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PSORIASIS - FROM TRANSCRIPTOME TO MIRNA FUNCTION AND BIOMARKERS

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**Karolinska
Institutet**

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The cover picture represents a fusion of Vincent van Gogh's paint "The Starry Night", with a psoriasis skin section imaged by fluorescence microscopy. The undulating structure, with intermittent protrusions (rete ridges), defines the epidermis (upper) from the dermis (lower). In blue the cells' nuclei and in yellow the single molecules of a long non-coding RNA studied in our lab.

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Psoriasis - From Transcriptome to miRNA Function and Biomarkers

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To everyone who loves me, unconditionally.

*“On Earth, I have experienced highs and lows, turbulence and peace, success and suffering.
I have been rich and poor, I have been able-bodied and disabled.*

*I have been praised and criticized, but never ignored. I have been enormously privileged,
through my work, in being able to contribute to our understanding of the universe.*

*But it would be an empty universe indeed if it were not for the people I love, and who love
me. Without them, the wonder of it all would be lost on me.*

*Be brave, be curious, be determined, overcome the odds.
It can be done!”*

Stephen Hawking – “Brief Answers to the Big Questions”

ABSTRACT

Psoriasis is a chronic inflammatory, immune-mediated skin condition that affects in average 2 to 3% of the world population, phenotypically characterized by red and scaly plaques on the skin of affected patients. It is a multifactorial disorder, in which both genetic predisposition and environment play key roles. Psoriasis lesional skin is characterized by abnormal keratinocyte differentiation and proliferation, as well as dermal immune cell infiltration. Psoriasis is associated with several comorbidities, e.g. arthritis, however, currently no biomarkers exist that could be used to predict or identify these at an early stage. Many studies aimed to characterize the psoriasis transcriptome, but few studies have been focusing on elucidating the gene alterations in keratinocytes in this disease. In this thesis, we explored the transcriptomic landscape of epidermal cells from lesional and non-lesional skin of patients with psoriasis, as well as from healthy volunteers' skin and investigated the biomarker-potential of circulating microRNAs.

In our **first study**, we investigated the alterations of the protein-coding transcriptome in the psoriasis epidermal compartment. The separation of the epidermis from the dermis and sorting for CD45-neg cells allowed us to exclude dermal signatures including those from fibroblasts, endothelial cells, dendritic cells and T cells, but also from immune cells infiltrating the epidermis, known to populate at increased ratio the psoriasis lesional skin. We have identified biological pathways related to immune responses, cell cycle and keratinization involved in the epidermal alterations, as well as the enrichment and dominance of psoriasis-associated cytokine signatures. Moreover, we established that genetic variations associated with psoriasis may contribute to the keratinocyte transcriptomic changes in the disease.

In our **second study**, we investigated the alterations of the non-protein-coding transcriptome in psoriasis and identified a set of long non-coding RNAs differentially expressed in psoriasis epidermal cells. Several had genomic localization overlapping psoriasis-associated SNPs, suggesting their potential implication in the genetic susceptibility to psoriasis. We validated the over-expression of the lncRNA LINC00958 in CD45-neg cells from psoriasis lesions compared to non-lesional and healthy skin and determined its expression in different skin cell types and subcellular localization.

In our **third study**, we focused on psoriatic arthritis, the major psoriasis comorbidity, affecting about 1/3 of the patients with cutaneous psoriasis. In particular, we investigated the potential of circulating microRNAs as biomarkers for early diagnosis of psoriatic arthritis symptoms in patients with cutaneous psoriasis. We have identified two circulating microRNAs, let-7b-5p and miR-30e-5p, with

significantly reduced levels in plasma-derived extracellular vesicles of patients with confirmed psoriatic arthritis, compared to cutaneous-only psoriasis patients.

Finally, in our **fourth study**, we investigated the role and functions of miR-378a, previously found overexpressed in psoriasis lesional keratinocytes compared to non-lesional and healthy skin. In vivo, in a mouse model of psoriasis-like skin inflammation, the injection of miR-378a resulted in increased clinical signs of inflammation, increased skin thickness and number of proliferating cells in the epidermis. In vitro, in cultured primary human keratinocytes, miR-378a overexpression enhanced the expression of pro-inflammatory chemokines CXCL8/IL8 and CCL20, as well as reduction of NFKBIA proteins levels.

LIST OF SCIENTIFIC PAPERS

I. **The keratinocyte transcriptome in psoriasis: pathways related to immune responses, cell cycle and keratinization**

Lorenzo Pasquali, Ankit Srivastava, Florian Meisgen, Kunal Das Mahapatra, Ping Xiag, Ning Xu Landén, Andor Pivarsci, Enikő Sonkoly

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III. **Circulating microRNAs in extracellular vesicles as potential biomarkers for psoriatic arthritis in patients with psoriasis**

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IV. **miR-378a is up-regulated in psoriasis keratinocytes and enhances their response to IL-17A**

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LIST OF ABBREVIATIONS

3'-UTR	Three prime untranslated region
AMP	Antimicrobial peptide
BP	Biological Processes
CARD14	Caspase recruitment domain family member 14
CCL	Chemokine (C-C motif) ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
circRNA	CircularRNA
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAMP	Danger-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell-sorting
FFPE	Formalin-Fixed, Paraffin-Embedded
GEO	Gene Expression Omnibus
GO	Gene Ontology
GSEA	Gene set enrichment analysis
GWAS	Genome-wide association study
HKGS	Human keratinocyte growth supplement
IFN	Interferon
IKK	I κ B-kinase
IL	Interleukin
I κ B	Inhibitor of kappa B
JAK	Janus kinase
lncRNA	Long non-coding RNA
LTP	Lipid-transfer protein
MACS	Magnetic-activated cell-sorting
MAPK	Mitogen-activated protein kinase
miRNA	microRNA

MMP	Matrix metalloproteinases
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated
NHEK	Normal Human Epidermal Keratinocytes
NIK	NF- κ B-inducing kinase
NKT cells	Natural killer T cells
NLR	Nucleotide-binding domain, leucine-rich repeat
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cells
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PRR	Pattern recognition receptor
PsA	Psoriatic arthritis
PsC	Cutaneous-only psoriasis
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RHE	Reconstructed human epidermis
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA-seq	RNA-sequencing
SALT	Skin-associated lymphoid tissue
SCID	Severe combined immunodeficiency
scRNA-seq	Single-Cell RNA-sequencing
siRNA	Small interfering RNA
SIS	Skin immune system
SNP	Single nucleotide polymorphism
SOCS-3	Suppressor of cytokine signaling 3
STAT	Signal transducer and activator of transcription
TH	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPM	Transcript-per-million
TRAF	TNF receptor-associated factor 6
Treg	Regulatory T cells
UV	Ultraviolet

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1 INTRODUCTION

“The finest clothing made is a person’s own skin, but, of course, society demands something more than this” - *Mark Twain*.

Between us and the rest of the world there is an interface that makes up about 16 percent (~1/7th) of the total body weight: the skin. Largest organ in our body, it accounts for way more functions than just holding our organs inside. Its surface protects, regulates and senses the surrounding environment and it processes hundreds to thousands physical sensations every day.

Many skin conditions could arise from anything that irritates, clogs or inflames the skin, provoking symptoms such as burning, swelling, itching and redness. These environmental factors, combined with specific genetic predisposition, might result in an inadequate immune response. If not quickly rebalanced, this can escalate into cancer-formation, pathogens’ infection, autoimmunity and prolonged inflammation that damages healthy cells. All these, not only can afflict the skin’s appearance but also, most critically, endanger people’s lives.

This thesis summarizes the effort made to further elucidate the transcriptomic changes that shape the transformation of keratinocytes in psoriasis lesions. We profiled protein-coding genes, as well as microRNAs and long non-coding RNAs, in isolated cells from the epidermis of patients with psoriasis, with the hope that one day some of these could be implemented in RNA-targeted therapies of psoriasis itself. Moreover, we screened for circulating microRNAs as potential biomarkers to easily diagnose psoriatic arthritis in patients with psoriasis. This would lead to timely treatments of patients at risk, promptly reducing the severity of the symptoms and improving their overall quality of life.

1.1 The Skin

The skin protects the body against infection, extreme temperatures, external traumas, irritation and UV radiation, maintaining the balance of fluids and synthesizing vitamin D, functioning in this way as a physical, chemical and immunological barrier [1-3]. In just a square centimeter of skin we can find millions of cells and nerve endings that allow to sense the outside world, while its sweat glands and blood vessels help maintain a proper temperature and communicate about health and emotions, through events like blushing, flushing and sweating. Skin accounts for about 3 to 5 kilograms of our body weight and, although just few millimeters thick (from the thinnest on the eyelids to the thickest of palms and soles), if it could be spread out it would measure up to two square meters [2]. It comes in lots of different pigmentations and together with hair, nails, and specialized sweat and

sebaceous glands, skin forms the integumentary system. The key to the integumentary system is layers: the skin has three main layers, from the outermost to the innermost represented by epidermis, dermis and hypodermis, each with particular types of cells that have specific functions (*Figure 1*).

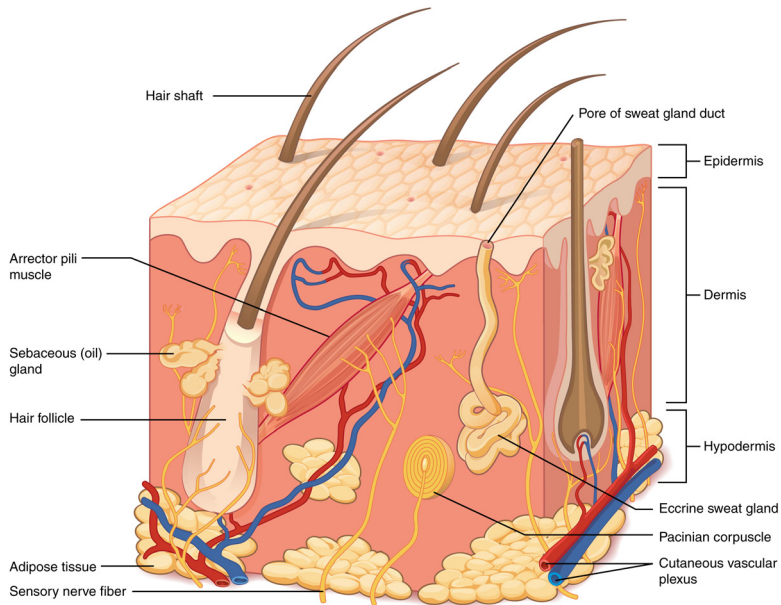


Figure 1. The structure of the skin. Access for free at <https://openstax.org/books/anatomy-and-physiology/pages/1-introduction> Copyright© Jan 16, 2020 OpenStax. Creative Commons Attribution 4.0 International License.

1.1.1 The Epidermis

Assuming the skin is intact, the epidermis is the only layer visible. It's made of keratinized, stratified squamous epithelial tissue – multiple layers (from four to five depending on the body location) set on top of each other, like bricks – which is composed of more than 90% of keratinocytes and the remaining of melanocytes, immune cells such as T lymphocytes and Langerhans cells and Merkel cells [2, 3]. The epidermis itself is composed of multiple layers (*Figure 2*), further described from the outside inwards. *Stratum corneum*, the main component of the skin barrier is the one facing the surface of the skin. It is made of about 15 to 30 sheets of dead keratinocyte cells which undergone keratinization (or cornification), and it offers basic protection from pathogens and other environmental threats. In addition, at this level lipid chains contribute to the physical barrier formation too, preventing the dehydration of underlying tissues. *Stratum lucidum* - also known as

“clear layer”- consists of few rows of flat, dead keratinocytes that are only found in the thick skin of palms, soles and digits. The high content of clear proteins rich in lipids called eleidin, fills these cells providing their translucent appearance and creating a hydrophobic barrier. The *Stratum granulosum*, or “granular layer”, contains living keratinocytes, which have markers for late differentiation stage (involucrin, loricrin, filaggrin). It is called granular layer because the cells here get compressed and flattened, maturing as they move up through the epidermal layers. The membrane of the cells thickens and they start producing a fibrous structure called keratohyalin rich in histidine and cysteine proteins. The two major constituents of the keratohyalin are the profilaggrin (precursors of filaggrin) and the involucrin proteins. The first is responsible for binding the filaments of keratin together [4], while the second for building of the cells’ envelope in the cornified layer [5]. Moving towards the *Stratum spinosum*, or “spiny layer”, we reaching a point where cell regeneration, or mitosis, is active. Made of eight to ten layers of keratinocytes, cells here look spiny when they’re dehydrated for microscope slide preparation and that’s because they contain filaments that help them hold to each other called desmosomes. Another cell type we can find in this layer is the Langerhans cell, tissue resident dendritic-cells which role is not fully understood yet but they’re thought to be protectors of the skin by processing microbial antigens and act as antigen-presenting cells [6]. Finally, the deepest, thinnest epidermal level is the *Stratum basale* or “basal layer” (also known as *stratum germinativum*). It’s just a single layer of cuboidal-shaped cells representing the precursors of keratinocytes, where most of the new-cell production occurs. In this layer, two other important cell types can be found: Merkel cells, overrepresented on hands and feet skin surfaces, with the main responsibility of translating the “touch” sensation to the brain through activation of sensory nerves; and the melanocytes, cells that produce melanin, a pigment that color hair and skin and protects the genomic content from being damaged by UV radiation. This stratum is also what connects the epidermis to the basal lamina, below which lie the other layers of the skin, the dermis and hypodermis [1, 7, 8].

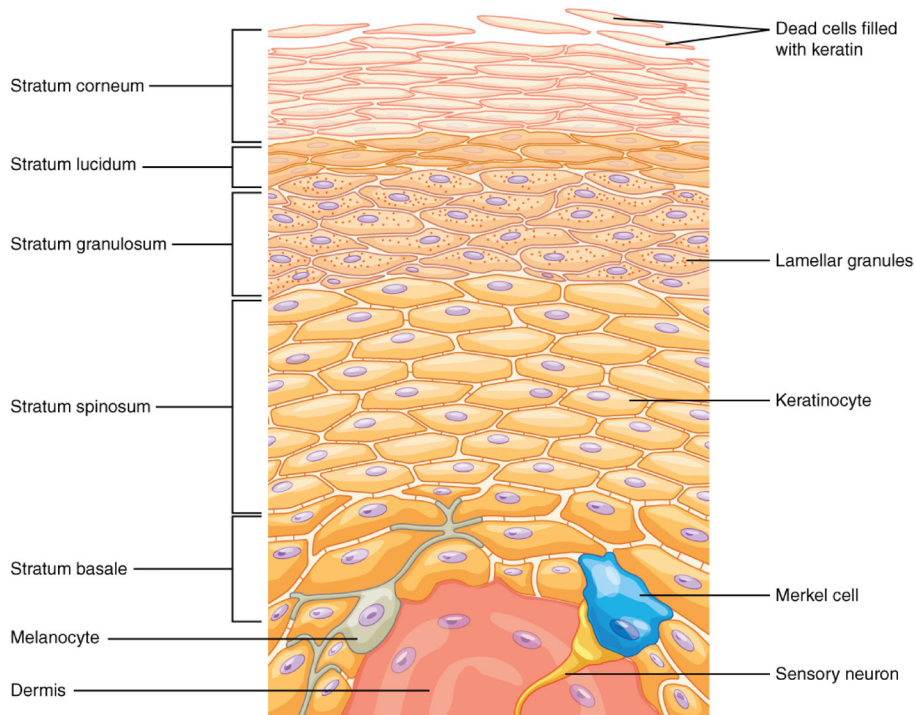


Figure 2. Layers of the epidermis. Access for free at <https://openstax.org/books/anatomy-and-physiology/pages/1-introduction> Copyright© Jan 16, 2020 OpenStax. Creative Commons Attribution 4.0 International License.

1.1.1.1 Keratinocytes

The bulk of epidermis is made of cells called keratinocytes (KCs), which are the building blocks of that tough, fibrous protein keratin that gives structure, durability, and waterproofing to hair, nails, and outer skin. These cells are constantly dying and being replaced -- millions of them every day are lost, enough to completely replace the epidermis every 4 to 6 weeks [9]. Keratinocytes are encoded to undergo an event called “terminal differentiation”, a type of controlled programmed journey, which slowly takes place as the keratinocytes moves up through all layers of epidermis. During this process of differentiation, the keratinocytes make multiple changes in both its morphology and function, until they reach the surface. The epidermal differentiation program is regulated at several levels including signaling pathways, transcription factors, and epigenetic regulators that establish a well-coordinated process of terminal differentiation [10]. As the basal keratinocytes move upward the keratin intermediate filament structures formed by keratin 5 (K5) and keratin (K14) is replaced by keratin 1 (K1) and keratin 10 (K10) filaments, the markers of early differentiation that start to get expressed at the suprabasal layer [8, 11, 12].

1.1.2 The Dermis

The dermis, just below the epidermis, is composed of two layers [13]. The upper papillary layer is composed of a thin sheet of areolar connective tissue that is riddled with little peg-like projections called dermal papillae. Just below, we find the deeper and thicker reticular layer, which makes up 80 percent of the dermis, made of dense irregular connective tissue. All of the dynamic parts contained within the dermis –like blood vessels (capillaries), collagen, elastic fibers, and extracellular matrix– are distributed between both its layers. The dermis is responsible for the elasticity and resilience of the skin and the presence of mechanoreceptors and thermoreceptors provides respectively the sense of touch and heat. Cells here are characterized by a reduced ability to regenerate, resulting in a more difficult healing from wounds. The dermal dense extracellular matrix (ECM) is populated primarily by sparsely distributed fibroblasts, but other cell types are also found, including Schwann cells, macrophages, mast and stem cells [14].

1.1.3 The Hypodermis

The most basal layer of the skin is the subcutis or hypodermis. It consists of mostly adipose (or fatty) connective tissue and it provides insulation, energy storage, shock absorption, and helps anchor the skin. Drugs are commonly injected in this layer due to the high vascularity of the tissue which allows a fast absorption of the drug itself [15]. Adipose tissue can contribute to immune response and the role of adipocytes has been shown to be relevant for epidermal homeostasis during hair follicle regeneration and wound healing [16-18].

1.1.4 Skin Immune Cells

The skin represents the primary mechanical and immunological barrier with features that protect us from microbial pathogens' penetration and physical insults. It also represents an exclusive environment in which skin cells interact with the immune cells for the maintenance of the body's homeostasis and, in certain cases, inducing immune responses [19, 20]. Heterogeneous and highly specialized immune-resident cells and immunocompetent skin-trophic lymphocytes that cooperate to create a network in the skin, establishing what was previously known as SALT (skin-associated lymphoid tissue), successively renamed as SIS (skin immune system) [3, 21-23].

In the epidermis, a specific subset of **dendritic cells** (DCs), the antigen-presenting **Langerhans cells**, project their dendrites toward the epithelial layer and sample bacterial antigens such as toxins [6]. These can function as anti-inflammatory and activator of the inflammation depending on the context. In the dermis, the dendritic cells have higher efficiency in detecting dead cells and presenting antigens

such as viruses, other intracellular pathogens and/or skin associated self-antigens to T cells [24]. If the dendritic cells are considered the immune system sentinels, **T cells** are the immune system effectors.

Healthy skin presents more than twice the number of T cells which are found in the blood [25]; most of them are considered memory T cells which have previously encountered antigens and, therefore, can rapidly reactivate. T cells in the epidermis are mainly **CD8+ T cells**, a subtype that can become cytotoxic and kill target cells upon their activation [26]. Their prolonged permanence in the epidermis is mostly disconnected from the circulation [27]. In contrast, in the dermis there are mainly **CD4+ T cells**, with a predominant modulatory role in the immune response. Even in healthy non inflamed skin, beside dendritic cells, the dermis contains other types of immune cells such as, $\alpha\beta$ T cells, $\gamma\delta$ T cells, natural killer (NK) cells, B cells, mast cells, and macrophages, which can be involved in the allergic reaction of the skin [2].

Skin dendritic cells and keratinocytes can sense tissue damage derived from different sources (i.e. wounds), through evolutionary conserved receptors that recognize pathogen derived molecular patterns or host derived molecules that have been exposed by cell death (i.e. DNA) [28, 29]. The activated dendritic cells migrate to the lymph nodes where they present antigens to naïve T cells, preparing them to differentiate into effector T cells (process known as “sensitization”) [30]. These activated T cells can then migrate towards the epidermis and suppress the affected keratinocytes to control the infection or secrete signals that recruit additional immune effector cells [31]. The immune response operated by **helper T lymphocytes** including **Th1** (T-helper cell type 1) cells, is followed by the production and release of type II interferons (IFNs) such as IFN- γ , by **Th2** releasing IL-4, IL-5 and IL-13 and by the more recently discovered T-helper cells **Th17** with their production of IL-17 and IL-22 and **Th22**, producing IL-22, IL-13, TNF- α and FGF- β (*Figure 3*) [32-34]. Further insights about these cells in the psoriasis context will be provided later.

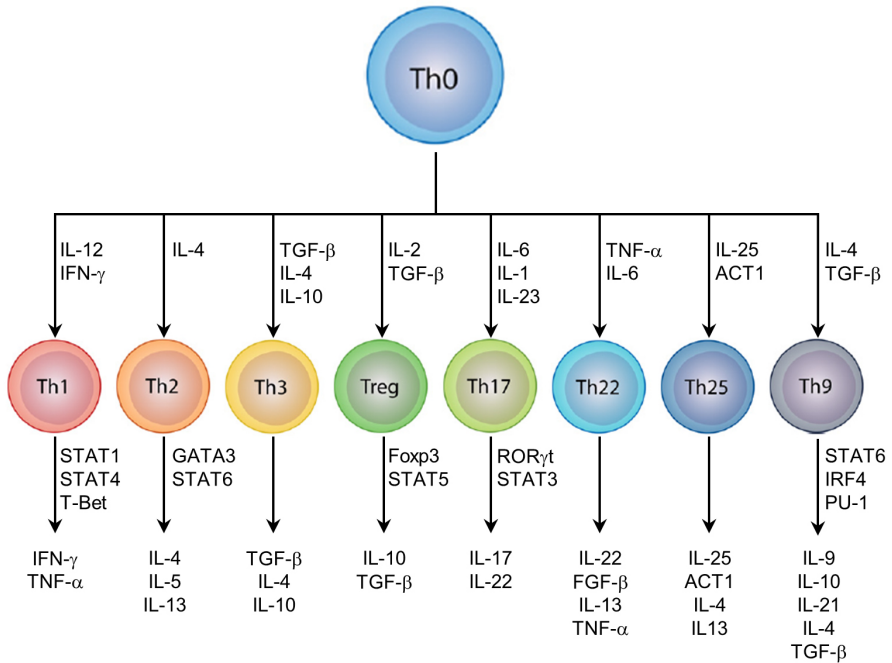


Figure 3. Profile of T helper lymphocytes, polarization from naïve to more specialized, and related cytokines production. Reproduced and adapted with permission from Quaresma JAS, 2019. Copyright© 2019 American Society for Microbiology.

Keratinocytes, in addition to provide a physical barrier, they are the first line of immunological defense in the skin. They can recognize pathogens through pathogen-associated molecular patterns (PAMPs) via their pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) -expressed on both their cell surface (TLR1, 2, 4 and 5) and on endosomes (TLR3, 9)- and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [2, 35-37]. Upon pathogen recognition or under specific inflammatory conditions, keratinocytes produce chemokines and cytokines such as CXCL1-5, CXCL8 (IL-8), IL-1, IL-6, IL-10, IL-17C, IL-22, IL-18, IL-36 and TNF- α , as well as defensive antimicrobial factors including b-defensins, LL37 and S100 proteins [2, 38-41]. Release of inflammatory mediators such as IL-1 by keratinocytes induce the activation of dendritic cells, while chemokines (i.e. the chemotactic factor CXCL8/IL-8) recruit primarily neutrophils, but also macrophages and T cells [42, 43].

Other cells have fundamental roles in the intricate organization of the skin immune complex.

Neutrophil granulocytes, the most abundant immune cells, in situations of injury or infection travel through blood vessels and capillaries attracted by chemokines through chemotaxis [44], and communicate with antigen-presenting cells (APCs) where the inflammation takes place [45]. They participate in removing foreign particles or pathogens through processes of phagocytosis and endocytosis, as well as amplify inflammatory responses by releasing large quantities of antimicrobial proteins (AMPs) [46, 47].

Eosinophils, granulocytes originated in the bone marrow, are recruited by Th2-cytokines in the latest stages of the inflammatory reaction. These contains granules with toxic arginine-rich proteins and are sources of a large variety of cytokines such as TGF- α , TGF- β , IL-13, and VEGF, contributing to tissue healing in case of injuries and tissue remodeling and angiogenesis in case of chronic inflammation [48].

Basophils, which have a relatively short life span (1-2 days), are developed in response to cytokines such as IL-3, IL-4, IL-33 and IL-8 and their infiltration in the skin occurs often together with eosinophils, in a ratio that can differ [49]. Presence of immunoglobulin type E (IgE) receptors on their surface makes them suitable in skin immunity to fight infectious or dangerous agents [50].

Macrophages in the skin play important roles in different stages of wound repair and tissue regeneration, with the ability to migrate towards lymph nodes in particular immunologic situations. Moreover, they regulate processes involving skin homeostasis, inflammation, autoimmunity and skin cancer [51]. Recently, a newly discovered subset of nerve-associated macrophages has been shown to take part in the nerves' regeneration process after an injury [52].

Mast cells are very similar to the basophils in terms of mechanism of action and type of mediators but are predominantly found in tissues instead of circulating in the blood stream [53]. Activated by inflammatory peptides released by nerve fibers, mast cells establish with these a feedback mechanism that gives raise to pain and itch [54].

