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Molecular Alterations in the Pathogenesis of Two Chronic Dermatoses – Vitiligo and Psoriasis

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II **Šahmatova L**, Sügis E, Šunina M, Hermann H, Prans E, Pihlap M, Abram K, Rebane A, Peterson H, Peterson P, Kingo K, Kisand K. Signs of innate immune activation and premature immunosenescence in psoriasis patients. Scientific Reports 2017; 7: 7553.

III **Šahmatova L**, Tankov S, Aab A, Hermann H, Reemann P, Karelson M, Abram K, Kisand K, Kingo K, Rebane A. MicroRNA-155 is dysregulated in the skin of patients with vitiligo and inhibits melanogenesis-associated genes in melanocytes and keratinocytes. Acta Dermato-Venereologica 2016; 96: 742–747.

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Paper II: Participating in the collection of the material (enrolling participants, taking informed consent, gathering clinical information, taking skin biopsy samples); conducting laboratory experiments (separating plasma from whole blood, extracting RNA from skin biopsy samples, conducting cytokine testing); analysing the data and preparing the manuscript.

Paper III: Participating in the collection of the material (enrolling participants, taking informed consent, gathering clinical information, taking skin biopsy samples); conducting laboratory experiments (extracting RNA from skin biopsy samples and cells; conducting cell culture experiments); analysing the data and preparing the manuscript.

2. ABBREVIATIONS

ΔΔCt method	comparative Ct method
2D	two-dimensional
3'UTR	3' untranslated region
AIM2	absent in melanoma 2
AMP	adenosine monophosphate
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
BSA	body surface area
CARD	caspase activation and recruitment domain
CCL	C-C motif chemokine ligand
cDNA	complementary DNA
cGAMP	cyclic guanosine monophosphate–adenosine monophosphate
cGAS	cyclic guanosine monophosphate–adenosine monophosphate synthase
CXCL	C-X-C motif chemokine ligand
DAMP	damage-associated molecular pattern
DAPI	4′,6-diamidine-2′-phenylindole dihydrochloride
DC	dendritic cell
DGCR8	DiGeorge syndrome critical region 8
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EOMES	eomesodermin
FLICA	fluorochrome-labelled inhibitor of caspases
G-CSF	granulocyte–colony stimulating factor
GM-CSF	granulocyte-macrophage–colony stimulating factor
GMP	guanosine monophosphate
GO	Gene Ontology
HP	Human Phenotype Ontology
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
ISH	*in situ* hybridisation
JNK	c-JUN N-terminal kinase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LC3	microtubule-associated protein light chain 3
MAPK	mitogen-activated protein kinase
MDA5	melanoma differentiation-associated protein 5
MHC	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
miRNA	microRNA
moLC	monocyte derived Langerhans cells
mRNA	messenger ribonucleic acid

mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-kappa B
NLR	nucleotide-binding domain leucine-rich repeat-containing
NLRC	NLR family, CARD-containing
NLRP	NLR family, PYD-containing
PAMP	pathogen-associated molecular pattern
PASI	psoriasis area and severity index
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PRR	pattern recognition receptor
PYCARD	PYD and CARD domain containing
PYD	pyrin domain
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
SFM	serum-free medium
STING	stimulator of interferon genes
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF	tumour necrosis factor
Tregs	regulatory T cells
TSLP	thymic stromal lymphopoietin
TYRP1	tyrosinase related protein 1
UV	ultraviolet
UVRAG	UV radiation resistance-associated gene protein
WIPI1	WD repeat domain, phosphoinositide interacting 1

3. INTRODUCTION

Vitiligo is an acquired chronic idiopathic disease manifesting mainly as white macules on the skin due to the destruction of melanocytes (Ezzedine et al, 2015a). It is the most common depigmenting disorder affecting 0.5–2% of the population worldwide (Bergqvist & Ezzedine, 2020). Both sexes are equally affected, and there are no differences in rates of occurrence according to phototype or race (Boniface et al, 2018b). Although the depigmentation of the skin and hairs rarely causes any physical symptoms, vitiligo is not merely a cosmetic disease. Vitiligo may cause psychosocial problems and impair the quality of life (Ezzedine et al, 2015b; Silverberg & Silverberg, 2013). Therapeutic options for vitiligo are limited and the results are often unsatisfactory. This is partially caused by uncertainty in the etiopathogenetic mechanisms (Bergqvist & Ezzedine, 2020; Taieb et al, 2013). Multiple mechanisms have been considered to contribute to the destruction of melanocytes. These include autoimmune mechanisms together with genetic and environmental factors as well as metabolic, oxidative stress and cellular abnormalities. However, the biological mediators and the molecular mechanisms that lead to metabolic defects, melanocyte degeneration and autoimmunity remain not fully understood (Bergqvist & Ezzedine, 2020; Boniface et al, 2018b; Ezzedine et al, 2015a). MicroRNAs (miRNA), the regulators of gene expression, have been the objects of research since the early 1990s (Gebert & MacRae, 2019; Jonas & Izaurralde, 2015). To date, much uncertainty still exists about the role of miRNAs in the pathogenesis of vitiligo (Yan et al, 2020).

Psoriasis is a common inflammatory skin disease with a complex pathogenesis (Boehncke & Schon, 2015). The prevalence of psoriasis in adults is 0.91–8.5% depending on the ethnicity and geography, being higher at higher latitudes, and in Caucasians compared to other ethnic groups (Parisi et al, 2013). However, psoriasis is equally prevalent in both sexes. Most commonly, psoriasis manifests as red scaly patches on the skin, but nails and joints can be affected as well (Boehncke & Schon, 2015). Moreover, multiple associated comorbidities have been described, with cardiometabolic diseases being the most prevalent ones (Takeshita et al, 2017). Pathogenetically, psoriasis is characterized by inflammation as well as abnormal proliferation and differentiation of keratinocytes. Within inflammation, the tumour necrosis factor (TNF) and the interleukin (IL)-23/T helper (Th) cell 17 axis play a central role (Boehncke & Schon, 2015; Harden et al, 2015b; Ogawa et al, 2018). Knowledge about the pathogenesis has enabled the development of several effective conventional and biological therapeutic agents (Amatore et al, 2019). However, every therapeutic agent is not equally effective for all psoriasis patients and the markers predicting individual responses before treatment still need to be identified (da Rosa et al, 2017; Hoffmann et al, 2011). Non-invasive diagnostic methods for skin diseases are being developed (Falcone et al, 2017; Orro et al, 2014), but the accurate diagnostic and prognostic markers for predicting the course of

psoriasis have not been developed yet. Therefore, extensive research on the pathogenetic mechanisms of psoriasis is still in progress (Boehncke & Schon, 2015; Christophers et al, 2014; Fanoni et al, 2019; Liang et al, 2017; Sweeney et al, 2011).

This study attempts to unravel the molecular mechanisms of vitiligo and psoriasis, focusing on local and systemic inflammation, the involvement of innate immunity and miRNAs in disease pathogenesis.

4. REVIEW OF THE LITERATURE

4.1. Overview of vitiligo

4.1.1. Clinical manifestation of vitiligo

Clinically, depigmented macules on the skin and mucosa as well as depigmented hairs are characteristic to vitiligo (Ezzedine et al, 2015a). The first symptoms usually occur in young people; specifically, in 50% of cases vitiligo appears at an age younger than 20 (Ezzedine et al, 2015a) and in 25% of cases below the age of 10 (Boniface et al, 2018b). Based on the clinical picture, there are two types of vitiligo – non-segmental and segmental vitiligo (Ezzedine et al, 2012). In the case of the most common form, non-segmental vitiligo, white macules affect multiple parts of the body in a symmetrical pattern, usually starting from the fingers, hands and face (Boniface et al, 2018b; Ezzedine et al, 2012) (Figure 1). Segmental vitiligo lesions locate unilaterally in a segmental pattern and are commonly accompanied by depigmented hairs. This form of vitiligo progresses rapidly and is less responsive to the treatment than non-segmental vitiligo (Bergqvist & Ezzedine, 2020).

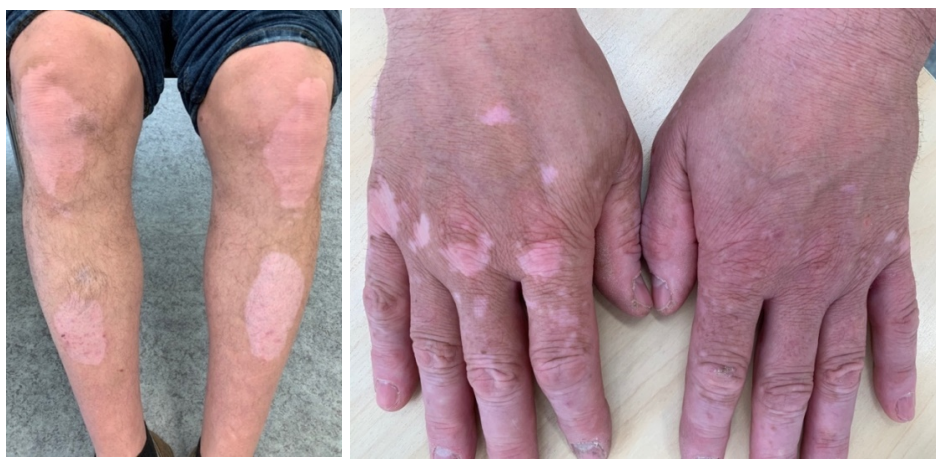

Figure 1. Non-segmental vitiligo (photographs taken by the author)

The onset of non-segmental vitiligo is usually insidious but severe sunburn, pregnancy, skin trauma or emotional stress may precede the occurrence (Alikhan et al, 2011). Generally, the course is slowly progressive (Ezzedine et al, 2012). A progressive disease, also defined as an active disease, is marked by the development of new lesions and the extension of the old lesions (Ezzedine et al, 2012). This is characterised by hypomelanotic lesions with poorly defined borders (Benzekri & Gauthier, 2017), numerous 1- to 5-mm depigmented

lesions as well as the Koebner phenomenon, the appearance of lesions on the lines of trauma. Inflammatory lesions are less common (Aboul-Fettouh et al, 2017).

Although there are rarely any physical symptoms other than depigmented macules on the skin, vitiligo patients, especially dark-skinned patients, have an impaired quality of life (Ezzedine et al, 2015b; Karelson et al, 2013; Silverberg & Silverberg, 2013). Moreover, the prevalence of depression as well as anxiety is higher in vitiligo patients in comparison to the general population (Lai et al, 2017; Osinubi et al, 2018).

In addition to psychological problems, patients with vitiligo exhibit an elevated frequency of autoimmune diseases, including the autoimmune thyroid disease, type 1 diabetes, Addison's disease, pernicious anaemia, *alopecia areata*, systemic lupus erythematosus, systemic sclerosis, Sjögren's syndrome and rheumatoid arthritis (Alikhan et al, 2011; Choi et al, 2017). Thyroid diseases are the most common, affecting 14.3% of patients with vitiligo. Furthermore, 20.8% of vitiligo patients have thyroid-specific autoantibodies (Vrijman et al, 2012). However, vitiligo can be a part of all types of autoimmune polyendocrine syndromes that are present with a combination of Addison's disease, hypoparathyroidism, ectodermal dysplasia, mucocutaneous candidiasis, *alopecia areata*, gonadal failure and/or pernicious anaemia (Alikhan et al, 2011). Besides, vitiligo can be associated with ophthalmological and auditory problems (Alikhan et al, 2011). Specifically, up to 20% of vitiligo patients are affected by hearing impairment which may be caused by functional disorders in the intermediate cells (melanocytes) of the *stria vascularis* (Alikhan et al, 2011; Hong et al, 2009). Ocular anomalies, including choroidal abnormalities, uveitis, iritis or retinal findings, are caused by pigment disturbances and occur in up to 40% of patients (Alikhan et al, 2011; Karadag et al, 2016).

Frequent comorbidities for vitiligo are also chronic inflammatory skin disorders such as psoriasis and atopic dermatitis (Alikhan et al, 2011; Mohan & Silverberg, 2015). Overall, 11.7% patients with vitiligo have a history of atopic dermatitis (Mohan & Silverberg, 2015). The prevalence of psoriasis among vitiligo patients varies from 4.0% (Powell & Dicken, 1983) to 7.6% (Sheth et al, 2013). A recent meta-analysis reported that compared to controls, vitiligo patients are 3.43-fold more likely to be diagnosed with psoriasis. Explanations for this association remain speculative (Yen & Chi, 2019).

4.1.2. Etiopathogenesis of vitiligo

According to histopathological and immunohistochemical studies, melanocytes and consequently melanin are absent in the lesional skin of vitiligo (Ezzedine et al, 2015a). However, sometimes occasional melanocytes can persist (Kim et al, 2008). Multiple mechanisms are supposed to be involved in melanocyte destruction, including a genetic predisposition, environmental triggers, meta-

bolic abnormalities, an impaired renewal of melanocytes, and altered inflammatory and immune responses (Boniface et al, 2018b).

Epidemiological studies have shown that vitiligo tends to aggregate in families. Around 20% of vitiligo patients have at least one first-degree relative with vitiligo (Nath et al, 1994), and the risk ratio for first-degree relatives is increased up to 18-fold (Alkhateeb et al, 2003). A twin study in Caucasians found that the concordance of vitiligo was 23% in monozygotic twins (Alkhateeb et al, 2003). Genetic susceptibility to vitiligo is determined by several loci. Genome-wide association studies have identified many vitiligo-associated genes. Most of them encode immunoregulatory components (*HLAI*, *HLAII*, *IFIH1*, *IL2RA*, *PTPN22*, *CTLA4*), proteins involved in melanocyte function (*TYR*, *OCA2*, *MC1R*) and apoptotic regulators (*CASP7*, *SERPINB9*, *BCL2L11*, *BAD*) (Jin et al, 2016; Jin et al, 2012; Jin et al, 2010).

As elevated levels of reactive oxygen species have been observed in vitiligo lesional skin and in melanocytes isolated from the skin of vitiligo patients (Li et al, 2017), it is believed that in genetically susceptible individuals, environmental factors such as ultraviolet (UV) radiation, chemical agents or mechanical trauma may lead to an uncontrolled production of reactive oxygen species and oxidative stress (Boniface et al, 2018b). The stressed melanocytes (Rezk et al, 2017) and surrounding keratinocytes (Richmond et al, 2017) can release proinflammatory cytokines and chemokines, such as the C-C motif chemokine ligand (CCL) 5, C-X-C motif chemokine ligand (CXCL) 8, CXCL9, CXCL10, CXCL12, CXCL16, which are important for the recruitment of immune cells (Bastonini et al, 2019; Rezk et al, 2017; Richmond et al, 2017). Moreover, nucleic acids that can be released from damaged melanocytes and/or keratinocytes can be recognised by different pattern recognition receptors (PRR) leading to the activation of innate immunity and an inflammatory response that in turn activates adaptive immunity (Boniface et al, 2018b; Wang et al, 2015a). Indeed, melanocyte-specific antibodies, which are uncommon in healthy persons, have been found to circulate in the blood and are deposited in the skin of vitiligo patients (Sandoval-Cruz et al, 2011). Additionally, skin-homing melanocyte-specific cytotoxic T lymphocytes have been found circulating in the blood of vitiligo patients (Boniface et al, 2018a; Cheuk et al, 2017; Richmond et al, 2018; van den Boorn et al, 2009).

Although vitiligo is clinically not an inflammatory disease, skin deposits of inflammatory cells and an increased expression of inflammatory cytokines are characteristic, especially to the marginal zone of a vitiligo lesion, also known as perilesional skin (Sandoval-Cruz et al, 2011). Perilesional skin and to a lesser extent lesional skin is infiltrated by T lymphocytes, B lymphocytes, Langerhans cells, dendritic cells (DCs) and histiocytes (Montes et al, 2003; Sandoval-Cruz et al, 2011; Wang et al, 2011). The population of T cells consists of both, CD8+ and CD4+ T cells, with an increased CD8+/CD4+ ratio. It has previously been observed that these cells are polarised towards a type-1-like subset, producing the TNF and interferon (IFN)-γ (Wankowicz-Kalinska et al, 2003). Van den Boorn et al. showed that CD8+ T cells isolated from perilesional skin are

cytotoxic against melanocytes (van den Boorn et al, 2009). Previous results demonstrate an increased expression of several cytokines (TNF, IFN-γ, IL-1b, IL-6, IL-10, IL-17, IL-22, IL-23A, transforming growth factor [TGF]-β, CCL5, CXCL10, CXCL12) and their receptors (IL10RA, IL10RB, IL20RB, IL22RA2, IL28RA, IFNA1, IFNB1) in the skin, peripheral blood mononuclear cells (PBMC) and/or the serum of vitiligo patients (Bhardwaj et al, 2017; Grimes et al, 2004; Mansuri et al, 2016; Moretti et al, 2002; Regazzetti et al, 2015; Reimann et al, 2012; Rezk et al, 2017; Rätsep et al, 2008; Singh et al, 2016).

Melanocytes and infiltrating cells are not the only cell populations that are altered in vitiligo pathogenesis (Bastonini et al, 2019). Keratinocytes in the depigmented epidermis are more vulnerable to apoptosis and produce smaller amounts of melanogenic mediators than in normal skin. Furthermore, the expression of genes involved in keratinocytes' differentiation and cornification is dysregulated in the vitiligo lesional epidermis (Moretti et al, 2009; Singh et al, 2017). In addition, fibroblasts show a senescent phenotype in the skin of patients with vitiligo (Bastonini et al, 2019).

However, the exact mechanisms underlying vitiligo still remain unknown and the initial events that breach the self-tolerance to melanocyte-specific antigens and the factors that cause the destruction of melanocytes still remain unidentified.

4.1.3. Management of vitiligo

The diagnosis of vitiligo is usually made based on the clinical picture (Ezzedine et al, 2015a). The Wood's lamp, a handheld UVA emitting device, facilitates determining the extent and activity of vitiligo, as well as monitoring the response to therapy (Alghamdi et al, 2012). However, sometimes distinguishing between vitiligo and other diseases manifesting as depigmented or hypopigmented patches can be difficult. Common disorders that can have a similar presentation to vitiligo include *nevus depigmentosus*, idiopathic guttate hypomelanosis, *pityriasis versicolor* and chemically-induced leukoderma. In diagnostic difficulties a biopsy can be required (Alikhan et al, 2011).

For the treatment of vitiligo, immunosuppressive and pigmentation stimulating measures can be used. These include topical corticosteroids, topical calcineurin inhibitors, phototherapy, oral steroids and grafting procedures (Taieb et al, 2013). Recently, afamelanotide (Lim et al, 2015), Janus kinase inhibitors (Liu et al, 2017; Rothstein et al, 2017), simvastatin (Vanderweil et al, 2017), apremilast (phosphodiesterase 4 inhibitor) (Majid et al, 2019) and IL-15 signalling blockade (Richmond et al, 2018) have shown promising treatment results but further studies are required to clarify the efficacy and safety of these measures (Abdel-Malek et al, 2020; Frisoli et al, 2020). The therapeutic options for vitiligo depend on the extent, the distribution and the activity of the disease as well as on the patient's age, phototype and intention to treat the disease (Bergqvist & Ezzedine, 2020). Unfortunately, the care often extends over a long period of time and patients are frequently frustrated by the failure of treatments.

Therefore, camouflage techniques, depigmenting agents and psychological interventions are important parts of the global management of vitiligo (Rodrigues et al, 2017; Taieb et al, 2013).

There have been attempts to find biomarkers that allow for an early accurate diagnosis, prognosis, and for the prediction of treatment response of vitiligo (Speeckaert et al, 2017). Histopathology is considered the gold standard in the assessment of disease activity. The presence of an inflammatory infiltrate with the gradual loss of melanocytes in perilesional biopsies is the most straightforward sign of the disease activity in vitiligo (Yadav et al, 2016), but skin biopsies are too invasive for biomarker purposes (Speeckaert et al, 2017). The most evidence for circulating biomarkers is currently available for cytokines IL-1β, IL-17, IFN-γ and TGF-β (Bhardwaj et al, 2017; Singh et al, 2016), chemokines CXCL10 and CXCL9 (Rashighi et al, 2014; Wang et al, 2016c), the soluble cell surface marker CD25 (Speeckaert et al, 2016), autoantibodies (Harning et al, 1991) and oxidative stress markers (Mitra et al, 2017). However, further studies are necessary to find novel biomarkers, to validate the use of biomarkers for clinical trials and daily practice.

4.2. Overview of psoriasis

4.2.1 Clinical manifestation of psoriasis

The most common form of psoriasis is plaque psoriasis. It manifests itself as monomorphic, sharply demarcated erythematous plaques covered by silvery scales (Figure 2). There can be a few plaques, which can extend over larger areas, and they can also occur as erythroderma affecting the entire body's surface (Boehncke & Schon, 2015). Based on the extent of the disease, the activity of the erythema, the intensity of the infiltration and scaling of psoriatic plaques, the psoriasis area and severity index (PASI) is calculated (Schmitt & Wozel, 2005). Although there is no international consensus on the categorisation of the severity of psoriasis (Strober et al, 2020), psoriasis with PASI > 10 (Finlay, 2005; Mrowietz et al, 2011) or > 12 (Schmitt & Wozel, 2005) can be classified as moderate to severe or severe. It is now well established that patients with an early onset of the disease (< 40 years) have a more severe course, whereas patients with a late onset (≥ 40 years) tend to have a milder form of the disease (Boehncke & Schon, 2015).

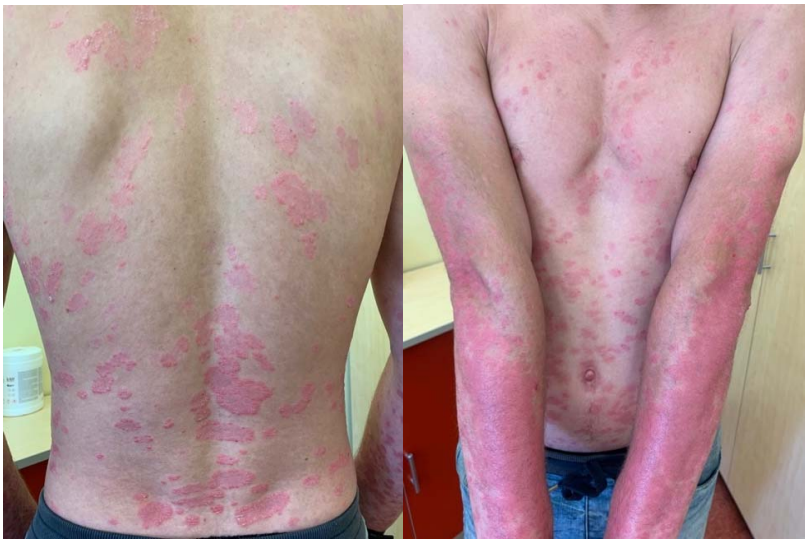

Figure 2. Plaque psoriasis (photographs taken by the author)

Although psoriasis affects the skin, it is not merely a skin-restricted disease but a systemic inflammatory disorder, especially in the case of severe psoriasis (Boehncke & Schon, 2015; Dowlatshahi et al, 2013; Reich, 2012). Among patients with psoriasis, 50% have concomitantly nail involvement and 30% have psoriatic arthritis. Furthermore, many co-morbidities are associated with psoriasis, e.g., obesity, diabetes, cardiovascular diseases, non-alcoholic fatty liver disease, Crohn's disease, lymphoma, cancer, anxiety and depression, many of which can be the result of a persistent inflammation in the body in the case of severe psoriasis (Boehncke & Schon, 2015; Reich, 2012).

