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DISSERTATION

VOLUME AND DIFFERENTIATION OF SEBACEOUS GLANDS IN PSORIASIS

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"*Always be the first, always excel all others: your envious soul shall love no one but the 'friend'- that made the soul of the Greek quiver and led him to the path of the glory."*

Friedrich Nietzsche

"Thus spoke Zarathustra"

To my beloved parents Ilias and Maria

and my beloved sister Chryssoula

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ABBREVIATIONS

APC	antigen-presenting cell
Blimp-1	B lymphocyte-induced maturation protein-1
BMI	body mass index
CD	cluster of differentiation
CE	coefficient error
CK	cytokeratin
3D	three dimensional
EMA	epithelial membrane antigen
ESM	epithelial sialomucin
FABP	fatty acid binding protein
FADS	fatty acid desaturase
HE	hematoxylin eosin
HMFG	human milk fat globulin
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
INV	involucrin
IQR	interquartile ranges
kDa	kilodaltons
MAM-6	milk fat globule membrane antigen 6
MCA	mucin-like carcinoma antigen
MC-5R	melanocortin-5 receptor
MHC	major histocompatibility complex
NFkB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

OCT	optical coherence tomography
PASI	psoriasis area and severity Index
PGA	psoriasis global assessment
PPAR	peroxisome proliferator-activated receptor
PSORAS	psoriatic arthritis susceptibility gene
PSORS	psoriasis susceptibility gene
SCD-3	stearyl CoA desaturase-3
SD	standard deviation
SG	sebaceous gland
SGA	sebaceous gland antigen
SPASI	simplified psoriasis area and severity Index
SURS	systematic uniform random sampling
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor

SUMMARY

Psoriasis is a chronic inflammatory skin disease, with the main clinical characteristics of desquamation, infiltration, and inflammation. Microscopic findings in previous studies have also revealed some degree of atrophy of the sebaceous gland in psoriasis and alopecia psoriatica in patients with psoriasis capillitii. The aim of this thesis was to investigate the role of sebaceous glands in the pathogenesis of psoriasis and especially in the pathogenesis of alopecia psoriatica. Skin specimens from psoriasis-involved and healthy looking skin of 14 patients were obtained in order to perform histological and stereological analysis. I chose the method of stereology, which is a method that provides the quantification of 3-dimensional structures ex vivo, in order to be able to estimate and, for the first time, calculate the volume of sebaceous glands in psoriasis, and compare it with the healthy skin of the same patient. My results revealed a significant reduction of the number of sebaceous glands as well as of the volume of each separate sebaceous gland in the lesional compared to the non-lesional skin of our patients. These results led us to examine further the differentiation process of the sebocytes in psoriasis and compare it with the healthy skin of psoriasis patients. To achieve this goal, skin specimens from psoriasis-involved and healthy looking skin of another 14 patients were obtained in order to perform immunohistochemical analysis. I used four immunohistochemical markers to compare the differentiation stages of sebocytes in those two groups. I could show that the sebocytes in psoriasis do not differentiate properly. These findings bring the sebaceous gland -for the first time- to the front of the research in psoriasis and reveal a possible new role of the sebaceous gland that needs to be further investigated.

ZUSAMMENFASSUNG

Psoriasis ist eine chronische entzündliche Hauterkrankung und wird klinisch durch Plaques mit scharf begrenzter Schuppung, Infiltration und Entzündung charakterisiert. Vor ca. 20 Jahren hat man beobachtet, dass die Talgdrüse eine auffällige Atrophie bei der histologischen Untersuchung der psoriatischen Läsionen zeigt. Das Ziel meiner Arbeit war es, die Rolle der Talgdrüsen in der Pathogenese der Psoriasis, und besonders der Alopecia psoriatica, zu untersuchen. Hautbiopsien aus läsionaler und nicht-läsionaler Haut von 14 Patienten mit Psoriasis vulgaris wurden entnommen, um histologische und stereologische Ananlyse durchzuführen. Um das Volumen der Talgdrüsen bei Psoriasis erstmalig zu berechnen und ihm mit dem Volumen der Talgdrüsen nicht-läsionaler Haut der Patienten zu vergleichen, wurde die Methode der Stereologie ausgewählt, die die Quantifizierung von 3-dimensionalen Strukturen ex vivo erlaubt. Unsere Ergebnisse zeigten eine deutliche Reduzierung der Anzahl der Talgdrüsen sowie des Volumens jeder einzelner Talgdrüse in der läsionalen Haut im Vergleich zur nicht-läsionalen Haut unserer Patienten. Diese Ergebnisse haben mich motiviert, weiter die Differenzierung der Sebozyten bei der Psoriasis zu erforschen. Um dieses Ziel zu erreichen, habe ich erneut Hautbiopsien aus läsionaler und nicht-läsionaler Haut von 14 neuen Psoriasis-Patienten entnommen, um immunohistochemische Analysen durchzuführen. Ich habe vier immunhistochemische Marker benutzt, um die Stadien sebozytärer Differenzierung in beiden Gruppen zu vergleichen. Meine Ergebnisse zeigten, dass wahrscheinlich die Differenzierung der Sebozyten bei der psoriatischen Läsion gestört ist. Diese Befunde bringen die Talgdrüsen zum ersten Mal im Focus der Psoriasis-Forschung und stellen eine mögliche neue Rolle der Talgdrüse in der Psoriasis vor.

INTRODUCTION

Psoriasis

Psoriasis is a chronic, recurrent inflammatory skin disease, characterized by rapid proliferation and abnormal differentiation of epidermal keratinocytes and a mixed inflammatory infiltrate with epidermotropism. The patients typically present with the clinical picture of sharply margined, red, scaly, and raised plaques of the skin (Figure 1a), mainly on contact-exposed surfaces, such as elbows and knees (1). The condition can also affect palms, soles, capillitium, skin folds, the genital area, face, nails, and joints. It is distinguished as psoriasis vulgaris of the chronic stationary plaque type, which is the most common form, and the exanthematic forms of guttate, pustular, and erythrodermic psoriasis, and it can be associated with psoriatic arthritis. Additional limited forms are psoriasis inversa, psoriasis capillitii and nail psoriasis.

Psoriasis has a worldwide distribution, but its impact varies among nations, ranging up to 3% (1). The highest rates in Europe were recorded in Denmark and Iceland, and people in Northern Europe are more affected than in the Mediterranean countries. In Caucasians, generally, the rate reaches 2%, while in the United States, it appears to reach 2.2–2.6%, with about 150,000 new diagnoses per year (2). Histologically, psoriasis is characterized by thickening of the corneal layer of the epidermis (hyperkeratosis), retention of nuclei in corneocytes (parakeratosis or hyperparakeratosis), thickening of the spinous layer (acanthosis), reduction or absence of the granular layer, dilatation of blood vessels, and infiltration of inflammatory cells into the papillary dermis (Figure 1b).

Psoriasis has a multi-factorial pathogenesis, and although the disease has been thoroughly investigated during the past four decades, it is not yet fully understood. The main pathological features are the increased proliferation of keratinocytes and their faster but abnormal maturation (1); i.e., the keratinocyte cell cycle is 8 times shorter (36 instead of 311 hours), and its proliferation rhythm twice as fast (3). The strong immunological basis of the disease and the involvement and hyperactivity of the immune system are also major characteristics.

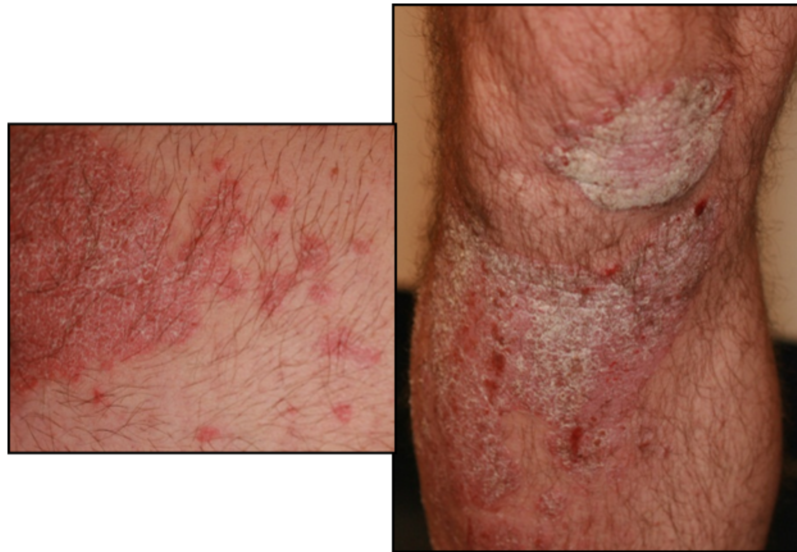

Figure 1a. The typical phenotype of the plaque type psoriasis includes erythematosquamous skin lesions, mainly at knees and elbows.

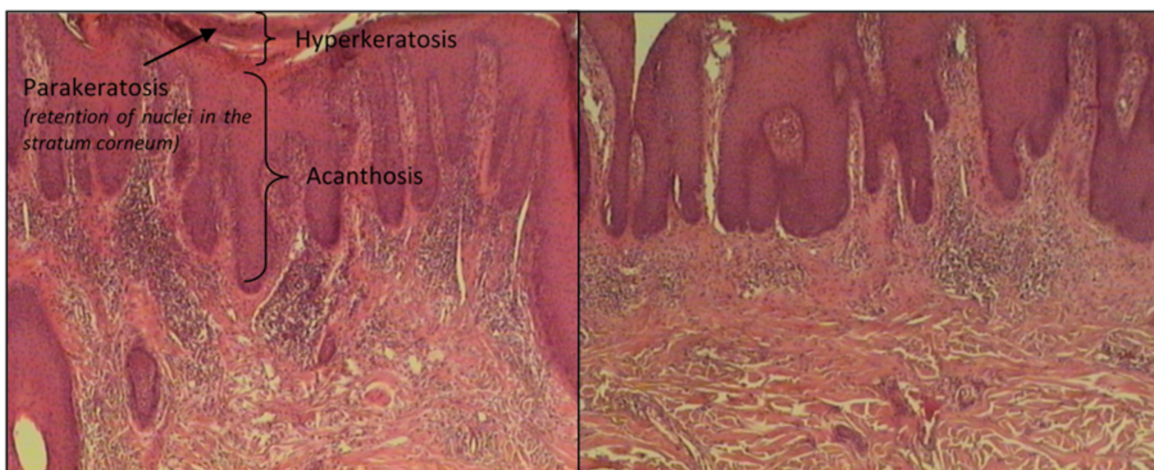

Figure 1b. Hyperkeratosis, parakeratosis and acanthosis are the main histological characteristics of plaque type psoriasis.

The main cytokines involved in the pathogenesis of psoriasis are interferon (IFN)-γ, interleukin (IL) 2, and tumor necrosis factor (TNF)-α factor. These are produced by T helper 1 (Th1) cells. Other molecules involved, but mainly produced by keratinocytes, are IL6, IL8, and IL1, and tumor growth factors (TGF)-α and TGF-β (3). However, the newly revealed ability of a different subset of T-lymphocytes to produce IL-17 adds to the understanding of the pathogenesis of the disease; these are the CD4+Th17 and CD8+Th17 cells (4). Stimulation of the IL-17 producing cells by IL-23 from antigen-presenting cells (APCs) leads to increased levels of IL-17 and IL-22 from either Th17 cells or dedicated IL-22-producing T cells (Th22 cells) (5–7). All these molecules facilitate the increasing proliferation of keratinocytes, inhibit apoptosis, enhance the growth of T cells, and stimulate the production of more cytokines from T lymphocytes and macrophages. Furthermore, they promote angiogenesis, chemotactic attraction of neutrophils and increased production of intercellular adhesion molecule 1 (ICAM)-1 from endothelial cells (3, 8–10).

However, the stimuli that trigger the cascade of the immunological events and lead to the classic phenotype of psoriasis remain unclear. Some authors suggest a wide variety of external and internal stimuli, such as infection, drugs, and physical and psychological stress (11, 12). Others propose a chronic activation of the transcriptional factor “nuclear factor kappa-light-chain-enhancer of activated B cells” (NFkB), such as in other autoimmune diseases, derived by a lack of inhibition of NFκB and consequent transcription of pro-inflammatory genes (13). Still others believe that the stimulation of resident dendritic cells to produce IFN-α following local trauma or infection can implicate the initiation of psoriasis (14). The important role of the immunological cascade in the pathogenesis of the disease is underlined by the fact that allogeneic bone marrow transplantation in psoriasis patients appears to cause a reduction of the psoriatic lesions. On the other side, psoriasis can develop in previously non-psoriasis patients who are subject to bone marrow transplantation from a donor with psoriasis (3).

Psoriasis has also been shown to have a strong genetic component. This is shown by studies of twins, which have reported that 67% of monozygotic twins both develop psoriasis, whereas only 18% of dizygotic twins both contract the disease (3). A genetic basis has been implicated in the locations (loci) of at least 9 genes, PSORS1–9, and

with one extra for psoriatic arthritis, PSORAS1 (15, 16). The most studied is PSORS1, which

sits on chromosome 6 in the major histocompatibility complex MHC. Genetic correlations have further been made between psoriasis and other diseases, and a strong correlation has been found with the genes for atopic dermatitis (loci on chromosomes 1q21, 3q21, 17q25, and 20p), rheumatoid arthritis (3q21 and 17q24–25), and Crohn`s disease (chromosome 16) (3).

Previously, psoriasis was considered primarily as a disease of the keratinocytes. During the last several years, some studies have reported that skin appendages may also play a role or show some changes in psoriasis. The first report was in 1994, which described a remarkable atrophy of the sebaceous gland in psoriasis; however, it provided only a clinical/ histopathological observation, and no quantitative analysis was conducted (17). A 2008 study confirmed this observation, and a 2012 work also reported a 60% absence and 25% atrophy of the sebaceous glands in patients with psoriasis (18, 19). However, all these studies are qualitative estimations and depend on the critical approach of the investigator.

Alopecia psoriatica in psoriasis capillitii

Psoriatic alopecia was first described by Shuster in 1972 (19, 20) and it was initially considered to be associated exclusively with chronic plaque type psoriasis, and not with other psoriasis forms such as acute erythrodermic or generalized pustular psoriasis (21, 22). At the beginning, there were conflicting data whether the alopecia in psoriasis is a scarring or non-scarring process. Some histopathological studies have indicated a scarring process (i.e., reduction in hair follicle density and presence of a peri-infundibular lymphocytic infiltrate with destruction of the follicle) (21-25), whereas clinical reports of complete hair re-growth following topical anti-psoriatic treatments favoured a non-scarring process (26).

Shuster concluded that scalp psoriasis results in 3 distinct types of alopecia: 1) hair loss confined to lesional skin as confirmed by hair pluck revealing dystrophic bulbs (most common); 2) acute hair fall with a predominance of telogen hairs; and 3) "destructive or scarring alopecia" associated with decreased hair density and "perifollicular

inflammation with destructive folliculitis and fibrous tissue replacement" (least common) (19).

