulosum at baseline (**a**), at 30 minutes after histamine iontophresis (**b**), and at 90 minutes after histamine iontophoresis (**c**). **a**) The first nucleated cells delimitating the border between the SC and the stratum granulosum are well-defined. The surrounding areas present a low reflectance level typical of normal skin. **b**) At 30 minutes, the boundaries between the first nucleated cells are ill-defined, possibly because of the presence of intracellular fluid. The surrounding areas are higly reflective, possibly indicating an hydration effect of the active electrode or of the underlying edema. **c**) At 90 minutes, the boundaries of the first nucleated cells are better defined and the reflectance is lower compared to the situation at 30 minutes, indicating the decrease of the effects of histamine iontophoresis on the SC.

DISCUSSION

The aim of this pilot study was to explore the feasibility of using TAP to objectively detect subtle changes in skin surface mediators in two well-established *in vivo* models of skin irritation, tape stripping and histamine iontophoresis. In both models, IL-1α, IL-1RA and hBD-1 could be consistently measured, as reported previously [22], albeit with very different dynamics. In the first hour after tape stripping, a higher amount of IL-1α, IL-1RA and hBD-1 was detected, while in the first 80 minutes after histamine iontophoresis, lower amounts were detected. This likely reflects the different nature of the two models. In tape stripping, the removal of SC disrupts the skin barrier completely, thus making the pool of IL-1α, IL-1RA and hBD-1 more accessible for TAP measurements. In histamine iontophoresis, the SC remains intact while its thickness increases due to hydration from the active electrode and, possibly, from the underlying edema, originated as part of the triple response to histamine, with the consequent "dilution" of the biomarkers [15], resulting in less amount detected by TAP. A "wiping" effect of the superficial biomarkers due to the application of the active electrode cannot be excluded either. However, despite the influence of the nature of the two models, we believe that the measurements with TAP reflect the underlying dynamics of these mediators following irritation. This hypothesis is supported by the following observations. Firstly, it was shown that the release of a pre-formed pool of IL-1α is one of the first steps in the inflammatory cascade following skin barrier disruption by tape stripping [9]; accordingly, in our study, IL-1α was upregulated in the first 24

hours after tape stripping, but it was back at baseline at 72 hours, a time point in which the skin barrier was not fully recovered as demonstrated by TEWL measurement in this study and by immunohistochemistry in previous studies [23, 24], possibly reflecting a downregulation of the inflammatory response at later time points. Secondly, the trend to higher IL-1RA/IL-1α ratio at 24 and 72 hours after tape stripping is in agreement with the notion that this ratio is increased in cutaneous inflammatory processes both in irritated and diseased skin [14, 17, 18]. Third, the somewhat delayed upregulation of hBD-1 and, to a lesser extent, of hBD-2, is in line with previous findings in which upregulation of the microbial barrier of the skin was found from 24 hours after barrier disruption [11, 25]. Fourth, iontophoresis was shown to trigger the release of IL-1α as a consequence of the loss of the epidermal calcium gradient [26, 27]: the release of IL-1α might explain why this mediator was slighly increased in the first 20 minutes after stimulation, in contrast to the lower levels of IL-1RA and hBD-1 measured becaused of the dilution effect of the edema.

The additional mediators currently available with TAP were rarely detected in the tape stripping study and, quite surprisingly, mostly not detectable in the histamine iontophoresis study. The reason for this discrepancy might be two-fold. Firstly, experiments were performed in the summer time in the second study and in autumn in the first study. Secondly, experiments in the second study were performed under less strictly-controlled ambient conditions, allowing temperature to be around two degrees higher than during experiments in the first study in which biophysical measurements were used. A higher moisture or sweat/sebum production on the surface of the skin, caused either by the seasonal and/or ambient room conditions, might have prevented the detection of other mediators already present at lower concentrations in the skin. It is therefore advisable that future studies with TAP are performed in controlled ambient temperature and humidity, similar to the conditions required for biophysical measurements. The influence of seasonal effects should nevertheless be further investigated, also considering that greater protein recovery in summer than in winter with the Sebutape adsorption method has been previously reported [18].

Based on the literature data on *in vivo* tape stripping, we had expected to detect upregulation of TNF-α, IL-8, and TSLP [9, 10, 28, 29]. Low or undetectable levels of TNF-α and IL-8, in contrast to the availability of IL-1α and IL-1RA, have been reported with several minimally and non-invasive approaches [3, 4, 12, 15, 18-20]. TSLP was also not detected in the interstitial fluid extracted by microporation [16]. The difficulty in sampling IL-8 and TNF-α, as well as the other biomarkers available with TAP, might be due to a variety of reasons, including wrong time point of sampling, in which the levels might have already decreased below the limit of detection; rapid uptake by target cells; or absence within the upper skin layers [15, 16]. With respect to CXCL-3, and other inflammatory mediators sporadically measured at baseline, their presence in non-irritated skin has been proposed to be caused by a process called "immune-surveillance", consisting in the continuous attraction of inflammatory cells to guard against

pathogens and tumors [15]. CRP has been recently shown to be present in the basal layer, possibly due to extrusion from the underlying dermal vasculature [30].

The high variability in biomarkers levels measured with TAP is common to other approaches of cytokine sampling [3, 4, 14, 15, 17-19]. The advantage of TAP with respect to a tape stripping approach lies in the reproducible method of cytokine sampling and extraction, not depending on sources of variation such as tape adhesiveness, application and removal force, angle and speed [31], making TAP measurements comparable between experiments performed in different laboratories. In addition, TAP does not damage the SC, unlike tape stripping, and as such does not affect the experimental model for skin irritation. Also, multiple markers can be simultaneously assessed and, in particular, the possibility to measure antimicrobial peptides (hBD-1, hDB-2) opens opportunities to evaluate both the recovery of the microbial and permeability barriers following perturbation, concomitantly and non-invasively, possibly allowing to gain new insights into their interdependence [6, 7]. However, it is necessary to consider that TAP, as other minimally and non-invasive methods, allows extraction of biomarkers mostly from the upper surface of the skin, and that biomarkers level there might not always be representative of the level in the whole skin [3]. In addition, our preliminary results on the use of TAP in skin irritation suggest that the immediate re-sampling from the same skin spot following irritation might lead to lower amounts of extracted biomarkers (supplementary Figure S3): future studies should thus consider to sample a slightly different location, or leave more time between successive samplings.

In conclusion, this pilot study confirmed that TAP can measure IL-1α, IL-1RA and hBD-1 consistently from the skin surface in both normal and irritated skin, and that it is sensitive enough to differentiate between different modalities of skin irritation and to show dynamics of skin irritation. Other biomarkers available with TAP could be more consistenly detected using other sampling time points, modalities of skin irritation or diseased skin. Further studies will need to take into account the control of ambient conditions, the avoidance of immediate re-sampling at the same location and the inclusion of a sufficient number of volunteers to manage inter-subject variability [19]. The further characterization of the role of cytokines, chemokines and antimicrobial peptides by TAP in healthy and diseased skin might constitute a potential novel and non-invasive tool in several applications, such as in the assessment of sensory irritation, characterized by the development of sensations in absence of objective clinical signs of inflammation [2], in the quantitifation and monitoring of therapeutic treatments [17], and, ultimately, in the definition of a refined personalized diagnostic method and customized treatment [32].

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SUPPLEMENTARY FIGURES

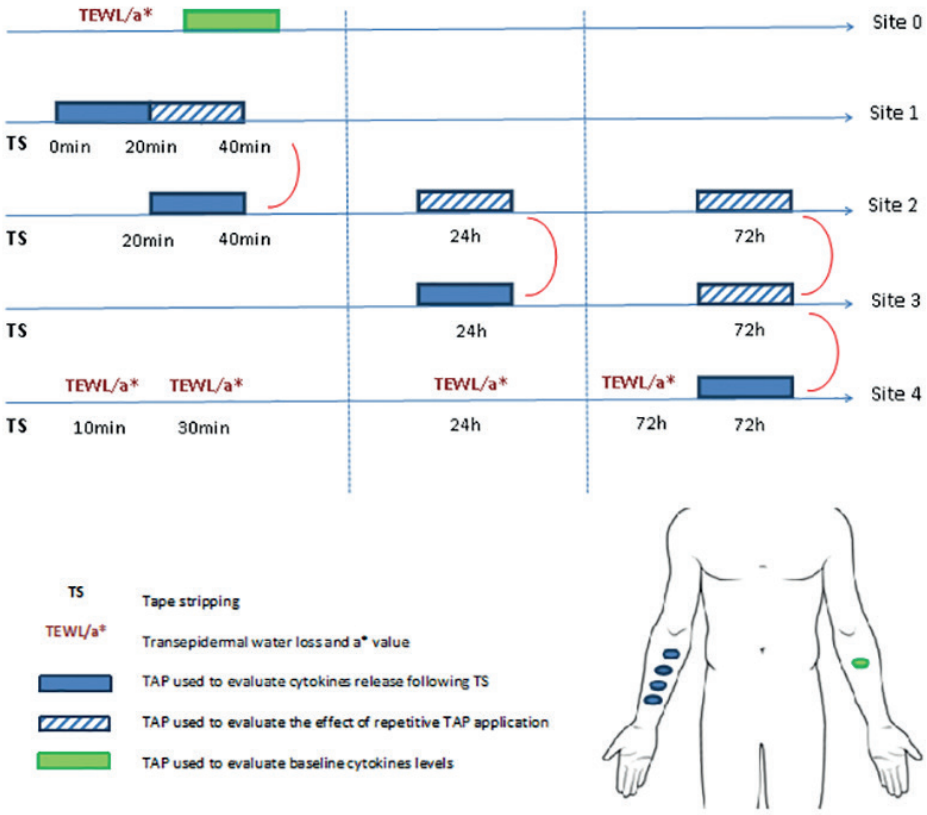

Figure S1. Scheme of the study protocol of the tape stripping experiment.

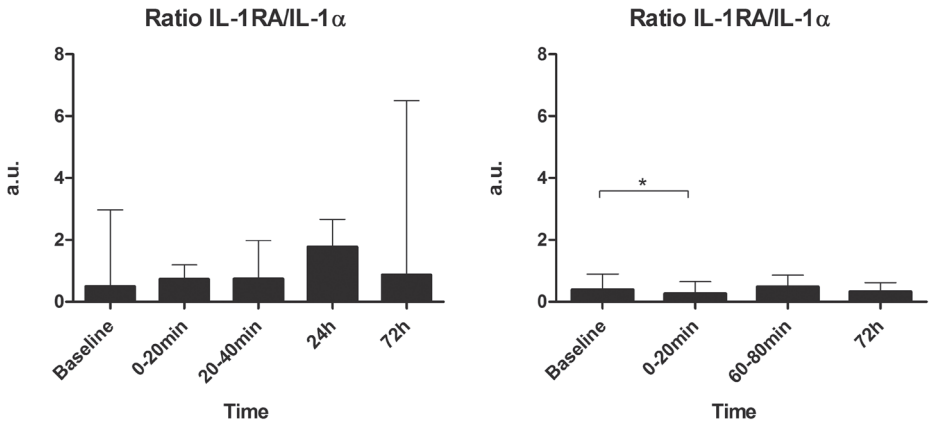

Figure S2. Ratio between IL-1RA and IL-1α at baseline and at several time points after (**a**) tape stripping (n=7; median and range), and (**b**) histamine iontophoresis (n=10; median and range). * = p ≤0.05.

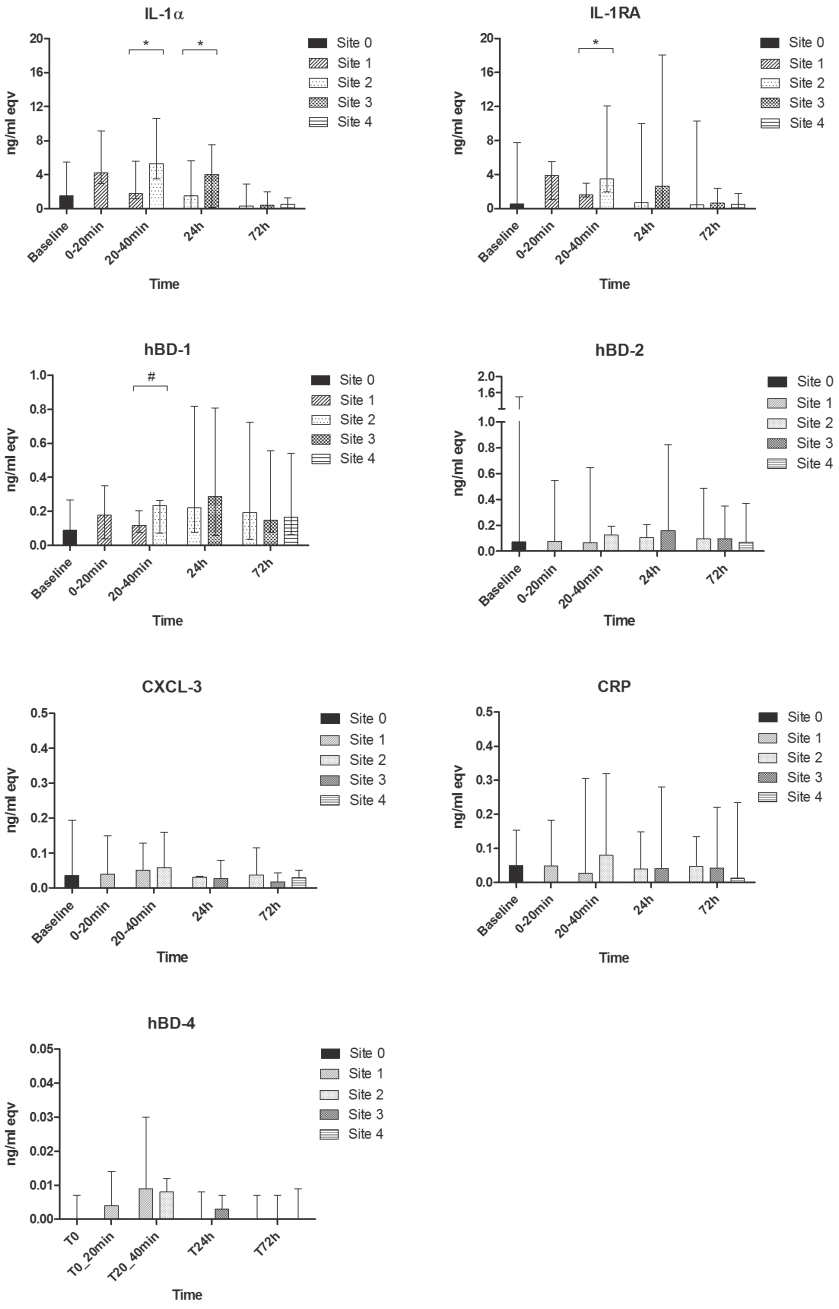

Figure S3. Differences between biomarkers measured on different sites on the volar forearm at baseline and at several time points after tape stripping (n=7/biomarker; median and range). * = $p \leq 0.05$; # = $0.05<p<0.08$.

ABBREVIATIONS

AD	Atopic dermatitis
CI	Confidence interval
CIE	Commission Internationale de l'Éclairage
CW	Continuous irradiation mode
DRS	Diffuse reflectance spectroscopy
DT	Delayed tanning
Hb	Hemoglobin
hBD	Human beta defensin
IL	Interleukin
IPD	Immediate pigment darkening
ITA	Individual tipology angle
LED	Light emitting diode
NIR	Near-infrared
NO	Nitric oxide
OPN	Opsin
oxy-Hb	Oxigenated hemoglobin
PV	Psoriasis vulgaris
PW	Pulsed irradiation mode
RCM	Reflectance confocal microscopy
ROS	Reactive oxygen species
SC	Stratum corneum
TAP	Transdermal analyses patch
TEWL	Transepidermal water loss
UV	Ultraviolet

5.3

Effects of blue light on inflammation and skin barrier recovery following acute perturbation. Pilot study results in healthy human subjects

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ABSTRACT

Background/aims: While growing evidence supports the therapeutic effect of 453-nm blue light in chronic inflammatory skin diseases, data on its effects on acutely-perturbed human skin are scarce. In this study we investigated the impact of 453-nm, narrow-band LED light on healthy skin following acute perturbation.