Natural Killer (NK) and Natural Killer T (NKT) cells, after being exposed to the cytokine IL-12, they activate, mature and produce consistent volumes of IFN- γ [55]. NK cells are fundamental components of the innate immune system, while NKT have a role in both innate and acquired immune response, besides promoting the differentiation and enhancing the biological features of NK cells [56]. After infiltrating the skin, NKT cells can behave in different ways, either stimulating or suppressing the immune system [57].

Innate lymphoid cell (ILCs), class of immune cells recently identified in skin, which shares transcription factors (TFs) and cytokine profile similarities with T cell subsets but don't seem to have antigen-specific receptors on their surface [58]. ILCs quickly respond to infections, they control skin homeostasis and responses of the adaptive immune system, being categorized into three subgroups named ILC1 (adaptive response mediated by Th1 cells), ILC2 (adaptive response mediated by Th2 cells) and ILC3 (adaptive response mediated by Th17 cells) [59, 60].

B lymphocytes, which main function is to secrete the proteins that are bound to their membrane, known as antibodies (Ab) or immunoglobulins (Ig). Members of the TNF (tumor necrosis factor) ligand family promote the activation and development of B cells into mature B lymphocytes [61]. These play a significant role in systemic autoimmune diseases but they can also present autoantigens or promote the interruption of peripheral T-cells tolerance [23].

Endothelial cells, present in the vascular and lymphatic systems of the skin, have the ability to regulate different adhesion molecules such as intercellular adhesion molecules-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (ELAM-1) and the vascular cell adhesion protein VCAM-1, in response to stimuli from TNF- α [23, 62]. Endothelial cells prevent the formation of blood clots (thrombi), permeabilize the blood vessels controlling the blood pressure, they are involved in tissue aging, presentation of antigens, process of wound repair and formation of blood vessels from pre-existing vasculature (angiogenesis) [63].

Table 1. Immune role of skin cells.

Cell	Location(s)	Function(s)	Activating factors	Cytokine(s) produced
Keratinocyte	Epidermis	Mechanical barrier, keratin production, immune function, innate immunity	IFN- γ , infections	IL-1, IL-6, IL-10, IL-12, IL-15, IL-18, IL-19, IL-20, CCL20, CXCL8/IL-8, CXCL9, CXCL10, CXCL11, TNF- α , TGF- β , IL-23, GM-CSF, G-CSF
Langerhans cell	Epidermis	Antigen-presenting cell, migration and recruitment to the secondary lymphoid organs, and antigenic presentation to T lymphocytes	Infectious and noninfectious antigens	IL-12, IL-23, IL-6, TNF- α
Dermal dendrocyte	Dermis	Antigen-presenting cell, phagocytosis, regulation of collagen synthesis, and homeostasis of the dermis	Infectious and noninfectious antigens	IL-12, IL-23, IL-6, TNF- α
Plasmacytoid dendritic cell	Dermis	Antigen-presenting cell	IL-3, CD40, virus, bacterium, CpG oligonucleotides	IL-12
Inflammatory dendritic cell	Dermis, epidermis	Antigen-presenting cell	Microbial and endogenous antigens	IL-12
T CD4 Th1 cell	Mainly in the dermis; rare in the epidermis	T helper lymphocyte; coordinates immune function	IL-12	IFN- γ
T CD4 Th2 cell	Dermis; rare in epidermis	T helper lymphocyte; coordinates immune function	IL-4	IL-4, IL-5, IL-13
T CD4 Th3 cell	Dermis	T helper lymphocyte; coordinates immune function	IL-4, IL-10, TGF- β	IL-4, IL-10, TGF- β
T CD4 Th9 cell	Dermis	T helper lymphocyte; coordinates immune function	IL-4, TGF- β	IL-9, IL-10, IL-21
T CD4 Th17 cell	Dermis	T helper lymphocyte; coordinates immune function	IL-6, IL-1, IL-23	IL-17, IL-22
T CD4 Th22 cell	Dermis	T helper lymphocyte; coordinates immune function	TNF- α , IL-6	IL-22, FGF- β , IL-13, TNF- α
T CD4 Th25 cell	Dermis	T helper lymphocyte; coordinates immune function	IL-25, Act1	IL-4, IL-13, IL-25, Act1
Treg cell	Dermis	Control of the immune response	IL-2, TGF- β	IL-10, TGF- β
T CD8 cell	Dermis, epidermis	Elimination of intracellular microorganisms and infected cells	IL-2, IL-12, IFN type I	IFN- γ

T $\gamma\delta$ cell	Dermis	Elimination of intracellular microorganisms and infected cells; cell death	IL-2, IL-12	IL-17, IFN- γ
NKT cell	Dermis	Elimination of lipid antigens	IL-12, IL-18	IL-4, IL-17, IFN- γ
NK cell	Dermis	Innate immunity against viruses and intracellular bacteria	IL-12, IL-15	IFN- γ
ILC	Dermis	Innate immunity	IL-12, IL-18, IL-23, IL-25, IL-33, TGF- β	IL-1, IL-23, IL-25, IL-33, TSLP
B cell	Dermis	Humoral immune response	IL-2, IL-4, IL-6, IL-11, IL-13, TNF- α , BAFF	Antibodies
Plasma	Dermis	Synthesis and release of antibodies	IL-4, IL-6, IL-10, IFN- γ , BAFF	Antibodies
Breg cell	Dermis	Control of the immune response	IL-2, IL-4, IL-5	IL-10, TGF- β
M1 macrophage	Dermis	Phagocytosis, antigen presentation, bactericidal action	IFN- γ	IL-6, IL-12, TNF- α , iNOS
M2 macrophage	Dermis	Phagocytosis, antigen presentation, regenerative effects	IL-4, IL-13	IL-10, TGF- β , arginase-1
Mast cell	Dermis around the vessels	Hypersensitivity reaction, vasodilation, chemotaxis, inflammation	IL-3, IL-5, IL-13	TNF- α , IL-1, IL-4, IL-5, IL-6, IL-13, CCL3, CCL4, IL-3, GM-CSF
Basophil	Dermis	Hypersensitivity reaction, vasodilation, chemotaxis, inflammation	IL-3, IL-5, GM-CSF, histamine releasing factor	IL-4, IL-13
Eosinophil	Dermis	Hypersensitivity reaction, vasodilation, chemotaxis, inflammation, IgE production	IL-5, IL-13, GM-CSF	IL-3, IL-5, IL-8, IL-10, leukotrienes, GM-CSF, hydrolases
Neutrophil	Dermis, epidermis	Innate immunity, phagocytosis	C3, IFN- γ , TNF- α , GM-CSF	ROS, proteolytic enzymes
Endothelial cell	Dermis	Inflammation, immune response, infections	IL-1, IL-6, TNF- α	TNF- α , IL-1, IL-6, IL-8, IL-15, IL-17, IL-18, G-CSF, GMCSF, VEGF
Fibroblast	Dermis	Inflammation, immune response, infections	IL-1, TNF- α	PGE ₂ , GM-CSF, CXCL8/IL-8, MIP-2, PDGF, TGF- β , FGF- β

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1.1.5 NF- κ B Signaling Transduction

In both immune and non-immune cells, the **NF- κ B signaling pathway** has been found to play substantial roles in homeostasis regulation and promotion of inflammation. The basic components of this pathway include receptors (pro-inflammatory cytokines such as TNF- α and IL-1), signal adapters (TNF receptor associated factors –TRAFs and receptor interacting proteins –RIPs), IKK complexes (IKK α or IKK1, IKK β or IKK2 and IKK γ or NEMO), I κ B proteins (I κ B α , I κ B β , I κ B ζ , I κ B ϵ , BCL-3, p100 (precursor of p52), p105 (precursor of p50)) and NF- κ B dimers (p50 (NF- κ B1), p52 (NF- κ B2), p65 (RelA), c-Rel and RelB) [64-66]. In homeostatic conditions, homodimers and heterodimers are kept inactivate in the cytoplasm of the cells, associated to the inhibitory proteins I κ B. When cells receive intra- and extra-cellular stimuli, the IKK complexes are activated, resulting in phosphorylation and ubiquitination of I κ B proteins that, in turn, are degraded and NF- κ B dimer released. Upon further post-translational modifications, the NF- κ B dimer is transported to the nucleus, where it can finally bind to its target genes and promote their transcription [67, 68]. This summarizes the canonical (or classical) pathway of NF- κ B, which results in the mechanism of signal transduction. Alternatively, in response to a subset of tumor necrosis factor (TNF) family members such as CD40L, BAFF, LTB, RANKL and TWEAK, the NF- κ B-inducing kinase NIK activates IKK α , which in turn phosphorylates p100, precursor of p52. Activated p52 forms a heterodimer with Rel-B and the complex RelB/p52 translocate into the nucleus of the cell, inducing the expression on non-canonical NF- κ B related genes, such as the anti-apoptotic genes Bcl2 and Bcl-xl (involved in B cell maturation and survival), promoting cell survival [69-71].

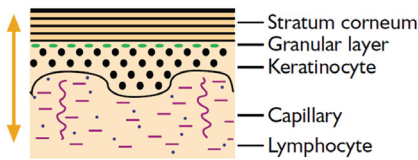
In general, a dysregulation of the immune responses cause skin disorders such as psoriasis [72-74], atopic dermatitis [75, 76], cancers [77, 78] and many other skin related diseases.

1.2 Psoriasis

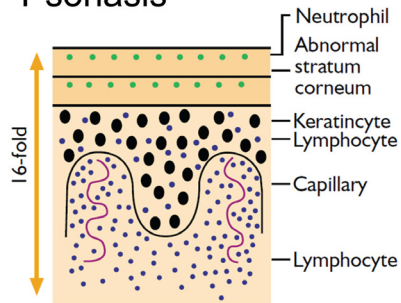
Psoriasis is a common immune-mediated skin disease with a strong genetic background, which affects approximately 0.5%–1% of children and 2-3% of the adult population (>125 million people worldwide) [79], reported at higher rates in locations distant from the equator [80] and for which there is no clear cause or cure. Psoriasis may begin at any age, however, there are two peaks: at 20-30 years and 50-60 years. This means that 50% of the cases in psoriasis starts before the age of 25 [81]. Psoriasis is recognized as a skin disease with significant impact on quality of life and emotional well-being of patients suffering from it [82]. Indeed, depression is very common among these patients [83]. It waxes and wanes throughout the life of a patient, and without treatment a spontaneous remission is highly unlikely [84]. Although the exact etiology of psoriasis is unknown, it is a widely held view that can be provoked by non-specific triggers such as mild trauma, drugs (lithium, interferon-alpha, antimalarial medications etc.), stress (which is probably the strongest environmental trigger of psoriasis) but also viral infections (e.g. HIV) can start the inflammatory processes which lead to the development of the disease [85]. This chronically relapsing inflammatory disease is thought to be multifactorial, involving both environmental and genetic factors [86, 87]. It is characterized by aberrant interaction between keratinocytes and infiltrating immune cells, which leads to hyperproliferation and altered differentiation of the keratinocytes themselves and formation of psoriatic plaques [88, 89]. The severity of the psoriatic plaques is quantified by two major scoring systems: the psoriasis area and severity index (PASI) and the physician's global assessment (PGA). In addition, the Dermatology Life Quality Index (DLQI) represents a ten-question questionnaire which assesses how psoriasis is affecting well-being and quality of life of psoriasis patients [90].

The characteristic histological features of psoriasis are epidermal hyperplasia and an inflammatory cell infiltration in both the dermis and the epidermis. The rapid proliferation of immature keratinocytes in psoriasis, which may increase more than ten times over the normal rate, is combined with an impaired cellular differentiation, while the retention of the keratinocytes' nuclei in the stratum corneum results in a phenomenon called "parakeratosis" [91] (*Figure 4*). Keratinocytes, once activated by different triggers (environment, injuries, stress, cytokines, viral infection etc.), have been shown to produce a large number of cytokines, which may induce further proliferation of these cells and have other pro-inflammatory and immunomodulatory effects [92, 93].

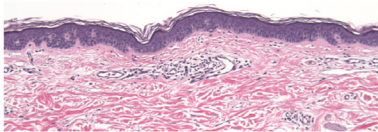
Normal



Psoriasis



Normal



Psoriasis

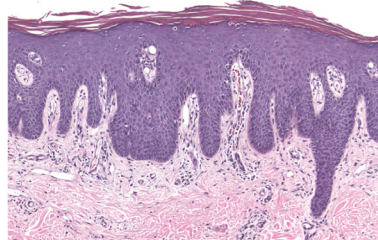


Figure 4. Normal skin vs. established psoriasis; both the diagrams (upper) and the histology (bottom) illustrate increased epidermal thickness and dermal lymphocyte infiltration. Reproduced and adapted with permission from “An Atlas of Psoriasis, Second Edition - 2004” by Lionel Fry (upper) (Copyright© 2004 Taylor & Francis Group) and Michigan Medicine (bottom) Copyright 2020 Regents of the University of Michigan.

1.2.1 Psoriasis clinical presentation

Psoriasis can manifest as various phenotypes clinically (*Figure 5*). The most common subtype – about 85-90% – is the chronic plaque-type psoriasis (also known as *psoriasis vulgaris*), red scaly plaques on scalp, knees and elbows. The guttate psoriasis, counting for the 10%, it’s an interesting subtype which differs not in the aspect but in its course: many little red spots that can be spread along the whole body, which can spontaneously (or with the help of some treatments) heal and in many cases never come back. Inverse psoriasis (<5%), which does not manifest scaly plaques but just red patches on the skin folds (inguinal area, armpit, intergluteal, etc.) and differentiate it from candidiasis it is often challenging. Pustular psoriasis, which includes palmoplantar pustulosis, acrodermatitis continua of Hallopeau and generalized pustular psoriasis, is characterized by blisters filled with pus and broad areas of red, inflamed skin. Finally, the erythrodermic psoriasis (1% to 2% of all cases) can involve up to 90% of the whole body and has the typical sunburn looking pruritic and inflamed skin [85, 94, 95].



Figure 5. Clinical features of psoriasis (PsO) –pictures of psoriasis subtypes and their body location. Reproduced and adapted with permission from Lionel Fry, 2004, “An Atlas of Psoriasis” Second Edition. Copyright© 2004 Taylor & Francis Group.

1.2.2 The genetics of psoriasis

Psoriasis has a strong genetic background. Results from twin and population studies have shown higher incidence of psoriasis (either vulgaris or psoriatic arthritis) in monozygotic rather than in dizygotic twins (probandwise concordance rate of 0.33 in monozygotic vs 0.17 dizygotic) [96, 97] and in first- and second-degree relatives of patients than in the general population [88, 98-100]. Linkage and genetic association studies comprising large case-control datasets revealed potential candidate susceptibility variants known as single nucleotide polymorphisms (SNPs) which are linked to psoriasis [101-104]. The allele HLA-C*06:02 is considered the major genetic determinant of psoriasis [105-107]. It is present in the psoriasis susceptibility-1 (PSORS1), a region that includes genes in the major histocompatibility complex (MHC). This contributes approximately to 40-50% of the psoriasis

heritability and encode for human leukocyte antigens (HLAs) [105-110]. Regarding psoriatic arthritis, the most shared comorbidity among patients with psoriasis, it has been shown that HLA-B alleles are associated with higher risk of developing psoriatic arthritis; in particular HLA-B*27 [107, 111, 112], while HLA-C*06:02 has lower association risk to this comorbidity [111].

Approximately 36 genomic susceptibility loci have been associated with psoriasis, some of them (PSORS1, PSORS2 and PSORS4) giving higher contribute to the disease than the others [105, 113, 114]. Mutations of the gene CARD14, localized in the PSORS2 region on chromosome 17 (17q25.3), are responsible for the enhanced pro-inflammatory effect of NF- κ B and overexpression of genes in keratinocytes which have been associated to psoriasis [115-117]. However, despite the proven genetic predisposition in psoriasis, the link between environmental triggers, gene expression alterations and genetic heterogeneity is not clear yet [118]. The majority of susceptibility genes identified in genome-wide association studies (GWAS) are related to adaptive immunity (cytokines, cytokine receptors and inflammatory pathways), while some are related to innate immunity, keratinocyte functions and skin barrier, suggesting that the genetic predisposition involves both the skin and the immune system [101, 102, 119]. One example for the skin-related genes are late cornified envelope (LCE) genes that are part of the epidermal differentiation complex (EDC) which is involved in the terminal differentiation of the epidermis [120, 121]. Meta-analysis studies reported an association between psoriasis and the common deletion LCE3C/LCE3B-del in the late-cornified envelope cluster, which is known to be on chromosome region 1q21.3, where the PSORS4 locus was identified [122, 123]. Since localized in the cornified layer of the epidermis, the assumption was that LCE genes encode for structural proteins involved in physical barrier function [8], even though other studies have provided hypothesis about their potential role as antimicrobial peptides [124].

1.2.3 Immunopathogenesis of psoriasis

Both the innate and the adaptive immune system play fundamental roles in the initiation and maintenance of psoriasis [125]. Immunologic changes are caused partially by the genes – since behind the skin inflammation in psoriasis, thousands of protein-coding genes are known to be differentially expressed – and partially by the environment. One of the key participants in the innate immunity are the keratinocytes, which can respond to different danger signals and recruit T cells to the skin, which are important in sustaining disease activity [34, 38]. Keratinocytes produce AMPs such as LL-37, defensins, and S100 proteins and, once injured or subjected to inflammatory negative feedback from certain cytokines, they can produce high doses of the chemokine CCL20 and CXCL8/IL-8. CCL20 has the purpose to recruit myeloid dendritic cells (DCs) and Th17 cells into psoriasis skin, while IL-8/CXCL8 functions as chemoattractant for neutrophils to recruit them to the

site of the lesion [34, 38, 126, 127]. Fundamental transcription factors in psoriasis lesions are cyclic adenosine monophosphate (cAMP), Janus kinase (JAK), activator protein-1 (AP-1), CCAAT enhancer binding protein beta (C/EBP- β) and nuclear factor- κ B (NF- κ B). These enhance the expression of pro-inflammatory cytokines upon modulation of TNF- α and IL-17 production, sustaining the inflammation loop in the lesional epidermis of psoriasis patients [128, 129]. A simplified representation of the immunopathogenic mechanisms in psoriasis is illustrated in *Figure 6*.

1.2.3.1 Cellular and molecular immunologic circuits in psoriasis

From a general point of view, the model of pathogenesis of psoriasis starts with a genetically predisposed individual, which encounters one or many potential environmental triggers inducing keratinocytes stress. It is known today that psoriasis is not exclusively a T cell-dependent disease but keratinocytes play a pivotal role in triggering the early stages of the pathogenesis and perpetuating the chronic inflammation in psoriasis, in a mutual interaction with the T cells [34, 93, 130, 131]. Stressed keratinocytes begin to release self-DNA and self-RNA complexes into the extracellular compartment. Plasmacytoid dendritic cells (pDCs) are activated by these complexes and initiate the production of IFN- α [132]. The presence of IFN- α lead to the activation and maturation of dendritic cells (DCs) which circulate in the lymph nodes where they present the putative antigen to the naïve T cells. Activated dendritic cells produce IL-23 and IL-12, which stimulates the three populations of CD4⁺ T-helper cells Th1 and Th17 [133]. IL-23 from inflammatory DCs activates Th17 cells to produce IL-17A and IL-17F, which drive keratinocyte responses [133-135]. Once activated, the epidermis can produce abundant cytokines and inflammatory mediators, including IL-8/chemokine CXCL8, MCP-1/CCL2, CXCL1, CXCL2, and CXCL3, and CCL20 [38, 88, 136]. These chemokines attract leukocytes such as neutrophils, DCs, and CCR6⁺ Th17 cells. CXCL9, CXCL10, and CXCL11 are also produced and they recruit additional circulating Th1 cells expressing CXC-chemokine receptor 3 (CXCR3⁺) and CC-chemokine receptor 4 (CCR4⁺) and Th17 cells expressing CCR4 and CCR6 into the dermis and epidermis. T cell-derived cytokines act on epidermal keratinocytes as proximal inducers of these inflammatory circuits [3, 38, 88, 136].

1.2.3.2 Autoantigens and psoriasis autoimmunity

More recently, activation of autoimmune pathways in psoriasis has emerged. Prinz et al. in 2015 has proven that antigen-specific CD8⁺ T cell mediate autoimmune response against melanocytes, hypothesizing that psoriasis is an autoimmune disease that depends on HLA class I. In the study they show that the main psoriasis risk allele HLA-C*06:02 confers susceptibility to psoriasis by promoting melanocytes-specific autoimmunity through presentation of **ADAMTSL5** (ADAMTS Like 5), autoantigen generated by ERAP1 (Endoplasmic Reticulum Aminopeptidase 1). If an inflammatory trigger meets under pathogenic predisposition related to

gene variants from interferon signaling pathway, NF- κ B activation pathway and others, then this would generate an increased inflammatory response, upregulation of pro-inflammatory signals and ligands-dependent recruitment of immune cells. The CD8⁺ T cells that infiltrate the epidermis allow the recognition of the autoantigen ADAMTSL5, which increases the risk for psoriasis in epistasis with HLA-C*06:02 [137, 138].

Besides ADAMTSL5, also the antimicrobial peptide LL37 (alternatively known as CAMP) was found having increased expression in keratinocytes of psoriasis lesions as well as in psoriasis-associated immune cells (e.g. macrophages and dendritic cells) [139]. Moreover, LL37 has the ability to convert inert self-DNA into condensed structures complexed with AMPs, activating TLR9 that allows plasmacytoid dendritic cells sensing viral and microbial DNA (event that does not occur in normal conditions). This results in the production of IFN- α , which provides inner stimuli for dendritic cells and T cells, leading to the development of psoriatic plaques. Since this represents a unique inflammatory pathway, which is normally used in antiviral immune responses, LL37 has been proposed as psoriasis autoantigen, responsible of triggering the inflammatory feedback loop [140, 141].

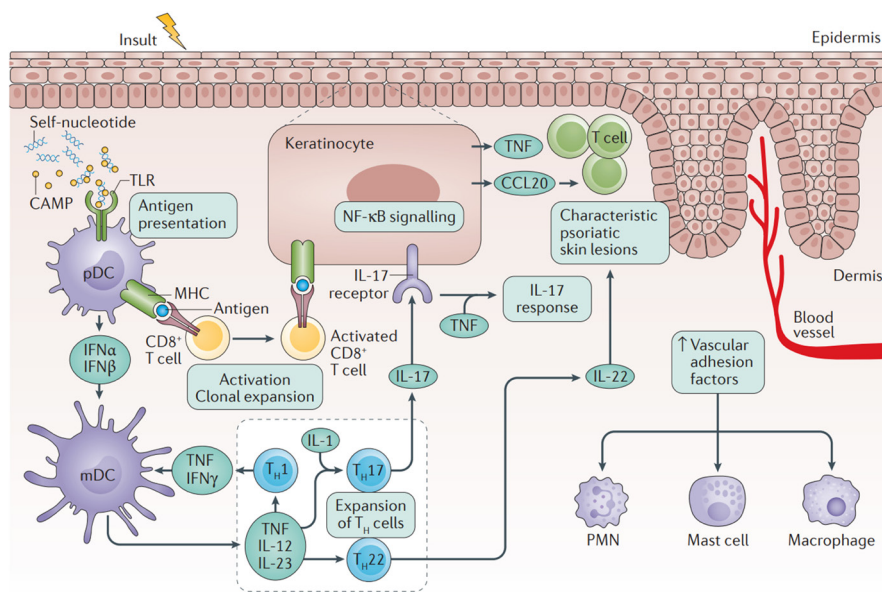


Figure 6. The immunopathogenesis of psoriasis. Interplay of cutaneous cell types, which is dependent on macrophages, dendritic cells, T cells, and other cells of the immune system, involves many cytokines and chemokines that orchestrate the pathological changes normal (left side) to psoriatic (right side) skin. Reproduced and adapted with permission from Greb, J. E., et al. (2016). "Psoriasis". Copyright© 2016 Macmillan Publishers Limited, part of Springer Nature.

1.2.3.3 Cytokine and chemokine imbalance in psoriasis

Cytokines such as IL-12 and IL-23, both released by dendritic cells and macrophages, induce the polarization of several CD4⁺ T cells which, in turn, produce some of the most important cytokines in psoriasis: interleukin-17A (IL-17A) and IL-17F, interleukin (IL-22), interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α), [38, 136, 142]. IL-23 in particular, has been recognized as a key player in the pathogenesis of chronic autoimmune diseases [143] and has a special role in psoriasis, through activation of Th17 lineage followed by release of IL-17 and IL-22 pro-inflammatory cytokines [144].

IL-17A is produced by CD4⁺ T cells which are polarized by IL-1 β and IL-6 cytokines into Th17 cells but also by the CD8⁺ T cells subset Tc17 [145]. It acts on keratinocytes leading to an increased expression of chemokines such as CCL20, CXCL1, CXCL3, CXCL5, CXCL6 and CXCL8 (IL-8), and it has been shown to play a role in recruiting myeloid dendritic cells, more T-helper 17 cells and neutrophils to the psoriatic lesion. IL-17A induces production of AMPs (such as β -defensin-2, S100 protein A7, A8, A9) and pro-inflammatory cytokines, as well as producing granulocyte colony stimulating factor (G-CSF). These, in turn, may help sustain immune responses in the skin, as well as inflammation and plaques development in psoriasis [38, 142, 146-148]. IL-17A has also the positive role in protecting skin from *Staphylococcus aureus* infections but also from gastrointestinal infections due to *Escherichia coli* and activating immune response against the bacterium responsible for tuberculosis (*Mycobacterium tuberculosis*) and against fungal infections [149-151]. Therefore, excessive IL-17A-related inflammation that could lead to chronic inflammatory diseases and the absence or dysfunctional IL-17A due to therapies with specific blockers while the body is infected by pathogens, represent a balance between two conditions that requires fine regulation in order to maintain the overall epithelia homeostasis [152].