Since then, several studies for psoriatic alopecia have been published. Most of them describe infundibular dilatation, perifollicular inflammation and fibrosis, thinned follicular epithelium and fibrous tracts as the main histopathological changes of alopecia psoriatica. The sebaceous gland-related changes include atrophy, reduction of size or number and complete absence (19).

The question that has been raised through the years is whether there is a difference in the clinical and histopathological findings between those psoriatic patients who present alopecia and those who do not have alopecia. Female tend to show psoriatic alopecia earlier that male patients, but there are no measurement data (19). Another question is whether psoriatic alopecia has specific histological changes or if similar findings are present in other lymphocyte-induced scarring alopecias, like central centrifugal cicatricial alopecia, lichen planopilaris, frontal fibrosing alopecia and lupus alopecia. Histopathological features seen in these entities include decreased or absent follicular units, perifollicular lymphocytic infiltrates, distention of follicular ostia (lupus), infundibular hyperkeratosis (LPP/ FFA), fibrous tracts, and naked hair shafts. It is certainly obvious that the frequency of these changes varies with the diagnosis and the stage of disease and that some findings are more specific for certain diseases; however, the follicular changes noted in psoriatic alopecia are not unique and are often present in other lymphocytic alopecia types (18).

The etiology of psoriatic alopecia has not been yet clarified. One theory suggests that psoriatic plaque “strangles” the hair shaft. This theory could explain the reversible effect of alopecia, when the disease is in regression and the plaque is thinner or smaller. However, this theory has been abandoned since several years and other explanations have come to the fore, such as inflammation, vascular abnormalities and fibrosis.

Interestingly, latest studies have shown that a big majority of patients with psoriatic have atrophy or complete absence of sebaceous glands at time of presentation. In recent series of patients, it has been observed that atrophy of sebaceous glands was the only statistically significant histopathological finding present in the psoriatic groups (27, 28).

The reason why sebaceous gland atrophy/loss occurs in psoriasis is yet unclear. One possible explanation could be that psoriasis has an extremely complex cytokine milieu. Another reason for sebaceous gland atrophy/loss relates to the perifollicular inflammation of the upper "permanent" portion of the hair follicle that is present in psoriatic alopecia, it is also common to all scarring alopecias (21). Near this site (particularly where the arrector pili attaches), the bulge region contains stem cells that give rise to multipotent progenitor cells. It is thought that these multipotent cells give rise to the hair shaft, as well as the sebaceous gland. In addition, there is unconfirmed evidence that these cells may give rise to adjacent epidermis. Thus, damage to this region compromises the sebaceous gland (29, 30, 31). In this question, the exact knowledge of sebocyte function, proliferation and differentiation could play an important role.

Sebocytes and their differentiation

Sebaceous gland and hair follicle constitute the pilosebaceous unit of the skin (Figure 2). During late embryogenesis, developing hair follicles display several bulges (hair peg stage), one of which will give rise to the sebaceous gland and is located just above the hair follicle stem cell bulge and below the infundibulum of the developing follicle. The sebaceous gland arises as an outgrowth of the outer root sheath of the hair follicle, while undifferentiated sebocytes emerge from peripheral basal cells and then move centrally as first partially, and later fully, differentiated sebocytes. The fully developed sebaceous gland remains attached to the hair follicle throughout adult life. Differentiated sebocytes produce and secrete lipid-rich sebum into the hair canal that empties to the skin surface (32–34).

The sebaceous gland is constantly renewed throughout adult life by a population of progenitor cells responding to a coordinated interplay of various signals, thereby controlling the balance between proliferation and differentiation of the tissue (35). Two models have been proposed to describe how sebaceous gland cells might arise and regenerate. One is that bulge stem cells of the hair follicle may produce multipotent progenitor cells that migrate upwards to generate the sebaceous gland (36, 37). Another possibility is that self-renewing stem or progenitor cells may reside within the sebaceous gland itself to generate and maintain the mature sebocytes (38, 39).

Recently, the molecular signatures characteristic of the hair follicle stem cell compartment have been unraveled (40, 41).

During the last several decades, many studies have revealed immunohistochemical (and other) markers of sebocytes and sebaceous glands. Some of these markers are specific to certain differentiation stages of sebocytes. It has been shown that, in the very early stages, the hair follicle stem cells express the molecule Sox9, whereas other data have shown that the sebocyte progenitors express Blimp-1 (39, 42), although Blimp-1 has also been shown to represent a marker of terminal differentiation, so that the findings are controversial (43). In early differentiation, the sebocytes express sebaceous gland antigen (SGA), cytokeratin (CK)7, CK10, and c-myc (44–46). During their maturation, they express markers such as epithelial membrane antigen (EMA), perilipin A, and fatty acid desaturase 2 (FADS2) (47–49). The late or fully differentiated sebocytes express more markers like melanocortin-5 receptor (MC-5R), stearyl CoA desaturase-3 (SCD-3), squalene, and epithelial sialomucin (ESM or MAM-6) (50–53).

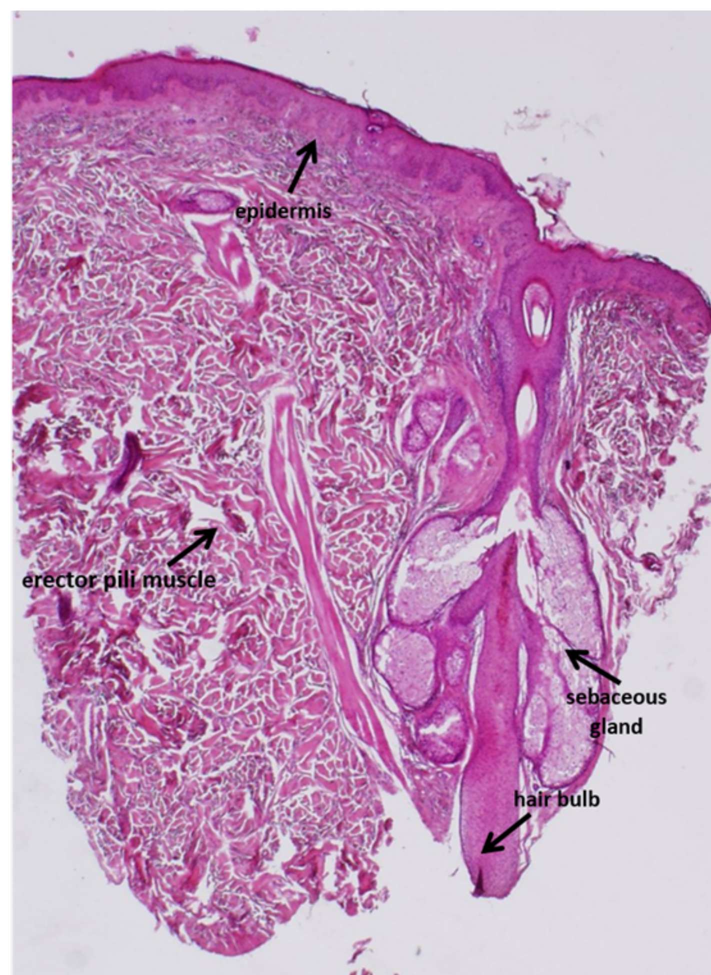

Figure 2. The pilosebaceous unit of the skin.

In addition, it has been shown that peroxisome proliferator-activated receptor(s) (PPAR) have an important role in the expression and differentiation of sebocytes, and especially that PPAR-γ helps the differentiation of early to mature sebocytes and PPAR-β/δ aids the differentiation of mature to late differentiated sebocytes (54). There are also several markers of sebocytes that are not specific to particular differentiation stages, such as fatty acid binding protein 4 and 5 (FABP4, FABP5), mucin-like carcinoma antigen (MCA), and human milk fat globulin 1 and 2 (HMFG1, HMFG2) (46, 53).

Figure 3 summarizes the main differentiation markers of sebocytes and their stage of expression. Finally, some theories suggest that sebocytes may become, under certain circumstances, subject to a kind of "keratinization", i.e., express markers of keratinocytes and lose their ability to produce sebum. These theories have not yet been fully supported, but they can be effectively explained under the theory that sebocytes and keratinocytes do, in fact, derive from the same stem cells (55).

The Ki-67 antigen is a nuclear protein that is preferentially expressed during all active phases of the cell cycle (G1, S, G2, and M-phases), but it is absent in resting cells (G0-phase) (56, 57). During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis, most of the protein is relocated to the surface of the chromosomes. The antigen is rapidly degraded as the cell enters the non-proliferative state (58, 59), and there appears to be no expression of Ki-67 during the DNA repair processes (60, 61).

CK7 belongs to the intermediate filaments that create a cytoskeleton in almost all eukaryotic cells. In contrast to other intermediate filaments, CKs are composed of a highly complex multigene family of polypeptides with molecular masses ranging from 40 to 68 kDa. CKs are generally held to belong to the most fundamental markers of epithelial differentiation and, until now, 20 distinct CK polypeptides have been revealed in various human epithelia (62, 63). The CKs can be divided into acidic type-A (class I) and neutral-basic type-B (class II) subfamilies. CK7, a 54 kDa protein, belongs to the neutral-basic type-B subfamily, and its distribution is confined to glandular and transitional epithelia (62).

Epithelial membrane antigen (EMA) belongs to a heterogeneous population of human milk fat globule (HMFG) proteins. HMFG is a complex secretory product of mammary epithelium, and these proteins are present in a variety of epithelia of both normal and neoplastic types (64). EMA labels epithelial cells in a wide variety of tissues and is a useful tool for the identification of neoplastic epithelia, and especially of breast carcinoma metastases in histological sections of liver, lymph node, and bone marrow. It is also useful for the recognition of spindle cell epithelial malignancies (65, 66).

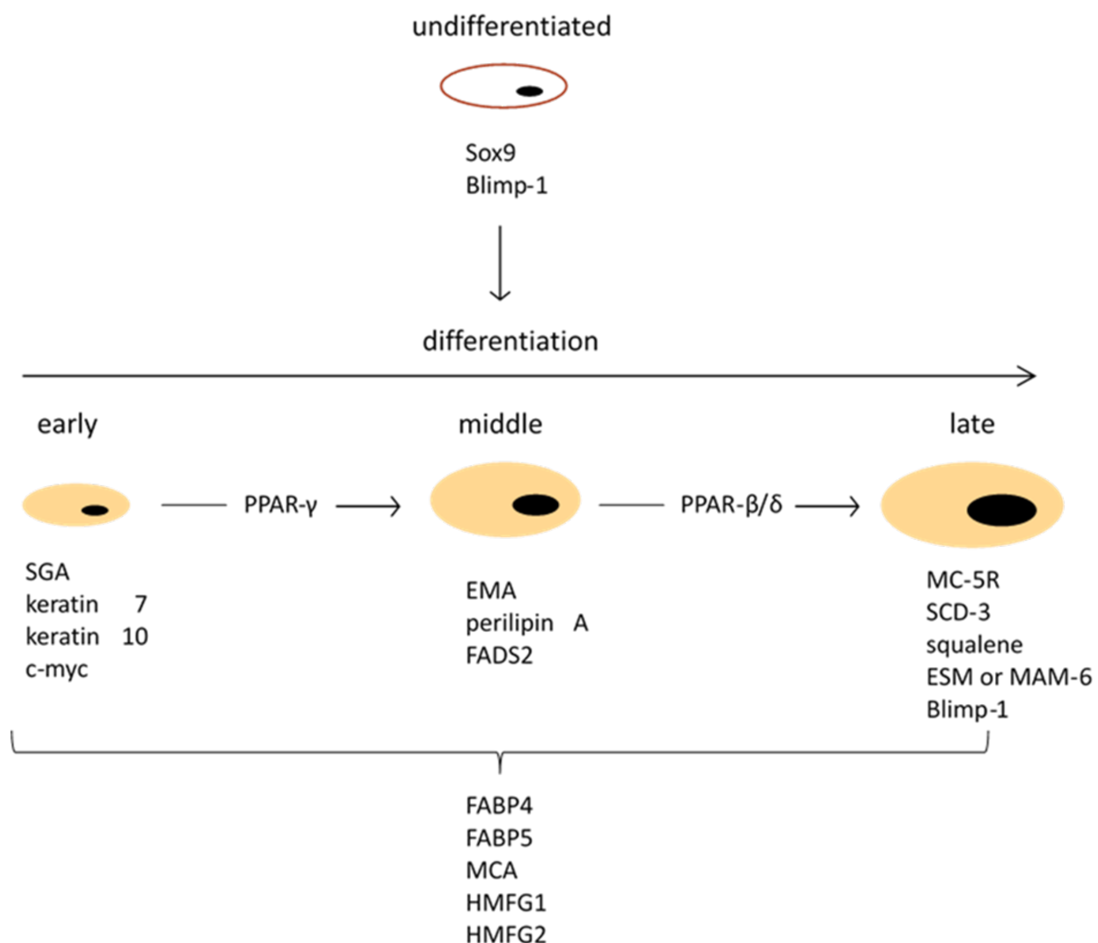

Figure 3. Summary of the main differentiation markers of sebocytes and the stage of their expression. Blimp-1 may be both an early and terminal differentiation marker. There are markers (s. at the bottom of the figure) that may be expressed during the whole differentiation process of sebocytes. PPAR-γ helps the differentiation of early to mature sebocytes and PPAR-β/δ helps the differentiation of mature to late differentiated sebocytes. *For abbreviations please consult the abbreviations list.*

Epithelial sialomucin (ESM), also known as milk fat globule membrane antigen 6 (MAM-6), is a tumor marker found at normal levels in noncancerous tissues and at higher levels in a variety of epithelial carcinomas. Located in the glycocalyx of normal glandular epithelial cells and in malignant glands of endocervical and endometrial tissues, epithelial sialomucin is a mucus glycoprotein that functions as a marker for mammary and other skin appendage/epithelial tumors (67, 68).

Involucrin is a 68 kDa precursor protein of the keratinocyte cornified envelope that is formed beneath the inner surface of the cell membrane during terminal differentiation. Involucrin first appears in the cell cytosol but ultimately becomes cross-linked to membrane proteins by transglutaminase. During keratinocyte terminal differentiation, glutamine residues of involucrin become covalently cross-linked to other envelope precursors via covalent ε-(γ-glutamyl) lysine bonds. Moreover, its large size allows involucrin to cross-link molecules that are separated by substantial distances in the cornified envelope. These properties allow a single involucrin molecule to form multiple cross-links in multiple spatial planes with other envelope precursors (69, 70).

Stereology

Stereology is an interdisciplinary field that provides quantitative information about three-dimensional structures from measurements made on two-dimensional planar sections of materials or tissues. It is based on fundamental principles of geometry (e.g., Cavalieri's principle) and statistics and utilizes random, systematic sampling to provide unbiased and quantitative data. It is a developing and promising tool, applied already in the biosciences, histology, and neuroanatomy fields (71).

