DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
14

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RANNO RÄTSEP

Genetics of psoriasis and vitiligo, focus on IL10 family cytokines

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Department of Physiology, University of Tartu, Tartu, Estonia

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II Kingo K, Mossner R, Traks T, **Ratsep R**, Raud K, Reimann E, Kruger U, Silm H, Vasar E, Reich K, Koks S. Further association analysis of chr 6q22–24 suggests a role of IL-20RA polymorphisms in psoriasis. J Dermatol Sci. 2009.

III Kingo K, Mossner R, Koks S, **Ratsep R**, Kruger U, Vasar E, Reich K, Silm H. Association analysis of IL19, IL20 and IL24 genes in palmoplantar pustulosis. Br J Dermatol. 2007 Apr; 159(4):646–52.

IV **Rätsep R**, Kingo K, Karelson M, Reimann E, Raud K, Silm H, Vasar E, Kõks S. Gene expression study of IL10 family genes in vitiligo skin biopsies, peripheral blood mononuclear cells and sera. Br J Dermatol. 2008 Dec;159(6):1275–81.

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IV cDNA synthesis and detection using TaqMan® QRT-PCR, statistical analysis. Author wrote the manuscript and handled correspondence.

ABBREVIATIONS

ARMS-PCR	amplification refractory mutation system polymerase chain reaction
CI	confidence interval
DC	dendritic cells
ELISA	enzyme-linked immunosorbent assay
EM	expectation-maximization
HLA	human leukocyte antigen
HPRT1, *HPRT1*	hypoxanthine phosphoribosyltransferase-1, gene encoding HPRT1
IFNγ	interferon gamma
IFNγR1, *IFNGR1*	interferon-gamma receptor 1, gene encoding IFNγR1
IL10, *IL10*	interleukin-10, gene encoding IL10
IL19, *IL19*	interleukin-19, gene encoding IL19
IL20, *IL20*	interleukin-20, gene encoding IL20
IL22, *IL22*	interleukin-22, gene encoding IL22
IL24, *IL24*	interleukin-24, gene encoding IL24
IL20Rα, *IL20RA*	interleukin-20 receptor alpha subunit, gene encoding IL20Rα
IL20Rβ, *IL20RB*	interleukin-20 receptor beta subunit, gene encoding IL20Rβ
IL22Rα1, *IL22RA1*	interleukin-22 receptor alpha 1, gene encoding IL22Rα1
IL22Rα2, *IL22RA2*	interleukin-22 receptor alpha 2, gene encoding IL22Rα2
IRF4	interferon regulatory factor 4
LD	linkage disequilibrium
LPS	lipopolysaccharide
OR	odds ratio
PASI	psoriasis area and severity index
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PPP	palmoplantar pustulosis
QRT-PCR	quantitative real-time polymerase chain reaction
SNP	single nucleotide polymorphism
STAT	signal transduction and activators of transcription
TF	transcription factor
TGFβ	transforming growth factor beta
Th	T helper cells
TNFα	tumour necrosis factor alpha
PAX	paired box

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease with inflammatory scaly plaques as the main clinical feature. Vitiligo, a depigmentation disorder, is a skin disease with white patches with macular depigmentation as the main clinical feature. The two diseases seem completely different but they both affect the organ of the human body, which provides frontmost protection against environment – the skin. Being exposed to a wide variety of environmental agents, the skin is prone to the development of autoimmune conditions, such as psoriasis and vitiligo. The exact causes of these disorders remain unclear. Throughout the numerous hypotheses suggesting causes and processes leading to these chronic disorders a unifying theory emerges. Along with environmental factors and genetic predisposition another protective system of the human body – the immune system – has a major role in the pathogenesis of these disordes. Failure to maintain epigenetic homeostasis in the immune response due to factors including environmental influences leads to aberrant gene expression, contributing to immune dysfunction and in some cases the development of autoimmunity in genetically predisposed individuals. The significance of the genetic factor is accepted and has been investigated, but we still have no DNA analysis that would help to predict or diagnose these disorders, and we still cannot clearly tell why some of us develop these disorders. The more profound knowledge we have on the pathogenesis, the better means can be developed to relieve symptoms of these disorders and some day it may be even possible to cure them.