Methods: Tape stripping and histamine iontophoresis were performed on the forearm of 22 healthy volunteers over two consecutive weeks. In one week, challenges were followed by irradiation for 30 minutes. In the other week (control), no light was administered. Reactions were evaluated up to 72 hours thereafter by transepidermal water loss (TEWL), diffuse reflectance spectroscopy, and skin surface biomarkers.

Results: At 24 hours after tape stripping, upregulation of interleukin-1α (IL-1α) emerged in the non-irradiated control ($p=0.029$). This was not the case in the irradiated location ($p>0.05$), which also displayed higher TEWL *versus* non-irradiated control ($p=0.034$). The b* value was higher at 72 hours post light exposure compared to control ($p=0.018$). At 30 minutes following histamine iontophoresis and light treatment, a trend towards a higher a* value was observed ($p=0.051$).

Conclusion: We provide the first, *in vivo* evidence that blue light at 453 nm exerts biological effects on acutely-perturbed healthy human skin.

INTRODUCTION

Light and optical techniques have made a profound impact on photodermatology, where therapeutic applications span aesthetic and medical domains [1, 2]. Depending on wavelength, power density, exposure time, and optical properties of the skin compartments, the therapeutic action of light can be based on photomechanical, photothermal, photochemical, and photobiological interactions [3]. In particular, the therapeutic effects of ultraviolet (UV)-free visible and near-infrared (NIR) optical radiation relying on non-thermal photochemical and photobiological interactions [4] have been the object of increasing consideration. Since the first pioneering studies using low-photon-density, red-laser light sources in the late 1960s [5], this field has expanded to embrace the more recently developed light emitting diodes (LEDs) with optical output covering the whole visible and NIR spectrum [4].

While perhaps the vast majority of applications focuses on red and NIR wavelengths, UV-free blue light, corresponding to the spectral range of both violet (400-450 nm) and blue (450-495 nm), has been attracting increasing attention. A solid body of scientific data has been accumulated in the last decade, stemming from *in vitro* experiments on human cutaneous cells performed using well-defined irradiation parameters [6-13]. The current evidence attributes the high therapeutic potential of UV-free, 453-nm blue light to its anti-proliferative capacity on human endothelial cells, keratynocytes and fibroblasts [9, 13], often ascribed to initiation of cell differentiation triggered by the release of nitric oxide (NO) from nitrosated proteins. Interestingly, while fluences up to 500 J/cm^2 were non-cytotoxic when applied to *in vitro* cell cultures of human endothelial cells and keratynocytes, a dose-dependent depletion of T-lymphocytes was observed [9], providing initial evidence for an anti-inflammatory effect of blue light. Additional evidence in this respect came from *in vitro* studies using HaCaT cells, where gene expression analysis showed downregulated pathways implicated in inflammatory responses [6], and using dendritic cells, where a reduced ability to release pro-inflammatory cytokines was shown [8, 11]. At the clinical level, the recently reported positive impact of blue light on aleviating symptoms of psoriasis vulgaris (PV) and atopic dermatitis (AD), chronic inflammatory skin diseases characterized by hyperproliferation and inflammatory infiltrates, holds promise for its therapeutic potential [14-17].

In parallel with this *in vitro* and clinical works focusing on combating PV and AD, *ex vivo* studies on human hair follicles and *in vitro* studies on outer root sheath keratinocytes demonstrated positive effects of low-fluence (<5 J/cm^2) blue light on the prolongation of the anagen phase and on the proliferation of hair follicle matrix keratinocytes [7]. Low-fluence blue light was also reported to have a stimulatory effect on the metabolism of human dermal fibroblasts, leading to increased production of collagen, whereas higher levels had an inhibitory effect on cell metabolism and protein synthesis [18]. These emerging data show the importance of dose selection and could possibly pave the path for therapies adressing hair loss and different stages of wound healing.

Intriguingly, however, the same dose of blue light was shown to have differential effects on distinct lineages of human dermal fibroblasts [10]. In addition to differential biological responses elicited by different cell lineages and irradiation parameters [10, 18], identifying effective therapeutic parameters for blue light-based therapies is further complicated by the still incomplete knowledge of the photoacceptors in the skin [19]. Besides nitrosated proteins [9, 12], the effects of blue light could be mediated by photoacceptors in mitochondria, including cytochrome c oxydase, but also by cryptochromes and other flavoproteins, and all could lead to the generation of NO and reactive oxigen species (ROS) [10, 20] or to the activation of G-protein coupled receptors [7]. The latter, and, more specifically, the opsin (OPN) protein family, were recently reported to be present in the skin and its appendages, and to be potential mediators of the effects of blue light [7]. All these different mechanisms could activate several downstream pathways and, ultimately, impact cellular responses. As a consequence, the entry into the mainstream of therapies of blue light requires additional fundamental and clinical research, identifying photoacceptors and molecular pathways, linking irradiation parameters to cellular events and proving therapeutic effects based on clinical outcomes.

Setting the focus on the latter, the primary objective of this study was to investigate the impact of UV-free blue light at 453 nm on the recovery of the skin barrier and on the cutaneous inflammatory response elicited by acute perturbation of the skin of healthy human volunteers. Whilst blue light has already proven effective in clinical studies on diseased skin [2, 14-17], data on its biological effects on perturbed but otherwise healthy skin are scarce. Skin reactions were evaluated up to 72 hours after stimulation using several non-invasive biophysical measurements. As a seconday objective, we aimed to investigate whether skin reactions differed significantly if irradiation was delivered in continuous or pulsed mode [21]. By underpinning the study with well-defined irradiation parameters, *in vivo* skin models and a combination of read-outs, we aimed to obtain novel insights into the effects of UV-free blue light on acutely pertubed healthy human skin.

PATIENTS AND METHODS

Study participants

Twenty-two healthy volunteers were included and randomized in two groups, where irradiation with blue light was delivered in continuous (CW) or pulsed (PW) mode. The mean age was 21.9 (± 1.9) years old in the CW group (10 females, 1 male) and 22.5 (± 3.6) years old in the PW group (10 females, 1 male). Criteria for inclusion were: age between 18 and 40 years, Fitzpatrick skin type I, II or III, and willingness to give a written informed consent. Exclusion criteria were: pregnancy or breastfeeding; presence of implanted electrical devices (*e.g.* cardiac pacemakers); history of skin diseases, including conditions causing photosensitivity; atopic or allergic predisposition (*i.e.* atopic dermatitis, asthma, allergic rhinoconjuctivitis);

use of immunosuppressive drugs and compromised skin at the experimental sites (both volar forearms). Volunteers were asked not to apply toiletries on their forearms starting from 24 hours before the experiments and not to sunbathe or use a tanning bed starting from two weeks before the experiments and during the study. Experiments were performed at the dermatology department of the Radboud University Medical Center in Nijmegen, the Netherlands, in a room with controlled temperature and relative humidity conditions. The study, approved by the ethics committee Regio Arnhem-Nijmegen (protocol ID: NL56421.091.16), was performed according to the Declaration of Helsinki and was carried out between November 2016 and April 2017.

Acute skin perturbation

Skin perturbation was performed on two different locations of the volar forearm by means of tape stripping and histamine iontophoresis, as previously described [22]. Tape stripping (area: 2.9 cm^2) was performed approximately 4 cm apart from the electrode for iontophoretic histamine delivery (active area: 7.2 cm^2), in distal direction, so that both stimuli would cover a length of less than 15 cm on the volar forearm. Skin perturbation with both stimuli was completed within 15 minutes.

Irradiation with blue light

Irradiation of the volar forearm was delivered with a proprietary investigational medical device of Class IIa. The device comprised a lamp unit containing 84 high-power LEDs (LUXEON Rebel LXML-PR02, Lumileds Holding B.V., the Netherlands) with quasimonochromatic emission centered at 453 nm (bandwidth: 19 nm). Lenses in front of the LEDs collimated light beams, minimizing the dependence of the irradiance at the skin surface from the exact distance from the lamp unit. The LEDs panel was positioned approximately 7 cm above the forearm. The irradiation window allowed uniform irradiation of a 15 cm-long section of the volar forearm. Two pre-defined, calibrated irradiation settings were used, both delivering a fluence of 18 J/cm^2 at 10 mW/cm^2 average irradiance during a 30-minute treatment time. The difference between the settings was that one delivered the fluence in CW mode, and the other in PW mode with 200 mW/cm^2 peak irradiance, 5% duty cycle and 100 Hz repetition frequency. A similar dose of blue light was demonstrated to be safe in human subjects [23]. Moreover, the 200 mW/cm^2 peak irradiance had already been successfully applied in clinical studies on PV and AD [14, 16]. Skin surface temperature was measured throughout irradiation by means of two thermocouples (Omega Engineering, USA) connected to a data logger (TC-08, Pico Technology, UK). The thermocouples were attached to the skin in between the sites stimulated with tape stripping and histamine iontophoresis. Data from the two thermocouples were averaged to obtain a single 30-minute reading of skin surface temperature during irradiation. Throughout irradiation, participants wore safety glasses eliminating the risk of potential retinal damage.

Table 1. Summary of the study protocol and measurements. Acute perturbation with tape stripping and histamine iontophoresis was performed during the first visit of each week. At 30 and 60 minutes after histamine iontophoresis, and at 60 minutes after tape stripping, DRS was used to measure the L*a*b* values, ITA and the complete reflectance spectra. At 60 minutes after tape stripping, TEWL was additionally measured. TEWL, L*a*b* values, ITA and the reflectance spectra were measured also at 24 and 72 hours only on this site, as the reaction to histamine iontophoresis faded within a few hours, leaving no clinically visible signs. At 24 hours, measurement of IL-1α, IL-1RA, hBD-1 and hBD-2 by means of TAP was performed. At 72 hours, skin morphology was assessed by RCM. The difference between the two eeks was that, in one of the two, skin perturbation was immediately followed by irradiation with blue light, thus assuring an "irradiation" and a "control" week. The irradiation week (first or second) and the irradiation forearm (left or right) were randomized between volunteers. In both weeks, baseline TEWL, L*a*b* values, ITA and the reflectance spectra were measured on intact skin during the first visit, on the forearm contra lateral to the one on which the perturbation methods were applied. Baseline TAP and RCM assessments were performed only once, during the "control" week. The "control" week was planned one week apart from the "irradiation" week in order to avoid possible systemic effects elicited by irradiation, a possibility which could not be excluded at this stage. In this respect, an additional "control" was added during the "irradiation" week by performing tape stripping also on the contra lateral, non-irradiated forearm. Skin surface biomarkers were not measured on this additional control. In total, each volunteer underwent stimulation with histamine iontophoresis twice (in the "control" and "irradiation" week) and stimulation with tape stripping three times (two in the "irradiation" week – irradiated and non-irradiated – and one in the "control" week).

				IRRADIATION WEEK		**CONTROL WEEK**	
				Tape Stripping*	Histamine iontophoresis	Tape Stripping	Histamine iontophoresis
VISIT 1	Acute perturbation			✓	✓	✓	✓
	Irradiation with blue light			✓	✓		
	TEWL	Baseline**			✓		✓
		Post stimulus	1 h	✓		✓	
	DRS	Baseline**			✓		✓
		Post stimulus	0.5 h		✓		✓
		Post stimulus	1 h	✓	✓	✓	✓
VISIT 2 (24 h after VISIT 1)	TEWL	Post stimulus	24 h	✓		✓	
	DRS	Post stimulus	24 h	✓		✓	
	TAP	Baseline**					✓
		Post stimulus	24 h	✓		✓	
VISIT 3 (72 h after VISIT 1)	TEWL	Post stimulus	72 h	✓		✓	
	DRS	Post stimulus	72 h	✓		✓	
	RCM	Baseline**					✓
		Post stimulus	72 h	✓		✓	

DRS: diffuse reflectance spectroscopy; hBD: human beta defensin; IL: interleukin; ITA: individual typology angle; TAP: transdermal analyses patch; TEWL: transepidermal water loss; RCM: reflectance confocal microscopy.

* In the control tape stripping performed in the irradiation week, the same measurements were made as for the other tape stripping spots except for TAP.

**Baseline measurements were performed in the forearm contra lateral to the one where acute perturbation was applied.

Study protocol and skin measurements

The study protocol was repeated over two consecutive weeks, accomodating a total of six visits. Tape stripping and histamine iontophoresis were performed during the first visit of each week. Skin reaction were evaluated by transepidermal water loss (TEWL) (Aquaflux AF200, Biox, UK), diffuse reflectance spectroscopy (DRS) (Spectrophotometer 2600d, Konica Minolta, Japan), skin surface biomarkers, and reflectance confocal microscopy (RCM). DRS included measurement of the Commission Internationale de l'Éclairage (CIE) L*a*b* values and the complete reflectance spectrum in the range 360-740 nm. The individual typology angle (ITA) was additionally computed [24]. Skin surface biomarkers consisted in interleukin (IL)-1α, IL-1RA, human beta defensin (hBD)-1 and hBD-2, and were measured by means of transdermal analyses patch (TAP) (FibroTx LLC, Estonia) [22]. RCM (VivaScope 1500 system, Lucid Inc., USA) was employed to assess the thickness of the stratum corneum (SC) and living epidermis, as described previously [22]. The study protocol is schematically described in Table 1.

Statistical analysis

The primary objective (*i.e.* to investigate whether effects of blue light on skin barrier recovery and/or inflammation were detectable) was tested by comparing repeated measures in the irradiation and control weeks with the paired-samples t-test. The normality of these differences was checked with the Kolmogorov-Smirnov test. The second objective (*i.e.* to investigate whether differential effects by irradiating in CW or PW mode were detectable) was tested by the independent-samples t-test. TEWL measurements in the irradiation and control week were compared at 1, 24 and 72 hours, after subtracting the baselines measured in the respective weeks (ΔTEWL). For the L*a*b* values and ITA, the respective baselines were not subtracted, as these differed between the control and irradiation week in the PW group (see Results). No subtraction of the baseline was made for the thickness of SC and living epidermis either. Given the non-normal distribution of skin surface biomakers, the Wilcoxon signed rank test for matched pairs was used to analyse differences between baseline and 24 hours after tape stripping in both the irradiation and control week, as well as between the post-stimulus values. The reflectance spectra were converted to absorbance spectra [25] and qualitatively compared. Statistical analyses were performed with SPSS Statistics version 20 for Windows (IBM SPSS Inc., USA). A p value ≤0.05 was considered statistically significant. Due to the exploratory nature of this study, no corrections for multiple comparisons were applied.

RESULTS

Randomization and irradiation with blue light

The radomization in irradiation group (CW or PW), irradiation week (first or second) and irradiation forearm (left or right) is schematically summarized in supplementary Figure S1. No

differences between the CW and PW groups were present with respect to age, Fitzpatrick skin type (five type II and six type III in both groups) and skin temperature during irradiation with blue light. The average skin temperature during the 30-minute irradiation was 32.2 (± 1.3) °C in the CW group and 31.9 (± 1.2) °C in the PW group ([-0.8; 1.3] 95% CI, *p=0.643*). The average increase in skin temperature between the last five minutes and the first five minutes of irradiation was 0.9 (± 0.7) °C in the CW group and 0.8 (± 0.5) °C in the PW group ([-0.5; 0.6] 95% CI, *p=0.887).*

Baseline measurements: TEWL, L*a*b* values and ITA

On the first visit in the irradiation and control week, baseline TEWL, L*a*b* values and ITA were measured on intact skin, on the forearm contra lateral to the one where acute perturbation was performed. Baseline TEWL did not differ between the irradiation and control week in either group. In the CW group, no differences were present for the L*a*b* values and the ITA. In the PW group, higher a* and b*, and lower ITA, emerged in the control compared to the irradiation week. Values are presented in Table 2.