To conduct the three-dimensional interpretation of a few planar sections of material, the sections must be typical or representative of the entire material. There are two ways to ensure this. One is to assume that any planar section is typical (e.g., assume that the material is completely homogeneous); this is called model-based stereology and is currently obsolete because of the incorrect assumptions entailed. The other is to select planar sections at random according to a specified random sampling protocol; this is called design-based stereology (71-73).

Design-based stereology was renewed during the 1980s through the work of Gundersen et al., which made it possible to obtain reliable three-dimensional data without making incorrect assumptions about the structures under investigation (74-76). Design-based stereology can be applied to materials that are inhomogeneous, and it has been increasingly applied in the biomedical sciences (77, 78).

Recent years have also seen the application of stereology in the field of dermatology, primarily including investigations of skin structure, skin tumors, and skin wound healing and scarring. Lately, the volume of sebaceous glands in patients with hidradenitis suppurativa/ acne inversa has been estimated stereologically, showing a reduction of the volume of the sebaceous glands in this disease (71, 72, 79).

Stereological analysis involves a two-step process. Statistical sampling principles are employed to obtain statistically valid histological sections from an organ; then appropriate geometric probes (e.g., points, lines) are superimposed on sections to determine the interactions of the probes with the structural feature being estimated.

The first requirement for obtaining a representative random sample from an organ is that the entire organ must be available for sampling. Stereologists generally sample an organ using systematic uniform random sampling (SURS). With SURS, the organ is sliced at regular uniform intervals (T) with the first cut positioned randomly within the first interval (0–T) by using a random number table to select the position within 0–T. The latter step constitutes the random element of the sampling. This sampling method ensures all positions in the organ are given equal probability of being sampled and is essentially always more efficient than simple random sampling (80, Figure 4).

By analogy to opinion polling, the ‘‘questioning’’ in stereology- asking how much of a geometric feature (volume, surface area, length, or number of objects) is present in a tissue structure is represented in the section- is done by using geometric probes. Each geometric structural feature has an associated geometric probe that must be used to accurately estimate the quantity of geometric feature in the 3D tissue space (Table 1).

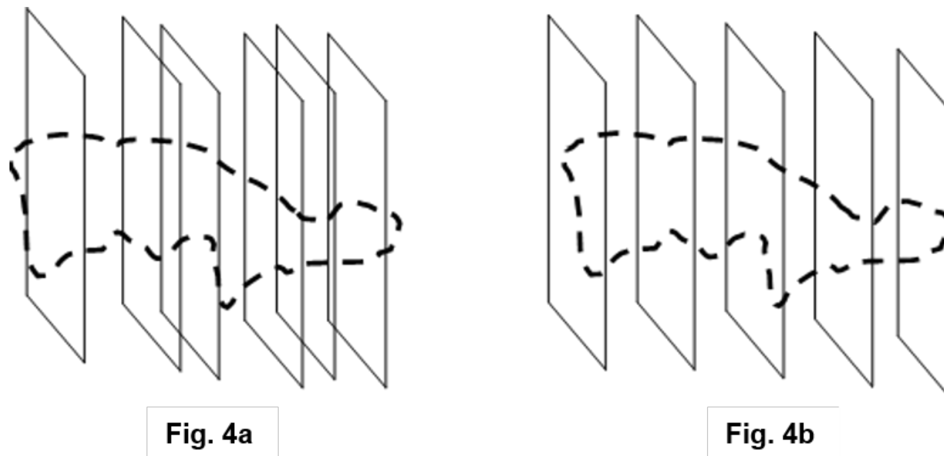

Figure 4. Simple uniform random versus systematic uniform sampling of a structure (modified from Boyce et al., Toxicol Pathol, 2010).

A scientific strength of design-based stereology is that, if performed properly, it guarantees accurate (unbiased) and precise quantitative estimates of tissue structure in an efficient manner. Here it is crucial to distinguish between accuracy and precision. A method can be precise in a statistical sense by having a small standard deviation but still be biased, just as an imprecise method can be unbiased (Figure 5).

Feature	Probe
Number	Volume
Length	Planes
Surface	Cycloids
Volume	Points

Table 1. The probes that are used for the stereological estimation of certain morphological features (modified from Kamp et al., Exp Dermatol, 2009).

The precision of a method can be improved by increasing the sample size, e.g., taking more biopsies, including more sections, or counting more fields of vision, whereas accuracy can only be obtained through uniform random sampling and the use of unbiased estimators. The processing of the specimens and the cutting are equally crucial. Processing-induced shrinkage is particularly problematic for paraffin-embedding, which can cause up to a 40%-50% reduction in tissue volume (81-83). Plastic-embedded sections must be cut essentially dry, and paraffin sections must be prepared at a constant block temperature (easiest to achieve by cutting at room

temperature) to avoid expansion or contraction of the block face, which can cause variation in section thickness. Shrinkage is minimized if plastic, frozen, or vibratome sections are used and carefully processed (84).

As mentioned, total structural quantity in an organ or object can be estimated in a two-step process: (1) determining mean density (volume, surface, length, or number per unit volume), and (2) multiplying density by the reference volume, that is, the volume of the organ. There is a simple and robust estimator, known as Cavalieri's Principle, that allows for estimation of volume, regardless of how irregularly shaped the object is (85, 80). Cavalieri stated that the total volume of an arbitrarily shaped object can be estimated in an unbiased manner from the product of the distance between the systematic random planes (sections) through the object and the sum of the areas of the transects of the object within these planes (Figure 6a).

The areas of the transects can easily be determined by point counting so that each point intersecting the reference plane (Pi) is associated with a given area (Ap), and the estimation of the area of the transect (At) in each section is thus

$$A_t = \sum_{i=1}^{n} P_i \cdot A_{\mathrm{p}}$$

The total volume of the object can thus be expressed as

$$V_{\mathrm{object}} = \sum_{t=1}^{n} A_t \cdot t$$

where t represents the distance between sections.

The Cavalieri principle provides the investigator with a tool to precisely estimate the entire volume of the specimen microscopically or macroscopically. This ability is essential to many stereological designs (71).

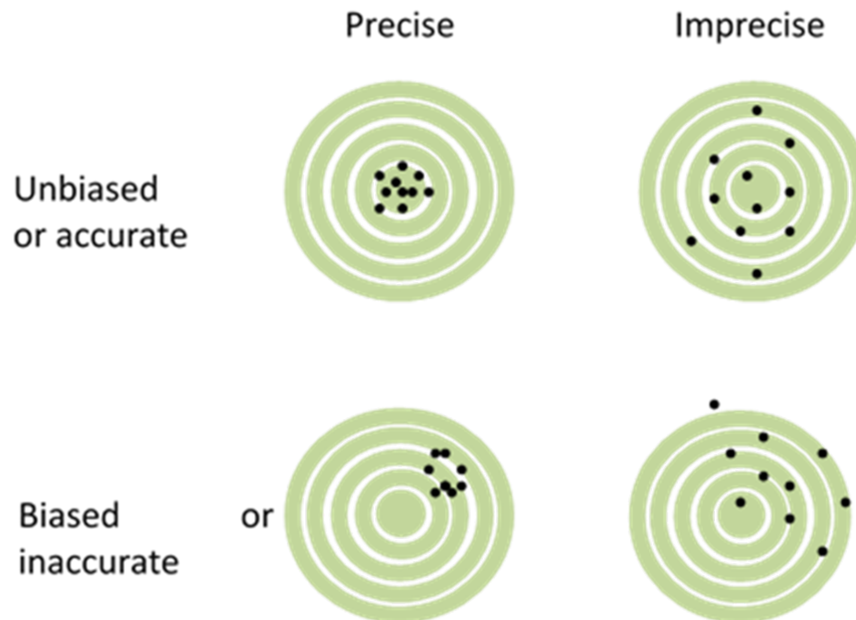

Figure 5. Illustration of the statistical terms of precision, biasedness and accuracy (modified from Boyce et al, Toxicol Pathol, 2010).

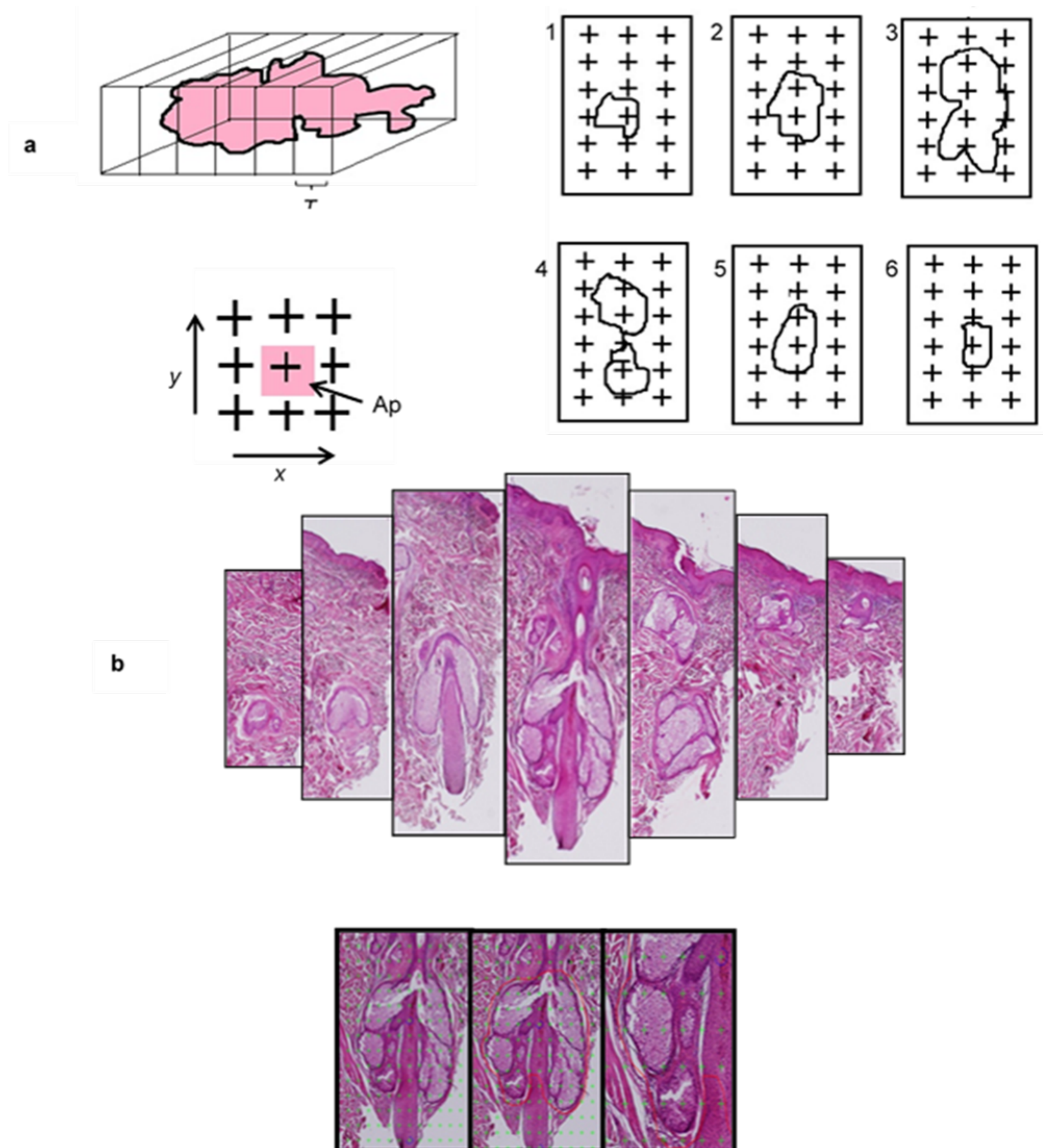

Figure 6a. Illustration of the Cavalieri method. An object is sectioned exhaustively and a series of parallel planes with a T thickness is produced. A number of points are randomly superimposed by the software, and the number of points hitting the profile is counted. Each point grid has been shifted randomly in the x and y direction. **6b:** Application of the method ex vivo. A sebaceous gland is sectioned exhaustively and the number of grids hitting each profile is recorded.

AIMS OF THE THESIS

The main aim of this study was to investigate the characteristics of sebaceous glands in patients with psoriasis and psoriatic alopecia capillitii, and compare those characteristics between psoriatic lesional and non-lesional skin.

More analytically, the aims were the following:

- to detect changes of the sebaceous glands in psoriatic lesional compared to non-lesional skin;
- to reveal the morphology of sebaceous glands in psoriatic lesional skin and compare it with psoriatic non-lesional skin;
- to measure the number, size and volume of sebaceous glands in psoriatic lesional skin and compare them with psoriatic non-lesional skin
- to examine the differentiation characteristics of human sebocytes in psoriatic lesional skin and compare them with psoriatic non-lesional skin;
- to elucidate the role of sebaceous glands in the pathogenetic sequence of psoriasis and of psoriatic alopecia capillitii.

To my knowledge, this study undertook the first quantitative analysis of the volume of the sebaceous glands and provided the first evidence of absence and atrophy of the sebaceous gland in psoriasis, which has been until now only a clinical observation. Such a quantitative analysis became possible with the revolution of the field of stereology, which currently provides the capability to estimate three-dimensional (3D) structures and to perform with a high degree of accuracy surface, volume, and area measurements.

MATERIALS AND METHODS

Patients

I recruited 28 patients with the obvious clinical diagnosis of psoriasis vulgaris and capitis from the Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, Germany. The half of the patients were included in the histological/stereological study and the other half in the immunohistochemical study. The demographic and clinical data of the patients are shown in Table 2. Each patient signed a consent form prior to inclusion. The study was approved by the Ethics Committee of the Charité-Universitaetsmedizin Berlin der Freien Universitaet Berlin.

Biopsies

I performed 5 mm punch biopsies from the head area of 7 of the psoriasis patients and from the back area of the other 7 psoriasis patients. The biopsy localizations are also summarized in Table 2. All 14 biopsies were performed under local anesthesia with a 0.5% solution of prilocaine (Xylonest® 0,5%). The head and back areas were chosen as the most seborrheic areas of the human body with a high frequency of sebaceous glands. From each patient 2 biopsies were taken, one from lesional and one from the neighboring non-lesional skin. The biopsies were taken perpendicular to the skin surface and, thus, parallel to the vertical axis chosen by the investigator.

The procedure of removing the biopsy from the patient and placing it between polyester pads was considered a random rotation around the vertical axis. Subsequently, the biopsies were fixed for 24 h in 4% formalin solution and embedded in paraffin. All paraffin blocks were sectioned exhaustively, uncooled and perpendicular to the surface of the epidermis with a thickness of 10 microns using a Leica rotary microtome RM 2035, serial number 08860729. The microtome must be calibrated prior to the study to ensure the exact thickness of the sections. The sections were cut uncooled because temperature changes may affect the volume of the tissue and consequently influence the thickness of the sections. All sections were collected on SuperFrost® color Vogel glass slides and stained with hematoxylin-eosin (duration of staining 6 minutes instead of the usual 4 because of the thickness of the sections).

a.