Skin is not only the largest organ of the body, but by hosting T-cells, Langerhans cells, monocytes, granulocytes and mast cells it functions as an immune organ as well. Langerhans cells are the major resident antigen presenting cells of the skin and form an extensive network in the epidermis. Similarly to the immune cells, keratinocytes are also capable of producing various cytokines with many of those being pro-inflammatory and directly influencing the immune response. Genetic and epigenetic factors that influence keratinocyte functions, both barrier and immunologic, can have a profound impact on both innate and adaptive immune responses. Thus, the skin environment is an orchestra of cells and factors that direct immune and inflammatory responses to infectious agents, chemicals and self-antigens.

Interleukin(IL)-10 has a dominant role in several immune reactions and its inadequate expression has been demonstrated in autoimmune diseases, infections, and malignancies. Increasing evidence indicates that IL10 itself and recently identified interleukins of the IL10 family are involved in the function of epidermis and psoriasis. Elevated expression of inflammatory cytokines has also been reported in blood and involved skin of vitiligo patients. Recent insights into immunocytic infiltration and epidermal hyperplasia indicate a major role of Th17 cells in inflammatory autoimmune disorders. Upregulation of Th17 derived cytokines, including IL22 in psoriatic skin, leads to the production of anti-microbial peptides and chemokines. IL22, as an effector

cytokine produced by T-cells, mediates the crosstalk between the immune system and epithelial cells. Based on such reports we chose to investigate the roles of IL10 family interleukins in the pathogenesis of psoriasis and vitiligo.

The aim of this research was to continue scientific investigation based on our prior results on IL10 family genes in predicting risk of psoriasis vulgaris. To take the research on the roles of interleukins further, we investigated the next step in the interleukin signalling pathway – receptors of the IL10 family interleukins. As interleukins are connected to the inflammation in the pathogenesis of psoriasis, we were interested to investigate the roles of the studied interleukins in the pathogenesis of other chronic inflammatory skin disorders (palmoplantar pustulosis and vitiligo).

REVIEW OF LITERATURE

Psoriasis and vitiligo

Although the association between psoriasis and vitiligo has been described since 1890, the relationship between these two diseases is not clear. It has been suggested that the decline or absence of melanin might be a predisposing factor for developing scaly erythematous plaques of psoriasis. The incidence of psoriasis in individuals with dark skin is lower than that in the white population, thus suggesting that the resistance of pigmented skin in patients with vitiligo to psoriasis could be an important biological response rather than chance.[1]

Profiles of pro-inflammatory cytokines reveal similarities in the pathogenesis of psoriasis and vitiligo. Keratinocytes, similarly to immune cells, are capable of producing various cytokines including IL1α/β, IL6, IL7, IL10, IL12, IL13, IL15, tumour necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ).[2] Many of these are pro-inflammatory and directly influence the immune response. Enhanced levels of neurotensin-induced TNFα production from the melanocytes were demonstrated in vitiligo lesions.[3] Similarly, elevated level of TNFα was found in the perilesional skin of patients with vitiligo.[4] Furthermore, TNFα is known to be one of the key cytokines in the pathogenesis of psoriasis and the level of TNFα is elevated in psoriatic lesions.[5]

Koebner phenomenon, where lesions appear at the site of injury, is seen in both, psoriasis and vitiligo.[6] Injury of a previously healthy skin triggers the release of fibroblast growth factor, which leads to the proliferation of endothelial cells and subsequently to lesions characteristic of psoriasis or vitiligo.