Table 2. Measurements on intact skin (baseline) in the irradiation and control week in the two groups of healthy volunteers included in the study (n=11 in CW, n=11 in PW).

	Irradiation week		**Control week**			
	Mean	SD	Mean	SD	95% CI	p value
CW group						
TEWL [g/m²h]	10.5	2.6	10.6	2.0	(-0.8; 0.7)	0.865
a* value [a.u.]	5.1	0.6	5.0	1.3	(-0.5; 0.6)	0.856
b* value [a.u.]	14.9	1.5	15.2	1.8	(-0.8; 0.3)	0.299
L* value [a.u.]	69.2	2.2	68.9	2.2	(-0.6; 1.2)	0.461
ITA [°]	52.1	5.3	51.2	5.8	(-1.2; 3.0)	0.361
PW group						
TEWL [g/m²h]	9.8	2.0	10.7	1.8	(-2.5; 0.8)	0.273
a* value [a.u.]	5.4	0.8	5.9	0.9	(-0.8; -0.2)	**0.009**
b* value [a.u.]	14.7	2.4	15.5	2.4	(-1.0; -0.2)	**0.010**
L* value [a.u.]	67.8	2.3	67.2	2.1	(-0.1; 1.3)	0.095
ITA [°]	50.0	7.5	48.0	7.5	(0.7; 3.3)	**0.007**

a.u.: arbitrary units; CI: confidence interval of the difference between irradiation and control week; CW: continuos irradiation; ITA: individual typology angle; PW: pulsed irradiation; TEWL: transepidermal water loss.

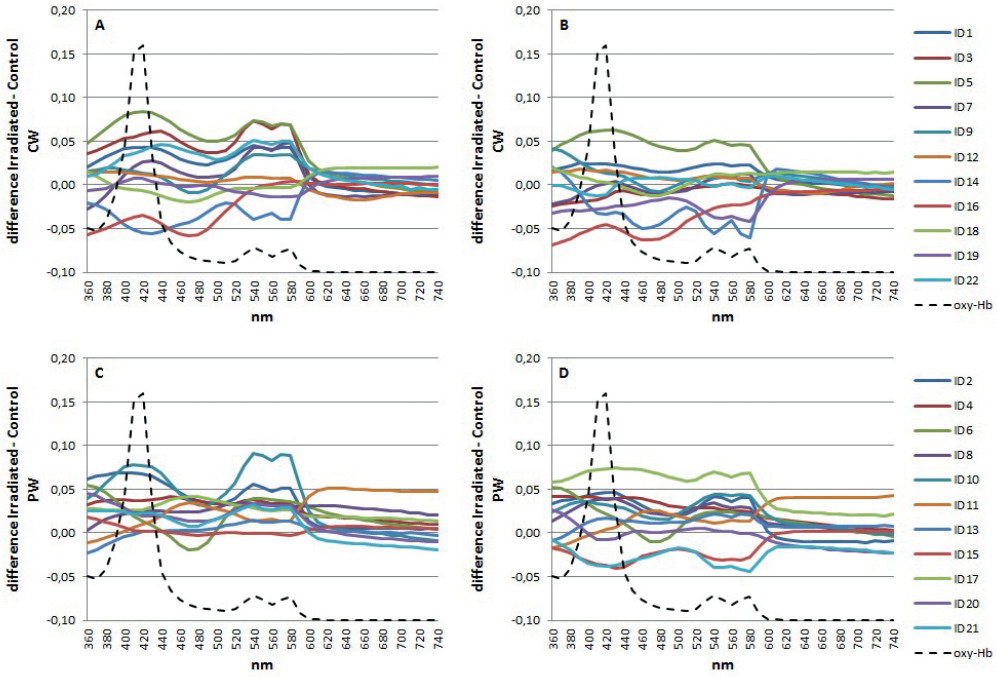

Figure 1. Difference between the absorbance spectra measured in the irradiated and control forearm in the control week at 30 minutes (**a**, **c**) and 60 minutes (**b**, **d**) after histamine iontophoresis in the CW group (**a**, **b**) and PW group (**c**, **d**). The positive absorption peak of oxy-hemoglobin (oxy-Hb) between 540 and 580 nm is visible at 30 minutes in about 50% of volunteers (IDs 1, 2, 3, 5, 7, 9, 10, 20, 21, 22). At 60 minutes, this effect is starkly decreased. The absorbance spectrum of oxy-Hb is added, rescaled and shifted on the y axis for clarity.

Histamine iontophoresis: L*a*b* values, ITA and reflectance spectra

All 22 participants underwent stimulation with histamine iontophoresis. DRS measurements were performed at 30 and 60 minutes after histamine iontophoresis, corresponding to immediately and 30 minutes after irradiation with blue light, respectively. The b* and L* values and the ITA were lower in the irradiated forearm compared to the control forearm at 30 minutes after histamine iontophoresis, whereas an opposite trend was present for the a* value. No differences emerged at 60 minutes after histamine iontophoresis. Values are presented in Table 3. Albeit the effect at 60 minutes was higher in the PW than in the CW group with respect to the a* and L* values and the ITA, the difference did not reach statistical significance. The values for the comparison between CW and PW groups are presented in supplementary Table S1.

The difference between the absorbance spectra measured in the irradiation and control week is shown in Figure 1.

Table 3. Measurements at 30 minutes and 60 minutes after histamine iontophoresis in the irradiation and control week (n=22; n=11 in CW, n=11 in PW).

Histamine iontophoresis	Irradiation week		Control week			
	Mean	SD	Mean	SD	95% CI	p value
30 minutes						
a* value [a.u.]	11.0	1.6	10.2	2.3	(-0.003; 1.5)	0.051
b* value [a.u.]	17.1	1.9	17.7	2.3	(-1.2; -0.05)	**0.034**
L* value [a.u.]	62.9	2.6	64.1	2.3	(-1.7; -0.7)	**0.000**
ITA [°]	37.1	7.9	38.8	7.5	(-3.3; -0.1)	**0.035**
60 minutes						
a* value [a.u.]	10.4	1.5	10.3	1.5	(-0.5; 0.8)	0.724
b* value [a.u.]	16.9	2.1	17.1	2.3	(-0.7; 0.2)	0.215
L* value [a.u.]	63.9	2.6	64.3	2.2	(-1.0; 0.2)	0.167
ITA [°]	39.4	8.1	39.9	7.1	(-2.1; 1.1)	0.490

a.u.: arbitrary units; CI: confidence interval of the difference between irradiation and control week; CW: continuos irradiation; ITA: individual typology angle; PW: pulsed irradiation.

Tape stripping: TEWL, L*a*b* values, ITA and reflectance spectra

In 3 out of 22 volunteers, tape stripping could not be performed until complete removal of the SC and these volunteers were therefore excluded from the analysis. In the remaining volunteers, ΔTEWL measured 24 hours after tape stripping was higher in the irradiated forearm than in the non-irradiated forearm in the control week, whereas no differences emerged at 1 and 72 hours. Values are presented in Table 4. Albeit the effect at 24 hours was higher in the PW compared to the CW group, the difference did not reach statistical significance (supplementary Table S1). No differences were present between ΔTEWL measured in the irradiated and non-irradiated forearm in the irradiation week, nor between the ΔTEWL measured in the non-irradiated forearm in the irradiation and control week (data not shown).

In the irradiated forearm compared to the non-irradiated forearm in the control week, L* value and ITA were lower at 1 hour after tape stripping, b* value was higher at 24 hours and 72 hours and ITA was lower at 72 hours. Values are presented in Table 4. No significant differences emerged between the two groups (supplementary Table S1). Similarly to the data in Table 4, in the irradiated forearm compared to the non-irradiated forearm in the irradiation week, L* value was lower at 1 hour after tape stripping ([-1.7; -0.04] 95% CI and *p=0.042*), b* value was higher and ITA was lower at 24 hours ([0.2; 1.1] 95% CI and *p=0.005* for b* value, [-3.1; -0.3] 95% CI and *p=0.022* for ITA) as well as at 72 hours ([0.5; 1.5] 95% CI and *p=0.000* for b* value, [-3.9; -0.3] 95% CI and *p=0.023* for ITA). In addition, at 1 hour, a* value was higher in the irradiated forearm compared to the non-irradiated forearm ([0.1; 1.3] 95% CI, *p=0.034*). In

the comparison between the non-irradiated forearm in the irradiation and control week, no differences were present at any time point (data not shown).

The difference between the absorbance spectra measured in the irradiation and control forearm in the control week is shown in Figure 2.

Table 4. Measurements at 1 hour, 24 hours and 72 hours after tape stripping in the irradiation and control week (n=19; n=10 in CW, n=9 in PW).

Tape stripping	Irradiation week		Control week			
	Mean	SD	Mean	SD	95% CI	p value
1 hour						
ΔTEWL [g/m²h]	62.8	14.2	60.3	16.9	(-2.5; 7.5)	0.308
a* value [a.u.]	9.4	1.5	9.1	1.6	(-0.3; 1.0)	0.340
b* value [a.u.]	15.3	1.9	14.9	1.7	(-0.1; 1.0)	0.099
L* value [a.u.]	65.7	2.9	66.5	2.4	(-1.5; -0.01)	**0.047**
ITA [°]	45.4	8.3	47.8	6.5	(-4.1; -0.6)	**0.011**
24 hours						
ΔTEWL [g/m²h]	54.2	15.7	47.8	18.2	(0.6; 12.3)	**0.034**
a* value [a.u.]	8.9	1.6	9.2	1.3	(-1.0; 0.4)	0.416
b* value [a.u.]	16.6	1.8	15.8	1.9	(0.3; 1.3)	**0.003**
L* value [a.u.]	66.7	2.3	66.8	2.8	(-0.9; 0.9)	0.958
ITA [°]	45.1	6.2	46.4	6.5	(-2.9; 0.3)	0.107
72 hours						
ΔTEWL [g/m²h]	21.1	10.8	21.3	11.6	(-5.3; 4.9)	0.934
a* value [a.u.]	8.0	1.2	7.9	1.0	(-0.6; 0.8)	0.743
b* value [a.u.]	17.5	2.0	16.9	1.7	(0.1; 1.1)	**0.018**
L* value [a.u.]	66.0	2.4	66.7	2.4	(-1.5; 0.1)	0.085
ITA [°]	42.4	7.0	44.5	6.0	(-4.0; -0.2)	**0.033**

a.u.: arbitrary units; CI: confidence interval of the difference between irradiation and control week; CW: continuos irradiation; ITA: individual typology angle; PW: pulsed irradiation; TEWL: transepidermal water loss.

TAP: IL-1α, IL-1RA, ratio IL-1RA/IL-1α, hBD-1 and hBD-2

All biomarkers were upregulated at 24 hours after tape stripping in the control week compared to baseline ($p<0.05$). This was the case also at 24 hours after tape stripping in the irradiation week compared to baseline for all biomarkers except IL-1α: in this case, no significant difference with respect to baseline emerged. No differences were present between the levels of biomarkers measured in the irradiation and control week. Values are shown in Figure 3.

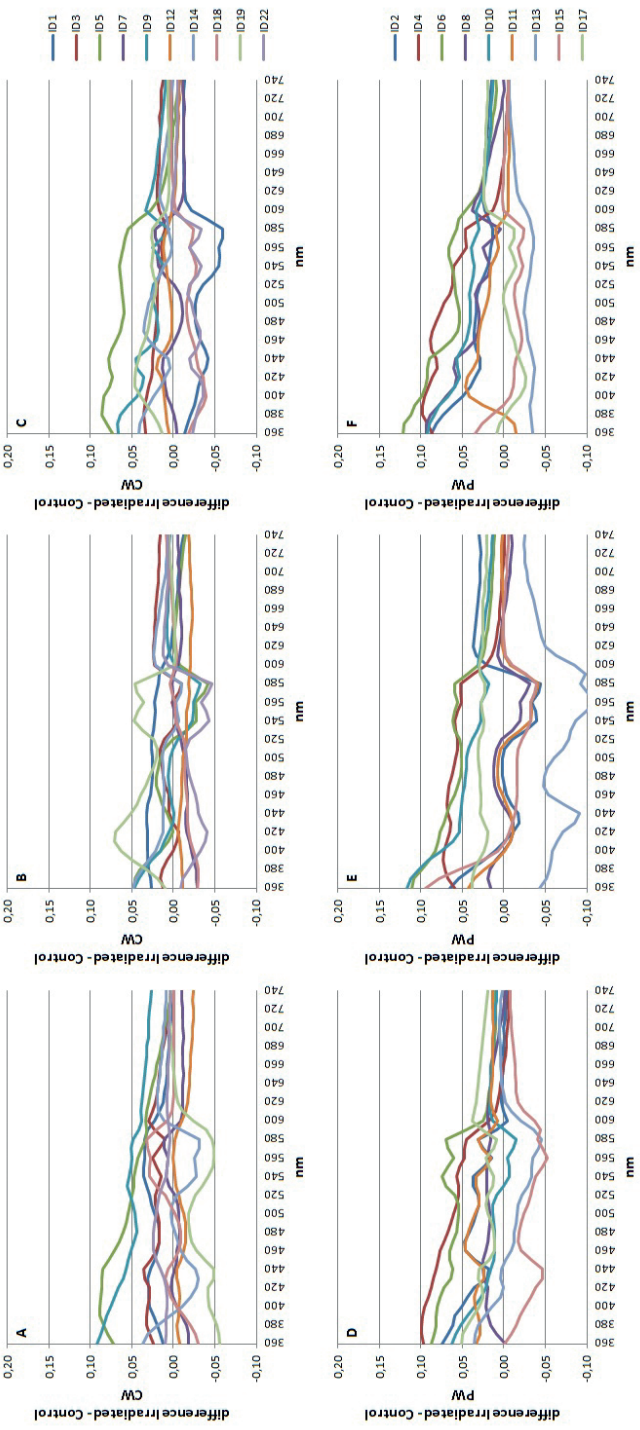

Figure 2. Difference between the absorbance spectra measured in the irradiated and control forearm in the control week at 1 hour (**a**, **d**), 24 hours (**b**, **e**) and 72 hours (**c**, **f**) after tape stripping in the CW group (**a**, **b**, **c**) and PW group (**d**, **e**, **f**). The stronger absorption at wavelenghts lower than 400 nm is visible in some volunteers at 1 hour (IDs 2, 4, 5, 6, 9 and 10), 24 hours (IDs 2, 4, 6, 10 amd 15) and 72 hours (IDs 2, 4, 5, 6, 8, 9 and 10). This effect is more marked in the PW group.

