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Description of the cytokines and cutaneous neuroendocrine system in the development of vitiligo

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Description of the cytokines and cutaneous neuroendocrine system in the development of vitiligo

UNIVERSITY OF TARTU
PRESS
1632

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The dissertation was accepted for the commencement of the degree of *Doctor Philosophiae* in neurosciences on September 24, 2012 by the Council of the Commencement of Doctoral Degrees in Neurosciences

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Commencement: December 7, 2012

This research was supported by the European Regional Development Fund. Publication of this dissertation is granted by the University of Tartu

ISSN 1736–2792
ISBN 978–9949–32–155–1 (print)
ISBN 978–9949–32–156–8 (pdf)

University of Tartu Press
www.tyk.ee
Order No. 518

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LIST OF ORIGINAL PUBLICATIONS

I **Reimann, E**., Kingo, K., Karelson, M., Salum, T., Aunin, E., Reemann, P., Abram, K., Vasar, E., Silm, H., Kõks, S. Analysis of the expression profile of CRH-POMC system genes in vitiligo skin biopsies. Journal of Dermatological Science. 2010 Nov; 60(2): 125–8.

II **Reimann, E.,** Kingo, K., Karelson, M., Reemann, P., Loite, U., Sulakatko, H., Keermann, M, Raud, K., Abram, K., Vasar, E., Silm, H., Kõks, S. The mRNA expression profile of cytokines connected to the regulation of melanocyte functioning in vitiligo skin biopsy samples and peripheral blood mononuclear cells. Human Immunolog. 2012 Apr; 73(4): 393–8.

III **Reimann, E.,** Kingo, K., Karelson, M., Reemann, P., Loite, U., Keermann, M., Abram. K., Vasar, E., Silm, H., Kõks, S. The expression profile of genes associated to dopamine pathway in vitiligo skin biopsies and blood sera. Dermatology. 2012 May; 224(2): 168–76.

Author's contribution:

I Study design, RNA extraction from skin biopsies, cDNA synthesis and detection using TaqMan QRT-PCR method, statistical analysis. The author wrote the manuscript and handled the correspondence.

II Study design, extracting peripheral blood mononuclear cells (PBMCs) from total blood, RNA extraction from skin biopsies and PBMCs, cDNA synthesis and detection using TaqMan QRT-PCR method, statistical analysis. The author wrote the manuscript and handled the correspondence.

III Study design, extracting blood sera from total blood, RNA extraction from skin biopsies, cDNA synthesis and detection using TaqMan QRT-PCR method, homogenizing skin biopsies for protein measuring, protein expression measuring applying ELISA, statistical analysis. The author wrote the manuscript and handled the correspondence.

ABBREVIATIONS

4a-OH-BH_4	4a-hydroxy- tetrahydrobiopterin
$6BH_4$	6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin
ACTH	adrenocorticotropin
AGRP	agouti-related protein
APC	antigen presenting cell
ASIP	agouti signalling protein
ATRN	attractin
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
CAT	catalase
$CD4^+$, 8^+	cluster of differentiation 4^+, 8^+
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COMT	catechol-*O*-methyltransferase
CRH	corticotropin-releasing hormone
CRHR1	corticotropin-releasing hormone receptor type 1
DA	dopamine
DBH	dopamine beta-hydroxylase
DDC	dopa decarboxylase
DRD1-5	dopamine receptor 1-5
E	epinephrine
GM-CSF	granulocyte macrophage colony-stimulating factor
Gp130	glycoprotein 130
GPX1	glutation peroxidase
GR	glucocorticoid receptor
GWAS	genome wide association study
HPA axis	hypothalamic-pituitary-adrenal axis
HPRT1	hypoxanthine phosphoribosyl-transferase 1
ICAM1	intercellular adhesion molecule 1
IFN	interferon
INFA	interferon alpha
INFB	interferon beta
INFG	interferon gamma
IL28A (*IFNL2*)	interleukin 28 alpha (interferon lambda 2)
IL28B (*IFNL3*)	interleukin 28 beta (interferon lambda 3)

IL29 (*IFNL1*)	interleukin 29 (interferon lambda 1)
IFNAR	interferon alpha receptor
IL	interleukin
IL10RA	interleukin-10 receptor alpha
IL10RB	interleukin-10 receptor beta
IL20RA	interleukin-20 receptor alpha
IL20RB	interleukin-20 receptor beta
IL22RA1	interleukin-22 receptor alpha 1
IL22RA2	interleukin-22 receptor alpha 2
IL28RA	interleukin-28 receptor alpha
IP3	inositol triphosphate
L-DOPA	L-3,4-dihydroxyphenylalanine
L-Phe	L-Phenylalanine
L-Tyr	L-Tyrosine
LIF	leukemia inhibitory factor
LPH	gamma-lipotropin
LPS	lipopolysaccharide
MAOA	monoamine oxidase A
MAOB	monoamine oxidase B
MC1-4R	melanocortin 1-4 receptor
MCH	melanin concentrating hormone
MCHR1, 2	melanin concentrating hormone receptor 1 and 2
MDM1	nuclear protein homolog (mouse) gene
MHC	major histocompatibility complex
MSH	melanogenesis stimulating hormone
NE	norepinephrine
NLRP1	NLR family pyrin domain containing 1
NSV	non-segmental vitiligo
NURR1	nuclear receptor related 1 protein
OPRD1	delta 1 opioid receptor
OPRK1	kappa 1 opioid receptor
OPRL1	opioid receptor like 1
OPRM1	mu 1 opioid receptor
OSM	oncostatin M
PAH	phenylalanine hydroxylase
PBMC	peripheral blood mononuclear cells
PCD	pterin-4a-carbinolamine dehydratase.
PDYN	prodynorphin

PENK	proenkephalin
PKA	protein kinase alpha
PMCH	pro-melanin-concentrating hormone
PNMT	phenylethanolamine N-methyltransferase
PNOC	prepronociceptin
POMC	proopiomelanocortin
PTPN22	protein tyrosine phosphatase, non-receptor type 22
qBH_2	quinonoid dihydrobiopterin
QRT-PCR	quantitative real-time polymerase chain reaction
SCF	stem cell factor
STAT1/2	signal-transducer and activator of transcription protein 1/2
SV	segmental vitiligo
TGFB	transforming growth factor beta
TH	tyrosine hydroxylase
TNFA	tumour necrosis factor alpha
URC	urocortin
UV radiation	ultraviolet radiation
VMAT1, 2	vesicular monoamine transporters 1 and 2
XBP1	X box binding protein 1

1. INTRODUCTION

The skin, the largest organ in the body, plays a critical role in maintaining internal homeostasis, serving as a barrier between the external environment and the internal milieu. [1] Because of its location, the skin is exposed continuously to a fluctuating environment with potentially noxious stimuli. [1, 2] It thus requires a precise and focused mechanism for the immediacy of its interactions with environmental stressors; preferably, such a mechanism should already be activated while cellular/tissue damage is still contained and of low magnitude. Such mechanisms are formed by the epidermal barrier, the secretory activity of adnexal structures, the local pigmentary and immune systems, vascular and mesenchymal components of the dermis. [1, 3–8] During the last few years, a modern concept of an interactive network between cutaneous nerves, the neuroendocrine axis, and the immune system has been established. This response system would restrict tissue damage and restore local homeostasis. [1]

One of the most important systems for maintaining the basal and stress-related homeostasis is the classical hypothalamic-pituitary-adrenal (HPA) axis. The required elements for the pathway are produced in hypothalamus, anterior lobe of the pituitary gland, and adrenal cortex. [9] The skin is also able to express all the elements of the HPA axis including the corticotropin-releasing hormone (CRH), proopiomelanocortin (POMC), adrenocorticotropin (ACTH) and β-endorphin with corresponding receptors, the glucocorticoidogenic pathway (primarily cortisol, a potent anti-inflammatory agent), and the glucocorticoid receptor (GR). Thus, there is a cutaneous HPA axis, which affects the immune system locally, but most probably influences the whole organism systemically. [9–13] Similarly to the classical HPA axis, the functioning of the local HPA axis is also regulated by different mediators, i.e. cytokines and neurotransmitters. [13–22]

Vitiligo is an idiopathic disorder, where selective destruction of the skin melanocytes results in the development of depigmented patches. It affects 0.5–2% of the general population without any racial, sexual, or regional differences in prevalence. [23, 24] In addition, patients often have other autoimmune diseases; furthermore, they suffer from psychological stress and the depigmented patches are more susceptible to sunburns, and thus skin cancer may develop. [23–28] Until now, the cause of vitiligo has not been fully understood; thus, different factors potentially participate and there are several theories – autoimmune, neural, and biochemical hypotheses. [29–34]

In the case of vitiligo pathogenesis, the pathways, which participate or potentially affect the cutaneous HPA axis, have been demonstrated to be altered to some extent. Our group has previously demonstrated significant differences in POMC system peptides and receptors in vitiligo patients compared to controls. [35] The expression of POMC, the key pro-peptide for HPA axis, decreased in patients' involved skin and also the melanocortin receptor level decreased in lesions. [9, 35]

Additionally, the expression of cytokines and the amount of neuromediators, which regulate the activity of HPA axis, i.e. interleukin-1 (IL1), IL6, and tumour necrosis factor alpha (TNFA), dopamine, norepinephrine, has altered in the blood and skin of vitiligo patients. [14, 22, 34, 36–40] Our group has previously studied the IL10 cytokine family and their receptors in vitiligo patients. We were not able to see any significant decrease in anti-inflammatory IL10 expression as shown by previous studies. [37, 41] Thus, we found that the pro-inflammatory IL22 expression had increased in the blood of vitiligo patients; also the expression of pro-apoptotic IL24 and receptor subunits IL10 receptor A and B (IL10RA and IL10RB) had increased in patients' blood. [37] In addition, we found associations between the polymorphisms of *IL19* and vitiligo pathogenesis. [42]

The aim of this research was to obtain further information about the different participating pathways in the functioning and possibly regulating the cutaneous HPA axis; the author intended to demonstrate the expression pattern changes of the studied genes in the case of vitiligo pathogenesis. The endogenous opioids pathway was analysed together with CRH and melanin-concentrating hormone (MCH) pathways, IL10 cytokine family and associated pathways and the dopamine pathway.

2. REVIEW OF LITERATURE

2.1. Skin as a neuroimmunoendocrine organ

The strategic location of the skin as the barrier between the environment and the internal milieu determines its critical function in the preservation of body homeostasis. [1] The skin is continuously exposed to many hostile environmental factors and to acute transfers of solar, thermal, or mechanical energy. [2] There are several instant mechanisms for the restoration of the structural and functional integrity of the skin upon disruption. Such mechanisms include the barrier-forming properties of the epidermis, the secretory activity of adnexal structures, the immune and pigmentary systems of the skin, as well as vascular and mesenchymal components of the dermis. [3, 5–8]

Skin is composed of three primary layers: the epidermis, the dermis, and the hypodermis. In addition to the barrier and defensive functions, skin as the largest organ in humans has several other important purposes among which the sensation of heat and cold, touch, pressure, vibration and tissue injury are mostly connected to the central nervous system (CNS) directly via efferent nerves or CNS derived mediators or indirectly via the adrenal glands or immune cells. Both sensory and autonomic (sympathetic) nerves influence a variety of physiological (vasocontraction, vasodilation, body temperature, barrier function, secretion, cell growth, differentiation, nutrition) and pathophysiological (inflammation, immune defence, apoptosis, proliferation, wound healing) functions in the skin. [43, 44]

In the skin, cutaneous nerve fibres are principally sensory with additional complement autonomic nerve fibres. [45] Most nerve fibres are found in the mid-dermis and the papillary dermis, but also in the epidermis, blood vessels, hair follicles, sebaceous glands, sweat glands, and apocrine glands forming a three-dimensional network. [46, 47] In contrast to sensory nerves, autonomic nerves never innervate the epidermis in mammals. [45] Autonomic nerve fibres in the skin almost completely derive from sympathetic (cholinergic) and, in the face, rarely from parasympathetic (also cholinergic) neurons. [48] Although very effective, they constitute only a minority of cutaneous nerve fibres compared with sensory nerves. Autonomic nerves innervate blood vessels, anterovenous anastomoses, lymphatic vessels, erector pili muscles, eccrine glands, apocrine glands and hair follicles. [49] Both autonomic as well as sensory nerve fibres are involved in hair follicle cycling and inflammation. [50]

As is common to neurons, cutaneous cells, such as keratinocytes, melanocytes, microvascular endothelial cells, Merkel cells, fibroblasts, leukocytes are also capable of releasing neuropeptides. All epidermal cells express sensor proteins and neuropeptides regulating the neuro immuno-cutaneous system. The epidermis can be considered a true sensory tissue where sensor proteins and neuron-like properties enable epidermal cells to participate in the skin surface perception through interactions with nerve fibres. [51, 52] Dermal blood vessels

are highly innervated by sensory and autonomic nerve fibres, they also synthesize certain neuropeptides after activation and express receptors for neuropeptides, which suggests that a complex autocrine and paracrine neuroendocrine system may exist in the skin. [53]

Langerhans cells and mast cells are key cells between neuroendocrine and immune systems in skin. They participate in the endocrine system through the metabolism of vitamin D and production of neurohormones. [54, 55] Langerhans cells are closely connected to Merkel cells and with sensory neurons, the latter interaction inhibits the antigen presenting function of Langerhans cells, acting as an immunomodulator. [56] [57, 58] Merkel cells are tightly interacted with neurons and are the only excitable cells within the epidermis, in addition to neurons; however, the activating stimuli are still not clear. [59–61] Langerhans and Merkel cells produce different mediators, which influence keratinocytes and melanocytes, i.e. IL10 family cytokines IL19, IL20, IL24, IL28 and IL29. [62, 63]

Keratinocytes modulate the innate and adaptive immune responses; they produce antimicrobial peptides and chemokines (independently from epidermal or dermal immune cells) and are able to activate T cells directly, but also indirectly via antigen presenting cells (APCs; dendritic cells and Langerhans cells) in the skin. [64–69] For that purpose, keratinocytes express different cytokines e.g. IL1α, IL8, IL25, TNFA. [68, 70–73] They also produce mitogens, such as basic fibroblast growth factor (bFGF), endothelins, stem cell factor (SCF), hepatocyte growth factor, nerve growth factor, granulocyte macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), α-melanogenesis stimulating hormone (αMSH), ACTH, which are essential for regulating differentiation, growth, and survival of melanocytes. [74–76] Additionally, they produce IL10 family cytokines i.e. IL10, IL19, IL20, IL24. [77–79]

Melanocytes synthesize melanin pigments when the skin is exposed to the sun. Ultraviolet (UV) radiation-stimulated melanocytes produce POMC. [80] POMC derived α-, β-, γ-MSH, ACTH, β-, γ-lipotropin (LPH), and β-endorphin [81] can activate melanogenesis, stimulate epidermal cell proliferation, induce melanocytes and Merkel cells to rise to a suprabasal location, have immunosuppressive and anti-inflammatory effects, probably through calcitonin gene-related peptide (CGRP) and IL10, or can even elevate the intensity of the cutaneous innervations. [82, 83] Furthermore, in skin the melanocytes are the principal source of anti-inflammatory IL10. [84]

2.1.1. Neuroendocrine axes: cutaneous HPA axis

In addition to endocrine activities, where hormones, neurotransmitters, neuropeptides, and the corresponding receptors are produced by epidermal, adnexal, and dermal cells or released in situ from cutaneous nerve endings,

adnexal structures, e.g. eccrine, apocrine and sebaceous glands and hair follicles also perform cutaneous exocrine activities. [1, 6, 8] The function of the latter is to strengthen the epidermal barrier, to regulate thermoregulation, or to participate in the defence against microorganisms or in social communication. [1, 5–8] All these activities can be organized into cutaneous neuroendocrine axes. [85] Special attention should be paid to a cutaneous equivalent of the HPA axis that would regulate local responses to stress independently from the central level. [86] Among others are local cholinergic, catecholaminergic, and serotoninergic/melatoninergic systems, the steroidonergic pathway in the skin and cutaneous expression of the pituitary-thyroid axis elements. [1, 87–95].
The classical HPA axis mediates the main adaptive responses to systemic stress (Figure 1). [9] Activation of the HPA system starts with hypothalamic production of CRH, which in the anterior activates CRH receptor type 1 (CRHR1) and induces production and release of POMC-derived peptides ACTH, αMSH and β-endorphin. [10–12] ACTH stimulates production and secretion of cortisol by the adrenal cortex. [9–12]

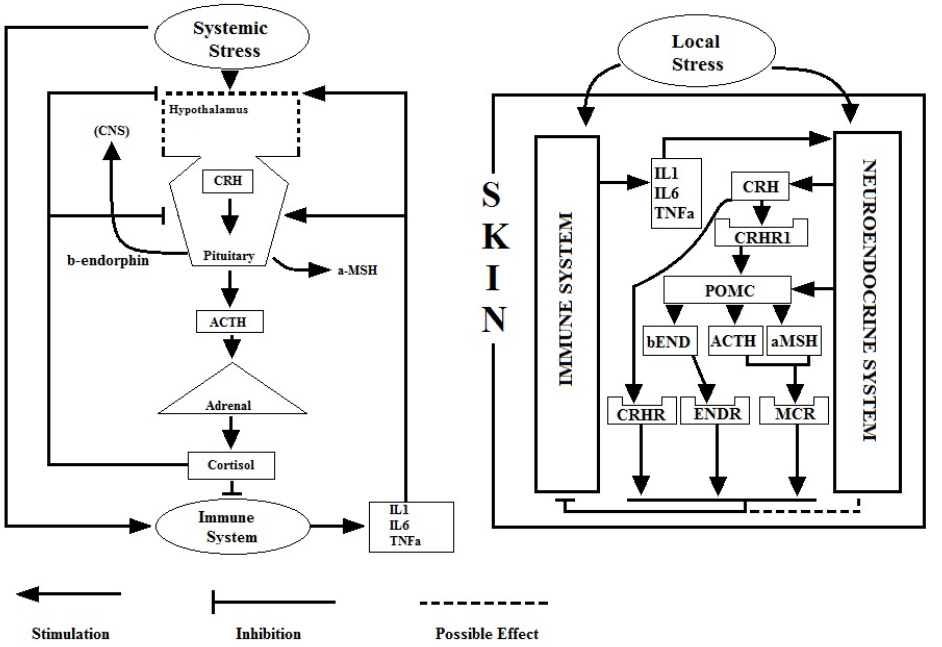

Figure 1. Classical and cutaneous HPA axis. [96]