Clinical features and pathogenic mechanisms of the diseases

Psoriasis

Psoriasis, once considered a skin disorder, is now known as a chronic systemic disease, which in addition to skin aberrations may eventually cause nail changes and affect joints. It is a universal inflammatory disease first described by Hippocrates using the term ‘psora’, meaning ‘to itch’. The estimated prevalence of psoriasis varies from 0.5% to 4.6%. While rates vary between countries and races (more frequent at higher latitudes and affects Caucasians more than other races), the prevalence is almost equal in men and women.[7] The clinical variants of psoriasis include psoriasis vulgaris (chronic plaque psoriasis), which is the most common form, and pustular psoriasis, which can be divided into subgroups of palmoplantar pustulosis (PPP) and generalized pustular psoriasis.

Two forms of psoriasis vulgaris differing in age of onset have been proposed, with early onset disease (onset $\leqq$ 40 years) more likely to be familial,

with severe course and associated with Human leukocyte antigen (HLA)-Cw6.[8] The severity of psoriasis can be graded as mild, moderate or severe according to the Psoriasis Area and Severity Index (PASI).[9] The PASI scores are calculated on the basis of the degree of erythema, desquamation and infiltration of lesions, combined with surface extension.

Main symptoms of psoriasis (inflammatory infiltrate and epidermal hyperproliferation with abnormal keratinocyte differentiation) are believed to result from a complex interaction between genetics and environmental factors.[10] One hypothesis, unifying different theories for the unknown cause of psoriasis pathophysiology, is the cytokine network model.[11] In this model, different exogenous and/or endogenous stimuli, such as bacterial, viral or drug-derived antigens,[12,13] initiate a cytokine cascade leading to the events underlying psoriasis. Anti-microbial peptides and pro-inflammatory cytokines such as TNFα promote the activation of dendritic cells (DCs) within the skin. TNFα is a cytokine that is both produced by and targets almost every cell population present in the skin. While involved and uninvolved psoriatic skin express similar TNFα mRNA levels, TNFα protein is selectively increased in lesions, indicating post-transcriptional regulation.

Recently, new insights into the link between immunocytic infiltration and epidermal hyperplasia have been presented, indicating a major role of Th17 cells in psoriasis as well as other inflammatory autoimmune disorders. Under the influence of DC-derived IL12 and IL23 T-cells become activated and acquire respectively a Th1- or Th17-like immune phenotype.[14–16] IL23, but not IL12 expression, is increased in psoriatic lesions, as shown by up-regulated expression of IL23 alpha subunit p19, IL12p40, but not IL12p35.[17,18] Elevated counts of circulating Th1-cells, and especially Th17-cells, were found in blood of patients with psoriasis.[19] Up-regulation of Th17 derived cytokines IL17, IL22, IL17F, IL26, and of IFNγ in psoriatic skin lead to the production of anti-microbial peptides and chemokines. Keratinocytes express high levels of IL22 receptors and are highly responsive to IL22, as well as to IL19, IL20 and IL24.[20,21] IL22 is distinctive among these cytokines in that it is primarily expressed by activated T-cells and not keratinocytes[22] and is therefore well-positioned as a bridge between the two. Such inflammatory setting, through its action on epithelial cells, will contribute to epithelial barrier disruption and recruitment of immune cells and causes chronic inflammation.[15–17,20]

Examination of individuals from the Danish Twin Registry has shown concordance for psoriasis in 63% of monozygotic twins compared with 15% for dizygotic twins.[23] However, it is evident that the concordance rates do not reach 100%, indicating that the environment plays a significant role in disease expression.[10]

The precise set of genes making individuals predisposed to psoriasis has not been identified, but several candidate genes have been suggested. Recent genome-wide association studies have provided us with many novel clues concerning psoriasis pathogenesis, in both immune and non-immune pathways. The MHC locus (HLA-Cw6), the major locus involved in the immune reactions

of human immune diseases, has consistently been shown to be associated with psoriasis.[12,24] Multiple recent large-scale studies have provided novel results showing late cornified envelope gene cluster[25–27] and genes encoding IL12B (IL12 p40 subunit), and IL23R (one of the IL23 receptor subunits) having strong association with psoriasis.[24,28,29] Zhou et al. (2003)[30] identified, with the help of 63,100-element oligonucleotide array, 1,338 genes with potential roles in psoriasis pathogenesis/maintenance, and revealed many perturbed biological processes, including immune and inflammatory responses. One of the loci, 1q31–32 – including *IL10*, *IL19*, *IL20* and *IL24* – has been shown to be associated with several autoimmune diseases and has also been shown to be associated with psoriasis based on transmission/disequilibrium test.[31]