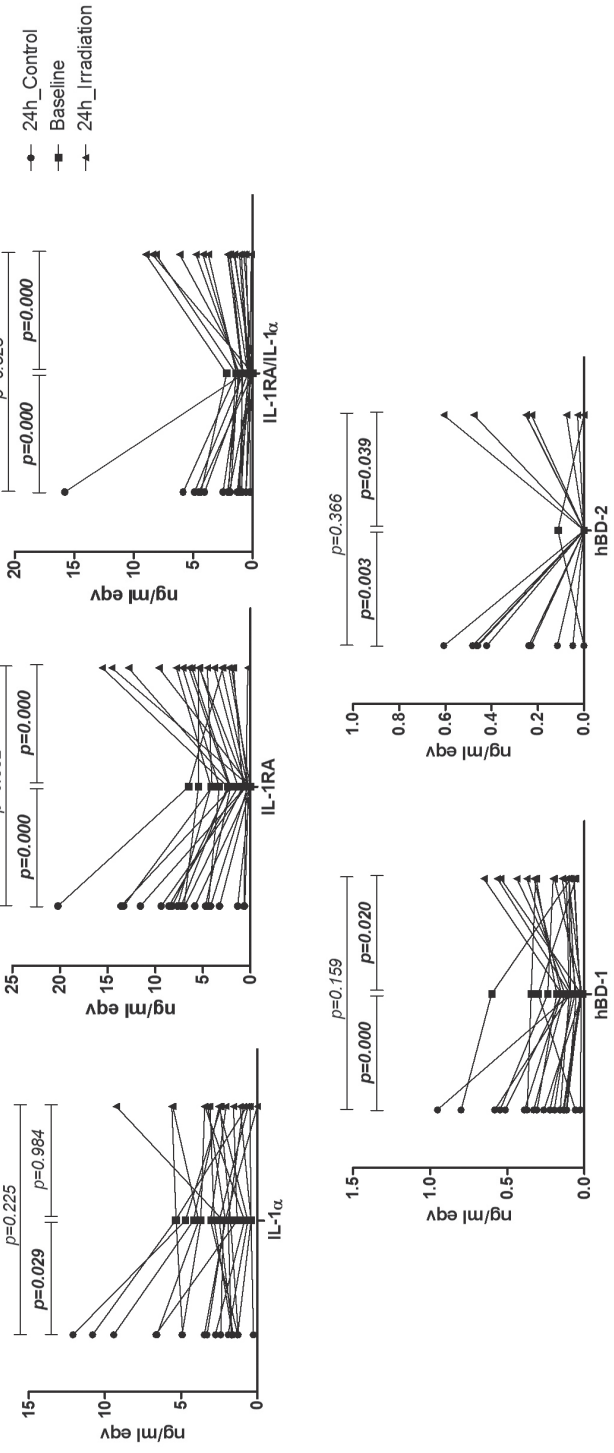

Figure 3. Levels of interleukin-1α (IL-1α), interleukin-1 receptor antagonist (IL-1RA), their ratio IL-1RA/IL-1α and the human beta defensins (hBD)-1 and hBD-2 measured on intact skin (baseline) and at 24 hours after tape stripping in both the irradiated forearm and the non-irradiated forearm in the control week. Comparisons were made with the Wilcoxon signed rank test for matched pairs.

RCM: SC and living epidermis thickness

The SC and the living epidermis were thicker at 72 hours after tape stripping compared to baseline in all experimental sites. No differences emerged between the irradiated forearm and the controls ($p>0.05$, data not shown).

DISCUSSION

The primary objective of this study was to investigate the biological effects of blue light at 453 nm on the recovery of the skin barrier and on the cutaneous inflammatory response following acute perturbation of healthy skin. This objective was achieved by comparing skin reactions to two *in vivo* skin models, namely tape stripping and histamine iontophoresis, in an "irradiation" week (stimuli followed by irradiation with blue light) with skin reactions in a "control" week (stimuli not followed by irradiation).

In the irradiation week, the overall dermal blood concentration was increased at 30 minutes after histamine iontophoresis, compared to the same time point in the control week. This was underlined by the trend to higher a* value, the lower b* and L* values and by the positive peaks between 540 and 580 nm in the difference absorbance spectra, corresponding to the absorption of oxygenated hemoglobin (oxy-Hb) [24]. Increased dermal blood flow following irradiation with blue light at 453 nm and 30 J/cm^2 fluence was already reported *in vivo* and attributed to the vasodilatory effect of NO release [12]. At 60 minutes after histamine iontophoresis, the overall dermal blood concentration was not different compared to the control stimulation, in line with the previously reported short-lived hemodinamic effects of blue light, fading within 15 minutes after irradiation [12].

TEWL was higher at 24 hours after tape stripping in the irradiated forearm compared to the control forearm in the control week. A delaying effect of blue light (430-510 nm) on the recovery of TEWL following acute barrier perturbation was reported in a study on mice [26]: using electron microscopy, the authors showed absence of lipids at the boundary between the SC and stratum granulosum, possibly indicating an inhibitory effect of blue light on the release of lamellar bodies. In our study, the higher TEWL after tape stripping in the irradiated forearm may be linked to the lack of significant increase of IL-1α compared to baseline found on this site, in contrast to the control stimulation where there was a significant upregulation of IL-1α compared to baseline. As measurements with TAP at baseline and on the irradiated tape-stripped site were performed one week apart, we cannot exclude a day-to-day variability effect on biomarkers levels; however, this was demonstrated to be low (coefficient of variation: 20%) on a five-day period in healthy volunteers [27]. In addition, the hypothesis on the impact of blue light on lamellar body release is supported by increasing evidence about the influence of IL-1α on lipid synthesis and epidermal differentiation [28]. Unfortunately, the difference in IL-1α levels did not reach statistical significance between the irradiated and the control tape-

stripped sites, probably due to the low number of subjects combined with the high variability in skin surface biomarkers measured by TAP [22]. At 72 hours, TEWL and the thickness of the living epidermis were not different in the irradiated forearm compared to the controls, indicating absence of an anti-proliferative response of one single treatment at such relative long times scale compared to the irradiation time [9]. More pronounced and long-lasting effects on the barrier function might have been obtained with repetitive irradiations instead of a single irradiation [14, 16]. Interestingly, no differences in TEWL emerged between the irradiated tape-stripped site and the control tape-stripped site in the irradiation week: this may indicate a systemic anti-inflammatory effect. Previous clinical studies on PV and AD observed amelioration of the control plaques together with the irradiated plaques. While the authors were keen to attribute this effect to the use of topical emollients and to a placebo effect [14, 16], one can also hypothesize that changes in skin surface biomarkers might also lead to alterations in their blood plasma levels, thereby introducing a systemic effect of otherwise locally administered light therapy. In order to infer systemic anti-inflammatory effects of the latter, blood plasma biomarkers should also be carefully inspected [20].

After tape stripping, b* was higher and L* and ITA were lower in the irradiated site compared to the controls. It might be tempting to speculate that irradiation with blue light generated immediate pigment darkeing (IPD), a phenomenon which has already been reported for UVA irradiation (315-400 nm) [29]. Indeed, human epidermal melanocytes are known to express OPN2 and OPN4 [30]: while OPN2 mediates UV response leading to melanogenesis, OPN4 in blood vessels (mouse aorta) was shown to be responsive to blue light irradiation with maximum aborption band in the range 430-460 nm [31]. This finding could potentially explain the effect observed in this study. IPD occurs in the first hours after irradiation, and recent evidence suggests synthesis of new melanin [32]. IPD might evolve in delayed tanning (DT), appearing within 3-5 days after exposure and resulting in neo-melanogenesis [29]. The occurrence of this phenomenon is also suggested by the monotonic decrease in the difference absorbance spectra of some volunteers, a trend similar to the absorbance spectrum of melanin in the skin [24, 25].

The secondary objective of this study was to investigate whether irradiation in CW or PW mode leads to differential biological effects of blue light on skin recovery. Differences in biophysical measurements between the irradiated and non-irradiated forearm in the control week were more marked in PW group than in the CW group after both stimuli, albeit not reaching statistical significance. An enhanced neo-melanogenesis triggered by blue light in PW mode could also explan the differences in a*, b*and ITA baseline values between the irradiation and control week in the PW group: indeed, when irradiation was performed in the first week, the baseline b* value and ITA measured in the control week *(i.e.* on the forearm where irradiation was performed the week before) were significantly higher and lower, respectively ($p<0.05$; n=6). When irradiation took place in the second week, no significant differences emerged ($p>0.05$; n=5). Previous studies have observed a higher biological response when light was delivered in

pulsed mode [21, 33]. Using blue light at 453 nm, a peak intensity of 200 mW/cm^2 led to better clinical outcomes in the treatment of PV compared to a peak intensity of 100 mW/cm^2 [16]. Blue light in pulsed mode with high peak intensity is therefore suggested to elicit stronger biological effects, albeit the mechanistic pathway remains to be unraveled.

In conclusion, this pilot study demonstrated that a single irradiation with blue light at 453 nm exerts biological effects on acutely perturbed skin. These consisted in an enhanced but short-lived hemodynamic response after histamine iontophoresis and, following barrier disruption, higher TEWL at 24 hours and a subclinical increase in pigmentation. Our results suggest a conditioning effect of blue light on skin barrier formation, possibly mediated by epidermal cytokines. These findings could be linked to the positive outcomes of the blue spectral band on alleviating symptoms of inflammatory skin diseases involving an impaired skin barrier [14-17]. In perspective, clinical studies aimed at unraveling the impact of blue light would benefit from larger sample populations, the combined use of several biophysical techniques and the local as well as systemic analysis of inflammatory biomarkers. This will bring us one step further in unlocking the so much aspired therapeutic potential of blue light for the treatment of both cutaneous and systemic conditions with a strong inflammatory component.

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SUPPLEMENTARY MATERIAL

Table S1. Comparison between the post-stimulus measurements in the irradiation and control week in healthy volunteers irradiated in CW mode and PW mode.

Difference irradiated-control in control week	CW group		PW group			
	Mean	SD	Mean	SD	95% CI	p value
Histamine iontophoresis						
30 minutes						
a* value [a.u.]	0.7	1.9	0.8	1.5	(-1.6; 1.4)	0.885
b* value [a.u.]	-0.7	1.4	-0.5	1.1	(-1.3; 0.9)	0.724
L* value [a.u.]	-0.9	1.4	-1.5	0.9	(-0.4; 1.6)	0.246
ITA [°]	-0.9	4.5	-2.6	2.2	(-1.5; 4.8)	0.278
60 minutes						
a* value [a.u.]	-0.3	1.6	0.5	1.3	(-2.1; 0.5)	0.192
b* value [a.u.]	-0.4	1.1	-0.1	0.8	(-1.1; 0.6)	0.582
L* value [a.u.]	0.0	1.2	-0.9	1.4	(-0.3; 2.1)	0.122
ITA [°]	0.5	3.6	-1.6	3.4	(-0.9; 5.3)	0.162
Tape stripping						
1 hour						
ΔTEWL [g/m^{2}h]	1.2	11.2	3.9	9.8	(-13.0; 7.6)	0.587
a* value [a.u.]	0.2	0.9	0.4	1.8	(-1.7; 1.2)	0.697
b* value [a.u.]	0.0	1.1	0.9	1.0	(-1.9; 0.1)	0.070
L* value [a.u.]	-0.7	1.5	-0.9	1.8	(-1.4; 1.7)	0.846
ITA [°]	-1.4	3.7	-3.4	3.4	(-1.5; 5.5)	0.244
24 hours						
ΔTEWL [g/m^{2}h]	2.5	10.6	10.7	12.9	(-19.6; 3.2)	0.146
a* value [a.u.]	0.1	1.0	-0.1	2.5	(-1.8; 2.2)	0.817
b* value [a.u.]	0.5	1.2	1.1	0.8	(-1.5; 0.4)	0.244
L* value [a.u.]	0.1	1.0	-0.1	2.5	(-1.8; 2.2)	0.817
ITA [°]	-0.9	2.7	-1.8	4.1	(-2.4; 4.2)	0.567
72 hours						
ΔTEWL [g/m^{2}h]	1.1	8.9	-1.7	12.6	(-7.7; 13.3)	0.581
a* value [a.u.]	0.0	1.4	0.2	1.6	(-1.7; 1.2)	0.729
b* value [a.u.]	0.4	0.7	0.8	1.2	(-1.3; 0.6)	0.403
L* value [a.u.]	-0.4	1.6	-1.0	1.7	(-0.9; 2.2)	0.393
ITA [°]	-1.3	3.4	-2.9	4.4	(-2.1; 5.4)	0.378

a.u.: arbitrary units; CI: confidence interval of the difference between CW and PW groups; CW: continuos irradiation; ITA: individual typology angle; PW: pulsed irradiation; TEWL: transepidermal water loss.

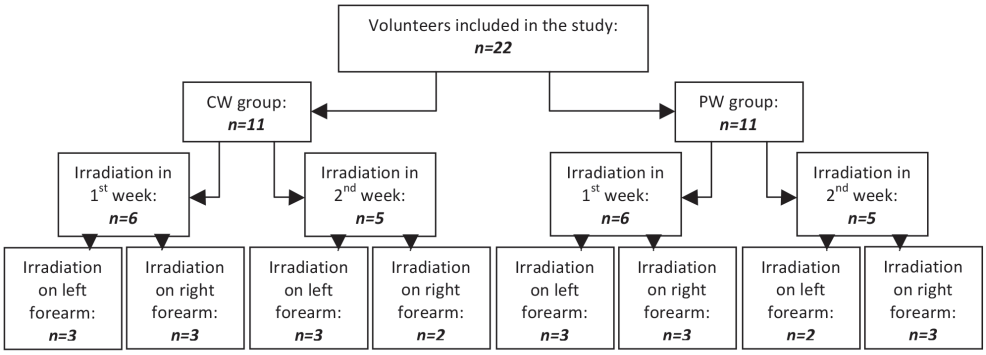

Figure S1. Schematic represenation of the randomization of the 22 volunteers included in the study in irradiation group (continous delivery mode - CW - or pulsed delivery mode - PW), irradiation week (first or second) and irradiation forearm (left or right).

ABBREVIATIONS

a*	CIE spectrum (Commission Internationale de l'Éclairage): a*, b* and L* values
CRS	Confocal Raman microspectroscopy
DRS	Diffuse reflectance spectroscopy
hBD	Human beta defensin
HE	Hematoxylin and eosin
IL	Interleukin
NIR	Near-infrared
NMF	Natural moisturizing factor
NSS	Non-sensitive skin
RCM	Reflectance confocal microscopy
SC	Stratum corneum
SDS	Sodium docecyl sulphate
SS	Sensitive skin
TAP	Transdermal analyses patch
TEWL	Transepidermal water loss
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
TRPV	Transient receptor potential vanilloid
VAS	Visual analogue scale

Chapter 6

Summary and general discussion

This thesis focused on two research areas, namely sensitive skin and skin irritation, and on their evaluation by means of non-invasive bioengineering techniques. Evaluation addressed both skin barrier properties as well as cutaneous inflammation and was performed *in vivo* in healthy human volunteers. In this chapter, the results are summarized and discussed for each research area, and future perspectives on the use of bioengineering techniques in dermatology and in cosmetic sciences are given.

SENSITIVE SKIN

The *first objective* of this thesis was to investigate three possible pathomechanisms leading to self-assessed sensitive skin (SS): impairment of the skin barrier function and enhanced vascular and sensory reactivities.

Is the skin barrier function impaired in sensitive skin?

The possible impairment of the skin barrier was addressed in **chapter 2.2**, in which confocal Raman microspectroscopy (CRS) was used to measure the stratum corneum (SC) levels of water, ceramides/fatty acids and natural moisturizing factor (NMF) in subjects with self-assessed SS. The indirect assessment of the thickness of the SC was added. These measurements were compared to those of subjects claiming their skin as non-sensitive (NSS). No differences were found between SS and NSS subjects in either the volar forearm, thenar of cheek, except for a trend to lower levels of ceramides/fatty acids in the cheek in SS subjects. These results demonstrated that the skin barrier in SS is not impaired in terms of thinner SC or lower levels of water, ceramides/fatty acids and NMF. In **chapter 2.2**, CRS was additionally used to track the penetration kinetics of a solution of glycerol in water, applied topically on the volar forearm. This test was performed to establish whether topicals penetrate faster in SS due to an impaired skin barrier function. Lower levels of glycerol were found in SS compared to NSS subjects in the first minutes after application, reaching statistical significance at one minute. In **chapter 3.1**, a solution of histamine dihydrochloride was applied topically on the volar forearm and lower back of subjects with SS and NSS. Histamine is a well-known pruritogen substance, and the perception of itch following application, indicative of its penetration into the SC, was used as further test for the impairment of the skin barrier. In this case, however, only a trend to more intense itch perception was reported by SS compared to NSS subjects in the volar forearm, and no differences emerged in the lower back. In addition, in the volar forearm, SS subjects showed a higher transepidermal water loss (TEWL) than NSS subjects. Overall, these findings do not support a major barrier impairment in SS, but the lower glycerol levels in SS compared to NSS subjects suggest that subtle alterations of the skin barrier in SS could be responsible for faster and/or increased penetration of topicals in the skin.