Stereology

Histological examination

I conducted a crucial qualitative histological study of my biopsies to describe the number, shape, and size of the sebaceous glands, as well as the differences between lesional and non-lesional skin. The presence or absence of the sebaceous glands, their distribution, and their morphology were studied and described for all biopsies.

Limitations

This first rough analysis is certainly very individual, and it is only a first qualitative observation about the differences between lesional and non-lesional skin. However, it is still quite clear, even with a first observation, that there are remarkable differences in the number and size of sebaceous glands between lesional and non-lesional psoriasis skin. To demonstrate this statistically, a quantitative analysis was performed.

Stereological volume estimation

I estimated the volume of the sebaceous glands by superimposing a software-generated grid of points over the HE-stained sections of skin and counting all points falling within the structure of interest, i.e., sebaceous gland tissue. Each point was associated with a known and predetermined area a(p), and the area of the structure of interest on each section can be estimated by multiplying a(p) by the number of points counted. By analyzing parallel sections throughout the entire biopsy with known and fixed systematic intervals, the total volume of the analyzed structure can be expressed simply as the sum of the transect areas multiplied by the distance between sections.

This is also referred to as the Cavalieri principle and can be expressed as

$$V_{\text{object}} = \sum_{t=1}^{n} A_t \cdot t$$

where At is the area of each transect equal to a(p) x ΣP, t is the average distance between the sections and k is the inverse fraction of sections analyzed (**Figure 6b**).

Patient No.	Gender	Age	Localization	PASI score
p1	M	35	back	11.4
p2	M	49	back	15.8
p3	F	64	back	17.6
p4	F	70	back	17.0
p5	M	36	back	9.0
p6	F	51	back	15.8
p7	M	61	back	14.0
p8	F	55	head	16.0
p9	M	45	head	18.8
p10	F	52	head	8.2
p11	M	33	head	19.6
p12	F	75	head	21.0
p13	M	59	head	20.2
p14	F	59	head	7.2

b. **Immunohistochemistry**

Patient No.	Gender	Age	Localization	PASI score
p1	M	49	head	15.8
p2	M	60	head	11.8
p3	F	67	head	24.3
p4	M	63	head	12.2
p5	F	39	head	7.0
p6	F	64	Head	6.0
p7	F	69	Head	4.2
p8	F	65	Back	15.8
p9	F	55	Back	24
p10	M	73	Back	20.2
p11	M	54	Back	30.6
p12	F	30	Back	12.4
p13	M	45	Back	21.8
p14	F	75	Back	21.0

Table 2. Summary of the clinical data of the patients, who were recruited for the stereological and immunohistochemical analysis.

For stereological analysis, sections were analyzed using a PC with Visiopharm NewCast software v. 4.4.5.0 connected to an Olympus BX51 light microscope with a motorized prior stage with spacing for 8 slides through an Olympus DP 70 digital camera and Olympus Objectives (4x U Plan Apo NA=0.18 and 10x U Plan Apo NA=0.40) (Figure 7).

Figure 7. Illustration of the Visiopharm NewCast software v. 4.4.5.0 connected to Olympus BX51 light microscope with a motorised Prior stage with spacing for 8 slides.

Limitations

The tissue shrinkage during formalin fixation and tissue expansion during paraffin infusion certainly result in an overall shrinkage of the studied tissue (4, 82, 86, 87) that is difficult to precisely quantify. Studies have shown that the majority of the shrinkage occurs immediately after the biopsy procedure and is independent of subsequent tissue preparation. It occurs because of the contraction of elastic fibers in the skin and collapse of blood and lymph vessels immediately after removal from the skin. The magnitude of the shrinkage is estimated to be 21% in length and 12% in width in excision biopsies immediately after excision (88). Consequently, the absolute stereological volume and other estimations are probably underestimated compared to in vivo volumes. This problem is minimized with the standardized tissue sampling and processing, which allows estimates about the relative volume changes.

This method of stereological volume estimation is designed to provide precise and unbiased estimates (80, 89). However, coefficient error (CE) does occur, which can be calculated and minimized by increasing the sample intensity (90) (i.e., more biopsies, more sections per biopsy, and more points counted). In my study, with an approximate count of 100–200 events on about 7–9 sections from each punch biopsy, the CE is

roughly 5%. This is a compromise to achieve a balance between sampling intensity and a reasonable study design that can be used for regularly shaped objects, like cylindrical tissue specimens.

Stereological estimation of the epidermis thickness

The biopsy and tissue processing procedure was performed as described above. Epidermal thickness was estimated by first superimposing a software-generated grid of sine-weighted lines over the sections (Figure 8) such that the lines crossed the epidermis 5 times in each sample. Then the 5 points where the lines crossed the basal layer of the epidermis were chosen and the shortest distance between the basal and corneal layers of the epidermis was estimated. The thickness of the epidermis was evaluated as the average of the 5 results. In that way, I ensured that the points for measuring the thickness were chosen randomly by the software and not by the investigator.

Limitations

There are many variations in the thickness of the epidermis of psoriatic skin, especially in comparison with healthy skin. Therefore, the 5 thickness measurements of a sample sometimes varied largely; this resulted in a systemic error confronting the standardized measurement method used in all samples. Furthermore, the investigator took care to preserve the vertical axis throughout the process, which is not difficult in skin specimens in which the layered tissue makes identification of the vertical axis easy. This consistent orientation is important because epidermis thickness estimation requires line probes with isotropic orientations in 3D space. Tissue shrinkage also constitutes a limitation, as explained above.

Immunohistochemistry

A second group of 14 patients with clinical diagnoses of psoriasis vulgaris and capitis from the Departments of Dermatology, Venereology, Allergology and Immunology, Dessau Medical Center, Dessau, Germany, was recruited for the immunohistochemical study. I performed 5 mm punch biopsies from the head area of 7 psoriasis patients and from the back area of the other 7 psoriasis patients; I took 2 biopsies from each patient,

one from lesional and one from the neighboring non-lesional skin. All 14 biopsies were performed under local anesthesia with a 0.5% solution of prilocaine (Xylonest® 0,5%).

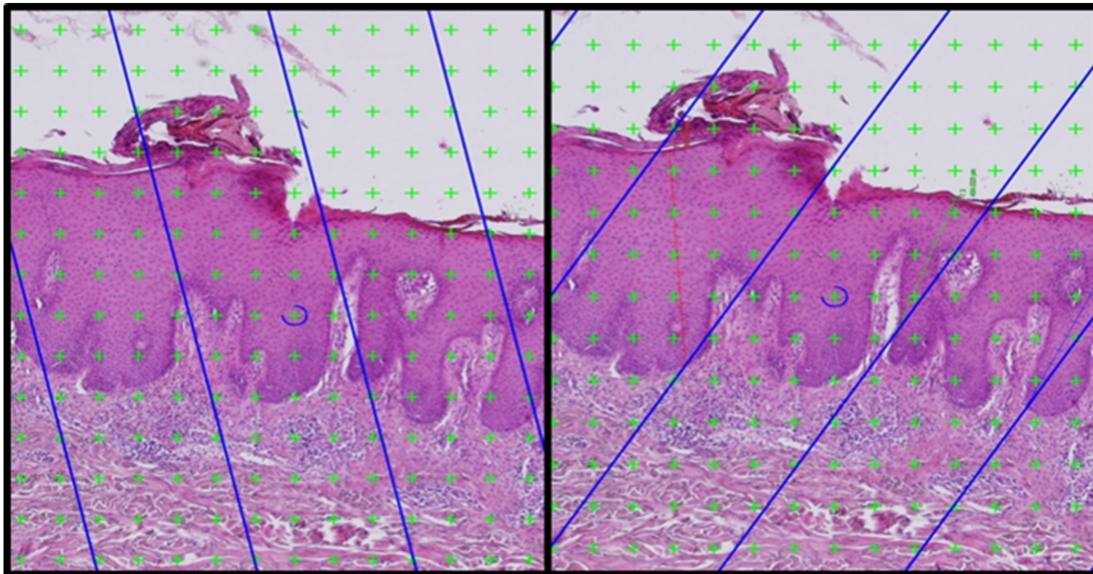

Figure 3. A software-generated grid of sine weighted lines is superimposed over the sections, so that the lines cross the epidermis 5 times in each sample. The shortest distance between basal and corneal layer of the epidermis is estimated and the thickness of the epidermis is calculated by summarizing the 5 results and dividing by 5.

The biopsies were fixed for 24 h in 4% formalin solution and embedded in paraffin. All sections were cut randomly in thicknesses of 4 microns using the Leica rotary microtome RM 2035, serial number 08860729, and collected onto EnVision FLEX microscope slides, K8020 21, DAKO. During the cutting procedure, the investigator took care to collect from all biopsies sections that included at least one sebaceous gland and- if possible- the largest one.

In my study, I chose to use particular biomarkers to show immunohistochemically each differentiation stage of sebocytes ex vivo. As a proliferation marker (the very early stage of sebocytes, before differentiation begins) I selected the common proliferation marker Ki67 (56, 57). As an early differentiation marker, I selected keratin 7 (58); as a medium differentiation marker the epithelial membrane antigen (EMA) (58); and as a late differentiation marker the epithelial sialomucin (ESM) (46, 53). As a possible marker of the keratinizating tendency of sebocytes, I selected involucrin, which is one of the most

typical markers of keratinocytes and which has also been shown to be sometimes expressed by sebocytes (59, 60).

For immunohistochemistry, I used the following antibodies: monoclonal mouse anti-human Ki-67 antigen IgG1 (Clone MIB-1, Code M7240, DAKO) in a dilution of 1:400 and a pretreatment with pH 6.1; monoclonal mouse anti-human cytokeratin 7 IgG1 (Clone OV-TL 12/30, Code M7018, DAKO) in a dilution of 1:1000 and pH 9; monoclonal mouse anti-human epithelial membrane antigen IgG2a (EMA, Clone E2, Code-Nr. M 0613, DAKO) in a dilution of 1:2000 and pH 6.1; monoclonal mouse antibody epithelial sialomucin IgG1 (ESM or MAM-6 140 C1, Code Nr. sc-52329, Santa Cruz Biotechnology Inc.) in a dilution of 1:1000 and without pretreatment; monoclonal mouse antibody involucrin IgG1 (SY5, Code Nr. sc-21748, Santa Cruz Biotechnology Inc.) in a dilution of 1:100 and pH 6.1. As negative control, I used the mouse antibody negative control IgG1 (X0931, DAKO) in a dilution 1:100 and without pretreatment. For all experiments (pretests and main tests), I used negative controls that were always negative. All antibodies were diluted in the antibody diluent with background-reducing components S3022 83 from DAKO. As target solution, I used the target retrieval solution from DAKO (10x, S1699 84, DAKO). The staining was conducted with a DAKO autostainer device (Table 3)

All antibodies were first tested and titrated on paraffin sections with sebaceous glands, which were chosen randomly from paraffin sections of other patients. Ki67 was titrated in 1:160 (standard concentration), 1:200 and 1:400, always in pH 6.1 (pH used regularly for Ki67), and I found that the titration was still working very well in the higher concentrations. CK7 was titrated in 1:100, 1:200, 1:1000, 1:2000, and 1:5000, always in pH 9 (pH used regularly for CK7). EMA was titrated in 1:500, 1:1000, 1:2000, 1:5000, and 1:10000, always in pH 6.1 (pH suggested by the firm). ESM was titrated in 1:100, 1:500, 1:1000, 1:2000, and 1:5000, always without pretreatment (as suggested by the firm). Involucrin was titrated in 1:50 and 1:100 and was tested in pH 6.1 and pH 9, and with an enzymatic pretreatment (proteinase K) because of the slightly weak expression of the antigen in sebaceous glands. Regarding this point, it is useful to remember that I used the antibody Ki 67 as a proliferative marker, cytokeratin 7 as an early differentiation marker, EMA as a middle differentiation marker, and ESM as a late

differentiation marker. The antibody involucrin was used a marker of a differentiation of sebocytes towards keratinocytes.

Limitations

I have selected four antibodies as representative markers of each differentiation stadium of sebocytes. This processing has two important limitations. First, data in the literature are controversial about some differentiation markers; e.g., the antibody BLIMP-1 has been shown to be expressed by both early and terminal differentiated sebocytes, so it is specific to certain differentiation stages.

Antibody	Dilution	Pre-treatment
Ki67	1:400	pH 6.1
CK7	1:1000	pH 9
EMA	1:2000	pH 6.1
ESM	1:1000	none
INV	1:100	pH 6.1
Neg. Control	1:100	none

** CK7= Cytokeratin 7, EMA= Epithelial Membrane Antigen, ESM= Epithelial Sialomucin, INV= Involucrin*

Table 2. The antibodies that were used for the immunohistochemistry and their dilution and pre-treatment conditions, after being several times titrated and tested in all possible conditions.

Second, the differentiation stages of sebocytes are not discernible; i.e., an antibody that may be expressed at the end of the early differentiation stadium may also, of course, be expressed at the beginning of the middle differentiation stadium, etc. There are antibodies like EMA that may be expressed during the whole differentiation processing of sebocytes and cannot be discriminated; it is, however, not expressed in the very early and very late stages. Some authors have only chosen to discriminate the sebocyte differentiation between early and late/terminal differentiation, which is certainly more

reliable (59). I have chosen three antibodies as differentiation markers (CK7, EMA, and ESM) and a very well-known proliferation marker, Ki67, to detect the phase before differentiation begins because the literature suggests that these are representative markers. Additionally, I tested them before staining my biopsies, and I was able to demonstrate that each marker is, indeed, representative of each differentiation stage. Other markers that can be used include melanocortin 5 receptor for terminal differentiation and CK10 for early differentiation (s. introduction, differentiation of sebaceous glands).

Another consideration is the fixation in paraffin; a possible improvement of the method may be staining the specimens in frozen sections. Finally, as I cut the biopsies, I chose sections with the sebaceous gland approximately in the middle of its differentiating procedure, and I repeated the staining on 5 sections per sample; however, I cannot be sure that the result would not have been different if the glands had been cut for another differentiation point. Perhaps a solution would be to cut the gland exhaustively and stain all the sections immunohistochemically, but that would increase the costs of the study enormously.