Palmoplantar pustulosis

Palmoplantar pustulosis (PPP), a subtype of psoriasis, is a chronic inflammatory skin disorder in which erythematous, scaly plaques with sterile pustules develop on palms and soles. There are several similarities between PPP and psoriasis vulgaris. Both diseases present with sharply delineated scaly and inflammatory plaques that are characterized histopathologically by epidermal hyper- and parakeratosis and the sterile accumulation of neutrophils. Cytokine imbalance with increased levels of pro-inflammatory cytokines such as IL6, interferon (IFN) -γ and TNFα is regarded as an important pathophysiological element in both diseases.[32]

Despite these similarities the relationship between PPP and psoriasis vulgaris is still controversial. Up to 24% of patients with PPP concomitantly suffer from psoriasis vulgaris.[33,34] However, until now, no common genetic background of plaque-type psoriasis and PPP has been confirmed. PSORS1, the major susceptibility locus for psoriasis vulgaris located on 6p21, was not linked with PPP in a recent study.[35] Of several other genetic variations that have been reported to be associated with psoriasis vulgaris, including polymorphisms of the genes encoding TNFα, TNFβ, IL1β,[36–38] only the NcoI polymorphism of the lymphotoxin alpha gene was also associated with PPP in a Japanese population.[39] Supported by similarities between psoriasis vulgaris and PPP, the present case-control study was conducted to test the hypothesis that these polymorphisms also influence the risk of PPP and that variations of the IL19 subfamily members therefore constitute a common genetic element of psoriasis vulgaris and PPP.

Vitiligo

Vitiligo is known as an idiopathic acquired depigmentation disorder, which is characterized by the loss of functional melanocytes from the epidermis. The frequency of vitiligo in different populations is from 0.1% to 2% without racial, sexual or regional differences.[40] In about 25% of the patients vitiligo develops

before the age of 10 years, in half of the individuals before the age of 20 years, and in the majority before the age of 40 years.[41] Vitiligo is thought to be a disorder in which genetic and environmental factors contribute to the autoimmune destruction of melanocytes. While most cases of generalized vitiligo are sporadic, 15–20% of cases have an affected first degree relative, and the inheritance pattern is suggestive of a polygenic, multifactorial disease.[42] Recent genome-wide association studies of vitiligo have identified several susceptibility loci;[43] [44] although each of the vitiligo susceptibility loci thus far identified accounts for only a small increase in relative risk, the biological pathways they highlight provide insights into the pathogenesis of the disease. Vitiligo presents as white patches with macular depigmentation on any part of the integument. Occasionally lesions have inflammatory borders. Any pattern of onset and involvement can be seen. Most commonly affected are fingers, hands, feet, and face, but also genitalia, mucosal surfaces, inner ear, and pigment layers of the eyes. It typically spreads from the acral sites of onset to the central integument. The spread of vitiligo can stop at any time or continue to involve the entire integument. In some cases, spontaneous repigmentation of some lesions has been observed. By its clinical presentation vitiligo is classified into two major forms with several subtypes. The generalized form is characterized by depigmented macules on both sides in a symmetrical pattern (bilateral, symmetrical vitiligo vulgaris), in contrast to the segmental form (unilateral, asymmetrical vitiligo). The segmental vitiligo is not likely to repigment with any therapy and possibly has a different pathopysiology than the generalized form of vitiligo.[45] Vitiligo is considered to be in the active stage if new areas of depigmentation have been observed during the previous three months, or stable stage, if no new depigmentation or enlargement of depigmentation have been observed for more than three months.