This notion is in line with previous findings of Richters and coworkers. In two clinical studies, tape stripping and the surfactant sodium dodecyl sulphate (SDS) were used as *in vivo* models to disrupt the skin barrier of the lower back. In the tape stripping study [1], a lower number of tape strips was necessary for the complete removal of the SC in SS compared to NSS subjects. In the SDS study [2], the SC thickness, measured in hematoxylin and eosin (HE) histologic images, was thinner at 72 hours after SDS application in SS than in NSS subjects. These results indicate that the skin barrier in SS is more vulnerable to mechanical and chemical stimuli.

We suggest that further investigations aimed at unraveling the altered skin barrier properties in SS should focus on the intercellular lipids and on the morphological and biochemical characteristics of the SC. The intercellular lipids might differ in terms of amount, ratio and lateral organization. CRS has been shown to be able to measure the overall amount of lipids as the ratio between the Raman signal of lipids to that of protein in the high wavenumber region [3]. In addition, features in the Raman spectra providing information on the lateral organization of the lipid chains have been identified in both the fingerprint and high wavenumber regions [4]. Besides CRS, a recent study demonstrated that information about the morphological and biochemical characteristics of the SC in SS subjects can be gained by analyzing the material extracted by tape stripping [5]. Material collected from tapes allowed to measure the amount of protein (used as marker of SC cohesion), the amount of enzymes responsible for correct maturation and desquamation of corneocytes, and the average dimension of corneocytes, whose smaller size may lead to a shorter penetration pathway for exogenous substances.

Of note, TEWL measured in the volar forearm was higher in SS than in NSS subjects in **chapter 3.1**, whereas an opposite trend was found in **chapter 2.2**. As the selection method, inclusion/exclusion criteria, age range, Fitzpatrick skin type and model device to measure TEWL were the same between the two studies, this discrepancy might be ascribed to a seasonal effect. The study using histamine iontophoresis (**chapter 3.1**) was carried out between April and November, while the study using CRS (**chapter 2.2**) between January and mid-March, thus within the winter months. It is tempting to speculate that SS might have different compensatory or adaptive mechanisms to the seasonal climatic changes than NSS, which in turn influence TEWL. Several parameters linked to the status of the skin barrier have been found to change across seasons, among which TEWL, SC hydration, sebum secretion, intercellular lipids, NMF components and corneocyte number and size [6]. It is generally believed that the low indoor humidity and cold outdoor temperature occurring in winter have a negative influence on the skin barrier [6]. Yet, an epidemiological study reported higher percentages of self-assessed SS in summer than in winter [7]. Other epidemiological as well as clinical studies have shown differences in SS prevalence and intensity of skin irritation between subjects living in different climatic and latitude regions [8, 9]. Season might thus constitute another confounder responsible for causing ambiguity in the understanding of SS [10].

Are vascular and/or sensory reactivities enhanced in sensitive skin?

The vascular and sensory reactivities in SS were addressed in **chapter 3.1** by application of histamine iontophoresis on the lower back. The intensity of the erythema resulting from the vasodilatory effect of histamine was measured by diffuse reflectance spectroscopy (DRS) and expressed as the a* component of the Commission Internationale de l'Éclairage (CIE) Lab color space, since erythema induces a significant increase in this component, making it particularly suited for its evaluation [11]. To estimate the extent of the flare reaction resulting from the neurogenic inflammation induced by histamine, a simple segmentation algorithm based on well-established image processing techniques and on a low cost acquisition set-up of skin photographs was devised. The sensory reactivity was expressed as the subjects' self-assessed perception of itch on a Visual Analogue Scale (VAS), and was assessed both during histamine iontophoresis and up to 60 minutes following its application. No differences emerged with respect to the intensity of erythema or the extent of the flare, whilst higher itch perception was reported by SS compared with NSS subjects following application of histamine, reaching statistical significance at 30 minutes.

Enhanced vascular reactivity was shown by Richters and coworkers; specifically, a flare response was observed in SS and not in NSS subjects during mechanical stimulation with tape stripping [1]. Despite this, a lower a* value was measured in SS compared to NSS subjects at 30 minutes after stimulus: the authors attributed this finding to the more intense spongiosis in the former, visible in HE histologic images, since edema is known to decrease redness [11]. The lack of differences in the vascular reactivity elicited by histamine might have been due to the low sample size, of by the fact that the chosen readout parameters were not sensitive enough to detect subclinical changes in blood flow, vasodilation and edema; further studies using histamine iontophoresis should therefore use more sensitive and specific bioengineering techniques. On the other hand, the higher itch perception reported by SS subjects was in keeping with the previous findings of Richers and coworkers, in which SS subjects reported more intense dryiness, burning and stinging perceptions in response to stimuli [1, 2, 12]. The fewer tryptase-positive mast cells in SS than in NSS subjects, consistently found in these studies [1, 2, 12], is also a strong argument in favor of the involvement of immune cells in SS, which in turn could explain the occurrence of the altered vascular reactivity and enhanced sensory perceptions in subjects with this condition.

The involvement of the cutaneous nervous system in SS has been the frequent object of evaluation of other authors as well. Stimulation of C and Aδ fibers, the cutaneous nerve endings responsible for mediating perceptions of pain, itch, cold, and heat [13], has been targeted by application of electric currents at 5 Hz and 250 Hz, respectively [14, 15]. During stimulation, a faster onset of sensory perceptions emerged in SS compared to NSS subjects. Further evidence on the neuronal basis of SS came from a study which demonstrated a greater cerebral activation in SS than in NSS subjects during a skin irritative test, as measured by functional magnetic resonance imaging [16]. Currently, research efforts are homing in on the molecular

basis, focusing on sensory proteins and inflammatory mediators [17, 18]. Among these, the transient receptor potential vanilloid 1 (TRPV1) is thought to play a role [18], given its obiquitous expression in human skin [19], its responsiveness to a wide range of stimuli [20] and its role in mediating pain, itch, burning, and warmth sensations [18, 20]. Activation of TRPV1 results in the release of neuropeptides, such as substance P and calcitonin gene-related peptide, from cutaneous sensory nerves [18, 19]. These would, in turn, bind to receptors on dermal blood vessels, leading to neurogenic inflammation and resulting in edema and vasodilation [19]. Neuropeptides would also activate keratinocytes and recruit other immune cells by inducing the release of pro-inflammatory cytokines and chemokines, thereby activating the cutaneous immune system [18]. As these neuropeptides are also known mast cells liberators, it would be of interest to investigate whether a causative link exists between TRPV1 activation and the fewer tryptase-positive mast cells in SS subjects found by Richters and coworkers [1, 2, 12]. Mast cells degranulation may also be directly triggered by activation of TRPV1 expressed on these cells [19]. Recent clinical studies have started to investigate an over-expression of the TRPV1 receptor in SS subjects at the protein level through immunohistochemistry and at the mRNA level through quantitative polymerase chain reaction [21, 22].

To complete the discussion over the sensitive skin phenomenon addressed in this thesis, the following discussion points are given.

Is the selection method of study participants appropriate?

The validity of the clinical studies evaluating differences between SS and NSS subjects described in **chapter 2.2** and **chapter 3.1** relies on the capacity of the multifactorial questionnaire to adequately select study participants. The questionnaire can be deemed an appropriate selection tool, since the symptoms and stimuli used to calculate the score according to which participants were classified into the SS and NSS groups were shown to discriminate between the two categories in a large survey-based study [23]. This is strenghtened by the differences between SS and NSS subjects at the clinical, biophysical and immunohistochemical level found by Richters and coworkers [1, 2, 12]. Other authors consider the use of questionnaires and patient-reported scales as an appropriate selection tool for SS studies [18]. A scale assessing the severity of SS has also been proposed [24].

The selection of study participants in **chapter 2.2** and **chapter 3.1** prevented possible confounders by excluding subjects with concomitant atopic diathesis and skin diseases and subjects with very fair skin phototypes (Fitzpatrick skin type I), as well as darker skin phototypes (Fitzpatrick skin type IV, V and VI). In **chapter 4.1** it is shown that another confounder which should be considered in SS studies is the possible role of fluctuating hormone levels. In the digital survey, distributed to a sample population of 300 women residents in the Netherlands, 42% of premenopausal women declared to perceive (increased) skin sensitivity just before and during the menstrual cycle, citing presence of bumps/pimples, dryness and itching as the

mot frequenly occurring symptoms which make them realize an increased SS. Interestingly, this effect was perceived also by women who do not normally describe their skin as sensitive. Whether oestrogen and, possibly, progesterone and testosterone, lead to objective changes in skin barrier properties and cutaneous inflammation during the menstrual cycle, remains to be evaluated in clinical studies. The effects of decreased sex steroid hormones levels in menopause are worth evaluation as well, since almost 32% of peri- and postmenopausal women reported perception of increased SS since the menopause. The digital survey was also used to evaluate risk factors leading to an increased likelihood to report SS, reported in **chapter 4.2**. Presence of atopic diathesis, skin diseases and fair skin phototype (Fitzpatrick skin type I and II) emerged as the risk factors increasing the likelihood of reporting SS, in line with previous findings of Richters and coworkers [23] and of several other authors (Table 2 in chapter 1.4). Of note, current or past smoking and low history of sun exposure showed a trend to make a significant contribution to the final model. As these and other lifestyle factors have been shown to influence skin properties, their possible role in SS should be taken into account. This is in keeping with the increased awareness of the need to study diseases not just on the basis of the genetic background of an individual, but also in terms of the environmental exposures occurring throughout one's life, the so-called exposome [25].

Is there a primary pathomechanism involved in sensitive skin?

As of today, it has not been established whether a primary pathomechanism responsible for SS exists, or if several pathomechanisms may occur concomitantly. Should the altered skin barrier be the primary pathomechanism, the cutaneous nerve endings would be more exposed to penetration of external irritants and lead to the onset of sensory hyper-reactivity and cutaneous inflammation ("outside-in" mechanism). On the other hand, an underlying dysfunction in cutaneous inflammation may also undermine the skin barrier function ("inside-out" mechanism). For example, increased release of histamine from mast cells may reduce the expression of differentiation-specific proteins, necessary for the correct formation of the skin barrier [26]. Some authors have proposed that different primary pathomechanisms might exist, which would lead to a classification of SS subjects according to the primary pathomechanism involved [17, 27]. Irrespective of classification, the use of *in vivo* skin models in the clinical studies of Richters and coworkers [1, 2, 12] and in **chapter 3.1** demonstrated that SS can be elicited by stimuli of different nature, confirming the findings of epidemiological studies (chapter 1.4). In addition, the lack of differences in SC levels of water, NMF and ceramides/fatty acids between subjects with SS and NSS found in **chapter 2.2**, together with the consistently higher sensory perceptions reported by the former, indicate that treatments for SS solely based on hydration and ceramides supplementation may not bring sufficient benefits to subjects with SS. Accordingly, increased research efforts have been directed towards the addition of anti-inflammatory active ingredients and inhibitors of sensory hyper-responsiveness (*e.g.* TRPV1 antagonists) to cosmetic formulations aimed at soothing SS symptoms [28-30].

Is sensitive skin generalized or localized?

Previously, SS was considered to be limited to the face. However, this view is now subject to change. An epidemiological study showed that, abeit sensitive facial skin was claimed by the majority of responders (77.3%), a still high percentage claimed to have sensitive body skin (60.7%) [31]. Both self-assessments of sensitive facial and body skin were consistent with self-assessments of a "general" SS. In another epidemiological study, 85% of responders declared that they had SS on the face, and 70% had SS in another body area [32]. These findings are in agreement with the results in **chapter 4.1**, where highest percentages of SS were found for the face (53.6%), and lower but still fair percentages emerged for the legs (27.7%) and the hands (27.3%). Variations in SC structure and biochemical composition, cutaneous nerve fiber density, and mast cells number, among other factors, are present between body sites [33, 34]. It is reasonable to believe that SS is a generalized condition, whose pathomechanisms are influenced by the peculiarities of each body site. In this respect, facial skin, characterized by a weaker skin barrier function and a higher density of cutaneous nerve fibers than other body sites [27, 33], is likely to be more severely impacted by external insults in SS compared to NSS subjects. This view is supported by the trend to lower ceramides/fatty acids in SS than in NSS subjects found in the cheeks but not in the volar forearm in **chapter 2.2**.

SKIN IRRITATION

The *second objective* of this thesis was to provide novel insights into the non-invasive and *in vivo* evaluation of skin irritation at the molecular level.

Step 1: To review the existing methods to sample molecular markers of inflammation from human skin in a minimally-invasive fashion.

A systematic literature review was performed to provide an overview of the existing minimally-invasive methods to sample interleukin (IL)-1 alpha and its receptor antagonist IL-1RA from human skin *in vivo*, presented in **chapter 5.1**. Ten different methods were found, with varying degrees of invasiveness and collection times; common to each was the possibility to sample additional biomarkers besides IL-1α and IL-1RA and the high inter-subject variability in the yield of biomarkers. The possibility to sample biomarkers with minimally-invasive methods is likely to facilitate the approval of clinical studies by the regulatory authorities and the inclusion of larger sample sizes. This would benefit not only research on skin irritation, but also research on drug discovery and development. In this case, the retrospective evaluation of how well one or more biomarkers predicted clinical benefits or risks related to an intervention could lead to their validation as surrogate endpoints, as defined by the Biomarkers Definition Working Group [35]. The advantage of biomarkers as surrogate endpoints in clinical trials is that they do not require long periods before being achieved, in contrast to clinical outcomes:

this could fasten the development of safe and effective therapies [35]. Another advantage is that the same biomarkers used as surrogate endpoints in clinical trials could be extended to clinical practice to measure disease responses [35]. This would be especially beneficial for psoriasis, a chronic inflammatory skin disease for which, up to now, no biomarkers that can accurately predict its progression and therapeutic response have been validated [36].

Step 2: To explore whether transdermal analyses patch (TAP) is able to detect dynamic changes in skin surface biomarkers following skin perturbation.

In **chapter 5.2**, a pilot study was carried out to explore the potential of transdermal analyses patch (TAP) to detect skin surface biomarkers following tape stripping and histamine iontophoresis as *in vivo* models of skin irritation. The cytokines IL-1α and IL-1RA and the antimicrobial peptide human beta defensin (hBD)-1 were consistently measurable following perturbation with both models. At the same time, the dynamics showed opposite results. Following tape stripping, an upregulation of all biomarkers was observed, which was somewhat slower for hBD-1. Immediately after histamine iontophoresis, no changes in IL-1α and a downregulation in IL-1RA and hBD-1 emerged. While these findings could be related to the increased availability due to a disrupted barrier following tape stripping, and to a dilution effect due to a swollen skin barrier following histamine iontophoresis, the different dynamics of the three biomarkers within the same model of irritation likely reflect a different release pattern, which TAP was sensitive enough to detect.

The pilot study also confirmed observations emerged from the literature review of **chapter 5.1**. The high inter-individual variability in the yield of biomarkers found with TAP was a common feature of other minimally-invasive methods included in the review. It appears thus that high inter-individual variability is a common feature of skin biomakers research: further studies should include a suitable number of participants in order to draw meaningful conclusions. The fact that additional biomarkers could be measured in the tape stripping experiment, performed in autumn, whereas no other biomarkers were detectable in the histamine iontophoresis study, performed in summer, suggests a seasonal effect on the yield of biomarkers. This aspect emerged in other studies included in the review [37, 38]. The effects of temperature and relative humidity should also not be overlooked: control of these parameters in the enviroment where biomarkers are collected is advisable, especially when minimally-invasive methods sampling biomakers from the skin surface are used.