Psoriasis Area and Severity Index (PASI)

The Psoriasis Area and Severity Index (PASI) is a widely used tool for measuring the severity of psoriasis. It combines the assessment of the severity of lesions and the area affected into a single score ranging from 0 (no disease) to 72 (maximal disease). The human body is divided into four sections: head (H) (10% of all skin), upper extremities (U) (20%), trunk (T) (30%), and lower extremities (L) (40%). For each body section, the percent area of skin involved is graded from 0 to 6, where grade 0 is 0% of affected skin, grade 1 is <10%, grade 2 10-29%, grade 3 30-49%, grade 4 50-69%, grade 5 70-89% and grade 6 90-100% of affected skin. The severity of involved skin is estimated by three clinical signs- erythema, induration, and desquamation- and these severity parameters are measured on a scale of 0 to 4 (0: none, 1: mild, 2: moderate, 3: severe, and 4: very severe). The sum of all three severity parameters is calculated for each section of skin, then multiplied by the area score for that area and multiplied by the weight of the respective area (0.1 for head, 0.2 for arms, 0.3 for body, and 0.4 for legs (Appendix I) (91). Thus, the PASI score is defined as

$$PASI = 0.1 \cdot (E + I + D) \cdot A + 0.2 \cdot (E + I + D) \cdot A + 0.3 \cdot (E + I + D) \cdot A + 0.4 \cdot (E + I + D) \cdot A$$

where E is the degree of erythema, I induration, D desquamation, A the area involved in each of the four areas, and the subscript designations represent H head, U upper extremities, T trunk, and L lower extremities.

Limitations

It is clear that higher PASI scores indicate more severe psoriasis; however, it is not always easy for the practitioner to describe the clinical severity that corresponds to specific PASI scores. Also, some authors suggest that PASI is flawed because patients rarely score higher than half the maximum score (92). The inter-rater reliability of the PASI index has been estimated to be 83–97%, and the intra-rater reliability to be 86–100% (93). Attempts have been made to provide other tools for evaluating psoriasis severity, such as the simplified PASI (SPASI) and the Psoriasis Global Assessment (PGA).

Statistics

Descriptive statistics are presented as means ± SD, medians with interquartile ranges (IQR), or percentages, as appropriate. Continuous variables were compared by using nonparametric tests: the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the matched pairs Wilcoxon signed-rank test. Correlations were assessed using the nonparametric Spearman's rank correlation coefficient and the respective p-value. It is generally considered that Spearman's rho of <0.20 indicates very weak correlation, 0.21 to 0.40 weak correlation, 0.41 to 0.60 moderate correlation, 0.61 to 0.80 strong correlation, and >0.81 indicates very strong correlation. For hypothesis testing, I considered a probability level of <0.05 to indicate statistical significance. All statistical tests were two-sided. Stata software was used for the statistical analyses (Stata Corp., College Station, TX, USA).

RESULTS

Histology-Stereology

Histology

Conducting estimates of the volume of the sebaceous glands, I found that 3 of the 14 patients had no visible gland tissue in the lesional skin, although there were definitely sebaceous glands in biopsies of their non-lesional skin. From the biopsies of the 11 patients, who had visible gland tissue in the lesional skin, 5 were from the back and 6 from the head. Figure 9 demonstrates the obvious absence or shrinkage of sebaceous glands in the lesional skin.

Stereological volume estimation

The volume of the sebaceous gland tissue is shown in Table 4 and Table 5, as well as in Figure 10 , and the mean volume in lesional skin was on average approximately one-fifth (20%) of that of healthy controls (0.018 mm3 vs. 0.057 mm3; p=0.001). When only considering the biopsies in which both lesional and non-lesional skin contained sebaceous gland tissue (Figure 11), the volume of sebaceous gland tissue was approximately one fourth (25%) of that of healthy controls (0.023 mm3 vs. 0.062 mm3; p=0.003). Figure 12 shows schematically the differences in sebaceous gland volume between lesional and non-lesional skin, in a point diagram. Table 4 summarizes also the differences in and the statistical significance of sebaceous gland volume between male and female patients between back and head.

Stereological estimation of the epidermis thickness

The mean epidermis thickness of the lesional skin was compared to the non-lesional skin (Table 4). There is a statistically significant strong inverse correlation between epidermis thickness and sebaceous gland volume as shown in Table 4D. The results suggest that there is a positive correlation between the activity of the disease and the atrophy of the sebaceous glands (the more active the disease was, the more atrophic sebaceous glands were detected).

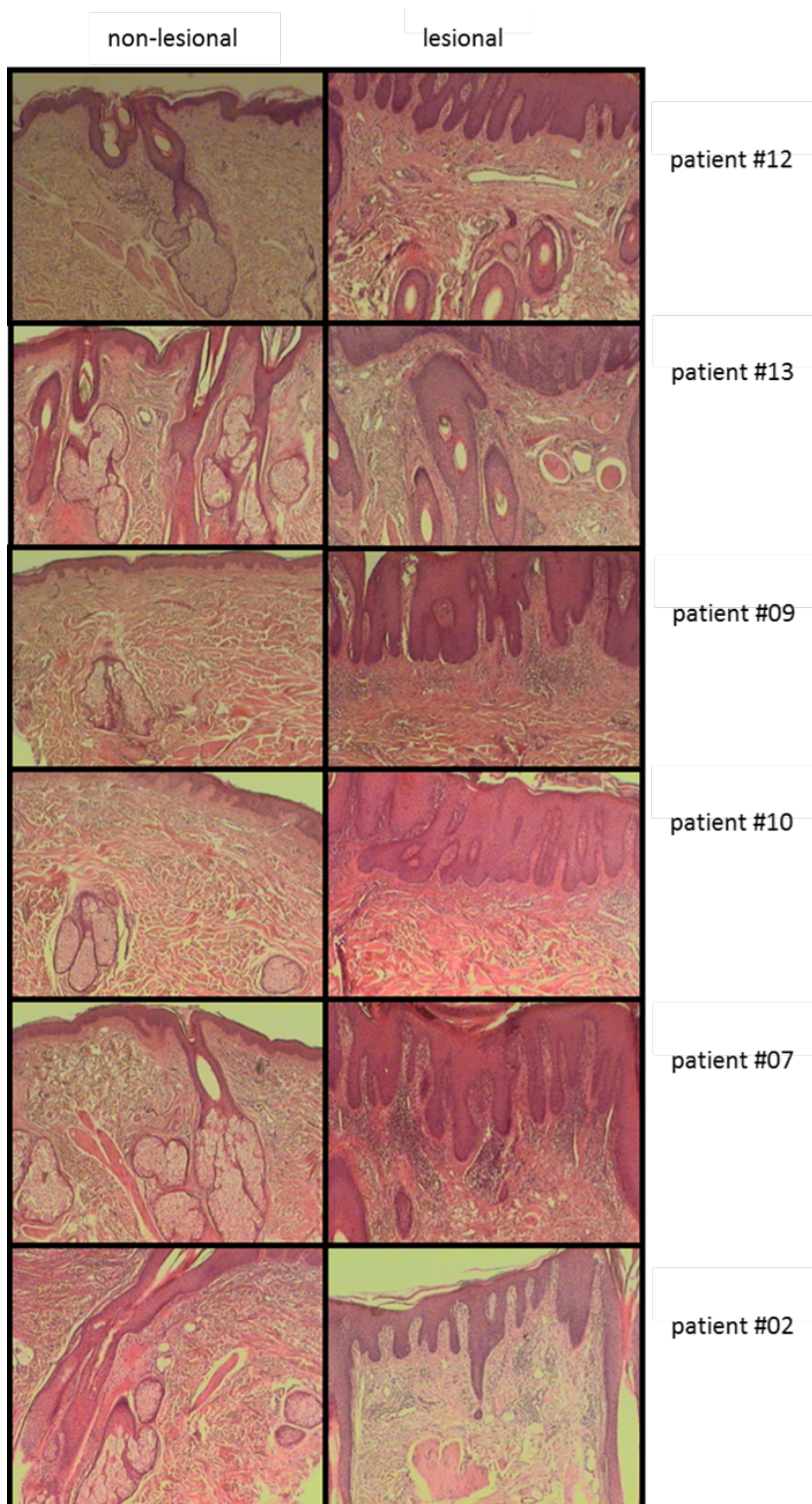

Figure 9. Examples of the absence and shrinkage of sebaceous gland tissue in the lesional compared to the non-lesional psoriatic skin.

		psoriasis													
		back							head						
		p1	**p2**	**p3**	**p4**	**p5**	**p6**	**p7**	**p8**	**p9**	**p10**	**p11**	**p12**	**p13**	**p14**
age		35	49	64	70	36	51	61	55	45	52	33	75	59	59
sex		m	m	f	f	m	f	m	f	m	f	m	f	m	f
	SG1 (mm^3)	0.001388	0.026196	0	0	0.028838	0.004903	0.001052	0.000985	0	0.089569	0.08661	0.004523	0.001478	0.006046
	SG2	0.03213				0.019211	0.001299					0.08296	0.00403	0.002284	0.004359
	SG3	0.003224					0.004344								
	Average	0.012247	0.026196	0	0	0.024024	0.003515	0.001052	0.000985	0	0.089569	0.084785	0.004276	0.001881	0.005203
epidermis psoriasis		*328.5121*	*335.3894*	*449.5276*	*	*205.8532*	*	*456.9433*	*325.107*	*477.7345*	*201.0271*	*264.831*	*255.1846*	*351.8502*	*340.4221*
		healthy													
		back							head						
		p1	**p2**	**p3**	**p4**	**p5**	**p6**	**p7**	**p8**	**p9**	**p10**	**p11**	**p12**	**p13**	**p14**
age		35	49	64	70	36	51	61	55	45	52	33	75	59	59
sex		m	m	f	f	m	f	m	f	m	f	m	f	m	f
	SG1 (mm^3)	0.029465	0.061302	0.00309	0.033249	0.006941	0.017912	0.046706	0.003291	0.017128	0.167045	0.16872	0.03213	0.076773	0.030719
	SG2		0.018559			0.047104	0.020509	0.017196	0.002239	0.101846	0.086616	0.165346	0.021584	0.047104	0.037078
	SG3		0.358108			0.092381	0.018024			0.101239				0.022216	0.044289
	Average	0.029465	0.14599	0.00309	0.033249	0.048809	0.018815	0.031951	0.002765	0.073405	0.12683	0.167033	0.026857	0.048698	0.037362
SG healthy-psoriasis		0.017218	0.119793	0.00309	0.033249	0.024784	0.0153	0.030898	0.00178	0.073405	0.037262	0.082248	0.02258	0.046817	0.032159
epidermis healthy		*56.4638*	*93.95569*	*63.15852*	*54.88412*	*78.34486*	*50.10517*	*83.54107*	*103.2759*	*61.40725*	*113.5996*	*119.5835*	*89.73941*	*89.65701*	*69.91259*
epi pso-epi healthy		272.0483	241.4337	386.369		127.5084		373.4022	221.8311	416.3273	87.42756	145.2475	165.4452	262.1932	270.5095

* epidermis fold, not able to measure

Table 3. Stereological estimation of the sebaceous gland (SG) volume between pathological psoriatic (psoriasis/ lesional) and healthy psoriatic (healthy/ non-lesional) skin. The maximum number of SG found in a biopsy was three (SG1, SG2, SG3). The non-lesional skin appears to have less SGs. Furthermore, the volume estimates appear to be much higher in the non-lesional compared to lesional skin. The patient Nos. 3, 4 and 9 appear to lack SGs in the lesional skin. A stereological estimation of the epidermis thickness by biopsy follows (epidermis psoriasis versus epidermis healthy). The reader can also observe the difference in SG volume between lesional and non-lesional skin per patient (SG healthy-psoriasis), as well as the difference in the epidermis thickness (epi pso-epi healthy). It is interesting to notice that the larger the epidermis difference between lesional/ non-lesional (i.e. the more severe psoriasis), the smaller the SG volume in the lesional skin.

A. Comparison of sebaceous gland volume between psoriatic and healthy skin

All patients (n=14)	
	Mean ± SD; median (IQR)
Sebaceous gland volume, psoriatic skin (mm^3)	0.018 ± 0.030; 0.004 (0.001–0.024)
Sebaceous gland volume, healthy skin (mm^3)	0.057 ± 0.053; 0.035 (0.027–0.073)

Matched pairs Wilcoxon signed-rank test; ***p = 0.001***

Only 11 of 14 patients (3 patients with no gland found were excluded)	
	Mean ± SD; median (IQR)
Sebaceous gland volume, psoriatic skin (mm^3)	0.023 ± 0.033; 0.005 (0.002–0.026)
Sebaceous gland volume, healthy skin (mm^3)	0.062 ± 0.056; 0.037 (0.027–0.127)

Matched pairs Wilcoxon signed-rank test; ***p = 0.003***

Back, all patients (n=7)	
	Mean ± SD; median (IQR)
Sebaceous gland volume, psoriatic skin (mm^3)	0.010 ± 0.011; 0.004 (0.000–0.024)
Sebaceous gland volume, healthy skin (mm^3)	0.044 ± 0.047; 0.032 (0.019–0.049)

Matched pairs Wilcoxon signed-rank test; ***p = 0.018***

Back, only 5 of 7 patients (2 patients with no gland found were excluded)

	Mean ± SD; median (IQR)
Sebaceous gland volume, psoriatic skin (mm^3)	0.013 ± 0.011; 0.012 (0.004–0.024)
Sebaceous gland volume, healthy skin (mm^3)	0.055 ± 0.052; 0.032 (0.029–0.049)

Matched pairs Wilcoxon signed-rank test; ***p = 0.043***

Head, all patients (n=7)

	Mean ± SD; median (IQR)
Sebaceous gland volume, psoriatic skin (mm^3)	0.027 ± 0.041; 0.004 (0.001–0.085)
Sebaceous gland volume, healthy skin (mm^3)	0.069 ± 0.059; 0.049 (0.027–0.127)

Matched pairs Wilcoxon signed-rank test; ***p = 0.018***

Head, only 6 of 7 patients (1 patient with no gland found was excluded)

	Mean ± SD; median (IQR)
Sebaceous gland volume, psoriatic skin (mm^3)	0.031 ± 0.043; 0.005 (0.002–0.085)
Sebaceous gland volume, healthy skin (mm^3)	0.068 ± 0.064; 0.043 (0.027–0.127)

Matched pairs Wilcoxon signed-rank test; ***p = 0.028***

B. Comparison of sebaceous gland volume between back and head

All skin samples (Psoriatic and Healthy)

	Mean ± SD; median (IQR)
Back (n=14), sebaceous gland volume (mm^3)	0.027 ± 0.037; 0.021 (0.003–0.032)
Head (n=14), sebaceous gland volume (mm^3)	0.048 ± 0.053; 0.032 (0.003–0.085)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test; ***p = 0.36***

Only **psoriatic** skin samples

	Mean ± SD; median (IQR)
Back (n=7), sebaceous gland volume (mm^3)	0.010 ± 0.011; 0.004 (0.000–0.024)
Head (n=7), sebaceous gland volume (mm^3)	0.027 ± 0.041; 0.004 (0.001–0.085)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test; ***p = 0.65***

Only **healthy** skin samples

	Mean ± SD; median (IQR)
Back (n=7), sebaceous gland volume (mm^3)	0.044 ± 0.047; 0.032 (0.019–0.049)
Head (n=7), sebaceous gland volume (mm^3)	0.069 ± 0.059; 0.049 (0.027–0.127)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test; ***p = 0.41***

C. Comparison of sebaceous gland volume between males and females

All skin samples (Psoriatic and Healthy)