Although vitiligo might be considered to be a minor disorder, it may have a devastating impact on patients' self-esteem and social interactions and cause significant distress.[46] There is no consensus about the mechanisms involved in the dysfunction and degeneration of melanocytes in vitiligo, but there are three prevailing hypotheses for the pathogenesis of vitiligo: an autoimmune mechanism, an auto-cytotoxic mechanism, and an abnormality in melanocytes or in surrounding keratinocytes-producing factors necessary for the survival and function of melanocytes.[47–49] Among these, the autoimmune hypothesis has the widest support. Vitiligo is often associated with other autoimmune disorders, such as Hashimoto's thyroiditis, Graves' disease, type 1 insulin-dependent diabetes mellitus and Addison's disease.[50] However, as none of the three prevailing hypotheses is sufficient to explain fully the mechanisms of vitiligo, the convergence theory is proposed, stating that stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment and impaired melanocyte migration and proliferation can all contribute in varying proportions to the aetiopathogenesis of vitiligo.[51]

Vitiligo is primarily a depigmentation disorder, but the significance of the inflammatory component is demonstrated by increased expression of inflam-

matory cytokines in the involved skin[52] and blood.[53] Compared with controls, an elevated expression of pro-inflammatory cytokines and their receptors (IL1β, IL6, IL8, TNFα, IFNγ and IL2R) has been reported in blood.[53–58] A significant increase of IL2, TNFα and IFNγ has also been reported in the involved skin of vitiligo patients.[49,57] IL1β, IL6, TNFα are involved in the pathogenesis of vitiligo as paracrine inhibitors of human melanocyte proliferation and melanogenesis.[59] Considering the existing data on the cytokines' involvement in the disease pathogenesis, we were interested to investigate the effects of cytokines of the IL10 family in vitiligo.

IL10 family cytokines and receptors

IL10, also known as cytokine synthesis inhibitory factor, and the other members of IL10 family cytokines discovered more recently (IL19, IL20, IL22, IL24 and IL26)[60] share limited primary sequence identity, structural homology and receptor subunits, and are involved in the regulation of inflammatory responses in various tissues. Despite homology and sharing receptor complexes, interleukins of the IL10 family have distinct physiological roles.[61] According to current concepts, the IL10 family can be divided into two subfamilies of more closely related ligands: the IL10 subfamily (IL10, IL22, and IL26) and the IL19 subfamily (IL19, IL20, and IL24).[62] Lipopolysaccharide (LPS) administration induces secretion of IL10 family cytokines, suggesting their involvement in innate immune response.[22]

IL10 is an immunosuppressive cytokine in the immune system – by suppressing the release and function of a number of pro-inflammatory cytokines such as IL1, TNFα, and IL6, it is an endogenous feedback factor for the control of immune responses and inflammation.[63] IL10 has pleiotropic effects in immunoregulation and inflammation; it is associated with many autoimmune and inflammatory diseases[64] and is reported to be overexpressed in metastatic melanoma.[65,66] IL10 signals over IL10Rβ/IL10Rα receptor complex. From this receptor complex the IL10Rβ subunit can pair with IL10Rα subunit to bind IL10 as ligand, with IL22Rα1 subunit to bind IL22,[67] or with IL20Rα subunit to bind IL26 is suitable as ligand.[68]

IL22, belonging to IL10 subfamily, shares IL10Rβ subunit with IL10 to signal through IL10Rβ/ IL22Rα1 receptor complex, but has also an agonistic soluble class II cytokine receptor IL22Rα2. In contrast to IL10, the IL22 has pro-inflammatory activities. IL22 strongly induces hyperplasia of reconstituted human epidermis and plays an important role in skin inflammatory processes and wound healing.[69,70] IL22 is preferentially produced by Th17 cells and mediates the IL23-induced acanthosis through the activation of signal transduction and activators of transcription 3 (STAT3) in vivo.[16] Elevated levels of IL22 have been found in the blood of psoriatic patients.[21]