The HPA axis is regulated at several levels, including stimulatory or inhibitory signals from the brain mediated through neurotransmitter systems and the suppressive feedback influence of corticosteroids themselves. Cortisol affects the HPA axis through binding to the GR located in hypothalamus and pituitary. [14] In addition, the axis is regulated by the endogenous opioid system – the opioid receptor μ (OPRM) activation by β-endorphin inhibits both production of CRH in the hypothalamus and POMC peptides in the anterior pituitary. [97, 98] Other neuromediators also participate in regulating the HPA axis activity. In the case of dopamine it has been found that during stress it has an adaptive, negative feedback capacity preventing excessive HPA axis activation in the rat brain. [14] The norepinephrine system as a quick stress response pathway has synergistic effects with a slower responding HPA axis. [15] Norepinephrine can excite CRH production and activate the HPA axis. [16] Serotonin activates the HPA axis in mice by increasing the expression of CRH. [17]

The neuroendocrine and immune systems communicate bidirectionally. The neuro-immune-endocrine interface is mediated by cytokines acting as auto/paracrine or endocrine factors regulating pituitary development, cell proliferation, hormone secretion, and feedback control of the HPA axis. [18] Complex interactions have been described for cytokine actions, including overlapping, synergistic, and antagonistic activities. By stimulating the HPA axis cytokines antagonize their own peripheral pro-inflammatory action. Excess HPA axis stimulation leads to immunosuppression and, therefore, increased susceptibility to infection. [13, 18] The concept of distinct groups of pro-inflammatory and anti-inflammatory cytokines has emerged on the basis of their peripheral action. [19] Glycoprotein 130 (Gp130), cytokine family (LIF, IL6, IL11, ciliary neurotrophic factor (CNTF), and oncostatin M (OSM)) participate in ACTH regulation and mediates the immuno-neuroendocrine interface. [20, 21] The most thoroughly studied cytokines are IL1, IL6, and TNFA, which activate the HPA axis in response to various threats to homeostasis. [22]

Skin cells are able to produce CRH and related peptides urocortin (URC) 1 and 2. In humans, the CRH and its receptor CRHR1 expression are stimulated by UV radiation. [2, 5, 86, 99, 100] CRHR1, which is activated by both CRH and URC, is predominantly expressed in the epidermis; the signal transduction pathways are coupled to cAMP, IP3, and Ca^{2+}. [96, 101–103] POMC and receptors for its derivates are also produced and processed in skin. The process is upregulated by UV radiation and increasing cAMP levels. Activation of the melanocortin receptors regulates or modifies several skin functions including pigmentation and local and possibly systemic immune activity. [2, 4, 104, 105] Cortisol and corticosterone are synthesized in hair follicles and melanocytes and fibroblasts. [101, 106, 107] Thus, Slominski et al. have proposed that the cutaneous defence against stressors is organized as an equivalent of HPA axis that operates as a coordinator and executor of the local responses to stress (Figure 1). [108]

In skin the cascade possibly goes as follows: CRH activates CRHR1 and further POMC expression and ACTH production in fibroblasts and melanocytes but not in epidermal keratinocytes. [107] In response to CRH and ACTH, melanocytes produce higher levels of cortisol and corticosterone, while fibroblasts only enhance the production of corticosterone, but not cortisol, which is produced continuously. [107] In the case of hair follicle keratinocytes, the CRH-POMC-cortisol cascade has been demonstrated to be functionional. [109]

As with the classical HPA axis, cytokines also control the activity of the cutaneous HPA axis. The cytokine IL1 has significant stimulatory effects on POMC gene expression and on the production of POMC-derived ACTH, αMSH, and βLPH peptides by resident skin cells and circulating immune cells. [110–113] IL1 also stimulates melanocortin 1 receptor (MC1R) expression. [114–117] TNFA stimulates POMC gene expression in dermal fibroblasts; transforming growth factor beta (TGFB) has the opposite effect, inhibiting POMC gene expression in the same cell system and in keratinocytes. [118] Endothelin-1, interferon alpha, beta and gamma (INFA, INFB, and INFG) can also stimulate the expression of functional MSH receptors on melanocytes. [105, 114, 115, 119–121] Thus, the cutaneous immune system also participates, through the opposing action of selected cytokines, in the regulation of the local HPA axis.

2.2. Vitiligo

Vitiligo is characterized by progressive disappearance of skin pigment cells, with straightforward clinical translation – white macules or white hair appear, usually without any accompanying clinical symptoms. Vitiligo occurs worldwide with an estimate prevalence of 0.5–2% in most populations. In almost half of the patients vitiligo starts before the age of 20 years, and males and females are affected with approximately equal frequency. [23, 24] Vitiligo has been associated with concomitant occurrence with a number of other autoimmune diseases, as well as a wide range of psychosocial difficulties, significantly, impacting quality of life. [25–27]

In the involved vitiligo, skin melanocytes are partially or completely lost, and no melanin is synthesized in this area. The cause of destruction of epidermal or follicular melanocytes is complex and not yet fully understood; however, there are several theories (autoimmune, neural, and biochemical hypotheses). [29, 30] Melanocyte death may occur due to the factors from inside and/or outside the cell and many potential systems could be involved. Histological data have demonstrated that at the perilesional skin in patients with non-segmental vitiligo an inflammatory infiltrate of low intensity made of mononuclear cells occurs in the upper dermis and the dermal-epidermal interface. [122] The initiation mechanism of this microinflammatory reaction is still unknown, but it has been suggested that various local triggers alert the skin innate immune system

and may precede adaptive immune responses targeting melanocytes. [123, 124] The absence of melanocytes affects skin homeostasis, as noted for skin sensitization, skin dermal neural responses, photoadaption, and redox status. [122]

The genome wide association studies (GWAS) account for a limited part of the heritability of the disease but may mediate a crucial part of disease phenotype, such as progression or age at onset. [125] Most vitiligo susceptibility loci encode immunoregulatory proteins or melanocyte components that are likely to mediate immune targeting and the relationships among vitiligo, melanoma, and eye, skin, and hair coloration. [126] For example, associations have been established between vitiligo pathogenesis and polymorphisms in tyrosinase gene (*TYR*), catalase gene (*CAT*), *MC1R*, major histocompatibility complex genes (*MHC*), protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*), NLR family pyrin domain containing 1 gene (*NLRP1*), and X box binding protein 1 gene (*XBP1*). Both protective and susceptibility increasing effects have been found in the case of different polymorphisms in these genes. [127, 128]

Additionally, Koebner's phenomenon is considered, according to which the induction of an isomorphic lesion follows a trauma. However, the significance of the phenomenon is controversial: does it indicate an intrinsically poor melanocyte attachment (melanocytorrhagy theory), or is it related to local inflammatory activity and overall disease progression. [129, 130]

Vitiligo has been classified on the basis of clinical grounds into two major forms: segmental (SV) and non-segmental vitiligo (NSV), the latter including generalized, acrofacial, and universal vitiligo. [131] The term *vulgaris* (synonymous with "common", a subgroup of generalized vitiligo) should not be used according to the Vitiligo Global Issues Consensus Conference (VGICC; held in 2011) (Table 1). However, while the samples for our studies had been collected prior to this decision, the term is still valid in the present dissertation. [132] NSV is characterized by depigmented macules that vary in size from a few to several centimetres in diameter, often involving both sides of the body with a tendency towards symmetrical distribution. SV has a limited, segmental distribution, has an earlier onset than NSV; its course is rapidly progressive but limited – depigmentation spreads within the segment over a period of 6–24 months and then stops. In contrast to NSV, SV has early involvement of melanocytes of hair follicles. [133] Mixed vitiligo is coexistence of SV and NSV. Focal vitiligo refers to an acquired, small, isolated hypopigmented lesion that does not fit a typical segmental distribution and which has not evolved into NSV after a period of 1–2 years. [132] Mucosal vitiligo involves oral and/or genital mucosae. [132] Universal vitiligo corresponds to complete or nearly complete depigmentation of the skin. [32, 132] The neural hypothesis is thought to be involved in SV whereas the autoimmune hypothesis is commonly related to NSV or focal vitiligo. [30] Regardless of this complicated classification, different studies suggest rather a continuum bridging all forms of vitiligo around a transient auto-inflammatory phase, which is associated with the wiping out of melano-

cytes, and a predisposing background to develop autoimmune responses that follow a risk gradient from SV to NSV. [122, 129, 130, 134, 135]

Table 1. Vitiligo classification according to the Vitiligo Global Issues Consensus Conference (held in 2011) [132]

	Subtypes
Non-segmental vitiligo	Acrofacial Mucosal (more than one mucosal sites) Generalized Universal Mixed Rare variants
Segmental vitiligo	Uni-, bi-, or plurisegmental
Undetermined/unclassified vitiligo	Focal Mucosal (one site in isolation)

2.2.1. Vitiligo, endogenous opioids and CRH-POMC system

Neuropeptides, including endogenous opioids and hormones, are mainly produced by neurons to communicate with each other. They may also be synthesized locally in skin involving the perception of pain and itch sensation and regulating melanogenesis and melanin transport to surrounding keratinocytes. [136, 137] We have previously studied the melanocortin system, which is an essential coordinator and executor of responses to stress. It consists of *POMC*, agouti signalling protein (*ASIP*), agouti related protein (*AGRP*) and the family of five melanocortin receptor genes (*MC1-5R*). The melanocortin system is important part of the CRH-POMC system, which is highly organized in skin-generating functions analogous to the HPA axis. [104, 138] We showed that the *POMC* mRNA expression level is significantly lower in the involved skin of vitiligo patients compared to uninvolved skin. The mRNA expression level of *MC1R* and *MC4R* decreased in involved skin comparing to uninvolved skin; however, the expression is evidently higher in uninvolved skin than in the skin of controls. [138]

The system of endogenous opioids includes several groups of different neuromediators – endorphins, enkephalins, dynorphins, endomorphins, nociceptins, and their receptors. Endogenous opioids are synthesized from different precursors; in addition to the *POMC* gene, there are prodynorphin (*PDYN*), prepronociceptin (*PNOC*), and proenkephalin (*PENK*) genes (Figure 2). *PDYN* codes leu-enkephalin, dynorhin A and B, α- and β- neodynorphin and 'big dynorphin' (consists dynorphin A and B), which bind to different receptors – mu 1, delta 1, kappa 1 opioid receptors (*OPRM1*, *OPRD1*, *OPRK1* genes, respectively). *PNOC* codes Noc II, nociceptin and nocistatin, all three bind to

opioid receptor like 1 (OPRL1). *PENK* codes both met-and leu-enkephalin petides, which bind to OPRM1, OPRD1, and OPRK1. [139]

Not much is known about the functions of products of *PDYN* and *PNOC* genes, which may involve the skin. Nociceptin is believed to play a role in nociceptive functions and increased adaptation to stress. [140, 141] Dynorphin A is known to participate in nociception and hyperalgesia and in inducing migration of keratinocytes and wound healing. [142–144] It has been suggested that OPRM1 and OPRD1 may have a role in keratinocyte proliferation and differentiation. [145] B-endorphin may stimulate melanocyte proliferation through OPRM1. [146, 147] Opioids may affect the skin also through immune cells. For example, OPRD1 is upregulated in T cells during activation in vitro and in vivo. [148] Endogenous opioids reduce leukocyte proliferation through OPRM1, which has a positive correlation with human melanoma progression. [149] In psoriatic skin the level of dynorphin A and OPRK1 has decreased compared to healthy skin. [150] OPRM1 is down-regulated by β- endorphin and its expression has decreased in psoriatic skin. [151] No associations with vitiligo pathogenesis have been demonstrated.

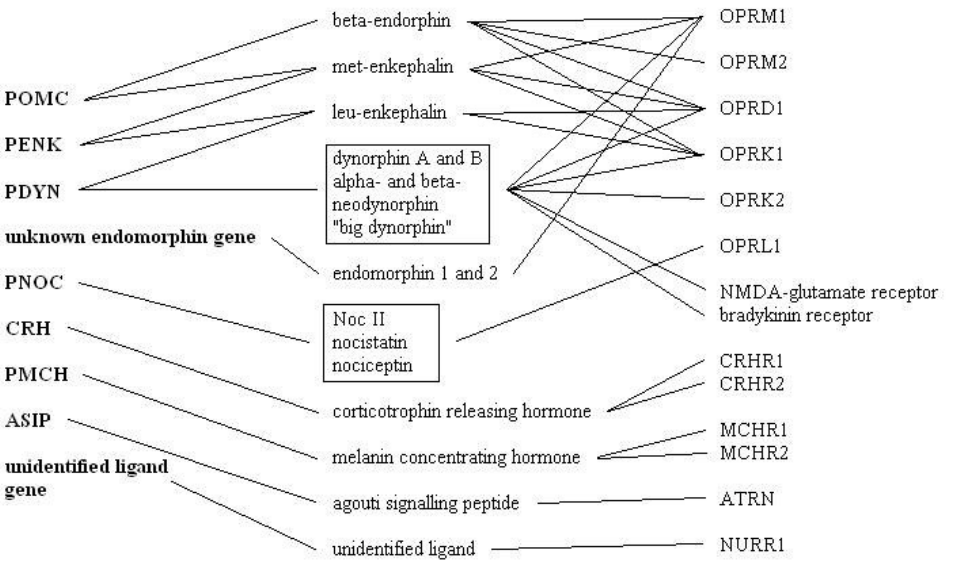

Figure 2. Components connected to the CRH-POMC system: POMC, proopiomelanocortin; PENK, proenkephalin; PDYN, prodynorphin; PNOC, prepronociceptin, CRH, corticotropin-releasing hormone; PMCH, pro-melanin-concentrating hormone; ASIP, agouti signalling protein; OPRM1 and -2, opioid receptor mu 1 and 2; OPRD1, opioid receptor delta 1; OPRK1 and -2, opioid receptor kappa 1 and 2; OPRL1, opioid receptor like 1; NMDA-glutamate receptor, N-methyl-D-aspartic acid-glutamate receptor; CRHR1 and 2, corticotropin-releasing hormone receptor 1 and 2; MCHR1 and 2, melanin-concentrating hormone receptor 1 and 2; ATRN, attractin; NURR1, nuclear receptor related 1

Until December 2010, there was no data about *PENK* mRNA expression in the skin; however, antibodies against met-enkephalin have been found. [152] Slominski et al. demonstrated *PENK* expression in fibroblasts, keratinocytes, and melanoma cells. [153] Depending on the site of production or target cells, the *PENK*-derived peptides can act in a para- or autocrine manner affecting immune activities, having direct antimicrobial activities, regulating cell proliferation and differentiation. [154–159] It was discovered in vitiligo patients that the met-enkephalin level in the patients' blood sera had increased and the daily circadian rhythm had changed. [160] There is no data available about any associations between leu-enkephalin with vitiligo pathogenesis.

CRH and its receptor CRHR1 are the central components of the HPA axis and important in coordinating systemic stress. [161] While CRH and CRHR1 are expressed also in human skin, they are believed to regulate various functions there. In the skin the CRH system is closely connected to the POMC system; CRH enhances the production and secretion of POMC and its peptides (MSH, ACTH, endorphin). POMC, however, is an important regulator of melanogenesis and survival of the melanocytes. This CRH-POMC skin system seems to have a major role in maintaining skin integrity and homeostasis. [162–166]

In mammals, the melanin-concentrating hormone (MCH) serves mainly as a neuropeptide, which is found in many regions of the brain [167]. Using different methods melanin-concentrating hormone receptor 1 (MCHR1) expression has been detected in melanocytes but not in keratinocytes or fibroblasts. [168] No MCHR2 expression has been demonstrated in any skin cells. [169, 170] In the skin, the MCH system is connected to the POMC system; MCH is known to inhibit the αMSH induced cAMP accumulation in cultured human melanocytes, resulting in reduced melanin production. This finding suggests that MCH may oppose the actions of the melanocortin system. [168, 171] Associations between the MCH-MCHR1 system and vitiligo pathogenesis have been investigated – autoantibodies against MCHR1 have been found in the patients' blood sera; however, the amount of antibodies is not correlated to the activity of the disease. [172, 173]

Also connected to the POMC system, attractin (ATRN) is a receptor for melanogenesis inhibiting agouti protein in mice. [174] ATRN is rapidly upregulated on the membrane of activated T cells and the soluble form is also released from there. It is known that loss of ATRN results in repercussion of skin pigmentation in mice. [175] The nuclear receptor related 1 protein (NURR1) induces and functions downstream MC1R signalling in melanocytes. [176] NURR1 is thought to mediate the effects of CRH and is involved in at least two signalling pathways mediating inflammatory signals. [177, 178] It has a key role in maintaining the dopaminergic system of the brain. [179]

2.2.2. Vitiligo and genes connected to IL10 family cytokines

The autoimmune theory of the destruction of melanocytes has a wide range of supportive evidence – abnormalities in both humoral and cell-mediated immunity have been documented in vitiligo patients. [30, 180] Additionally, vitiligo is associated with an increased local and systemic cytokine production. [181–186]

The IL10 cytokine family contains interleukins with a limited primary sequence similarity and structural homology and common receptor subunits, but distinct physiological roles. [187–189] In addition to IL10, the superfamily consists of IL19, IL20, IL22, IL24, and IL26. [187] IL28A (interferon lambda 2 (*IFNL2*)), IL28B (*IFNL3*) and IL29 (*IFNL1*) are recent members of the IL10 cytokine family (also categorized under the interferon family). [190]