IL-17F (known also as CANDF6 or ML1), shares the same locus and receptors as IL-17A, having the highest homology to this cytokine, being co-regulated and, therefore, often co-expressed [153]. Although only one polymorphism (rs763780 (His161Arg)) in the IL-17F gene has been recently associated to increased psoriasis susceptibility [154], high levels of IL-17F cytokine have been found in psoriasis lesional skin compared to non-lesional and healthy [155] as well as in the serum of the psoriasis patients [156]. In psoriasis-like model (imiquimod mice), IL17F has been found to promote skin inflammation upon infiltration of $\gamma\delta$ T cells and ROR γ t⁺ innate lymphocytes [157].

Other members of the IL-17 family (*Figure 7*) include IL-17C which, together with IL-17A and IL-17F, has the highest expression in lesional skin of psoriasis patients [158], while IL-17B, IL-17D show reduced expression in psoriasis skin [155], most likely providing a minor contribution to the inflammation occurring

in psoriasis. Finally, IL-17E (also known as IL-25) is produced by several different cell types including epithelial and endothelial cells, T cells, macrophages, dendritic cells and type-2 myeloid cells. It's upregulated in psoriasis skin lesions (produced by involved keratinocytes) but also atopic dermatitis and contact dermatitis [159-162].

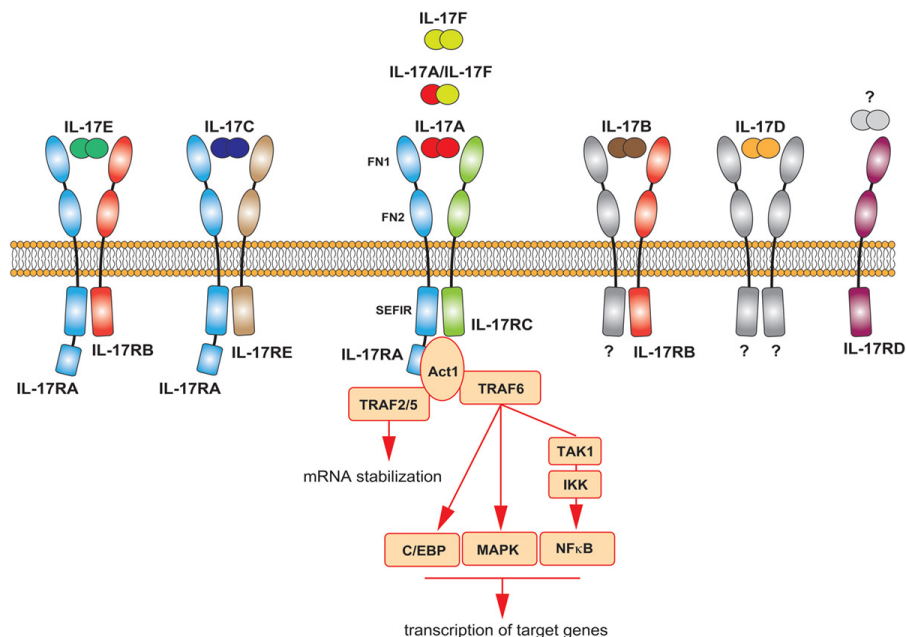


Figure 7. The IL-17 family members with respective receptors. Reproduced and adapted with permission from Brembilla NC et al., 2018; *Frontiers in Immunology*. Copyright© 2018 Brembilla, Senra and Boehncke.

IL-22, member of the IL-20 family and produced by T-helper 22 cells (and in part by Th17 cells), it inhibits epidermal differentiation leading to a disturbed skin barrier and induces pro-inflammatory gene expression and migration of human keratinocytes. Binding its receptors IL-22R1/IL-10R2 it activates JAK/STAT3 pathway, contributing to the stimulation of inflammatory responses [38, 136, 163-165]. Moreover, the activation of this pathway by IL-22 leads to a reduction in the expression levels of keratinocyte differentiation markers such filaggrin (FLG), loricrin (LOR), involucrin (IVL) and class I/II cytokeratin members (CKs) such as keratin 1 (KRT1) and keratin 10 (KRT10) [165, 166].

IFN- γ activates a signaling pathway that was considered to be the major player in the pathogenesis of psoriasis vulgaris [167]. Nowadays, IFN- γ has been redefined

as more relevant for the initiation phase of the disease [168] and its increased levels in the serum of psoriasis patients has been proposed as a marker of disease prognosis and severity [169]. It belongs to the interferon family type II and can be produced by T-helper 1 cells but also by T-helper 17 and NK cells [170-172]. Moreover, a population of T cells co-producing IL-17A and IFN- γ was identified in psoriasis lesional skin [173]. Keratinocytes, which carry the IFN- γ receptor on their membrane, in presence of the ligand respond by originating inflammatory and anti-viral responses through the JAK1-2/STAT1 signaling pathway [174].

TNF- α , member of the TNF superfamily and mainly stored by activated mast cells and macrophages in the skin, it can also be produced by CD4+ T cells NK cells, neutrophils and keratinocytes [175, 176]. The gene encoding for TNF- α is located on chromosome 6, nearby the Major Histocompatibility Complex (MHC) [177]. The TNF- α protein is released upon cleavage of its precursor pro-TNF- α expressed on the cell membrane, and it has a tight relationship with matrix metalloproteases (MMPs), which inhibition has been shown to prevent the pro-TNF- α processing [178]. Moreover, evidences of a potential synergism between IFN- γ and TNF- α in inflammatory atherogenesis have been proven [179], providing a rationale for dual cytokine antagonism since psoriasis and atherosclerosis share many similar underlying inflammatory mechanisms.

Collectively, the cytokines IL-17, IFN- γ , IL-22, and TNF can cause keratinocyte proliferation as well as production of chemokine, cytokine, and antimicrobial peptides, acting both independently or in exerting a synergistic effect. This becomes a self-amplifying loop, where these products act back on the DCs, T cells, and neutrophils to perpetuate the cutaneous inflammatory process [148, 164, 180-182].

1.2.4 Treatment of psoriasis

Depending on the severity of the disease, appropriate treatment can be initiated. Topical therapies (corticosteroids, vitamin D analogues and calcineurin inhibitors) are used for the treatment of mild-to-moderate psoriasis without PsA, while moderate-to-severe psoriasis patients are generally treated with phototherapy, methotrexate, retinoids or biologics [88, 183-185]. Phototherapy involves the use of UVB or psoralen plus UVA (PUVA) [84], with an efficacy that leads from 50% to 70% of patients achieving at least 75% PASI improvement after 4-6 weeks [186]. The phototherapy mechanisms of action are multiple: promotion of inflammatory cells (e.g. APC) apoptosis, stimulation of anti-inflammatory cytokine IL-10 production, with Th17 cell suppression and Th2 and regulatory T-cells activation [187]. Retinoids, analogs of vitamin A with the ability to inhibit epidermal cell proliferation and differentiation [188], have been used since the 80's [189] for the treatment of psoriasis vulgaris, alone or combined with UV light treatment [190].

Two of the traditional systemic therapeutics are methotrexate, synthetic analog of folic acid which downregulates the psoriatic key inflammatory cytokines and chemokines IL-17, IL-22, IL-23 and CCL20 [191], and cyclosporine, which acts by inhibiting T cell activity through the inhibition of calcineurin, a phosphatase that promotes the expression of the pro-inflammatory cytokine IL-2 [192]. Other well-known systemic therapeutics are acitretin, apremilast and fumarates [193-195].

Newest treatment targets discovered with the latest research on the immunopathogenesis of psoriasis opened new exciting therapeutic options with biological agents (or biologics). Biologics are drugs that selectively target specific molecules and are manufactured in living systems such as cells [183, 184, 196, 197]. TNF- α blockers, firstly implied in the treatment of rheumatoid arthritis (RA), were rapidly expanded to the treatment of psoriasis and psoriatic arthritis too, due to their ability to act on the IL-23/IL-17A axis and reduce the inflammatory effects by downregulation of IL-17A and/or its signaling pathway [198]. In 2004, the TNF- α antagonist etanercept (a recombinant human fusion protein rather than a monoclonal antibody) was the first biologic agent approved to treat psoriasis [199, 200]. After this, other TNF- α blockers have been released, such as infliximab, adalimumab and certolizumab pegol [201-204]. Secukinumab, ixekizumab (approved anti-IL-17A ligand monoclonal antibodies) and brodalumab (approved anti-IL-17RA monoclonal antibody) are biological antagonist of IL-17 pathway [129, 183, 184, 197]. Ustekinumab, on the other end, it's a monoclonal antibody directed against the subunit- β (p40) shared by both IL-12 and IL-23 cytokines, which inhibits the downstream signaling pathways (including IL-17) [205].

Newest biologics for the treatment of psoriasis through targeting the p19 subunits specific for IL-23 cytokine are guselkumab (FDA approved in 2016), a human immunoglobulin G1 lambda (IgG1) monoclonal antibody able to get efficacy 91% PASI 75 [206], tildrakizumab which, alike ustekinumab, it requires follow-up injection doses every 12 weeks [207], risankizumab [208, 209] and mirikizumab [210]. Bimekizumab, the first biologics able to neutralize simultaneously IL-17A and IL-17F, has shown extremely promising results already at phase IIb clinical trials, being able to reach efficacy 94% PASI 75 at week 12 and 60% PASI 100 at week 12 [211].

Finally, blocking IFN- γ pathway with specific antibodies has shown little or no therapeutic effect [212], another reason why, as mentioned earlier, it is believed that this cytokine plays a major role in the activation of antigen-presenting cells in the initiation phase of psoriasis, rather than maintaining the lesional psoriasis phenotype [168].

Nevertheless, due to limitations in the actual therapies, newer treatments (both systemic and topical) are still needed. For example, patients treated with IL-17A

blockers have increased predisposition to develop *Candida albicans* infections [213] or have inflammation at the gut mucosa, where the cytokine IL-17A has a protective role [150]. On the other hand, the selective IL-23p19 inhibitors have shown an impressive safety profile with no contraindications nor side effects reported within the data collected in the last few years [214]. In an ideal situation, the best treatment for psoriasis would be the one which gives a perfect clearance of the disease, with 100% PASI improvement in all the treated patients, with no side effects, that's convenient and easily accessible at low cost.

1.2.5 Comorbidities of psoriasis

Psoriasis is equally prevalent in both sexes, although it has been recently shown to be present in a more severe form on average in men [215] and it is considered an immune-mediated systemic inflammatory disease [216] associated with different comorbidities [217-219]. Among the classic comorbidities, we can find psoriatic arthritis (PsA), inflammatory bowel disease (IBD), metabolic syndrome (hypertension, obesity, diabetes, hyperlipidemia), cardiovascular diseases (CVD), psychological and psychiatric disorders (especially depression) and uveitis [219]. Those instead considered as emerging comorbidities in psoriasis are chronic liver diseases such as hepatitis and nonalcoholic fatty liver disease (NAFLD), lymphomas, chronic obstructive pulmonary disease, sexual dysfunction, obstructive sleep apnea (OSA), osteoporosis and even Parkinson's disease [218-220].

Up to one third (~30%) of patients with psoriasis develop **psoriatic arthritis** during the course of the disease; today, the term "psoriatic disease" is used which includes both cutaneous psoriasis and PsA being part of the same disease [221]. PsA is a seronegative inflammatory polyarthropathy with an age of onset in between 30 to 55 years, it affects men and women equally and it involves the peripheral joints (arthritis), axial skeleton (spondylitis), insertion of tendons and ligaments into bone (enthesitis), inflammation of whole digits (dactylitis) [222]. In the majority of cases, PsA appears years after the onset of cutaneous psoriasis –5 to 10 years for the manifestations to take place [223]. However, even in patients with known psoriasis, PsA is difficult to diagnose and it is common that patients get a diagnosis many years after the onset of joint symptoms [224]. The unspecific symptoms make the diagnosis challenging, and it is estimated that the prevalence of undetected psoriatic arthritis among patients with cutaneous psoriasis is to be between 10% and 15% [225]. Moreover, genetic susceptibility regions for psoriasis vulgaris and psoriasis arthritis are partially shared but not identical [226].

In recent years, tools to accurately measure the disease activity and questionnaires for patient's self-assessment have been made accessible to clinicians attempting to perform accurate diagnosis of psoriatic arthritis [227, 228]. The CASPAR

(CLASSification for Psoriatic ARthritis) criteria [229] is an accurate classification method that collects previously published principles to classify psoriatic arthritis and further develop them to improve their sensitivity and specificity for the diagnosis of the disease. The DAS28 (28-joint Disease Activity Score) [230], originally created to assess rheumatoid arthritis progression and improvement upon treatments, consists of the measurement of twenty-eight joints including proximal interphalangeal joints, metacarpophalangeal joints, wrists, elbows, shoulders and knees, taking into account both tender and swollen joints. The BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) is a score that quantifies the disease activity in axial psoriatic arthritis [231]. Despite the positive correlation of this with other disease activity scores, it is not able to discriminate patients with active peripheral psoriatic arthritis only from those with active axial and peripheral musculoskeletal disease. Recently, BASDAI has been implemented by combining it with the results from the CRP (C reactive protein) of the patients with cutaneous psoriasis, producing a new score system called ASDAS (Ankylosing Spondylitis Disease Activity Score) [232].

The currently available clinical markers for psoriatic arthritis such as scalp and intergluteal psoriasis, nail changes [233] as well as dactylitis, juxta-articular bone formation and negativity for the rheumatoid factor are not enough to obtain an accurate prediction of the disease progression (*Figure 8*). More recently soluble biomarkers including pro-inflammatory cytokines such as IL-6, CXCL10, as well as MMP3, high-sensitivity CRP and CPII have been proposed as screening markers for the identification of psoriatic arthritis in patients with cutaneous psoriasis [234, 235]. However, at the time of writing, no reliable biomarkers are available for this purpose in clinical practice.



Figure 8. Clinical features of psoriatic arthritis (PsA). (a) Involvement of the distal interphalangeal (DIP) joint in psoriatic arthritis. (b, c) Patient with arthritis mutilans displaying polyarthritis, evident psoriatic nail changes, and reduction in length of the first digit on the right hand. Reproduced and adapted with permission from P.J. Mease and P.S. Helliwell, 2008, “Atlas of Psoriatic Arthritis”. Copyright© Springer-Verlag London Limited 2008.

1.2.6 Psoriasis-like animal models

Psoriasis is a skin disease that primarily affects the human species and, besides few primates, no other animals present natural occurrence of skin inflammation or phenotype that resemble psoriasis [236]. Aiming to obtain better models to study the pathogenesis of the disease and support in vitro cell culture results, an effort was made by the research community to develop murine models with characteristics as similar as possible to psoriasis in human. The mice models can be represented in three major groups: (i) genetically engineered, (ii) humanized xenograft and (iii) acute or inducible [237-240].

The first group represents a mouse model with perturbations (transgenic or knock-out) of specific genes associated to psoriasis, and it can be either whole-body or conditional tissue-specific (skin-specific in this case) [239, 241]. Few examples are transgenic mice for *Vegf* [242], *Tgf- β* [243], *Il-36* [244], *Stat3* [245], *Ppar β /d* [246], and knockout mice for *Cd18* [247], *Il-1rn* [248], *Ikk2* [249], *JunB* and *Jun (c-Jun)* [250], *Ifnr-2* [251] or *Il-36RA* [252].

The second group consists of immunodeficient mice receiving human lesional or non-lesional psoriatic skin xenotransplanted on their back, which can be followed by a step of transferring T cells [239, 253, 254]. Boyman et al. in 2004 described a new xenotransplantation model, showing spontaneous formation of psoriasis-like skin lesions when human psoriasis non-lesional skin was transplanted onto AGR129 mice [255]. Here healthy skin grafted onto mice as control did not show psoriasis-like phenotype. A reason for this could be the increased number of T cells in non-lesional compared to healthy skin [256], although it has been proven that injecting T cells in healthy skin could not convert it into non-lesional psoriatic skin [257]. A possible explanation might be related to the absence of auto-antigens triggering the dendritic cells, which further promote the psoriasis-like phenotype [258].

The third group, in particular the imiquimod-induced model, is one of the most used psoriasis-like model to study this disease. Characterized and reported for the first time in 2009 [259], this model consists in first shaving the back of the mice in order to expose their skin, followed by the topical application of 62.5 mg of imiquimod cream (5% IMQ, Aldara, 3M Pharmaceuticals, St Paul, MN) [260]. The topical re-application of the synthetic imidazoquinolone amine compound occurs daily and lasts for about 5 to 7 days [259]. Use of Aldara cream was initially exclusive to treat skin malignancies such as basal cell skin carcinoma (BCC), actin skin keratosis and, occasionally, melanoma [261, 262]. Although the mechanism of action of imiquimod is not completely understood, it is now well established its role as ligand of the toll-like receptors TLR-7 and, to a smaller extent, TLR-8 [263]. The agonistic activity exerted by imiquimod towards these TLRs promotes infiltration of plasmacytoid dendritic cells (pDCs) and activation of IL-23/IL-17

axis, followed by downstream inflammatory signaling events, including the translocation to the nucleus of the transcription factor NF- κ B. This results in the production of the pro-inflammatory cytokines IL-2, IL-6, IL-8, IL-12, IFN α , TNF- α , chemokines CCL2, CCL4, CCL4, as well as NF- κ B-independent cytokines such as IFN- γ [264]. The application of imiquimod on the skin of mice results in hyperplasia of the epidermis with keratinocytes increased proliferation accompanied by erythema, scaling, acanthosis and impaired epidermal differentiation [259], all characteristic features of human psoriasis which make this model widely accepted as model of lesional skin development [239, 265]. From the transcriptomic point of view, different strains used to recreate this model have shown differences in the gene signature upon imiquimod application, with the C57BL/6 J (B6) being the most consistent, best-replicating the gene alterations found in human psoriasis lesions [266].

Direct intradermal injection of IL-23 cytokine in mice was also shown to induce inflammation and phenotypic effects similar to psoriasis, through the CCL20 receptor CCR6 [267, 268]. A study showed that ear injection of IL-23 in IL-22^{-/-} mice resulted in low ear swelling and epidermal hyperplasia but same results were obtained in IL-17^{-/-} normally expressing IL-22, suggesting the importance of IL-17A in mediating psoriasis-like inflammation and phenotype in this mouse model [135]. On the other hand, mice lacking the CCL2-ligand receptor CCR2 – important for different immune diseases, including psoriasis– following injection with IL-23 had increased production of Th2-dependent cytokines such as IL-4, characteristics of atopic dermatitis-like inflammation [269]. This suggests careful consideration when using this model for psoriasis related studies.

1.3 Protein-coding RNAs

The human genome is constituted of ~2-3% of DNA which is transcribed into RNA and this, once exported from the nucleus to the cytosol, is translated into proteins [270-272]. Protein-coding genes could be in single copy or in tandem clusters – a collection of identical copies of genes which are transcribed simultaneously, in order to increase the transcription output of these genes [273, 274]. The proteins resulting from the transcription of protein-coding genes belong to different categories, i.e. cytokines, growth factors, receptors, transcription factors, phosphatases, structural proteins and transporters (*Figure 9*). The exact number of these protein-coding genes is not clearly understood yet (approximately 20-25,000 in the human genome) and can be different between individuals, as well as between different cell types in the same individual [275, 276]. The gene transcription rate can be altered by variations in our DNA, serving as a marker for the onset of complex traits or disorders [277, 278]. An example is TP53 (OMIM #191117), probably the gene with the highest frequency of mutation in the human genome, that leads to the altered expression of its p53 protein. This, in turn, loses its activity as tumor

suppressor and gains functions that promote the development of cancer in different cells and organs [279-281]. A variety of protein-coding genes are known to be altered in the skin, having potential implications in skin aging [282, 283] and being directly implicated in the pathogenesis of multiple skin diseases [284, 285].

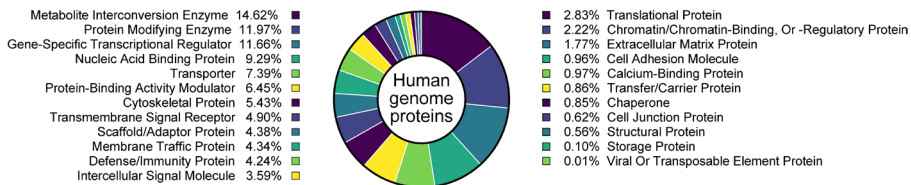


Figure 9. Human genome classification according to functions of its genes transcribed into proteins. Percentage of genes serving for each function it is given.

Some parts of the genome are transcribed into RNA which, on the other hand, is not translated into proteins. This give raise to a class of RNA called non-coding RNA.

1.4 Non-coding RNAs

About 97-98% of the approximately 3 billion base pairs that compose the human genome was referred to as “junk DNA” [286] and simply considered useless for decades. Our understanding of the genome has advanced tremendously with improved genomic technologies that have enabled scientists to finally bring the picture into focus. An important role in the RNA revolution occurred in this field was played by the ENCODE [287, 288] and FANTOM projects [289, 290]. Thanks to these consortiums, it was clear that a large part of the non-protein-coding genome is indeed transcribed into RNA [291]. Since the discovery of the small regulatory RNA which does not code for proteins called microRNAs (such as lin-4 and let-7) in the 1990s, our understanding of ncRNAs as regulatory molecules continuously evolved [292-294]. Following studies shed some light on new categories of non-coding RNAs, such as small nucleolar RNA (snoRNAs), responsible for chemical modifications of rRNAs (most abundant RNA molecules in the cell) and tRNAs (decode the mRNA sequence into peptide or protein), and PIWI-interacting RNAs (piRNAs), which preserve the genomic integrity by suppressing mobile genetic elements [295-297]. Moreover, more than 10,000 RNA molecules longer than 200 nucleotides, called long non-coding RNAs (lncRNAs) have been reported in human [298-300]. Still largely uncharacterized category, recent works suggested multiple roles of the lncRNAs, such as the epigenetic control of gene expression, promoter-specific gene regulation, X-chromosome inactivation, imprinting, maintenance of nuclear architecture, often in accordance to their cellular localization (nuclear or cytoplasmic) [301-304]. A simplified classification of the coding and non-coding RNA classes is shown in *Figure 10*.

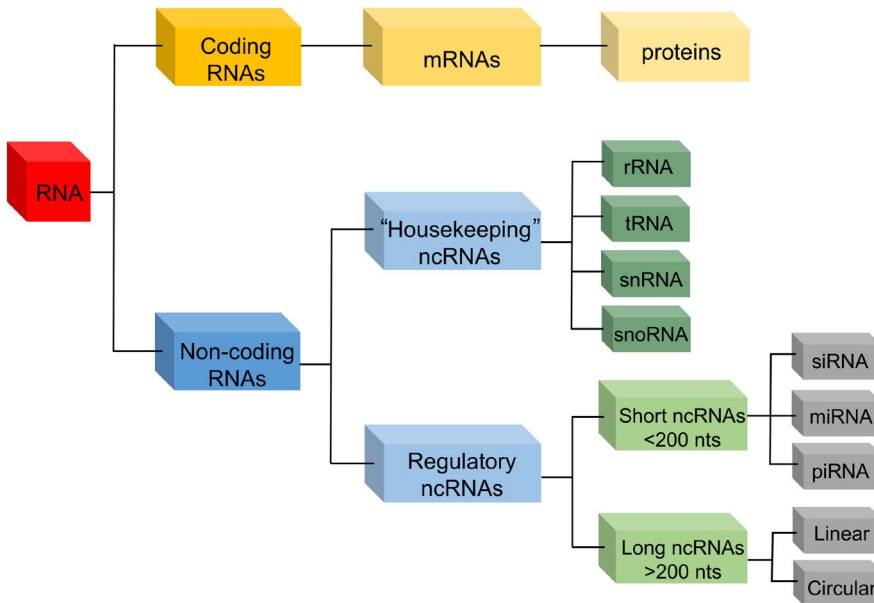


Figure 10. Major classes of coding and non-coding RNAs in the eukaryotes.

1.4.1 MicroRNAs

Among the non-coding RNAs, microRNAs (miRNAs) are the most well characterized and most conserved among species. miRNAs are short RNAs (~19-25 nucleotides) which previously were not considered to have any function [305]. The interest for microRNAs began with the discovery of lin-4, a small RNA molecule of 22 nucleotides which interferes with a specific developmental transition phase in *Caenorhabditis elegans* [292]. For almost seven years no studies were published in the field, until a second similar short RNA, called let-7 (lethal-7), was discovered [294]. The peculiarity of let-7 is that it is one of the most conserved elements in the animal kingdom (100% conserved sequence between flies and humans), suggesting a role of this small molecule family in the genome. MicroRNAs have extremely high tissue-, cell- and sometimes species-specificity compared to protein-coding genes [306]. Today, microRNAs are considered to be fundamental regulators of gene expression, in addition to epigenetic, transcriptional, translational regulation and other kind of modifications [292]. In human more than 2,000 miRNA genes are known (even more than any other transcription factor family).

1.4.1.1 *MicroRNA biogenesis and functions*

MicroRNAs, alike protein-coding genes, are encoded in the DNA and transcribed by RNA polymerase II into a long primary transcript (pri-miRNA) [307]. This forms hairpins which is cleaved-off by a microprocessor complex composed of Drosha (RNase III endonuclease) [308] with its cofactor DGCR8 and exported to the cytoplasm in the form of precursor microRNA (pre-miRNA; 60-70 nucleotides) by the carrier exportin 5 (XPO5) [309]. Here the terminal ends are further cleaved by the RNase III enzyme DICER (with TRBP or PACT) [310], processing the pre-miRNA into a double-stranded RNA (~22 nucleotides); one strand of the resulting microRNA-duplex is incorporated into a larger protein-complex called RISC (RNA Induced Silencing Complex), which includes the argonaut protein 2 (AGO2) [310, 311]. This complex mediates the binding of the mature miRNA seed sequence to the 3' untranslated region (UTR) of the target messenger RNA(s) [292]. Therefore, if a microRNA is expressed in the cell, the protein synthesis will be inhibited since the ribosome cannot translate the RNA when RISC is bound to it [312, 313]; alternatively miRNA-induced mRNA degradation can occur [314, 315]. MicroRNA biogenesis is summarized in *Figure 11*.