4.2.2. Etiopathogenesis of psoriasis

Based on the clinical picture and histopathological findings, inflammation and the abnormal proliferation of keratinocytes are the hallmark features of psoriasis (Boehncke & Schon, 2015). Histopathologically, the epidermis is markedly thickened and in the dermis blood vessels are dilated. The inflammatory infiltrate in the skin mostly consists of T lymphocytes, DCs and neutrophils (Lowes et al, 2014). A genetic predisposition and environmental factors are important in the disease pathogenesis (Harden et al, 2015b).

The importance of genetic factors is supported by twin and family studies as well as genome-wide studies. The concordance rate for monozygotic twins is approximately 70% and for dizygotic twins it is about 20% (Harden et al, 2015b). Genome-wide scans for psoriasis-associated genes have identified genes that mostly regulate antigen presentation (*HLA-Cw6*, *ERAP1*, *ERAP2*, *MICA*), the IL-23 axis (*IL12B*, *IL23A*, *IL23R*, *JAK2*, *TYK2*), T cell development

and polarisation (*RUNX3*, *STAT3*, *TAGAP*, *IL4*, *IL13*), innate immunity (*CARD14*, *REL*, *TRAF3IP2*, *DDX58*, *IFIH1*) and the negative regulators of immune responses (*TNIP1*, *TNFAIP3*, *NFKBIA*, *ZC3H12C*, *SOCS1*) (Ellinghaus et al, 2012; Ellinghaus et al, 2010; Tsoi et al, 2012; Tsoi et al, 2017).

From the immunopathogenetic mechanisms, the TNF-α pathway and IL-23/Th17 pathway have a central role in the development of psoriasis (Ogawa et al, 2018). It is proven that complexes of host deoxyribonucleic acid (DNA) and the epidermis-produced antimicrobial peptide cathelicidin LL-37 stimulate dermal plasmacytoid DCs. Activated DCs produce proinflammatory mediators such as IFN-α, TNF-α and IL-23. TNF-α is additionally released by a broad range of other cell types including macrophages, lymphocytes, keratinocytes and endothelial cells and it exerts its activities on several cell types. IL-23 drives the differentiation of Th17 cells that produce several mediators such as IL-17A, IL-17F and IL-22, which in turn induce inflammation and keratinocyte proliferation (Bettelli et al, 2007; Lowes et al, 2014). From these cytokines, IL-17A, the most abundant in psoriatic skin, is additionally produced by other skin infiltrating cells such as the CD8+ T cells, γδT cells, neutrophils and mast cells (Cai et al, 2013; Hijnen et al, 2013; Kim et al, 2016; Laggner et al, 2011; Lin et al, 2011). Furthermore, antimicrobial peptides, cytokines and chemokines secreted by keratinocytes act as chemoattractants for the infiltrating immune cells (Buchau & Gallo, 2007). Thus, in the case of psoriasis a positive feedback loop exists between the cells of the immune system and the resident epithelial cells. Changes are most prominent in the lesional skin of psoriasis, but there are also clear signs of inflammation and immune cell infiltration in the seemingly healthy skin of the patients (Chiricozzi et al, 2015; Gudjonsson et al, 2010; Keermann et al, 2015).

There is a growing body of evidence about the importance of innate immunity in psoriasis pathogenesis (Christophers et al, 2014; Liang et al, 2017; Sweeney et al, 2011). Although knowledge about TNF-α, IL-23 and IL-17 as the key cytokines in the pathogenesis of psoriasis has enabled the development of novel effective drugs that block the associated pathways (Liang et al, 2017; Ogawa et al, 2018), it is still debated whether psoriasis is autoimmune, i.e., caused by autoantigen-specific T cells, or rather autoinflammatory due to the excessive stimulation of innate immune receptors and inflammasomes.

4.2.3. Management of psoriasis

A diagnosis of plaque psoriasis can be made by physical examination in the vast majority of patients. A skin biopsy can be helpful for challenging cases (Boehncke & Schon, 2015). Disorders that may be mistaken for plaque psoriasis include parapsoriasis, *pityriasis rubra pilaris*, seborrheic dermatitis, *lichen simplex chronicus*, atopic dermatitis, nummular eczema, *lichen planus*, *tinea corporis* and subacute cutaneous lupus erythematosus (Bordignon et al, 2011; Lebwohl, 2003; Napolitano et al, 2016).

Psoriasis has no known cure but many therapies can reduce or nearly stop symptoms (Amatore et al, 2019). Topical therapy is the primary mode of treatment for plaque psoriasis in patients with limited skin involvement (Menter et al, 2009) but is often insufficient for patients with moderate to severe psoriasis (Menter et al, 2020; Menter et al, 2019). The most commonly used topical agents for plaque psoriasis are corticosteroids, vitamin D analogues, calcineurin inhibitors, salicylic acid, anthralin and emollients (Menter et al, 2009). The major treatment options for moderate to severe psoriasis include phototherapy and systemic medications (Amatore et al, 2019; Elmets et al, 2019). There are two types of systemic therapeutic options: non-biological and biological agents (Amatore et al, 2019). Among non-biological medications, methotrexate, cyclosporine and apremilast work by targeting the immune system, whereas acitretin works predominantly by decreasing keratinocyte hyperproliferation and restoring the normal epidermal differentiation (Menter et al, 2020). In the past decade, several biologics have been developed and approved for the treatment of psoriasis (Amatore et al, 2019). With some exceptions, biologics are monoclonal antibodies. The biological agents currently in use include TNF-α, IL-17A, IL-23, or IL-12/IL-23 inhibitors (Menter et al, 2019). A treatment to which all patients respond adequately is not available yet (Kaushik & Lebwohl, 2019a; b).

The prediction of treatment response and overall prognosis of psoriasis has remained a challenge (Amatore et al, 2019; Kaushik & Lebwohl, 2019a; b). Moreover, making accurate diagnosis and differentiating psoriasis from other chronic inflammatory skin diseases can be difficult (Boehncke & Schon, 2015). Therefore, multiple biomarker candidates have been identified at the genome, transcriptome, proteome and metabolome level (Jiang et al, 2015). Some of the numerous potential biomarkers include *HLA-Cw6* (Gudjónsson et al, 2002), *CCL27* (Gudjonsson et al, 2010), *C10orf99* (Gudjonsson et al, 2009; Keermann et al, 2015), *NOS2* (Quaranta et al, 2014), *S100A7* and S100A7 (Gudjonsson et al, 2009; Williamson et al, 2013), *IL36G* and IL-36γ (D'Erme et al, 2015; Keermann et al, 2015), miR-143, miR-223 (Løvendorf et al, 2014), glutamate, phenylalanine, urea and spermidine (Kang et al, 2017; Pohla et al, 2020). As no single marker has shown to be robustly specific to psoriasis, combining several potential biomarkers into a single multicomponent biomarker results in a more accurate diagnostic tool. However, molecular diagnostic methods have not been used yet in the clinics (Reimann et al, 2019).

4.3. Inflammasomes

Inflammasomes are multimolecular complexes that assemble in the cytosol of cells in response to exogeneous as well as endogeneous danger signals, and protelytically generate active forms of the inflammatory cytokines IL-1β and IL-18 (Guo et al, 2015). Inflammasomes are made up of three components: sensors binding with different exogenous and endogenous danger signals;

proteolytic enzymes called caspases; and the adaptor protein PYD and CARD domain containing (PYCARD, also known as ASC) that enables the sensors to bridge effector caspases, of which caspase 1 is the most common (Rathinam & Fitzgerald, 2016) (Figure 3).

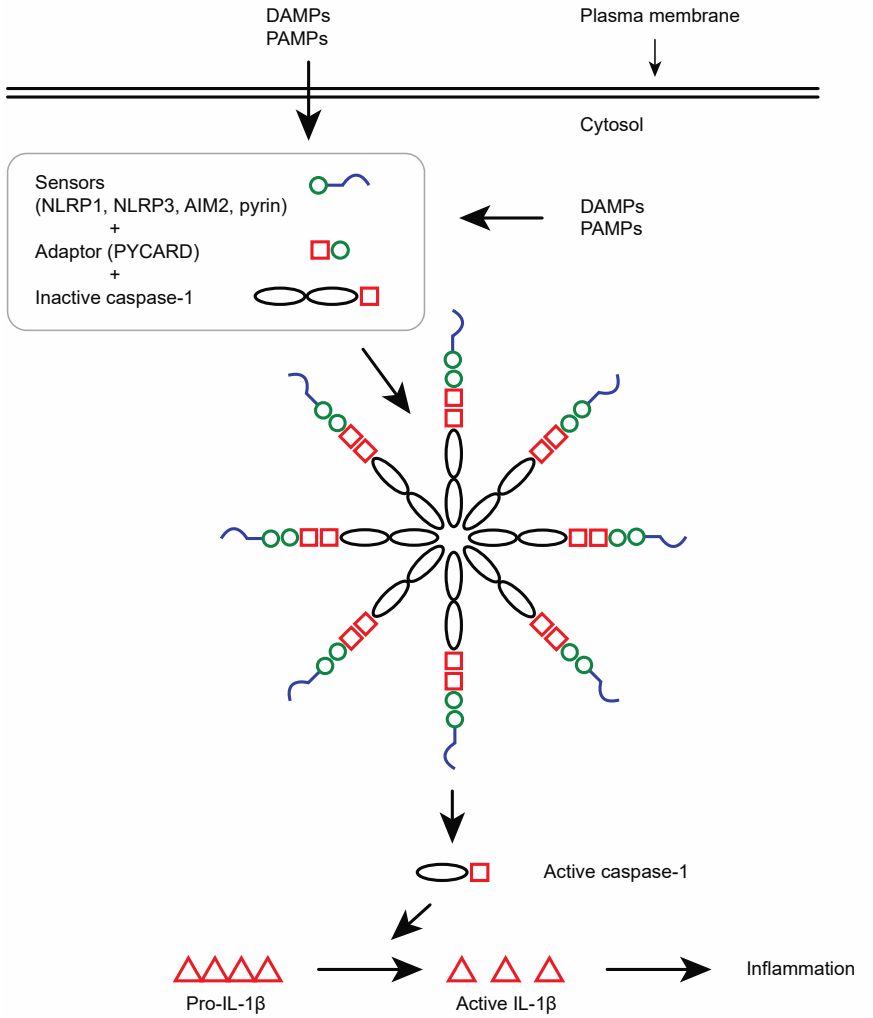

Figure 3. The activation of the inflammasome. The formation of the inflammasome and the synthesis of pro-IL-1β is induced by various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that are recognized by sensors. The subsequent production of biologically active IL-1β is mediated by inflammasomes. NLRP1, nucleotide-binding domain leucine-rich repeat-containing (NLR) family, pyrin domain (PYD)-containing; AIM2, absent in melanoma 2; PYCARD, PYD and CARD domain containing.

The most well-established inflammasomes, named by their receptors, are NLRP1 (nucleotide-binding domain leucine-rich repeat-containing [NLR] family, pyrin domain [PYD]-containing 1), NLRP3 (NLR family, PYD-containing 3), NLRC4 (NLR family, caspase activation and recruitment domain [CARD]-containing 4), AIM2 (absent in melanoma 2) and pyrin (Rathinam & Fitzgerald, 2016). The classic outcome of inflammasome activation is inflammation through the cleavage of proinflammatory IL-1 family cytokines into their bioactive forms, IL-1β and IL-18, and pyroptosis, which is a type of inflammatory cell death (Guo et al, 2015). However, many alternative functions have been associated with inflammasomes. Therefore, in addition to inflammation, inflammasomes can regulate many other cellular processes, including cell proliferation, gene transcription and tumourigenesis (Rathinam & Fitzgerald, 2016).

The conditions that are characterised by an aberrant innate immune response to endogenous signals due to the dysregulation of inflammasomes are called autoinflammatory disorders (Havnaer & Han, 2019). There are several monogenic and multifactorial autoinflammatory syndromes that along with other symptoms manifest in dermatological signs. Well-known examples are the PAPA syndrome, including pyogenic arthritis, *pyoderma gangrenosum* and acne, and the PASH syndrome, consisting of *pyoderma gangrenosum*, acne and *hidradenitis suppurativa* (Havnaer & Han, 2019). However, alterations in inflammasomes have been observed in the pathogenesis of several diseases that are not classical autoinflammatory diseases, including diabetes, Alzheimer's disease, atherosclerosis and cancer (Rathinam & Fitzgerald, 2016).

There is much uncertainty about the relationship between the pathogenesis of vitiligo and inflammasomes. In patients with vitiligo, polymorphisms of *NLRP1* have been demonstrated (Jin et al, 2007). The expression of *IL1B* mRNA as well as the protein levels of NLRP1 and IL-1β have been found to be increased in the perilesional skin of active vitiligo (Marie et al, 2014; Wang et al, 2011). Although in normal skin NLRP1 is known to be expressed in Langerhans cells, in vitiligo perilesional skin NLRP1 is additionally expressed in melanocytes and keratinocytes (Kummer et al, 2007).

In comparison to vitiligo, more is known about the associations between inflammasomes and psoriasis. Polymorphisms of *NLRP1*, *NLRP3* and *CARD8* have been linked to an increased susceptibility to psoriasis (Carlström et al, 2012; Ekman et al, 2014). The upregulation of *AIM2* (Dombrowski et al, 2011), *CASP1*, *CASP5*, *CASP4* and *PYCARD* (Johansen et al, 2007; Salskov-Iversen et al, 2011) has been identified in psoriatic lesional skin. Furthermore, *CARD14*, which encodes the caspase recruitment domain-containing protein 14, has been identified as the gene responsible for the association of the psoriasis susceptibility locus 2 (PSORS2) with psoriasis, and mutations in *CARD14* have been reported in patients with psoriasis (Jordan et al, 2012). Mutations in *CARD14* are associated with an increased nuclear-factor kappa B (NF-κB) activation and an upregulation of psoriasis-associated genes in keratinocytes leading to clinical manifestations of the disease (Liang et al, 2017). However, it is still unclear

whether innate or adaptive immunological mechanisms dominate in the pathogenesis of psoriasis.

4.4. Autophagy

Autophagy is the enzymatic digestion of cytoplasmic contents to maintain cellular homeostasis. In this process, cytoplasmic components are trapped within vesicles called autophagosomes, which fuse with lysosomes and lead to the proteolytic degradation of the trapped components (Figure 4). Depending on the cellular organelles that are being degraded, specialised forms of autophagy have been described, including mitophagy (degradation of mitochondria), nucleophagy (degradation of nuclei), and ER-phagy (degradation of the endoplasmic reticulum). Autophagy is controlled by macromolecular signalling complexes, among which beclin 1 with the UV radiation resistance-associated gene protein (UVRAG), WD repeat domain, phosphoinositide interacting 1 (WIPI1) and microtubule-associated protein light chain 3 (LC3) are positive regulators, and the mammalian target of rapamycin (mTOR) is a negative regulator (Mizushima et al, 2010; Yu et al, 2015). In addition, WIPI1 and LC3 are also some of the key components of an autophagosome (Mizushima et al, 2010). Although autophagy is primarily a mechanism for degrading damaged cellular organelles and proteins to maintain cellular homeostasis, it is shown that the dysregulation of autophagy can be involved in pathological processes including autoimmunity, infections and malignant tumours (Yu et al, 2015; Zhou & Zhang, 2012). Moreover, autophagy plays a critical role in normal epidermal development, and an impaired autophagy may contribute to the pathogenesis of diseases with a disturbed epidermal differentiation, such as psoriasis (Akinduro et al, 2016).

The idea that the dysfunction of autophagy can be involved in vitiligo pathogenesis is supported by Jeong et al. who demonstrated that variation in the *UVRAG* gene contributes to the risk of non-segmental vitiligo in the Korean population (Jeong et al, 2010), and by Wang et al. who found that several genes involved in the autophagy process were dysregulated in the leukocytes of generalised vitiligo patients (Wang et al, 2016b). Furthermore, autophagy deficiency leads to premature senescence and a decreased proliferation of melanocytes (Zhang et al, 2015). Melanosomal autophagy in stressed melanocytes mediates antigen presentation and DC maturation (Xie et al, 2016). Actually, melanosomes are also lysosome-related organelles whose maturation is controlled by molecules overlapping with autophagy regulators like WIPI1 and LC3 (Ho et al, 2011; Yun et al, 2016). However, little is currently known about the dysregulation of autophagy in the pathogenesis of vitiligo as well as psoriasis.

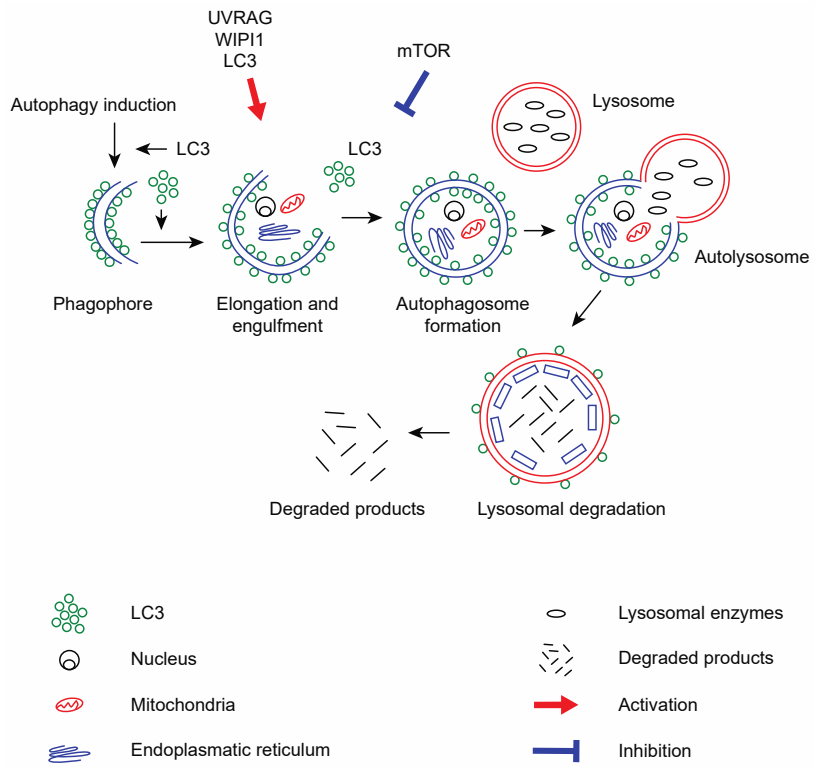

Figure 4. The process of autophagy. The autophagy inducing signal causes the formation of a sequestering membrane called phagophore. The microtubule-associated protein light chain 3 (LC3) conjugates with the sequestering membrane and controls the elongation of phagophore. As the phagophore expands, cytoplasmic constituents, including organelles, such as mitochondria and endoplasmic reticulum, are enfolded, and it results in the formation of an autophagosome. These vesicles fuse with lysosome, and the cytoplasmic proteins are proteolytically degraded. UVRAG, beclin 1 with the UV radiation resistance-associated gene protein; WIPI1, WD repeat domain, phosphoinositide interacting 1; mTOR, mammalian target of rapamycin.

4.5. Biological functions of miRNAs

MiRNAs are small, approximately 22 nucleotide-long, non-coding RNAs that regulate gene expression. MiRNAs inhibit gene expression through translational repression or messenger RNA (mRNA) destabilisation. In order to suppress gene expression, in most cases, miRNAs bind with partial complementarity to sequences in the 3` untranslated region (3`UTR) of target mRNAs (Figure 5) (Bartel, 2004). As one miRNA can bind to hundreds of genes and multiple miRNAs can regulate the same gene simultaneously, the biological effect of miRNAs can be remarkable (Jonas & Izaurralde, 2015). It should be noted that miRNAs can also activate the expression of genes, for example indirectly

through suppressing inhibitory proteins or more directly through binding to the 3'UTR and hindering the binding of proteins that otherwise would cause rapid degradation of corresponding miRNAs (Gebert & MacRae, 2019).

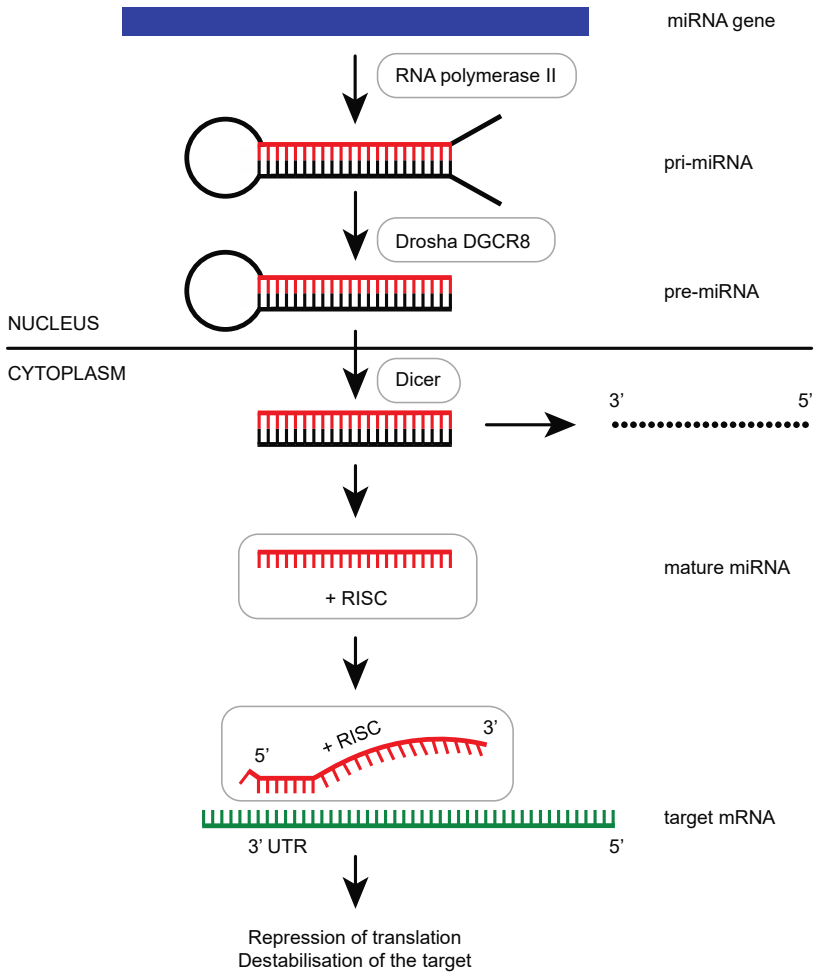

Figure 5. The biogenesis and mechanism of the action of miRNAs. MiRNAs are synthesised in the nucleus by RNA polymerase II as part of longer transcripts. These long pri-miRNAs are cleaved by the ribonuclease III enzyme Drosha and RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) complex to hairpin-like pre-miRNAs. Pre-miRNAs are exported to the cytoplasm, where they are further processed by the RNase III endonuclease Dicer to a short RNA duplex. One strand of the duplex is incorporated into the RNA-induced silencing complex (RISC). The second strand can be loaded into another RISC or it is degraded. MiRNA binds with the partial complementarity in the 3' untranslated region (UTR) of the target messenger RNA (mRNA), followed by the inhibition of translation or destabilisation of mRNA.