IL10 is most abundantly produced by monocytes and type 2 T helper cells; however, also by mast cells, keratinocytes, dendritic cells, and eosinophiles. [191–193] It is an important immunomodulatoy cytokine, which has an anti-inflammatory effect while inhibiting the synthesis of pro-inflammatory cytokines produced by type 1 T helper cells. Thus, it may also stimulate certain T cells, mast cells as well as antibody production and maturation of B cells. [41, 194, 195] IL10 is associated with many autoimmune and inflammatory diseases, such as rheumatoid arthritis, psoriasis, melanoma and systemic lupus erythematosus. [196–200] IL19 is produced by monocytes, B cells, and keratinocytes, and it has been reported to have a direct effect on immune cells. [187, 201, 202] It is associated with psoriasis and asthma. [203, 204] IL20 is synthesized in monocytes and keratinocytes, and its over-expression provokes psoriasis-like skin lesions, which suggests its involvement in inflammatory response in the skin. [205, 206] IL22, which is produced by natural killer cells, T helper cells, fibroblasts, macrophages, mast cells, and dendritic cells, induces the expression of different pro-inflammatory molecules. [202, 205, 207–209] While mostly derived from T cells, IL22 may have a role in innate immunity of the skin and is associated with rheumatoid arthritis and psoriasis. [37, 207, 208, 210] IL24 is mainly synthesized by monocytes, macrophages, and type 2 T helper cells; it is known as pro-apoptotic cytokine and is cytotoxic for various tumour cells, including melanoma. [202, 205, 211–213] IL26 is mainly produced by T cells, monocytes, and natural killer cells [202], and it is involved in the activation of signal-transducer and activator of transcription protein 1 and 3 (STAT1 and STAT3) pathways, which are important for the stimulation of IL10, IL8, and intercellular adhesion molecule 1 (ICAM1) production. [214] IL28A, IL28B and IL29 are produced by T cells, dendritic cells, and virus-infected cells; they are believed to have antiviral, antiproliferative, and anti-tumor activity. [190] In addition, lipopolysaccharide (LPS) stimulation appears to induce the secretion of IL10 family cytokines suggesting their involvement in the innate immune response. [205, 215]

Our previous study failed to detect IL10 mRNA expression in the skin, and PBMCs did not reveal any significant differences between vitiligo patients and

controls. The protein level has a tendency to decrease in sera (statistically insignificant). [37] Thus, according to other studies, the IL10 protein level in vitiligo skin has decreased. [41] In the PBMCs of vitiligo patients the expression of IL19 has elevated, and there are associations between the polymorphisms of *IL19* and vitiligo pathogenesis. [37, 42] LPS increases the IL20 mRNA level in the PBMCs of controls but not in vitiligo patients. [37] The mRNA and protein expression of IL22 is elevated in patients' PBMCs and sera, respectively. [37, 207] Similarly to IL20, LPS stimulation affects the IL24 mRNA expression only in the PBMCs of controls but not in vitiligo patients. [37]

The receptor subunits for IL10 family cytokines form different receptor complexes, on which the ligands can bind (Figure 3). IL10RB is expressed in most cells; thus, IL10RA primarily in lymphocytes, monocytes, natural killer cells, and dendritic cells. [202] IL20RA, IL20RB, and IL22RA1 levels are the highest in epithelial and stromal cells. They are expressed together in considerable levels only in skin and lung tissue. [202, 205] IL22RA2 is mostly produced by plasma cells, monocytes, B and T cells, and dendritic cells. [202, 216] IL28RA is expressed in monocytes and dendritic cells, thus, most probably also in keratinocytes and melanocytes. [62, 63]

The expression of the receptors of IL10 family cytokines has altered in different pathogeneses, such as psoriasis, melanoma, and chronic atopic eczema; also UV radiation affects the expression pattern. [217–222] Our previous studies showed that the mRNA expression level of IL10RB and IL22RA1 had not altered in vitiligo skin. IL10RA has a tendency towards increased expression in vitiligo skin. mRNA expression of IL20RA decreased in patients' skin. The mRNA expression of both IL10RA and IL10RB increased in patients' PBMCs, the IL20RA and IL22RA1 mRNA levels were under detection limits. [37] Until now there is no data about IL22RA2 (inhibits IL22 activity [202, 216]) and IL28RA (the receptor complex combining IL28RA and IL10RB forwards the signals of IL28A, IL28B, and IL29 [190]) associations with vitiligo pathogenesis.

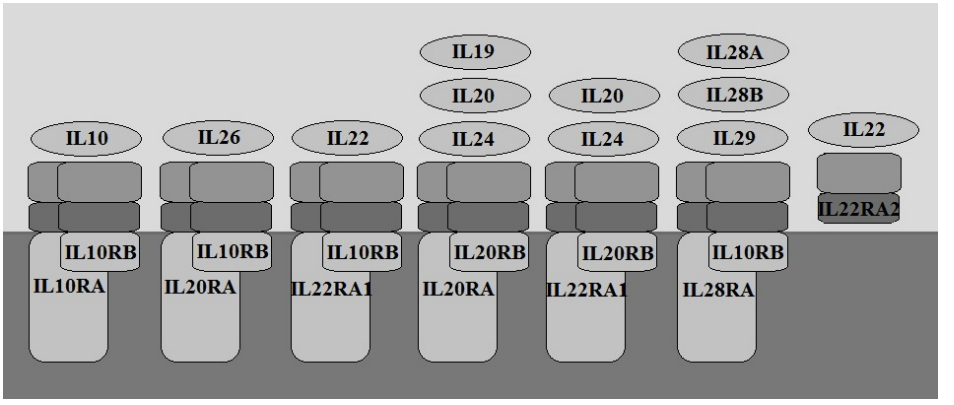

Figure 3. The receptor complexes for the IL10 family cytokines. [62, 63, 214, 223]

There are some genes that most probably are to some extent connected to IL10 family cytokines. Nuclear protein homolog (mouse) gene (*MDM1*) is located together with *IL22* and *IL26* in the locus 12q15; however, the biological function of MDM1 is unknown. [224, 225] IFNA and IFNB bind to the same cell surface receptor (IFNAR). IFNA is produced by leukocytes and keratinocytes while IFNB is synthesized by a variety of cells (fibroblasts, epithelial cells, and macrophages). [226, 227] Both *IFNA* and *IFNB* mRNAs have been detected in normal human melanocytes. [228] IFNA might be important in enhancing biological defence activities against oxidative stress and may cause induction of anti-melanocyte autoantibodies or activation of cytotoxic T cells. [229–231] IFNB is thought to induce apoptosis and to have anti-proliferative effects on malignant cells. [232] IFNA, IFNB, and also IFNG enhance human B cell proliferation. [233] Both IFNA and IFNB therapies may cause vitiligo. [230, 234, 235] IFNG is predominantly produced by natural killer cells, cluster of differentiation 4^+ and 8^+ ($CD4^+$ and $CD8^+$) T cells. [236] The association between IFNG and several skin conditions, such as atopic dermatitis, dermatomyositis, systemic lupus erythematosus, psoriasis, and melanoma, has been demonstrated. [237–241] Its mRNA expression has increased in vitiligo-involved and uninvolved skin compared to control skin. [194, 242] IFNG stimulates the expression of ICAM1, which is important for activating T cells and recruiting leukocytes. [243, 244] ICAM1 protein expression is upregulated in vitiligo skin and in melanocytes from perilesional vitiligo skin. [245]

2.2.3. Vitiligo and Dopamine pathway

There is considerable evidence that the melanocortin pathway, the most important melanogenesis regulator, is functionally linked with the dopamine system. [5, 246] Dopaminergic compounds inhibit the secretion of αMSH and β-endorphin and affect POMC mRNA expression level in pituitary. [247, 248] Classically, catecholamines (dopamine, norepinephrine, and epinephrine) act as neurotransmitters; however, they are also involved in regulative processes in the skin, which has a full capacity to synthesize these agents (Figure 4). [249, 250] Vesicular monoamine transporters 1 and 2 (VMAT1, VMAT2) participate in the removal of the dopamine from the cells. Also, several enzymes degrading catecholamines, such as monoamine oxidase A and B (MAOA, MAOB) and catechol-*O*-methyltransferase (COMT), are present in the epidermis. [251, 252]

The dopamine pathway is potentially associated to melanoma – there are possible associations between L-DOPA and malignant melanoma; and psoriasis – immunoreactivity to PNMT increased ten times in lesional skin compared to unlesional skin; L-DOPA treatment relieves psoriasis in Parkinson's disease patients. [253–256] Furthermore, until now the associations between vitiligo pathogenesis and genes connected to the dopamine pathway have been proven to some extent; however, the relations have not been fully understood as yet.

Both phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH), which participate in catecholamine synthesis, use (6R)-L-erythro-5,6,7,8-tetrahydro-biopterin ($6BH_4$) as a cofactor. PAH and pterin-4a-carbinolamine dehydratase (PCD; essential for regenerating 6BH4 in the PAH system) activity has decreased in the skin and sera of vitiligo patients compared to controls, whereas the activity of TH has increased. [257, 258] Autoantibodies against TH have been found in patients, especially at an active stage of vitiligo. [259] Auto-antibodies against dopa decarboxylase (DDC) have been found in the sera of 80% of type I patients of the autoimmune polyendocrine syndrome, who also have vitiligo. [260] Also, the protein expression in patients' epidermis is significantly lower in association with decreased serotonin and melatonin levels. [261] The activity of $6BH_4$ dependent enzyme phenylethanolamine N-methyltransferase (PNMT) has decreased in keratinocytes obtained from the involved skin of vitiligo patients and also when measured from whole skin samples. [251]

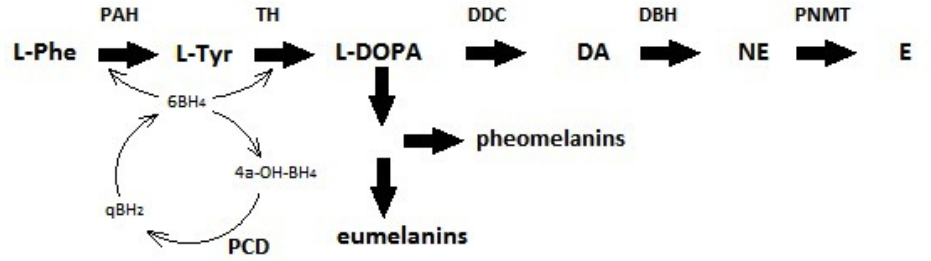

Figure 4. Schematic representation of the catecholamine biosynthesis cascade, showing major intermediates and enzymes involved in the process. L-Phe – L-Phenylalanine, L-Tyr – L-Tyrosine, L-DOPA – L-3,4-dihydroxyphenylalanine, DA – dopamine, NE – norepinephrine, E – epinephrine, $6BH_4$ – 6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin, 4a-OH-BH_4 – 4a-hydroxy- tetrahydrobiopterin, qBH_2 – quinonoid dihydrobiopterin, PAH – Phe hydroxylase, TH – Tyr hydroxylase, DDC – dopa decarboxylase, DBH – dopamine beta-hydroxylase, PNMT – phenylethanolamine N-methyltransferase, PCD – pterin-4a-carbinolamine dehydratase.

In vitiligo skin the elevated H_2O_2 level is the main cause of $6BH_4$ recycling errors. [262] The activity of enzymes important to metabolize H_2O_2 has impaired in patients' skin and also systemically – the latter has been proven in the case of CAT and glutation peroxidase (GPX1). [263–265] The elevated $6BH_4$ level stimulates the activity of TH, causing an increase of catecholamines in vitiligo skin and plasma. [39, 257] The higher level of catecholamines induces the activities of catecholamine degrading enzymes, which has been demonstrated in vitiligo skin in the case of MAOA and COMT. [251, 252]

Dopamine actions are mediated through five specific cell surface receptors coupled to G proteins and belonging to two main families (D1-like and D2-like receptors). [266] These receptors participate in the regulation of melanogenesis. The competitive interactions between αMSH and the receptor agonist on dopamine receptor D1 (DRD1) have been demonstrated. [267] DRD1B, DRD2, and DRD4 participate in the regulation of pigment synthesis and transport in photoreceptor cells. [268, 269] DRD2 agonist has been shown to inhibit hair follicular melanogenesis in mice, and DRD2 is present in a significant proportion of melanomas. [253, 270]

3. AIMS OF THE STUDY

The main purpose of the studies presented here was to receive more information about the nervous and immune system interactions in the skin of vitiligo patients. The aim of these studies was to evaluate the possible role of different pathways in vitiligo pathogenesis: the endogenous opioids pathway together with the CRH and MCH pathway, IL10 cytokine family and associated pathways, the dopamine pathway. Most likely all these pathways participate in the function of the cutaneous HPA axis. For these purposes the following goals were set:

- To evaluate the possible role of the CRH-POMC system and associated systems (MCH, endogenous opioids) in vitiligo pathogenesis by analysing the mRNA expression of the following genes in skin biopsies: *CRH*, *CRHR1, PMCH*, *MCHR1*, *ATRN*, *NURR1*, *PDYN, PNOC, OPRD1*, *OPRK1*, *OPRM1*, and *OPRL1*.
- To analyse various cytokines, their receptors and some other genes potentially related to the development, proliferation, and survival of melanocytes and the regulation of melanogenesis (*IL20RB*, *IL22RA2*, *IL26*, *IL28A*, *IL28B*, *IL29*, *IL28RA*, *MDM1*, *IFNA1*, *IFNB1*, *IFNG* and *ICAM1*) and to evaluate their possible role in vitiligo pathogenesis. For that purpose the mRNA levels in PBMCs and skin biopsies were observed.
- To analyse the mRNA and protein expression of dopamine pathway associated genes in blood sera and skin biopsies (*PAH, PCD, TH, DDC, DBH, PNMT, GPX1, MAOA, MAOB, COMT, DRD1-DRD5, VMAT1* and *VMAT2*) and to observe the possible connections to vitiligo pathogenesis.

4. MATERIALS AND METHODS

4.1. Ethical considerations

The protocols and informed consent forms used in all studies were approved by the Ethical Review Committee on Human Research of the University of Tartu. All the participants signed a written informed consent.

4.2. Characteristics of study participants

All the patients and control subjects of these studies were Caucasians living in Estonia. Unrelated patients with vitiligo from the Department of Dermatology at the University of Tartu were included. The diagnosis of vitiligo was based on the following clinical signs – loss of pigmentation with typical localization and white colour on the skin lesions under Wood's lamp. All the patients included in this study have vitiligo vulgaris and none of them had received specific therapy in the previous six months. Patients were classified as having active vitiligo if new areas of depigmentation were observed during the previous 3 months and as stable if no new depigmentation or enlargement of depigmentation had been observed for more than 3 months. The control group consisted of volunteers free from a positive family history of vitiligo and other chronic dermatoses. The control subjects were recruited from among health care personnel and medical students and patients present at the dermatological outpatient clinic with either facial teleangiectasis or skin tags. The main characteristics of the participants in all three studies are presented in Table 2–4.

Table 2. Main characteristics of the participants in the study "Analysis of the expression profile of CRH-POMC system genes in vitiligo skin biopsies"

	Total number of individuals	Stage of vitiligo	Sex female/ male	Average age (years)	Mean age of vitiligo onset (years)	Patients with family history*
Vitiligo patients**	18	13 active, 5 stable	14/4	51.4	27.4	3
Controls	14	–	12/2	44.9	–	–
Controls	14	–	12/2	44.9	–	–

* The family history of vitiligo is based on the information received from the patients.
** Thirteen patients in this study have other autoimmune diseases (autoimmune thyroid disease (n=5), psoriasis (n=3), rheumatoid arthritis (n=2), pernicious anemia (n=2), and alopecia areata (n=1))

Table 3. Main characteristics of the participants in the study "The mRNA expression profile of cytokines connected to the regulation of melanocyte functioning in vitiligo skin biopsy samples and PBMCs."

	SKIN			PBMC			
	Control	Vitiligo uninvolved	Vitiligo involved	Control	Control + L*	Vitiligo	Vitiligo + L*
Group size	15	15	15	17	17	15	15
Age range	19–79	19–77	19–77	23–69	23–69	22–69	22–69
Average onset age (years)	–	26.7	26.7	–	–	27.4	27.4
Male/Female ratio	4/11	5/10	5/10	6/11	6/11	5/10	5/10
Stage of vitiligo	–	9 active, 6 stable	9 active, 6 stable	–	–	12 active, 3 stable	12 active, 3 stable

*L – LPS stimulated PBMCs. Vitiligo is considered to be in an active stage if new lesions appeared or the present lesions expanded within three months and in a stable stage if no changes occurred within three months.

Table 4. Main characteristics of the participants in the study "The expression profile of genes associated to the dopamine pathway in vitiligo skin biopsies and blood sera."

	mRNA expression analysis						Protein expression analysis				
	Skin			Skin (*gDNA)			Skin			Blood sera	
	Control	Vitiligo uninvolved	Vitiligo involved	Control	Vitiligo uninvolved	Vitiligo involved	Control	Vitiligo uninvolved	Vitiligo involved	Control	Vitiligo
Group size	20	27	27	14	16	16	10	6	6	14	14
Age range	22–70	20–71	20–71	19–79	22–70	22–70	21–66	22–58	22–58	23–63	20–62
Average onset age (years)	–	28.9	28.9	–	27.1	27.1	–	30.7	30.7	–	21.9
Male/Female ratio	5/15	6/21	6/21	3/11	6/10	6/10	5/5	1/5	1/5	5/9	7/7
Stage of vitiligo	–	18 active, 9 stable	18 active, 9 stable		10 active, 7 stable	10 active, 7 stable	–	4 active, 2 stable	4 active, 2 stable	11 active, 3 stable	11 active, 3 stable

*Samples marked with gDNA were used in case of assays detecting genomic DNA (GPX1, DRD1, DRD5).

4.3. Analysis of mRNA expression

4.3.1. Sample collection and RNA extraction from skin biopsies and PBMCs

One skin biopsy (ø 4 mm) from non-sun-exposed skin was taken from control subjects. Two skin biopsies (ø 4 mm) were obtained from each patient with vitiligo – one from the involved skin and the other from non-sun-exposed uninvolved skin. All biopsies were instantly frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

For total RNA extraction from skin biopsies the RNeasy Fibrous Tissue Mini Kit (QIAGEN Sciences, Maryland, USA) was used according to the manufacturer's protocol. Biopsies were homogenized with T10 basic homogenizer (IKA Labortechnik, Germany). Extracted RNA was dissolved in RNase free water and stored at –80 °C until cDNA synthesis.

Blood was collected from subjects between 8:00 and 12:00 in the morning to limit the effect of circadian variation of cytokine production. BD Vacutainer CPT tubes (BD, New York, USA) were used to separate PBMCs from other blood cells. The cells were centrifuged at 1500 g for 30 minutes at 20 °C. After that the blood sera were collected from the top of the PBMCs. PBS (Phosphate Buffered Saline) was used to wash the isolated PBMCs twice, after which they were centrifuged at 190 g for 10 min at 20 °C. Half of the PBMCs were cultivated in the presence of LPS (L2630, Sigma, MO, USA) for 12 hours; and the other half was cultivated without LPS stimulation using RPMI-1640 medium (PAA Laboratories GmbH, Austria) with 10% Foetal Calf Serum (PAA Laboratories GmbH, Austria) and 1% Pen/Strep (PAA Laboratories GmbH, Austria). LPS was used to induce the expression of pro-inflammatory cytokines. [271]

For RNA extraction from the PBMCs, the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) was used according to the manufacturer's protocol. The RNA sediment was dissolved in RNase free water and incubated at 55 °C for 10 min. The RNA was stored at –80 °C until cDNA synthesis.