Step 3: To explore whether photobiomodulation with blue light at 453 nm affects skin recovery following acute perturbation.

In **chapter 5.3**, a pilot study is presented in which photobiomodulation with blue light centered at 453 nm was used immediately after tape stripping and histamine iontophoresis to investigate possible conditioning effects on skin recovery and cutaneous inflammation. Experiments were repeated over two consecutive weeks: in one week, irradiation was used to

condition skin responses, while in the other week no light was used (control). The cytokines IL-1α and IL-1RA and the antimicrobial peptides hBD-1 and hBD-2 were measured with TAP on normal skin and at 24 hours after tape stripping in the irradiated and in the control sites. As in the pilot study reported in **chapter 5.2**, these biomarkers were consistently measurable, and in the control site they were upregulated with respect to baseline. However, while a similar increase was present in the irradiated site for IL-1RA, hBD-1 and hBD-2, no upregulation emerged for IL-1α. This finding might be related to the previously reported anti-inflammatory effects of blue light, albeit the photoacceptor(s) and the mechanisms responsible for transducing light absorption into cellular responses remain to be unraveled.

This pilot study fosters the measurement of biomarkers locally in the skin by minimally invasive methods. The possibility to detect different dynamics in small scale pilot studies, shown in **chapter 5.2** and **chapter 5.3**, holds promise for the use of TAP in clinical studies in which the effect of therapies is tested, or a biomarker blueprint of different inflammatory skin diseases and subtypes thereof is sought.

To complete the discussion over skin irritation, the relationship between its assessment at the molecular level and at the macroscopic level by means of bioengineering techniques deserves attention.

A number of articles in the review of **chapter 5.1** added macroscopic assessment of skin irritation by bioengineering techniques in addition to the sampling of IL-1α and/or IL-1RA. In some studies, lower TEWL and higher SC hydration were in agreement with decreased inflammatory biomarkers, indicating improvement of the clinical status of the skin [38-40]. On the other hand, the association was not straightforward in other studies. In some cases, decreased TEWL and increased SC hydration following topical treatments were not mirrored by decreased levels of inflammatory biomarkers [41, 42]. In other cases, increased TEWL and erythema, indicators of skin irritation, were accompanied by lower yield of IL-1α compared to control [43-45]. Other articles described varying associations between bioengineering techniques and inflammatory biomarkers according to the type of irritant used, or to the single or repeated application of the same irritant. In this respect, substances causing mild increase in TEWL and erythema could determine higher yields of IL-1α and/or IL-1RA than substances for which bioenginneering techniques indicated a more pronounced inflammatory reaction [46-48]. An inverse relationship between the severity of cutaneous inflammation measured by bioengineering techniques and the recovered amounts of IL-1α and/or IL-1RA could be due to different kinetics of release, dependent on the type of irritant used and the extent of irritant-induced keratinocyte injury [46, 48].

In **chapter 5.2** and **chapter 5.3**, bioengineering techniques including TEWL, DRS and reflectance confocal microscopy (RCM) were used to further characterize skin irritation following the application of tape stripping and histamine iontophoresis. In **chapter 5.2**, upregulation

of IL-1α, IL-1RA, hBD-1 and hBD-2 could be related to increased availability following skin barrier disruption; however, at 72 hours after tape stripping, IL-1α and IL-1RA levels were back at baseline, whereas this was not the case for TEWL. This indicates that the upregulation measured with TAP could not exclusively be related to the disrupted barrier, but also to an underlying dynamics of release. Similarly, downregulation of IL-1RA and hBD-1 immediately after histamine iontophoresis could be related to increased SC thickness assessed by RCM; however, the absence of downregulation of IL-1α might reflect a release of this biomarker. In **chapter 5.3**, higher TEWL in the irradiated tape-stripped site compared to the control tape-stripped site was found, concomitantly to the absence of upregulation of IL-1α. Both results indicate an effect of blue light on the recovery of the skin barrier following acute perturbation: in particular, an inhibited inflammatory response could undermine the normal recovery of skin homeostasis, thereby explaining the increased TEWL.

Chapters 5.1, **5.2** and **5.3** show examples of how the assessment of skin irritation could be improved by a synergic combination of measurements: inflammatory biomarkers at the molecular level, and skin biophysical properties at the macroscopic level. Together, such *in vivo* and non-invasive assessments could allow a deeper understanding of skin responses not only following irritation, but also in diseased conditions and following therapeutic interventions.

FUTURE PERSPECTIVES ON BIOENGINEERING TECHNIQUES IN DERMATOLOGY AND IN COSMETIC SCIENCES

The sensitivity and specificity of the analysis of cells and tissue morphology offered by skin biopies make them the gold standard to assess skin physiology and pathology. Also in **chapter 3.1**, skin biopsies were taken to thoroughly characterize the effects of histamine iontophoresis on the epidermal and dermal compartments using immunohistochemistry. However, the importance of objective skin assessments offered by bioengineering techniques, obtained non-invasively and therefore without discomfort, cannot be underestimated. At times, moreover, bioengineering techniques may prove more advantageous that skin biopsies. In **chapter 5.2**, RCM revealed an increased SC thickness following histamine iontophoresis, likely indicating an hydrating effect of the active electrode or of the underlying edema. This effect was marked at 30 minutes, with a median increase of approximately 4 μm, and halved at 90 minutes. Yet, in **chapter 3.1**, the analysis of skin biopsies taken at 60 minutes after stimulation did not reveal increased SC thickness. This indicates that minor changes in SC thickness might have been made undetectable by the preparatory processing of the tissue prior to staining.

Recent developments have increased the specificity and sensitivity of information offered by bioengineering techniques. An example is given in **chapter 2.1**, in which a systematic literature review was performed to compare skin barrier assessments with established, macroscopic biophysical methods (TEWL and electrical methods for estimating the SC hydration) and with

CRS. While the former provide an indirect assessment of the status of the skin barrier, the latter can directly measure the biochemical components and their distribution in the SC. In the studies included in the review of **chapter 2.1**, a relationship between the two assessments was not always straightforward; accordingly, in **chapter 2.2**, only weak correlations were found between macroscopic biophysical methods and CRS. Besides CRS, successful attempts at directly measuring SC components have been reported with other top-notch techniques. A novel prototype based on near-infrared (NIR) microspectroscopy has been recently proposed for simultaneous and quantitative measurement of water and lipid levels in the skin [49]. Another study has demonstrated, for the fist time, the feasibility of time-of-flight secondary ion mass spectrometry (ToF-SIMS), a surface analysis technique characterized by high chemical sensitivity and specificity, to analyze SC lipids in tape-stripped samples collected *in vivo* [50]. Advances have been made also in the assessment of cutaneous inflammation. For example, it is known that, when linearly polarized light is illuminated on the skin, a part is reflected by the skin surface, retaining its polarization state, whereas another part undergoes diffuse scattering in the underlying dermis, becoming depolarized [51]. By detecting the light re-emitted from the skin using a cross-polarization filter, an image is obtained containing information on the skin color originating from the microcirculation at a depth of approximately 0.5-1 mm, whereas effects from the skin surface are suppressed [51]. Analysis of cross-polarized images was able to detect subclinical changes in erythema, not visible with the naked eye, following mild skin irritation [52]. Enhanced visualization of erythema and edema in cross-polarized images was also achieved by exploiting the characteristic absorption bands of hemoglobin and water in narrow-band spectral imaging [53]. At the same time, red blood cells concentration could be estimated from cross-polarized, RGB images after a post-processing algorithm [54]. Another approach employed NIR femtosecond lasers to excite multiphoton absorption in endogenous skin fluorophores: besides obtaining "optical biopsies" with subcellular resolution, *in vivo* and non-invasively, clear changes in the cellular metabolism of inflamed compared to normal skin could be detected by concomintant measurement on the lifetime of the autofluorescence [55]. Efforts have also been directed at overcoming the limitation of single-point measurements given by TEWL and electrical methods for estimating SC hydration. Information on the heterogeneity of the status and hydration of the skin barrier was obtained by imaging the skin with contact-based capacitance sensors [56] and by interpolating single-point measurements to generate continuous color maps [57].

Despite their potential, bioengineering techniques still face some challenges before widespread application in clinical dermatological practice. As mentioned in **chapter 2.1** for CRS, albeit the same applies for other technologies, a potential way forward could be to promote collaborations between technology experts, dermatologists and skin scientists. The aim would be to determine how to include the information provided by the instrumentation in routine clinical or product testing protocols, and how it could affect the decision-making processes. This view is shared by other authors [58], who also highlighted the necessity to raise

awareness of the existing technologies within the medical community, standardize protocols, determine clinically relevant parameters such as sensitivity and specificity in large clinical trials, and determine the cost-effectiveness of the technologies. This approach has been proved successful for RCM, for which a protocol for the diagnosis of chronic plaque psoriasis in dermatological practice has been proposed [59]. The protocol was based on the knowledge of disease features recognizable in RCM images, gained through a series of preliminary clinical studies in psoriasis expertise centers, and on a sound knowledge of relevant literature. As such challenges will be overcome, and more affordable technological implementations will be brought forward, chances are high that the widespread application of bioengineering techniques in dermatological practice and in cosmetic sciences will prove invaluable in offering personalized and non-invasive skin treatment solutions.

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AFKORTINGEN

CIE	Commission Internationale de l'Éclairage
CRS	Confocale Raman microspectroscopie
DRS	Diffuus reflectie spectrofotometer
hBD	Humaan beta defensine
IL	Interleukine
LED	Lichtgevende diode
NMF	Natural moisturizing factor
RCM	Reflectie confocale microscopie
RGB	Rood-blauw-groen
TAP	Transdermal analyses patch
TEWL	Transepidermaal waterverlies
TRPV1	Transient receptor potential vanilloid 1

Chapter 7

Nederlandse samenvatting

De doelstelling van dit proefschrift was om onderzoek te verrichten naar gevoelige huid en huidirritatie door middel van niet-invasieve meetmethoden. Zowel traditionele biofysische methoden als transepidermaal waterverlies (TEWL) en capaciteit (Corneometer) werden gebruikt, als ook nieuwe technieken zoals confocale Raman microspectroscopie. Metingen werden uitgevoerd om kenmerken van zowel de huidbarrière als van huidontsteking in gezonde proefpersonen te karakteriseren.

GEVOELIGE HUID

Het *eerste doel* van dit proefschrift was om inzicht te krijgen in drie mogelijk onderliggende pathomechanismen van gevoelige huid: een verminderde barrièrefunctie en een verhoogde sensitiviteit en vasculaire reactiviteit. In een aantal klinische studies werden proefpersonen die een subjectief ernstig gevoelige huid en proefpersonen die een niet-gevoelige huid rapporteerden geselecteerd op basis van een nieuwe vragenlijst [1]. In de vragenlijst stonden vragen over sensaties, huidreacties en de ernst hiervan op verschillende prikkels, zoals toiletartikelen, kou en stress. Deze sensaties behoorden tot de discriminerende factoren voor gevoelige huid, die gevonden werden in een eerdere studie [1].

Is er een verminderde huidbarrière in gevoelige huid?

De barrièrefunctie werd onderzocht in **hoofdstuk 2.2** met behulp van een nieuwe optische techniek, confocale Raman microspectroscopie (CRS), en in **hoofdstuk 3.1** door middel van topicale applicatie van een oplossing van histamine. CRS werd gebruikt om de dikte van het stratum corneum en de hoeveelheid water, lipiden en natural moisturizing factor (NMF) in die laag direct te meten. Deze techniek werd ook gebruikt om de penetratie kinetiek van een oplossing van glycerol in het stratum corneum te onderzoeken. Histamine is een stof die jeuk, zwelling en roodheid opwekt als het in de huid doordringt. In beide klinische studies beschreven in **hoofdstuk 2.2** en **3.1** werd tevens gebruik gemaakt van traditionele biofysische meetmethoden zoals TEWL en Corneometer om een indirecte schatting van de hydratiestatus van de huidbarrière te maken. In **hoofdstuk 2.2** vonden wij, behalve een trend naar minder lipiden in de wang van proefpersonen met gevoelige huid vergeleken met proefpersonen met niet-gevoelige huid, geen significante verschillen tussen de twee groepen. Dat betekent geen verschil in de dikte van het stratum corneum en in de hoeveelheid water, NMF en lipiden in de onderarm, wang en handpalm. Het enige significante verschil was een kleinere hoeveelheid glycerol in het bovenste deel van het stratum corneum en in de eerste minuten na applicatie in de groep met gevoelige huid. In **hoofdstuk 3.1** vonden wij een trend naar verhoogde jeuksensaties na topicaal histamine in de groep met gevoelige huid in de onderarm maar niet in de onderrug. Hieruit concluderen wij dat de barrièrefunctie in mensen met gevoelige huid niet significant verminderd is; toch is de kleinere hoeveelheid glycerol in deze groep waarschijnlijk een bewijs

van een sneller doordringen van stoffen in de huid. Eerdere klinische studies die dezelfde vragenlijst hebben gebruikt voor de selectie van proefpersonen met en zonder gevoelige huid hebben aangetoond dat het stratum corneum gemakkelijker beschadigd wordt bij de gevoelige huid [2, 3]. Onze aanbeveling is dat aanvullend onderzoek naar de mate van betrokkenheid van de huidbarrière in de gevoelige huid gericht zou moeten zijn op adhesie en grootte van corneocyten en op de organisatie van de intracellulaire lipiden [4, 5]. Verder onderzoek naar de invloed van seizoen op de gevoelige huid is ook wenselijk, omdat wij een hogere TEWL vonden in de onderarm van mensen met gevoelige huid vergeleken met mensen zonder gevoelige huid in de lente/zomer (**hoofdstuk 3.1**) en niet in de winter (**hoofdstuk 2.2**). De invloed van seizoen op kenmerken van de huidbarrière is al bekend in de literatuur [6].

Is er verhoogde sensitiviteit en vasculaire reactiviteit in gevoelige huid?

De verhoogde sensitiviteit en vasculaire reactiviteit werden onderzocht in **hoofdstuk 3.1** met behulp van een *in vivo* huidmodel, histamine iontoforese. In dit huidmodel wordt elektrische geleiding gebruikt om histamine gemakkelijker in de huid te laten doordringen, met een minimaal effect op de huidbarrière. Wij vonden geen significante verschillen tussen proefpersonen met gevoelige en niet-gevoelige huid wat betreft de mate van roodheid, gemeten met behulp van een diffuus reflectie spectrofotometer (DRS) als de a* waarde van de *Commission Internationale de l'Éclairage* (CIE) kleurruimte [7]. Ook de uitbreiding van de flare respons, gemeten in macroscopische foto's met behulp van beeldverwerking en een segmentatie algoritme, was niet afwijkend. Wel hadden proefpersonen met gevoelige huid een hogere subjectieve perceptie van jeuk, die significant werd op 30 minuten na histamine iontoforese.