Since the mRNA interaction with the seed sequence of the miRNA is short (6 to 8 nucleotides), hundreds or even thousands of genes can be targeted by the same miRNA, which can in this way simultaneously regulate different biological processes and pathways [316].

Very often miRNAs have relatively mild effect on selective mRNAs, but the target itself is not randomly chosen from the transcriptome; indeed, a miRNA may regulate a receptor, some components of the signal transduction pathway and transcription factors, inducing rather dramatic phenotypes [317, 318].

Some microRNAs reside in introns of both protein-coding genes and long non-coding RNAs, while few exceptions reside in the exons [319-321]. Another interesting feature of the miRNAs is their coexistence in more copies in the genome; they can be present on different chromosomes and in multiple copies, confirming their robust transcription (they are abundantly present) [322].

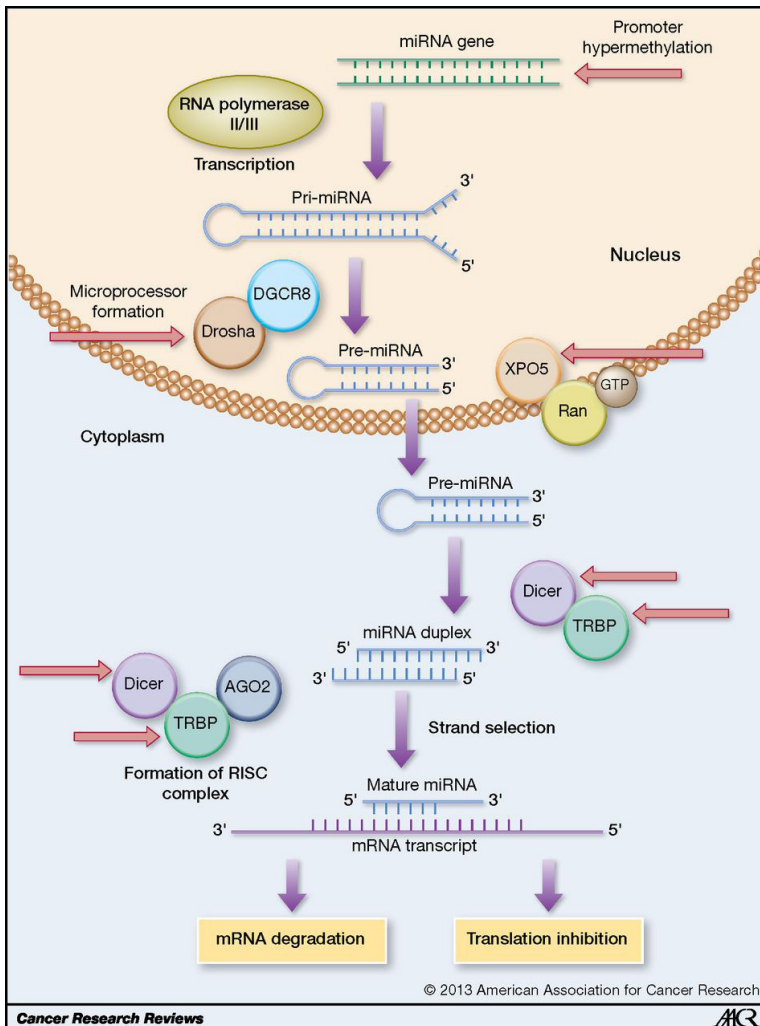


Figure 11. Model of microRNA biogenesis and posttranscriptional regulation of genes. Reproduced and adapted with permission from Mulrane L et al., 2013; Cancer Research. Copyrights© 2013 AACR

1.4.1.2 MicroRNAs in skin and psoriasis

The potential of microRNAs in the global regulation of gene expression and developmental transitions during mammalian skin development, led to an increased interest in the function of these messenger-RNA regulators in stem cell biology and developmental biology [323]. Some of the most abundant microRNAs such as miRNA-200 and miRNA-19/miRNA-20 families, have epidermis-specific

expression while the miRNA-199 family is largely expressed in the hair follicles [324]. In 2007, our research group showed for the first time altered expression of a set of miRNAs in psoriasis skin, with a microRNA signature distinct from healthy skin and also from atopic dermatitis, another inflammatory skin disease. Further studies from our group have demonstrated that differentially expressed miRNAs regulate cellular processes with relevance for psoriasis, such as keratinocytes proliferation and differentiation (miR-125b, miR-203) [325], apoptosis of activated T cells (miR-21) [326], keratinocyte-immune cell cross talk (miR-31) [327] and regulation of IL-17- and Toll-like-receptor (TLR)-mediated inflammatory responses in keratinocytes [328, 329]. Other groups have identified additional miRNAs in the context of psoriasis such as miR-135b, which regulates keratinocyte differentiation and proliferation [330, 331], miR-136 that provide regulation of TGF- β 1-induced keratinocyte proliferation arrest [332, 333]. Zibert et al. miRNA profiling studies in psoriasis identified miR-21, -205, -221 and -222 targeting mRNAs having a role in cellular growth, epidermal development, proliferation, apoptosis, degradation of the extracellular matrix and immune and inflammatory responses [333]. In addition, combining NGS and LCM, Løvendorf et al. identified miR-193b down-regulation and miR-223 up-regulation in Th17 cells of dermal infiltrates of psoriatic plaques [334].

MicroRNAs have a therapeutic potential in patients with psoriasis. For example, the role of miR-146a in psoriatic patients was assessed by our group, using miR-146a^{-/-} mice in conjunction with the imiquimod-induced mouse model of psoriasis [329], and by others [335, 336], as well as by Rebane et al. in atopic dermatitis [337]. miR-21 inhibitor study on the xenograft model have proven impaired transcriptional activity of Jun/activating protein 1 (AP-1), followed by activation of IL-6 and Stat3 signaling pathways in psoriatic lesion [338].

1.4.1.3 Circulating microRNAs as potential biomarkers

While miRNAs act within the cell, they are also present in cell-free body fluids such as blood, serum, plasma, saliva etc., partially within extracellular vesicles (EVs), partially bound to lipids or proteins (LTPs, lipid-transfer proteins) representing non-vesicular carriers [339, 340]. MiRNAs circulating within extracellular vesicles in body fluids are very stable since resistant to degradation by RNases. This makes them ideal candidates as biomarkers [341, 342]. Beside their hypothesized role as mediator of intracellular communications [343], circulating miRNAs have also been proposed as biomarkers in several diseases, in particular cancer but also in inflammatory and metabolic diseases [344-348], as well as ageing and age-associated disorders [349]. More recently, circulating microRNAs have found interest as potential biomarkers in skin-related diseases such as melanoma, for either diagnosis, recurrence and/or progression [350, 351]. Very little is known about microRNAs as biomarkers in psoriasis but detection of some of them (such

as miR-223 and miR-143) has been proven to provide potential clinical applications for diagnosis, prognosis, and treatment responses of psoriasis [352, 353]. Increased serum levels of miR-33 and miR-424 have been found in psoriasis patients compared to healthy controls [354, 355]. A recent study proposed six miRNAs (miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p and miR-21-5p) with significant up-regulation in serum of psoriatic arthritis patients compared to healthy controls, as potential biomarkers for PsA [356]. Finally, in 2013 our group observed four circulating miRNAs whose levels were significantly altered (increased: miR-128a; decreased: let-7d, miR-142-3p, miR-181a) in psoriasis compared to healthy serum, as well as upon anti-TNF- α treatment [357].

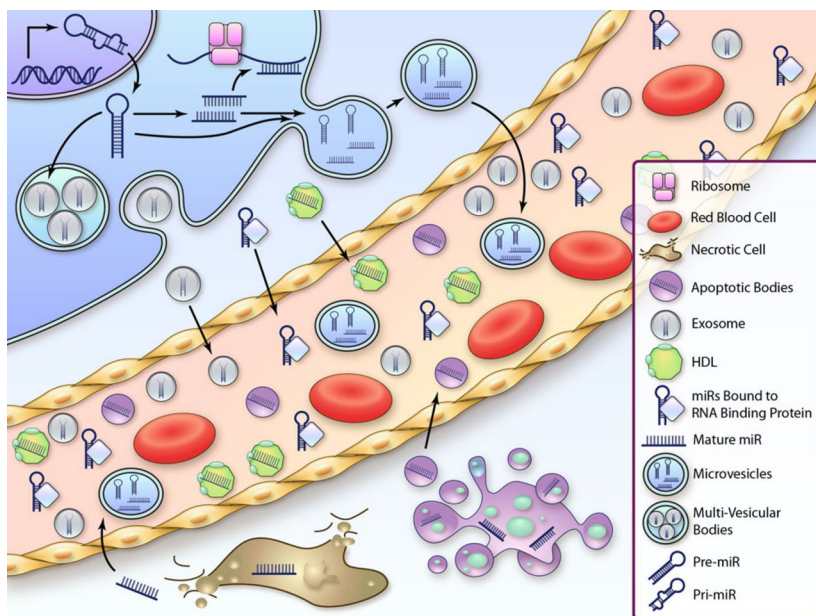


Figure 12. Circulating microRNAs enclosed in different compartments including vesicles (exosomes, microvesicles, multi-vesicular and apoptotic bodies), protein and lipid complexes. Reproduced and adapted with permission from Kumarswamy R et al., 2013; *Circulation Research*. Copyrights© American Heart Association.

1.4.2 Long non-coding RNAs

LncRNAs are transcripts longer than 200 nucleotides in length, they are mostly (but not all) transcribed by the RNA polymerase II, 5'-capped, spliced, sometimes polyadenylated and they appear not to have conserved open reading frames (ORFs), thus without protein production capacity, but still sharing traits with their protein-coding mRNA cousins, such as exhibiting intron-exon splicing and

regulated transcriptional control [358, 359]. In 2001, Mattick et al. proposed long non-coding RNAs and introns as part of a unique network with properties like supporting communications between genes, hypothesizing a superior complexity of the eukaryotic genome in carrying out multiple tasks simultaneously [360, 361]. In 2005, the FANTOM3 project started shading some light on the transcriptional complexity of mammalian genomes. Thanks to the so-called CAGE (Cap Analysis Gene Expression) and GIS (Gene Identification Signature), it was possible to precisely map the 5' and 3' ends of transcripts from 237 full length cDNA libraries prepared from a wide collection of mouse cells and tissues [358]. According to the ENCODE project [287, 288], between 10,000 and 200,000 lncRNAs are estimated to be present in the mammalian genome [362, 363]. Some of the most well-known lncRNAs are Xist (X-inactive specific transcript), discovered in 1991, with a role in the chromosome X inactivation [301] and H19, the very first lncRNA discovered in Eukaryotes, with multitasking properties [364].

Transposable elements (TEs), which represent about 40-45% of the genome [272], are considered the main contributors of long non-coding RNAs origins and divergences among different species [365-367] (*Figure 13*). Although a broad spectrum of lncRNAs are also known to originate from pseudogenes [368-370].

Since lncRNAs often have little homology across species, model systems where to study them must be chosen carefully. They are typically expressed at much lower levels than mRNAs and very sensitive detection methods are required to measure changes in expression [371, 372]. They can exist in complex genome sites between protein coding genes, within genes or even overlapping other genes, making crucial the specificity of the assay used for detection. Despite being a relatively new field, lncRNAs are gaining enormous interest from researchers, principally because they can play significant roles in our biology such as (1) cell development and differentiation [373], (2) epigenetic regulation [363] and (3) pathogenic disease [374], all processes where they might represent a new class of biomarkers or druggable targets for cancer, heart disease, inflammation, diabetes and neurodegeneration just to name a few.

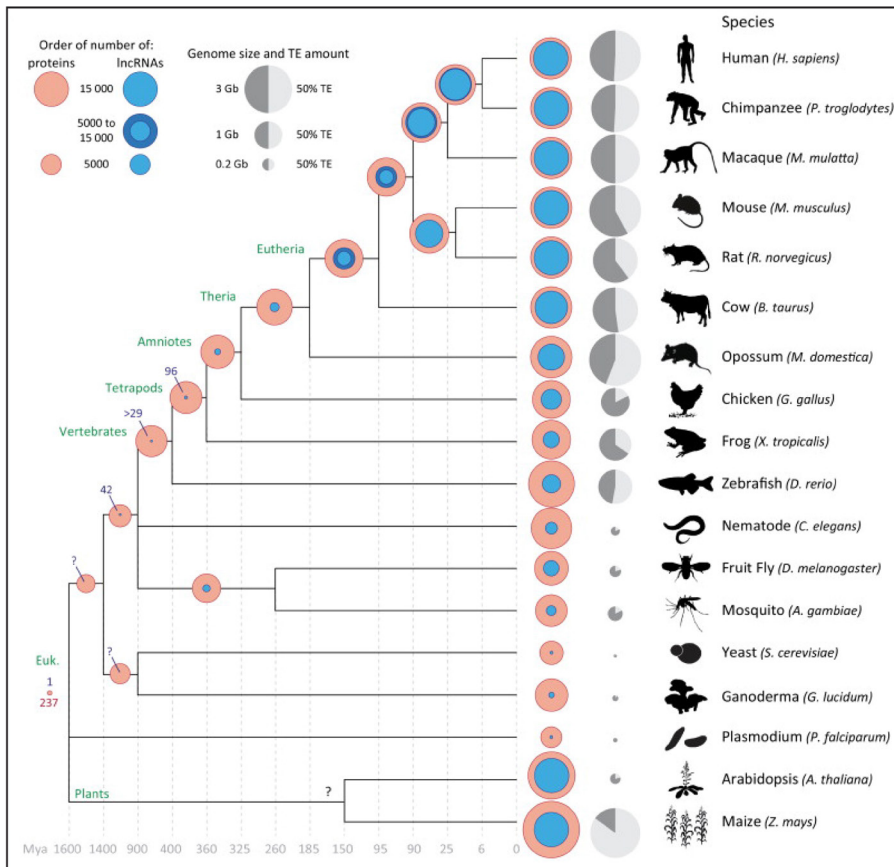


Figure 13. *lncRNA* expression divergence between species. Content of protein-coding (red) and long non-coding RNAs (blue) in different species during evolution. Transposable elements (TEs) and size of the genome are shown in gray. Reproduced and adapted with permission from Kapusta A & Feschotte C, 2014; *Trends in Genetics*. Copyrights© 2014 Elsevier Ltd.

1.4.2.1 Genomic location of *lncRNAs*

According to where they are found relative to nearby protein-coding genes, long non-coding RNAs are transcribed from intergenic regions, exons, introns, and overlapping regions [372]. Intronic *lncRNAs* are actively transcribed and spliced from the intron of a protein-coding gene, while long intergenic non-coding RNAs (*lincRNAs*) are transcribed from intergenic regions [375, 376]. Antisense *lncRNA* are generally transcribed from the antisense strand of genes coding for proteins [377], while sense *lncRNA* are transcribed from the sense strand and can overlap with part of protein-coding genes or covering the entire sequence of a protein-

coding gene through an intron [378]. Conservation among species has been shown to have direct association with the genomic location of lncRNAs [379].

1.4.2.2 Mechanisms of action of lncRNAs

The features of the lncRNAs allow them to direct epigenetic complexes in *cis* (on the chromosome from which they are transcribed) or in *trans* (other chromosomes from which their transcription occurred) [380]. They are unique in their ability to act in an allele-specific way, as they are tethered to the locus in *cis* during the transcription. Because the RNA molecule is functional and its structure will often dictate function, the secondary and tertiary structures of lncRNAs help to determine their role(s) [381, 382]. Long non-coding RNAs can function in multiple ways, according to their nuclear or cytoplasmic localization and whether they are *in cis*- or *in trans*-acting lncRNAs [383].

Enhancers: linking three-dimensional chromosome conformations by taking enhancers to their target promoters or activator sites along the genome, mediating the activation of neighboring genes or transcripts. The expression of these enhancer RNAs (eRNAs) usually correlates with the expression of neighboring genes [384, 385].

Guides (e.g. XIST in *cis* and HOTAIR in *trans*): the lncRNA can bind the epigenetic complex in a sequence-specific manner, “guiding” the epigenetic machinery in its processes, for example histone methylation [301, 303, 386].

Scaffolds (e.g. HOTAIR and NEAT1): they mediate the formation of large ribonucleoprotein complexes, binding other RNAs through base pairing and so acting as sensors for mRNAs, miRNAs or other lncRNAs; they can also bind one or more proteins through separate domains. Essentially when the lncRNA acts as a scaffold, rather than just recruiting one complex it tends to recruit more, bringing them somewhere else [386].

Decoys (e.g. MALAT1): provide binding sites for DNA binding proteins or other regulatory molecules, preventing them from binding their targets. For example, they can act as a “sponge” on specific microRNAs, moving them away and determining their gene regulation [386, 387].

Signals: where basically is the transcription itself running through a locus which has the function, rather than from the lncRNA that’s being produced [387].

Reservoirs: such as H19 which acts as reservoirs for specific miRNAs in order to release them in a secondary moment [388].

Other lncRNAs features are represented by the formation of RNA-protein complexes which influence the regulation of gene expression [389], and their interaction with DNA [390] and other RNAs [391] through their secondary structures. Moreover, studies have shown that long non-coding RNAs can have a role in dosage compensation [392], genomic imprinting [393] and they can act as transcriptional activators [394] or repressors [395].

This section has the aim to give more insights to the long non-coding RNAs mechanisms of action previously described, according to their subcellular localization (*Figure 14*).

Generally, **nuclear** lncRNAs are involved in gene regulatory processes [396], including transcriptional regulation through the interaction with the transcription preinitiation complex at the promoter-specific level –in turn, this alters the local chromatin accessibility. The lncRNAs Evf-2, also known as DLX6-AS1 and LED (LncRNA activator of Enhancer Domains), appears to act as a transcriptional activity modulator at enhancers sites [397]. In the cell nucleus they can also simultaneously activate/repress multiple gene loci through the modulation of the chromatin accessibility, promoting epigenetic gene regulation (examples are the lncRNAs FENDRR and HOTAIR) [363, 398, 399]. Finally, several nuclear lncRNAs might be capable to regulate the nuclear architecture. Examples of this type of regulation are the X chromosome inactivators FIRRE and XIST [400, 401] and NEAT1, a lncRNA associated to paraspeckles in the nucleus [402, 403]. Upon specific inflammatory signals, it can dissociate from the paraspeckles, translocate to the cytoplasm and regulate the expression of genes involved in the activation of the inflammasome in macrophages [404] or in different cancers [405, 406].

Long non-coding RNAs residing in the **cytoplasmic** compartment have been shown to be involved in the control of mRNA stability through targeting double stranded binding regions of the mRNA target, providing positive and negative post-transcriptional gene modulation [407]. An example is the cytoplasmic lncRNA TINCR (terminal differentiation-induced ncRNA) shown by Kretz et al., which directly interacts with ds-binding protein Staufen-1 (STAU1), promoting mRNA degradation [408]. This reflects the role of certain lncRNAs as regulators of the translation. For example, lncRNA-p21 can directly modulate the translation of messenger RNAs such as JUNB and CTNNB1 through the RNA-binding protein HuR; if HuR is not available, lncRNA-p21 can repress the translation of these mRNAs [409]. At last, cytoplasmic lncRNAs can also regulate gene expression by acting as microRNA sponges, also known as competing endogenous RNAs (ceRNAs) [410]. This is because the lncRNA-miRNA complex does not allow the microRNA to reach the actual target, modulating miRNA-mediated silencing of the messenger RNA [411, 412]. Other examples of miRNA sponges are the circularRNAs (circRNAs) [413], such as circNRP1, which promotes gastric cancer progression by sponging the miRNA miR-149-5p [414].

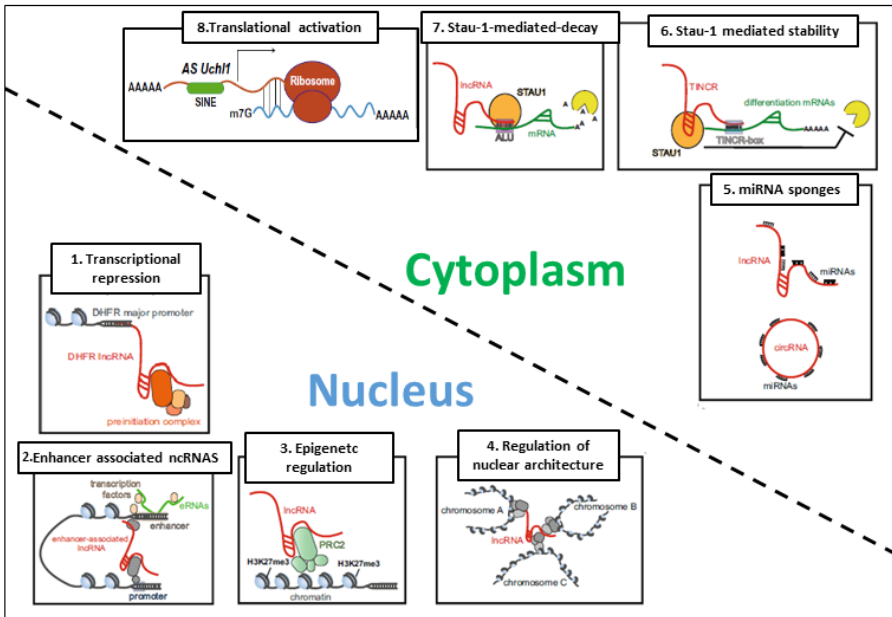


Figure 14. Functions of nuclear and cytoplasmic long non-coding RNAs. Nuclear localized lncRNAs can act as 1. Transcriptional repressor by interacting with both DNA element and transcription machinery 2. Enhancer by docking transcription factors to regulate gene expression in cis and trans. 3. Epigenetic regulators by guiding chromatin modifiers to different genomic location 4. Regulator of nuclear architecture by mediating inter chromosomal interaction. Cytoplasmic lncRNAs function as 5. MiRNA sponges to hijack several miRNAs at a time. 6 and 7. Regulator of mRNA stability by interacting with different mRNA binding proteins 8. Translational activator by recruiting ribosomes to 5' cap. Reproduced and adapted with permission from Hombach S and Kretz M, 2016; "Non-coding RNAs: Classification, Biology and Functioning". Copyrights© Springer International Publishing Switzerland 2016.

1.4.2.3 Long non-coding RNAs in skin and psoriasis

Several lncRNAs appear to be expressed only in specific conditions, such as precise developmental time points [415, 416] or in response to stress and other external signals [417]. To date, little is known about the role of lncRNAs in normal skin biology and in psoriasis. The first lncRNA described in psoriasis was PRINS (Psoriasis Susceptibility-Related RNA Gene Induced by Stress), a lncRNA showing up-regulated expression already in non-lesional psoriatic epidermis, which regulates the stress response of keratinocytes and it has equal expression throughout all the epidermal layers [418] (Figure 15). Studies by Kretz et al. have characterized the role of two lncRNAs in epidermal differentiation. TINCR (terminal differentiation-induced ncRNA) [419], which is mostly expressed in the differentiation

compartment (suprabasal layer of the epidermis) and ANCR (anti-differentiation ncRNA) [420], more abundant in the basal layer of the epidermis, where progenitor cells reside (*Figure 15*). A recent study from Gupta et al. characterize the long noncoding RNA transcriptome in lesional full-depth skin from psoriasis patients before and after treatment with adalimumab monoclonal antibody. RNA-Seq analysis revealed that almost one thousand lncRNAs were significantly deregulated in the psoriasis lesion after the treatment with adalimumab [421]. Similarly, weighted gene co-expression network analysis (WGCNA) on RNA-seq data from psoriasis patients (before and after treatment with adalimumab) and healthy controls, allowed the detection of specific networks and pathways previously not identified through differential expression analysis, with potential roles for lncRNAs [422]. The RNA-Seq study from Tsoi et al. aimed to identify deregulated lncRNAs in psoriasis patients, including full-depth skin biopsies from lesional and non-lesional/uninvolved psoriasis, as well as from healthy donors [423]. Their computational approach resulted in the detection of about three thousand lncRNAs with known annotation and about thousand novel deregulated lncRNAs with skin-specificity, enrichment in the EDC (epidermal differentiation complex) and potential functions in the immunopathogenesis of psoriasis [423].

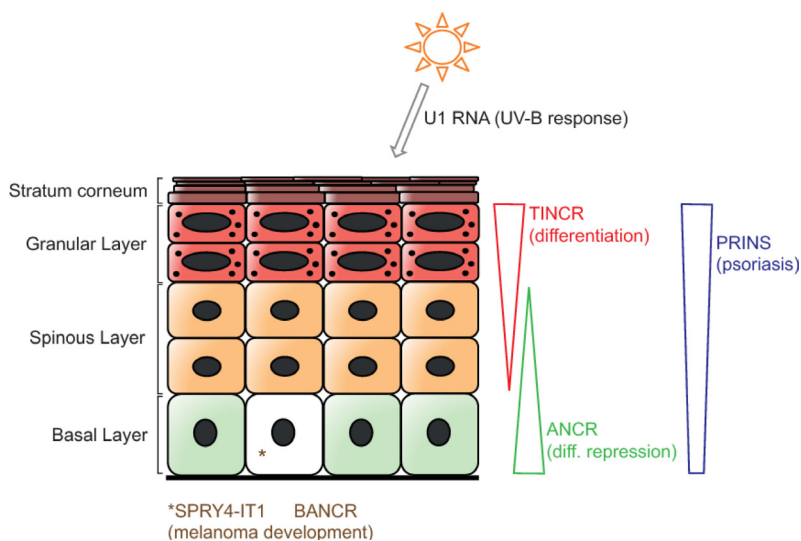


Figure 15. Long non-coding RNAs PRINS, TINCR and ANCR identified as potential regulators of the epidermal homeostasis. Reproduced and adapted with permission from Hombach S and Kretz M, 2013; *BioEssay*. Copyrights© 1999-2020 John Wiley & Sons.