4.3.2. cDNA synthesis

For cDNA synthesis, 500 ng of total RNA and High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) was used. In the case of TaqMan® QRT-PCR assays also amplifying genomic DNA (for genes *IFNA1*, *IFNB1*, *IL28A*, *IL28B* and *GPX1*, *DRD1*, *DRD5*) QuantiTect Rev. Transcription Kit (QIAGEN Sciences, Maryland, USA) was applied. Both cDNA synthesis kits were used according to the manufacturer's protocol.

4.3.3. Quantitative real-time polymerase chain reaction analysis

cDNA was used as a template for TaqMan® QRT-PCR analysis in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Two primers and a labelled probe were used to detect the mRNA expression level of the reference gene hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*) (HPRT1 exon 6, 5'-GACTTTGCTTTCCTTGGTCAGG-3'; HPRT1 exon 7, 5'-AGTCTGGCTTATATCCAACACTTCG-3'; VIC-5'- TTTCA-CCAGCAAGCTTGCGACCTTGA-3'-TAMRA) in all studies. The assays and primers used for the studied genes in each study are as follows.

Analysis of the expression profile of CRH-POMC system genes in vitiligo skin biopsies: Two primers and a labelled probe were used for studying *OPRM1* (Fw, 5'- ATTGGTCTTCCTGTAATGTTCA-3'; Rv, 5'- CAGGTTG-GATGAGAGAATGTTAGTGT-3; FAM-5'-AGCTACAACAAAATACAGG-CAAGGTTCCATAGATTG-3'-MGB) [272]; *OPRD1* (Fw, 5'-GGGCAAC-GTGCTTGT-3'; Rv 5'-TCTGGAAAGGCAGCGTG-3'; FAM-5'-ACGGCC-ACCAACATCTACATCT-3'-MGB); and *CRH* (Fw, 5'-GCCTCCCATCTCC-CTGGAT-3'; Rv, 5'-TGTGAGCTTGCTGTGCTAACTG-3'; FAM-5'-TCC-TCCGGGAAGTCTTGGAAATGGC-3'-MGB) [273] and commercial assays for measuring *PDYN* (Hs00225770_m1), *PNOC* (Hs00173823_m1), *OPRK1* (Hs00175127_m1), *OPRL1* (Hs00173471_m1), *CRHR1* (Hs01062290_m1), *PMCH* (Hs00173595_m1), *MCHR1* (Hs00538798_m1), *ATRN* (Hs00390610_m1), *NURR1* (Hs01118813_m1) mRNA expression.

The mRNA expression profile of cytokines associated with the regulation of melanocyte function in vitiligo skin biopsy samples and peripheral blood mononuclear cells: Two primers and a labelled probe were used to detect the mRNA expression level of *IL28B* (Fw, 5'- AGAGGGCCAAA-GATGCCTTAG -3'; Rv, 5'- GGGAGCGGCACTTGCA -3; FAM-5'- AGA-GTCGCTTCTGCTG -3'-MGB). The expression levels of other genes under investigation were detected using the following commercial assays: *IL20RB* (Hs00376373_m1), *IL22RA2* (Hs00364814_m1), *IL26* (Hs00218189_m1), *IL28A* (Hs00820125_g1), *IL29* (Hs00601677_g1), *IL28RA* (Hs00417120_m1), *MDM1* (Hs00220015_m1), *IFNA1* (Hs00256882_s1), *IFNB1* (Hs01077958_s1), *IFNG* (Hs00989291_m1), *ICAM1* (Hs99999152_m1).

The expression profile of genes associated with the dopamine pathway in vitiligo skin biopsies and blood sera: the expression levels of genes under investigation were detected using the following commercial assays: *PAH* (Hs00609359_m1), *TH* (Hs00165941_m1), *PCD* (Hs00165396_m1), *DDC* (Hs00168031_m1), *DBH* (Hs01089840_m1), *PNMT* (Hs00160228_m1), *GPX1* (Hs00829989_gH), *MAOA* (Hs00165140_m1), *MAOB* (Hs01106246_m1), *COMT* (Hs00241349_m1), *DRD1* (Hs00377719_g1), *DRD2* (Hs00241436_m1), *DRD3* (Hs00364455_m1), *DRD4* (Hs00609526_m1), *DRD5* (Hs00361234_s1), *VMAT1* (Hs00915191_m1), *VMAT2* (Hs00161858_m1).

4.3.4. mRNA expression data analysis

All reactions were carried out in four replicates. Data are expressed as a mean ΔCT value relative to HPRT1 ± S.E.M. The results of different groups were compared pairwise and no adjustment for multiple testing was performed. Adjustment to normal distribution was tested by the Kolmogorov-Smirnov test. The data of the studied genes following normal distribution were parametrically tested with unpaired t test and the data not following the normal distribution with the Mann-Whitney test. During statistical analysis vitiligo patients were observed as a whole group or the active and stable form separately. For statistical analysis Microsoft Office 2008 Excel application for Mac (Microsoft Corporate, Redmond, WA, USA) and GraphPad Prism 4 and 5 software (GraphPad Software, San Diego, CA, USA) were applied.

4.4. Protein level measurements

4.4.1. Tissue homogenization for protein measurements

In order to measure protein levels in skin biopsies, the tissue pieces were homogenized in PBS buffer including 1 mM ethylenediaminetetraacetic acid and 1 mM phenylmethanesulfonylfluoride using Precellys 24 homogenizer with the Cryolys system (BERTIN TECHNOLOGIES, Montigny le Bretonneux, France).

4.4.2. ELISA

To detect the protein levels of DDC, MAOA, MAOB, DRD1 and DRD5 in blood sera and skin biopsie homogenates, commercial ELISA 96-well plate ready-to-use kits were applied (Cat. No HU9011, HU9062A, HU9062B, HU8296, HU8292, respectively; BIOTANG INC, Waltham, MA, USA).

4.4.3. Protein expression data analysis

Adjustment to normal distribution was tested by the Kolmogorov-Smirnov test. The data of all the studied proteins following normal distribution were parametrically tested by the unpaired t test and the data not following the normal distribution by the Mann-Whitney t test. During statistical analysis vitiligo patients were observed as a whole group or the active and stable form separately.

5. RESULTS

5.1. Analysis of the expression profile of CRH-POMC system genes in vitiligo skin biopsies

The present study focused on twelve genes (*CRH*, *CRHR1, PMCH*, *MCHR1*, *ATRN*, *NURR1*, *PDYN, PNOC, OPRD1*, *OPRK1*, *OPRM1* and *OPRL1*) associated with the CRH-POMC system. The mRNA expression levels were measured in punch biopsies of the involved and uninvolved skin of vitiligo patients and from the non-sun-exposed skin of healthy subjects.

5.1.1. mRNA expression in skin biopsies

PNOC and its receptor *OPRL1* expression levels were 2.99 and 1.83 times higher, respectively, in vitiligo involved skin when compared to control skin ($P < 0.05$) (Fig. 5, a and b). The expression of *OPRL1* was increased only in the

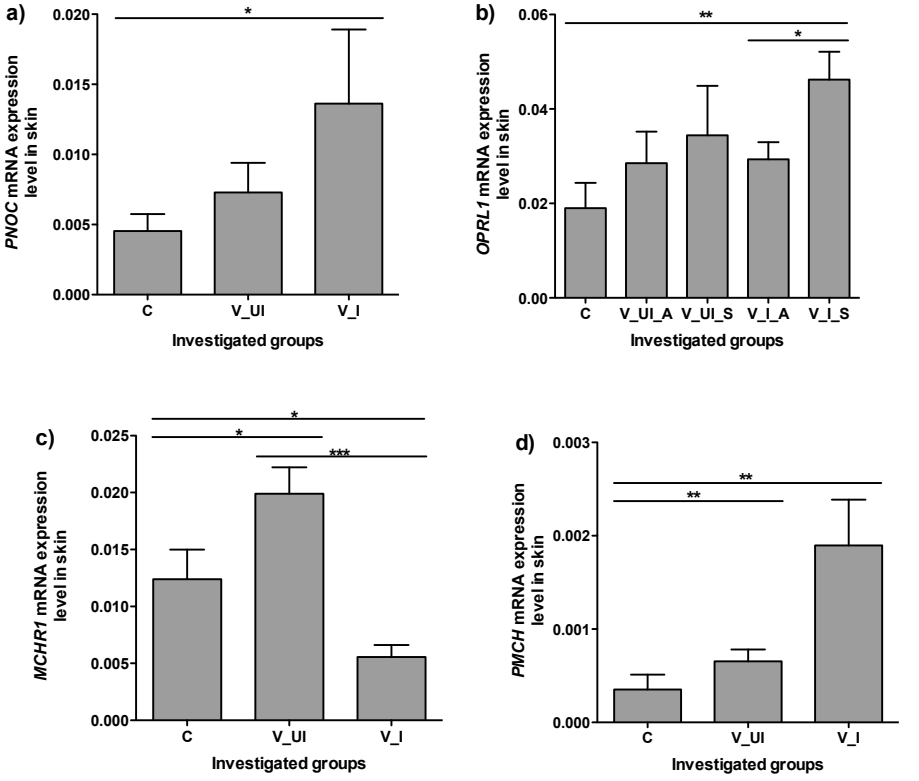

Figure 5. Expression levels of *PNOC* (a), *OPRL1* (b), *PMCH* (c), and *MCHR1* (d) in skin biopsies by comparison with groups of control subjects (C) and uninvolved (V_UI) and involved (V_I) skin of patients with vitiligo. Data are expressed as a mean ΔCT value relative to HPRT1 ± S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

case of stable form – 2.43 times ($P < 0.01$). The difference between the active and stable form is 1.58 times ($P < 0.05$). *PMCH* mRNA expression level was increased 1.85 times ($P < 0.01$) in vitiligo uninvolved skin and 5.36 times ($P < 0.01$) in involved skin compared to control skin (Fig. 5, c). The expression level of its receptor gene *MCHR1* was 1.6 times higher in vitiligo uninvolved skin ($P < 0.05$) and 2.23 times lower in involved skin ($P < 0.05$) than in control skin (Fig. 5, d). Comparing vitiligo involved and uninvolved skin, the expression level of *MCHR1* differed 3.57 times ($P < 0.001$) and was higher in uninvolved skin.

PDYN, OPRK1, CRHR1, ATRN, and *NURR1* expression levels did not differ in vitiligo or control skin samples. The expression levels of *OPRD1*, *OPRM1*, and *CRH* mRNA exceeded the detection limit in skin when 125 ng of cDNA was used in QRT-PCR reaction; these levels were not sufficient for an accurate statistical analysis.

5.2. The mRNA expression profile of cytokines related to the regulation of melanocyte functioning in vitiligo skin biopsy samples and peripheral blood mononuclear cells

This study focused on twelve genes (*IL20RB*, *IL22RA2*, *IL26*, *IL28A*, *IL28B*, *IL29*, *IL28RA*, *MDM1*, *IFNA1*, *IFNB1*, *IFNG*, and *ICAM1*) related to immunomodulation, melanogenesis, and development and growth of melanocytes. The mRNA expression levels were measured in punch biopsies of involved and uninvolved skin of vitiligo patients and non-sun-exposed skin of healthy subjects and also in PBMCs (with or without LPS stimulation) derived from patients and controls.

5.2.1. mRNA expression in skin biopsies and PBMCs

This study showed that the mRNA expression of *IL20RB* was in vitiligo uninvolved skin 1.4 times higher ($P < 0.01$) than in controls (Fig. 6, a). The expression was also increased in vitiligo involved skin; however, it is statistically significant only in the case of stable form (1.5 times ($P < 0.05$)). In blood the mRNA expression of *IL20RB* was increased in vitiligo patients' cells when compared to controls (32.3 times ($P < 0.001$) without LPS stimulation and 9.6 times ($P < 0.001$) with LPS stimulation) (Fig. 6, b). LPS stimulation decreased the *IL20RB* expression level in vitiligo patients' PBMCs 4.3 times ($P < 0.001$); this was not found in controls.

The mRNA expression level of *IL22RA2* was 1.5 times higher ($P < 0.05$) in vitiligo uninvolved skin compared to control skin (Fig. 6, c). The expression was 1.6 times lower ($P < 0.05$) in involved skin when compared to uninvolved

skin. This effect was observed only in the case of the active stage of vitiligo – the *IL22RA2* expression was 1.7 times ($P < 0.05$) higher in controls and 2.7 times ($P < 0.05$) higher in uninvolved skin than in involved skin. In PBMCs the mRNA level of *IL22RA2* was at the detection limit and proper statistical analysis was not possible (data not shown).

The mRNA expression level of *IL26* did not differ significantly in skin when comparing patients and controls (data not shown). In PBMCs the mRNA level of *IL26* was near the detection limit and accurate statistical analysis was not possible (data not shown).

The mRNA expressions of *IL28A*, *IL28B*, *IL29*, and *IL28RA* were detected in skin and in PBMCs; however, the levels were too low for accurate statistical analysis in the case of *IL28A*, *IL28B*, and *IL29* in skin and *IL29* in PBMCs (data not shown). The mRNA expression level of *IL28RA* was 1.7 times higher ($P < 0.01$) in vitiligo uninvolved skin compared to controls (Fig. 6, d). *IL28A* mRNA expression was 1.7 times ($P < 0.05$) lower in patients' PBMCs compared to controls when stimulated with LPS (Fig. 6, e). The mRNA expression levels of *IL28B* and *IL28RA* did not differ statistically relevantly in the PBMCs of vitiligo patients and controls (data not shown).

The expression level of *MDM1* mRNA was 4.2 times ($P < 0.001$) higher in uninvolved and 3.0 times ($P < 0.001$) higher in involved vitiligo skin when compared to controls (Fig. 7, a). In blood the expression of *MDM1* was higher in patients' PBMCs – 1.7 times ($P < 0.01$) without LPS stimulation and 1.5 times ($P < 0.01$) with LPS stimulation (Fig. 7, b).

In the skin the *IFNA1* mRNA expression level was too low for accurate statistical analysis (data not shown). In blood the expression level of *IFNA1* was 4.8 times ($P < 0.05$) higher in patients' PBMCs compared to control cells (Fig. 7, c). In the case of the stable form *IFNA1* expression was even 7.3 ($P < 0.05$) and 9.0 times ($P < 0.01$) higher without or with LPS stimulation, respectively. The increase was not statistically relevant with the active form.

The mRNA expression of *IFNB1* was 4.5 times ($P < 0.05$) higher in vitiligo uninvolved skin compared to control skin (Fig. 7, d) and 2.9 times ($P < 0.05$) higher in patients' PBMCs than in the cells of the controls (Fig. 7, e). In the case of the stable form, the expression in blood cells was 3.4 times ($P < 0.01$) higher without LPS stimulation and 3.0 times ($P < 0.05$) higher with LPS stimulation. The increase was not statistically relevant with the active form.

The mRNA expression of *IFNG* was 3.9 times higher ($P < 0.05$) in vitiligo involved skin than in control skin (Fig. 7, f). *IFNG* expression was higher in patients' PBMCs compared to controls without or with LPS stimulation (3.5 times ($P < 0.01$) and 3.3 times ($P < 0.01$), respectively) (Fig. 7, g).

ICAM1 mRNA expression was higher in both uninvolved and involved vitiligo skin (1.7 times ($P < 0.01$) and 1.4 times ($P < 0.05$), respectively) by comparison with control skin (Fig. 7, h). *ICAM1* mRNA expression at the stable stage had decreased in involved skin compared to uninvolved skin (1.7 times ($P < 0.01$)); this pattern did not occur at the active stage. The mRNA expression

of *ICAM1* was at the same level in the PBMCs of patients and controls (data not shown).