Een flare respons in proefpersonen met gevoelige huid werd ook waargenomen in een ander *in vivo* huidmodel, tapestrippen [3]. Deze huidreactie werd niet opgewekt in proefpersonen met niet-gevoelige huid. Bovendien werd een lagere a* waarde in de gevoelige huid gemeten op 30 minuten na tapestrippen: dit resultaat zou verklaard kunnen worden door de aanwezigheid van een ernstige spongiose in deze groep vergeleken met de niet-gevoelige huid, omdat bekend is dat zwelling roodheid vermindert [7]. Onze hypothese is dat de afwezigheid van significante verschillen in vasculaire reactiviteit na histamine iontoforese zou kunnen toegewezen worden aan de lage aantal proefpersonen geïncludeerd in de studie of aan de sensitiviteit van de metingen. Meer geavanceerde en sensitieve meetmethoden zouden gebruikt moeten worden om subtiele verschillen in vasculaire reactiviteit in gevoelige huid vast te stellen. Toch komt de hogere jeuk perceptie na histamine iontoforese in proefpersonen met gevoelige huid overeen met de hogere huidpercepties waargenomen in andere studies [2, 3, 8]. Deze studies hebben ook een lager aantal tryptase positieve mestcellen per mm dermis aangetoond in de gevoelige groep vergeleken met de niet-gevoelige groep, daarmee suggererend dat het innate immuunsysteem een rol speelt in het pathomechanisme van gevoelige huid. Uitgebreid onderzoek naar de verhoogde sensitiviteit in gevoelige huid werd

uitgevoerd in het verleden, bijvoorbeeld met behulp van elektrische geleidingen om bepaalde zenuwen in de huid te stimuleren [9], of met behulp van functionele magnetische resonantie imaging om de activering van de hersenen na huidirritatie in kaart te brengen [10]. Huidige onderzoeken zijn meer gericht op de moleculaire mechanismen van sensitiviteit en vasculaire reactiviteit [11, 12]. In het bijzonder wordt verondersteld dat de transient receptor potential vanilloid 1 (TRPV1) een rol speelt bij de gevoelige huid, vanwege zijn aanwezigheid in meerdere cellen en structuren in de huid en vanwege zijn interactieve rol bij cutane percepties [13]. Verder onderzoek naar de betrokkenheid van TRPV1 en mestcellen, en hun betrokkenheid, is van belang in de ontrafeling van het fenomeen van gevoelige huid, en het zou mogelijk de verhoogde sensitiviteit en vasculaire reactiviteit van deze aandoening verklaren.

De volgende opmerkingen zijn relevant om de discussie over gevoelige huid in dit proefschrift volledig te maken.

Is de vragenlijst een passend selectiemiddel in klinische studies?

De vragenlijst die wij hebben gebruikt om proefpersonen met en zonder gevoelige huid te includeren in de klinische studies van **hoofdstuk 2.2** en **3.1** is een passend selectiemiddel gebleken. Eerdere studies die deze vragenlijst hebben gebruikt hebben verschillen aangetoond tussen proefpersonen met en zonder gevoelige huid middels klinische, biofysische en immunohistochemische methoden [2, 3, 8]. Verder hebben wij exclusiecriteria toegepast die een invloed zou kunnen hebben gehad op de uitslag, zoals atopie, co-existerende huidziekten en een zeer licht of donker huidtype (Fitzpatrick schaal I, IV, V en VI) [1]. In **hoofdstuk 4.1** hebben wij gevonden dat ook de verandering van vrouwelijke hormonen ten grondslag aan gevoelige huid zou kunnen liggen. In deze studie hebben wij een vragenlijst digitaal toegestuurd naar een populatie van 300 vrouwen van verschillende leeftijden en wonende in Nederland. Ongeveer 42% van vrouwen in pre-overgang en 32% van vrouwen in overgang of post-overgang meldden een (verhoogde) gevoelige huid tijdens de menstruatiecyclus en vanaf de overgang, respectievelijk. De invloed van oestrogeen, en in mindere mate progesteron en testosteron, op kenmerken van de huidbarrière en van huidontsteking, en indirect op gevoelige huid, zou onderzocht moeten worden in klinische studies. Met dezelfde vragenlijst, in **hoofdstuk 4.2** hebben wij ook de onafhankelijke bijdrage van verschillende factoren om het risico op gevoelige huid te voorspellen onderzocht. Deze waren atopie, huidziekten en licht huidtype (Fitzpatrick schaal I en II); dit was in overeenstemming met de literatuur (zie tabel in hoofdstuk 1.4). Opmerkelijk was de trend naar een significante bijdrage van een voorgeschiedenis van lage blootstelling aan zonlicht en roken op dit moment of in het verleden. Het is bekend dat deze en andere leefstijlfactoren invloed op de huid hebben: het is dus van belang dat toekomstige studies rekening houden met deze factoren in het ontrafelen van gevoelige huid.

Is er een primair mechanisme dat ten grondslag ligt aan gevoelige huid?

Het is nog niet bekend dat een verminderde barrièrefunctie indirect zou kunnen zorgen voor een verhoogde sensitiviteit en vasculaire reactiviteit, of dat de verhoogde sensitiviteit en vasculaire reactiviteit de primaire onderliggende factor zouden kunnen zijn voor de verminderde barrièrefunctie, bijvoorbeeld vanwege het vrijkomen van histamine door mestcellen [14]. Eerdere studies hebben voorgesteld dat er verschillende primaire mechanismen zouden bestaan, en dit zou leiden tot een classificatie van mensen met gevoelige huid volgens het primaire pathomechanisme [11]. Onafhankelijk van deze classificatie, heeft het gebruik van reproduceerbare *in vivo* huidmodellen en uitlezingen over tijd, zoals in Richters *et al.* [2, 3, 8] en in **hoofdstuk 3.1**, aangetoond dat gevoelige huid kan worden opgewekt door verschillende factoren. Verder wijzen de hogere huidpercepties in gevoelige huid die gevonden zijn in deze studies en het gebrek aan verschillen in de hoeveelheid water, NMF en lipiden in de huidbarrière aangetoond in **hoofdstuk 2.2** op het feit dat behandelingen exclusief gebaseerd op suppletie en herstel van de huidbarrière niet genoeg zijn om gevoelige huid te herstellen. Mede daardoor heeft onderzoek naar persoonlijke verzorging in mensen met gevoelige huid zich nu gericht op anti-ontstekingsingrediënten en TRPV1-antagonisten [15-17].

Is gevoelige huid gelokaliseerd of diffuus?

In het verleden werd gevoelige huid hoofdzakelijk in het gezicht gerapporteerd. Nieuwe studies hebben echter aangetoond dat mensen die aangeven gevoelige huid te hebben dit ook ervaren op een aantal specifieke andere plaatsen op het lichaam [18, 19]. Ook in **hoofdstuk 4.1** hebben wij gevonden dat, terwijl de meeste vrouwen rapporteerden gevoelige huid te hebben in het gezicht (53.6%), een redelijk percentage benen (27.7%) en handen (27.3%) noemden als locatie voor gevoelige huid. Het is bekend dat kenmerken van de huid, zoals de barrièrefunctie, de densiteit van cutane zenuwen en het aantal mestcellen, variabel zijn in verschillende plekken van het lichaam [20, 21]. Onze hypothese is dat gevoelige huid een diffuse aandoening is, en dat de specifieke eigenschappen van een willekeurige huidlocatie de pathomechanismen zullen beïnvloeden. De huid van het gezicht bijvoorbeeld, die in het algemeen een verminderde barrièrefunctie en een grotere densiteit van cutane zenuwen toont vergeleken met andere plekken van het lichaam [21], zou meer beschadigd kunnen zijn door externe factoren in mensen met gevoelige huid dan in mensen zonder deze aandoening. Deze hypothese wordt ondersteund door de trend naar minder lipiden gevonden in proefpersonen met gevoelige huid dan in degene zonder gevoelige huid in de wangen en niet in de onderarm in **hoofdstuk 2.2**.

HUIDIRRITATIE

Het *tweede doel* van dit proefschrift was om meer inzicht te krijgen in de *in vivo* en minimale-invasieve meting van ontstekingseiwitten na huidirritatie.

Stap 1: Het verkrijgen van een overzicht van de bestaande methoden om ontstekingseiwitten in de menselijke huid te meten op een minimaal-invasieve manier.

In **hoofdstuk 5.1** werd een uitgebreid systematisch literatuuronderzoek uitgevoerd om een overzicht te krijgen van de bestaande minimaal-invasieve methoden om interleukine (IL)-1 alpha (IL-1α) en zijn antagonist IL-1RA te meten in de menselijke huid *in vivo*. Wij hebben tien methoden gevonden, met verschillende mate van invasiviteit en tijd nodig om de meting uit te voeren. Algemene eigenschappen waren de mogelijkheid om andere eiwitten samen met IL-1α en IL-1RA te meten en de hoge variabiliteit in de hoeveelheid eiwitten tussen mensen. Omdat deze minimaal-invasieve meetmethoden geen of minimaal huidongemak geven, zou het gebruik in klinische studies makkelijker zijn dan methoden waarvoor het nemen van een huidbiopt nodig is. Dat kan een voordeel zijn niet alleen voor onderzoek naar huidirritatie, maar ook voor onderzoek naar nieuwe behandelingen en geneesmiddelen. De eiwitten die gemeten worden op de huid zouden als biomarkers gebruikt kunnen worden om de respons op bepaalde therapieën en behandelingen te voorspellen [22]. Het gebruik van deze biomarkers zou in de toekomst kunnen leiden tot volledig op de patiënt toegesneden therapie.

Stap 2: Het exploreren van de bruikbaarheid van transdermal analyses patch (TAP) in het bestuderen van dynamische veranderingen in de hoeveelheid ontstekingseiwitten op het oppervlak van de huid na acuut kunstmatige schade.

Kennis over de dynamiek van ontstekingseiwitten na huidirritatie kan ons van belangrijke informatie voorzien over de ontstekingsprocessen in de huid. De in **hoofdstuk 5.2** beschreven resultaten tonen aan dat transdermal analyses patch (TAP), een nieuwe methode gebaseerd op een pleister gecoat met antilichamen, gebruikt kan worden om de hoeveelheden ontstekingseiwitten op het oppervlak van de huid kwalitatief en kwantitatief te meten. In deze studie hebben wij gevonden dat IL-1α, IL-1RA en de antimicrobiële peptide humaan beta defensine (hBD)-1 (hBD-1) consistent gemeten konden worden en verschillende dynamiek lieten zien na kunstmatige schade met tapestrippen en histamine iontoforese als *in vivo* huidmodellen. Bovendien, was de dynamiek binnen een huidmodel voor de drie eiwitten verschillend: dat suggereert een verschillend expressiepatroon en dat TAP gevoelig genoeg was om dat te meten.

De hoeveelheden ontstekingseiwitten gemeten door TAP waren heel variabel tussen proefpersonen, wat ook al opgemerkt werd in het systematisch literatuuronderzoek van **hoofdstuk 5.1** voor de andere meetmethoden. Vervolgstudies die de minimaal-invasieve meting van ontstekingseiwitten gaan gebruiken zullen dus genoeg proefpersonen moeten includeren om betrouwbare conclusies te trekken. Opmerkelijk was dat in de studie van **hoofdstuk 5.2** meer typen van ontstekingseiwitten gemeten konden worden in de lente vergeleken met de zomer. Het is aantrekkelijk te speculeren dat seizoen een invloed heeft op de hoeveelheid van ontstekingseiwitten in de huid, zoals gesuggereerd door andere studies [23, 24]. De temperatuur en vochtigheid van de kamer waar experimenten worden gedaan moet ook gestandaardiseerd zijn.

Stap 3: Het exploreren van de invloed van blauw lichtgevende diode (LED)-licht (453 nm) op het herstel van de huid na acute kunstmatige schade.

Eerdere klinische studies hebben de positieve effecten van blauw LED-licht (453 nm) op de verbetering van de symptomen van plaque psoriasis en atopische eczeem aangetoond [25, 26]. Huidige kennis wijst naar het verminderen van de ontsteking van de huid en het afremmen van de versnelde aanmaak van huidcellen, beide kenmerken van deze chronische inflammatoire huidziekten. In een immunohistochemische studie met gezonde proefpersonen werd ook aangetoond dat de bestraling met blauw licht op intacte huid veilig was [27]. Niettemin ontbreekt onderzoek naar de invloed van blauw licht op huidirritatie in gezonde proefpersonen nog steeds. In **hoofdstuk 5.3** hebben wij dus de effecten van blauw LED-licht (453 nm) na acuut kunstmatige schade door middel van tapestrippen en histamine iontoforese in gezonde proefpersonen onderzocht. De experimenten in deze twee huidmodellen werden uitgevoerd op twee opeenvolgende weken: in een week werd blauw licht gebruikt om de huidreacties te beïnvloeden, terwijl in de andere week geen licht werd gebruikt (controle). In beide weken werd op 24 uur na tapestrippen TAP gebruikt om IL-1α, IL-1RA, hBD-1 and hBD-2 te meten. In overeenstemming met **hoofdstuk 5.2** vonden wij een toename na tapestrippen in vergelijking met intacte huid in alle biomarkers behalve IL-1α. Met betrekking tot deze cytokine werd geen significante toename gemeten vergeleken met baseline na bestraling met blauw licht. Dit is aanvullend bewijs voor de invloed van blauwe licht op de ontstekingsprocessen van de huid.

Hoofdstuk 5.2 en **5.3** geven voorbeelden van de bruikbaarheid van TAP als minimaal-invasieve methode om de dynamiek van ontstekingseiwitten lokaal in de huid te karakteriseren. Naar aanleiding van dit proefschrift, hopen wij dat deze meetmethode toegepast zal worden in grotere klinische onderzoeken naar huidziekten zoals psoriasis en atopisch eczeem: dit zou nieuwe biomarkers aan het licht kunnen brengen om de klinische respons op topicale behandelingen of biologische agentia te voorspellen, en uiteindelijk om per individu te beslissen welke behandeling de voorkeur geniet.

De volgende opmerking is relevant om de discussie over huidirritatie in dit proefschrift volledig te maken.

In sommige artikelen geïncludeerd in het systematisch literatuuronderzoek van **hoofdstuk 5.1** werden ook niet-invasieve biofysische methoden voor de indirecte schatting van de status en hydratie van de huidbarrière in combinatie met de meting van IL-1α en/of IL-1RA gebruikt, zoals TEWL en capaciteit. In enkele artikelen wezen zowel de ontstekingseiwitten als de biofysische metingen op een verbetering van de klinische status van de huid [24, 28, 29]. Daar tegenover toonden andere artikelen geen duidelijke associatie: bijvoorbeeld, een lagere TEWL en hogere hydratie van de huidbarrière werden niet gevolgd door een vermindering van ontstekingseiwitten [30, 31]. In sommige gevallen veranderde bovendien de associatie met het type van huidirritant of met het eenmaal of meermaals gebruik van dezelfde huidirritant [32].

Reden voor deze discrepantie zou kunnen zijn dat er een verschillende dynamiek of mate van schade van keratinocyten is [32].

Ook wij hebben in **hoofdstuk 5.2** en **5.3** een combinatie van biofysische methoden (TEWL, DRS en reflectie confocale microscopie - RCM) gebruikt, samen met TAP, om huidirritatie na tapestrippen en histamine iontoforese te bestuderen. In **hoofdstuk 5.2** kon de toename van IL-1α, IL-1RA, hBD-1 and hBD-2 verklaard worden door de beschadigde huidbarrière na tapestrippen, die aangetoond werd door een hogere TEWL. Niettemin was op 72 uur na stimulatie TEWL nog hoger dan in normale huid, terwijl IL-1α en IL-1RA weer op baseline waren. Dit suggereert een dynamiek van deze ontstekingseiwitten na kunstmatige huidschade, die niet alleen verklaard kan worden door TEWL veranderingen. Op dezelfde manier kon de daling van IL-1RA en hBD-1 direct na histamine iontoforese verklaard worden door het dikkere stratum corneum, gemeten met behulp van RCM. Tegelijkertijd wijst het feit dat de niveaus van IL-1α niet veranderd waren op het verhoogd vrijkomen van deze biomarker na iontoforese. In **hoofdstuk 5.3**, op 24 uur na tapestrippen en in de controle week, was de hoeveelheid IL-1α verhoogd vergeleken met baseline, zoals ook gevonden werd in **hoofdstuk 5.2**. In tegenstelling werd op hetzelfde tijdstip en na bestraling met blauw licht een gebrek aan toename van IL-1α vergeleken met de niveau op baseline (intacte huid) door ons gevonden. Op 24 uur na tapestrippen was TEWL hoger in de onderarm waar de bestraling had plaatsgevonden vergeleken met de controle onderarm. Deze resultaten wijzen op een effect van blauw licht op de huidbarrière en op het cutane ontstekingsproces, wat in overeenstemming is met de resultaten van klinische studies bij psoriasis en atopische eczeem.