	Mean ± SD; median (IQR)
Males (n=14), sebaceous gland volume (mm^3)	0.050 ± 0.052; 0.031 (0.012–0.073)
Females (n=14), sebaceous gland volume (mm^3)	0.025 ± 0.038; 0.005 (0.003–0.033)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test; ***p = 0.15***

Only **psoriatic** skin samples

	Mean ± SD; median (IQR)
Males (n=7), sebaceous gland volume (mm^3)	0.021 ± 0.030; 0.012 (0.001–0.026)
Females (n=7), sebaceous gland volume (mm^3)	0.015 ± 0.033; 0.004 (0.000–0.005)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test; ***p = 0.40***

Only **healthy** skin samples

	Mean ± SD; median (IQR)
Males (n=7), sebaceous gland volume (mm^3)	0.078 ± 0.056; 0.049 (0.032–0.146)
Females (n=7), sebaceous gland volume (mm^3)	0.036 ± 0.042; 0.027 (0.003–0.037)

Two-sample Wilcoxon rank-sum (Mann-Whitney) test; ***p = 0.048***

D. Correlations

Correlation of **"sebaceous gland volume in psoriatic skin"** with **"thickness of psoriatic epidermis"**

Spearman's rho = –0.73; p=0.007 (strong inverse statistically significant correlation)

Correlation of **"sebaceous gland volume in psoriatic skin"** with **"[psoriatic epidermis – healthy epidermis]"**

Spearman's rho = –0.75; p=0.005 (strong inverse statistically significant correlation)

Correlation of **"[sebaceous gland volume in psoriatic skin – sebaceous gland volume in healthy skin]"** with "[psoriatic epidermis – healthy epidermis]"

Spearman's rho = –0.10; p=0.75 (not correlated variables)

E. Characteristics of the study population (n=14).

	Mean ± SD; median (IQR) or n (%)
Age (yrs)	53.1±12.8; 53.5 (45–61)
Gender (males, %)	7 (50.0%)
Sebaceous gland volume, psoriatic skin (mm^3)	0.018±0.030; 0.004 (0.001–0.024)
Sebaceous gland volume, healthy skin (mm^3)	0.057±0.053; 0.035 (0.027–0.073)
Thickness of psoriatic epidermis (mm)	332.7±92.8; 332.0 (260.0–400.7)
Thickness of healthy epidermis (mm)	80.5±22.3; 80.9 (61.4–94.0)

Table 1. Summary of the results of the stereological analysis of sebaceous gland volume and its correlation with the epidermis thickness, i.e. the activity of the disease.

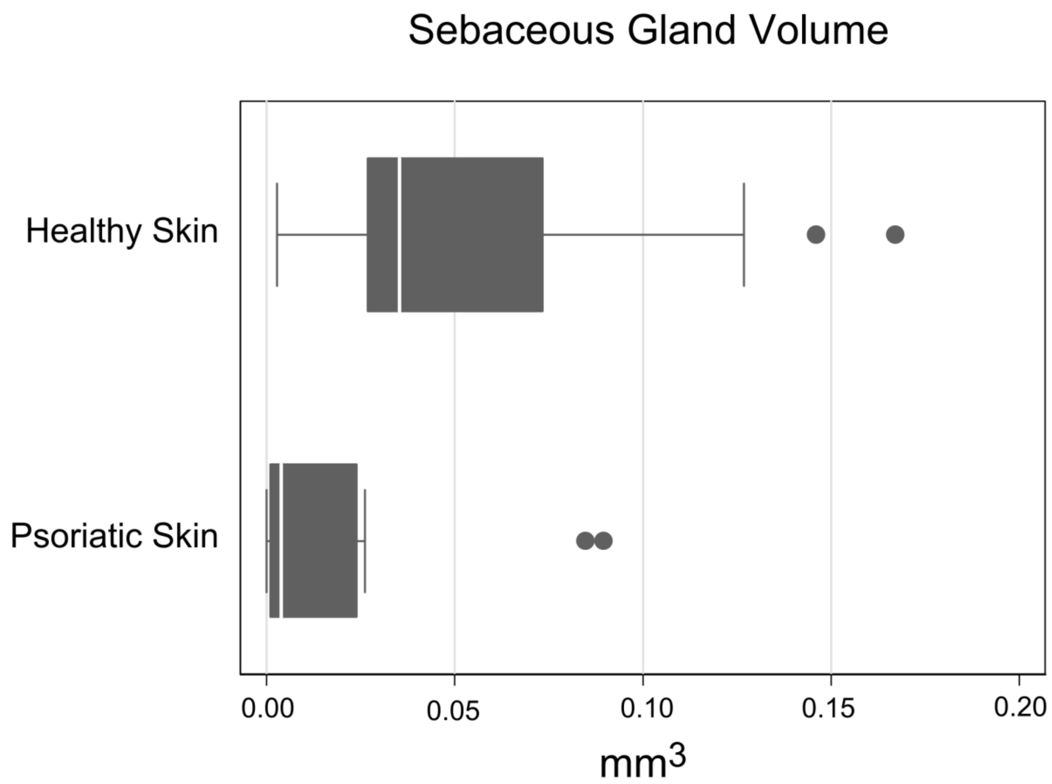

Figure 10. Comparison of sebaceous gland volume between healthy and psoriatic skin (n=14). Results are presented with box-and-whisker plots. Sebaceous gland volume was significantly lower in psoriatic skin (p=0.001).

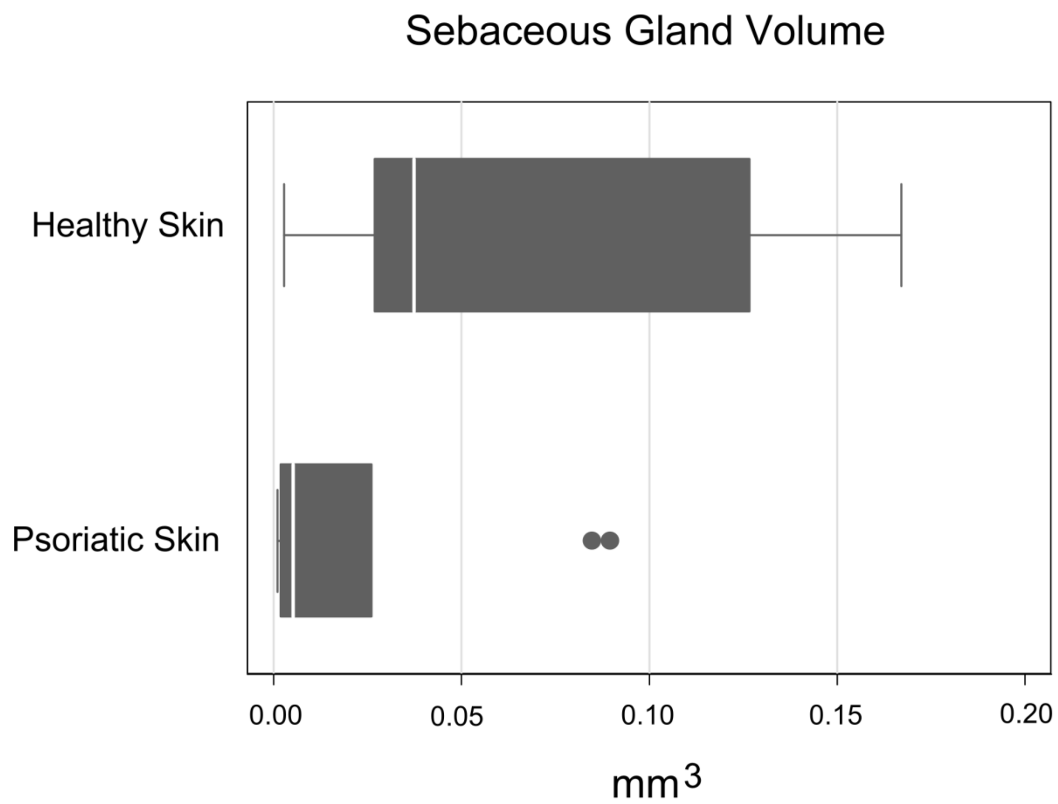

Figure 11. Comparison of sebaceous gland volume between healthy and psoriatic skin (n=11). Results are presented with box-and-whisker plots. Sebaceous gland volume was significantly lower in psoriatic skin (p=0.003).

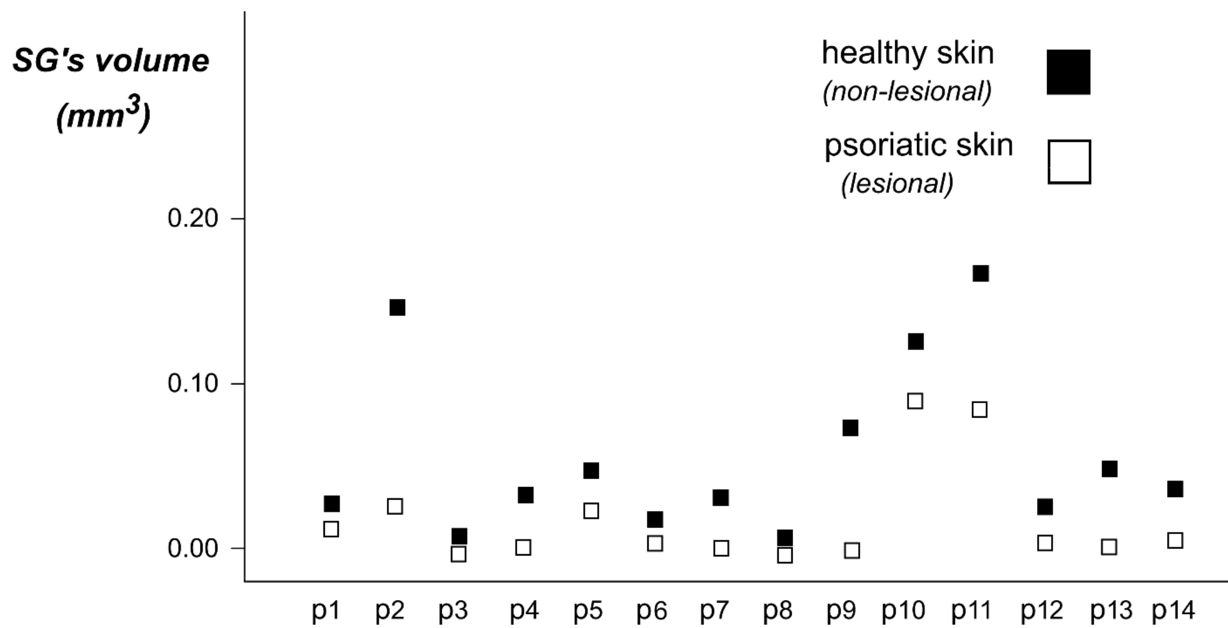

Figure 12. Schematic presentation of the differences of sebaceous gland volume between psoriatic (lesional) and healthy (non-lesional) skin in psoriasis patients. Sebaceous glands are smaller in lesional psoriatic skin in almost all patients.

Immunohistochemistry

Preliminary experiments

After titrating and testing all antibodies, I concluded that CK7, EMA, and ESM are useful as early, middle, and late differentiation markers, respectively, of sebaceous glands ex vivo in a concentration of CK7 1:1000, EMA 1:2000, and ESM 1:1000. This finding is not of minor importance because, to my knowledge, this is the first time this has been tested and shown so clearly (Figure 13). It is also interesting to note that EMA and ESM positively stained the basal layer of the epidermis, whereas CK7 did not (Figure 13). On the other hand, CK7 very intensively stained the sweat glands in the dermis (data not shown). Ki67 is a well-known proliferation marker that is used routinely in a concentration of 1:160 and a pre-treatment of pH 6,1. In this experiment, I tested various concentrations, and discovered that a concentration of 1:400 gave the same results as lower concentrations, and it was, indeed, a very strong marker of proliferation (Figure 13). Involucrin is normally used as a keratinocyte marker. I tried to test it in sebaceous gland tissue ex vivo. The results showed a slight expression of involucrin in the ductus seboglandularis (the point of insertion of the sebaceous gland into the hair shaft) (Figure 13). The epidermis was stained positively (Figure 14).

Main experiment

CK7: Out of the 14 patients, one showed a clear difference between lesional and non-lesional skin with higher expression of CK7 in the lesional skin (Figure 15). Two patients showed a slight difference with CK7 being again more highly expressed in the lesional compared to non-lesional skin. The rest of the patients (11/14) showed no obvious difference between lesional and non-lesional skin (Table 6). The results suggest a possible tendency of sebocytes to be less differentiated in the lesional skin, but this needs to be verified in further experiments.

EMA: Similarly, of the 14 patients, one showed a clearly higher expression of EMA in the non-lesional compared to lesional skin (Figure 15); this was the same patient who showed a clear difference in CK7 expression. Two patients showed a slight difference, with EMA again being higher expressed in the non-lesional than the lesional skin; these were the same two patients who showed a slight difference in CK7 expression in the

two skin areas. The rest of the patients (11/14) showed no obvious difference between lesional and non-lesional skin (Table 6). The results suggest a possible tendency of sebocytes to be less differentiated in the lesional skin, but this needs to be verified in further experiments.

ESM: Out of the 14 patients, one, the same patient mentioned above (s. CK7 and EMA), showed a clear difference between lesional and non-lesional skin, with higher expression of ESM in the non-lesional skin (Figure 15). Two patients, the same 2 patients mentioned above (s. CK7 and EMA), showed a slight difference, with ESM again being more highly expressed in the non-lesional skin. The rest of the patients (11/14) showed no obvious difference between lesional and non-lesional skin (Table 6). The results suggest a possible tendency of sebocytes to be less differentiated in the lesional skin, but this needs to be verified in further experiments.

Ki67: Of the 14 patients, one, the same patient mentioned above (s. CK7, EMA, and ESM), showed a small but clear difference between lesional and non-lesional skin with Ki67 showing a higher expression in the lesional skin (Figure 16). The rest of the patients (13/14) showed no obvious difference between lesional and non-lesional skin (Table 6). The results are controversial, and although it is known that in lesional psoriatic skin there is a high rate of proliferation, this remains to be proved.

INV: No differences were shown between lesional and non-lesional skin regarding the expression of involucrin (Figure 16, Table 6). The theory of less sebum production by sebocytes in the lesional skin because of immature differentiation of sebocytes could be considered, but the theory of keratinization of sebocytes, i.e., differentiation of sebocytes toward keratinocytes, could not be verified through my experiments.