The first miRNA lin-4 was described in 1993 in nematode *Caenorhabditis elegans* (Lee et al, 1993). Since then new miRNAs have been discovered at an extremely high speed, and 48,860 different miRNAs in different species have been described so far. The human genome has suggested to contain over 2,500 miRNAs (Kozomara et al, 2019), and more than 60% of human protein-coding genes own predicted miRNA target sites indicating that miRNAs are essential for the normal functioning and development of all organisms (Gebert & MacRae, 2019). Accordingly, these small RNAs have been shown to regulate numerous cellular processes, including cell proliferation, differentiation, development, signal transduction, metabolism, homeostasis, apoptosis and immune responses (Gebert & MacRae, 2019; Jonas & Izaurralde, 2015; Rebane & Akdis, 2013). For instance, miR-155 targets the suppressor of the cytokine signalling *SOCS1* and thereby activates IFN signalling in CD8+ cytotoxic T cells (Dudda et al, 2013). In the skin, through targeting *CTLA4*, which suppresses the inhibitory cytotoxic T lymphocyte associated protein, miR-155 enhances T cell responses (Sonkoly et al, 2010). Based on this and several other studies, it can be concluded that, miRNA dysregulation can be associated with the pathogenesis of various diseases, including common dermatoses, such as psoriasis (Hermann et al, 2017; Sonkoly et al, 2007), atopic dermatitis (Rebane & Akdis, 2013; Rebane et al, 2014; Sonkoly et al, 2010; Vaher et al, 2019) and allergic contact dermatitis (Vennegaard et al, 2012).

One of the interesting characteristics of miRNAs is that they are secreted and can be found in quantitative levels in all extracellular fluids, for example, serum, urine and tears (Gebert & MacRae, 2019). Accordingly, a growing number of reports have shown that subsets of extracellular miRNAs may have clinical relevance as biomarkers for a variety of diseases, including cancer, viral infections, nervous system disorders, cardiovascular disorders and skin disorders (Hanna et al, 2019; Hayes et al, 2014; Lv et al, 2014; O'Brien et al, 2018; Wang et al, 2016a). These biomarkers can be potentially used to indicate the presence, the stage and the prognosis of the disease, as well as the therapy response (Condrat et al, 2020; Hanna et al, 2019). For the application of miRNAs as biomarkers, numerous ongoing clinical trials have been initiated (https://clinicaltrials.gov).

In addition, due to their gene-regulation properties, miRNAs have become attractive targets for novel therapeutic approaches. MiRNA mimics and molecules targeted at miRNAs, called antimiRs, have shown promise in preclinical development (Rupaimoole & Slack, 2017). Several miRNA therapeutics have reached clinical trials (Bajan & Hutvagner, 2020), including a mimic of miR-29 for treating scleroderma (Gallant-Behm et al, 2019), and antimiRs targeted at miR-122 for treating chronic hepatitis C (Bajan & Hutvagner, 2020) and antimiR-155 for treating cutaneous T cell lymphoma (Seto et al, 2018). One of the biggest challenges in developing miRNA-based therapeutics is to identify the best miRNA candidates or miRNA targets. Other challenges include safe, specific and efficient *in vivo* delivery methods and enhancing the stability of RNA-based therapeutic agents. Therefore, miRNA-based therapeutics have not

been developed and used as widely as antibody-based therapeutics. Even so, in future, miRNA overexpression and suppression may be applied clinically in addition to other biological therapeutics (Rupaimoole & Slack, 2017).

4.6. MiRNAs in psoriasis and vitiligo

In psoriatic patients, miRNA expression has been extensively studied and so far the dysregulation of more than 250 miRNAs either in the skin or blood cells has been determined (Hawkes et al, 2016). Moreover, the functions of multiple miRNAs have been associated with psoriasis, including miR-21 (Meisgen et al, 2012), miR-31 (Peng et al, 2012), miR-99a (Lerman et al, 2011), miR-125b (Xu et al, 2011), miR-135b (Joyce et al, 2011), miR-138 (Fu et al, 2015), miR-146a (Srivastava et al, 2017), miR-155 (Wang et al, 2020), miR-203 (Sonkoly et al, 2007), miR-210 (Zhao et al, 2014), miR-221, miR-222 (Zibert et al, 2010) and miR-424 (Ichihara et al, 2011). Among these, miR-146a is one of the most highly upregulated miRNAs in psoriatic skin (Sonkoly et al, 2007). miR-146a is a well-known anti-inflammatory miRNA that inhibits the NF-κB pathway by targeting *TRAF6* and *IRAK1* (Taganov et al, 2006). In addition to its anti-inflammatory role, miR-146a also suppresses the proliferation of keratinocytes (Hermann et al, 2017). Other psoriasis-associated miRNAs also regulate the proliferation and differentiation of keratinocytes, including miR-31 (Peng et al, 2012), miR-99a (Lerman et al, 2011), miR-125b (Xu et al, 2011), miR-135b (Joyce et al, 2011), miR-203 (Sonkoly et al, 2007), miR-221, miR-222 (Zibert et al, 2010) and miR-424 (Ichihara et al, 2011). Inflammation in psoriasis is believed to be regulated in addition to miR-146a (Hermann et al, 2017; Taganov et al, 2006) by miR-21 (Meisgen et al, 2012), miR-138 (Fu et al, 2015), miR-155 (Wang et al, 2020) and miR-210 (Zhao et al, 2014). However, studies on miRNAs to find potential diagnostic biomarkers and treatment targets for psoriasis are still ongoing (Hawkes et al, 2016).

In patients with vitiligo, the aberrant expression of 66 miRNAs in the skin (Mansuri et al, 2014; Vaish et al, 2019), 69 miRNAs in the serum (Shi et al, 2016; Shi et al, 2013) and 100 miRNAs in the whole blood (Shang & Li, 2017) has been demonstrated by the microarray analysis. From these miRNAs, miR-1, miR-184, miR-328, miR-383 and miR-577 are similarly dysregulated in the whole blood and skin of vitiligo patients (Mansuri et al, 2016). The dysregulation of multiple miRNAs has been confirmed using the quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Namely, the upregulation of miR-25 (Shi et al, 2016) and miR-9 (Su et al, 2019) as well as the down-regulation of miR-211 (Sahoo et al, 2017) has been demonstrated in the skin of vitiligo patients. Also, the dysregulation of several miRNAs has been shown to appear in the serum (let-7b, miR-15b, miR-16, miR-25, miR-451) (Shi et al, 2016), in the PBMCs (miR-224-3p, miR-4712-3p, miR-3940-5p, miR-155) (Lv et al, 2019; Wang et al, 2015b) and in the whole blood (miR-1238-3p, miR-202-3p, miR-630 miR-766-3p) (Shang & Li, 2017) of vitiligo patients. For

aberrantly expressed miRNAs, several putative target genes have been identified (Mansuri et al, 2016; Sahoo et al, 2017; Vaish et al, 2019). Most of them are associated with autoimmune mechanisms (*IL1B*, *IL2RG*, *PTPN22*), melanocyte functioning (*TYRP1*, *TYR*, *TRPM1*, *EDN1*) or oxidative stress and cellular energy metabolism (*HSP60*, *HSP70*, *G6PD*, *PPARGC1A*) (Dwivedi et al, 2013; Mansuri et al, 2016; Sahoo et al, 2017; Shi et al, 2016; Vaish et al, 2019; Wang et al, 2016d).

It has been shown that miR-25, the miRNA upregulated in the skin and serum of vitiligo patients, is induced by oxidative stress in melanocytes. miR-25 suppresses the level of *MITF*, a transcription activator of melanogenesis-related genes, which leads to the dysfunction of melanocytes and facilitates the oxidative stress-induced apoptosis. Moreover, miR-25 suppresses the production and secretion of growth factors in keratinocytes (Shi et al, 2016). Another upregulated miRNA in vitiligo skin, miR-9, has been found to inhibit adhesion and migration of melanocytes (Su et al, 2019). miR-211 is thought to regulate mitochondrial energy metabolism in melanocytes (Sahoo et al, 2017) and also melanocyte migration (Su et al, 2020).

Single nucleotide polymorphisms in miRNA genes can affect the phenotypes or the development of diseases. As the computational analysis predicted that miR-196a-2 could putatively target the *TYRP1* mRNA, whose protein is the key enzyme in the synthesis of melanin, *miR-196a-2* was selected for genetic study in the Han Chinese population. The CC genotype of rs11614913 in *miR-196a-2* was found to decrease the susceptibility to vitiligo through regulating oxidative stress by targeting *TYRP1* and *TYR* (Cui et al, 2015; Huang et al, 2013). However, although recent studies have emphasised the implication of miRNAs in vitiligo, there is still little information about the role of particular miRNAs in the pathogenesis of this disease (Yan et al, 2020).

5. SUMMARY OF THE LITERATURE REVIEW

Vitiligo is a chronic skin disease that manifests as depigmented macules on the skin due to the destruction of melanocytes (Ezzedine et al, 2015a). As the onset of vitiligo is often during childhood or adolescence and the white macules mostly locate on visible body regions (Boniface et al, 2018b; Ezzedine et al, 2015a), vitiligo affects the quality of life (Karelson et al, 2013; Lai et al, 2017; Osinubi et al, 2018; Silverberg & Silverberg, 2013). A lack of knowledge about the pathogenesis of vitiligo has impeded the development of effective methods of treatment (Boniface et al, 2018b; Ezzedine et al, 2015a; Taieb et al, 2013). Although vitiligo is generally considered to be an autoimmune disorder, the mechanisms that lead to the break-down of self-tolerance, the destruction of melanocytes and the maintenance of the disease progression still remain unclear (Boniface et al, 2018b). In addition, so far, little attention has been paid to the role of miRNAs in the pathogenesis of vitiligo (Yan et al, 2020).

Psoriasis is a common chronic inflammatory disease that is characterized by erythematous and scaly plaques on the skin and often also by nail changes and psoriatic arthritis (Boehncke & Schon, 2015). Biological therapy has improved the results of the treatment but the therapeutic agents are not equally effective for all psoriasis patients. To date we are unable to predict the treatment outcome and the course of the disease (Amatore et al, 2019; da Rosa et al, 2017; Hoffmann et al, 2011). A cross-talk between the innate and the adaptive immune system in the pathogenesis of psoriasis has been a popular subject of research. However, it is still unknown whether psoriasis is rather autoimmune or autoinflammatory disease (Boehncke & Schon, 2015; Christophers et al, 2014; Liang et al, 2017; Sweeney et al, 2011).

6. AIMS OF THE STUDY

The general purpose of this study was to acquire additional knowledge about the pathogenesis of the two chronic dermatoses – vitiligo and psoriasis – to uncover novel factors and pathways that could be associated with these diseases and used as possible diagnostic or prognostic markers or therapeutic targets in the future.

The specific aims of the study were:

1. to investigate the local and systemic inflammatory response in vitiligo and psoriasis;
2. to get an insight into the immune cell infiltration in the lesional and non-lesional skin of vitiligo and psoriasis patients;
3. to test the involvement of inflammasomes and autophagy in the studied dermatoses;
4. to study the expression changes and role of miRNAs in vitiligo pathogenesis.

7. SUBJECTS AND METHODS

7.1. Ethical considerations

Case-control studies were conducted at the Dermatology Clinic of Tartu University Hospital. The studies were approved by the Research Ethics Committee of the University of Tartu. The numbers of the approvals are as follows: 207/T-12, 219/T-28, 236/M-26 and 244/M-14. All of the participants signed a written informed consent form.

7.2. Characteristics of the study participants

Individuals at the age of ≥ 18 years were invited to participate in the study. A total of 23 patients with non-segmental vitiligo (6 males and 17 females, ages ranging from 19–60 years); 43 patients with plaque psoriasis (33 males and 10 females, ages ranging from 20–65 years) and 32 control individuals (8 males and 24 females, ages ranging from 22–57) were included (Table 1). All of the participants were unrelated Caucasian individuals living in Estonia.

Table 1. Characteristics of the study participants (Paper I, II, III)

	Control individuals	**Vitiligo patients**	**Psoriasis patients**
Number of participants	32	23	43
Gender, n			
Male	8 (25%)	6 (26.1%)	33 (76.7%)
Female	24 (75%)	17 (73.9%)	10 (23.3%)
Age range (y)	22–57	19–60	20–65
Mean age±SD (y)	36.9±10	35.9±12.4	46.9±11.8
Mean duration of disease±SD (y)	–	15.6±11.3	23.4±11.2
Activity of vitiligo, n	–		–
Active		8 (34.8%)	
Stable		15 (65.2%)	
Body surface area affected by vitiligo, n			–
<10%		9 (39.1%)	
≥10%		14 (60.9%)	
Patients with psoriatic arthitis, n	–	–	13 (30.2%)
Patients with psoriatic nail involvement, n	–	–	33 (76.7%)
Onset of psoriasis, n	–	–	
Early onset psoriasis			37 (86%)
Late onset psoriasis			6 (14%)
PASI range	–	–	2–44
PASI, n	–	–	
<12			14 (32.6%)
≥12			29 (67.4%)

The patients with non-segmental vitiligo were enrolled from the outpatient department of the Dermatology Clinic. The diagnosis of vitiligo was based on the occurrence of depigmented macules on the skin with the typical localisation. Depigmentation was confirmed with a Wood's lamp. Eight of the vitiligo patients had active and 15 had stable vitiligo. In the patients with active vitiligo, the development of new lesions or the extension of old lesions manifested in three months before the examination. None of the patients had received any treatment for their vitiligo for at least a month before their recruitment.

The patients with a clear clinical diagnosis of plaque psoriasis were recruited at the outpatient or inpatient department of the Dermatology Clinic. Concomitant psoriatic arthritis was diagnosed in 13 patients. Nail involvement was present in 33 patients. Early onset psoriasis, occurring before the age of 40, was diagnosed in 37 patients. PASI was calculated and it ranged from 2–44. None of the patients had received any treatment for their psoriasis for at least a month before their recruitment.

The control individuals were recruited from among healthcare personnel, medical students and patients who turned to the dermatological outpatient clinic for the surgical excision of a nevus. None of the control individuals had any history of chronic skin diseases nor vitiligo or psoriasis in their family.

7.3. Data collection

7.3.1. Collection of clinical information

A questionnaire about the age, gender, nationality, skin phototype, comorbidities and diseases in the family was filled by a dermatovenereologist for all participants. Information about the onset and course of vitiligo or psoriasis was collected. An objective status was assessed in the vitiligo and psoriasis patients. The body surface area (BSA) affected by vitiligo was measured and PASI was calculated for the patients with psoriasis.

7.3.2. Collection of material

For RNA purification (Paper I, II, III) and immunofluorescence microscopy (Paper I, II), two skin punch biopsy samples (3–4 mm in diameter) were collected from the patients with vitiligo and psoriasis, one from the marginal zone of the lesional skin (hereinafter called lesional skin) and another from the non-sun-exposed non-lesional skin. From the patients with psoriasis, punch biopsy samples from the lesional and non-lesional skin were also gathered for Caspase-1 assay (Paper II). For *in situ* hybridisation (ISH), one punch biopsy sample was taken from the lesional skin of the vitiligo patients (Paper III). From the control individuals, one skin punch biopsy sample (3–4 mm in diameter) from non-sun-exposed skin was taken for RNA analysis, immunofluorescence microscopy, Caspase-1 assay as well as for ISH (Paper I, II, III). Skin samples

taken for RNA extraction were instantly frozen in liquid nitrogen or in dry ice and stored at -80°C until further experiments (Paper I, II, III). For immunofluorescence microscopy (Paper I, II), Caspase-1 Assay (Paper II) and ISH (Paper III), skin biopsy specimens were embedded into the Tissue-Tek (Thermo Scientific, Waltham, MA, USA) before freezing.

Each study participant gave 16 ml venous blood, which was collected into BD Vacutainer® CPT™ Cell Preparation Tubes with sodium heparin (BD Biosciences, Franklin Lakes, NJ, USA). The plasma was collected for cytokine testing (Paper I, II).

Different skin cell types, including pooled normal human epidermal keratinocytes (Promocell, Heidelberg, Germany) (Paper I, III), foreskin-isolated melanocytes (Paper I, III) and fibroblasts (Paper I) as well as monocyte derived Langerhans cells (moLC) (Paper I) were used for cell culture experiments. Melanocytes and fibroblasts were harvested from paediatric foreskin (approval number 178/T-19) and cultured as described by Reemann et al. (Reemann et al, 2014).

7.4. Laboratory methods

7.4.1. RNA extraction

For RNA extraction from the skin biopsy samples, the specimens were homogenised by a gentleMACS^{TM} Dissociator (Miltenyi Biotec, Heidelberg, Germany) using M tubes and 700 µl of the QIAzol Lysis Reagent (Qiagen, Valencia, CA, USA) or using Precellys24 (Bertin, Montigny-le-Bretonneux, France) (Paper I, II, III).

Total RNA from skin was isolated by using RNeasy Fibrous Tissue Mini Kit (Qiagen) or miRNeasy Mini Kit (Qiagen) (Paper I, II, III). For RNA extraction from the cells, QIAzol Lysis Reagent (Qiagen) was directly added to the cells and total RNA was isolated using the miRNeasy Mini Kit (Qiagen) (Paper I, III). The concentration and quality of the RNA were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA) (Paper I, II, III).

7.4.2. Cell culture, stimulation and transfection

Pooled, normal human epidermal keratinocytes (Promocell, Heidelberg, Germany) were cultured in a Keratinocyte-serum-free medium (SFM) with supplements (Life Technologies, Grand Island, NY, USA) (Paper I, III). Melanocytes and fibroblasts were isolated and cultured as described by Reemann et al (Reemann et al, 2014) (Paper I, III). MoLCs were generated as follows. PBMCs were isolated from a buffy coat by Ficoll-Hypaque Plus (Amersham Biosciences, Piscataway, USA) density gradient centrifugation. Monocytes were isolated using MACS® anti-CD14 beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol up to purity over 95%. The

isolated cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% penicillin and streptomycin (PAA Laboratories, Pasching, Austria) at a density of 1–1.5×10^6 cells/ml. MoLC were differentiated for seven days in the presence of granulocyte-macrophage-colony stimulating factor (GM-CSF) (50 ng/ml), IL-4 (25 ng/ml), TGF-β (10 ng/ml) and thymic stromal lymphopoietin (TSLP) (5 ng/ml) (R&D Systems, Minneapolis, USA) (Geissmann et al, 1998) (Paper I).

For two-dimensional (2D) cultures, keratinocytes with the density of 2x10^4 cells per well on 24-well plates were seeded; for 3D keratinocyte culture in the air-liquid interface, 5×10^4 cells per well were seeded on ThinCert Cell Culture Inserts (0.4μm pore, 0.33 cm^2) (Greiner Bio-One, Kremsmünster, Austria). For the 3D culture, Keratinocyte-SFM medium with supplements and Dulbecco's Modified Eagle Medium (Life Technologies) containing High Glucose, GlutaMAX™ and Pyruvate in 1:1 ratio were used. For the 2D culture, Keratinocyte-SFM medium with supplements (Life Technologies) was used (Paper I).

For cell stimulation, 2×10^4 melanocytes and keratinocytes per one well in a 24-well plate were seeded for each stimulation for 5 h, 24 h and 48 h with 10 ng/ml of TNF-α, 20,000 U/ml of IFN-α2a, 20,000 U/ml of IFN-γ and 10 ng/ml of IL-1β (Paper III).

For transfections, 2×10^4 melanocytes in 1ml of Medium 245CF supplemented with the Human Melanocyte Growth Supplement-2 (Life Technologies) or 2×10^4 keratinocytes in 1ml of the Keratinocyte-SFM medium (Life Technologies) were seeded per one well in a 12-well plate. After 24 hours, the cells were transfected with the miRIDIAN microRNA hsa-miR-155-5p mimic and miRIDIAN microRNA Mimic Negative Control #1 (GE Healthcare Life Sciences, Fairfield, CT, USA) at the final concentration of 60 nM using siPORT NeoFX (Life Tecnologies) according to the manufacturer's protocol. When indicated, after 24 h, the transfected melanocytes and keratinocytes were stimulated with IFN-γ for an additional 48 h (Paper III).

7.4.3. qRT-PCR

All amplifications and analyses were carried out on the ViiA™ 7 Real-Time PCR system (Life Technologies, Grand Island, NY, USA). The relative gene expression levels were calculated using the comparative Ct (ΔΔ*C*t) method (Livak & Schmittgen, 2001).

For analysing mRNA expression in the skin samples, cDNA was synthesised from 5 μg of total RNA using oligo-dT and SuperScript® III Reverse Transcriptase (Life Technologies) according to the manufacturer's protocols. For PCR, SYBR® Green master mix (Life Technologies) was used. Data were normalised to the housekeeping gene *ACTB* expression. The names of the genes are listed in Table 2 (Paper I, II, III).