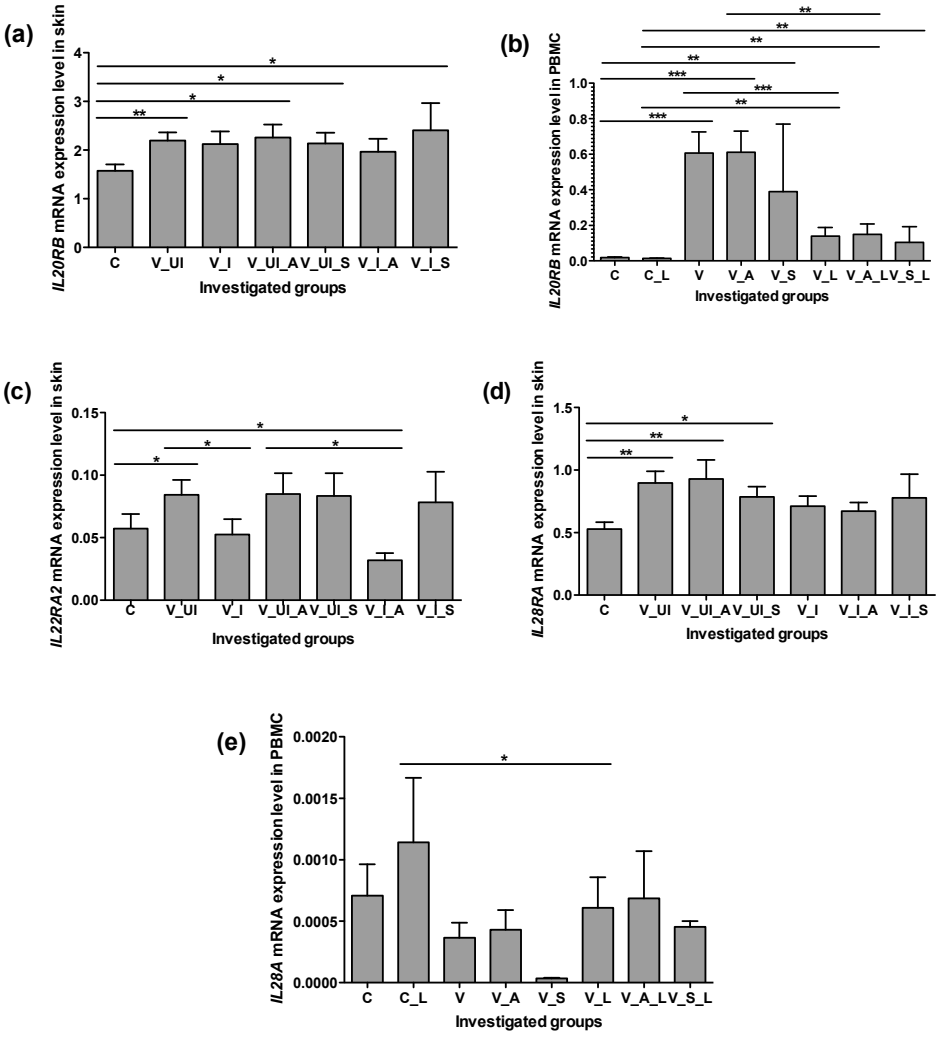

Figure 6. mRNA expression levels of genes associated with IL10 family cytokines in vitiligo patients' uninvolved (UI) and involved (I) skin and the skin of controls (C) and patients (V) and controls (C) PBMCs with (L) or without LPS stimulation. Additionally, patients were divided into subgroups with the active (A) and stable (S) forms of vitiligo. Data are expressed as a mean ΔCT value relative to HPRT1 ± S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

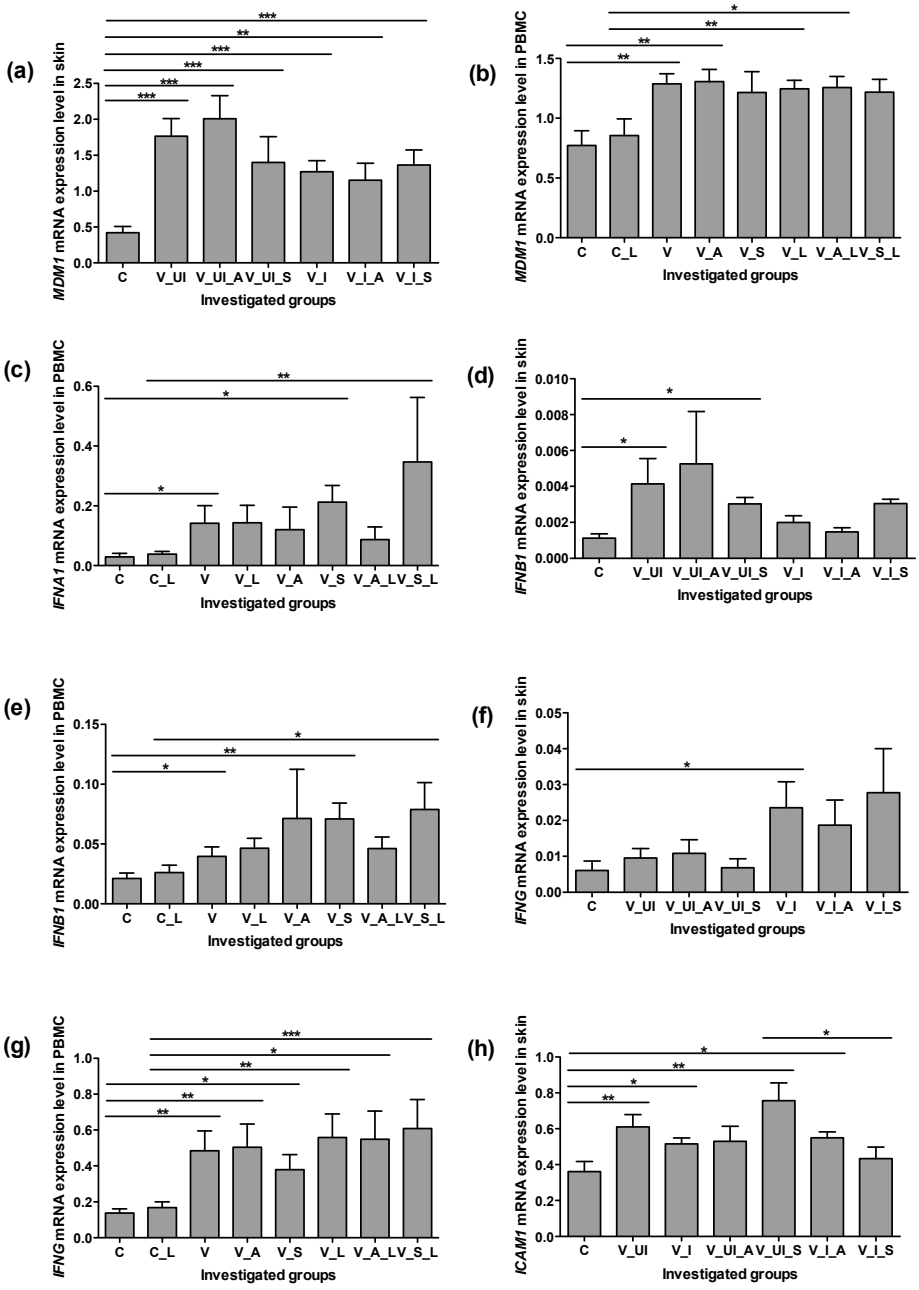

Figure 7. mRNA expression levels of genes possibly regulating the functioning of melanocytes in the uninvolved (UI) and involved (I) skin of vitiligo patients and the skin of controls (C) and in the PBMCs of patients (V) and controls (C) with (L) or without LPS stimulation. Additionally, the patients were divided into subgroups with the active (A) and stable (S) forms of vitiligo. Data are expressed as a mean ΔCT value relative to HPRT1 ± S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

5.3. The expression profile of genes associated with the dopamine pathway in vitiligo skin biopsies and blood sera

To study the possible associations between vitiligo and genes in dopamine pathway, the mRNA expression levels of seventeen genes (*PAH, PCD, TH, DDC, DBH, PNMT, GPX1, MAOA, MAOB, COMT, DRD1-DRD5, VMAT1*, and *VMAT2*) were measured in punch biopsies from the involved and uninvolved skin of vitiligo patients and from the non-sun-exposed skin of control subjects. Subsequently, the protein levels of five genes were measured in homogenates of skin biopsies and blood sera. DDC, MAOA, DRD1, and DRD5 were chosen while their mRNA expression level in the skin differentiated between patients and controls; also MAOB was included while the latter is sequentially and functionally similar to MAOA. The protein level of other genes was not analysed due to the limited amount of biopsy material available.

5.3.1. mRNA expression in skin biopsies

We were able to detect *PAH* and *TH* mRNA in skin biopsies; however, the levels were too low for accurate statistical analysis. The mRNA expression of *PCD*, *DBH*, and *PNMT* did not differ significantly between vitiligo and control skin. The mRNA expression of *DDC* was 5.2 times lower ($P < 0.05$) in uninvolved and 7.6 times lower ($P < 0.05$) in involved vitiligo skin by comparison with control skin (Fig 8. A).

mRNA expression level of *GPX1* has a tendency to increase in vitiligo skin compared to control skin. The difference was significant only in the case of involved skin of patients with stable vitiligo – 2.7 times higher ($P < 0.05$) compared to control skin. Furthermore, *GPX1* mRNA level increased 2.7 times ($P < 0.01$) in the involved skin of patients with stable vitiligo compared to patients with the active form (Fig 8. B). The expression level of *MAOA* mRNA was higher in the skin of vitiligo patients than in controls – in uninvolved skin the difference was significant only in the case of the stable form (1.8 times; $P < 0.05$); in involved skin the differences were 2.5 times ($P < 0.001$) and 2.2 times ($P < 0.05$) in patients with active and stable vitiligo, respectively (Fig 8. C). In the case of *MAOB* (Fig 8. D) and *COMT*, the mRNA expression levels did not differ between vitiligo skin and controls.

DRD1 and *DRD5* mRNA expression levels were higher in vitiligo skin compared to controls. In uninvolved skin the *DRD1* expression was elevated 4.2 times ($P < 0.001$) and *DRD5* 1.9 ($P < 0.01$) times (Fig 8. E and 8. F). In involved skin the difference from controls was significant only in the case of *DRD1* (3.2 times; $P < 0.05$). The expression level of *DRD2* did not differ when controls and vitiligo patients were compared. mRNA expression of *DRD3* and *DRD4* was detected in skin; however, the expression level was too low for

accurate statistical analysis. The mRNA expression levels of *VMAT1* and *VMAT2* were equivalent in the skin samples of controls and vitiligo patients.

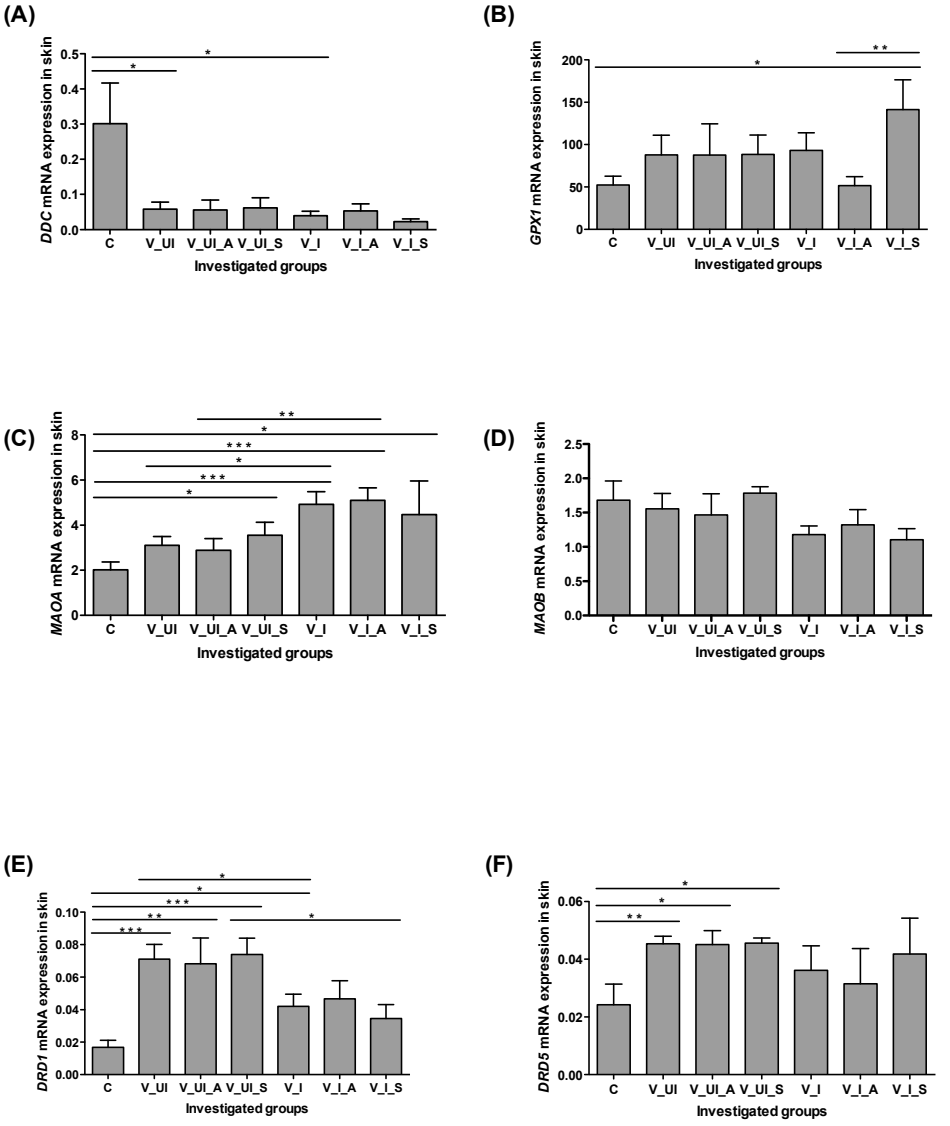

Figure 8. mRNA expression of the studied genes in the uninvolved (UI) and involved (I) skin of vitiligo patients and controls (C). Additionally, the groups of vitiligo patients with uninvolved skin and involved skin were divided into subgroups of patients with the active (A) and stable (S) forms of vitiligo. Data are expressed as a mean ΔCT value relative to HPRT1 ± S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

5.3.2. Protein expression level in skin biopsies

The protein levels of all five genes (*DDC, MAOA, MAOB, DRD1, DRD5*) were different in controls and involved and uninvolved skin of vitiligo patients.

The expression of DDC protein was higher in vitiligo skin than in controls – the difference was statistically significant in uninvolved and involved skin of patients with active vitiligo (2.7 times; $P < 0.05$ and 1.8 times; $P < 0.05$, respectively). It was reflected also in the whole group of involved skin – the difference amounted to 1.9 times ($P < 0.05$) (Fig 9. A).

The protein levels of MAOA and MAOB increased in vitiligo patients compared to controls. In patients with active vitiligo the MAOA protein level is 2.8 times ($P < 0.05$) and 1.8 times ($P < 0.05$) higher in uninvolved and involved skin, respectively (Fig 9. B). The effect can also be seen in whole groups: it is 2.4 times ($P < 0.05$) and 1.9 times ($P < 0.05$) higher in vitiligo uninvolved and involved skin, respectively. The MAOB level is 2.7 times ($P < 0.05$) higher in uninvolved and 2.0 times ($P < 0.05$) higher in the involved skin of patients with active vitiligo (Fig 9. C). When observing whole groups: MAOB protein expression is 2.4 times ($P < 0.05$) higher in uninvolved and 2.1 times ($P < 0.01$) higher in the involved skin of vitiligo patients compared to controls.

Both DRD1 and DRD5 protein expression levels are higher in vitiligo skin than in controls. The level of DRD1 protein is 2.6 times ($P < 0.05$) higher in uninvolved and 1.8 times ($P < 0.05$) higher in the involved skin of patients with active vitiligo (Fig 9. D). It is reflected also in the whole group of involved skin – the protein level is 1.8 times ($P < 0.05$) higher. The DRD5 protein level increased 3.0 times ($P < 0.01$) in uninvolved skin and 2.1 times ($P < 0.05$) in the involved skin of patients with active vitiligo (Fig 9. E). The effect can be seen also in whole groups – the protein level is 2.6 times ($P < 0.05$) higher in vitiligo uninvolved and 2.2 times ($P < 0.05$) higher in involved skin compared to controls.

5.3.3. Protein expression level in blood sera

The protein level of DDC, MAOA, MAOB, and DRD5 did not differ statistically relevantly in the sera obtained from the blood of controls and vitiligo patients. The expression of DRD1 protein decreased 1.4 times ($P < 0.01$) in patients' sera compared to controls (Fig 9. F).

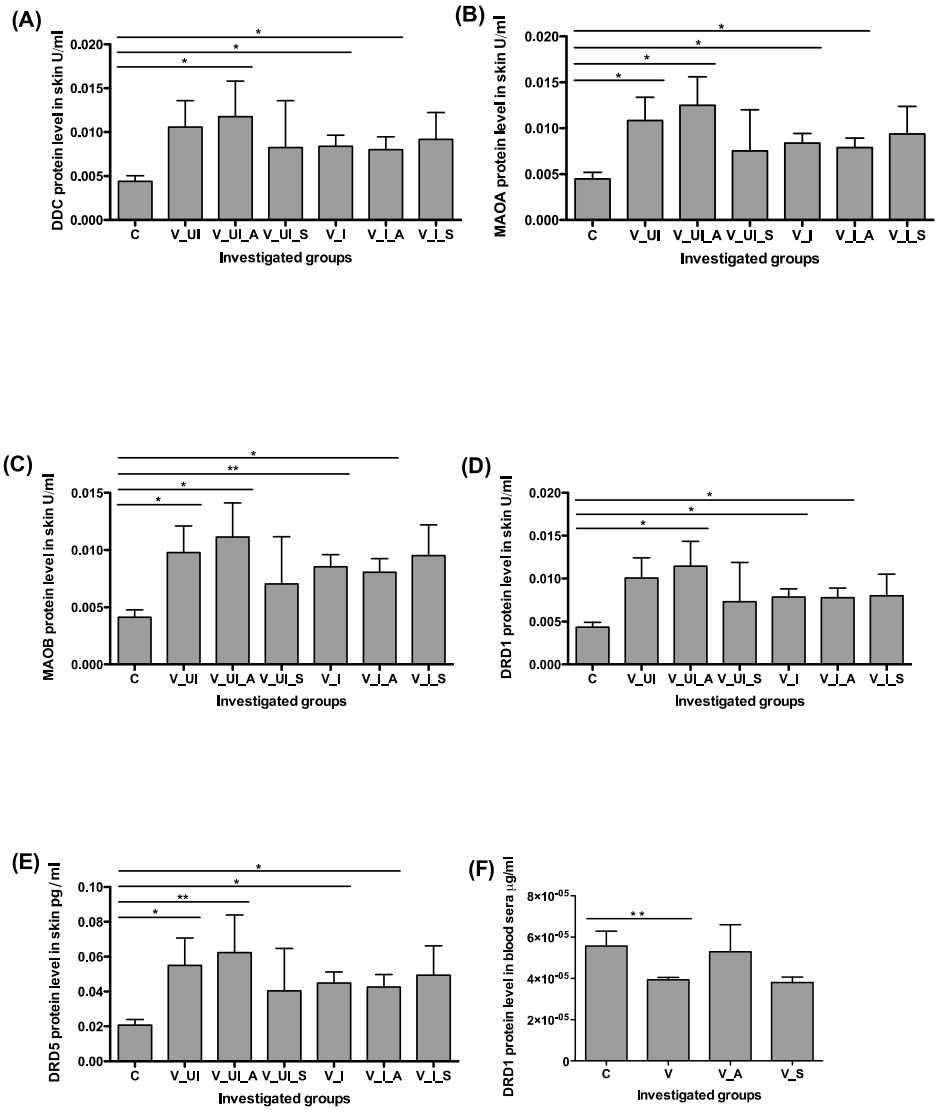

Figure 9. Protein expression of the studied genes in the uninvolved (UI) and involved (I) skin of vitiligo patients and controls (C), also in patients (V) and controls (C) blood sera. Additionally, different patient groups of skin and blood serum studies were divided into subgroups of patients with the active (A) and stable (S) forms of vitiligo. The columns show mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6. DISCUSSION

6.1. Analysis of the expression profile of CRH-POMC system genes in vitiligo skin biopsies

Vitiligo is a pigmentation disorder and the mechanisms leading to it are still not completely understood. The associations between vitiligo pathogenesis and the CRH-POMC system are becoming more and more evident. [160, 172] The present study focused on possible interactions between vitiligo and neuropeptides and their receptors related to the CRH-POMC system. For that purpose, twelve different genes were studied – *CRH, CRHR1, PMCH, MCHR1, ATRN, NURR1, PDYN*, *PNOC*, *OPRM1*, *OPRD1*, *OPRK1*, and *OPRL1* (Table 5).