1.5 Transcriptomic profiling technologies

The DNA, with its nearly 3 billion base pairs, in absence of specific alterations (e.g. sun-damage induced mutations) remains unchanged in each cell of an organism. What changes is how each part of the DNA (genes) that encodes a specific function is expressed and, therefore, how the cell alternates the expression of its genome to make proteins, the effectors that allow the modifications. We can then refer to “gene expression” talking about a whole series of events that allow certain portions of the DNA to be read out and to contribute to a certain function in the cell [424].

We call the mechanism of measuring the levels of each gene in the genome at a precise moment “gene expression profiling”. This allows a deeper understanding of the cell’s functions at a certain time [425, 426]. In recent years, several techniques have been developed to perform accurate analysis of gene expression and identify differences between cell types, their stage of development or their function in presence of a pathological condition, as well as producing diagnostic tools for specific diseases.

1.5.1 Microarray

A microarray (or DNA chip) is an array of oligonucleotide probes closely arranged on a small solid surface [427]. The probes, which represents oligonucleotide sequences of known genes, are synthesized on the solid surface in a way that both position and the nucleotide sequence (between 15 to 25 nucleotides in length) are known. They can be used to identify genes by hybridization of the DNA from the organism to the oligonucleotide probes in the microarray. The DNA of the organism being studied is fragmented and tagged with a fluorescent dye; the fragments are then incubated with the chip and the DNA which is complementary to the probes in the microarray bind together, while the unbound DNA is washed away. The surface of the microarray is then scanned with a laser beam and the data obtained is used to produce a visible image. The intensity of fluorescence is used to indicate the extent of hybridization of cDNA (obtained from mRNA) to different probes. Over 200 thousand probes can be constructed on a single microarray, giving the possibility to quantify an arbitrarily large number of different nucleic acid sequences in solution [427, 428].

Being economically advantageous, microarrays are suitable for processing large number of samples or for having microarray-based diagnostic tests with clinical utility. Moreover, microarray data storage and analysis is less challenging compared to the latest technologies. Despite background hybridization is a limitation in terms of accurate expression measurements of transcripts low in abundance, microarray are still adequate tools to answer specific questions through the investigation of well annotated genes, for which probes are designed [429]. In addition, a recent

study comparing Affymetrix HTA 2.0 microarray with Illumina 2000 RNA-seq for analysis of patient samples identified highly consistent results of gene expression analysis between the platforms, but discordant results in terms of alternative splicing identification, with higher stochastic variability for the RNA-seq data, lacking reads for short and low abundant genes [430].

1.5.2 Next-generation sequencing

Genome-wide coverage of transcripts is possible with RNA-sequencing. RNA-seq is a high-throughput profiling technology at single base resolution with low background noise, which can be used to simultaneously map transcribed regions of gene expression as well as distinguishing different isoforms and allelic expression. Compared to microarray, Next-generation sequencing technology does not require probes which are species- or transcript-specific (no prior knowledge of genome sequence) and has minimal or absent background signal. This allows the detection of novel, unannotated transcripts (with deep coverage and high resolution), novel isoforms from alternative splicing events, fusion genes and single nucleotide variants [431-434].

Briefly, the workflow consists first in converting long RNAs into a cDNA fragments library through nucleic acid fragmentations. This is required since the current sequencing machines can handle 200-300 bp long fragments. Sequencing adaptors are then attached to each cDNA fragment, allowing (i) the sequencing machine to recognize the fragments and (ii) to sequence different samples simultaneously. PCR will amplify only those fragments with sequencing adaptors. Following quality control, which includes verification of library concentration and fragments length, sequencing data is generated at high throughput manner from both ends. The resulting sequence reads are then aligned with the reference genome or transcriptome and are classified into three types: exotic reads, junction reads and poly(A) reads. Expression profile can be generated by using these three types of reads and the number of reads that align to each full-length transcript represents the expression level of each gene [435, 436].

However, RNA-seq has faced some challenges too, such as short reads and PCR-bias during amplification of the adapter-ligated library. Since the introduction of this technology, the system has been updated with increased reads length and strand-specificity, which can be used for high levels of mappability and *de novo* assembly of transcriptomes. Moreover, a third generation of NGS abolishes the need of genome fragmentation, enabling full-length transcript sequencing and directly detecting epigenetic modifications on native DNA [437, 438]. Another concern is related to the impact of PCR amplification on the accuracy of gene expression quantitation by RNA-seq; this seems to be solved by the new technologies including

PCR-free library preparation methods for sequencing, such as the Illumina TruSeq® DNA PCR-free [439].

With the advent of the RNA-sequencing technology, new implementation combining molecular biology and biochemistry emerged [440]. A summary of some of the currently most used sequencing applications to study the transcriptome can be found in Table 2.

Table 2. Summary of the RNA-sequencing applications for transcriptome study.

Methods	Description	Usage
mRNASeq	messengerRNA Sequencing	To identify messenger RNAs (mRNAs)
miRNASeq	microRNA Sequencing	To identify micro RNAs (miRNAs)
GROSeq	Global Run On Sequencing	To identify nascent RNAs that are actively transcribed by RNA Pol II
PROSeq	Precision nuclear run-on sequencing	
ChIRPSeq	Chromatin Isolation by RNA Purification	To discover regions of the genome bound by a specific RNA
RiboSeq	Ribosome profile Sequencing	To identify RNAs that are being processed by the ribosome and hence this method helps to monitor the translation process
CLIPSeq	Cross-Linking and Immunoprecipitation Sequencing	To identify the binding sites of cellular RNA-binding proteins (RBPs) using UV light to cross-link RNA to RBPs without the incorporation of photoactivatable groups into RNA
PAR-CLIP Seq	Photoactivatable-Ribonucleoside-Enhanced Cross-Linking and Immunoprecipitation Sequencing	To identify and sequence the binding sites of cellular RNA-binding proteins (RBPs) and microRNA-containing ribonucleoprotein complexes (miRNPs)
NETSeq	Native Elongation Transcript Sequencing	It sequences and captures nascent RNA transcripts after immunoprecipitation of RNA Pol II elongation complex
TRAPSeq	Targeted Purification of Polysomal mRNA Sequencing	To detect and identify translating mRNAs
PARESeq	Parallel Analysis of RNA Ends Sequencing	To detect and identify miRNA cleavage sites and uncapped transcripts that undergo degradation
GMUCT	Genome-wide Mapping of Uncapped Transcripts	
TIFSeq	Transcript Isoform Sequencing	RNA isoforms are identified after 5' and 3' paired-end sequencing
PEAT	Paired-End Analysis of Transcription start site	
CELSeq	Cell Expression by Linear amplification and Sequencing	Single-cell transcriptomics methods
SMARTSeq	Switching Mechanism At the 5' end of the RNA Template Sequencing	
STRT	Single-cell Tagged Reverse Transcription	

2 AIMS

This thesis aims to characterize global changes in coding and non-coding gene expression of keratinocytes in psoriasis skin, as well as to investigate potential circulating biomarkers for the diagnosis of psoriatic arthritis in patients with cutaneous psoriasis.

The objectives of the doctoral studies were:

- To dissect the keratinocyte-specific protein-coding transcriptome of psoriasis skin (*Paper I*)
- To identify the long non-coding RNA landscape of psoriasis keratinocytes and uncover their contribution to the epidermal alterations in the disease (*Paper II*)
- To explore whether plasma-derived extracellular vesicles microRNAs may serve as biomarkers for psoriatic arthritis in patients with psoriasis (*Paper III*)
- To characterize the role of miR-378a in keratinocytes in psoriasis (*Paper IV*)

3 MATERIALS AND METHODS

Table 3. Summary of projects' related material and methods.

Method	Sample material	Study
Ethics statement	Human skin and blood, mice skin	I, II, III, IV
Patients, biopsies and study design	Human skin and blood	I, II, III, IV
Isolation of CD45-negative epidermal cells and extracellular vesicles	Human skin and blood	I, II, III, IV
RNA extraction	Human skin and blood, mice skin, cultured cells	I, II, III, IV
Microarray gene expression analysis	Human skin, cultured cells	I, II, IV
Next-generation sequencing	Human skin and blood	III, IV
Gene ontology and gene set enrichment analyses	Human skin, cultured cells	I, IV
Mapping genes in proximity of psoriasis-associated SNPs	Human skin	I, II
Analysis of transcription factor binding site motifs	Human skin	I
Cell culture, treatments and transfections	Human skin, cultured cells	I, II, IV
Quantitative real-time PCR	Human skin and blood, cultured cells	I, II, III, IV
RNAscope in situ hybridization assay	Human skin	II
Western blotting	Cultured cells	IV
Mice and the IMQ (imiquimod)-induced mouse model of psoriasis	Mice skin	IV
Histology and immunohistochemistry	Human skin, mice skin	IV
Statistical analysis	All types of samples	I, II, III, IV

3.1 Ethics statement

All the studies which includes human sample material (skin biopsies or blood) (study **I**, **II**, **III**, **IV**) were approved by the Stockholm Regional Ethics Committee and conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from all participants (patients and/or healthy donors) prior to enrolment. All animal experiments (study **IV**) were performed only upon approval by local ethics committee of Stockholm, Sweden (Swedish Board of Agriculture) and in the respect of the welfare of the animals. The ethical permits identified by the codes 2007/20-31/2, 01-258 and N153/14, were in favor of the studies included in this thesis.

3.2 Patients, biopsies and study design

For study **I**, **II** and **IV**, four mm full-depth punch biopsies were taken from the skin of healthy volunteers and from age-matching patients with chronic plaque psoriasis, from their lesional plaques and healthy-looking non-lesional skin. Biopsies were taken at the Department of Dermatology, Karolinska University Hospital (Stockholm, Sweden) and at the Swedish Psoriasis Association (Psoriasisföreningen). Biopsies from non-lesional/non-irritated skin were taken at least 10 cm from any psoriasis lesion. The patients had moderate to severe chronic form of psoriasis and were subject to examination by expert dermatologists which determined their PASI (Psoriasis Area and Severity Index) score at the time of sample collection. Psoriasis patients had not received systemic immunosuppressive treatment or UV-B/PUVA treatment for at least 4 weeks and topical therapy for at least 2 weeks before skin biopsy. All the subjects included in the study were of Caucasian origin. For study **III**, patients in the Stockholm Psoriasis Cohort (SPC) [441] with cutaneous-psoriasis developed within one year were enrolled and 500 µL of plasma samples were collected from them. Plasma was also collected from the same patients after ten-years of continuous duration of the disease. These patients were closely monitored and a clinical examination by expert dermatologists determined whether they developed psoriatic arthritis symptoms at the ten-years follow-up visit. Psoriasis Area and Severity Index (PASI) was used to quantify the severity of the skin disease and each of these patients were assessed for potential psoriatic arthritis symptoms. Rheumatologists further examined the patients with subjective joint problems and, if fulfilling all the CASPAR (Classification Criteria for Psoriatic Arthritis) [229] criteria, they were included in the study as “PsA” (psoriatic arthritis) group. This only in case the joint symptoms were not the result of other apparent causes. Patients who received rheumatologic examination are in line with the Swedish Early Psoriatic Arthritis Registry [442]. Due to the lack of radiographs, evidence of juxta-articular new-bone formation was excluded from the assessment of CASPAR in this study. Disease activity for patients in the psoriatic arthritis (PsA) group was measured by the Disease Activity Score 28 (DAS28), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). Patients included in the cutaneous-only psoriasis (PsC) group had no signs of subjective joint symptoms and no prior psoriatic arthritis diagnosis, or this was conclusively ruled out by the rheumatologist. None of the patients received any systemic biological or conventional treatment for at least four weeks prior the samples collection.

3.3 Isolation of CD45-negative epidermal cells and extracellular vesicles

For study **I, II, IV**, skin biopsies from psoriasis lesion and non-lesional skin, as well as from healthy volunteers, were incubated in dispase (5U/ml) (Thermo Fisher Scientific) overnight (about 14-16 hours) at the temperature of 4 °C. Following, manual separation of epidermis and dermis was possible using sterile forceps. Smaller pieces of epidermis were digested with trypsin/EDTA (Thermo Fisher Scientific) to obtain a single-cell suspension. This was purified by depleting CD45-positive cells through incubation with CD45-labeled microbeads (Miltenyi Biotec) for 15 min at 4 °C and using MACS MS magnetic columns according to the manufacturer's instructions (Miltenyi Biotec). CD45-positive cells were retained in the column, while CD45-negative cells (predominantly keratinocytes) could easily pass and get harvested.

For study **III**, the process of extracellular vesicles (EVs) isolation from patients with cutaneous-only psoriasis (PsC) and with established psoriatic arthritis (PsA) was performed at Qiagen Genomic Services, Germany, according to their standard protocols. Extracellular vesicles were precipitated using miRCURY™ Exosome Isolation Kit – Cells, Urine and CSF (Exiqon A/S, Qiagen brand). Before proceeding to RNA isolation, the presence of EVs pellet was determined by the operators through visual examination of each sample.

3.4 RNA extraction

For study **I, II and IV**, total RNA extraction from the sorted CD45-negative cells from psoriasis patients and healthy donors was performed using the miRNeasy Mini Kit (Qiagen). Briefly, QIAzol Lysis Reagent was added to the samples followed by vigorous shake. Upon chloroform addition, the samples were centrifuged for 15 min at 12,000 g, 4 °C. Upper phase was collected and the samples were alternatively centrifuged and washed with specific buffers. The RNA pellet was resuspended in RNase-free water. Nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine the concentration of the extracted RNA, Agilent 2100 Bioanalyzer chip (Agilent) was used to assess RNA quantity and RIN (RNA integrity number). RNA samples with a RIN \geq 9.0 were used for microarray analysis (study **I, II, IV**), microRNA sequencing (study **IV**) and further quantitative real-time reverse transcriptase-PCR. Extraction of total RNA from cultured cells is generally performed using TRIzol (Thermo Fisher Scientific) and chloroform (Merck KGaA) according to the phenol-chloroform manual nucleic acids separation method. For study **III**, RNA extraction was performed at Qiagen Genomic Services, Germany, using their standard protocols. Total RNA was extracted from extracellular vesicles (EVs) using the miRCURY™ RNA isolation kit – Cell and

Plants (Exiqon A/S, Qiagen brand), and RNA spike-in mix (UniSp2, UniSp4 and UniSp5, Exiqon A/S, Qiagen brand) were incorporated in the purification step in order to keep track of the efficiency of the RNA extraction.

3.5 Microarray gene expression analysis

For study **I**, for microarray gene expression profiling, 100 ng of total RNA extracted from CD45-negative cells was hybridized to the Affymetrix GeneChip® HTA (Human Transcriptome Array) 2.0 (Affymetrix; Thermo Fisher Scientific brand). R and the Bioconductor package LIMMA (Linear Models for Microarray Data) [443] were used for preprocessing of the data, identification of differentially expressed protein-coding genes and related statistical analysis. Significantly deregulated genes were selected using threshold for linear fold-change ≤ 0.67 or ≥ 1.50 and False Discovery Rate (FDR) ≤ 0.05 after multiple hypothesis correction with Benjamini-Hochberg method.

For study **II**, Affymetrix HTA 2.0 array was used, which includes more than six million probes and allows the identification of both coding and non-coding transcripts. Further annotation of these probes through Ensembl BioMart [444] allowed the identification of the associated gene and transcript biotypes, on the bases of which we could select long non-coding RNAs only. Transcriptome Analysis Console (TAC) software (Thermo Fisher Scientific) was used for data analysis and transcripts with fold-change ≤ 0.67 or ≥ 1.50 and False Discovery Rate (FDR) ≤ 0.05 were considered significantly differentially expressed.

For study **IV**, Affymetrix Human Gene 2.0 ST Array provided complete gene expression profiling and Transcriptome Analysis Console (TAC) software was used to identify protein-coding transcripts having fold-change ≤ 0.77 or ≥ 1.30 and nominal P -value ≤ 0.05 .

3.6 Next-generation sequencing

For study **III**, upon reverse transcription of the RNA samples into cDNA and test for the expression of few microRNAs and synthetic spike-in RNAs as quality control, these samples proceed to library preparation. Small RNA sequencing was performed at Qiagen Genomic Services, Germany, with the Illumina NextSeq500 (Illumina). Next-generation sequencing analysis of small RNAs were conducted with using R (version 3.6.3, www.r-project.org/) and the Bioconductor (Release 3.9, www.bioconductor.org/) [445] package edgeR (version 3.26) [446]. MicroRNAs low in abundance were excluded from further analysis. To be included they had to have TPM (transcript per million) ≥ 5 in at least one of the two investigated cohorts. Significantly deregulated microRNAs passed the thresholds of fold-change ≤ 0.8 or ≥ 1.2 and P -value ≤ 0.05 . Exact test on the negative binomial distribution was used to determine the P -value for the significantly differentially expressed miRNAs.

For study **IV**, small RNA next-generation sequencing was performed at Beijing Genomics Institute, China. Briefly, adaptor sequences were trimmed from fastq files and together with low-quality tags were removed from the sequencing data. Sequences (clean reads) were aligned to the reference human miRBase v.21 precursor dataset (www.mirbase.org/) [447]. Based on the length of 22-25 nucleotides and precursor analysis giving information about the secondary structure, novel microRNA transcripts were predicted using MIREAP tool (github.com/liqb/mireap); these were then mapped to the human genome. Transcript Per Million (TPM) data normalization was followed by differential expression analysis of microRNAs, using R (version 3.6.3) and its Bioconductor (Release 3.10) package edgeR (version 3.14) [446]. Psoriasis lesional *vs.* healthy, psoriasis lesional *vs.* psoriasis non-lesional, and psoriasis non-lesional *vs.* healthy group comparisons were performed and the threshold for microRNAs selection was set to fold-change ≤ 0.7 or ≥ 1.4 and false discovery rate (FDR) $< 10\%$. Besides these criteria, miRNAs, which had average 1 transcript per million (TPM) in at least one of the groups and which were expressed in at least half of the samples in any group were considered to be expressed.

3.7 Gene ontology and gene set enrichment analyses

Analysis of Gene Ontology Biological Processes (GO-BP) were performed for study **I** and **IV** using the online tool Enrichr (<https://amp.pharm.mssm.edu/Enrichr>) [448, 449]. Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis were performed for study **I**. Moreover, in study **I** we performed Gene Set Enrichment Analysis (GSEA) (www.gsea-msigdb.org/gsea/) [450, 451] to identify which cytokine-responsive gene signatures were enriched in CD45-negative epidermal cells from psoriasis lesional skin. We based this study on 36 experiments available in GEO (Gene Expression Omnibus) database (www.ncbi.nlm.nih.gov/geo/), in which keratinocytes monolayer and 3D reconstructed epidermis were treated with different cytokines. We extracted the lists of genes up-regulated by the cytokines' treatments having fold-change ≥ 1.5 and nominal *P*-value ≤ 0.05 . Analysis were carried out using Gene Set Enrichment Analysis software (GSEA, powered by Broad Institute) [450].

3.8 Mapping genes in proximity of psoriasis-associated SNPs

For study **I** and **II**, we aimed to determine whether deregulated protein-coding or non-coding transcripts identified with microarray analysis were overlapping regions in proximity to SNPs (single nucleotide polymorphisms) associated to psoriasis susceptibility loci (PSORS). The genomic coordinates of psoriasis-associated SNPs were collected from four previous publications [101, 104, 452, 453]. We

then used the R based Bioconductor package “GenomicRanges” [454] to overlap the genomic coordinates of significantly deregulated protein-coding genes in study **I**, with a range of 500 Kbp (250 Kbp upstream and 250 Kbp downstream) from the exact genomic position of PSORS-associated SNPs. Similarly, due to the extensive length of long non-coding RNAs, in study **II** we calculated a range of 1 Mbp (500 Kbp upstream and 500 kbp downstream) from the exact genomic position of PSORS-associated SNPs and we overlapped to this region the significantly differentially expressed long non-coding RNAs detected in our profiling study.

3.9 Analysis of transcription factor binding site motifs

In study **I**, the software MetaCore™ (Thomson Reuters; Clarivate Analytics brand), which uses data from literature (predicted and validated), allowed us to determine the transcription factor binding motifs among the significantly deregulated genes in psoriasis lesion (PP) vs. healthy controls (H).

3.10 Cell culture, treatments and transfections

Normal human epidermal keratinocytes (NHEK) (Thermo Fisher Scientific, Catalog# C0055C) were cultured in 5% CO₂ at 37 °C in EpiLife serum-free medium (Thermo Fisher Scientific) containing Human Keratinocyte Growth Supplement (HKGS) (Thermo Fisher Scientific) and penicillin/streptomycin (Thermo Fisher Scientific) antibiotics. Keratinocyte cultures between the third and fourth passage were used to perform all the experiments. When the keratinocytes' monolayer reached about 70% confluence in 24-well plate, medium was removed and replaced with 0.5 ml of EpiLife serum-free medium (Thermo Fisher Scientific) in order to not affect further treatments and/or microRNA transfections.

In study **I**, NHEK were treated with recombinant Epigen (rEPGN) protein (Novus-Biologicals (Bio-Techne brand), Catalog# NBP2-34987) at three different concentrations (50, 100, 200 ng/mL) and cell growth was determined with IncuCyte ZOOM® Live-Cell Analysis System (Essen BioScience, Sartorius brand), while area among the proliferating cells was analyzed with the IncuCyte ZOOM® software (Essen BioScience, Sartorius brand). In study **II**, cells were treated with the cytokines IL-17A (100 ng/ml), IL-22 (20 ng/ml) and TNF- α (50 ng/ml) or with a combination of the three (R&D Systems, Bio-Techne brand) for 1, 3, 6, 24, 48, 72 and 96 hours. In study **IV**, both monolayer of cultured keratinocytes and 3D reconstructed human epidermis were used. For RNA extraction and quantitative real-time PCR experiments, monolayer of keratinocytes were seeded in 24-well plates at a density of 40,000 cells/well. For western blotting, cells were seeded in 6-well plates at a density of 200,000 cells/well. Primary human keratinocytes in monolayer cultures were treated with IL-17A (100 ng/ml), IL-1 β (10 ng/ml),

IL-36 α (10 ng/ml), IL-22 (20 ng/ml), TNF- α (50 ng/ml), or a combination of IL-17A, IL-22 and TNF- α (R&D Systems, Bio-Techne brand). Keratinocytes were then transfected with 50 nM mirVana™ miRNA Inhibitor for miR-378a (to induce down-regulation) or Negative Control #1 (Thermo Fisher Scientific); or 1 nM mirVana™ miRNA Mimic miR-378a (to induce overexpression) or Negative Control #1 (Thermo Fisher Scientific) using Lipofectamine 2000 (Thermo Fisher Scientific). Upon transfection, cells were treated with IL-17A (R&D Systems, Bio-Techne brand) at a concentration of 100 ng/ml and collected at different time points. To inhibit the NF- κ B pathway, the anti-inflammatory compound BAY 11-7082 (Sigma-Aldrich; Merck KGaA brand) –inhibitor of I κ B kinase (IKK)– was used to treat monolayer cultures of NHEK at a concentration of 10 μ M. Dimethyl sulfoxide (DMSO) was used as control. For C/EBP- β inhibition, a set of four pre-designed short interference RNAs (siRNAs) for C/EBP- β gene (Dharmacon, Cat. #LQ-006423-00-0002) mixed together in a pool or siRNA Negative Control No. 1 (Thermo Fisher Scientific) were transfected at a concentration of 30 nM using Lipofectamine 2000 (Thermo Fisher Scientific), following the manufacturer's instructions.

Three-dimensional (3D) reconstructed human epidermis (RHE) used in study **IV** were obtained from MatTek (Ashland) and cultured according to manufacturer's instructions at the liquid-air interphase in hydrocortisone-free medium. IL-17A (20 ng/ml) (R&D Systems, Bio-Techne brand) was added to the cultured three-dimensional epidermal equivalents for 72 hours. A different three-dimensional epidermal equivalent model was generated using primary normal human epidermal keratinocytes (NHEK) isolated from the epidermis of juvenile foreskin (same donor) and were kindly provided by our collaborator Prof. Bernhard Homey (Heinrich Heine University, Düsseldorf, Germany). 3D epidermal equivalents were treated with a mix of cytokines associated with psoriasis (IL-17A (30 ng/ml) + TNF α (5 ng/ml) + IFN γ (20 ng/ml)) (R&D Systems, Bio-Techne brand) and collected 72 h after treatment.

3.11 Quantitative real-time PCR

In study **I**, **II**, **IV**, for mRNAs, total RNA extracted from CD45-negative human epidermal cells, from keratinocytes monolayer, from 3D reconstructed human epidermis and from mice skin biopsies was reverse transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). For microRNAs, total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) including primers for miR-378a and RNU48 (Thermo Fisher Scientific). TaqMan® PrimeTime pre-designed primers for mRNAs were purchased from Integrated DNA Technologies (IDT). Long non-coding RNAs TaqMan-based primers for quantitative RT-PCR were design using the IDT PrimerQuest®

Tool (Integrated DNA Technologies) and were based on their transcripts' sequences. QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific) was used to quantitatively amplify the reverted cDNA. Normalization against the housekeeping gene 18S (18S, forward primer: CGGCTACCACATCCAAGGAA; reverse primer: GCTGGAATTACCGCGGCT, probe: TGCTGGCACCAGACTTGCCCTC) for human mRNAs and RNU48 for human miRNA miR-378a was calculated using the DDcT-method. Expression of mouse messengerRNAs were normalized against the averaged expression of the housekeeping genes *Gapdh*, *B2m* and *Actb*, and mouse microRNA miR-378a against the housekeeping gene *Rnu6*, with the DDcT-method.