To measure mRNA levels in melanocytes and keratinocytes, cDNA was synthesised from 100 to 900 ng of total RNA using oligo-dT, RevertAid

Reverse Transcriptase, deoxyribonucleotide triphosphates, reaction buffer and RiboLock RNase Inhibitor from Thermo Fisher Scientific. qRT-PCR was performed using 5 HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia). *EEF1A1* was used as a housekeeping gene (Paper III). The primers were designed with the assistance of Primer 3 and were ordered from TAG Copenhagen (Copenhagen, Denmark) (Paper III).

Table 2. List of the genes studied by qRT-PCR (Paper I, II, III)

Short name	**Long name**
ACTB	actin beta
AIM2	absent in melanoma 2
CASP1	caspase-1
CCL2	C-C motif chemokine ligand 2
CCL5	C-C motif chemokine ligand 5
CCL20	C-C motif chemokine ligand 20
CCL27	C-C motif chemokine ligand 27
CTLA4	cytotoxic T-lymphocyte associated protein 4
CXCL1	C-X-C motif chemokine ligand 1
CXCL2	C-X-C motif chemokine ligand 2
CXCL8	C-X-C motif chemokine ligand 8
CXCL10	C-X-C motif chemokine ligand 10
EEF1A	eukaryotic translation elongation factor alpha 1**
EOMES	eomesodermin
FOXP3	forkhead box 3
IFIH1	interferon induced with helicase C domain 1
IFITM1	interferon-induced transmembrane protein 1**
IFNAR1	interferon alpha and beta receptor subunit 1
IFNG	interferon gamma
IFNGR	interferon gamma receptor 1
IL10	interleukin 10*
IL17A	interleukin 17A
IL17F	interleukin 17F
IL1B	interleukin 1 beta
IL36A (alias IL1F6)	interleukin 36, alpha
IL1RN	interleukin receptor antagonist
IL20RA	interleukin 20 receptor subunit alpha
IL22	interleukin 22
IL22RA1	interleukin 22 receptor subunit alpha 1
IL22RA2	interleukin 22 receptor subunit alpha 2
IL26	interleukin 26*
IRF1	interferon regulatory factor 1**
KLRK1	killer cell lektin like receptor K1
LCN2	lipocalin 2*
MEF2A	myocyte enhancer factor 2A**
MICB	MHC class I polypeptide-related sequence B

Short name	Long name
NLRP1	NLR family pyrin domain containing 1
NLRP3	NLR family pyrin domain containing 3
PI3	peptidase inhibitor 3*
PYCARD	PYD and CARD domain containing
SDCBP	syndecan binding protein**
SOCS1	suppressor of cytokine signalling 1**
SOX10	SRY (sex determining region Y)-Box 10**
S100A8	S100 calcium binding protein A8*
S100A9	S100 calcium binding protein A9*
TNF	tumour necrosis factor
TRGC1	T cell receptor gamma constant 1
TYRP1	tyrosine-related protein 1**
WIPI1	WD repeat domain, phosphoinositide interacting 1
YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon
* measured only in the skin from the psoriasis patients comparatively to the skin from the control individuals (Paper II) ** measured only in melanocytes and keratinocytes (Paper III)	

MiRNA expression was analysed with TaqMan miRNA qRT-PCR assays (Life Technologies) according to the manufacturer's protocol with few modifications. Briefly, miRNA specific cDNAs were synthesised using the TaqMan MicroRNA Reverse Transcription Kit using 10 ng of the total RNA in a 5 µl reaction mix, out of which 2.5 µl was used per 10 µl of total qPCR mix containing additionally TaqMan® MicroRNA Assays (Thermo Fisher Scientific) and 5x HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne). For normalisation, the let-7a was chosen as one of the most common housekeeping miRNAs, with Ct values between 20.2 and 22.1 across all skin samples (Paper III).

7.4.4. Immunofluorescence assay

Immunofluorescence assay was conducted on 5 µm thick frozen sections of the skin biopsy samples. The skin sections were fixed with 4% formaldehyde, permeabilised with 0.2% Triton X-100 in phosphate-buffered saline (PBS) and blocked with the normal goat serum and bovine serum albumin (Paper I, II). After that, the Alexa Fluor™ 594 Tyramide SuperBoost™ Kit (Thermo Fisher Scientific, Wilmington, MA, USA) was used for signal amplification. Skin sections of the patients with vitiligo were incubated with mouse monoclonal antibodies for LC3 (nanoTools, Teningen, Germany) and mouse anti-human major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA)/MICB antibodies (BioLegend, San Diego, CA, USA). Next, the slides were incubated with the poly-horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody. For signal enhancement, the tyramide

working solution was added and the stop solution was used to halt the HRP reaction. To identify melanocytes, the skin sections were incubated with anti-tyrosinase related protein 1 (TYRP1) rabbit polyclonal antibodies (Atlas Antibodies, Sweden) and incubated with the Alexa Fluor® 488 conjugated Goat anti-Rabbit IgG (H+L) Secondary Antibody (1:1,000, ThermoFisher Scientific, 1:500) (Paper I). The antibodies used on the skin sections gathered from the patients with psoriasis were CD3 (mouse anti-human, Alexa Fluor 488-conjugated, UCHT1, Biolegend) and eomesodermin (EOMES) (rabbit polyclonal, Novus Biologicals). After incubation with antibodies, the skin slides were washed in PBS, and incubated with Alexa Fluor 594-conjugated F(ab')2-goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibodies (ThermoFisher Scientific, 1:500) (Paper II). All of these antibodies were used on the control skin (Paper I, II). For nuclear staining 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (1 μg/mL) was used. After that, the slides were washed in PBS and covered with a fluorescent mounting medium (Dako, Santa Clara, CA, USA) and coverslips (Paper I, II). The images were obtained with the FV1200 (Olympus, Tokyo, Japan) (Paper I) or LSM710 (Zeiss, Wetzlar, Germany) confocal microscope (Paper II). For the quantification of the fluorescence signal, marking LC3 and DAPI, Fiji (Schindelin et al, 2012) with built-in options was used (Paper I).

7.4.5. Caspase-1 assay

Caspase-1 activation was assessed in the lesional and non-lesional psoriatic skin and the control skin cryosections with the FAM-FLICA™ Caspase-1 Assay Kit (ImmunoChemistry Technologies, Bloomington, MN, USA) according to the manufacturer's manual. Briefly, 5 μm cryosections of the skin biopsy specimens were fixed with acetone and washed twice with PBS. For blocking, the 10% normal goat serum (Thermo Fisher Scientific) and 0.5% bovine serum albumin in PBS were used. The caspase-1 activity was determined by incubating with the fluorochrome-labelled inhibitor of caspases (FLICA) Caspase-1 Reagent (FAM-YVAD-FMK) followed by washing steps. The nuclei were stained with Hoechst 33342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-b enzimidazole trihydrochloride trihydrate). The slides were covered with the Fluorescence Mounting medium (Dako, Santa Clara, CA, USA) and analysed with the Leica DM5500 B microscope (Leica Microsystems, Wetzlar, Germany). The green fluorescence signal, indicating the activity of caspase-1, was quantified with the help of Fiji (ImageJ)64 in fixed areas of the *stratum spinosum*. Cell nuclei were counted in the same fixed areas and the fluorescence intensity measure was divided with the number of cells in the area (Paper II).

7.4.6. miR-155 *in situ* hybridisation

ISH was optimised and performed on 10 μm frozen sections of the lesional and non-lesional vitiligo skin as well as the control skin using the microRNA ISH Buffer and Controls Kit according to the manufacturer's instructions. For the detection of miR-155, the miRCURY LNA™ Detection Probe for hsa-miR-155 (88072-15) (Exiqon, Vedbaek, Denmark) was used. Prehybridisation, hybridisation and washings were performed at 50°C. The slides were incubated with a sheep anti-digoxigenin alkaline phosphatase conjugate (1:1,500, Roche, Basel, Switzerland) and stained by adding the BM Purple AP Substrate (Roche, Pleasanton, CA, USA). The nuclei were stained with the Nuclear Fast Red counterstain (Vector Laboratories, Burlingame, CA, USA). The images were obtained with the Leica DM5500 B microscope (Leica Microsystems, Wetzlar, Germany) (Paper III).

7.4.7. Cytokine testing

For separating the plasma from the blood cells, the blood tubes were centrifuged at 1,500 g for 30 minutes. The plasma was collected and stored at -20˚C. Similarly to the control individuals, the concentration of TNF-α, IL-1β, IL-1Ra/IL-1F3, IL-2, IL-5, IL-6, IL-8/CXCL8, CXCL10/IP-10, IFN-γ, Granulocyte–Colony Stimulating Factor (G-CSF) and GM-CSF was measured in the heparin treated plasma of the vitiligo patients (Paper I). From the plasma of the psoriasis patients, the levels of TNF-α, IL-1β, IL-1Ra/IL-1F3, IL-2, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-17A, IL-17F, IL-22, IL-31, IL-33, IL-36β, IFN-γ, CXCL10/IP-10, G-CSF, GM-CSF, and Lipocalin-2/NGAL were measured (Paper II). The xMAP technology on Luminex 200 (Luminex Corporation, Austin, Texas, USA) was used. The Milliplex MAP multiplex assay was conducted in a 96-well microplate format according to the manufacturer's instructions (Millipore, Billerica, MA, USA) (Paper I, II).

7.5. MiRNA target selection and pathway analysis

Putative targets for miRNAs were identified using the Targetscan 6.2 (http://www.targetscan.org/) (Friedman et al, 2009). Only highly conserved targets with a total context score below -0.15 were chosen. For the pathway analysis, the genes expressed in the skin according to the previously published (Rebane et al, 2012) dataset E-MTAB-729 (9,966 genes with an average signal >40.0 in the skin from healthy individuals) were selected. The pathway analysis was conducted using the g:Profiler (http://biit.cs.ut.ee/gprofiler) (Reimand et al, 2007). To estimate the significance of the overlap between the target genes and the indicated functional group, the Fisher exact test was used (Paper III).

7.6. Biostatistics and data visualisation

Statistical analysis was performed using the statistical software R (https://www.r-project.org/) (Paper I, II) or GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) (Paper III). The mRNA expression values and the fluorescence signal ratios in Papers I and II were log-transformed before the statistical testing in order to adhere to the assumptions of the normal distribution. The conformity to the normal distribution was assessed using the Kolmogorov–Smirnov test. Comparisons between the vitiligo patients and the control individuals were made using an unpaired Student's *t*-test (Paper I) and a paired Student's *t*-test or the Mann-Whitney *U* test (Paper III). The paired *t*-test was used, since it is required for comparing lesional and non-lesional skin. In Paper III, the results of the paired *t*-test did not differ significantly from the results gained with the unpaired Student's *t*-test. When comparing the vitiligo lesional skin with non-lesional skin (Paper I, III) or the expression of genes in cell cultures (Paper I, III), the paired Student's *t*-test was used. In the mRNA expression analysis and immunofluorescence signal quantification, if the vitiligo lesional and non-lesional skin was compared to the healthy control skin, Dunnett's correction was applied to adjust for multiple comparisons (Paper I). The mean and standard deviations of the log-transformed mRNA expression values were back-transformed to a linear scale for plotting, which is shown as the geometric mean ×÷ the geometric standard deviation on the graphs (Paper I). For comparing the lesional and non-lesional psoriatic skin and the control skin, the Linear Models for Microarray and RNA-Seq Data (*limma*) R package ver. 3.28.21 from Bioconductor (Ritchie et al, 2015) was used (Paper II). A *p*-value < 0.05 was considered significant.

To compare cytokine concentrations in the plasma of the vitiligo patients and control individuals, the unpaired Student's *t*-test was used, since the data approximately followed a normal distribution (Paper I). To identify statistically significant differences in the concentration levels of cytokines in the psoriasis patients and control individuals, the values were log2-transformed and the Wilcoxon signed-rank test was used. The results were considered significant at *p*-value < 0.05. Comparisons of the selected phenotypes in the plasma samples of the psoriasis patients were performed using multi-factor ANOVA, followed by Tukey's multiple comparison test to find the groups that were significantly different from each other. Results were then filtered based on the adjusted *p-value* ≤ 0.05. A phenotype comparison was carried out for the differentially expressed plasma cytokines which showed statistically significant changes between the psoriasis patients and the control group (Paper II).

8. RESULTS

8.1. Inflammatory markers are modestly upregulated among the patients with vitiligo (Paper I)

To study inflammation in the vitiligo skin, we measured the relative expression of genes encoding inflammatory cytokines, receptors and the components of inflammasomes in the lesional and non-lesional skin of 16 vitiligo patients (4 males, 12 females, ages ranging from 19–60 years) and 24 control individuals (8 males, 16 females, ages ranging from 24–57 years). From cytokines, only *TNF* was significantly upregulated both in the vitiligo lesional (adjusted $p < 0.05$) and non-lesional skin (adjusted $p < 0.05$) (Figure 6A). The expression of *IL36A* (alias *IL1F6*) (adjusted $p < 0.05$), *CCL5* (adjusted $p < 0.05$) and *CXCL10* (adjusted $p < 0.001$) was increased exclusively in the vitiligo lesional skin. Solely in the vitiligo non-lesional skin, *IL1RN* (adjusted $p < 0.05$) was up-regulated. Comparing the vitiligo lesional skin with the non-lesional skin, the *CCL27* expression was higher in the lesional skin (adjusted $p < 0.05$) (Figure 6A). The transcripts of *IFNG* and Th17 specific cytokines (*IL17A*, *IL17F*, *IL22*) remained undetectable in the control as well as in the vitiligo biopsy samples (data not shown). Despite this, similarly to the IFN-induced chemokine *CXCL10*, IFN-induced innate immune receptor *IFIH1* was upregulated in the vitiligo lesional skin (adjusted $p < 0.01$) (Figure 6B). Furthermore, the *IFIH1* expression was also increased in the non-lesional skin taken from the vitiligo patients (adjusted $p < 0.05$). The highly significant upregulation of *IL22RA1* (adjusted $p < 0.05$) was detected in the vitiligo non-lesional skin (Figure 6B). From the studied inflammasome-related genes, only *AIM2* was slightly elevated in the vitiligo non-lesional skin (adjusted $p < 0.05$) (Figure 6C). We also compared the gene expression between the patients with an active (5 patients) and a stable (11 patients) form of the disease; however, the data did not reveal a higher inflammatory marker expression in the active vitiligo skin (data not shown).

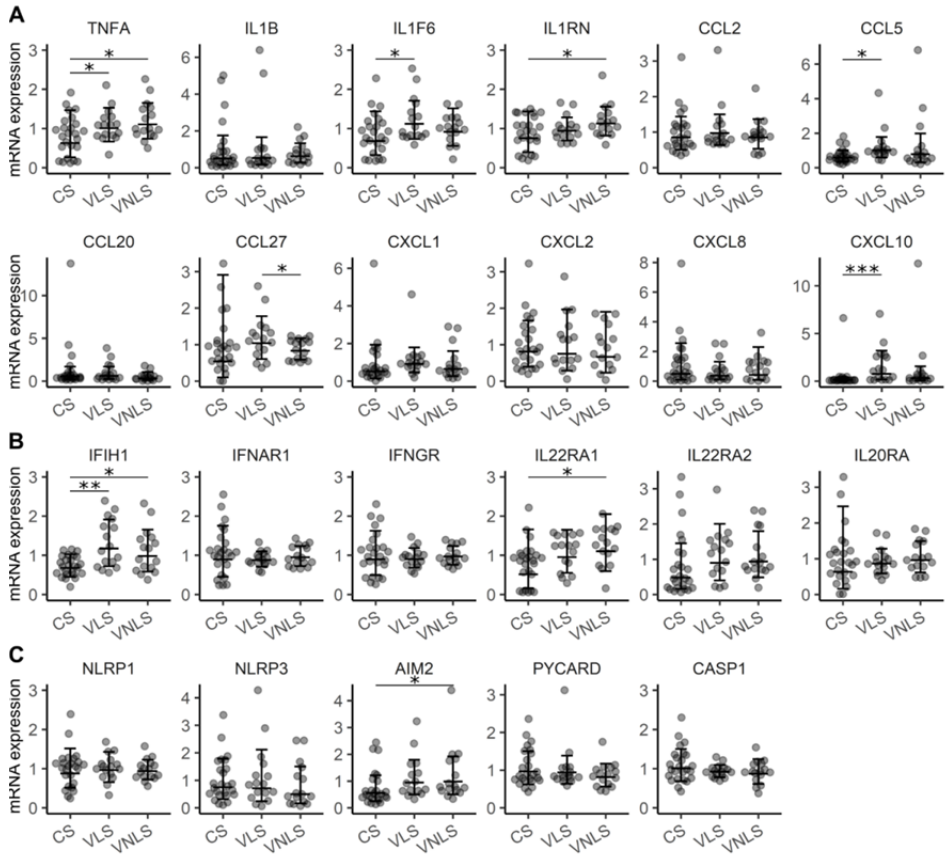

Figure 6. The relative expression of mRNAs encoding **(A)** cytokines, **(B)** receptors and **(C)** components of the inflammasome in the skin of the control individuals (CS), and in the lesional (VLS) and non-lesional skin (VNLS) of the vitiligo patients. The geometric mean ×÷ the geometric standard deviation is indicated. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

To check for possible signs of a systemic inflammation, we measured the concentration of inflammation-associated cytokines in the plasma of 18 vitiligo patients (5 males, 13 females, ages ranging from 19–60 years) and 24 control individuals (8 males, 16 females, ages ranging from 24–57 years). We found lower plasma concentrations of the IL-1 inhibitor IL1-Ra ($p < 0.05$) and G-CSF ($p < 0.01$) in the vitiligo patients compared to those in the control individuals (Figure 7).

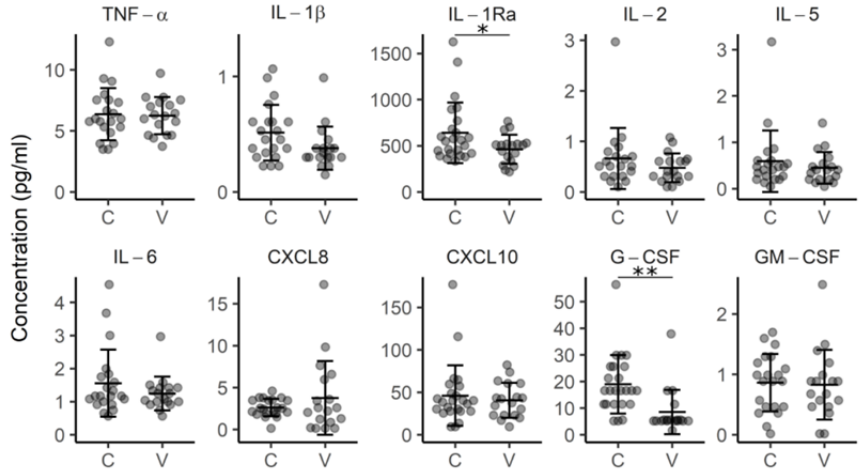

Figure 7. The concentration of cytokines in the plasma of the control individuals (C) and vitiligo patients (V). The arithmetic mean ± the standard deviation is indicated. *p < 0.05; **p < 0.01.

8.2. Markers of innate cell infiltration are dysregulated in the vitiligo skin (Paper I)

Next, we studied immune cell infiltration to vitiligo lesions based on the cell-type specific gene expression in skin. The expression of the T cell inhibitor *CTLA4* was increased in the vitiligo lesional (adjusted p < 0.001) and non-lesional skin (adjusted p < 0.001) (Figure 8A). Moreover, *EOMES*, a transcription factor characteristic of effector cytotoxic T cells, unconventional memory CD8+ T cells and NK cells (Collins et al, 2017; Jacomet et al, 2015; Martinet et al, 2015; White et al, 2016), was increased in both the vitiligo lesional (adjusted p < 0.001) and non-lesional skin (adjusted p < 0.001) (Figure 8A). *KLRK1*, encoding the activating NK cell receptor NKG2D, and *TRGC1,* which encodes a T cell receptor γ-chain, showed a tendency for an increased expression in the vitiligo lesional skin compared to that in healthy skin (adjusted p = 0.055 and p = 0.058, respectively) (Figure 8A). Notably, the stress molecule *MICA/MICB*, which can be bound by activating NK cell receptors (Ghadially et al, 2017), also showed a tendency for an increased expression in vitiligo lesions (adjusted p = 0.052) (Figure 8A). Next, we performed MICA/MICB immunofluorescence staining on sections from three vitiligo (1 male, 2 females, ages ranging from 28–47 years) and three control (3 females, ages ranging from 22–38 years) biopsy samples together with the melanocyte marker TYRP1 (Figure 8B). While the control skin and the vitiligo non-lesional skin were completely clean of MICA/MICB, we noted multiple positive cells in the sub-epidermal area of the vitiligo lesional skin. Often, the stained cells had a disturbed nuclear morphology, as can be seen in the middle panel of Figure 8B. There was no co-localisation of MICA/MICB with TYRP1.

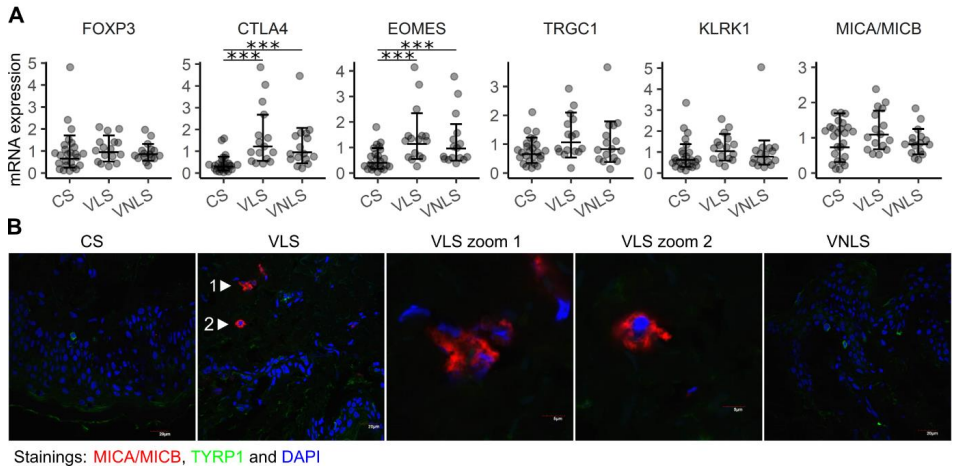

Figure 8. (A) The relative mRNA expression of the genes encoding the markers of the infiltrating immune cells and their target molecules. The geometric mean ×÷ the geometric standard deviation is indicated. ***$p < 0.001$. **(B)** An immunofluorescence image of MICA/MICB (MHC class I chain-related protein A and B) and TYRP1 (tyrosinase related protein 1) in sections of the biopsy samples from the control individuals (CS), and in the lesional (VLS) and non-lesional skin (VNLS) of the vitiligo patients. DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) counterstains cell nuclei.