Table 5. Expression level of the POMC-CRH-MCH pathway and other endogenous opioid associated genes in the skin. Data combined from our previous and present studies. [138] The mRNA levels of the studied genes in vitiligo patients compared to controls. ⇑ – expression in vitiligo patients has increased; ⇓ – expression in vitiligo patients has decreased; ⇔ – expression is not significantly different between patients and controls; — – expression was not observed; ND – expression was not detected; NDL – expression was near detection limit, statistical analysis not performed. * $P < 0.05$; ** $P < 0.01$; – $P > 0.05$.

	mRNA in skin			
	Uninvolved skin		Involved skin	
Gene name	active	stable	active	stable
POMC	⇔		⇓*	
MC1R	⇑**		⇓–	
MC2R	⇑–		⇓–	
MC3R	⇑–		⇓–	
MC4R	⇑**		⇓–	
MC5R	⇑–		⇓–	
ASIP	⇔		⇔	
AGRP	⇔		⇔	
CRH	NDL		NDL	
CRHR1	⇔		⇔	
PMCH	⇑**		⇑**	
MCHR1	⇑*		⇓*	
PDYN	⇔		⇔	
PNOC	⇔		⇑*	
OPRM1	NDL		NDL	
OPRD1	NDL		NDL	
ORRK1	⇔		⇔	
OPRL1	⇔		⇔	⇑**

The present study shows that in involved vitiligo skin the expression of PNOC mRNA has increased significantly compared to control skin. Furthermore, the level of its receptor OPRL1 mRNA expression increased relevantly in the involved skin of patients with stable vitiligo compared to control samples, which indicates the activation of this opioid-related system in vitiligo skin. The PNOC system has not been studied properly and possible associations between the PNOC system and vitiligo pathogenesis have not been observed previously. A study was published, where Staphylococcal enterotoxin A, which activates T-cells, was injected into male mice and it caused an increase in the PNOC mRNA expression level in the brain. [274] According to another study, nociceptin acting through OPRL1 receptors inhibits components of neurogenic inflammation in rat skin. [275] It has also been reported that nociceptin may in different conditions either stimulate or inhibit the proliferation and activation of T cells. [276] Hence, this system could participate in inhibiting the progression of lesions; PNOC and OPRL1 could have a substantial immunomodulatory role in skin as well in the nervous system and may be associated with the inflammation common to vitiligo.

The present study indicates that the mRNA expression level of *MCH* is detectable in skin although previous studies using reverse transcription-PCR detected MCH expression in the endothelial cells of cultured skin, but not in the entire skin or cultured melanocytes, keratinocytes, or fibroblasts. [168] One possibility is that mRNA of PMCH is derived from macrophages. [277] By comparison with control skin, the mRNA expression of PMCH was increased both in uninvolved and involved vitiligo skin. The considerable accumulation of MCH found in vitiligo involved skin contributes to the previous finding that MCH inhibits the melanogenic action of αMSH. [172]

Measuring the level of MCHR1 in different groups indicated that the expression is upregulated in uninvolved vitiligo skin and down-regulated in involved skin when compared to control skin. In the literature there is no information about the expression level of MCHR1 in vitiligo skin compared to control skin. However, as noted, autoantibodies against MCHR1 have been found in the sera of vitiligo patients. [171] Melanocytes have been found to be the only skin cells expressing MCHR1 [168], and vitiligo involved skin has fewer or no melanocytes, which explains the lower MCHR1 expression level. The increase of MCHR1 expression in uninvolved vitiligo skin is probably connected to the antagonizing mechanism of the POMC system and could be a part of the initiating process of the reduction of melanogenesis and destruction of melanocytes. However, further studies are needed to confirm how exactly MCH with its receptors and the CRH-POMC system are associated in skin.

6.2. THE mRNA expression profile of cytokines associated with the regulation of melanocyte functioning in vitiligo skin biopsy samples and peripheral blood mononuclear cells

Several cytokines have been suggested to participate in vitiligo pathogenesis. The present study assessed the importance of cytokines and their receptors and a few other genes connected to IL10 family cytokines and possibly involved in the regulation of the development and survival of melanocytes and melanogenesis. [181–186] See Table 6 for a better overview of our previous and present studies about IL10 family cytokines and their receptors in the skin and blood samples of vitiligo patients.

In addition to our previous studies, the present study revealed supplemental changes in the expression of cytokines and receptor genes associated with the IL10 cytokine family. The *IL20RB* mRNA level was higher in the skin samples of vitiligo patients. Previous work indicated that the *IL20RA* expression level decreased and the *IL22RA1* mRNA level remained the same in vitiligo skin compared to control skin. Ligands for these receptor complexes – IL19, IL20 and IL24 were not detected in the skin when QRT-PCR was used. [195] Here the increased IL20RB level may occur due to a compensating mechanism, which tries to retain the normal amount of the IL20RA-IL20RB complex; however, further studies are needed to see how the mechanism works in the skin.

As in the skin, the *IL20RB* mRNA level was also higher in patients' PBMCs compared to control cells. We have demonstrated earlier that the mRNA expression level of *IL20RA* and *IL22RA1* are near detection limit in PBMCs when using QRT-PCR. The IL19 mRNA expression increased; the IL20 mRNA and protein levels have a tendency to decrease, and IL24 mRNA expression remains the same in vitiligo patients' blood compared to controls. [195] The increased expression level of IL20RB in PBMCs may be somehow associated with the higher levels in the skin, while the regulation mechanism could be similar locally and systemically.

IL22RA2 (antagonizing IL22 receptor complex) mRNA expression level decreased in involved vitiligo skin comparing to uninvolved vitiligo skin and control skin – these changes appear only in the case of active vitiligo. We have showed earlier that *IL22* expression increased significantly in patients' PBMCs (these changes occur only at the active stage of vitiligo), and also the protein level is higher in patients' blood sera comparing to controls. [195] IL22 receptor complex genes *IL22RA1* and *IL10RB* expression levels are at the same level in the skin of patients and controls. [195] Thus, it is possible that a certain level of IL22RA2 is essential for binding the excess of IL22; and in the case of the active stage of vitiligo the lack of IL22RA2 may possibly lead to inflammation and thereby destruction of melanocytes.

Table 6. Expression level of IL10 cytokine family associated genes. Data combined from our previous and present studies. [37] The mRNA and protein levels of the studied genes in vitiligo patients compared to controls. ⇑ – expression in vitiligo patients has increased; ⇓ – expression in vitiligo patients has decreased; ⇔ – expression is not significantly different between patients and controls; — – expression was not observed; ND – expression was not detected; NDL – expression was near detection limit, statistical analysis not performed. * $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$; – $P > 0.05$.

	mRNA in skin				mRNA in PBMCs				Protein in blood sera	
	Uninvolved skin		Involved skin		Vitiligo		Vitiligo + LPS		Vitiligo blood sera	
Gene name	Active	Stable	Active	Stable	Active	Stable	Active	Stable	Active	Stable
IL10	ND				⇑–				⇓–	
IL19 isoform 1	ND				ND				—	
IL19 isoform 2	ND				⇑*	⇔	⇑*	⇔	—	
IL20	ND				⇓–				⇓–	
IL22	ND				⇑**	⇔	⇑–	⇔	⇑***	
IL24	ND				⇔		⇓*		—	
IL26	⇔				NDL				—	
IL28A	NDL				⇓–		⇓*		—	
IL28B	NDL				⇔				—	
IL29	NDL				NDL				—	
IL10RA	⇑–				⇑***				—	
IL10RB	⇔				⇑***		⇔		—	
IL20RA	⇓**				ND				—	
IL20RB	⇑**		⇔	⇑*	⇑***				—	
IL22RA1 (IL22RA)	⇔				ND				—	
IL22RA2 (IL22RB)	⇑*		⇓*	⇑–	NDL				—	
IL28RA	⇑**		⇑–		⇔				—	

It is known that IL20 and IL22 have similar functions – preventing terminal differentiation of keratinocytes and upregulating antimicrobial peptides to protect epithelial surface from invading pathogens. [278, 279] IL22 has been shown to induce the expression of IL20 and they also share one receptor subunit – IL22RA1, which is expressed similarly in the skin of controls and vitiligo patients. [189, 195, 279] According to previous findings, the IL20 and

IL22 are both upregulated in the skin of psoriasis patients and the blood levels correlate with the significance of the disease. [279–281] In the case of vitiligo the expression pattern of IL20 and IL22 is dissimilar – IL20 expression has a tendency to decrease and IL22 expression increased in patients' PBMCs and no expression is detected in the skin. [195] The receptors expression pattern does not match either. [195, 282] This indicates that in the case of various autoimmune diseases associated with skin, the IL20 and IL22 may act differently, the regulation methods may be dissimilar, and thus, the effect on skin cells varies.

The mRNA expression level of *IL28A* decreased in vitiligo patients' PBMCs and *IL28B* expression has a tendency to increase in patients' PBMCs (statistically insignificant) when comparing to controls. *IL28A*, *IL28B*, and *IL29* mRNA levels are near detection limit in the skin. *IL28RA* expression is higher in patients' uninvolved skin compared to controls and has a tendency to decrease in involved skin. Our previous studies showed that the mRNA expression of *IL10RB*, which together with IL28RA constructs a receptor complex for IL28A, IL28B, and IL29, increased significantly in vitiligo patients' PBMCs compared to controls; however, the level remained uniform in the skin. [195] It has been shown that both epidermal melanocytes and keratinocytes are major targets for IL28A, IL28B, and IL29. [283] Maturing dendritic cells and regulatory T cells may produce these cytokines; growth inhibition has been demonstrated in the case of keratinocytes. [62] The results of our study indicate the possibility that IL28A, IL28B, and IL29 could participate in vitiligo pathogenesis and could possibly influence also the proliferation of melanocytes. These cytokines and their receptor subunits should be studied further to achieve more confident implications.

A major finding in the case of IL10 family cytokines and their receptors is the controversial effects of LPS stimulation. In previous and present studies we have observed LPS stimulation in the case of PBMCs derived from patients and controls. [195] The LPS affects differently the mRNA expression levels of *IL20* (a tendency to increase in controls, a similar level in patients), *IL22* (an increase in controls, a tendency to decrease in patients), *IL24* (a tendency to increase in controls, a tendency to decrease in patients; a significant decrease of IL24 mRNA expression appears only in the case of LPS stimulation), *IL10RB* (the level remains the same in controls, an increased level in patients decreases to the control level) and *IL20RB* (a similar level in controls, a decreased level in patients). LPS is recognized by toll-like receptor 4 (TLR4), which further induces the production of proinflammatory cytokines to create the innate immune response. [284] Our findings may imply that in the case of vilitigo pathogenesis, in addition to the adaptive immune system, the innate immune response is somehow involved. Another finding is that the altered innate immune response may lead to the activation of autoimmunity, which was proven in the case of vitiligo. [285]

The present work demonstrated that *MDM1* mRNA expression is higher in vitiligo patients' skin and PBMCs when compared to controls. *MDM1* (located near *IL22* and *IL26* on chromosome 12) may have a common expression regulation mechanism with *IL22*, while both are upregulated in patients' PBMCs. [195] The function of MDM1 is still unknown; its connections to oncogenic processes, transcriptional machinery, and iron management have been suggested. [224, 225, 286, 287] Our work demonstrates that MDM1 is possibly related to vitiligo pathogenesis and needs further analysis. Additional support to its role in melanocyte survival comes from a recent study where MDM1 mutant mice exhibited pigmentary abnormalities and had an increased number of phagosomes in retinal pigment epithelium. [288]

The expression profiles of IFNA and IFNB were also changed – *IFNA1* and *IFNB1* mRNA expression were higher in vitiligo patients' PBMCs (especially in the case of stable form) compared to controls; *IFNB1* expression increased significantly in vitiligo uninvolved skin. Previously it has been demonstrated that IFNA and IFNB subcutaneous treatment could cause vitiligo. [230, 234, 235] The mechanism could move through both the innate and adaptive immune-system pathways while IFNA and IFNB as important agents against viral infection are also able to affect T and B cells. [230, 231, 233] Our findings suggest that vitiligo could be triggered also by the inner changes (both local and systemic) of the expression patterns of these interferons. The fact that the mRNA levels are even higher in the stable stage of vitiligo implies the possibility that IFNA and IFNB may participate in maintaining the depigmented lesions.

Our study confirmed that the *IFNG* mRNA expression level increased significantly in patients' involved skin and also in PBMCs compared to controls. *ICAM1* mRNA expression increased in both uninvolved and involved vitiligo skin compared to controls. Furthermore, in patients with stable vitiligo the ICAM1 expression decreased in involved skin compared to uninvolved skin, which did not occur in the case of patients with active vitiligo. These results are supported by previous findings – in vitiligo skin, the amount of cytotoxic T cells increased, which is probably due to an increased level of leukocytes recruiting ICAM1 and its expression stimulating agent IFNG. [244, 245, 289] Thus, ICAM1 is probably an important link between cytokines and T cells involved in vitiligo pathogenesis. The reason why the *ICAM1* mRNA level has a tendency to decrease in involved skin compared to uninvolved skin of stable vitiligo could be in the loss of melanocytes in depigmented areas. On the other hand, the epidermal cells express more ICAM1 than they should at the active stage of vitiligo, which leads to already proven T cell activation in vitiligo skin. [290] However, further research is needed.

6.3. The expression profile of genes associated with the dopamine pathway in vitiligo skin biopsies and blood sera

Melanogenesis can be initiated from either L-Phe or L-Tyr, which are hydroxylated to L-DOPA. The latter serves as a precursor for melanins and catecholamines, which both are synthesized in skin via separate pathways. These pathways share several enzymes, cofactors and are competitive on certain levels. [250] It has been shown that the levels of dopamine, norepinephrine, epinephrine, and their metabolites have increased in vitiligo patients' plasma and urine. [34, 39, 40] The increase in the catecholamine level even suggests that it might be an initiating event of vitiligo pathogenesis. [291] Thus, only dopamine has been proven to have an apoptotic effect on melanocytes, most probably while increasing the reactive oxygen species level in cells. [292, 293]

Our study revealed that the expression profile of several genes connected to the dopamine pathway has altered in vitiligo skin (Table 7). In the case of enzymes in the catecholamine synthesis pathway the DDC (essential to produce dopamine from L-DOPA) mRNA level decreased in patients' skin, which correlates with the findings that the protein level in epidermis is significantly lower, and the serotonin and melatonin levels have decreased. [261] Though, the findings of our study contradict the previous studies of mRNA expression and protein – the DDC protein level in vitiligo skin increased. However, the latter supports the fact that the level of dopamine increased in patients. [34, 39, 40] DDC is important for both dopamine and serotonin production, and these controversial results need additional studies; the present data do not enable us to make any further conclusions.

The studies of the activity of H_2O_2 degrading enzyme GPX1 in vitiligo patients have yielded controversial results – the activity has been higher, lower, or similar comparing to the blood and skin of controls. [265, 294–300] Our study found that the mRNA expression of GPX1 has a tendency to increase in patients' uninvolved skin compared to controls. On the other hand, we showed that the GPX1 mRNA level was at the same level in controls and involved skin of patients with active vitiligo; however, the level is significantly higher in the involved skin of patients with the stable form. These results correlate with the previous results, where among active vitiligo cases only 4% of the studied individuals had an increased GPX1 activity; however, in stable cases 20% had a higher GPX1 activity. [295] The increased transcription of *GPX1* may somehow be involved in inhibiting the progression of the disease; however, the differences between the active and stable forms needs further analysis.

Table 7. The mRNA and protein levels of the studied genes in vitiligo patients compared to controls. ⇑ – expression in vitiligo patients has increased; ⇓ – expression in vitiligo patients has decreased; ⇔ – expression is not significantly different between patients and controls; — – expression was not observed. * P < 0.05; ** P < 0.01; ***P < 0.001.

	mRNA in skin				Protein in skin				Protein in blood sera	
	Uninvolved skin		Involved skin		Uninvolved skin		Involved skin		Vitiligo blood sera	
Gene name	active	stable	active	stable	active	stable	active	stable	active	stable
DDC	⇓*		⇓*		⇑*	⇔	⇑*	⇔	⇔	
GPX1	⇔		⇔	⇑*	—				—	
MAOA	⇔	⇑*	⇑***		⇑*	⇔	⇑*	⇔	⇔	
MAOB	⇔		⇔		⇑*	⇔	⇑*	⇔	⇔	
DRD1	⇑***		⇑*		⇑*	⇔	⇑*	⇔	⇓**	
DRD5	⇑**		⇔		⇑**	⇔	⇑*	⇔	⇔	

The activity of catecholamine degrading enzymes COMT and MAOA has previously been demonstrated to be higher in vitiligo skin. [251, 252] We found that the mRNA expression of COMT was similar in the skin of vitiligo patients and controls, which may indicate the possibility that the increased activity is due to the changes in the protein level. In the case of MAOA and MAOB we found that the mRNA level of MAOA was higher in patients' skin compared to controls; the MAOB mRNA expression level did not differ significantly between the groups. Both MAOA and MAOB protein levels were higher in patients' uninvolved and involved skin. Our results support the previous findings that the activity of MAOA increased in patients' skin compared to controls. [251] While MAOA and MAOB equally metabolize dopamine, one might suggest that MAOB activity also increases in vitiligo skin, which needs to be confirmed. The fact that in vitiligo uninvolved skin the protein levels of MAOA and MAOB increased at the active stage compared to the stable form, may imply an attempt to reduce the dopamine level in the skin while new patches are developing on the skin.