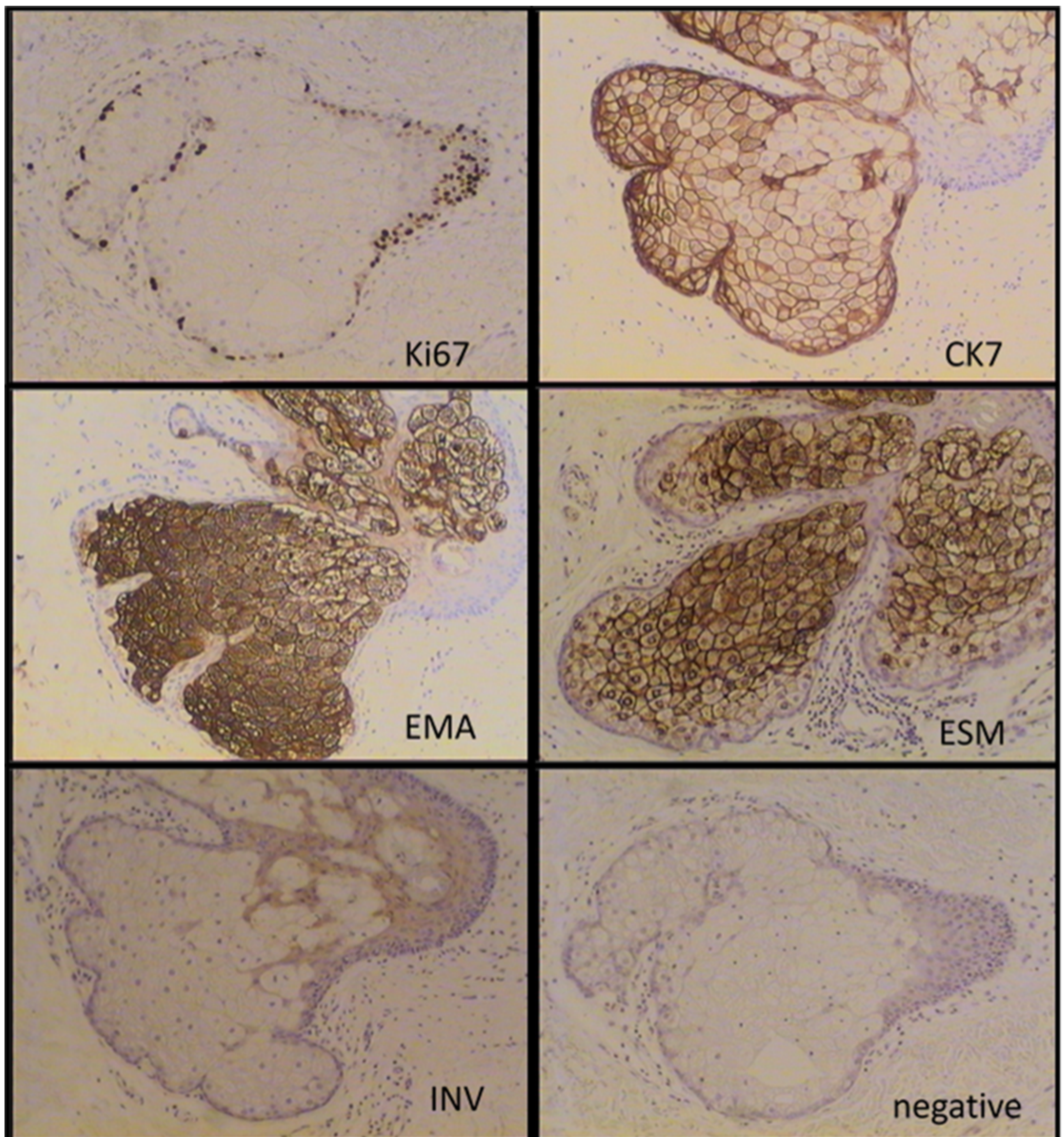

Figure 13. The five antibodies that were tested immunohistochemically prior to the experiment, to show the four main differentiation stages of sebocytes (proliferation with Ki67, early differentiation with CK7, middle differentiation with EMA and late differentiation with ESM), and to show the tendency of sebocytes to keratinization (INV). The last figure shows the negative control. CK7= cytokeratin 7, EMA= epithelial membrane antigen, ESM= epithelial sialomucin, IVN= involucrin

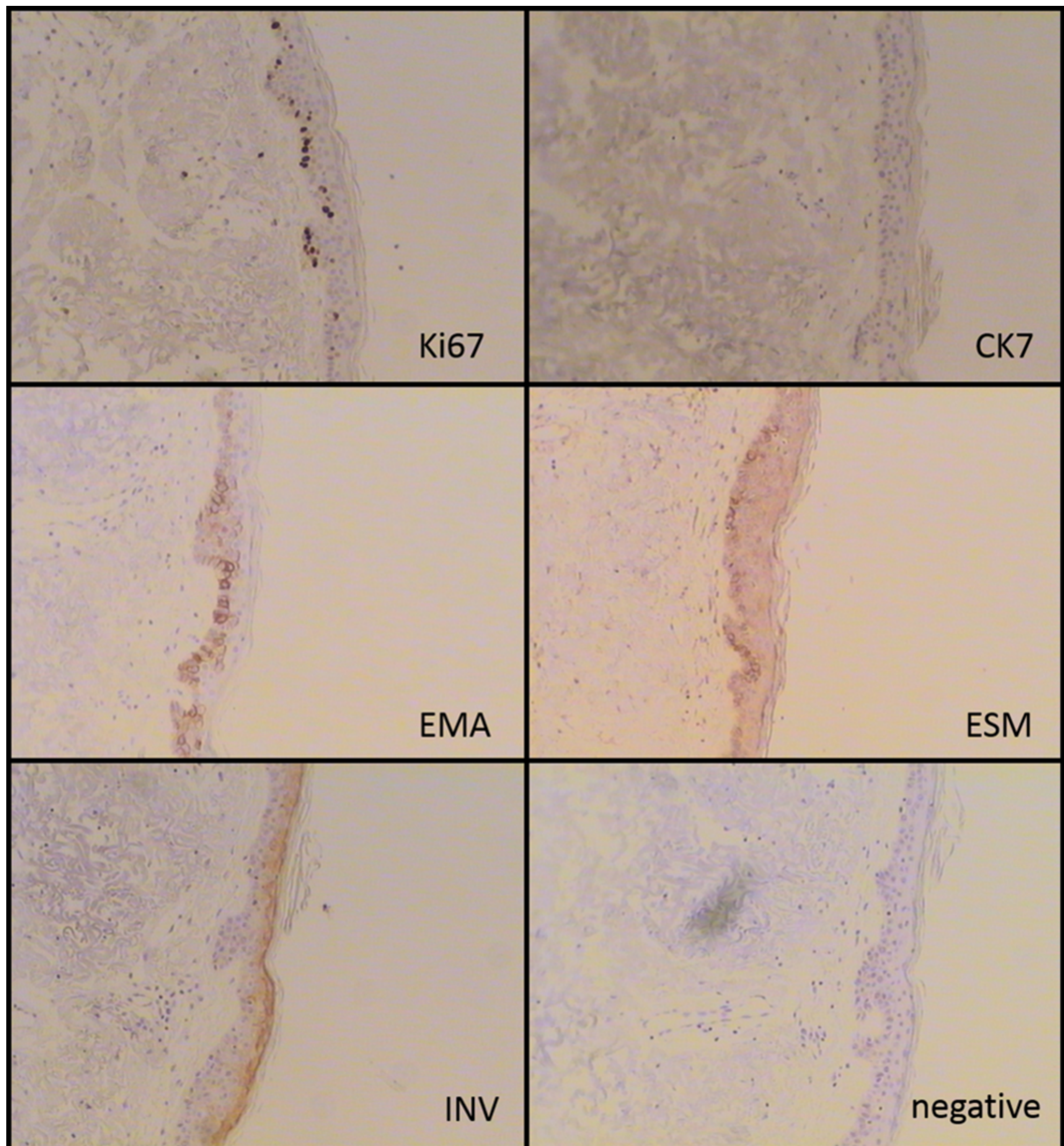

Figure 14. Positive staining of the basal layer of the epidermis with Ki67, EMA and ESM. Positive staining of the upper layer of the epidermis with involucrin, as expected. No staining of the epidermis with CK7. CK7 stained intensively the sweat glands (data not shown). CK7= cytokeratin 7, EMA= epithelial membrane antigen, ESM= epithelial sialomucin, IVN= involucrin, negative= negative control

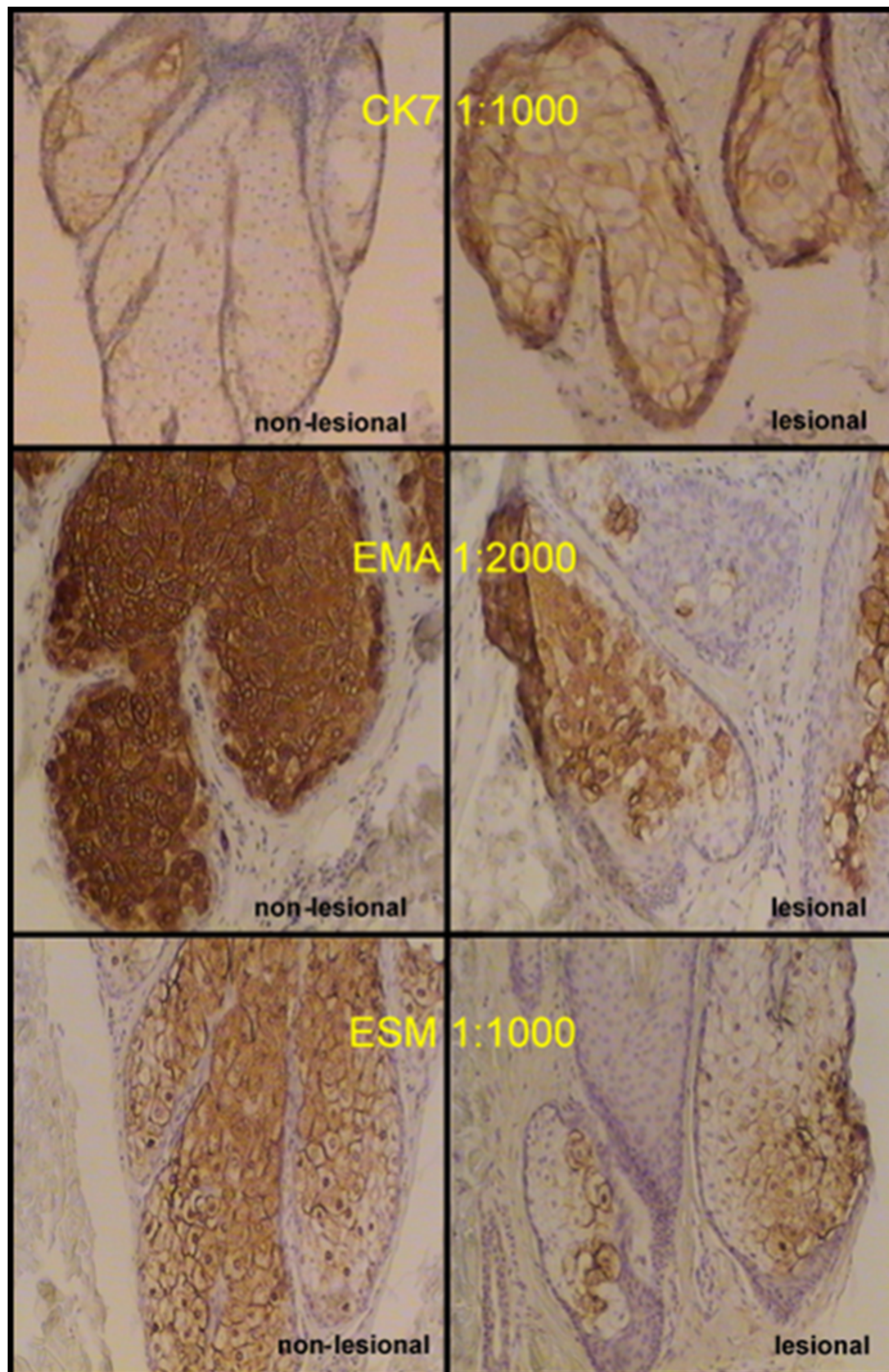

Figure 15. CK7 as early differentiation marker higher expressed in the lesional skin of patient #3. EMA and ESM as middle and late differentiation marker higher expressed in the non-lesional skin of the same patient. This patient shows a lack of mature differentiation in the lesional psoriatic skin, a finding that could explain the reduced sebum production at the psoriatic lesion. *CK7= cytokeratin 7, EMA= epithelial membrane antigen, ESM= epithelial sialomucin*

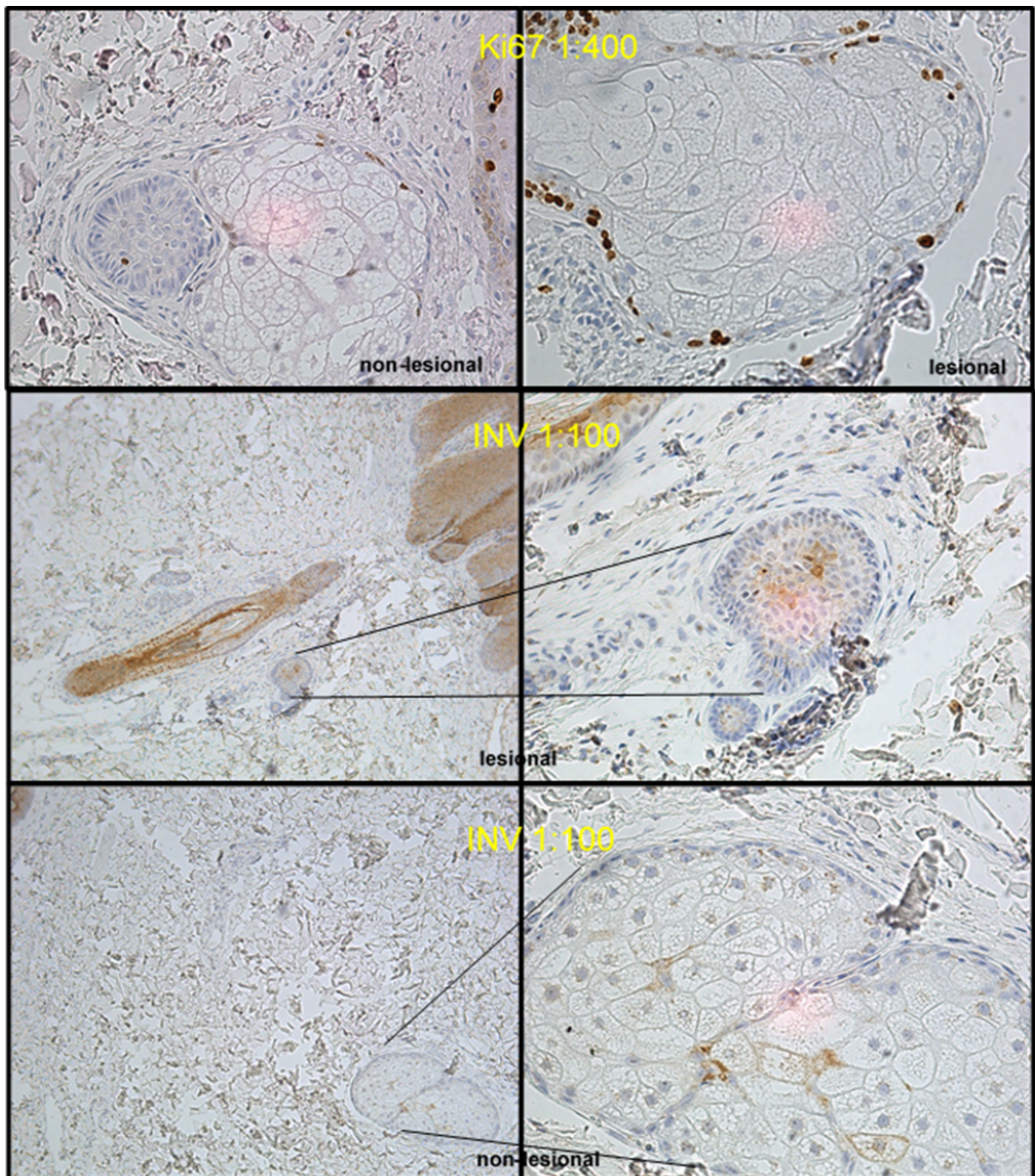

Figure 16. Slightly higher expression of Ki67 in the lesional skin compared to the non-lesional psoriatic skin. Unclear differences between the expressions of involucrin. This picture shows a possible elevated rate of proliferation in the lesional skin. INV= involucrin

a.	**Ki67**	**CK7**	**EMA**	**ESM**	**INV**
p1	0	0	0	0	0
p2	0	0	0	0	0
p3	**1**	**2**	**2**	**2**	0
p4	0	**1**	**1**	**1**	0
p5	0	0	0	0	0
p6	0	0	0	0	0
p7	0	**1**	**1**	**1**	0
p8	0	0	0	0	0
p9	0	0	0	0	0
p10	0	0	0	0	0
p11	0	0	0	0	0
p12	0	0	0	0	0
p13	0	0	0	0	0
p14	0	0	0	0	0