8.3. Hallmarks of autophagy are altered in the vitiligo skin (Paper I)

The gene *WIPI1*, regulating autophagy and melanosome maturation (Akinduro et al, 2016; Ho et al, 2011), was significantly downregulated in both the lesional (adjusted $p < 0.01$) and non-lesional (adjusted $p < 0.001$) skin taken from the patients with vitiligo (Figure 9A). We compared the *WIPI1* mRNA expression levels in various cell types present in the skin and found the highest expression level in melanocytes, followed by fibroblasts (Figure 9B). The *WIPI1* expression was relatively low in the moLCs and keratinocytes in a 2-dimensional culture but the expression increased during the differentiation of keratinocytes at the air-liquid interface (Figure 9B). To determine if the *WIPI1* downregulation in the vitiligo skin can be associated with impaired autophagy in melanocytes, we stained the tissue sections of the skin biopsy samples for LC3, a marker of autophagy (Mizushima et al, 2010) (Figure 9C). When autophagy is initiated, the cytosolic form of LC3 is conjugated with phosphatidylethanolamine, a cell membrane phospholipid, to form a lipidated LC3 conjugate, which is recruited to autophagosomal membranes. This is associated with the conversion of the dim homogenous fluorescence into a dotted pattern in the cytosol (Mizushima et al, 2010). In the control skin and in the vitiligo non-lesional skin, most of the melanocytes were devoid of LC3 dots, whereas in the vitiligo

lesions, the scarce remaining melanocytes with weak TYRP1 staining showed an LC3 pattern characteristic to autophagy (Figure 9C). Keratinocytes in the vitiligo lesions also had significantly more autophagic vacuoles throughout all the layers of the epidermis, in comparison to the control and non-lesional skin (Figures 9D, E).

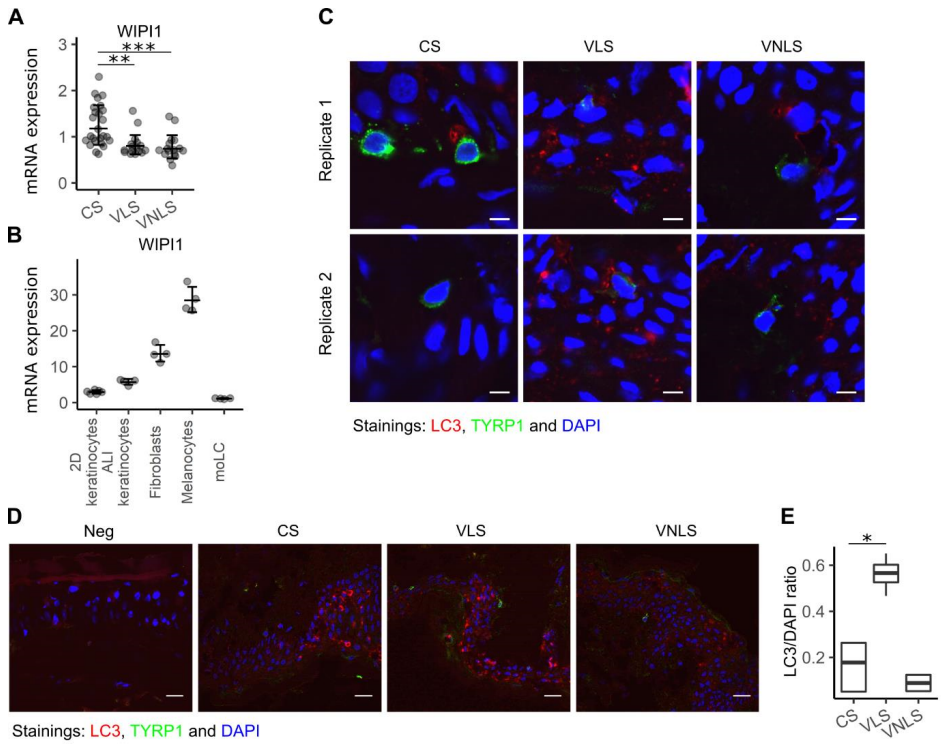

Figure 9. (A) *WIPI1* relative mRNA expression in the skin of the control individuals (CS), and in the lesional (VLS) and non-lesional skin (VNLS) of the vitiligo patients. The geometric mean ×÷ the geometric standard deviation is indicated. $^{**}p < 0.01$; $^{***}p < 0.001$. **(B)** *WIPI1* relative mRNA expression in various cell types of the skin. The geometric mean ×÷ the geometric standard deviation is indicated. 2D, two-dimensional; ALI, air-liquid interface; moLC, monocyte derived Langerhans cells. **(C)** An immunofluorescence image of LC3 (microtubule-associated protein light chain 3) and TYRP1 (tyrosinase related protein 1) in sections of the biopsy samples from the control individuals (CS), and in the lesional (VLS) and non-lesional skin (VNLS) of the vitiligo patients. DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) counterstains cell nuclei. The white bar represents 5 µm. **(D)** The immunofluorescence image as in panel C with the white bar representing 20 µm. Neg. refers to a control slide with secondary antibodies only. **(E)** The box-and-whisker plots show the median and interquartile range of the ratio between the fluorescence signal marking the LC3 expression and DAPI (cell nuclei) in CS, VNLS and VLS. Whiskers cover data points within a 1.5× interquartile range. $^{*}p < 0.05$.

8.4. Inflammatory cytokines, chemokines and antimicrobial peptides are upregulated, but *CCL27* is downregulated in psoriatic lesions (Paper II)

In order to assess inflammation in the psoriatic skin, we conducted a gene expression analysis in the lesional and non-lesional skin of 33 psoriasis patients (23 males, 10 females, ages ranging from 20–63 years) and 23 control individuals (8 males, 15 females, ages ranging from 24–57 years). We found that the Th17 cytokine gene expression (*IL17A*, *IL17F*, *IL22*, *IL26*) was significantly increased in the psoriatic lesional skin in comparison to the non-lesional and control skin (adjusted $p < 0.001$ for all these comparisons) (Figure 10A). The mRNA of other proinflammatory cytokines, *IFNG*, *TNFA, IL1B* and *IL36A* (alias *IL1F6*), was also markedly elevated in the psoriatic lesional skin in comparison to the control skin (adjusted $p < 0.001$ in all comparisons) and the psoriasis non-lesional skin (adjusted $p < 0.001$ in all comparisons except the case of *TNFA*). From these cytokines, *IL36A* was additionally upregulated in the psoriasis non-lesional skin (adjusted $p = 1.70 \times 10^{-5}$) (Figure 10A). The expression of chemokines *CXCL1*, *CXCL2*, *CXCL8*, *CXCL10*, *CCL2*, *CCL5* and *CCL20* was significantly elevated in the lesional skin of the psoriasis patients (adjusted $p < 0.001$ for all comparisons) and the expression of *CXCL10* (adjusted $p = 0.002$) and *CCL20* (adjusted $p = 0.022$) was also upregulated in the psoriasis non-lesional skin (Figure 10B). Only *CCL27* was significantly downregulated in the psoriatic skin (adjusted $p < 0.001$). With regard to cytokine receptors, only *IL22RA1* showed elevated mRNA signals in the psoriatic skin (adjusted $p < 0.001$) (Figure 11). Antimicrobial peptides *S100A8*, *S100A9*, *PI3* and *LCN2* were significantly upregulated in the psoriatic skin (adjusted $p < 0.001$ for all comparisons) (Figure 12).

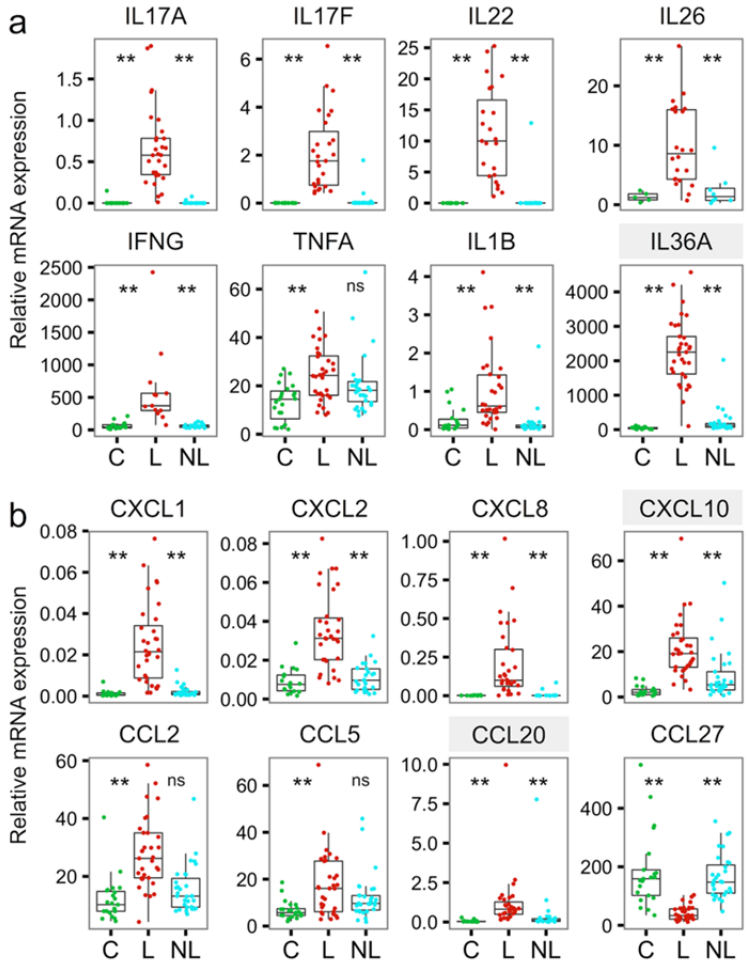

Figure 10. The relative expression of mRNAs encoding **(a)** inflammatory cytokines and (**b**) chemokines in the skin biopsy samples obtained from the lesional (L) and non-lesional (NL) skin of the psoriasis patients as well as from the control individuals (C). The box-and-whisker plots depict the median (central line), interquartile range (IQR, Q1-Q3, box), Q3 + 1.5 × IQR (upper whisker) and Q1 − 1.5 × IQR (lower whisker). Stars above the groups C and NL depict their significance level from L samples. *$p < 0.05$, **$p < 0.001$. Grey shading behind the gene name indicates statistically significant differences between the NL and C biopsy samples (for IL36A $p = 1.70 \times 10^{-5}$, CXCL10 $p = 0.002$, CCL20 $p = 0.022$).

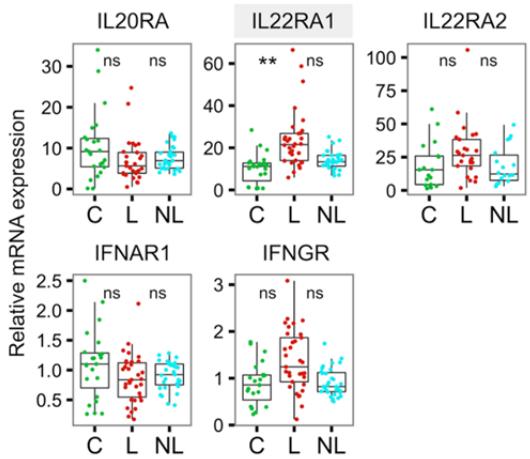

Figure 11. The relative mRNA expression of several cytokine receptors in the skin biopsy samples obtained from the lesional (L) and non-lesional (NL) skin of the psoriasis patients as well as from the control individuals (C). The box-and-whisker plots depict the median (central line), interquartile range (IQR, Q1-Q3, box), Q3 + 1.5 × IQR (upper whisker) and Q1 – 1.5 × IQR (lower whisker). Stars above the groups C and NL depict their significance level from L samples. ** $p < 0.001$. Grey shading behind the gene name indicates statistically significant differences between the NL and C biopsy samples (for IL22RA1 $p = 0.009$).

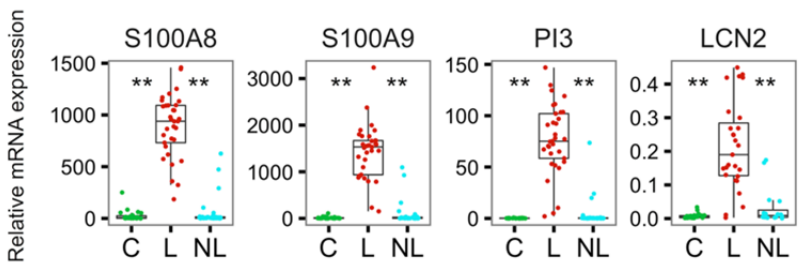

Figure 12. The relative mRNA expression of several antimicrobial peptides in the skin biopsy samples obtained from the lesional (L) and non-lesional (NL) skin of the psoriasis patients as well as from the control individuals (C). The box-and-whisker plots depict the median (central line), interquartile range (IQR, Q1-Q3, box), Q3 + 1.5 × IQR (upper whisker) and Q1 – 1.5 × IQR (lower whisker). Stars above the groups C and NL depict their significance level from L samples. ** $p < 0.001$.

8.5. Innate receptors and inflammasome components are dysregulated in the psoriatic skin (Paper II)

Next, we studied components of the autophagosome (*WIPI1*) and several innate receptors *(IFIH1, AIM2*) and inflammasome components (*AIM2*, *NLRP1, NLRP3, PYCARD, CASP1*) in the skin of psoriasis patients in comparison to control individuals. The *WIPI1*, *NLRP1*, *NLRP3* and *CASP1* expression levels were not significantly different in the studied groups (data not shown). However, the *IFIH1* (adjusted $p < 0.001$), *AIM2* (adjusted $p < 0.001$) and *PYCARD* (adjusted $p < 0.001$) genes showed an increased expression in the psoriatic lesional skin (Figure 13A). To determine whether the elevated expression of inflammasome components (*AIM2*, *PYCARD*) could be associated with the increased inflammasome activation and active caspase-1 accumulation in the psoriatic lesions, we used the FLICA reagent, which forms a covalent bond with active caspase-1. The lesional and non-lesional skin from two psoriasis patients (2 males, 47 and 65 years old) and the skin from two control individuals (2 females, 39 and 51 years old) was used for the assay. The FLICA staining of the healthy skin cryosections localised exclusively to the epidermis with the highest signals in the granular layer (Figure 13B). While the psoriatic non-lesional skin (Figure 13C) did not substantially differ from the healthy control skin, FLICA staining was detectable throughout the thickened psoriatic epidermis in the lesional skin and covered some dermal areas close to the epidermis (white arrow in Figure 13D). Due to the intercellular oedema in the psoriatic epidermis, the number of cells per fixed section area was lower, and therefore the green fluorescence intensity per keratinocyte was significantly higher in psoriatic lesions ($p < 0.05$) in comparison to the healthy skin or non-lesional skin of the psoriasis patients (Figure 13F).

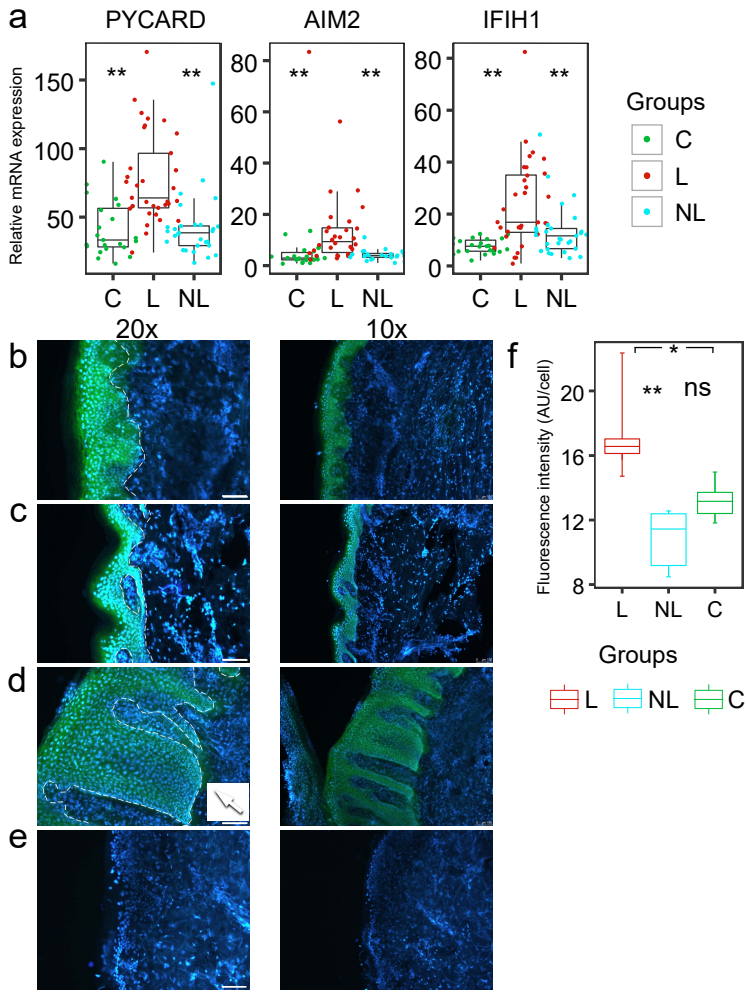

Figure 13. (**a**) The relative mRNA expression of genes encoding innate receptors in the skin biopsy samples obtained from the lesional (L) and non-lesional (NL) skin of the psoriasis patients and from the control individuals (C). The box-and-whisker plots depict the median (central line), interquartile range (IQR, Q1-Q3, box), Q3 + 1.5 × IQR (upper whisker) and Q1 – 1.5 × IQR (lower whisker). Stars above the groups C and NL depict their significance level from L samples. ** $p < 0.001$. (**b–e**) Fluorescence microscopic images illustrate caspase-1 activation as detected with the FAM-FLICA Assay Kit (green) in the control skin (**b**), psoriatic non-lesional skin (**c**) and psoriatic lesional skin (**d**) biopsy frozen sections with DAPI for counterstaining cell nuclei. (**e**) Represent control slides. White bars in the 20x images represent 75 µm. The white dotted lines indicate the basal membrane. (**f**) Green fluorescence intensity was measured from the slides in fixed areas of the *stratum spinosum* (from the slides of two different individuals and 2–3 different areas per slide) and divided by the number of cells in that area. *$p < 0.05$, ** $p < 0.001$.

8.6. Gene expression analysis reveals immune cell infiltration to the psoriatic lesional as well as non-lesional skin (Paper II)

Several immunoregulatory genes and marker transcription factors were upregulated in the skin of psoriasis patients in comparison to control individuals (Figure 14). Specifically, the expression of *CTLA4*, *FOXP3*, *IL10* and *IL1RN* was markedly elevated in the psoriatic lesions, while the expression of *CTLA4* and *IL10* was elevated also in the non-lesional skin of the psoriasis patients (adjusted $p < 0.001$ in all comparisons) (Figure 14A). We found a moderate increase in the stress molecule *MICB* expression in the psoriatic lesional skin (adjusted $p < 0.05$) (Figure 14B). From the markers of effector cells, only the *EOMES* expression was significantly increased in the psoriatic skin (adjusted $p < 0.001$) as well as the non-lesional skin (adjusted $p < 0.001$) (Figure 14B). The immunofluorescence microscopy of the skin sections from two psoriasis patients and two control individuals showed that in the psoriatic skin a fraction of CD3+ T cells contained eomesodermin in their nuclei (Figure 14C, left panels) while the control skin sections (Figure 14C, middle panels) contained very few T cells.

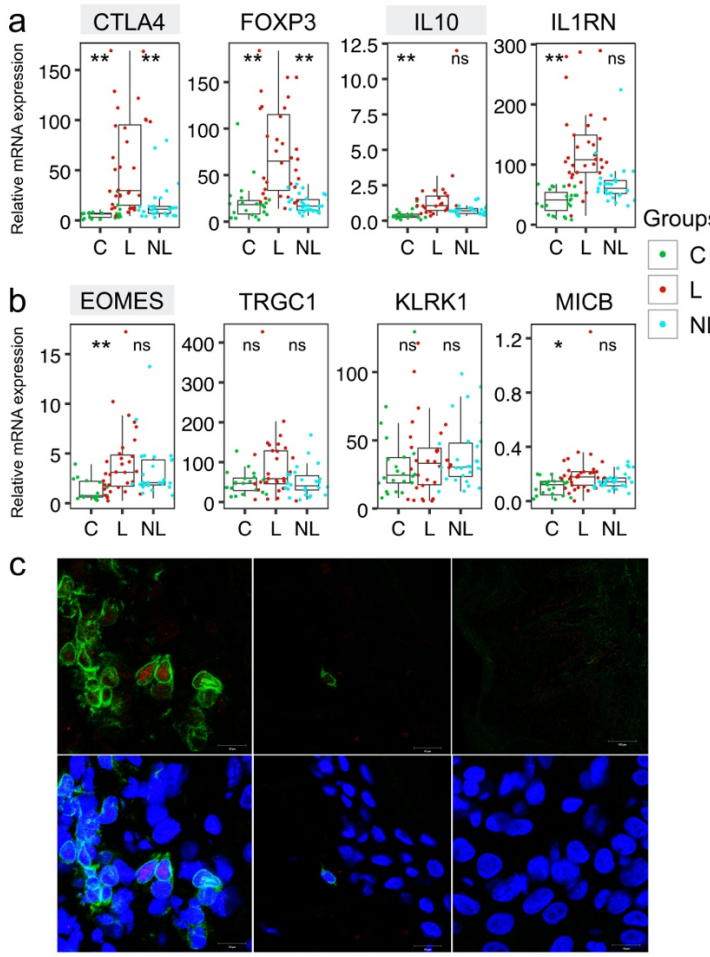

Figure 14. The relative mRNA expression of several **(a)** immunoregulatory genes and **(b)** genes specific for different cytotoxic effector cells in the skin biopsy samples obtained from the lesional (L) and non-lesional (NL) skin of the psoriasis patients and from the control individuals (C). The box-and-whisker plots depict the median (central line), interquartile range (IQR, Q1-Q3, box), Q3 + 1.5 × IQR (upper whisker) and Q1 – 1.5 × IQR (lower whisker). Stars above the groups C and NL depict their significance level from L samples. *$p < 0.05$; ** $p < 0.001$. Grey shading behind the gene name indicates statistically significant differences between the NL and C samples ($p < 0.001$). (**c**) Fluorescence microscopic images illustrate CD3 expression (green) and eomesodermin staining (red) in the psoriatic lesional skin biopsy frozen sections (left panels). The middle panels represent the healthy control skin and the right-hand panels the negative control staining (secondary antibodies only). The lower panels include DAPI for counterstaining cell nuclei (blue). The white bar represents 10 μm.