For study **III**, a volume of 2 μ L of RNA was reverse transcribed in 10 μ L reactions using the miRCURY LNATM Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon A/S, Qiagen brand). cDNA was then diluted 50X and analyzed in 10 μ L PCR reactions according to the manufacturer's protocol. Each microRNA was assayed once by RT-qPCR on the microRNA Ready-to-Use PCR custom Pick & Mix using ExiLent SYBR® Green master mix (Exiqon A/S, Qiagen brand). Exclusion of the template from the reverse transcription reaction was possible by performing negative controls, which were further profiled like the samples. The steps of amplification, detection and quantification were achieved in 384-well plates with a LightCycler® 480 Real-Time PCR System (Roche Diagnostics). Experiments were conducted at Qiagen Genomic Services, Germany. Haemolysis was monitored through the ratio of the expression of two microRNAs: miR-451a (expressed in red blood cells) and miR-23a-3p (relatively stable in serum and plasma and not affected by haemolysis), where a ratio above 7.0 indicates an increased risk of being affected by haemolysis [455]. None of the samples needed to be excluded based on this criterion. MicroRNA expression values obtained by quantitative RT-PCR were normalized to the average of three microRNA normalizers (hsa-miR-191-5p; hsa-miR-320a; hsa-miR-423-3p). These were identified to be expressed with high stability across our samples by the NormFinder algorithm (update January 2015) [456]. Each miRNA Ct value obtained from quantitative RT-PCR analysis was converted in the corresponding expression level ($EC_{q_{miR}} = 2^{-Cq}$), and the relative expression units were calculated as $DC_{q_{miR(target)}} = EC_{q_{miR(target)}} - \text{mean}(EC_{q_{miR(normaliser1)}}, EC_{q_{miR(normaliser2)}}, EC_{q_{miR(normaliser3)}})$. Finally, difference in the levels of the target miRNAs among the two groups (PsA vs. PsC) was calculated as $DDC_{q_{miR}} = (DC_{q_{miR(target)}} \text{ in PsA}) - (DC_{q_{miR(target)}} \text{ in PsC})$.

3.12 RNAscope in situ hybridization assay

In study **II**, 7 μ m thick formalin-fixed (4%) paraffin-embedded (FFPE) skin sections from healthy volunteers and patients with psoriasis were used to perform the manual RNAscope® in situ hybridization assay. For the long non-coding RNA LINC00958, predesigned double Z (ZZ) probes were purchased from Advanced

Cell Diagnostics, Inc. (ACDBio; Bio-Techne brand, Cat. No. 478601). Briefly, skin sections were deparaffinised with X-TRA-Solv© and ethanol at different concentrations. To block endogenous peroxidase activity (Hydrogen Peroxide), reducing the background noise signal, we used the RNAscope® Pretreatment reagents according to the manufacturer's instructions (ACDBio; Bio-Techne brand), while to better access the RNA-target molecule we performed heat induced target retrieval and treatment of the sections with Protease Plus (ACDBio; Bio-Techne brand). LINC00958 and Negative Control (ACDBio; Bio-Techne brand, Cat. No. 321831) probes were hybridized for 2 hours at 40 °C and three amplification steps (Amp1, Amp2, Amp3) were performed using RNAscope® Multiplex Fluorescent Reagent Kit v2 (ACDBio; Bio-Techne brand, Cat. No. 323100). Fluorescence detection was possible through probes labelling with TSA® plus fluorophore (PerkinElmer, Akoya Biosciences brand) and sections imaged with Confocal microscope Zeiss LSM 800 with Airyscan (Carl Zeiss).

3.13 Western blotting

In study IV, proteins were isolated from previously described transfected and treated cell cultures, using 60 µL lysis buffer. Analysis of western blotting were performed according to standard procedures using the following antibodies purchase from CTS (Cell Signaling Technology): anti-IκBα (mouse monoclonal, 1:1000, Cat. #4812), anti-phospho-IκBα (rabbit monoclonal, 1:1000, Cat. #2859). HRP-coupled β-Actin (1:20000) (Merck KGaA) was used as a loading control. The anti-rabbit secondary antibody (Cell Signaling Technology) was used following the manufacturer's instructions. The anti-rabbit IgG (Cell Signaling) served as negative control. Horseradish peroxidase-coupled isotype-specific anti-rabbit secondary antibodies were purchased from Dako (Agilent). Enhanced chemiluminescence detection system allowed the detection of the immune complexes according to the manufacturer's protocol (GE Healthcare).

3.14 Mice and the IMQ (imiquimod)-induced mouse model of psoriasis

In study IV, wild-type C57BL/6J mice were procured from Charles River. 5-7 µg of miR-378a mimic or scrambled control (mirVana) were injected intradermally into the shaved back skin of female C57BL/6J mice at 8 weeks of age to over-express miR-378a. The mimic injection was reinforced by the transfection agent Max Suppressor In Vivo RNA-LANCER II (Bio Scientific) and it was repeated at days 1, 2 and 3. At the same time of the repeated mimic injection (day 1, 2 and 3), a topical dose of 62.5 mg (5%) of Aldara cream (imiquimod/IMQ, Aldara cream; Meda Pharmaceuticals) was applied on the shaved back skin of the mice for 3 consecutive days and sacrificed at day 4, while control mice were treated with

the same amount of vehicle cream. Skin thickness was measured with a Vernier caliper (AgnTho's AB) and clinical scores were assessed by three independent researchers to assign a score of 0 to 4 (0, none; 1, mild; 2, moderate; 3, severe; and 4, very severe) for erythema, scaling, and thickness.

3.15 Histology and immunohistochemistry

In study **IV**, back-skin samples from mice were collected at the end of the experiments were fixed in neutral formalin. Seven μm paraffin-embedded sections were made and stained with the dyes haematoxylin and eosin (H&E) and epidermal thickness was measured using ImageJ (Fiji) software [457]. Analysis of cell proliferation was performed with immunostaining of anti-mouse Ki-67 marker (Cell Signaling Technology (CST), #12202) with rabbit as host species. Briefly, upon deparaffinization with X-tra Solv and ethanol, sections were incubated with Tris-EDTA buffer at 98 °C for 25 min to unmask the proteins. Hydrogen peroxidase (H_2O_2) at 0.3% concentration was applied for 15 min at room temperature (RT) in the dark. 10% normal blocking goat serum diluted in 1% BSA in PBS was applied for 45 min at RT, followed by overnight incubation at 4 °C with primary antibodies Ki-67 and rabbit IgG isotype control (Cell Signaling Technology (CST), #3900), both diluted 1:200 in 1% BSA in PBS. The following day, sections were incubated with anti-rabbit biotinylated secondary antibody (Cell Signaling Technology (CST), #14708) diluted 1:200 in 1% BSA in PBS for 45 min. Detection was possible using Vectastain ABC and AEC Peroxidase (HRP) Substrate Kit (Vector Laboratories). Number of positive cells/field of view was quantified using Fiji (ImageJ) software [457].

3.16 Statistical analysis

Most of the statistical analyses across all the experiments we've performed in study **I**, **II**, **III**, **IV**, were conducted using IBM SPSS Statistics for Mac ver. 25.0 (IBM Inc.), R software ver. 3.5.1 to 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism ver. 7.0a to 8.3.1 for Windows (GraphPad Software).

In particular, for study **III**, Spearman's correlation test was also used to determine the correlation between the significantly differentially expressed microRNAs let-7b-3p/miR-30e-5p in the two cohorts PsC/PsA, as well as their correlation with the psoriatic arthritis disease-related scores (PASI, DAS28, BASDAI, number of swollen and tender joints). To estimate the association between microRNA levels in plasma extracellular vesicles and the presence of arthritis, univariate and multivariate logistic regression analyses were performed. Receiver operating

characteristics (ROC) curves and area under the curve (AUC) assessed the accuracy of the classification of patients with and without the arthritis manifestations.

For different experiments, Mann-Whitney U test, Student's t-test or two-way ANOVA were used to determine significant difference between groups. *P*-values <0.05 were considered to be statistically significant. **P* <0.05; ***P* <0.01; ****P* <0.001 and *****P* <0.0001. Statistical analysis of microarray and RNA-seq data is described at the relevant sections in detail.

4 RESULTS AND DISCUSSION

The population of cells on which our studies were focused the most are CD45-negative cells isolated from the epidermis of patients with psoriasis, as well as from healthy donors. This population contains mainly keratinocytes and this approach allowed us to investigate keratinocyte-specific transcriptomic changes and exclude changes coming from immune cells, which are CD45-positive. The procedure leading to the isolation of CD45-negative epidermal cells from skin punch biopsies is illustrated in *Figure 16*.

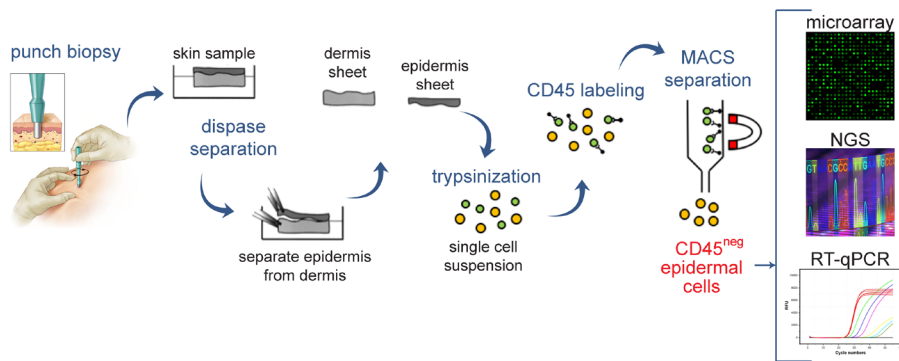


Figure 16. CD45-negative epidermal cell sorting from 4 mm punch skin biopsy. Pictures adapted from <https://www.mayoclinic.org/tests-procedures/skin-biopsy/about/pac-20384634>.

4.1 Keratinocyte-specific protein-coding transcriptome of psoriasis skin

In order to better characterize the contribution of keratinocytes in the altered transcriptome of psoriasis, we used sorted CD45^{neg} epidermal cells from psoriasis lesion (PP = 9), psoriasis non-lesion (PN = 9) and healthy donors (H = 9), in which dermal cells, as well as immune cells, were depleted. The approach we adopted is what makes our study unique compared to others, where full-depth skin biopsies [458-461] or epidermal sections obtained by laser capture microdissection [462] were used to perform psoriasis transcriptomic profiling studies.

4.1.1 Transcriptomic profiling of keratinocytes in psoriasis

Principal component analysis (PCA) in *Figure 17a* have shown separation of the CD45^{neg} cells from the three groups in consideration (PP, PN, H), with keratinocytes from the psoriasis lesion group that clearly dissociates from the non-lesional

and healthy ones. This was confirmed at the transcriptome level, where we have identified most of the changes in the PP vs. H group comparison (*Figure 17b*), with 2,365 genes with significantly altered expression (1,629 up- and 736 down-regulated) (*Figure 17c-d*). In lesional (PP) vs. non-lesional (PN) psoriasis, 1188 up- and 388 down-regulated genes were identified (*Figure 17d*), while just 36 significantly altered genes were found in PN vs. H group comparison (*Figure 17d*). Despite the relatively small number of altered genes found in non-lesional vs. healthy epidermal cells, this could suggest a “pre-psoriatic” state in the keratinocytes of the psoriasis non-lesional skin.

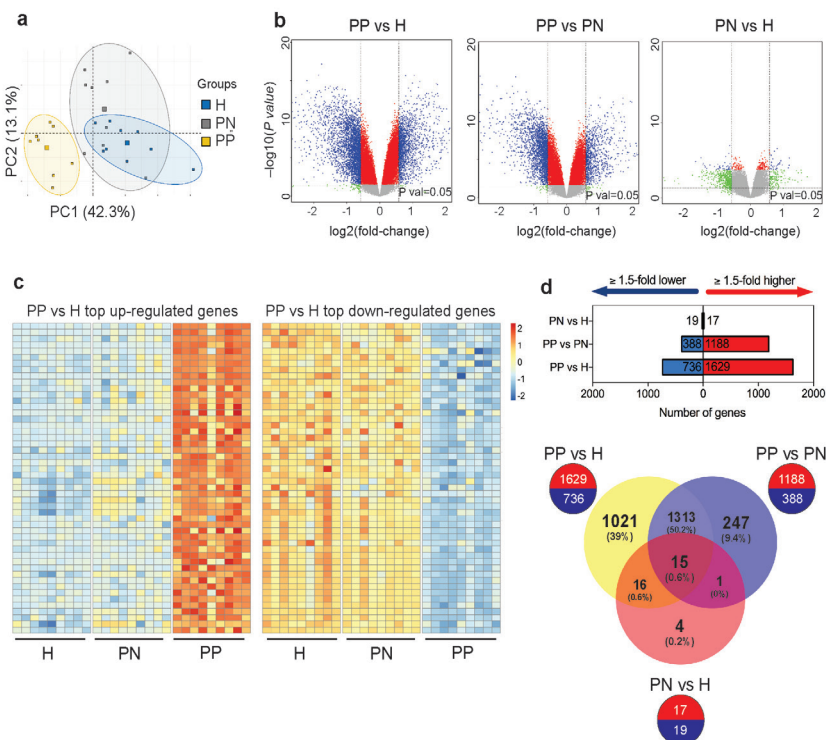


Figure 17. Transcriptomic changes in psoriatic keratinocytes. (a) Principal component analysis of deregulated genes in sorted CD45^{neg} epidermal cells from PP, PN, H. (b) Volcano plot showing the log₂(fold-change) for the identified transcriptomic changes. (c) Heatmaps for the top 50 up- and 50 down-regulated genes in PP vs. H group comparison. (d) Graph illustrating the number of deregulated transcripts –in blue down-regulated genes; in red up-regulated genes. Venn-diagram showing the extent of overlapping genes with fold-change >1.5 between the three groups. PP = psoriasis lesional keratinocytes, PN = psoriasis non-lesional keratinocytes, H = healthy keratinocytes, FC = fold-change. Reproduced and adapted with permission from Pasquali L et al, 2019; Copyrights© Acta Dermato-Venereologica.

4.1.2 Enrichment and signature dominance of deregulated genes in psoriatic keratinocytes

The altered gene expression found in psoriasis keratinocytes compared to healthy skin were enriched in pathways related to cellular proliferation, with potential contributors such as the previously uncharacterized gene Epithelial Mitogen (EPGN) [463], but also to innate immunity and response to inflammatory processes, such as type I interferon signature (IFI44, IFI44L and DDX60 genes), NF- κ B signaling pathway and DNA replication. This was consistent with previous studies exploring the transcriptome changes in full-depth skin biopsies [460, 461]. Moreover, genes previously thought to belong exclusively to immune cells, such as the negative regulator of NF- κ B signaling pathway TNIP3 (alternatively known as ABIN-3) in $\gamma\delta$ T cells [458], were identified in this study with altered expression in keratinocytes.

In particular, up-regulated gene in PP vs. H epidermal cells comparison, have shown enrichment in gene ontology terms related to keratinocytes development and differentiation, while down-regulated transcripts had enrichment in skin epidermis development. Pathways related to tight junctions and transforming growth factor beta (TGF- β) were the most significant KEGG pathways found for this group comparison. Similar pathways and gene ontology enrichment were found analyzing the transcriptomic changes in lesional vs. non-lesional keratinocytes. All these reflected a clear predisposition for altered differentiation in psoriatic keratinocytes.

The skin inflammation in psoriasis is driven by an over-production of pro-inflammatory cytokines from T cells infiltrating the epidermis. To understand which pro-inflammatory component had the biggest impact on the keratinocytes-specific transcriptome alterations, we performed Gene Set Enrichment Analysis (GSEA) using 36 publically available lists of genes up-regulated in cultured primary human keratinocytes and epidermal equivalents (GEO database). We have found that differentially expressed genes in PP vs. H had the most significant enrichment within the list of genes over-expressed by IL-17 and IL-22 treatment of primary human keratinocyte cell cultures, as well as by IL-20 subfamily and IL-1 α , IL-36, TNF- α , IFN- γ (*Figure 18a*). These results are in line with studies showing that Th17 cells secreted cytokines, IL-17, and IL-22, in synergy with IFN- γ and TNF- α , are central players in the psoriasis pathogenesis [88, 464]. Despite this, we have also found that just nearly 50% of the overexpressed genes in psoriasis lesional keratinocytes compared to healthy overlapped with cytokines-related induced gene lists, among which genes induced by IFN- γ have the highest proportion of overlap, followed by IL-22 and IL-17A. This suggests that the remaining 50% represents intrinsic changes in the epidermis of psoriasis, which do not depend on the cytokines' inflammation (*Figure 18b*).

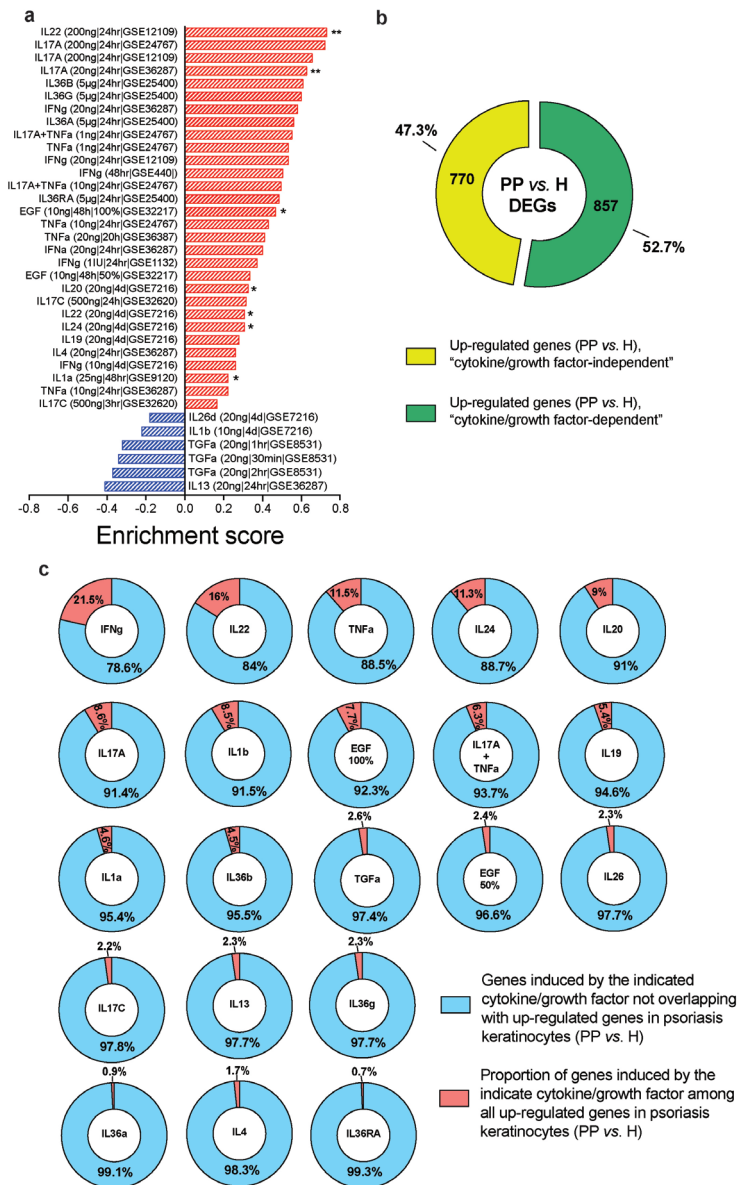


Figure 18. IL-17 and IL-22 gene signatures dominates the transcriptomic changes in psoriatic keratinocytes. (a) Gene Set Enrichment Analysis of 36 cytokines-induced gene lists and up-regulated genes in psoriatic keratinocytes compared to healthy. (b) up-regulated genes in psoriatic keratinocytes compared to healthy which are (dark green) or are not (light green) overlapping with any of the 36 cytokines-induced gene lists. (c) Proportion of significantly up-regulated genes by each cytokine/growth factor across all the overexpressed genes in psoriatic keratinocytes compared to healthy. PP = psoriasis lesional keratinocytes, H = healthy keratinocytes, DEGs = differentially expressed genes. Reproduced and adapted with permission from Pasquali L et al, 2019; Copyrights© Acta Dermato-Venereologica.

4.1.3 Altered genes in psoriatic epidermis may contribute to its genetic susceptibility

To understand the impact of the genetic component on the altered expression of transcripts in psoriatic keratinocytes, we overlapped the genomic coordinates of significantly deregulated transcripts in PP *vs.* H comparison, with regions nearby single nucleotide polymorphisms (SNPs) known to be associated to the susceptibility of psoriasis from previous GWAS studies [101, 104, 453, 465]. A total of 107 deregulated genes in psoriatic lesional *vs.* healthy keratinocytes were overlapping regions spanning the psoriasis-associated SNPs for 250 kbp up- and downstream, therefore being in close proximity. In lesional *vs.* non-lesional psoriatic keratinocytes 89 DEGs were overlapping SNPs associated to psoriasis susceptibility and only 2 in the non-lesional *vs.* healthy keratinocytes comparison (*Figure 19*). These results could partially explain the previously discussed 50% of alterations which are not associated to the cytokine milieu but that might be rooted in the genetic predisposition of psoriasis lesional keratinocytes.

The majority of these genes overlapping psoriasis-susceptibility SNPs were localized on chromosome 1, where the epidermal differentiation complex (EDC) [121] and the psoriasis susceptibility locus PSORS4 [466] reside. Few examples are the well-known late cornified envelope (LCE) genes [121], TNFAIP3 (regulator of NF- κ B signalling) [467] and the top down-regulated in our list C1orf68 (XP32) [468]. In addition, we have identified differentially expressed genes with unknown previous characterization in psoriasis overlapping genomic regions in proximity of psoriasis-susceptibility SNPs.

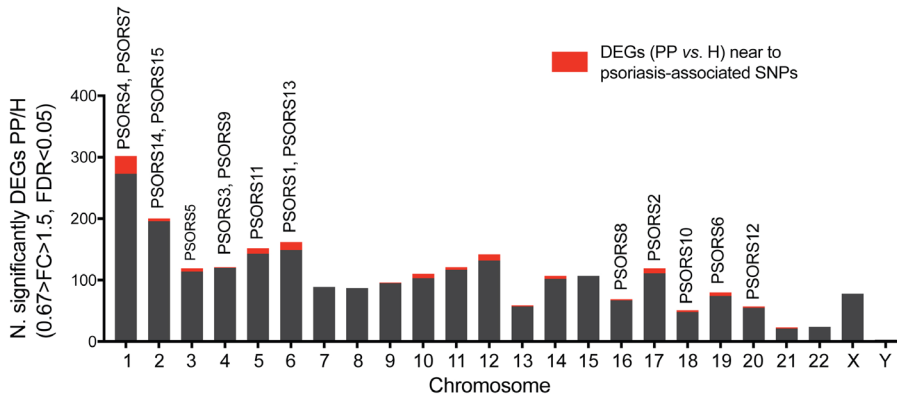


Figure 19. Distribution across chromosomes of the differentially expressed genes in psoriatic lesional vs. healthy keratinocytes comparison. In red is shown the proportion of genes overlapping psoriasis-associated SNPs. Known PSORS (psoriasis susceptibility loci) are reported above the respective chromosome where they belong to. Reproduced and adapted with permission from Pasquali L et al, 2019; Copyrights© Acta Dermato-Venereologica.

4.1.4 Transcription factors can act as upstream regulator of differentially expressed genes in psoriatic keratinocytes

Performing analysis of transcription factor binding sites of the differentially expressed genes in psoriatic lesional vs. healthy keratinocytes, we have determined a total of 338 transcription factors, of which 54 had altered expression at the mRNA level in the psoriatic lesional keratinocytes. Among these, we have found known TFs related to inflammation and epidermal differentiation –AP-1 subunits, important for the activation of cytokines expression in keratinocytes, followed by attraction of neutrophils and macrophages in the epidermis, leading to the typical psoriasis phenotypic alterations [250] and transcription factors associated to NF- κ B such as SOX4, KLF4, GATA3 and STAT1-3 [101], some of which have genomic location nearby PSORS-associated SNPs. Moreover, TRPS1, HEY2 and PAX3 are examples of TFs not characterized in the context of the disease yet, as well as FOXO1, previously thought to play roles in T cells only, now shown as differentially expressed in psoriatic lesional keratinocytes. Finally, the transcription factor NR4A3, which has been detected as one of the most down-regulated genes in psoriasis non-lesional compared to healthy keratinocytes in our study, was not identified as altered in previous psoriasis transcriptomic profiling studies.

4.2 Long non-coding RNA landscape of psoriasis keratinocytes

Long non-coding RNAs represent a relatively new field of study, which becomes even newer when it comes to investigate it in an inflammatory disease such as psoriasis. In addition, virtually nothing is known about the role of lncRNAs in keratinocytes alterations during psoriasis onset and/or progression. In this study, we selected the non-coding transcriptome from the previously sorted CD45^{neg} epidermal cells from psoriasis lesion/non-lesion and healthy donors, filtered for transcripts longer than 200 nucleotides and we inspected the differences in their expression levels across the three groups. Once more, we excluded the immune cells component known to infiltrate the epidermis of psoriasis patients [38], that could have masked the real expression of the keratinocytes-specific non-coding transcriptome.