** 0= no difference between lesional and non-lesional skin*
1= slight difference between lesional and non-lesional skin
2= strong difference between lesional and non-lesional skin

b.	**number of patients**		
	strong difference	**slight difference**	**no difference**
Ki67	0	1	12
CK7	1	2	11
EMA	1	2	11
ESM	1	2	11
INV	0	0	14

Table 2. Summary of the number of the patients who showed strong or slight differences in the innumohistochemical staining, between lesional and non-lesional skin.

DISCUSSION

My results clearly demonstrate and prove a reduction of the sebaceous gland tissue mass in lesional skin of psoriasis patients, confirming previous initial studies (17-19). Although this finding is innovative, it is compatible with common clinical experience, since it is well-known that psoriatic plaque is characterized by thickness, dryness, and scaling, which are major clinical findings of sebostasis. The question which arises, namely "does this finding indicate a role of sebaceous gland changes in the pathogenesis of psoriasis?", belongs to the hen and the egg principle, i.e., do pathologic sebaceous glands cause the reactive thickness and dryness of the epidermis or is this reduction of sebaceous glands an epiphenomenon of the epithelial pathology.

Although my findings confirm prior observations of reduced sebaceous gland tissue mass in psoriasis (17-19), this observation was verified and measured quantitatively for the first time in this study. A possible role of psoriatic inflammation, which is well-known to play a crucial role in the pathological cascade of psoriasis, in the epithelial skin structures - including the sebaceous glands - leading to these parallel pathological findings cannot explain where the inflammation comes from. My results additionally indicate that the reason why the sebaceous gland in psoriasis does not function properly is not only the reduction of its mass but also a modification of its differentiation. Indeed, the differentiation process of sebaceous glands in psoriatic lesions may also be pathological; the sebocytes receive a signal to stay undifferentiated or do not reach their terminal differentiation and cannot produce sebum in the magnitude the healthy, well-differentiated sebocytes do. In addition, and in a parallel manner to new findings in the development of acne, where the sebaceous gland plays a major role, an altered sebum composition towards the synthesis and release of proinflammatory lipids may initiate psoriatic inflammation, which is an interesting, provocative new hypothesis (94-96).

However, the question that certainly arises is why this may happen only in the psoriatic plaque. Therefore, these results should be further investigated, probably in a higher number of psoriatic patients and also in comparison with healthy, non-psoriatic skin. A hypothetical answer to this question could be, that signals targeting first the sebaceous gland, lead to alterations of its micro-environment, and progressively to local inflammation and development of the psoriatic lesion. Moreover, one should not forget

that the reason for these individual differences may be differences in the patients' clinical characteristics and medical histories. Factors such as first-diagnosed psoriasis, multi-therapies, gender, localization of the biopsy, cutting point of the sections, and others can affect the results showing the differentiation process of the gland.

In my study, I established and used four main differentiation markers of sebocytes. These markers have been mentioned several times in previous studies as possible or definite differentiation markers of sebocytes in vitro and ex vivo. However, my study definitely adds to these contributions by clearly showing the function of these four differentiation markers, which provides a useful tool in the study of sebaceous differentiation for the future. It should be noted that my study presents CK7 as a definite early differentiation marker and ESM as a definite late differentiation marker. Regarding EMA, several opinions suggest that this antigen is expressed by all sebocytes during the entire differentiation process, so it cannot be exactly distinguished from the other two differentiation markers. This means, practically, that sebocytes in all three differentiation stages may express EMA, including the ones that express CK7 or ESM. Thus, this study cannot definitely suggest that EMA is expressed in a certain differentiation stadium only, but it suggests that it is expressed either by all sebocytes or by the advanced differentiated sebocytes. Furthermore, a discrimination of the continuous differentiation process of sebocytes between three stages was made for the needs of this study; some previous studies have made two- or four-stage discrimination. Ki67 did not reveal any problems; it is a known proliferation marker used to visualize the proliferation of sebocytes in the basal layer. Sebocytes can proliferate and differentiate concomitantly; only undifferentiated sebocytes mostly proliferate, whereas mature ones only differentiate (97).

A further hypothesis was that the abnormal differentiation process of sebocytes in psoriatic lesions may represent a squamous keratinization, able to induce inflammation, as shown in experimental settings (97-99). It is well-established that sebocytes are highly specialized, sebum-producing epithelial cells that release their content by rupture of the cell membrane and cellular degradation with the function of holocrine secretion. Sebocytes are most commonly found in the skin in association with hair follicles (forming the pilosebaceous unit) where they arise from hair follicle keratinocytes. Sebum forms an integral component of the epidermal barrier, and the skin immune

system and its synthesis are strongly regulated by hormones, in particular by androgens. Sebocyte formation is controlled by multiple molecular pathways, such as Blimp1, Wnt, C-myc, and Hedgehog (1). Sebaceous glands extend from the upper hair follicle and are formed from a sebocyte progenitor that expresses Blimp 1, keratin 14, and keratin 5, and these cells produce a proliferative population of sebocytes that, in turn, differentiates to produce the lipid/sebum-producing cells (2, 3, 52). The derivation of sebocytes from hair follicle keratinocytes suggests the possible theory that there can exist a signal that forces the hair follicle keratinocytes to turn the normal proliferation/differentiation process toward keratinocytes instead of sebocytes and produce more keratinocytes instead of providing sebocytes to the sebaceous gland. This theory does not explain, however, where this signal of general squamous differentiation of dermis and epidermis comes from, and it does not provide any information about the sequence of the events or whether the squamous differentiation of sebocytes comes before the acanthosis and hyperkeratosis of the epidermis, or whether the pathology of the epidermis comes first and then, as a consequence, the atrophy of the sebaceous glands, or a signal forces all parts of the skin to a squamous differentiation.

A recent study by Kamp et al (79) on patients with hidradenitis suppurativa/ acne inversa revealed a very similar result: significantly more atrophy of the sebaceous glands in the involved skin compared to the uninvolved. This was the first stereological volume estimation of sebaceous glands in a skin disease. This finding is also very interesting in combination with my findings in psoriasis because it raises the question of what these two diseases have in common that results in this excessive sebaceous gland atrophy. These two skin diseases seem to have some characteristics in common, such as local hyperproliferation of keratinocytes, genetic background, chronic inflammation, and as a consequence, good response to anti-TNF agents and other biologics and increased risk of metabolic disease as comorbidity. In addition, both smoking and increased BMI impair the clinical picture of both diseases. However, several dermatological diseases share all or some of these characteristics. In fact, psoriasis and hidradenitis suppurativa/ acne inversa are clinically very different dermatological diseases that do not even belong in the same classification subgroup. On the other hand, one could raise the question of whether it is simply that sebaceous gland atrophy occurs in all dermatological pathologies, such as when the epithelium is

“ill,” it includes the sebaceous gland. A good and simple way to confirm this is to estimate stereologically the sebaceous gland volume in different skin diseases, not only in dermatoses and chronic inflammatory diseases but also in other skin pathologies, such as epithelial skin cancer (basal cell carcinoma or squamous cell carcinoma). It would be interesting to add to my study’s comparisons between psoriatic-involved and psoriatic-uninvolved skin further comparisons with completely healthy skin to investigate whether the sebaceous glands are smaller in psoriasis. Such a study would entail the ethical issue of using skin biopsies from a healthy population.

The findings of my study could be a stimulus for re-examining the role of sebaceous glands in psoriasis. For example, the existing theory of alopecia psoriatica being caused by the psoriatic plaque (hyperkeratotic plaque) strangling the hair follicle, can be for the first time revised. My results suggest that alopecia psoriatica is probably developed due to a secondary abnormality of sebaceous glands, rather than to the mechanical cause that has been hitherto proposed. On the other hand, however, the existence of psoriasis palmoplantaris, which selectively affects only the palmoplantar areas of the human body, may indicate that the involvement of sebaceous glands in psoriasis is a secondary phenomenon (100, 101).

In my study, I used the Cavalieri principle to estimate stereologically the volume of the sebaceous glands in psoriasis. The sampling in a stereological methodology itself, is a key part of the study. I tried to conduct the sampling in a way that avoided systematic bias by adhering strictly to a design-based protocol quantifying a sufficient number of systemic random sections with software-generated, randomly positioned grids of points or lines. However, the process of tissue preparation and the alterations in the tissue morphology make some degree of systematic bias inevitable. In the present study, I used paraffin embedding for my sections because it is fast and ubiquitous and produces sections with good resolution at high magnifications. But it is well-known that paraffin-embedding results in a certain degree of tissue shrinkage that is very difficult to predict. Some have suggested that cryo-preparation of tissue causes less shrinkage; however, cryo-sections have the disadvantages of poor preservation of morphology, lower resolution at high magnification, and they are more difficult to produce (4-6).

Other suggested options for tissue preparation include vibratome and celloidin sections, but it is unavoidable that a certain degree of alteration of tissue morphology is present

with every kind of tissue preparation. As a consequence, systematic bias due to morphological changes introduced by tissue preparation should be taken into consideration in all ex vivo stereological studies. However, through the strict sampling protocol and the unbiased estimators I used, high precision was achieved and conclusions can be drawn, if not for the absolute numbers, at least for their relative changes over time and among body locations.

In the same way, the immunohistochemistry was conducted in this study in paraffin-embedded sections with antibodies appropriate for this tissue preparation. A possible improvement of my methodology could be to perform the two methods, stereology and immunohistochemistry, on the same group of patients. The use of both methods with the same group of patients could entail sectioning the tissue, staining it immunohistochemically, and then estimating it stereologically. But the problem arises that these sections cannot be thicker than 5 microns because the immunohistochemistry demands very thin sections for proper results. As a result, much larger amounts of sections and slides would be required. Furthermore, the slides used for every method are different with much more expensive slides being needed for immunohistochemistry than for stereology. Moreover, sections stained immunohistochemically are perhaps not appropriate for stereological analysis because the detailed structure of the sebaceous gland may not be visible in sections with immunohistochemical staining. Consequently, such an alternative approach would increase the costs enormously without obtaining better results.

Another important consideration for avoiding systemic bias is the clinical characteristics of the patients. In most clinical studies of psoriasis, it is common practice to recruit patients in the first stage of the disease who have not previously been treated topically or systemically, or at least who have been treated only topically. In my study, I recruited patients in different stages of the disease, including those with and without previous treatments. I did this so that patients were their own controls, since in each case I compared the lesional skin with the non-lesional skin of the same patient. A possible improvement of the methodology, as I mentioned above, could be a parallel comparison with completely healthy non-psoriatic skin.

In this study, all stereological methods applied were based on samples obtained with invasive procedures. There is a non-invasive approach that can be adopted in the

stereological analysis that would avoid systematic bias (through the absence of morphological changes that result as a consequence of invasive procedures). These are imaging techniques that can visualize the object under investigation within the skin with a sufficiently high resolution to clearly identify its boundaries and clearly differentiate it from surrounding structures. These techniques include confocal microscopy, optical coherence tomography (OCT) (102–104), and high-frequency ultrasound (105, 106), listed according to increasing depth of imaging and decreasing resolution, two characteristics that are often inversely proportional. These techniques often have sufficient depth of imaging to visualize, for example, the dermo-epidermal junction, but they do not get deep enough to visualize structures that lie deep in the dermis, such as the sebaceous gland.

In my project, I applied the stereological principles mentioned above. Despite the limitations due to the tissue sampling and preparation, dermatologists should be encouraged to apply stereological quantitative analysis in dermatological studies. Through continuous improvement of techniques and a systematic plan, bias can be eliminated, and 3D quantities in the skin can be precisely estimated without bias.

The application of stereological principles in psoriasis revealed a clear reduction of sebaceous gland volume in the diseased skin. Further studies should investigate and expand these results to other skin diseases to determine whether my findings are isolated or a result of a common, more general pathology. My findings suggest a new approach in the effort to elucidate the complicated cascade in the pathogenesis of psoriasis, and they implicate -for the first time- not only the epidermal but also the dermal structures of this well-known, yet not well-understood, disease.

APPENDIX I

PATIENT'S NAME

DATE OF VISIT (YYYY / MM / DD)

LESION SCORE

Erythema (E) Induration (I) Scaling (S)	No Symptoms	Slight	Moderate	Marked	Very Marked
SCORE	0	1	2	3	4

AREA SCORE

AREA	0	1% - 9%	10% - 29%	30% - 49%	50% - 69%	70% - 89%	90% - 100%
SCORE	0	1	2	3	4	5	6

Lesion Score	Head (H)	Trunk (T)	Upper Limbs (UL)	Lower Limbs (LL) *including buttock*
Erythema (E)				
Induration (I) (thickness)				
Scaling (S)				
SUM: E + I + S				
Percentage of Affected Area				
Area Score				
SUBTOTAL: Sum x Area Score				
Body Area: Subtotal X amount indicated	x 0.1	x 0.3	x 0.2	x 0.4
TOTALS	H	T	UL	LL

PASI SCORE: H + T + UL + LL

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CURRICULUM VITAE

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

EIDESSTATTLICHE VERSICHERUNG

„Ich, Aikaterini I. Liakou, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Volume and differentiation of sebaceous glands in psoriasis“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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

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Datum Unterschrift

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