8.7. Plasma levels of cytokines are consistent with systemic inflammation in psoriasis (Paper II)

To study the signs of systemic inflammation in psoriasis, we measured the concentration of several cytokines and chemokines in the plasma samples of 41 psoriasis patients (31 males, 10 females, ages ranging from 20–63 years) and 23 control individuals (8 males, 15 females, ages ranging from 24–57 years). The levels of IL-17A (p = 3.6x10^{-5}), IL-6 (p = 0.002), TNF-α (p = 0.002), IL-1Ra (p = 4.9x10^{-4}) and CXCL8 (p = 4.3x10^{-5}) were significantly higher in the plasma of the psoriatic patients in comparison to the control individuals (Figure 15A). The IL-17A concentration was higher in moderate to severe psoriasis (p = 0.001), while higher IL-6 levels were associated with joint involvement (p = 0.042) and the sporadic form of psoriasis (p = 0.001) (Figure 15B).

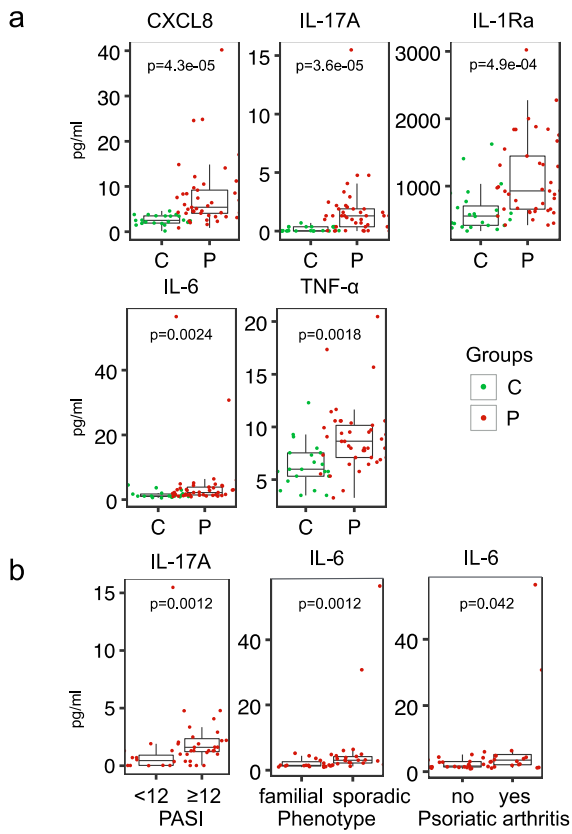

Figure 15. (**a**) The cytokine and chemokine concentrations in the plasma samples of the psoriasis patients (P) and the control individuals (C). (**b**) A comparison of the circulating cytokine levels in the psoriasis patients with different clinical features. The box-and-whisker plots depict the median (central line), interquartile range (IQR, Q1-Q3, box), Q3 + 1.5 × IQR (upper whisker) and Q1 − 1.5 × IQR (lower whisker).

8.8. MiRNAs are dysregulated in the vitiligo skin (Paper III)

To understand the potential role of miRNAs in vitiligo, we measured the expression of 12 miRNAs (miR-10a, miR-99b, miR-125a, miR-125b, miR-145, miR-146a, miR-146b, miR-155, miR-199a-3p, miR-203, miR-223 and miR-511) (Table 3) in the lesional and non-lesional skin of 15 vitiligo patients (4 males, 11 females, ages ranging from 19–60 years) and in the skin of 15 control individuals (6 males, 9 females, ages ranging from 25–53 years). The expression of all these miRNAs was measurable by qRT-PCR in the skin from the control individuals (Figure 16A). miR-125b was the most extensively expressed miRNA with the expression level approximately 3,800 times higher than that of miR-511, the miRNA with the lowest expression level (the average threshold cycle being 29.5) (Figure 16A). From the miRNAs examined, miR-99b, miR-155, miR-199a-3p, miR-125b, miR-145 and miR-146b were aberrantly expressed in the skin from the vitiligo patients (Figure 16B). The expression of miR-99b was increased in both the vitiligo lesional ($p < 0.001$) and non-lesional skin ($p < 0.01$). Exclusively in the vitiligo lesional skin, an upregulation of miR-155 ($p < 0.01$), miR-199a-3p ($p < 0.01$), miR-125b ($p < 0.05$) and a down-regulation of miR-145 ($p < 0.05$) was detected. miR-146b ($p < 0.05$) was differentially expressed in the lesional compared to the non-lesional skin from the vitiligo patients (Figure 16B). The expression levels of dysregulated miRNAs did not differ between the patients with active and stable vitiligo (data not shown). We did not detect statistically significant differences in the expression levels of miR-10a, miR-125a, miR-146a, miR-203, miR-223 and miR-511 between the groups (data not shown).

Table 3. The functions of the analysed miRNAs (Paper III)

miRNA	**Function**	**References**
miR-10a	Regulates the proliferation of different cells including keratinocytes	(Vaher et al, 2019)
miR-99b	Regulates cell proliferation and migration through targeting multiple genes in AKT/ mTOR signalling	(Jin et al, 2013)
miR-125b	Regulates the proliferation, differentiation and apoptosis in various cell types, including keratinocytes	(Xu et al, 2011)
	Suppresses proliferation and promotes the differentiation of keratinocytes by targeting *FGFR2*	(Xu et al, 2011)
miR-125a	Promotes differentiation; suppresses the proliferation and apoptosis of different cell types	(Potenza & Russo, 2013)
miR-145	Expression is reduced in cultured pigment cells after the induction of pigmentation	(Dynoodt et al, 2013)
miR-146a, miR-146b	Suppress inflammation through inhibiting the NF-kB signalling pathway by targeting *IRAK1* and *CARD10*	(Hermann et al, 2017; Rebane et al, 2014)
	Suppress keratinocyte proliferation	(Hermann et al, 2017)
miR-155	Induces inflammation through activating IFN signalling by targeting *SOCS1*	(Dudda et al, 2013)
	Increases the T cell proliferative response by targeting *CTLA4*	(Sonkoly et al, 2010)
miR-199a-3p	Regulates the proliferation and differentiation of different cell types by targeting *MTOR* and *CAV2*	(Fornari et al, 2010; Shatseva et al, 2011)
miR-203	Regulates the differentiation and functions of keratinocytes	(Sonkoly et al, 2007)
miR-223	Controls innate immune responses by targeting *NLRP3*	(Rebane & Akdis, 2013)
miR-511	Regulates the functions of dendritic cells and macrophages	(Tserel et al, 2011)

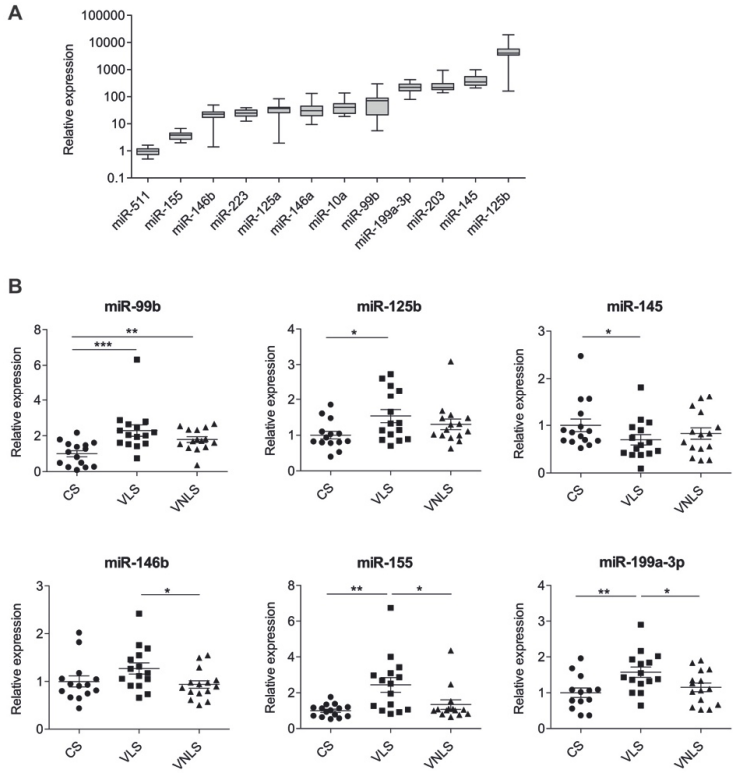

Figure 16. The relative expression of miRNAs in the skin of **(A, B)** the control individuals and **(B)** the patients with vitiligo. **(A)** The results in the control skin are represented as boxes with whiskers showing the minimum and maximum and are shown relative to the level of miR-511 (=1). **(B)** Selected individual miRNA levels in the skin of the control individuals (CS, =1) and in the lesional (VLS) and non-lesional skin (VNLS) of the patients with vitiligo. The mean ± the standard error of the mean is indicated. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

8.9. Dysregulated miRNAs in vitiligo have several putative targets related to melanocyte functioning (Paper III)

To examine whether the aberrantly expressed miRNAs (miR-99b, miR-125b, miR-145, miR-155, miR-199a-3p) can affect cellular processes associated with vitiligo, we conducted a pathway analysis for the conserved and best-scored miRNA targets expressed in the skin. We observed that miR-99b, miR-125b, miR-155 and miR-199a-3p have predicted direct targets belonging to either the Gene Ontology (GO) or Human Phenotype Ontology (HP) groups or to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) related to melanocyte differentiation, melanogenesis, melanosome structure and localisation, and skin pigmentation (Table 4). For miR-145, we identified predictive target genes involved in the regulation of stress-activated mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and the TGF-β signalling pathways.

Table 4. Pathway analysis of the putative targets of the miRNAs dysregulated in vitiligo

miRNA and targets (n)[a]	Functional group ID, name and genes in the functional group, n[b]	Putative targets in the functional group
miR-99b (40)	HP:0001000, abnormality of skin pigmentation, 39	LIFR, FGFR3, PTPN11
miR-125b (221)	GO:0033059, cellular pigmentation, 54 HP:0001000, abnormality of skin pigmentation, 268 KEGG:04916, melanogenesis, 101	BCL2, VPS33A, SS18 LIFR, ALDH3A2 DVL3, MAPK3
miR-155 (438)	GO:0030318, melanocyte differentiation, 28 GO:0042470, melanosome, 109 KEGG:04916, melanogenesis, 101	MEF2A, SOX10, TYRP1 RAB5C, SDCBP, SYPL1, SYTL2, TMEM33, TYRP1, YWHAE, YWHAZ CREB1, GNAS, GSK3B, TCF7L2, TYRP1
miR-199a-3p (321)	GO:0042470, melanosome, 109 GO:0030318, melanocyte differentiation, 28 GO:0032400, melanosome localization, 30 HP:0001000, abnormality of skin pigmentation, 268 HP:0007440, generalized hyperpigmentation, 24	SYTL2, TMEM33, YWHAE, NAP1L1, CALU, SYPL1, SLC2A1 ZEB2 VPS33A STK11, SRD5A3, PDGFRA, KIAA0319L, FOS, ALDH3A2, SPRED1 ALDH3A2
miR-145 (377)	GO:0051403, stress-activated MAPK cascade, 246	ARL6IP5, CRKL, DAB2, DUSP6, FOXO1, FZD4, FZD7, HIPK2, MAP2K4, MAP3K11, MAP3K2, MAP4K2, MAP4K4, NRAS, PDCD4, ZEB2, TAOK1, TNFSF19
	GO:0007254, JNK cascade, 192	CRKL, DAB2, FZD4, FZD7, HIPK2, MAP2K4, MAP3K11, MAP3K2, MAP4K2, MAP4K4, NRAS, PDCD4, ZEB2, TAOK1, TNFRSF19
	KEGG:04350, TGF-beta signalling pathway, 80	ACVR1B, ACVR2A, INHBB, RPS6KB1, SMAD3, SMAD4, SMAD5, SP1, TGFBR2

[a] Number of predicted putative direct targets expressed in the skin shown in parenthesis. [b]Pathways with significant overlap ($p<0.05$) with the predicted targets are presented.

8.10. miR-155 is upregulated in the vitiligo skin and is induced by inflammatory cytokines (Paper III)

As miR-155 was upregulated in the vitiligo lesional skin, predicted to target multiple important melanogenesis associated genes and has previously been shown to contribute to the activation of IFN signalling (Wang et al, 2010) playing a role in vitiligo pathogenesis (Bertolotti et al, 2014), we focused on miR-155 next. To get information about the localisation of miR-155 in the skin, we performed ISH on the skin samples from two vitiligo patients (2 females, 18 and 35 years old) and two control individuals (2 females, 27 and 47 years old). The expression of miR-155 was observed in the *stratum basale*, where melanocytes and proliferating keratinocytes are situated, as well as in the *stratum spinosum* of the epidermis of the vitiligo patients. In accordance with the qRT-PCR results (Figure 16B), no signal in one control skin and a faint positive signal of miR-155 in the epidermis of other control skin was detected (Figure 17A).

Next, we studied whether proinflammatory cytokines (TNF-α, IFN-γ, IFN-α, IL-1β) affect the expression of miR-155 in melanocytes and keratinocytes. These cytokines were selected because they are known to be associated with the pathogenesis of vitiligo (Bertolotti et al, 2014; Ezzedine et al, 2015a; Reimann et al, 2012). In foreskin-isolated melanocytes, miR-155 was significantly upregulated by TNF-α, IFN-γ, IFN-α and IL-1β after 24 h ($p < 0.001$, $p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively) and 48 h ($p < 0.001$, $p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively) of stimulation (Figure 17B). In pooled normal epidermal keratinocytes, the expression of miR-155 significantly increased after being treated by TNF-α and IFN-α ($p < 0.001$ in both cases) for 24 h and then decreased ($p < 0.01$ in both cases) at the 48 h timepoint. In reaction to IFN-γ, miR-155 was upregulated after 24 h ($p < 0.01$) and 48 h ($p < 0.05$) of stimulation. A significant upregulation of miR-155 by IL-1β was detected only after 48 h of keratinocyte stimulation ($p < 0.001$) (Figure 17C).

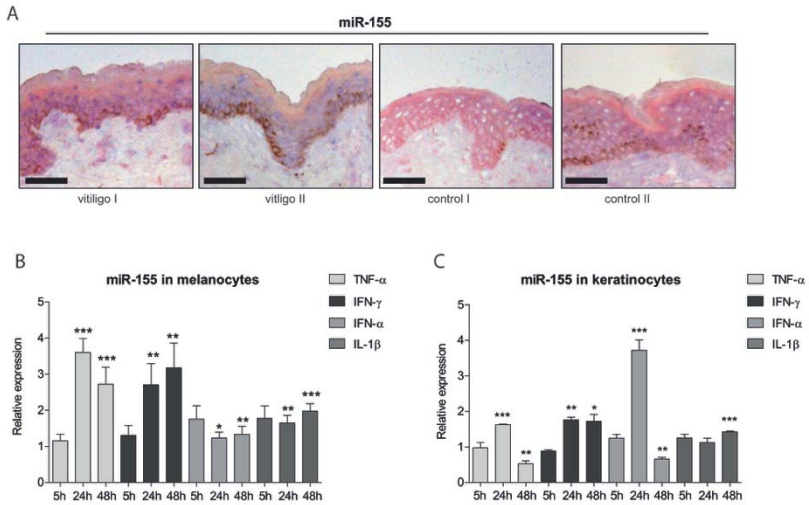

Figure 17. (A) The expression of miR-155 in the skin using *in situ* hybridisation. *Red colour* indicates nuclear fast red staining, *blue colour* the miR-155 expression, *bar* = 50 μm. The expression of miR-155 in **(B)** melanocytes and **(C)** keratinocytes. The expression of miR-155 in the tumour necrosis factor (TNF)-α-, interferon (IFN)-γ-, IFN-α- and interleukin (IL)-1β-treated melanocytes and keratinocytes is shown relative to the expression levels in unstimulated cells at each indicated time-point. Results are displayed as the mean ± the standard error of the mean. The data come from three different stimulations. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

8.11. miR-155 inhibits melanogenesis-associated targets and modulates interferon-inducible genes in melanocytes and keratinocytes (Paper III)

To determine whether miR-155 may affect the development of vitiligo by targeting the melanogenesis-associated and the IFN-regulated genes in the skin, we conducted miR-155 overexpression experiments in melanocytes and keratinocytes. In order to do so, we transfected human primary melanocytes with the miR-155 mimic and the control mimic (Figure 18A). Thereafter we stimulated the cells with IFN-γ or left them unstimulated, and measured the relative expression of selected putative miR-155 targets associated with melanocyte functions and IFN-regulated genes. The genes associated with melanogenesis, such as *TYRP1*, *SDCBP*, *YWHAE* (Figure 18B), and a gene associated with melanocyte differentiation, *SOX10* (Figure 18C), were involved in the analysis. From the IFN-γ-inducible genes, we analysed the effect of miR-155 on *IFITM1*, *IRF1* (Rebane et al, 2012) and the previously described miR-155 direct target *SOCS1* (Figure 18D).

The overexpression of miR-155 downregulated the expression of *TYRP1* ($p < 0.01$), *YWHAE* ($p < 0.01$) and *SOX10* ($p < 0.05$) in melanocytes that were left unstimulated (Figure 18B, C). The expression level of *SDCBP* was suppressed by miR-155 both in unstimulated ($p < 0.01$) and IFN-γ-stimulated ($p < 0.05$) melanocytes (Figure 18B). From IFN-inducible genes, *SOCS1* was down-regulated by miR-155 both in unstimulated ($p < 0.01$) and stimulated ($p < 0.05$) melanocytes. The *IRF1* expression was decreased ($p < 0.001$) and the *IFITM1* expression increased ($p < 0.05$) in unstimulated melanocytes transfected with miR-155 when compared to the control transfection. *IFITM1* and *IRF1* were strongly induced by IFN-γ, and *IFITM1* was further increased ($p < 0.01$) upon the overexpression of miR-155. No difference between miR-155- and control-transfected cells was found for *IRF1* when the cells were stimulated by IFN-γ (Figure 18D).

In a similar experiment in keratinocytes, the overexpression of miR-155 suppressed the expression of *YWHAE* ($p < 0.05$) in unstimulated cells (Figure 18E). *SOCS1* and *IRF1* were downregulated by miR-155 both in unstimulated ($p < 0.05$ and $p < 0.01$, respectively) and stimulated (both with $p < 0.05$) keratinocytes. As with the effect in melanocytes, the expression of *IFITM1* was elevated in unstimulated ($p < 0.001$) and IFN-γ-stimulated ($p < 0.001$) keratinocytes transfected with miR-155 when compared to the control transfections (Figure 18F).

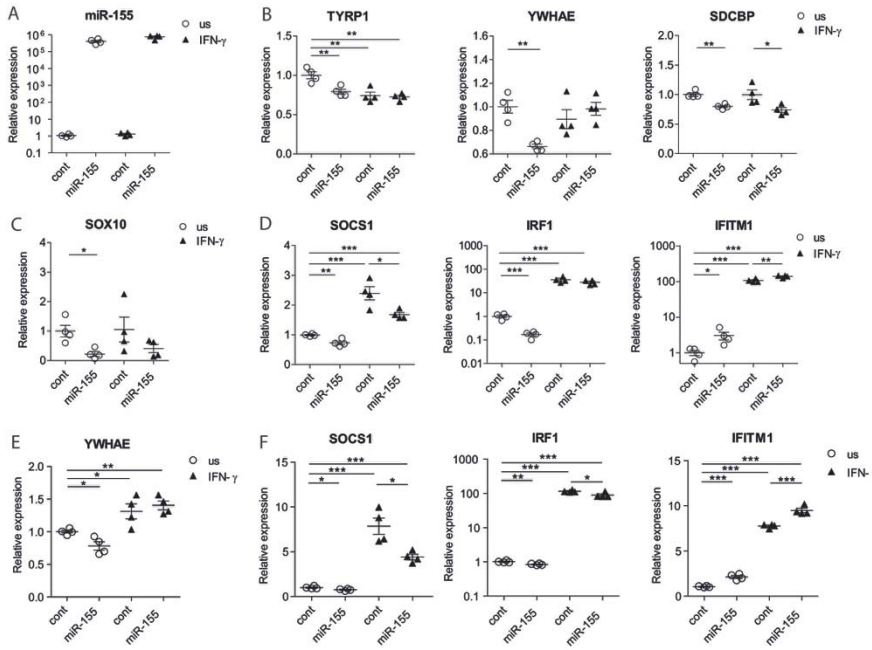

Figure 18. The relative expression of the melanogenesis-associated targets of miR-155 and interferon-inducible genes in **(A-D)** melanocytes and **(E, F)** keratinocytes. **(A–D)** Human primary melanocytes and **(E, F)** keratinocytes were transfected either with a control (cont) or hsa-miR-155-5p mimic for 24 h and then stimulated with the interferon (IFN)-γ for 48 h or left unstimulated (us). The relative expression compared with unstimulated control-transfected cells (=1) is shown. The data represent the mean ± the standard error of the mean. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

9. DISCUSSION

9.1. Inflammatory changes are modest and suggest the involvement of type I interferons in the pathogenesis of vitiligo (Paper I)

Although vitiligo is clinically a silent disease, at the cellular and molecular level moderate inflammatory changes still exist (Sandoval-Cruz et al, 2011). In accordance with multiple previous studies (Grimes et al, 2004; Moretti et al, 2002; Regazzetti et al, 2015; Reimann et al, 2012; Singh et al, 2017), we predominantly detected only a moderate upregulation of some inflammatory cytokines (*TNFA*, *IL36A*, *CCL5*) in the marginal areas of the vitiligo lesions where the destructive processes occur (van den Boorn et al, 2009). Exceptionally, *CXCL10*, an IFN-inducible chemokine, was very strongly upregulated while *IFNG* remained undetectable. The importance of CXCL10 together with the closely related CXCL9 (Strassner et al, 2017) in the pathogenesis of vitiligo has been demonstrated by previous studies in humans as well as in mouse models (Rashighi et al, 2014; Regazzetti et al, 2015; Richmond et al, 2017; Wang et al, 2016c). Although CXCL10 is mainly known as an IFN-γ-induced and Th1-associated chemokine (Rashighi et al, 2014), the CXCL10 expression can also be influenced by type I IFNs (Moll et al, 2011). Indeed, recent evidence suggests that type I IFNs play a role in vitiligo pathogenesis. Specifically, IFN-α producing plasmacytoid DCs infiltrate active vitiligo skin (Bertolotti et al, 2014; Jacquemin et al, 2017); pegylated IFN-α2a and IFN-α2b, which are used in the treatment of chronic hepatitis C, can induce depigmentation at the injection sites (Arya et al, 2010), and vitiligo patches have been observed at the site of the application of imiquimod, a Toll-like receptor (TLR)-7 and TLR-8 agonist that enhances IFN-α production (Bertolotti et al, 2014; Li et al, 2014).