Dopamine has five receptors (DRD1-DRD5) of which DRD1, DRD2, and DRD4 have been demonstrated to participate in the regulation of melanogenesis. [268–270] DRD1 and DRD5 both belong to the D1- like family; they activate adenylyl cyclase, which increases the intracellular cAMP level. The cAMP/PKA pathway is the main pathway through which melanogenesis enzymes can be reached, and it is used by melanocortins to modulate melanogenesis. [5, 301, 302] In addition to activating the cAMP/PKA pathway, both

dopamine receptors and melanocortin receptors have a similar expression pattern in vitiligo skin – our present and previous studies showed that the levels of *MC1R* and *MC4R* mRNA and dopamine D1-like family receptors' mRNA and protein increased in uninvolved skin and again decreased in the involved skin of vitiligo patients. [138] The mRNA expression of POMC decreased and the level of dopamine increased in involved vitiligo skin. [35, 303] Furthermore, previous studies demonstrated that αMSH binds to DRD1 and inhibits the attachment of the ligand – in the presence of both αMSH and dopamine the cAMP level decreased compared to the presence of these two mediators separately (indicated in the brain). [267] There is also data that αMSH might increase the dopamine level in nucleus accumbens through MC4R. [246] We did not find any data in the literature about dopamine binding or affecting the melanocortin receptors directly. We have suggested previously that the upregulation of the melanocortin system in vitiligo uninvolved skin is relevant to compensation of the loss of pigment in involved skin. [35] Nevertheless, the major challenge is to find out how exactly melanocortin and dopamine pathways are connected to each other while regulating melanogenesis in the skin and participating in vitiligo pathogenesis.

When observing from a different perspetive, it has been demonstrated that the level of dopamine and its metabolites increased in vitiligo patients' skin, urine, and peripheral blood. [34, 39, 264] In the case of DRD1 it has been shown that dopamine stimulates the DRD1 promoter and thereby mRNA expression. [304] Furthermore, dopamine acts on DRD1, which causes the negative feedback and a decrease in dopamine release (demonstrated in the brain). [305] In vitiligo uninvolved skin the elevation of D1-like receptors may also be caused by the increased level of dopamine in patients. Furthermore, the decrease of D1-like receptor expression in involved skin may be caused by the negative feedback pathway. However, these suggestions need further studies and more supportive evidence.

Our study showed that the DRD1 protein level decreased in patients' blood sera compared to controls. When dopamine activates D1-like receptors on regulatory T cells, they are less able to suppress the proliferation of effector T cells. Activated effector T cells increase the synthesis of cytokines and adhesion molecules and affect different target cells. [306] In vitiligo patients the decrease of DRD1 protein in blood sera may point to a possible compensating mechanism – an attempt to decrease the elevated level of activated effector T cells in the organism. Nevertheless, additional studies are required.

6.4. Local HPA axis in vitiligo skin

The main adaptive responses to systemic stress are mediated by the HPA axis. [9] Activation of this pathway proceeds through the hypothalamic production of CRH, which stimulates CRHR1 in pituitary. Anterior pituitary-derived POMC peptides activate the MC2R receptors in the adrenal gland and cortisol is released. The HPA axis is suppressed through negative feedback via cortisol receptors in hypothalamus and pituitary. [9–12, 104]

Skin expresses the neuroendocrine system using mediators similar to those involved in the classical endocrine systems at the brain and pituitary levels (CRH, URC, and POMC derived peptides); furthermore, skin cells express functional receptors activated by different neuropeptides. [1, 104, 307–309] Local effectors of this axis would regulate the pigmentary, immune, epidermal, dermal, and adnexal systems of the skin. Accordingly, physical stress (UV light) or biological or chemical stress would trigger multiple pathways involving structuralized or simultaneous local production of CRH, CRH-related peptides, and POMC derived messages. [86, 104] This complex response would be susceptible to heterotrophic modulation through feedback inhibition by cortisol. Thus, by analogy with the central axis, stress would stimulate the production and secretion of ACTH of cutaneous origin, which would further increase the level of glucocorticoids – recognized inhibitors of the immune system. [96]

In the case of vitiligo the mechanisms, which should ensure the homeostasis in the skin, have altered. In our studies, we observed different agents participating in cutaneous HPA axis. When moving along the signal cascade of the classical HPA axis the first agent is CRH, which through CRHR1 participates in cell proliferation, apoptosis, differentiation, and immune activities in the skin. [101, 103, 108, 310, 311] Our studies did not reveal any differences in *CRH* or its receptor *CRHR1* mRNA expression in vitiligo skin compared to control skin. CRH, which is produced in skin cells, is also suggested to be released from peripheral nerve endings. [312] The factors that have been demonstrated to affect the expression of *CRH* and *CRHR1* in the skin are UV radiation and an increased intracellular cAMP level. [162, 313, 314] Thus, in vitiligo patients, it is possible that the CRH system may have remained normal, and the effects are due to the further agents of this cascade.

Activated CRHR1 should stimulate the expression of POMC and the production of αMSH, ACTH, and β-endorphin, which are essential regulators of melanogenesis and survival of the melanocytes. [162–166] In vitiligo involved skin, the *POMC* mRNA expression level is significantly lower compared to uninvolved skin, and the mRNA expression level of *MC1R* and *MC4R* has decreased in involved skin comparing to uninvolved skin. However, the expression is evidently higher in uninvolved skin than in the skin of controls. [138] Studies from other groups showed that the αMSH level had decreased in patients' skin and sera. [315] The level of ACTH increased in vitiligo patients'

skin and plasma. [316, 317] The levels of β-endorphin in patients' plasma and tissue fluids from skin lesions increased, which could be an attempt to maintain the melanogenesis and number of melanocytes. [318, 319] Also, the met-enkephalin levels increased in patients' plasma. [160] The increased level of *POMC*-derived peptides could be explained by a systemic increase while locally the *POMC* expression decreased.

The decreased αMSH levels may be one of the reasons for higher inflammatory signals (IL1, IL6, IL8, IL22, and TNFA) and activation of adhesion molecules (ICAM1), as well as decreased IL10 levels in vitiligo patients. [36–38, 41] The causes of changed POMC and its derivates and also receptor levels may be different. In vitiligo skin the increased H_2O_2 level leads to the oxidation of epidermal ACTH, αMSH, and β-endorphin. In the case of β-endorphin it is known to lose its ability to induce melanogenesis. [320] The MC1R has been found to be expressed only in melanocytes, which explains the decrease in lesional skin. [37, 321] B-endorphin possibly stimulates the proliferation of melanocytes through OPRM1 [146, 147]. However, we were unable to evaluate the possible changes in *OPRM1* mRNA expression levels in the skin. Additionally, the increased expression of MCH and its receptor MCHR1 seem to inhibit the further signal pathway of αMSH in patients' skin. [171] Thus, the expression pattern and activity of genes associated with the POMC system have severely changed in the case of vitiligo pathogenesis, which may lead to the destruction of melanocytes.

In addition to the melanocortin system, the system of another endogenous opioid PNOC and its receptor OPRL1, have a potential role in maintaining the normal functioning of the inflammatory mechanisms in the skin. [275, 276] In vitiligo patients' skin, the activation of the PNOC system may not be sufficient to inhibit the increased inflammatory signals. [36–38] Thus, it may be a part of a cutaneous HPA axis regulation mechanism.

The next agents in the HPA axis are glycocorticoids, which are produced by melanocytes, fibroblast, and hair follicle keratinocytes. [107, 109] Glucocorticoid receptors are ligand-dependent transcription factors, which are highly expressed in keratinocytes, fibroblasts, and Langerhans cells and less expressed in melanocytes. [322] In vitiligo patients the cortisol levels in serum have not changed by comparison with controls. [160] So far there is no information about GR expression in vitiligo patients. However, glucocorticoids (corticosteroids) are used as immunosuppressive and anti-inflammatory agents in vitiligo therapy and repigmentation occurs. Combined with UV therapy, it induces the apoptosis of skin-infiltrating T cells and reduces the melanocyte associated antigen level. [323–326] Thus, according to the available data, one may suggest the importance of the glucocorticoid system in maintaining the melanocytes and protecting them from potential autoimmunity, but nothing further.

Cytokines have an important role in regulating the HPA axis. In the case of vitiligo, the expression pattern of pro- and anti-inflammatory cytokines has

altered. [36–38, 41] For example, the pro-inflammatory cytokine IL6, which increases the ICAM1 expression in melanocytes promoting melanocyte-leukocyte interaction and thus the apoptosis of melanocytes [182]; TNFA, which activates apoptotic pathways potentially destroying melanocytes; and IL1, which stimulates the proliferation of $CD4^+$ and $CD8^+$ T cells and B cells. [36] IL1, IL6, and TNFA also stimulate each other's expression. [22, 36, 182] The expression level of these three cytokines has increased in vitiligo patients. [22, 36, 182] All these cytokines stimulate the HPA axis; however, it is known that excessive activation of the classical HPA axis leads to immunosupression. [13, 18, 22, 182]

Among IL10 family cytokines, the expression of the pro-inflammatory cytokines IL19, which potentially stimulate apoptosis through IL6 and TNFA, and IL22, which initiates the innate immune system, has increased in patients' blood. [195, 327, 328] There is no information about any associations between these two cytokines and HPA axis regulation; thus, they may potentially stimulate the HPA axis through IL6, TNFA, and other mechanisms. The expression of the anti-inflammatory cytokine IL10 decreased in patients. [41] IL10, which inhibits the synthesis of pro-inflammatory cytokines, such as TNFA, is an important regulator of the HPA axis. [329] In the classical HPA axis, IL10 stimulates the production of CRH and ACTH, which may possibly be similar locally in the skin. [330]

Interferons are also important in regulating the HPA axis. The level of IFNG, which is essential for activating both the adaptive and the innate immune systems, has increased in vitiligo patients' skin. [242] It has been shown that it can inhibit and activate the HPA axis, depending on the concentration – higher levels stimulate the HPA axis. [331, 332] The pattern is similar in the case of IFNA and IFNB – the expression has increased in vitiligo patients, and they both activate ACTH and cortisol synthesis in the classical HPA axis. [333–335] Thus, assuming that in the classical and cutaneous HPA axis, the effects of different cytokines are similar, one might suggest that the IL-10 family cytokines, interferons, and other pro-inflammatory cytokines all support the elevated activation of the cutaneous HPA axis in the case of vitiligo pathogenesis.

Dopamine is known to prevent excessive HPA axis activation during stress (in the rat brain). [14] In vitiligo patients' skin, plasma, and urine, the dopamine level is elevated, possibly promoting the destruction of melanocytes. [34, 39, 40, 264, 292, 293] We also demonstrated that several genes associated with the dopamine pathway, are expressed differently in vitiligo patients' skin. Thus, if the dopamine effect on the cutaneous HPA axis is similar to the classical dopamine system, contrary to cytokines, it seems to inhibit the cutaneous HPA axis.

In vitiligo patients, the functioning of the cutaneous HPA axis (possibly the classical HPA axis, too) is somehow altered. Although the CRH system may have remained normal, the POMC system, which is essential for retaining melanogenesis active and melanocytes alive [162–166] is severely shifted – this pathway seems to be inhibited. According to the available data, the glyco-

corticoids system appears to be functioning tolerably. The cytokines seem to activate the HPA axis in vitiligo patients and the dopamine seems to inhibit it. Thus, in vitiligo patients' skin, the different mediators of the local HPA axis appear to have taken the system out of its ordinary course. The most important displacement, the melanocortin system, may potentially be the heart of this digression, and the activating and inhibiting agents may attempt to restore the normal state.

7. CONCLUSION

In vitiligo patients, the function of the cutaneous HPA axis has deviated from its normal state. The main inducer appears to be the changes in the melanocortin system, and the activating (cytokines) and inhibiting (dopamine) agents may attempt to restore the normal state of skin homeostasis.

- When evaluating the possible role of the CRH-POMC system and the associated systems (MCH, endogenous opioids) in vitiligo pathogenesis, we found the following results:
  - This study identified new genes, which may possibly be related to vitiligo pathogenesis – PNOC and its receptor OPRL1 are both upregulated in involved vitiligo skin compared to controls. Until now, there is no information about the POMC and PNOC derived peptides sharing their receptors with each other. The CRH-POMC system seems to have a major role in regulating different skin functions; it is highly likely that PNOC is also connected to this pathogenic network.
  - We were able to detect *PMCH* mRNA in whole skin biopsies, which has not been demonstrated before. We confirmed the previous results about the possible involvement of the MCH system in vitiligo pathogenesis – increased mRNA levels of *PMCH* in vitiligo skin and its receptor *MCHR1* in uninvolved vitiligo skin may be associated with the POMC system antagonizing mechanism and lead to decreased melanogenesis and destruction of melanocytes.
  - Until now the possible associations between vitiligo and CRH and its receptors CRHR1 and CRHR2 have not been investigated. The present study did not establish any, direct associations between the CRH system and vitiligo pathogenesis.
- When analysing different cytokines, their receptors, and a few other genes possibly related to the development, proliferation, and survival of melanocytes and regulation of melanogenesis, we demonstrated the following:
  - The present study found additional support that IL10 family cytokines and their receptors are important in vitiligo pathogenesis – there were significant changes in mRNA expression levels of *IL20RB*, *IL22RA2*, *IL28A*, and *IL28RA* in vitiligo patients' skin and/or PBMCs compared to controls.
  - This study supported our previous findings about the possible involvement of IL22 in vitiligo – the decreased level of IL22RA2 in the active stage vitiligo skin is unable to bind enough IL22, which potentially leads to increased inflammation.
  - We found additional information that LPS stimulation has different effects on the expression of IL10 family cytokines in the PBMCs of

patients compared to controls. This could indicate changes in the innate immunity system in the case of vitiligo pathogenesis.
  - We found that MDM1 may be associated with vitiligo pathogenesis, which has not been implied before. The possible involvement of IFNA and IFNB in the induction and maintenance of vitiligo lesions was supported by the present study. The study supplied additional information to previous work by other research teams about the involvement of IFNG together with ICAM1 in vitiligo pathogenesis.
- When evaluating the possible role of genes related to the dopamine pathway and vitiligo pathogenesis, we found additional support that the dopamine pathway may have an essential role in vitiligo pathogenesis:
  - In addition to influencing skin processes through the melanocortin pathway, the dopamine pathway potentially acts directly as well. In addition to enzyme activity data, we now have evidence of mRNA and protein expression changes in vitiligo patients' skin in the case of MAOA, MAOB, and DDC.
  - We demonstrated the changes in the expression pattern of D1-like family dopamine receptors in vitiligo patients' skin and blood.

8. FUTURE PROSPECTS

Our present studies are mostly based on on measuring the mRNA expression level of different genes in PBMCs and the skin. However, the mRNA profile does not necessarily reflect the protein levels. We need to conduct also protein studies for the IL10 family cytokines and their receptors and associated genes; also, the genes of the endogenous opioid system should be included.

In the case of the endogenous opioids pathway, we need to observe the PENK gene and protein expression in the future; recently it has been shown to be associated with vitiligo pathogenesis.

In dopamine pathway studies, we measured also the protein level of some genes in the skin and in blood sera. However, in the case of enzymes, the activity is also an essential indicator to consider. Thus, this could also be one of our future goals – to observe the activity of enzymes participating in the CRH-POMC pathway, other endogenous opioids pathways, the dopamine pathway, and the associated pathways of IL10 family cytokines.

We also would like to carry out some immunohistochemical studies with skin and blood cells from vitiligo patients and controls. It could provide additional information – in which cells exactly our study objects are expressed in different conditions.

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10. SUMMARY IN ESTONIAN

Tsütokiinide ja neuroendokriinse süsteemi osalemine vitiliigo patogeneesis

Üldteoreetiline taust

Nahk on keha suurim organ, mis koosneb kolmest kihist: epidermisest, dermisest ja hüpodermisest. Nahk on barjääriks keskkonna ja organismi sisemuse vahel ning selle peamiseks funktsiooniks on säilitada kehas tasakaal erinevate sisemiste ja väliste faktorite mõjuväljas. Kuna nahas asuvad ka immuunrakud ja närvirakud, siis lisaks füüsilisele barjäärile kasutatakse tasakaalu säilitamiseks sekretoorset aktiivsust, naha immuunsüsteemi ja pigmendi tootmist, samuti vaskulaarseid ja mesenhümaalseid komponente dermisest. Neuroendokriinsetest telgedest üks olulisemaid süsteemse stressi vastuse andjatest on hüpotaalamuse-hüpofüüsi-neerupealise telg (HPA telg), mis klassikaliselt koosneb CRH-POMC-glükokortikoidide signaalirajast. Kuna nahk on võimeline tootma kõiki vajalikke mediaatoreid ja vastavaid retseptoreid, siis võib öelda, et eksisteerib lokaalne HPA telg. HPA telje funktsioneerimist ja aktiivsust reguleerivad erinevad tsütokiinid ja neuromediaatorid, nagu IL1, IL6, TNFA, dopamiin, norepinefriin, serotoniin.

Vitiliigo on idiopaatiline haigus, kus seni veel täpselt teadmata põhjustel hävinevad melanotsüüdid ja selle tagajärjel tekivad nahale pigmenditud laigud. Peamised teooriad on autoimmuunne, neuraalne ja biokeemiline hüpotees. Arvatakse ka, et vitiliigo on teatud ulatuses pärilik. Vitiliigo võib esineda sõltumata soost, vanusest, rassist ning sageli koos teiste autoimmuunhaigustega. Patsiendid kannatavad psühholoogilise stressi all ja neil on suurenenud risk saada nahavähk.

Siiani on näidatud, et paljud geenid, mis osalevad lokaalse HPA telje töös, on vitiliigohaigetel teisiti ekspresseerunud, võrreldes kontrollidega. Meie töögrupp on varem näidanud POMC süsteemi geenide ekspressiooni langemist vitiliigohaigete nahas (nii POMC kui ka retseptorgeenid), samuti IL10 tsütokiinide perekonna geenide ekspressiooni muutusi (eelpõletikulise IL22, eelapoptootilise IL24 ja retseptorite alaühikute IL10RA ja IL10RB ekspressioon kasvanud). Lisaks on kirjandusest teada teiste põletikuliste tsütokiinide, nagu IL1, IL6, TNFA ning samuti dopamiini taseme tõus vitiliigohaigete nahas ja veres.