4.2.1 Profiling of psoriasis CD45^{neg} cells identifies altered long non-coding RNAs adjacent to psoriasis susceptibility loci

Our previous microarray study profiling of the keratinocytes transcriptome in psoriasis has shown substantial changes in the expression of messenger RNAs [130]. As expected, when reducing the dimensionality of the non-coding transcriptome data through principal component analysis (PCA), a clear segregation of the psoriasis lesional from the psoriasis non-lesional and healthy samples was identified (*Figure 20a*), with the majority of the alterations residing in the CD45^{neg} cells of psoriasis lesion (PP) vs. healthy donors (H) (*Figure 20b*). We decided to focus our study on the lincRNA (long intergenic non-coding RNA) class, due to their more established database annotation, as well as their potential role in the immune system development and function and common features with mRNAs [469, 470]. A total of 80 deregulated lincRNAs (43 up- and 37 down-regulated) were found comparing psoriatic lesional (PP) vs. healthy (H) epidermal cells (fold-change >1.5 and *P*-value <0.05). Comparing lesional (PP) vs. non-lesional (PN) psoriasis samples we have identified 65 deregulated lincRNAs (26 up- and 39 down-regulated) with the same fold-change and significance threshold criteria, while 2 up- and 2 down-regulated lincRNAs were found with altered expression levels in psoriasis non-lesion (PN) vs. healthy skin samples. Among these, we successfully identified lncRNAs previously known as altered in their expression in psoriasis, such as PRINS [418], in addition to lncRNAs which expression levels were found to be not changed in studies including full-depth skin samples [471]. An example is the lncRNA NEAT1.

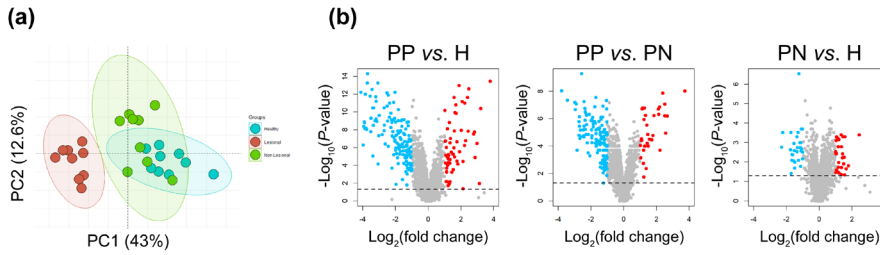


Figure 20. (a) Principal component analysis of the long non-coding RNA (lncRNA) transcriptome of CD45^{neg} epidermal cells from psoriasis lesional (PP, red) and non-lesional (PN, green) skin and skin from healthy controls (H, blue). (b) Volcano plot showing the log₂ fold-change and the nominal P-value threshold at 0.05 (horizontal line) for the lncRNAs. Blue and red color indicates transcripts differentially expressed with fold-change <0.5 and >2.0, respectively.

Further analysis of the genomic localization of the deregulated lncRNAs in our profiling study were intended to identify potential non-coding RNA players in the genetic of psoriasis. Genomic coordinates of significantly differentially expressed lncRNAs in our analysis were matched with coordinates from a 1 Mbp range across psoriasis-susceptibility SNPs (see Materials and Methods section) to identify potential overlaps. A total of 32 differentially expressed lncRNAs were found overlapping psoriasis-susceptibility SNPs in our profiling study. From these, 11 lncRNAs were deregulated exclusively in PP vs. H CD45^{neg} cells comparison, 11 differentially expressed exclusively in PP vs. PN comparison, while 10 were deregulated in both group comparisons. Several deregulate lncRNAs were overlapping SNPs on chromosome 6p21.3, where PSORS1 and the major histocompatibility complex (MHC) are located [472]. This region includes the main psoriasis risk allele HLA-C*06:02, which plays an essential role in the complex genetic predisposition of the disease [102]. In addition, several differentially expressed lncRNAs were found overlapping PSORS4 [473], together with genes involved in differentiation and cornification of the keratinocytes comprised in the epidermal differentiation complex (EDC) [121] on chromosome 1q21 (Figure 21). Taken together, these results denote the potential role of keratinocytes-specific lncRNAs in the genetic predisposition of psoriasis.

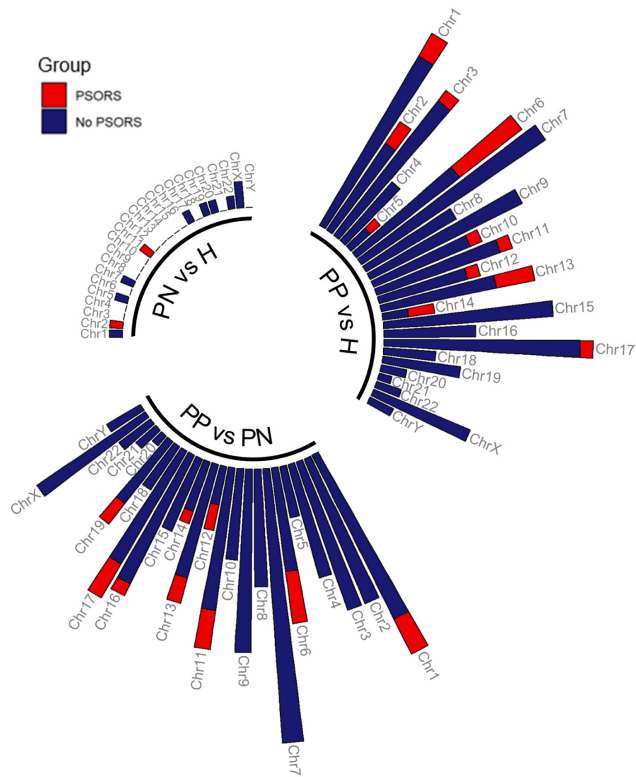


Figure 21. Genomic localization of deregulated lincRNAs in psoriasis lesional, non lesional and healthy CD45^{neg} sorted cells. In red the proportion of lincRNAs in proximity of psoriasis-susceptibility SNPs within their respective chromosome regions. H, healthy; PN, psoriasis non-lesion; PP, psoriasis lesion.

4.2.2 Epithelial tissue enriched LINC00958 is upregulated in psoriatic keratinocytes, with primarily cytoplasmic localization

LINC00958 was selected for further validation, since it was one of the top over-expressed lincRNAs in psoriasis lesional keratinocytes compared to healthy (fold-change = 2.14) in our microarray analysis (Figure 22a). Following RT-qPCR analysis confirmed the increased LINC00958 expression in isolated CD45^{neg} cells from psoriasis lesional, compared to paired non-lesional (fold-change = 2.24) and healthy skin samples (fold-change = 2.6) (Figure 22b). Interestingly, we did not find alterations of LINC00958 expression in cultured primary human keratinocytes treated with the pro-inflammatory cytokines IL-17A, IL-22, TNF- α , commonly found in psoriasis. This suggests the presence of other elements that could drive the up-regulation of this lincRNA in psoriasis lesions.

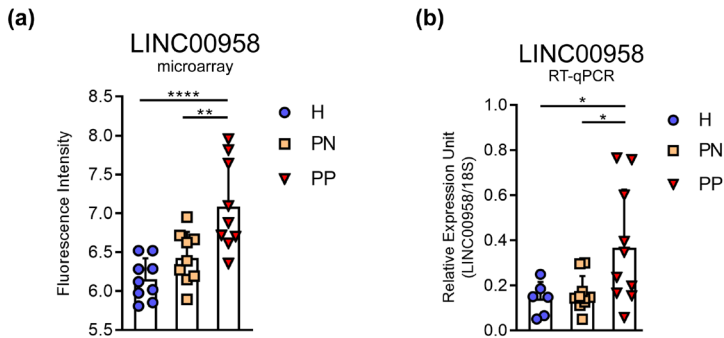


Figure 22. Affymetrix microarray (a) and RT-qPCR (b) gene expression analysis for LINC00958 in CD45^{neg} cells isolated from psoriasis lesion (PP), non-lesion (PN) and healthy (H) skin samples. H, healthy; PN, psoriasis non-lesion; PP, psoriasis lesion. RPKM, reads per kilobase per million reads. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

LINC00958, uncharacterized in the context of psoriasis at the time of writing, has been found linked to other diseases in different tissues (e.g. bladder cancer) [474-478]. In addition, we have determined the absence of protein coding potential for LINC00958 through different available algorithms, such as CPAT [479], CPC 2.0 [480], PORTRAIT [481] and PhyloCSF [482] (Figure 23a), and we have identified tissues with stratified squamous epithelia (such as the skin) as those with highest enrichment for this lncRNA (Figure 23b).

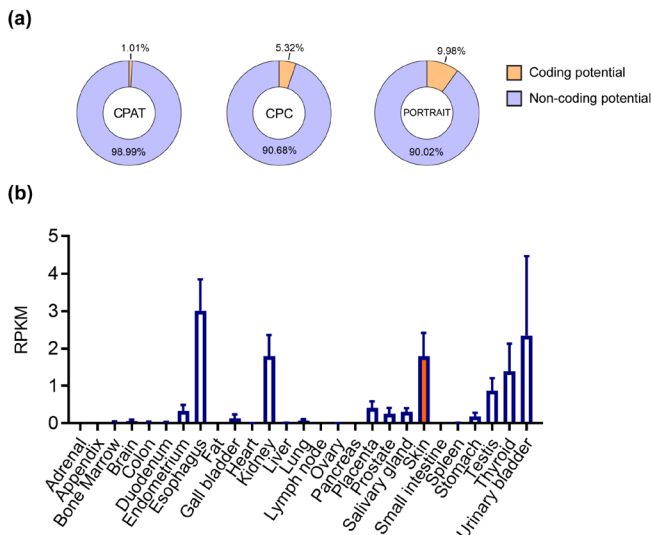


Figure 23. (a) LINC00958 protein-coding potential. (b) Expression of LINC00948 across 27 tissues from 95 normal human samples. CPAT, Coding Potential Assessing Tool; CPC, Coding Potential Calculator 2; PORTRAIT, Prediction of Transcriptomic ncRNA by Ab Initio Methods.

Using RNA-seq mapping reads from ENCODE database, we have determined peaks corresponding to enrichment in genomic regions for LINC00958 in different cell types. Its expression was limited to keratinocytes, while melanocytes, fibroblasts, adipose tissue and even immune cells (CD4+ and CD8+ T cells) show low or absent expression. This suggests that the overexpression of LINC00958 in psoriasis lesion is due to its increased levels in keratinocytes. This could also explain why LINC00958 expression was not detected as altered in previous non-coding RNA profiling studies in psoriasis [471].

Finally, using RNAscope in situ hybridization technique on psoriasis skin sections, we have identified the enrichment of LINC00958 mainly in the cytoplasm (*Figure 24*), with just sporadic single molecules detected at the nuclear level. This, as in general for cytoplasmic lncRNAs, could suggest a potential role of the lncRNA in for example regulating the stability or the translation of other mRNAs, as well as binding and interacting with certain proteins. Further functional studies of this and other differentially expressed lncRNAs in psoriatic keratinocytes could unveil their contribution in the epidermal homeostasis imbalance as well as in the development of the disease.

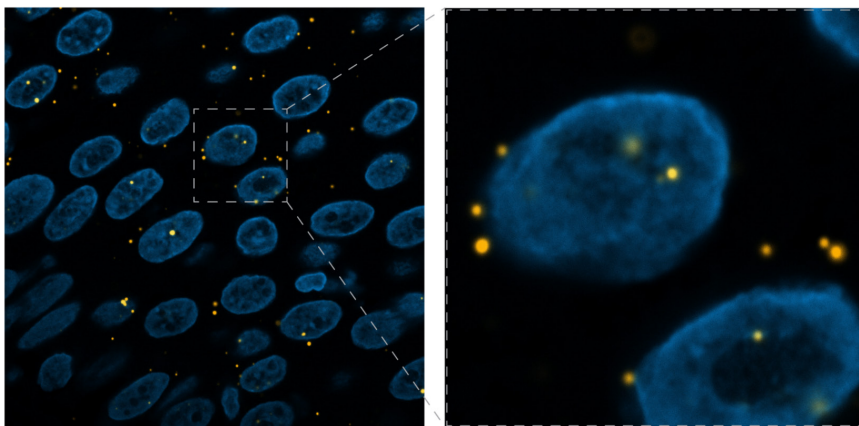


Figure 24. RNAscope in situ hybridization detection of LINC00958 at the single molecule level in psoriasis lesional skin sections. Dapi nuclei stained in blue; LINC00958 single molecules stained in orange.

4.3 Plasma-derived extracellular vesicles microRNAs as biomarkers for psoriatic arthritis in patients with psoriasis

As discussed in the introductory chapter of this thesis, psoriasis is rich in several comorbidities, among which the most common is psoriatic arthritis (PsA). This affects approximately 1/3 of the patients with cutaneous psoriasis, with manifestations visible on average no earlier than ten years from the diagnosis of the cutaneous psoriasis symptoms [483]. Because of the challenging diagnosis, it is estimated that up to 15% of the patients with psoriasis have undetected psoriatic arthritis [225]. Intergluteal psoriasis and nail changes clinical markers for psoriatic arthritis [233] do not result in an accurate prediction of disease onset. IL-6 and CXCL10 as soluble biomarkers [234, 235] as well as a genetic signature [484] have been proposed for assessing the risk of psoriatic arthritis onset in patients with cutaneous psoriasis. Despite the effort, at the time of writing, no consistent biomarkers for a clear detection of PsA are available in clinical practice.

New evidences have reported the ability of some cells to secrete miRNAs in extracellular vesicles (EVs) [485], creating a communication network between distant cells [486]. With this study we highlighted the role of circulating microRNAs in extracellular vesicles, as potential markers to distinguish cutaneous psoriasis patients predisposed to the development of psoriatic arthritis, from those who are not.

4.3.1 Next-generation sequencing identifies changed microRNAs levels in plasma extracellular vesicle of psoriatic arthritis patients

The study population comprised patients with cutaneous psoriasis which were followed up for about 10 years. At the ten years follow up first visit, a dermatologist established the progression of the diseases in absence of subjective joint pain. In patients with subjective joint symptoms, a second visit was done by a rheumatologist, assessing for PsA. This allowed the classification of 29 patients without joint symptoms as cutaneous-only psoriasis (PsC) and 28 patients with simultaneous cutaneous psoriasis and joint symptoms as psoriatic arthritis (PsA) (*Figure 25*). At the time of sample collection, none of the patient were on systemic treatments that could have influenced the circulating miRNAs levels.

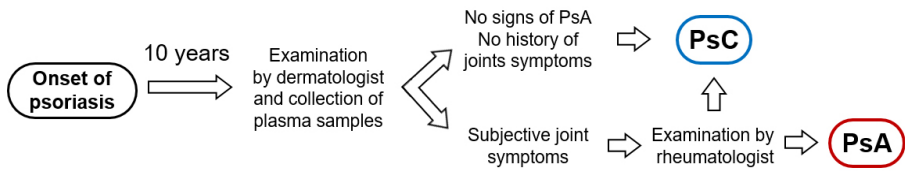


Figure 25. Flowchart showing the selection criteria for patients grouping and the timeline for collecting the samples. Reproduced and adapted with permission from Pasquali L et al, 2020; Copyright© 1999-2020 John Wiley & Sons.

In the discovery phase, we performed small RNA-sequencing on plasma samples from 14 cutaneous-only psoriasis (PsC) and 15 psoriatic arthritis (PsA) patients (see Materials and Methods section). After filtering the NGS data for microRNAs having TPM >5 in at least one of the two cohorts, we have identified 151 miRNAs with stable expression in circulating extracellular vesicles (Figure 26a). Applying filters for absolute fold-change >1.2 and *P*-value <0.05 we have found 19 miRNAs with significantly altered levels in extracellular vesicles (9 with increased and 10 with decreased levels) (Figure 26b).

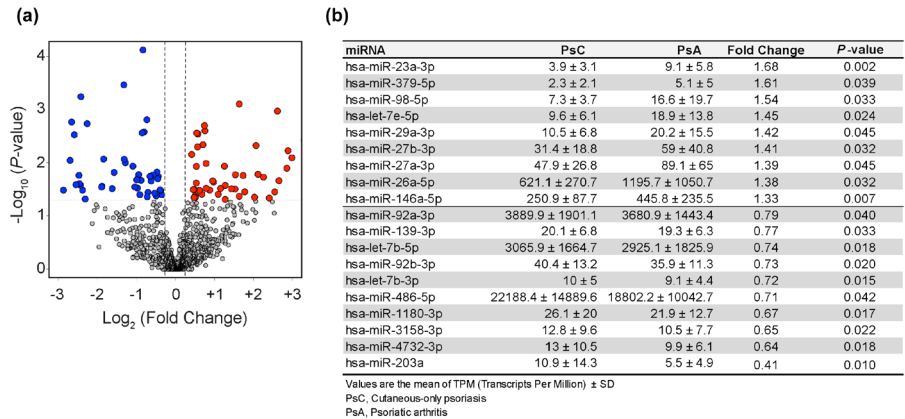


Figure 26. (a) Volcano plot showing miRNAs with down-regulated (blue) and up-regulated (red) levels in circulating EVs. (b) Table of the significantly altered miRNAs detected in our NGS analysis. Reproduced and adapted with permission from Pasquali L et al, 2020; Copyright© 1999-2020 John Wiley & Sons.

4.3.2 The miRNAs let-7b-5p and miR-30e-5p are potential biomarkers for psoriatic arthritis

We selected 41 miRNAs for validation with a RT-qPCR panel array; 15 miRNAs from the previous NGS analysis and 26 which have shown relevance in psoriasis, psoriatic arthritis and/or inflammation of the joints in previous publications [487-491]. In this validation phase, both patients' cohorts were extended, with a total of 29 PsC samples (including the 14 from the previous discovery phase) and 28 PsA samples (including the 15 of the discovery phase). RT-qPCR analysis have reported the levels of miRNAs let-7b-5p and miR-30e-5p being significantly lower in patients with psoriatic arthritis (PsA) compared to those with cutaneous-only psoriasis (PsC) (*Figure 27*).

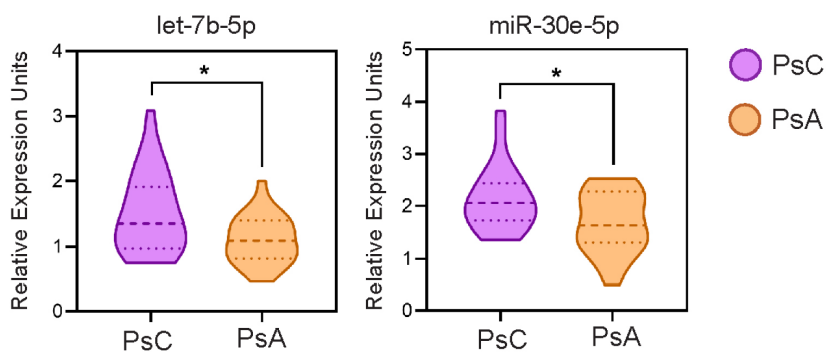


Figure 27. Violin plot showing RT-qPCR analysis results for the miRNAs let-7b-5p and miR-30e-5p circulating in EVs of PsC and PsA samples (extended cohorts). PsA, psoriasis with psoriatic arthritis; PsC, cutaneous-only psoriasis. * $P < 0.05$, Mann-Whitney U test. Reproduced and adapted with permission from Pasquali L et al, 2020; Copyright© 1999-2020 John Wiley & Sons.

In order to determine whether these microRNAs could be relevant markers for the diagnosis of psoriatic arthritis, we performed logistic regression analysis. Both let-7b-5p and miR-30e-5p have shown a significantly inverse association between their levels and the increased risk of developing arthritis manifestations in patients with cutaneous psoriasis (odds ratio = 0.270 for let-7b-5p; odds ratio = 0.255 for miR-30e-5p) (*Figure 28a*). Even after adjusting for variables like sex, age and PASI score, the inverse associations remained significant. Finally, ROC curves determined significant sensitivity/specificity for let-7b-5p (AUC = 0.68, $P < 0.05$) and miR-30e-5p (AUC = 0.69, $P < 0.05$) (*Figure 28b*), confirming the potential of these miRNAs in distinguish between the two study cohorts PsC and PsA.

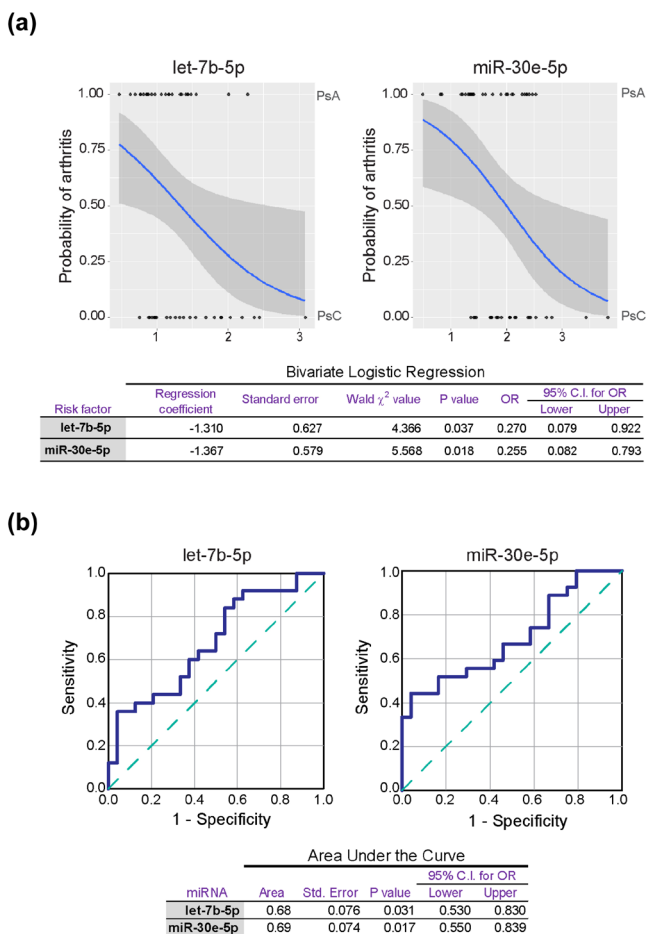


Figure 28. (a) Bivariate logistic regression analysis results illustrating the association between the circulating miRNAs let-7b-5p and miR-30e-5p and the psoriatic arthritis manifestations in patients with cutaneous psoriasis. (b) ROC curves plotting sensitivity/specificity for the extracellular vesicles levels of let-7b-5p and miR-30e-5p. PsA, psoriasis with psoriatic arthritis; PsC, cutaneous-only psoriasis; ROC, Receiver Operating Characteristic; AUC, Area Under the Curve; OR, Odds Ratio; C.I., confidence interval. Reproduced and adapted with permission from Pasquali L et al, 2020; Copyright© 1999-2020 John Wiley & Sons.

In agreement with our findings, miRNA let-7b-5p has been found targeting directly the pro-inflammatory cytokine IL-6 [492], which is known to be over-expressed in psoriatic arthritis patients compared to patients with cutaneous-psoriasis only [493]. Other targets of let-7b-5p are associated to joint inflammation [494, 495], osteoclasts formation and bone loss [496, 497], indicating a potential role of this

miRNA in the pathogenic context of psoriatic arthritis. The miRNA miR-30e-5p was found to be down-regulated in PBMC of rheumatoid arthritis patients compared to healthy controls [498] and directly inhibiting BMI1, an activator of NF- κ B-signaling pathway that promotes arthritis-like symptoms in mice models [499, 500].

Despite the relatively small number of samples that could reduce the statistical power of our analysis, we successfully validated two miRNAs with significantly lower levels in EVs of PsA compared to PsC patients, avoiding results biases by selecting patients not undergoing systemic treatments. Further studies could determine whether reduced levels of let-7b-5 and miR-30e-5p in circulating EVs of PsA patients promote joint inflammation and, therefore, contribute to the pathogenesis of the disease.

4.4 Role and function of miR-378a in psoriatic keratinocytes

In order to further characterize the role of keratinocytes in the pathogenesis of psoriasis, our group has previously identified microRNAs differentially expressed between epidermal cells from psoriasis lesional and non-lesional skin, as well as from skin of healthy volunteers [131]. The microRNA miR-378a (has-miR-378a-3p) was found as one of the top over-expressed in CD45^{neg} cells (mainly keratinocytes) from lesional psoriasis, compared to paired psoriasis non-lesional samples and healthy controls [131]. This microRNA is localized on chromosome 5 in human, within the intron of its host gene PPARGC1 β [501] and has conservation on chromosome 18 in mice [502].

4.4.1 miR-378a confirmed up-regulation in psoriatic lesional keratinocytes

From previous NGS analysis, miR-378a was detected significantly up-regulated in PP (psoriasis lesion) compared with PN (psoriasis non-lesion) keratinocytes, with a fold-change of 2.83, while in PP compared to H (healthy skin) keratinocytes with a fold-change of 3.69 (Figure 29a). RT-qPCR analysis confirmed the over-expression of miR-378a in keratinocytes from psoriasis lesion compared to psoriasis non-lesion (fold-change = 2.33) and healthy (fold-change = 2.42) skin (Figure 29b).

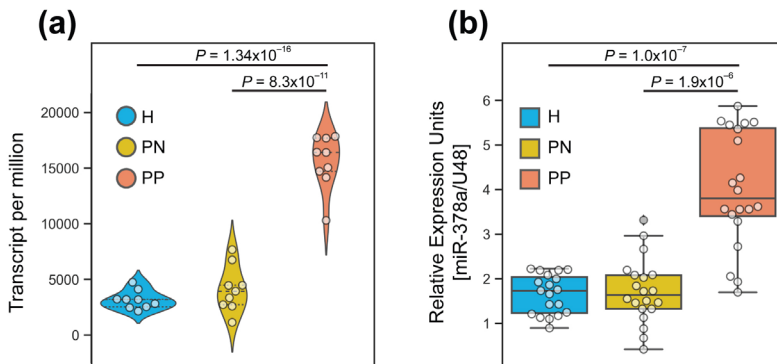


Figure 29. (a) small RNA-sequencing and (b) RT-qPCR expression analyses of miR-378a in sorted CD45^{neg} cells (mainly keratinocytes) from psoriasis lesional, non-lesional and healthy skin. H, healthy; PN, psoriasis non-lesion; PP, psoriasis lesion.