In addition to *CXCL10*, in the current study the IFN-induced *IFIH1* was upregulated in the vitiligo skin. *IFIH1* is a gene which encodes an intracellular innate immune receptor called the melanoma differentiation-associated protein 5 (MDA5) that binds double-stranded RNA (dsRNA). The expression of *IFIH1* has previously been observed to be induced by type I IFNs and to be implicated in autoimmune processes (Crampton et al, 2012). Moreover, *IFIH1* is one of the risk genes for vitiligo (Jin et al, 2016). It is possible that during the course of vitiligo, type I IFNs are induced by the cyclic guanosine monophosphate (GMP) – adenosine monophosphate (AMP) synthase – cyclic GMP-AMP – the stimulator of the interferon genes (cGAS–cGAMP–STING) pathway after oxidative stress generated DNA damage (Hartlova et al, 2015; Jian et al, 2014). In this pathway, unrepaired DNA lesions induce type I IFNs via the cytosolic DNA sensor STING to promote the activation of innate immunity (Hartlova et al, 2015). To sum up, these results corroborate that inflammatory changes in the vitiligo skin are moderate and indirectly support the association between type I IFNs and vitiligo. Therefore, IFIH1 and CXCL10 as well as other components

of the type I IFN signalling pathway can be potential biomarkers or treatment targets for vitiligo.

Among the studied cytokine receptors, only the *IL22RA1* expression was increased in the non-lesional skin of the vitiligo patients. Whether this implicates the IL-22 pathway in vitiligo pathogenesis remains unknown as the *IL22* transcripts were below the detection limit. However, an upregulation of *IL22* in the PBMCs of the vitiligo patients has been reported (Rätsep et al, 2008). Additionally, in the vitiligo non-lesional skin, the inhibitory *IL1RN* (Aksentijevich et al, 2009) showed a slight increase. This change could represent compensatory responses that rebalance the signalling to limit inflammatory processes (Aksentijevich et al, 2009). However, this is unlikely as the plasma concentration of IL-1Ra, the protein encoded by the *IL1RN* gene, was lower in the patients with vitiligo, in comparison to the control individuals.

In the plasma taken from the patients with vitiligo, we did not detect an increased concentration of any measured cytokines. Based on the findings of the skin gene expression analysis, these results are not unexpected. Interestingly, the concentration of G-CSF was significantly lower in the plasma of the vitiligo patients than in the control individuals. As G-CSF may have a pro-inflammatory or pro-repair influence depending on the context (Hamilton et al, 2017), the impact of this change is difficult to judge. To sum up, these results further support that strong systemic inflammation is probably not a part of vitiligo pathogenesis.

9.2. Lymphoid stress surveillance response contributes to the pathogenesis of vitiligo (Paper I)

The data reported here support the link between the lymphoid stress surveillance response and vitiligo. In lymphoid stress surveillance, lymphocytes are rapidly activated through their non-clonotypic receptors and unleash their effector mechanisms, like cytokine secretion and cytotoxic mediators, without any delays (Shafi et al, 2011; Strid et al, 2011). This response is mediated by an interaction between stress molecules and different stress molecule binding receptors (Shafi et al, 2011). Tissue dysregulation can be sensed by lymphocytes via the recognition of stress molecules (like MICA/MICB) that have been upregulated by damaging agents like oxidative stress (Ghadially et al, 2017; Shafi et al, 2011). Although the *MICA/MICB* mRNA only showed a tendency for an increased expression in the vitiligo lesions, we could clearly observe staining for the MICA/MICB protein in the dermal area of the vitiligo lesional skin but not in the healthy or non-lesional skin. The much more dramatic differences in the MICA/MICB protein expression in comparison to the mRNA expression can be explained by the posttranscriptional and posttranslational regulation of stress molecules (Ghadially et al, 2017). MICA/MICB are ligands for the activating NKG2D receptor encoded by the *KLRK1* gene and expressed by NK cells, γδ T cells and a subpopulation of cytotoxic αβ T cells that are a

part of tissue-resident immune cells in the skin (Cheuk et al, 2017; Strid et al, 2011).

Previously, the involvement of innate cells in the vitiligo pathogenesis has been suggested (Yu et al, 2012). We could not detect a significant upregulation of *TRGC1* that encodes a T cell receptor γ-chain (Hayday, 2019) arguing against the specific recruitment of γδ T cells to the vitiligo lesions. As this is a tissue resident population of innate-like T cells that is already located and ready to respond to stress signals by neighboring cells (Hayday, 2019), the lack of an increase in their number does not mean that they are not responding to the stress-ligands. However, as *CTLA4* is expressed in Tregs as well as in the activated T cells and *FOXP3*, a marker of Tregs, was not upregulated in the vitiligo skin, we suggest that *CTLA4* is upregulated in our samples due to the increased activation of the T cells in the skin of the vitiligo patients. Moreover, *EOMES*, a transcription factor characteristic of effector cytotoxic T cells, unconventional memory CD8+ T cells and NK cells (Collins et al, 2017; Jacomet et al, 2015; Martinet et al, 2015; White et al, 2016), was upregulated in both the vitiligo lesional and non-lesional skin. Unconventional memory CD8+ T cells is a heterogeneous subpopulation of the cells that acquire a memory phenotype in the absence of antigen exposure and once developed, they can respond immediately and vigorously to danger signals by releasing cytokines and cytotoxic mediators (Martinet et al, 2015; White et al, 2016). Innate and innate-like memory CD8+ cells as well as virtual memory T cells constitute the group of unconventional memory CD8+ T cells. The increase in the transcription factor *EOMES* is consistent with the involvement of unconventional memory cytotoxic cells that similarly to innate cells respond readily to cytokines and stress signals, and can be recruited by CXCL10 (Barbarin et al, 2017; Martinet et al, 2015). However, the precise cell type responsible for the lymphoid stress surveillance in vitiligo is yet to be identified.

9.3. Autophagy is activated in vitiligo (Paper I)

According to several hypotheses of vitiligo pathogenesis, melanocytes are not merely innocent bystanders that are attacked by cytotoxic cells but rather trigger the processes themselves by impaired oxidative stress responses and/or by dysregulated autophagic processes (Xie et al, 2016). Moreover, it has previously been shown that autophagy deficiency causes premature senescence and a decreased proliferation of melanocytes (Zhang et al, 2015), and melanosomal autophagy in stressed melanocytes mediates antigen presentation and DC maturation (Xie et al, 2016).

We demonstrated the highly significant downregulation of *WIPI1* in the lesional and non-lesional skin of the vitiligo patients. *WIPI1* is a gene that regulates autophagy as well as melanosome maturation (Akinduro et al, 2016; Ho et al, 2011). As the studied cell cultures revealed that *WIPI1* was most strongly expressed in melanocytes, then *WIPI1* downregulation in the vitiligo

skin could be the result of melanocyte loss in the lesions. Nevertheless, this is unlikely regarding the reduced *WIPI1* expression also in the vitiligo non-lesional skin. However, melanosomes are also lysosome-related organelles whose formation is regulated by molecules overlapping with autophagy regulators like WIPI1 and LC3 (Ho et al, 2011; Yun et al, 2016). Therefore, in the vitiligo skin, *WIPI1* downregulation could be linked with an impaired formation of melanosomes rather than with dysregulated autophagy.

According to our results, the downregulation of *WIPI1* in the vitiligo skin and the activation of autophagy are uncoupled processes as the *WIPI1* expression was reduced to a similar degree in the lesional and non-lesional skin whereas LC3 staining clearly indicated an increased autophagy only in the keratinocytes of the lesional skin. Whether the activated autophagy in the vitiligo keratinocytes represents a compensatory mechanism for the lack of melanin, is responsible for the faster degradation of the remaining melanin produced by residual melanocytes in the vitiligo skin (Tobin et al, 2000) or is the result of an increased stress-surveillance response in the skin remain unanswered at present. Autophagy and nucleophagy are suggested to be essential for normal epidermal development and differentiation (Akinduro et al, 2016). To the best of our knowledge, this is the first demonstration of excessive autophagy in the vitiligo keratinocytes. However, alterations in keratinocytes have been found by previous transcriptional studies (Singh et al, 2017). This suggests that the cross-talk between different skin cell types is relevant in the pathogenesis of vitiligo. Collectively, our results support the hypothesis that autophagy is activated in the vitiligo lesions involving melanocytes as well as keratinocytes. This evidence calls for further research into autophagy as a candidate treatment target for vitiligo.

9.4. A model to explain the pathogenesis of vitiligo (Paper I)

Based on the results of this study, we propose a model wherein different stressors like UV irradiation or altered melanosome maturation lead to an increased oxidative stress that can cause DNA damage (Figure 19). Damaged cells upregulate stress-ligands and the production of type I IFNs that in turn induce CXCL10 secretion from keratinocytes, which then attracts CXCR3 positive T cells. Stress-ligands can be bound by activating receptors on innate and innate-like T cells which deploy their effector mechanisms and cause the release of autoantigens from dying cells. This can prime the responses of antigen-specific T and B cells, which participate in the perpetuation of tissue damage. These findings reveal some informative traces that would help dissect the processes leading to melanocyte destruction.

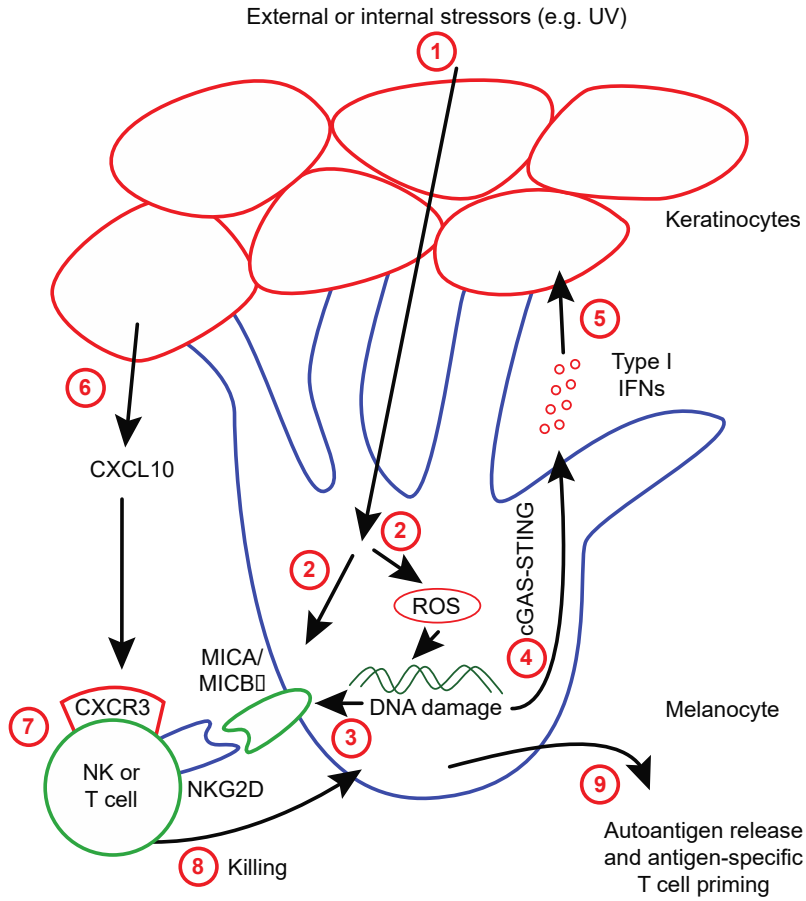

Figure 19. Model for the pathogenesis of vitiligo. Different external stressors like ultraviolet (UV) light or internal stressors like altered melanosome maturation **(1)** induce oxidative stress that damages DNA **(2)**. Damaged cells upregulate stress-ligands, such as a MHC class I polypeptide-related sequence A (MICA)/MICB **(3)**, and induce the production of type I interferons (IFNs) through the activation of the stimulator of interferon genes (STING)-pathway **(4)**. Type I IFNs in turn induce C-X-C motif chemokine ligand 10 (CXCL10) secretion from keratinocytes **(5, 6)**, which then attract chemokine receptor CXCR3 positive T cells **(7)**. Stress-ligands can be bound by activating receptors on unconventional memory CD8+ T cells and NK cells that deploy their effector mechanisms **(8)** and cause the release of autoantigens from dying cells. This can prime the responses of antigen-specific T and B cells **(9)**, which participate in the perpetuation of tissue damage.

9.5. Local and systemic inflammation is severe in psoriasis (Paper II)

Psoriasis is a prototypical Th17 pathway-mediated chronic inflammatory disease (Swindell et al, 2016; Xing et al, 2016) and biologicals blocking this pathway have shown high efficacy in the treatment of psoriasis (Blauvelt et al, 2017; Canavan et al, 2016; Lowes et al, 2014). The findings of this study confirm the central role of Th17 cytokines in psoriasis. In accordance with the upregulation of Th17 cytokines (*IL17A*, *IL17F*, *IL22*, *IL26*), the chemokines induced by IL-17A from keratinocytes (*CXCL1, CXCL2, CXCL8*) (Chiricozzi et al, 2011) were upregulated in the psoriatic skin. Additionally, other proinflammatory cytokines (*IFNG, TNFA, IL1B, IL36A*) and chemokines (*CCL2*, *CCL5* and *CCL20*) that recruit various inflammatory cells, including monocytes, DCs and Th17 cells (Ogawa et al, 2018), were highly expressed. Th17 cytokines are potent inducers of antimicrobial peptides, such as *S100A8*, *S100A9*, *PI3* and *LCN2* (Liang et al, 2006; Shao et al, 2016), all of which were upregulated in our study.

CCL27 was the only chemokine downregulated in the psoriatic skin. It is a skin specific chemokine that recruits CCR10+ lymphocytes to maintain skin homeostasis (Fu et al, 2016; Yang et al, 2016). In epithelial cell culture, *CCL27* is upregulated by IL-17 (Karakawa et al, 2014) and previous studies have convincingly shown its decrease in psoriatic lesions (Gudjonsson et al, 2010; Quaranta et al, 2014) and suggested this as a useful biomarker for the differentiation of psoriasis from atopic dermatitis in complicated differential diagnostic cases (Quaranta et al, 2014). The downregulation of *CCL27* in psoriatic skin would decrease the influx of immune cells that could counterbalance inflammation in the skin. Indeed, the expression of negative regulators of immune responses, specifically the Tregs marker *FOXP3*, coinhibitory molecules *CTLA4*, *IL10* and *IL1RN,* were markedly increased in the psoriatic lesions and IL-1Ra, the protein encoded by *IL1RN*, was elevated in the plasma of the psoriatic patients. However, FOXP3+ Tregs can lose their regulatory capacity in the inflammatory environment and start producing IL-17A which has been reported to happen in psoriatic skin (Bovenschen et al, 2011). In conclusion, these data are in line with multiple previous studies confirming the central role of Th17 cytokines in psoriasis (Boehncke & Schon, 2015).

Psoriasis is not a skin-restricted disease but in severe forms manifest many systemic features that in our study are exemplified by increased levels of several inflammatory mediators (CXCL8, IL-17A, IL-6, TNF-α) in the circulation. From the mediators with elevated serum levels, IL-17A was associated with the severity of the disease. This result confirms previous findings by Takahashi et al. (Takahashi et al., 2010). The IL-6 level was higher in the patients with psoriatic arthritis in our samples. Elevated levels of IL-6 in the serum and synovial fluid of patients with psoriatic arthritis have previously been reported and the efficiency of IL-6-targeted therapy in the treatment of

psoriatic arthritis is being studied (Mease et al, 2016). This supports the theory that IL-6 is a marker for psoriasis arthritis.

9.6. Autoinflammatory mechanisms are activated in the skin of the psoriasis patients (Paper II)

There is still no consensus about whether psoriasis is an autoimmune disease driven by autoantigen-specific T cells or an autoinflammatory disease provoked primarily by innate receptor signalling and innate-type cell activation in response to stress signals (Havnaer & Han, 2019). To clarify the importance of autoinflammatory mechanisms provoked primarily by innate receptor signalling in response to stress signals in psoriasis, we studied inflammasome components, several innate receptors and components of the autophagosome in the skin of the psoriasis patients.

Previous studies have revealed that mTOR signalling, which is known also as a negative regulator of autophagy, is hyperactivated in a psoriatic epidermis (Buerger et al, 2017) and the expression of LC3 as well as WIPI1 is reduced in a psoriatic epidermis (Akinduro et al, 2016). Contrary to our expectations, we did not find differences in the expression level of WIPI1 in the psoriasis patients compared to the control individuals. The possible explanation for this discrepancy might be related with the differences in the study participants or the methods used. Further research is needed to explain the role of impaired autophagy in psoriasis.

However, our results support recent discoveries that several innate receptors are involved in the pathogenesis of psoriasis (Tervaniemi et al, 2016). *IFIH1*, which encodes a receptor recognising dsRNA, showed elevated expression levels in the psoriatic lesions. It has previously been shown that the antimicrobial peptide LL37 induces the production of IFN-β by keratinocytes in response to dsRNA that is released from dying cells (Lande et al, 2015; Zhang et al, 2016). In our study, the upregulation of IFN-induced chemokine *CXCL10* in the psoriatic skin may indicate an enhanced IFN-signalling in psoriasis. Moreover, the therapeutic removal of the type I IFN receptor IFNAR by UV therapy has proven to be effective in the inflammation treatment of psoriatic skin (Gui et al, 2016). Additionally, *AIM2*, an innate receptor binding to dsDNA, was expressed at increased levels in the psoriatic skin in our study, being consistent with previous reports (de Koning et al, 2012; Dombrowski et al, 2011; Tervaniemi et al, 2016).

AIM2 is a component of inflammasomes and when activated, it recruits the adaptor protein ASC, which is encoded by the *PYCARD* gene to form inflammasomes and activate caspase-1, which cleaves pro-IL-1β to its bioactive form and induces pyroptosis (Man et al, 2016). Indeed, we showed the elevated expression of both genes – *PYCARD* and *IL1B* – in the psoriatic lesions. Previously, an increased level of active caspase-1 has been revealed in the lesional psoriatic epidermis via western blot (Dombrowski et al, 2011; Johansen

et al, 2007). Using the fluorescent detection of active caspase-1, we found caspase-1 in the granular layer of the normal healthy epidermis but not in the dermis, which indicates its physiological role in epidermal maturation. Psoriatic lesions revealed increased levels of active caspase-1 in the whole epidermis as well as in the subepidermal areas. Our data also confirm the recent finding that NLRP1 and NLRP3 inflammasomes are not differentially expressed in psoriasis (Tervaniemi et al, 2016), although single nucleotide polymorphisms in *NLRP3* are associated with wide-spread psoriasis (Carlström et al, 2012).

The source of the activating signal for AIM2 still remains to be identified. One possibility is that keratinocytes are the sources of the activating signal, as keratinocytes in psoriatic skin display free cytoplasmic dsDNA (Dombrowski et al, 2011). Another possibility is that the activating signal derives from neutrophils. Using immunofluorescence, de Koning et al. have demonstrated that there is AIM2 staining adjacent to Munro's microabscesses, which are the collections of neutrophils in *stratum corneum* of the psoriatic lesions (de Koning et al, 2012). Regarding the capability of neutrophils to undergo extracellular trap formation (NETosis) that releases huge amounts of immunostimulatory dsDNA, the location of AIM2 is perfect for responding to this signal. However, at this stage we can only speculate about the inflammasome type involved and the cause for its activation. Nevertheless, finding the involvement of inflammasomes and innate receptors in psoriasis strongly suggests the increased activation of innate immunity in psoriatic lesions and paves the way for the development of new effective treatment options for this serious inflammatory disease.

9.7. Innate and innate-like cells participate in the development of psoriasis (Paper II)

Multiple effector cells can mediate inflammation in psoriasis, such as cytotoxic T cells, NK cells, γδ T cells and neutrophils (Cai et al, 2013; Lowes et al, 2014). The most abundant effector cells in psoriatic skin are lymphocytes, out of which CD4+ cells mostly locate in the dermis and CD8+ cells in the epidermis (Cheuk et al., 2014; Di Meglio et al., 2016). Whereas the T cell response in psoriatic skin is highly polyclonal, it is unlikely that a single or a couple of autoantigens induce the disease (Harden et al, 2015a). To get information about innate cell infiltration to the skin of the psoriasis patients, we measured the expression of a stress molecule and several stress molecule binding receptors.

Our study is the first to describe an elevated level of *EOMES* in the psoriatic lesions and in the non-lesional skin at mRNA as well as protein levels, locating especially in the nuclei of the CD3+ T cells. As mentioned before, EOMES is an important transcription factor for CD8+ T cell development and effector function exertion (Knox et al, 2014; McLane et al, 2013). Moreover, *EOMES* is critical for the development of unconventional memory CD8+ T cells, which are T cells that activate rapidly without TCR stimulation, are IL-15 dependent and have innate-like functions, such as the capability to rapidly produce

inflammatory cytokines in the absence of antigenic recognition (Jacomet et al, 2015; Martinet et al, 2015; White et al, 2016). Recently, Collins et al. showed that *EOMES* is an essential factor for the development of NK cells as well (Collins et al, 2017). We did not see any statistically significant difference in the NK cell-specific gene *KLRK1* expression but interestingly, we found a moderate increase in the stress molecule *MICB* expression in the psoriatic skin which makes the cells vulnerable to the attack of NK cells, γδ T cell and tissue-resident cytotoxic αβ T cells (Cheuk et al, 2014; Hayday, 2009; Knight et al, 2012; Strid et al, 2011). Moreover, although the first suspects for making IL-17A in the psoriatic lesional skin were Th17 cells, several recent studies have pointed to CD8+ and γδ T cells as an important IL-17A source in this disease (Cai et al, 2013; Di Meglio et al, 2016; Hijnen et al, 2013; Kim et al, 2016; Laggner et al, 2011). While we were unable to see any significant differences in the T cell receptor gamma chain *TRGC1* expression in the psoriatic skin, we cannot rule out an increase in the proportion of IL-17A secreting cells. Indeed, Harden et al. detected several γ-chain sequences that were shared by the psoriasis patients' skin samples (Harden et al, 2015a).