Uurimustöö põhieesmärgid

Peamine eesmärk oli saada lisainformatsiooni närvisüsteemi ja immuunsüteemi interaktsioonidest vitiliigohaigete nahas. Tahtsime hinnata endogeensete opioidide, CRH, MCH, IL10 perekonna tsütokiinide, dopamiini raja ning nendega seotud radade võimalikku osalust vitiliigo patogeneesis. Kuna tõenäoliselt kõik need rajad osalevad naha HPA telje tegevuses, siis tahtsime hinnata muutusi

HPA telje töös vitiliigohaigetes, võrreldes kontrollidega. Selleks seadsime järgmised eesmärgid:

- CRH-POMC süsteemi ja nendega seotud radade (MCH ja endogeensete opioidide süsteemi) analüüsimine vitiliigohaigete ja kontrollide nahas. Selleks analüüsiti 12 geeni mRNA ekspressiooni.
- IL10 perekonna tsütokiinide ja teiste melanogeneesi ning melanotsüütide kasvu ja arenguga seotud geenide analüüs vitiliigohaigete nahas ja perifeerse vere mononukleaarsetes rakkudes (PBMC). Selleks mõõdeti 12 geeni mRNA ekspressiooni.
- Dopamiini rajaga seotud geenide analüüsimine vitiliigohaigete veres ja nahas. Selleks uuriti 17 geeni mRNA ja valgu ekspressiooni nahas ja vereseerumis.

Metoodika

Kõigi uurimustöös osalenud vitiliigo patsientide haigusvormiks oli *vitiligo vulgaris* ning kuus kuud enne vere ja nahaproovide kogumist ei saanud patsiendid ravi. Kontrollgrupis olevate indiviidide perekonnas pole teadaolevalt esinenud vitiliigot või teisi kroonilisi dermatoose. CRH-PMC süsteemi uuringus osales 18 vitiliigo patsienti ja 14 kontrollindiviidi. Melanogeneesi ja melanotsüütide funktsioneerimisega seotud tsütokiinide (IL10 perekonna tsütokiinid ja seotud geenid) uurimustöös osales naha uuringus 15 patsienti ja 15 kontrollindiviidi ning vere uuringus 15 patsienti ja 17 kontrollindiviidi. Dopamiini raja töös osales naha mRNA uuringus 27 patsienti ja 20 kontrollindiviidi, naha valgu uuringus 6 patsienti ja 10 kontrollindiviidi ning vere valgu uuringus 14 patsienti ja 14 kontrollindiviidi.

Naha biopsiatest ja PBMCdest mRNA ekspressiooni mõõtmiseks eraldati vastavatest proovidest totaalne RNA, millest sünteesiti cDNA, mida omakorda analüüsiti TaqMan- QRT-PCR meetodiga. Uuritavate geenide ekspressiooni normaliseerimisel kasutati koduhoidja geeni *HPRT1*. Kõiki proove analüüsiti neljas korduses ja omavahel võrreldi vitiliigohaigete kahjustatud ja kahjustamata nahka ning kontrollindiviidide nahka, lisaks vitiliigohaigete ja kontrollide PBMCdest mõõdetud mRNA ekspressiooni tasemeid.

Valgu ekspressiooni mõõtmiseks naha biopsia homogenaatidest ja vere seerumist kasutati kommertsiaalseid ELISA analüüsikomplekte (uuriti DDC, MAOA, MAOB, DRD1 ja DRD5 valgu taset). Omavahel võrreldi vitiliigohaigete kahjustatud ja kahjustamata nahka ning kontrollindiviidide nahka ning vitiliigohaigete ja kontrollide vereseerumeid.

Tulemused

CRH-POMC süsteemi geenide ekspressiooni profiil vitiliigohaigete nahabiopsiates

PNOC ja selle retseptori *OPRL1* mRNA ekspressioon oli vitiliigohaigete kahjustatud nahas vastavalt 2,99 ja 1,83 korda kõrgem kui kontrollide nahas

(mõlemal juhul P < 0,05). *OPRL1* puhul oli ekspressioon kasvanud vaid stabiilse vitiliigo vormiga patsientidel – erinevus 2,43 korda (P < 0,01). *PMCH* ekspressioon oli kahjustamata nahas kasvanud 1,85 (P < 0,05) ja kahjustatud nahas 5,36 korda (P < 0,01), võrreldes kontrollide nahaga. *MCHR1* tase oli vitiliigohaigete kahjustamata nahas 3,57 korda kõrgem kui kahjustatud nahas. *PDYN, OPRK1, CRHR1, ATRN* ja *NURR1* ekspressiooni tasemed ei erinenud patsientidel ja kontrollidel. *OPRD1*, *OPRM1* ja *CRH* mRNA tasemed olid liiga madalad usaldusväärse analüüsi teostamiseks.

Melanotsüütide funktsiooni reguleerivate tsütokiinide mRNA ekspressiooni profiil vitiligohaigete nahabiopsiates ja perifeerse vere mononukleaarsetes rakkudes

IL20RB mRNA ekspressioon oli vitiliigohaigete kahjustamata nahas kasvanud 1,4 korda (P < 0,01), võrreldes kontrollidega. Stabiilse vitiliigoga patsientide kahjustatud nahas oli *IL20RB* tase kasvanud 1,5 korda (P < 0,05). *IL20RB* tase oli vitiliigohaigete veres kasvanud 32,3 korda (P < 0,001) ja koos LPS stimulatsiooniga 9,6 korda (P < 0,001). LPS stimulatsioon langetas *IL20RB* taset patsientide veres 4,3 korda (P < 0,001); seda ei esinenud kontrollide puhul. *IL22RA2* mRNA ekspressioon oli patsientide kahjustamata nahas 1,5 korda (P < 0,05) kõrgem kui kontrollide nahas. Kahjustatud nahas oli tase langenud 1,6 korda (P < 0,05), võrreldes kahjustamata nahaga – see efekt esines vaid aktiivse vitiliigoga patsientidel. PBMCdes oli *IL22RA2* tase detekteerimise piiril ja usaldusväärset statistilist analüüsi ei saanud teostada. *IL26* ekspressioon ei erinenud patsientide ja kontrollide nahas ning PBMCdes oli tase liiga madal usaldusväärse analüüsi läbiviimiseks. *IL28A*, *IL28B* ja *IL29* nahas ning *IL29* tase PBMCdes oli liiga madal usaldusväärseks analüüsimiseks. *IL28RA* tase oli vitiliigohaigete kahjustamata nahas kasvanud 1,7 korda (P < 0,01), võrreldes kontrollidega. *IL28A* mRNA tase oli LPS stimulatsiooniga patsientide PBMCdes 1,7 korda madalam (P < 0,05) kui kontrollides. *IL28B* ja *IL28RA* tasemed ei erinenud patsientide ja kontrollide PBMCdes. *MDM1* mRNA ekspressiooni tase oli 4,2 korda kõrgem (P < 0,001) patsientide kahjustamata nahas ja 3,0 korda kõrgem (P < 0,001) kahjustatud nahas, võrreldes kontrollidega. Veres oli *MDM1* tase patsientide rakkudes LPS stimulatsioonita 1,7 korda kõrgem (P < 0,01) ja LPS stimulatsiooniga 1,5 korda kõrgem (P < 0,01). *IFNA1* mRNA ekspressiooni tase nahas oli liiga madal usaldusväärse analüüsi teostamiseks. Veres oli *IFNA1* tase 4,8 korda kõrgem (P < 0,05) patsientide rakkudes, võrreldes kontrollidega. Stabiilse vormiga haigetel oli *IFNA1* tase LPS stimulatsioonita 7,3 korda kõrgem (P < 0,05) ja LPS stimulatsiooniga 9,0 korda (P < 0,01) kõrgem – sellist erinevust ei esinenud aktiivse vormiga haigetel. *IFNB1* ekspressioon oli vitiliigohaigete kahjustamata nahas 4,5 korda kõrgem (P < 0,05) ja PBMCdes 2,9 korda kõrgem (P < 0,05), võrreldes kontrollidega. Stabiilse vormiga haigete PBMCdes oli LPS stimulatsioonite mRNA ekspressiooni taseme kasv 3,4 kordne (P < 0,01) ja LPS stimulat-

siooniga 3,0 kordne ($P < 0,05$) – sellist erinevust ei esinenud aktiivse vormiga patsientide puhul. *IFNG* mRNA tase oli patsientide kahjustatud nahas 3,9 korda kõrgem ($P < 0,05$), võrreldes kontrollide nahaga. LPS stimulatsioonita oli *IFNG* tase kasvanud patsientide PBMCdes 3,5 korda ($P < 0,01$) ja LPS stimulatsiooniga 3,3 korda ($P < 0,01$), võrreldes kontrollidega. *ICAM1* tase oli patsientide kahjustamata nahas 1,7 korda ($P < 0,01$) ja kahjustatud nahas 1,4 korda ($P < 0,05$) kõrgem kui kontrollide nahas. *ICAM1* tase oli stabiilse vitiliigoga patsientide kahjustatud nahas langenud 1,7 korda ($P < 0,01$), võrreldes kahjustamata nahaga – seda ei esinenud aktiivse vormi puhul. PBMCdes *ICAM1* tase patsientides ja kontrollides ei erinenud.

Dopamiini rajaga seotud geenide ekspressiooni profiil vitiliigohaigete nahabiopsiates ja vereseerumis

PAH ja *TH* mRNA tase oli nahas liiga madal usaldusväärse analüüsi teostamiseks. *PCD*, *DBH* ja *PMNT* mRNA ekspressioon ei erinenud patsientide ja kontrollide nahas. *DDC* mRNA tase oli patsientide kahjustamata nahas 5,2 korda ($P < 0,05$) ja kahjustatud nahas 7,6 korda ($P < 0,05$) madalam kui kontrollide nahas. *GPX1* tase oli stabiilse vitiliigoga patsientide kahjustatud nahas 2,7 korda kõrgem ($P < 0,05$) kui kontrollide nahas ning stabiilse vormiga haigetel 2,7 korda kõrgem ($P < 0,05$) kui aktiivse vormiga haigete kahjustatud nahas. *MAOA* mRNA tase oli stabiilse vitiliigoga haigete kahjustamata nahas 1,8 korda kõrgem ($P < 0,05$) kui kontrollide nahas. Kahjustatud nahas oli erinevus kontrollidega aktiivse vitiliigoga patsientidel 2,5 kordne (P 0,001) ja stabiilse vormiga haigetel 2,2 kordne ($P < 0,05$). *MAOB* ja *COMT* ekspressiooni tasemed ei erinenud patsientide ja kontrollide nahas. *DRD1* ja *DRD5* mRNA ekspressiooni tase oli vitiliigohaigete kahjustamata nahas kasvanud vastavalt 4,2 ($P < 0,001$) ja 1,9 ($P < 0,01$) korda. Kahjustatud nahas oli *DRD1* mRNA tase kasvanud 3,2 korda ($P < 0,05$). *DRD2* tase ei erinenud patsientidel ja kontrollidel. *DRD3* ja *DRD4* ekspressiooni tase oli detekteerimise piiril ja usaldusväärset analüüsi ei saanud teostada. *VMAT1* ja *VMAT2* tasemed ei erinenud patsientidel ja kontrollidel.

DDC valgu tase oli aktiivse vormiga haigetel kasvanud kahjustamata nahas 2,7 korda ($P < 0,05$) ja kahjustatud nahas 1,8 korda ($P < 0,05$). MAOA valgu tase oli aktiivse vormiga patsientide kahjustamata nahas 2,8 korda ($P < 0,05$) ja kahjustatud nahas 1,8 korda ($P < 0,05$) kõrgem kui kontrollide nahas. MAOB valgu tase oli aktiivse vormiga patsientide kahjustamata nahas 2,7 korda ($P < 0,05$) ja kahjustatud nahas 2,0 korda ($P < 0,05$) kõrgem kui kontrollidel. DRD1 valgu tase oli aktiivse vormiga haigete kahjustamata nahas 2,6 korda kõrgem ($P < 0,05$) ja kahjustatud nahas 1,8 korda kõrgem ($P < 0,05$) kui kontrollide nahas. DRD5 valgu tase oli kasvanud aktiivse vitiliigoga patsientidel 3,0 korda ($P < 0,01$) kahjustamata nahas ja 2,1 korda ($P < 0,05$) kahjustatud nahas, võrreldes kontrollidega.

Vereseerumis oli DDC, MAOA, MAOB ja DRD5 valgu tase patsientidel ja kontrollidel sarnane. DRD1 valgu tase oli patsientide seerumis 1,4 korda ($P < 0,01$) langenud, võrreldes kontrollidega.

Järeldused

Lokaalse HPA telje funktsioneerimine on vitiliigohaigete nahas oma normaalsest olekust kõrvale kaldunud. Peamiseks põhjuseks näivad olevat muutused melanokortiini rajas. HPA telje tööd aktiveerivate tsütokiinide ja inhibeeriva dopamiini süsteemi muutused võivad tuleneda katsest taastada normaalne olukord ja tasakaal.

- Käesoleva uurimustööga leidsime, et *PNOC* ja *OPRL1* geenide ekspressiooni taseme kasv vitiliigohaigete kahjustatud nahas võib viidata nende osalusele vitiligo patogeneesis.
- Suutsime esmakordselt detekteerida naha biopsiates *PMCH* mRNA ekspressiooni. Kinnitasime varasemaid tulemusi MCH osalemisest vitiligo patogeneesis – MCH ja MCHR1 taseme kasv võib olla seotud POMC süsteemi inhibeerimisega, mis viib melanogeneesi vähenemiseni ja melanotsüütide hävinemiseni.
- Oma tööga ei suutnud me leida otseseid seoseid CRH raja geenide ja vitiligo patogeneesiga.
- Käesolev töö tõestas veelkord, et IL10 perekonna tsütokiinid on olulised vitiliigo patogeneesis – näitasime *IL20RB*, *IL22RA2*, *IL28A* ja *IL28RA* ekspressiooni muutusi vitiliigohaigete nahas ja/või veres.
- Saime lisakinnitust IL22 olulisele rollile vitiliigo patogeneesis – tõenäoliselt ei suuda IL22RA2 vähenenud kogus siduda piisaval hulgal IL22, et vältida põletikulisi protsesse.
- Tööga näitasime veelkord, et LPS stimulatsioon mõjub IL10 perekonna tsütokiinidele vitiliigohaigetes ja kontrollides erinevalt, mis võib viidata vitiliigohaigete kaasasündinud immuunsuses muutustele.
- Näitasime esmakordselt *MDM1* geeni võimalikku seotust vitiliigo patogeneesiga. Lisaks toetasime varasemaid uuringuid IFNA ja IFNB osalusest vitiliigo tekkes ja püsimises, samuti IFNG ja ICAM1 osalusest vitiliigo patogeneesis.
- Käesolev töö andis lisakinnitust dopamiini raja võimalikule osalusele vitiliigo patogeneesis – võimalik, et lisaks tasakaalu mõjutamisele läbi melanokortiini raja võib dopamiin olukorda nahas ka otseselt muuta. Lisaks varasematele dopamiini raja ensüümide aktiivuse informatsioonile on meil nüüd olemas ka mRNA ja valgu ekspressiooni andmed. Lisaks näitasime D1-tüüpi dopamiini retseptorite perekonna geenide võimalikku osalust vitiliigo patogeneesis.

11. AKNOWLEDGEMENTS

This study was financially supported by the target-based funding from the Estonian Ministry of Education grants SF0180043s07 and SF0180148s08, by University of Tartu research grant PARFS 07915, by Estonian Science Foundation grants No. 6576, 7549 and 7479, by the European Social Fund grant No. 1.2.0401.09-0073 to the University of Tartu (Graduate School of Biomedicine and Biotechnology), by the European Union through the European Regional Development Fund and by the Archimedes Foundation.

My sincere gratitude to everyone who contributed to this work directly or indirectly:

- Professor **Sulev Kõks**, for supervising and encouraging to study different subjects in an outside this work, for organizing the fundings for the research, for the opportunity to visit great conferences around the world, for being an excellent motivator and example, how people can do a lot of great things.
- Professor **Külli Kingo** for supervising and giving the opportunity to participate in such an interesting research, for being able to correct the difficult writings I manage to generate, also for organizing all the samples and everything needed for the study.
- Professor **Eero Vasar** for collecting such a great team into the Department of Physiology.
- My corse-fellow **Paula Reemann**, with whom we had long days and sometimes evenings and nights in the lab to get things working. Even, if it was sometimes complicated, at least we could keep our heads up and do things with laughter.
- Gratitude to my corse-fellow from the bachelor times, **Kristi Raud**, who gave me an idea to join the science group at the Department of Physiology and with whom we started this research subject; who has been a great friend in- and outside the science.
- Special thanks to **Mario Plaas**, the head of the Transgenic Technology Core Laboratory for teaching me how to work with cell cultures and offering a lab space and technology for doing all that. And thanks for being a great friend.
- Major gratitude to our lab assistant **Annika Häling**, who is always ready to help and smiling and was very supportive during writing this dissertation.
- Labmates, corse-fellows, colleagues from and around Department of Physiology at the University of Tartu: **Ranno**, **Silva**, **Ele**, **Kersti**, **Mari-Anne**, **Jürgen**, **Alar**, **Ursel**, **Kati**, **Sirli**, **Anton**, **Ruth**, **Ülle**, **Tanel T**., **Tanel V**., **Triin**, **Indrek**, **Maarja**, **Urho**, **Este**, **Indrek**, **Kaido**, **Kertu**, **Riin**, **Sten**, **Marilin**, **Marite**, **Julia**, **Karin, Sander, Siiri**.
- People from the Institute of Veterinary Medicine and Animal Sciences at Estonian University of Life Sciences for the smiley faces and the lab space: **Ülle, Monika, Rutt, Aili, Pille**.

- I am grateful to all, who contributed to the studies and are the co-authors of the published articles: **Külli Kingo**, **Sulev Kõks**, **Maire Karelson**, **Tiit Salum**, **Eerik Aunin**, **Paula Reemann**, **Kristi Abram**, **Eero Vasar**, **Helgi Silm**, **Ulvi Loite**, **Helen Sulakatko**, **Maris Keerann**, **Kristi Raud**.
- Major gratitude to my family for being and supporting me in my studies.
- I thank my friends **Pille**, **Kati**, **Egle** and **Kuldar** for reminding me, that there is also life outside the laboratory.
- Heartfelt thanks to my friends in and around **MC39**, especially to: **Janek**, **Helena**, **Jäpe**, **Triin**, **Gunnar**, **Kaire**, **Marko**, **Triibu**, **Ranner**, **Rait**, **Ema**, **Isa**, **Miku**, **Matu**, **Päts**, **Vaino**, **Virxx**, **Punapea**, **Imre**, **Jax**, **Mait**, **Tom**. Thank you guys for the laughter and sunny days and events and warmness and kilometers.

PUBLICATIONS

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Publications:

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