Aan de hand van de resultaten beschreven in **hoofdstuk 5.1**, **5.2** en **5.3** kunnen wij de combinatie van niet-invasieve biofysische methoden en de minimaal-invasieve meting van ontstekingseiwitten aanbevelen om meer inzicht te krijgen in de mechanismen van huidirritatie in huidziekten en behandelingen daarvan.

TOEKOMSTIGE PERSPECTIEVEN MET BETREKKING TOT HET GEBRUIK VAN BIOENGINEERINGSTECHNIEKEN IN DE DERMATOLOGIE EN COSMETISCHE WETENSCHAPPEN

Huidbiopten bieden de hoogste sensitiviteit en specificiteit voor de analyse van de morfologie en bepaalde celtypen in fysiologie en pathologie. Ook wij hebben huidbiopten afgenomen in **hoofdstuk 3.1** om de effecten van histamine iontoforese op de epidermale en dermale compartimenten te bestuderen. Echter, het gebruik van bioengineeringstechnieken om de huid op een niet-invasieve manier te onderzoeken, zonder huidschade of huidongemak te veroorzaken, moet niet worden onderschat. Soms kunnen bioengineeringstechnieken zelfs voordelen hebben boven huidbiopten. Bijvoorbeeld, in **hoofdstuk 3.1** hebben wij de dikte van het stratum corneum in histologische beelden gemeten en wij hebben geen verschillen in de dikte

gevonden na histamine iontoforese. Wanneer wij daarentegen RCM gebruikten voor dezelfde meting in **hoofdstuk 5.2**, bleek het stratum corneum dikker te zijn onmiddellijk na iontoforese. Subtiele verschillen in de dikte van het stratum corneum zouden onzichtbaar kunnen worden vanwege de chemische procedure die huidbiopten ondergaan moeten.

Recente ontwikkelingen hebben de sensitiviteit en specificiteit van bioengineeringstechnieken verbeterd. Een voorbeeld daarvan is CRS voor de directe meting van stratum corneum componenten. In **hoofdstuk 2.1** hebben wij een uitgebreid systematisch literatuuronderzoek uitgevoerd om de associatie tussen metingen met CRS en traditionele biofysische technieken zoals TEWL en capaciteit te bestuderen. Deze associatie was niet altijd duidelijk, zoals ook wij vonden in **hoofdstuk 2.2** met de lage correlatie tussen CRS, TEWL en capaciteit. Recentelijk kwamen ook andere bruikbare geavanceerde technieken naar voren voor het karakteriseren van de huidbarrière, zoals nabij-infrarood spectroscopie [33]. Andere ontwikkelingen hebben zich gericht op het vasculaire systeem, bijvoorbeeld om een betere schatting van de ernst van het erytheem te maken met behulp van speciale filters bij het nemen van foto's van de huid [34, 35]. Weer andere technieken kunnen een schatting maken van de concentratie van bloedcellen uit rood-blauw-groen (RGB) beelden van de huid [36].

Ondanks hun bruikbaarheid in de niet-invasieve analyse van de huid in fysiologie en pathologie, zijn bioengineeringstechnieken nog niet algemeen toepasbaar als klinische hulpmiddelen binnen de dermatologie. Zoals beschreven in **hoofdstuk 2.1** voor CRS (en dit geldt ook voor andere technieken), is het belangrijk dat er meer samenwerking komt tussen dermatologen, ingenieurs en huidwetenschappers. Door die samenwerking zal er mee bewustzijn worden gekweekt om de voordelen van deze technieken in de medische wereld te verspreiden, de apparaten in de dagelijkse praktijk bruikbaar te maken, en protocollen te ontwikkelen voor de diagnose van huidziekten en het monitoren van therapieën. Deze strategie was succesvol voor RCM, voor welke een praktisch protocol voor de diagnose van stabiele en instabiele psoriasis plaques recent werd ontwikkeld [37]. Als meer bioengineeringstechnieken de transitie van onderzoeksapparaat naar klinische hulpmiddel doormaken, is de kans groot dat zij van grote waarde zullen zijn voor meer patiëntvriendelijke en patiënt-toegesneden therapieën in de dermatologie en voor toepassing in persoonlijke verzorging.

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Chapter 8

Short summary
List of publications
Research data management
PhD portfolio
Curriculum Vitae
Dankwoord

SHORT SUMMARY

This thesis focused on the non-invasive, objective and *in vivo* evaluation of skin barrier properties and cutaneous inflammation in healthy volunteers by means of novel and established techniques. This evaluation was applied in two research areas: sensitive skin (SS) and skin irritation.

The *first objective* of this thesis was to investigate three possible pathomechanisms of SS, namely impairment of the skin barrier and enhanced vascular and sensory reactivities. These were addressed by (i) measurements with established bioengineering techniques, including transepidermal water loss (TEWL), diffuse relfectance spectroscopy (DRS), and electrical methods to indirectly measure stratum corneum hydration (**chapter 2.2** and **chapter 3.1**); (ii) measurements with confocal Raman microspectroscopy (CRS), a top-notch bioengineering technique for the assessment of skin biochemical composition (**chapter 2.2**); (iii) iontophoretic and topical application of histamine, a vasoactive and pruritogen substance (**chapter 3.1**). The results did not show a major barrier impairment in SS, yet from CRS measurements evidence supporting a faster penetration of topicals in SS emerged. In SS subjects, histamine iontophoresis indicated higher sensory perceptions compared to NSS subjects. From these findings and in agreement with previous literature, it was concluded that further clinical studies aimed at unraveling pathomechanisms of SS should focus on the morphological and biochemical characteristics of the stratum corneum, on sensory proteins and on inflammatory mediators. In addition, by performing a cross-sectional digital survey in a population of women, it was shown that the role of female hormones and lifestyle factors on the onset or exacerbation of SS deserves further attention (**chapter 4.1** and **chapter 4.2**).

The *second objective* of this thesis was to provide novel insights into the minimally-invasive and *in vivo* evaluation of skin irritation at the molecular level. This was accomplished in three steps: (i) by performing a systematic literature review on the existing minimally-invasive methods to sample molecular markers of inflammation (IL-1α and IL-1RA) from human skin (**chapter 5.1**); (ii) by applying one of these methods (transdermal analyses patch – TAP), along with TEWL and reflectance confocal microscopy (RCM), to characterize the dynamics of molecular markers following two *in vivo* models of skin irritation (**chapter 5.2**); (iii) by employing TAP, TEWL, RCM, and the same two *in vivo* models, to evaluate the conditioning effects of photobiomodulation with blue light at 453 nm on skin recovery (**chapter 5.3**). In the review, ten minimally-invasive methods for the *in vivo* evaluation of skin irritation at the molecular level were described, and in the clinical studies it was shown that one of these, TAP, was sensitive enough to distinguish the dynamics of molecular markers following the two different *in vivo* models, or following photobiomodulation with blue light. It was concluded that further clinical studies aimed at unraveling the mechanisms of skin irritation would benefit from the local and minimally-

invasive sampling of molecular markers. In addition, the combined measurement of molecular markers and of skin properties by means of bioengineering techniques was recommended for a more thorough assessment of the skin status.

Together with these objectives, in **chapter 2.1** a future perspective for the non-invasive assessment of the skin barrier function in dermatology and cosmetic sciences was presented. This was done by seeking an association between measurements with established bioengineering techniques (TEWL and electrical methods) and CRS, and by showing the advantages of the latter compared to the former.

As a general conclusion, the work presented in this thesis supports the use of novel and established techniques in the non-invasive, objective and *in vivo* evaluation of skin barrier properties and cutaneous inflammation. Furthermore, it advocates the widespread application of such techniques in dermatological practice and in cosmetic sciences with the aim to offer personalized and non-invasive skin treatment solutions.

PUBLICATIONS RELATED TO THIS THESIS

Falcone D, Uzunbajakava NE, Varghese B, de Aquino Santos GR, Richters RJ, van de Kerkhof PC, van Erp PE. *Microspectroscopic confocal Raman and macroscopic biophysical measurements in the in vivo assessment of the skin barrier: perspective for dermatology and cosmetic sciences.* Skin Pharmacol Physiol, 2015. 28(6): 307-317.

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PUBLICATIONS NOT RELATED TO THIS THESIS

Falcone D, Uzunbajakava NE, van Erp PE, van de Kerkhof PC. *Confocal Raman microspectroscopy: a new paradigm in the diagnosis of sensitive skin?*, in *Sensitive Skin Syndrome, Second Edition.* Maibach HI, Andersen RM and Honari G, Editors. 2017, CRC Press. p. 73-82.

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RESEARCH DATA MANAGEMENT

The data management of the observational studies included in this thesis followed the guidelines for the handling of research data established by the Clinical Research Center Nijmegen (CRCN). The guidelines comply with the Dutch Personal Data Protection Act (*De Wet Bescherming Persoonsgegevens, Wbp*) and the Code of Proper Conduct (*Code Goed Gedrag - FEDERA*).

The data management plan was implemented in the following way:

1. Case Report Forms (CRFs) for data collection were designed by the investigators and approved by the ethics committee Regio Arnhem-Nijmegen (*Commissie Mensgebonden Onderzoek, CMO*). Each CRF contained demographics, code and research data of a single volunteer. CRFs were designed in both a paper and electronic version. The former was kept in a locked cabinet at the dermatology department of the Radboud University Medical Center. The latter was implemented in Castor EDC, a validated data management system complying with Good Clinical Practice (GCP) requirements.

2. The codelist was created in both a paper and electronic version. The former was kept in a locked cabinet at the dermatology department of the Radboud University Medical Center, in a location different from the one used for the paper CRFs. The latter was saved in a password-protected folder on a server of the dermatology department.

3. At the end of the study and after publication, the electronic CRFs were locked with the locking module of Castor EDC. The paper CRFs were kept in the locked cabinet at the dermatology department. The paper version of the codelist was signed by the principal investigator, scanned and saved in a password-protected folder on a server of the dermatology department. The electronic codelist was locked.

4. The paper CRFs and codelist are to be archived at the dermatology department for one year. Afterwards, they will be archived in an external paper archive. The locked electronic CRFs and codelist will remain in the password-protected folder on the server of the dermatology department.

5. At any time during and after the studies, access to the CRFs and codelist was restricted to the investigators, the principal investigator, the research nurse and the study monitors.

PHD PORTFOLIO

Institute for Molecular Life Sciences
Radboudumc

Name PhD candidate:	***Denise Falcone***	*PhD period:*	***01-04-2013 until 31-10-2017***
Department:	***Dermatology***	*Supervisor:*	***Prof. dr. PCM van de Kerkhof***
Graduate School:	***Radboud Institute for Molecular Life Sciences***	*Co-supervisor:*	***dr. PEJ van Erp***

		Year(s)	*ECTS*
	TRAINING ACTIVITIES		
a)	***Courses & Workshops at Radboud University, Nijmegen***		
	- *Scientific integrity*	*2014*	*1.0*
	- *Presentation skills*	*2014*	*1.5*
	- *Digital tools*	*2014*	*0.2*
	- *Academic writing*	*2014*	*3.0*
	- *Refresher course in statistics*	*2014*	*1.5*
	- *LinkedIn business*	*2014*	*0.1*
	- *Basic course on Regulations and Organisation for Clinical Investigators (eBROK)*	*2015*	*1.0*
	- *Advanced conversation*	*2015*	*1.5*
	- *Castor EDC*	*2015*	*0.2*
	- *Training in systematic reviews of animal studies*	*2017*	*0.2*
	- *Perfecting your academic writing skills*	*2017*	*1.5*
a)	***Seminars & lectures***		
	- *Career meeting "Get inspired" organized by Radboud University*	*2014*	*0.2*
	- *Journal club at the dermatology department, Radboudumc, Nijmegen*	*2014-2017*	*4.0*
	- *Radboud New Frontiers at Radboudumc, Nijmegen*	*2014*	*0.4*
	- *Radboud Research Rounds at Radboudumc, Nijmegen*	*2015*	*0.1*
b)	***(Inter)national Symposia & congresses***		
	- *Annual Science Day of the Nijmegen Institute for Infection, Inflammation and Immunity (N4i), at Radboudumc*	*2013*	*0.2*
	- *Dutch Society for Experimental Dermatology (NVED) in Lunteren, the Netherlands (poster presentation)*	*2016*	*0.5*
	- *British Society for Investigative Dermatology (BSID) in Dundee, UK (poster presentation)*	*2016*	*0.75*
	- *International Society for Biophysics and Imaging of the Skin (ISBS) in Lisbon, Portugal (oral and poster presentation)*	*2016*	*1.25*
	- *European Society for Dermatological Research (ESDR) in Munich, Germany (poster presentation)*	*2016*	*0.75*
	- *European Society for Dermatological Research (ESDR) in Salzburg, Austria (poster presentation)*	*2017*	*0.75*
c)	***Other***		
	- *Operator Training Skin Composition Analyzer gen2-SCA, RiverD International B.V., Rotterdam, the Netherlands (2 days)*	*2014*	*0.4*
	- *Microbiology and biosafety course, Fontys Hogeschool Eindhoven, the Netherlands (3 days)*	*2015*	*0.6*
	- *Intensive course in dermato-cosmetic sciences with focus on efficacy measurements, Vrije Universiteit Brussels, Belgium (5 days)*	*2015*	*1.0*
	- *Peer reviewer of scientific publications*	*2015-2017*	*0.5*
	TEACHING ACTIVITIES		
d)	***Lecturing***	*NA*	*NA*
e)	***Other***		
	- *Supervision of internship of two Master's students and one Bachelor's student (6 months)*	*2014-2017*	*6.0*
	- *Supervision of internship of four Bachelor's students ("Oriënterende onderzoeksstage 50MB2") (2 weeks)*	*2015*	*0.15*
TOTAL			***29.25***

CURRICULUM VITAE

Denise Falcone was born on February 24th 1988 in Domodossola, a town in Northwest Italy. After completing high school in 2007, Denise moved to Milan to study Biomedical Engineering at the Politecnico, graduating *cum laude* in 2012 with a thesis on the analysis of ventricolar repolarization using the T wave in electrocardiograms. During her Master's she spent a semester at the École Politecnique Fédérale de Lausanne, Switzerland. Willing to pursue a career abroad, Denise moved to the Netherlands, where in April 2013 she started a PhD project at the dermatology department of Radboud University Medical Center, Nijmegen, under supervision of prof. dr. Peter van de Kerkhof and dr. Piet van Erp and in collaboration with dr. ir. Natallia Uzunbajakava and dr. Jim Coombs at Philips Research Eindhoven. The results of her PhD project are described in this thesis. Denise presented her results at several (inter)national conferences, during which she was awarded a travel grant by the British Society for Investigative Dermatology (BSID) in Dundee, Scotland and the Albert Kligman Young Investigator Award at the International Society for Biophysics and Imaging of the Skin (ISBS) In Lisbon, Portugal. During her PhD, Denise was an enthusiastic attender of Radboud in'to Languages, where she participated in several courses to learn Dutch.

DANKWOORD

It seems hard to believe that more than four years have passed since the April morning when I first arrived at the dermatology department of Radboudumc to embark in my PhD adventure! Now older and (hopefully) wiser, I would like to thank the many people who helped me along the path.

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That's all Folks! Dear colleagues/lieve collega's/amici e parenti tutti, an heartfelt/een oprecht/un sentito

Thank you! Bedankt! Grazie!