The evidence of this study suggests the involvement of innate-type lymphocytes in the pathogenesis of psoriasis, but the precise role of T cells in psoriasis needs further clarification, including the possible role of unconventional cells, their potential therapeutic modification (e.g. by blocking IL-15) and their relation to tissue-resident memory cells (Cheuk et al, 2017; Watanabe et al, 2015). In conclusion, our data are in line with studies that support the auto-inflammatory pathogenesis of psoriasis which involves multiple innate receptors and innate cell types, probably including the recently described unconventional memory cytotoxic cells. It is therefore possible that also lymphoid stress surveillance response participates in the pathogenesis of psoriasis.

9.8. MiRNAs participate in the pathogenesis of vitiligo (Paper III)

One purpose of this thesis was to assess the expression changes and role of miRNAs in vitiligo pathogenesis. The current study found that miR-99b, miR-155, miR-199a-3p, miR-125b and miR-145 are dysregulated in the skin of the patients with vitiligo. Moreover, several putative targets associated with melanocyte differentiation and melanogenesis were predicted by pathway analysis for miR-99b, miR-125b, miR-155 and miR-199a-3p, and were proved experimentally for miR-155.

Previously, miRNA expression profiles in the PBMCs (Wang et al, 2015b), serum (Shi et al, 2013), whole blood (Shang & Li, 2017) and skin (Mansuri et al, 2014; Sahoo et al, 2017) of vitiligo patients have been investigated. We confirm Mansuri's finding (Mansuri et al, 2014) that the expression of miR-99b is increased in the skin of vitiligo patients. The finding that miR-125b, miR-155 and miR-199a-3p are dysregulated in the skin of vitiligo patients has previously

not been reported. Although the downregulation of miR-10a and the upregulation of miR-223 have earlier been observed in the serum of vitiligo patients (Shi et al, 2013), we did not detect any differences in the expression levels between the skin of the control subjects and the patients with vitiligo. This indicates that there could be differences in the expression levels of miRNAs in the skin and serum. Additionally, Mansuri et al. reported an upregulation of miR-145 in the vitiligo skin (Mansuri et al, 2014). In our study, miR-145 was downregulated in the vitiligo lesions. This discrepancy might be due to variation in study participants' characteristics or the technical approach, including differences in ethnicity, RNA extraction methods and miRNA expression evaluation methods. However, these results suggest that miRNAs are involved in the pathogenesis of vitiligo.

9.9. miR-155 contributes to the pathogenesis of vitiligo, modulating melanogenesis-associated and interferon-inducible genes in melanocytes and keratinocytes (Paper III)

Among dysregulated miRNAs, we found an increased expression of miR-155 in the epidermis of the vitiligo lesional skin, on the site where keratinocytes and melanocytes are located. In contrast, previous studies have demonstrated the expression of miR-155 mainly in immune cells (Dudda et al, 2013; Sonkoly et al, 2010). To understand whether proinflammatory vitiligo-associated cytokines can affect the expression of miR-155 in skin cells, we stimulated human primary melanocytes and keratinocytes with TNF-α, IFN-α, IFN-γ and IL-1β. From these cytokines, TNF-α, IFN-γ and IL-1β are dysregulated in the lesional skin, serum, PBMCs and the whole blood of vitiligo patients and these cytokines are known to inhibit melanocyte proliferation and melanogenesis (Camara-Lemarroy & Salas-Alanis, 2013; Laddha et al, 2014; Reimann et al, 2012; Tu et al, 2003). In addition, several indications that IFN-α plays a role in the pathogenesis of vitiligo (Arya et al, 2010; Bertolotti et al, 2014; Jacquemin et al, 2017; Li et al, 2014) are listed above (paragraph 9.1.). Thus, any miRNA modulating the pathways associated with the production or effect of these cytokines potentially influences the development of vitiligo. Indeed, miR-155 was induced in response to the vitiligo-associated cytokines in melanocytes as well as keratinocytes. It can thus be suggested that the presence of inflammatory cytokines in the skin of the patients with vitiligo modulates inflammatory responses and activates the expression of miR-155 in melanocytes and keratinocytes.

miR-155 is known as a proinflammatory miRNA, which among other targets suppresses *SOCS1*. This results in the activation of the Janus kinase-signal transducer and the activator of the transcription (JAK-STAT) pathway and, consequently, in type I and II IFN signalling (Wang et al, 2010). We found that

when overexpressed, miR-155 inhibited the expression of genes known to affect melanocyte differentiation and melanogenesis, such as *TYRP1*, *YWHAE*, *SDCBP* and *SOX10* in melanocytes, and *YWHAE* in keratinocytes. Additionally, the miR-155 overexpression altered the levels of interferon-regulated genes *SOCS1*, *IRF1* and *IFITM1* in melanocytes and keratinocytes. It is possible that miR-155 contributes to the pathogenesis of vitiligo, both through the targeting of melanogenesis-associated genes and via the modulation of IFN signalling. Multiple functions of miR-155 in vitiligo pathogenesis is supported by Lv et al who found that miR-155 is downregulated in the T cells isolated from the PBMCs of vitiligo patients and that the overexpression of miR-155 promoted the differentiation and function of Treg cells (Lv et al, 2019).

In addition to miR-155, other dysregulated miRNAs might contribute to the development of vitiligo. For example, miR-125b is downregulated after the induction of pigmentation in melanocytes (Dynoodt et al, 2013) and miR-125b mimics have been demonstrated to inhibit the expression of pigmentation-related genes (Kim et al, 2014). Similarly to miR-125b, the expression of miR-145 has been demonstrated to be decreased in cultured pigment cells after the induction of pigmentation (Dynoodt et al, 2013). The pathway analysis in our study detected possible direct targets for miR-145 among the MAPK, JNK and TGF-β pathways, which can disturb the viability and functionality of melanocytes (Bellei et al, 2013). Although no significant overlap was revealed between the miR-145 targets and genes associated with pigmentation, a computer analysis with Targetscan suggested the presence of binding sites for miR-145 in the mRNAs of melanogenesis-associated genes *RAB27A*, *SOX9* and *FCSN1* (data not shown). This is in line with the previous findings demonstrating the influence of miR-145 on genes involved in the pigmentation in miR-145-transfected cells (Dynoodt et al, 2013).

To summarise, we demonstrated that miR-99b, miR-125b, miR-145, miR-155 and miR-199a-3p are dysregulated in the skin of the patients with vitiligo, and showed that miR-155 has the capacity to modulate melanogenesis-associated and interferon-inducible genes in melanocytes and keratinocytes. The dysregulated miRNAs could be used as predictive markers in the diagnosis and treatment of vitiligo.

9.10. Concluding remarks and future prospects

The results of this study support several previous findings and provide much new information about the pathogenesis of vitiligo and psoriasis, but many questions still remain to be answered and need further investigation. First, the current data highlight the importance of innate immunological mechanisms in the development of both chronic dermatoses – vitiligo and psoriasis. Further studies need to be carried out in order to specify the role of innate immunity components in these diseases and to clarify the value of the components as possible therapeutic, diagnostic or prognostic targets. Special attention needs to

be paid to eomesodermin positive skin infiltrating cells in vitiligo as well as psoriasis. Second, the specific and/or overlapping roles of type I and II IFNs in vitiligo are yet to be substantiated. Third, considering the alteration of autophagy in the epidermis of the vitiligo patients, another aspect that needs to be elucidated is the pathogenic role of autophagy in developing vitiligo. Whether the activated autophagy in vitiligo keratinocytes represents a compensatory mechanism for the lack of melanin, is responsible for the faster degradation of the remaining melanin produced by residual melanocytes in the vitiligo skin or is the result of an increased stress-surveillance response in the skin, needs to be studied. Furthermore, studies are needed to clarify whether miR-155 and other dysregulated miRNAs described in this study are suitable diagnostic markers and/or targets for the treatment of vitiligo. Finally, considering the upregulation of EOMES, CXCL10, IFIH1 and MICB in the skin of the patients with vitiligo as well as psoriasis, we can propose that there are some overlapping mechanisms between these diseases that need to be uncovered in the future.

It needs to be acknowledged that our work has some limitations, which must be recognised for a more balanced interpretation of the results. Firstly, the number of participants in the study was relatively low, which could skew the data and give a distorted view of the results. Secondly, there were only few novel findings in our study as a substantial part of our gene expression analysis confirmed previously published results. On the other hand, the reproducibility of the previous findings strengthens their reliability. Despite these limitations, we believe that the results are still valuable for a better understanding of the pathogenesis of vitiligo and psoriasis.

10. CONCLUSIONS

1. We observed that a strong local and systemic inflammation is associated with psoriasis but not with vitiligo. While psoriasis is a Th17-mediated disease, indirect evidence suggests the involvement of type I IFNs in the disease process of vitiligo.
2. The gene expression and immunofluorescence analysis of skin biopsy samples identified several innate-like lymphocyte subpopulations and receptors in the lesions of psoriasis and vitiligo. These results support the role of the lymphoid stress surveillance response in vitiligo and psoriasis pathogenesis.
3. Inflammasomes were activated in the epidermis as well as the dermis in the psoriatic skin but the involvement of inflammasomes in vitiligo pathogenesis was not supported by this study. In contrast, autophagy was activated in the vitiligo lesions, involving melanocytes and keratinocytes.
4. Several miRNAs were dysregulated in the skin of the vitiligo patients and had various melanocyte-associated putative targets. From dysregulated miRNAs, the functional studies on miR-155 revealed its capacity to impact genes associated with melanogenesis and inflammatory responses in melanocytes and keratinocytes. These data suggest that miR-155 may contribute to the development of vitiligo.

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12. SUMMARY IN ESTONIAN

Molekulaarsed muutused kahe kroonilise dermatoosi – vitiliigo ja psoriaasi – patogeneesis

Üldteoreetiline taust

Vitiliigo on sagedaseim pigmendikaona väljenduv krooniline nahahaigus. Kuna vitiliigo avaldub tihti lapse- või noorukieas, lokaliseerub enamasti avatud kehapiirkondadesse ja puuduvad head ravivõimalused, mõjutab see oluliselt patsiendi elukvaliteeti. Pigmendi kadu on tingitud melanotsüütide hävinemisest, kuid senini on ebaselge, mis melanotsüütide vastase reaktsiooni vallandab ja kuidas seda reaktsiooni alal hoitakse.

Psoriaas on sage kompleksse patogeneesiga põletikuline nahahaigus, mis väljendub punetavate ja ketendavate naastudena nahal. Psoriaasi korral esineva põletiku süsteemset iseloomu toetab ~50%-l haigetest kaasnev küünekahjustus, ~30%-l kaasnev liigeshaaratus ja tavapopulatsioonist sagedamini esinevad kroonilised haigused, nagu kardiovaskulaarsed haigused, diabeet jt. Arengud psoriaasi ravis võimaldavad kasutada efektiivseid bioloogilisi preparaate, mis blokeerivad põletikulisi tsütokiine – tuumorinekroosifaktor (TNF)-α, interleukiin (IL)-23, IL-17. Senini ei ole aga täpselt teada, kas psoriaasi korral esineva põletiku tekkes omavad olulisemat rolli autoimmuunsed mehhanismid, mis on vahendatud autoantigeeni spetsiifiliste T-rakkude poolt, või pigem autoinflammatoorsed mehhanismid, mille aluseks on loomuliku ehk sünnipärase immuunsüsteemi aktivatsioon.

Uurimuse põhieesmärgid

Antud doktoritöö eesmärgiks oli hankida uut informatsiooni kahe dermatoosi – vitiliigo ja psoriaasi – patogeneesi kohta. Täpsemaks eesmärgiks oli uurida vitiliigo ja psoriaasi korral esinevat paikset ja süsteemset põletikulist vastust, saada informatsiooni nende haiguste korral nahka infiltreerivate immuunrakkude kohta, täpsustada inflammasoomide ja autofaagia kaasatust vitiliigo ja psoriaasi patogeneesis ning kirjeldada mikroRNA(miRNA)de ekspressioonimuutusi ja tähtsust vitiliigo tekkes.

Uuritavate grupid ja metoodika

Uuringusse kaasasime kokku 23 mittesegmentaalse vitiliigoga patsienti, 43 naastulise psoriaasiga patsienti ja 32 kontrollindiviidi. Uuritavate nahast määrasime põletikulisi tsütokiine, retseptoreid, immuunrakkude markereid ja nende ligande ning inflammasoomi ja autofaagia komponente kodeerivate informatsiooniRNAde (*messenger*RNA, mRNA) suhtelist ekspressiooni. Selleks

kasutasime kvantitatiivse reaalaja polümeraasi ahelreaktsiooni (qRT-PCR) meetodit. Plasmast mõõtsime põletikuliste tsütokiinide kontsentratsiooni Luminex meetodiga. Immunofluorestsentsuuringuga hindasime vitiliigohaigete nahas raku stressi ja autofaagia markeri avaldumist ning psoriaasihaigete nahas tsütotoksiliste lümfotsüütide transkriptsioonimarkeri eomesodermiini avaldumist. Psoriaasihaigete nahas hindasime inflammasoomi põhikomponendi kaspaas-1 aktiivsust.

Lisaks määrasime vitiliigohaigete nahas miRNAde suhtelist ekspressiooni qRT-PCRiga. miR-155 lokalisatsiooni nahas hindasime *in situ* hübridisatsiooniga. Potentsiaalsete miRNA märklaudgeenide tuvastamine toimus *TargetScan*'i abil ja signaalradade otsimiseks kasutasime *g:Profiler*'it.

Veel kasutasime erinevaid rakupopulatsioone – melanotsüüte, keratinotsüüte, fibroblaste ja Langerhansi rakke. Rakupopulatsioonides määrasime autofaagia markeri ja miRNAde avaldumist qRT-PCRiga. Melanotsüütidel ja keratinotsüütidel viisime läbi stimulatsioonikatse, milles hindasime põletikuliste tsütokiinide mõju miR-155 avaldumisele. Hindamaks miR-155 efekti melanogeneesiga seotud ja interferoonide poolt indutseeritavatele geenidele, teostasime melanotsüütide ja keratinotsüütide transfektsiooni miR-155-ga misjärel mõõtsime mRNAde ekspressiooni.

Tulemused ja arutelu

Leidsime, et vitiliigot põdevate patsientide nahas olid uuritud tsütokiinidest ja kemokiinidest kõrgelt ekspresseerunud *TNFA*, *IL36A* e *IL1F6*, *CCL5* ja *CXCL10*. Kuigi interferoon (IFN)-γ kodeeriv *IFNG* ei olnud uuritavate nahas määratav, oli vitiliigohaigete nahas kõrgelt avaldunud loomuliku immuunsüsteemi töös osalev retseptor *IFIH1* ja kemokiin *CXCL10*, mis mõlemad on teadaolevalt valdavalt IFN-γ poolt indutseeritavad. Kuna aga varasemast on teada, et *CXCL10* ekspressiooni võivad mõjutada peale IFN-γ ka I tüüpi interferoonid (IFN-α ja IFN-β) ning on mitmeid kaudseid viiteid I tüüpi interferoonide osalemisele vitiliigo patogeneesis, on võimalik, et *CXCL10* ja *IFIH1* ekspressiooni haigetel aktiveerivadki just I tüüpi interferoonid. Tsütokiinide määramine plasmast süsteemset põletikku vitiliigohaigetel ei toetanud – me ei täheldanud ühegi määratud tsütokiini kontsentratsiooni suurenemist vitiliigohaigetelt kogutud plasmas. Nahka infiltreerivate rakkude markeritest olid vitiliigot põdevate patsientide nahas kõrgelt avaldunud aktiveeritud T-rakkudel ja regulatoorsetel T-rakkudel ekspresseeruv *CTLA4* ja tsütotoksiliste lümfotsüütide transkriptsioonifaktor *EOMES*. Täpsemalt on EOMES e eomesodermiin tsütotoksliste efektor-T-lümfotsüütide, NK-rakkude ja sünnipäraste omadustega tsütotoksiliste T-lümfotsüütide transkriptsioonifaktor. NK-rakud ja sünnipäraste omadustega T-lümfotsüüdid ei vaja aktivatsiooniks T-raku retseptori (TCR) stimulatsiooni ja nendel rakkudel on valmisolek kiiresti reageerida ohusignaalidele. Kuna immunofluorestsentsuuringuga leidsime, et raku stressi korral avalduv ligand MICA/MICB on vitiliigo haiguskoldes subepidermaalselt

paiknevates deformeerunud rakkudes avaldunud, võime oletada, et stressligand aktiveerib sünnipäraste omadustega tsütotoksilisi T-rakke. Autofaagia ja melanosoomide küpsemise regulaator *WIPI1* oli nii vitiliigo haiguskoldes kui ka vitiliigo näiliselt terves nahas allareguleeritud. Selgitamaks välja, kas vitiliigo korral on *WIPI1* regulatsioonihäire tingitud pigem autofaagia häirumisest või melanosoomide küpsemise häirumisest, määrasime immunofluorestsentuuringu abil autofaagia markeri LC3 avaldumismustrit nahas. Vitiliigo haiguskoldes nii üksikutes jäänukmelanotsüütides kui ka keratinotsüütides kogu epidermise ulatuses oli LC3 võtnud kuju, mis on iseloomulik aktiveerunud autofaagiale.

Psoriaasi uuringu tulemused on kooskõlas paljude teiste töödega, mis toetavad nii lokaalse kui ka süsteemse põletiku esinemist psoriaasiga patsientidel. Leidsime, et psoriaasi põdevate patsientide nahas on kõrgelt avaldunud Th17-lümfotsüütide toodetavad tsütokiinid (*IL17A*, *IL17F*, *IL22*, *IL26*), IL-17A toimel keratinotsüütidest vabanevad kemokiinid (*CXCL1, CXCL2, CXCL8*), mitmed teised põletikulised tsütokiinid (*IFNG, TNFA, IL1B, IL36A*) ja kemokiinid (*CCL2*, *CCL5*, *CCL20*) ning Th17 tsütokiinide poolt indutseeritavad antimikroobsed peptiidid (*S100A8*, *S100A9*, *PI3* ja *LCN2*). Ainult *CCL27*, kemokiin, mis kinnitudes CCR10 retseptoriga vahendab lümfotsüütide liikumist nahka, oli psoriaasihaigete nahas madalalt avaldunud. See viitab põletiku kontrollimehhanismide aktiveerumisele, millele antud uuringus osutas ka põletiku negatiivsete regulaatorite (*FOXP3*, *IL10*, *CTLA4*, *IL1RN*) kõrge avaldumine psoriaasihaigete nahas. Põletikuliste tsütokiinide (CXCL8, IL-17A, IL-6, TNF-α) kõrge kontsentratsioon plasmas viitab süsteemsele põletikule. Lisaks leidsime toetust hüpoteesile, et psoriaasi tekkes on oluline osa häirunud loomulikul immuunsusel. Nimelt olid meie uuringus kõrgelt avaldunud mitmed sünnipärase immuunsuse töös osalevad molekulid – dsRNAga seonduv retseptor *IFIH1*, interferoonide poolt indutseeritav *CXCL10*, inflammasoomide komponendid *AIM2*, *PYCARD*, *IL1B* ning rakkude stressi korral avalduv ning NK-rakkude ja nahas persisteeruvate T-rakkudega seonduv ligand *MICB*. Inflammasoomi komponentide mRNAde ülesregulatsioon ja kaspaas-1 aktivatsioon psoriaasihaigete nahas epidermises ja dermise ülaosas osutavad autoinflmmatoorsete mehhanismide olulisusele psoriaasi patogeneesis. Leidsime, et tsütotoksiliste lümfotsüütide transkriptsioonimarker *EOMES* on psoriaasihaigete nahas ülesreguleeritud nii mRNA kui ka valgu tasemel eomesodermiinina T-lümfotsüütides. See võib olla põhjustatud loomuliku immuunsuse tsütotoksiliste rakkude aktivatsioonist. Autofaagia markeri *WIPI1* avaldumishäiret me psoriaasihaigete nahas ei tuvastanud.

MiRNAd on lühikesed mittekodeerivad nukleotiidide järjestused, mis seondudes mRNAga inhibeerivad geenide ekspressiooni. Uuringusse valisime immunoloogilisi protsesse, melanogeneesi ning rakkude proliferatsiooni, diferentseerumist ja apoptoosi reguleerivad miRNAd. Täpsustamaks mikroRNAde rolli vitiliigo patogeneesis, määrasime miRNAde (miR-10a, miR-99b, miR-125a, miR-125b, miR-145, miR-146a, miR-146b, miR-155, miR-199a-3p, miR-203, miR-223, miR-511) ekspressiooni vitiliigohaigete nahas. Leidsime, et

vitiliigohaigete nahas olid ülesreguleeritud miR-99b, miR-125b, miR-155 ja miR-199a-3p ning allareguleeritud miR-145. Ülesreguleeritud miRNAdele tuvastasime mitmeid melanogeneesiga seotud potentsiaalseid märklaudgeene. *In situ* hübridisatsiooni alusel oli miR-155 vitiliigohaigete epidermises tugevalt avaldunud. Stimulatsioonikatsete abil näitasime, et vitiliigo patogeneesis osalevad põletikulised tsütokiinid TNF-α, IFN-γ, IFN-α ja IL-1b aktiveerivad miR-155 ekspressiooni nii melanotsüütides kui ka keratinotsüütides. Et veelgi täpsustada miR-155 kaasatust vitiliigo patogeneesis, teostasime melanotsüütide ja keratinotsüütide transfektsiooni hsa-miR155ga. Selle tulemusena leidsime, et miR-155 toimel muutub mitmete interferoonidega seotud ja melanogeneesis osalevate geenide avaldumine nii melanotsüütides kui ka keratinotsüütides. Meie uuring näitab, et miRNAd osalevad vitiliigo patogeneesis ja nende funktsioon on häiritud nii melanotsüütides kui ka keratinotsüütides.

Järeldused

Meie töö tulemused kinnitasid varasemaid uuringuid, et psoriaasi korral on põletik eeskätt Th17-vahendatud, samas näitasime loomulikus immuunsuses osalevate 1. tüüpi interferoonide rolli vitiliigo puhul. Saime toetust hüpoteesile, et loomuliku immuunsuse talitlusega seotud rakkude funktsioon on nii vitiliigo kui ka psoriaasi tekkes olulise tähendusega. Tuvastasime, et psoriaasihaigete nahas on inflammasoomid aktiveerunud nii epidermises kui ka dermises. Autofaagia on aktiveeritud vitiliigohaigete melanotsüütides ja keratinotsüütides. Leidsime, et vitiliigoga patsientide nahas on häirunud mitmete miRNAde avaldumine. Nendest miRNAdest miR-155 mõjutab melanogeneesi ja põletikulises vastuses osalevate geenide avaldumist nii melanotsüütides kui ka keratinotsüütides.

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14. PUBLICATIONS

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