4.4.2 miR-378a enhances skin inflammation when locally injected in imiquimod-induced mouse model of psoriasis

Having found altered expression of miR-378a in psoriatic keratinocytes, we aimed to investigate further its role in the disease. We therefore injected miR-378a mimics in the intradermal layer of mice back skin, followed by topical application of imiquimod cream (IMQ, Aldara), known to induce psoriasis-like inflammation [259] (*Figure 30a*). Mice injected with miR-378a mimics and treated with IMQ cream were compared to mice injected with control oligos and treated as well with IMQ cream. Upon injection of miR-378a + IMQ application, mice have shown increased skin thickness (*Figure 30b*), as well as increased erythema and scaling (*Figure 30c*, macroscopic pictures), all evidences of ongoing inflammatory processes in the skin, typically found in psoriasis. An increased epidermal thickness was found analyzing skin sections from mice miR-378a mimic injected + IMQ compared to control oligos injection + IMQ (*Figure 30c*, H&E staining). In this group comparison, also increased number of cells in the basal layer expressing the proliferation marker Ki67 were detected in miR-378a mimic + IMQ mice compared to the control mice (*Figure 30c*, Ki67 staining). Finally, pro-inflammatory cytokines including *Cxcl1*, *Ccl20*, *Il-17c* and the psoriasis-associated marker *Krt16* were enhanced upon injection of miR-378a mimics and topical treatment with imiquimod cream (*Figure 30d*). These results support the potential role of miR-378a in the induction of skin inflammation in psoriasis by reproducing a psoriasis-like phenotype in mice.

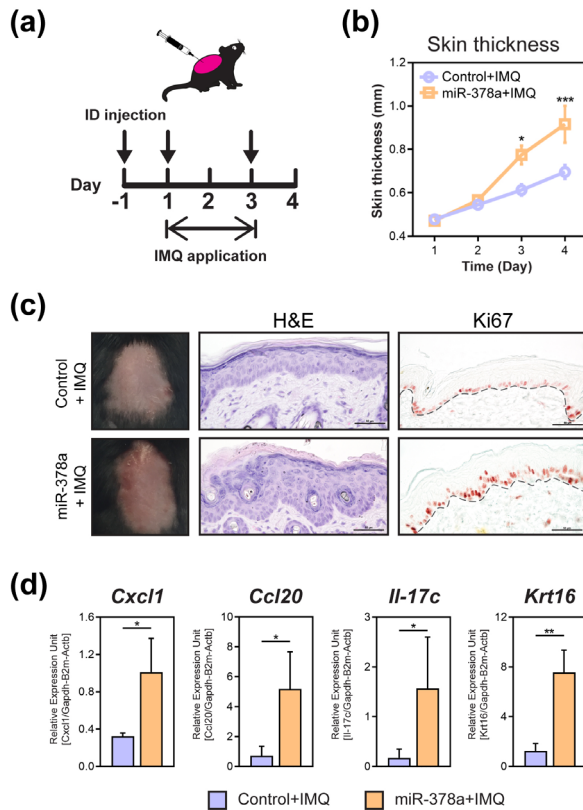


Figure 30. (a) Timeline for injection of miR-378a mimic or control oligos before imiquimod topical treatment on the shaved back skin of mice. (b) Measurement of skin thickness upon miR-378a mimic injection and IMQ topical treatment (orange) compared to control oligos injection and IMQ application (purple). (c) Quantitative RT-PCR analysis of *Cxcl1*, *Ccl20*, *Il-17c* and *Krt16* expression in back skin from mice samples. Scale bar = 50 μ m. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; $n = 6$ /group.

4.4.3 miR-378a is regulated by IL-17A via NF- κ B and C/EBP- β in primary human keratinocytes

We aimed to identify potential regulators of miR-378a by treating cultured primary human keratinocytes with known psoriasis-associated pro-inflammatory cytokines. RT-qPCR results have shown that expression levels of both pri-miR-378a and its mature form miR-378a were increased upon IL-17A treatment of keratinocytes monolayer cultures (Figure 31a). The increased expression of miR-378a compared to untreated control was significantly higher at 24 h after IL-17A treatment, increasing even more at 48 h, suggesting direct transcriptional induction of miR-378a by the cytokine IL-17A. Similarly, we have found overexpression of miR-378a also

in 3D epidermal reconstructs treated with IL-17A (Figure 31b) or treated with a combination of IL-17A, TNF- α and IFN- γ (Figure 31c), cytokines commonly found in psoriasis lesional skin.

miR-378a shares the transcription starting site (TSS) location with its host gene PPARGC1 β [290]. At this level, while further investigating the IL-17A pathway, we have found binding sites for the transcription factors NF- κ B and C/EBP- β . We tested whether these TFs were playing a role in the regulation of miR-378a by blocking NF- κ B signaling pathway with the inhibitor BAY 11-7082 or inhibiting C/EBP- β with a pool of specific siRNAs, in cultured monolayer of keratinocytes. The expression levels of miR-378a were reduced when NF- κ B inhibition was followed by treatment of IL-17A, while no expression changes were detected for miR-378a in absence of IL-17A treatment (Figure 31d). Alternatively, inhibition of miR-378a upon interruption of C/EBP- β signaling pathway was independent of IL-17A treatment (Figure 31e). Overall, we confirmed the importance of these TFs in promoting inflammatory responses through the regulation of miR-378a expression.

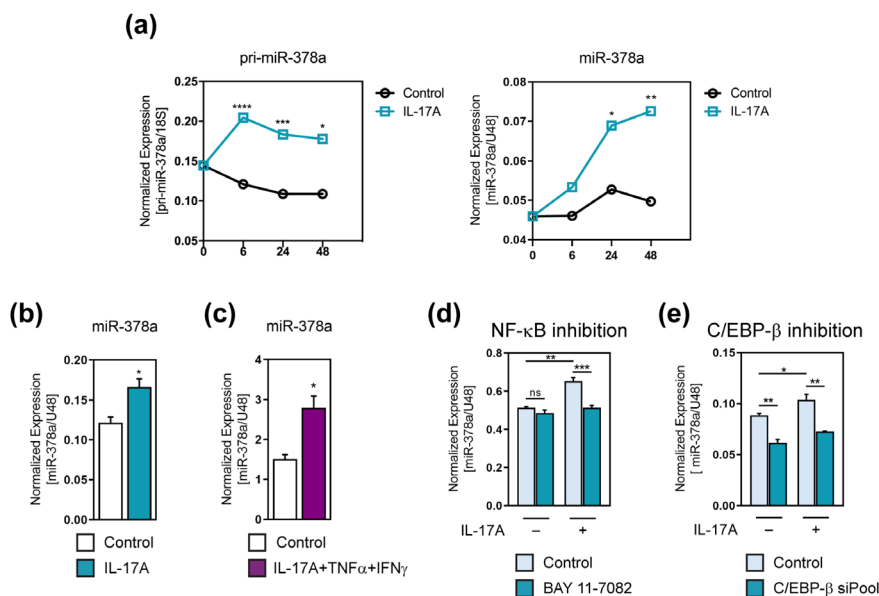


Figure 31. (a) RT-qPCR analysis of pri-miR-378a and miR-378a expression in cultured primary human keratinocytes treated with IL-17A at different time points. (b) RT-qPCR analysis of miR-378a in 3D epidermal reconstruct treated with IL-17A for 72 h. (c) RT-qPCR analysis of miR-378a in 3D epidermal reconstruct treated with a combination of IL-17A + TNF- α and IFN- γ for 72 h. (d) RT-qPCR analysis of miR-378a upon NF- κ B signaling pathway inhibition by BAY11-7082, in presence or absence of IL-17A treatment. (e) RT-qPCR analysis of miR-378a in cultured keratinocytes upon C/EBP- β inhibition by a pool of specific siRNAs, in presence or absence of IL-17A treatment.

4.4.4 miR-378a promotes inflammation through upregulation of the IL-17A-related chemokines IL-8/CXCL8 and CCL20

To investigate the effect of miR-378a overexpression in cultured keratinocytes, we transiently transfected monolayers of primary human keratinocytes with miR-378a mimics or control mimics, followed by adding or not IL-17A, and continued the treatment for 24 hours. The samples served for microarray transcriptomic profiling, which principal component analysis (PCA) has shown separation of the four groups including keratinocytes transfected with miR-378a mimics (with or without IL-17A treatment) as well as with control mimics (with or without IL-17A treatment) (*Figure 32a*). In the absence of IL-17A, 266 genes were significantly deregulated upon overexpression of miR-378a (186 were up- and 80 were downregulated) (*Figure 32b-left*). In the presence of IL-17A, 194 genes were found significantly differentially expressed by miR-378a overexpression (127 genes were up- and 67 downregulated) (*Figure 32b-right*). Gene ontology pathway analysis have shown the implication of these genes in biological processes related to skin development, epidermal differentiation and cytokines (in particular IL-6)-mediated signaling pathways. We could observe a significant increase in the expression of pro-inflammatory cytokines CXCL8/IL-8 and CCL20 by overexpressing miR-378a (*Figure 32c*), while a reduction in their expression levels was detected upon inhibition of miR-378a (*Figure 32d*). Given that these cytokines are typically released by triggered keratinocytes during inflammatory events occurring in psoriasis lesions [34, 38, 126, 127], the results obtained are in line with the up-regulation of miR-378a found in psoriatic keratinocytes.

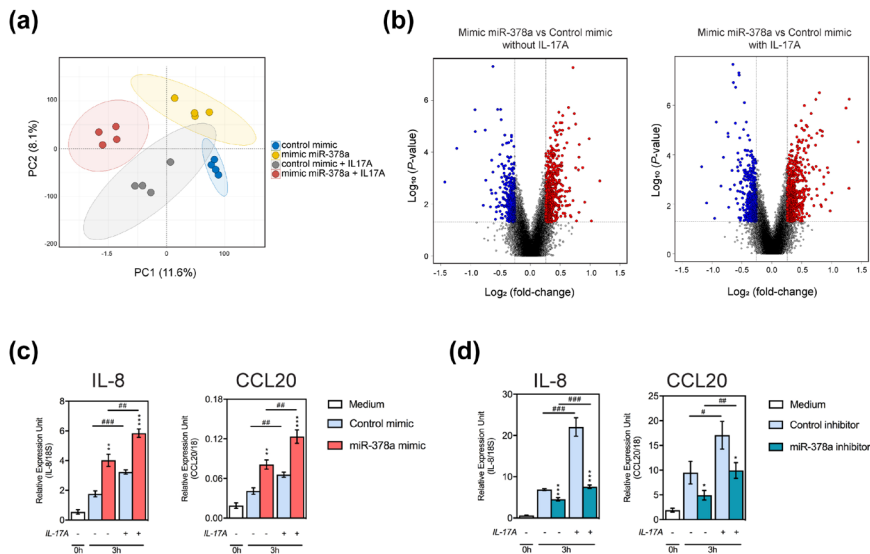


Figure 32. (a) PCA performed on microarray data obtained from treating cultured keratinocytes with oligo controls only (blue) and in presence of IL-17A (gray) or miR-378a mimic only (yellow) and in presence of IL-17A (red). (b) Volcano plot showing the proportion of differentially expressed transcripts detected by the microarray gene expression profiling in miR-378a mimic vs mimic control in absence (left) and presence (right) of IL-17A. Red dots represent down-regulated genes ($FC < 0.67$); red dots the up-regulated genes ($FC > 1.5$). (c) RT-qPCR analysis of CXCL8/IL-8 and CCL20 expression in cultured keratinocytes transfected with miR-378a mimic or mimic control, in presence or absence of IL-17A. (d) RT-qPCR analysis of CXCL8/IL-8 and CCL20 expression in cultured keratinocytes transfected with miR-378a inhibitor or inhibitor control, in presence or absence of IL-17A.

4.4.5 miR-378a suppresses the NF- κ B-inhibitor NFKBIA

The transcription factor NF- κ B plays an essential role in orchestrating the expression of genes coding for pro-inflammatory molecular mediators such as cytokines, chemokines and growth factors in human diseases [503-505]. In psoriasis, it is known to control inflammatory processes, as well as proliferation, differentiation and death for apoptosis of keratinocytes [506]. NF- κ B is kept inactive in the cytoplasm by the I κ B (inhibitor of kappa-B) protein family [507] and able to exert its function upon translocation into the nucleus after I κ B proteins get phosphorylated by a class of kinases named IKK (inhibitor of kappa-B kinase) [508].

To refine the mechanism through which miR-378a overexpression could promote inflammation in psoriatic keratinocytes, we studied its predicted targets. These were found using the online tool TargetScan [509], by identifying the presence of conserved sites on mRNAs that match the seed region of miR-378a. Among these,

we have found NFKBIA (I κ B α) as one of the most interesting, since involved in the interruption of the nuclear translocation signals of NF- κ B, holding it inactive in the cell cytoplasm [510, 511]. In addition, NFKBIA is also known to regulate inflammatory processes in skin diseases [512]. Due to the complementarity between miR-378a seed-sequence and the 3'UTR of NFKBIA (*Figure 33a*), we investigated whether inhibition or overexpression of miR-378a would have affected the expression of NFKBIA protein. As expected, we have seen a reduction in NFKBIA protein production when miR-378a was up-regulated in cultured keratinocyte, as well as an increased expression when miR-378a was knocked down (*Figure 33b*).

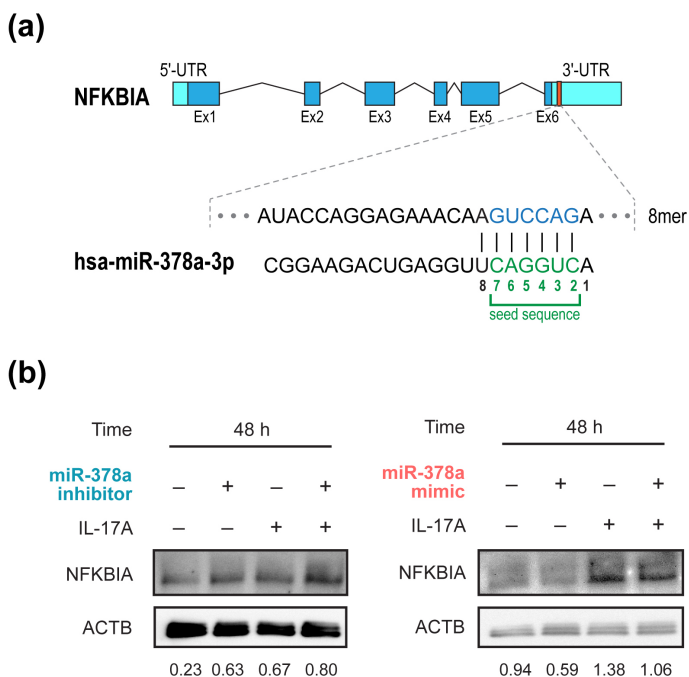


Figure 33. (a) Illustration of the 8-mer predicted complementarity between miR-378a seed sequence and 3'UTR of NFKBIA gene. (b) western blot analysis showing the effects of miR-378a inhibition and overexpression in cultured keratinocytes on the NFKBIA protein production.

Taken together, our results unfolded the importance of miR-378a in psoriasis-associated events, enhancing inflammation through the promotion of cytokines and chemokines expression in psoriatic keratinocytes, as well as by stimulating epidermal cells proliferation, which results in thicker skin.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Psoriasis has dramatic implications on the quality of life of about 125 million people worldwide. Despite the remarkable number of gene expression profiling studies attempting to clarify the impact of genomic alterations in the pathogenesis of psoriasis, the majority of these were performed without discerning specific cell populations in the skin. While aware of an altered cross-talk between keratinocytes and immune cells in psoriasis, the goal of this thesis was to gain a deeper understanding of the keratinocyte-specific transcriptomic changes in psoriasis lesional and non-lesional skin.

In our first study, we identified more than 2,000 protein-coding genes with significantly altered expression in psoriasis lesional keratinocytes compared to keratinocytes from healthy skin (**study I**). The most up-regulated genes were enriched for pathways related to innate immunity, cell proliferation and inflammation. This was consistent with previous profiling studies using full-depth skin biopsies. Type I interferon and NF- κ B were enriched among our set of deregulated genes in psoriatic keratinocytes compared to healthy skin, again in line with previous studies. Interestingly, we have also found a set of genes, for example TNIP3 (TNFAIP3 Interacting Protein 3) and TNFAIP3 (TNF Alpha Induced Protein 3), which were previously believed to be expressed by immune cells. We validated the overexpression of the previously uncharacterized gene EPGN, a growth factor that promotes keratinocytes proliferation and therefore potentially contributing to epidermal hyperplasia in psoriasis. Among the down-regulated genes in psoriatic keratinocytes, epidermis development and differentiation were pathways enriched, explaining the alteration of these processes in psoriasis. GSEA analysis using cytokine-induced gene signatures confirmed the important role of IL-17 and IL-22 signatures on the gene expression changes found in keratinocytes from the psoriasis lesions. Surprisingly, approximately 50% of the overexpressed genes in psoriatic keratinocytes did not overlap with cytokine-induced gene signatures, suggesting intrinsic alterations of the keratinocyte transcriptome. Therefore, we focused on potential genetic aspects of psoriasis. We have found a large share of differentially expressed genes in keratinocytes from psoriasis lesion compared to healthy skin overlapping SNPs associated to psoriasis susceptibility by previous GWAS studies. Many of them were localized in proximity or overlapping the epidermal differentiation complex (EDC) on chromosome 1, where PSORS4 is also located. These findings suggest that a part of the gene expression changes in psoriatic keratinocytes could be explained by genetic variation, contributing – together with immune cell functions – to the psoriasis susceptibility. We also analyzed the expression of transcription factors in psoriasis keratinocytes and identified 54 transcription factors had altered expression, including previously known

in the psoriasis context such as AP-1, RORA, SOX4, KLF4, NF- κ B, EGR and GATA3, some of which are located on psoriasis-susceptibility regions, supporting the link between genetic susceptibility and keratinocytes transcriptomic alterations in psoriasis. Other altered transcription factors identified in our study, including TRPS1, HEY2 and PAX3, were uncharacterized in the context of psoriasis and their role in the disease will need further study. Interestingly, several genes were found differentially expressed in psoriasis non-lesional compared to healthy epidermal cells, suggesting that keratinocytes contribute to the “pre-psoriatic state” of non-involved skin in psoriasis patients.

In **study II**, we investigated the expression of non-protein coding RNAs in the epidermal cells (CD45-negative cells) of patients with psoriasis, with a focus on the long intergenic non-coding RNA class. More than 450 deregulated non-coding transcripts were found in our analysis. In psoriatic keratinocytes, we have confirmed the overexpression of PRINS, the first lncRNA found altered in full-depth skin biopsies from psoriasis patients, as well as identified previously uncharacterized lncRNAs, e.g. NEAT1, which expression was not found significantly altered in other psoriasis transcriptomic studies using full-depth skin biopsies. We validated the expression of the lncRNA LINC00958, which was upregulated in keratinocytes from psoriasis lesion compared to both psoriasis non-lesion and healthy keratinocytes. This lncRNA has been described in several cancers affecting different organs but it has not been previously identified in psoriasis. Besides confirming its non-coding potential, we have also determined its expression in different skin cell types, excluding the possibility that the overexpression we observe in psoriasis lesions is attributable to dermal or immune cell components. The cytoplasmic enrichment of LINC00958 opens new possibilities for future experiments aiming to further characterize this lncRNA in the context of psoriasis. Functional studies are needed to shed light on its function in the disease, which according to its subcellular localization we could expect to be controlling mRNA stability or regulating mRNA translation.

Similar to mRNAs, the majority of the changes in non-coding transcripts expression were detectable comparing psoriatic lesional keratinocytes with healthy skin. However, changes in the expression of several lncRNAs were found also in uninvolved non-lesional keratinocytes of psoriasis patients compared to healthy skin. These alterations in the keratinocytes-specific non-coding transcriptome suggests a situation of “pre-psoriatic inflammatory stage” occurring in psoriasis non-lesional epidermis. Interestingly, a part of the differentially expressed non-coding RNAs were overlapping with psoriasis-associated genetic loci on chromosome 1 (PSORS4, epidermal differentiation complex regions), and on chromosome 6 (PSORS1, psoriasis risk allele HLA-C*06:02), suggesting the contribution of lncRNAs to the genetic susceptibility to psoriasis.

In **study III**, we aimed to investigate the potential of circulating miRNAs to become a diagnostic tool to use in clinical practice that could overcome the challenges of an early diagnosis of psoriatic arthritis manifestations, in patients with cutaneous psoriasis. We investigated whether changes in the expression levels of circulating microRNAs in extracellular vesicles could serve as biomarkers for this purpose. A cohort including patients with cutaneous-only psoriasis since ten years and patients with confirmed psoriatic arthritis upon ten years follow-up, was used for small RNA-sequencing profiling screening. This revealed microRNAs with altered expression levels in plasma extracellular vesicles. Some of the discovered miRNAs were included in the following validation phase and quantitative real-time PCR analysis confirmed the down-regulation of the miRNAs let-7b-5p and miR-30e-5p in patients with confirmed arthritis manifestations and cutaneous psoriasis compared with patients with cutaneous psoriasis only. Let-7b-5p, which has been associated to inflammatory events in atherosclerosis, directly targets IL-6, a pro-inflammatory cytokine overexpressed in patients with psoriatic arthritis compared to patients with cutaneous-only psoriasis, and other genes associated to joint inflammation (HMGA1 and HMGA2) or inflammatory bone loss (PRDM1). The level of miR-30e-5p in extracellular vesicles was found reduced in a study comparing PBMC from patients affected by rheumatoid arthritis with those from healthy donors. Down-regulation of miR-30e-5p leads to increased expression of its target BMI1, mediator of arthritis in mice through the activation of the NF- κ B signaling pathway. Limitations encountered in our study, which are common in these type of exploratory profiling studies, include the relatively small sample size within the two cohorts, as well as the technical and biological variability for example across the samples. However, a strength of this study is that patients did not receive any systemic treatments and, thus, we excluded the potential effect of systemic treatments on circulating miRNAs. Another strength is the use of extracellular vesicles instead of whole plasma. Since miRNAs are secreted selectively into EVs, these may therefore be more specific markers than those from whole plasma. Future studies on larger cohorts of patients would definitely provide more insights about the potential of these miRNAs as diagnostic tools for psoriatic arthritis.

In **study IV** we aimed to characterize functionally the miRNA miR-378a in psoriasis. Small RNA sequencing analysis of CD45-negative cells from psoriasis lesion/non-lesion and healthy skin performed by our group, identified miR-378a to be overexpressed in psoriasis lesional keratinocytes. Once more, this approach helped avoiding potential miRNA expression signature “contaminations”, coming from dermal cells or immune cells infiltrating the epidermis of psoriasis patients. As a model of psoriasis-like skin inflammation, we applied imiquimod (Aldara®) cream on shaved back skin of mice, which histologically and at the molecular level resembles human psoriasis. Injection of miR-378a into mouse skin together with induction of skin inflammation increased overall skin thickness, the number of

proliferating cells in the basal layer of the epidermis and increased the expression of the pro-inflammatory chemokines and cytokines *Cxcl1*, *Ccl20* and *Il-17c*. These results suggested that miR-378a actively participates in establishing inflammatory processes in the skin. We have also found that IL-17A can increase the expression of miR-378a in cultured primary human keratinocytes. Studying the alterations in its expression upon interruption of the IL-17A-related pathways NF- κ B and C/EBP- β , we have confirmed the importance of these transcription factors in establishing an inflammatory response through the action of miR-378a. Next, we performed Gene Ontology (GO) Biological Processes (BP) pathway analysis of genes differentially expressed upon miR-378a-overexpression and inhibition. Enrichment in skin development, regulation of epidermal differentiation, cytokines- and, specifically, IL-6-mediated signaling pathway were found among the genes regulated by miR-378a. In addition, inflammatory cytokines including IL-8/CXCL8 and CCL20 underwent enhanced expression in cultured primary human keratinocytes when miR-378a overexpression by mimic transfection was combined with IL-17A treatment. At last, upon miR-378a overexpression we have detected reduced NFKBIA protein expression, a predicted direct target of miR-378a which regulates the activation, and further translocation to the nucleus, of NF- κ B. We hypothesize that this could explain the mechanism through which miR-378a boosts the inflammation through the previously described psoriasis-associated cytokines. Further studies on the phosphorylation of NF- κ B upon miR-378a inhibition/overexpression will reveal the genuine answer to this question.

Overall, our studies identified specific transcriptomic changes in keratinocytes from psoriasis lesional and non-lesional skin. These included both coding and non-coding genes, complementing previous profiling studies in psoriasis and further highlighting the contribution of keratinocytes to molecular changes in the disease, in part genetically determined (intrinsic) and in part sustained by cytokines-related inflammatory processes (extrinsic). Future functional studies of the coding and non-coding transcripts, as well as the microRNAs, found deregulated in the epidermis of psoriasis lesions could reveal their role in skin-related processes, such as the maintenance of epidermal homeostasis, as well as in impairing keratinocyte-related functions in psoriasis. Furthermore, we have identified two microRNAs, let-7b-5p and miR-30e-5p, which require additional studies in larger cohorts in order to be established in clinical practice routines, for the diagnosis of early arthritis manifestations in patients with cutaneous psoriasis.

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There is no
Money without Labor
Loyalty without Trust
Appreciation without Education
Wisdom without Experience
Success without Sacrifice
Happiness without Peace of Mind
Beginnings without Endings
—Marc Chernoff

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