The members of the IL19 subfamily display a significant amino acid homology and all bind to the IL20 receptor beta chain,[62,71,72] indicating that they share the same receptor complex (composed of IL20Rα and IL20Rβ subunits) and that their biological activities could be partially overlapping. IL20 and IL24 can also bind to a receptor complex composed of IL20Rβ and IL22Rα1.[61] IL19 subfamily cytokines are preferentially expressed in inflamed tissues[15] and play a major role in the regulation of epidermal functions. The absence of either Rα chain for the two types of receptor complexes for these cytokines on immune cells implies that they cannot act on these cells. IL19, IL20 and IL24 do not induce activation of STAT molecules in immune cells. Instead, several tissues, particularly the skin, tissues from the reproductive and respiratory systems, and various glands appear to be the main targets of these mediators.[15] IL19 subfamily induces pro-inflammatory cytokines such as IL6 and TNFα that are elevated in psoriasis and vitiligo, and is excessively expressed in psoriatic lesions compared with normal skin.

IL20-overexpressing transgenic mice, showing psoriasis-like skin lesions, suggest the importance of IL20 in the inflammatory response in the skin.[73] Both IL19 and IL20 are expressed by distinct populations of keratinocytes.[74] Animal experiments and increased expression of IL19 and IL20 in human inflamed tissues support the assumption that these cytokines play an important role in the pathogenesis of inflammatory diseases.[75]

IL24 functions as a pro-apoptotic cytokine and is cytotoxic for various tumour cells, including melanoma, without inducing harmful effects in normal fibroblast or epithelial cells.[76,77]

Due to high level of expressed interleukin receptor complex subunits, skin is a potential target for IL10 family interleukins (IL10, IL19, IL20, IL22 and IL24).[78] We have previously described association of allelic variations of genes of the IL10 family with susceptibility to psoriasis vulgaris.[79–83] Research presented here extends our previous findings, elaborating roles of IL10 family interleukins, including their receptors, and investigating the roles of IL10 family interleukins and receptors in vitiligo and palmoplantar pustulosis.

Main goals of the project

The main interest of the studies gathered here was to elucidate the role of allelic variations in *IL20RA*, *IL20RB*, *IL22RA2* and *IFNGR1* in psoriasis vulgaris, and extend research on the IL10 family genes and receptors to other cutaneous illnesses, like palmoplantar pustulosis and vitiligo. For these purposes the following goals were set:

- To investigate, whether the genes encoding the two chains of IL20-RI – *IL20RA* and *IL20RB* – located on chromosomes 6q22.33–23.1 and 3q22.3, respectively, are associated with psoriasis vulgaris.

- To continue investigation with an additional set of single nucleotide polymorphisms in the chromosomal region 6q22–24, which contains *IL20RA*, interleukin-22 receptor alpha 2 (*IL22RA2*) and interferon-gamma receptor 1 (*IFNGR1*) for association with psoriasis vulgaris.
- To investigate whether variations in the *IL19*, *IL20* and *IL24* genes that have previously been associated with psoriasis vulgaris may also play a role in palmoplantar pustulosis.
- To evaluate the potential roles of the IL10 family cytokines and receptors in vitiligo using quantitative real-time polymerase chain reaction (QRT-PCR) to detect mRNA expression levels in samples extracted from skin biopsies and peripheral blood mononuclear cells (PBMC), and enzyme-linked immunosorbent assay (ELISA) to measure protein concentrations in serum.

MATERIALS AND METHODS

Ethical considerations

The study was approved by the Ethics Review Committee on Human Research of the University of Tartu and written informed consent was obtained from all participants.

Characteristics of study participants

Association analysis study of *IL20RA* and *IL20RB* genes in psoriasis vulgaris

Unrelated Caucasian patients living in Estonia with a clear clinical diagnosis of plaque psoriasis (n = 254; 119 women, 135 men, age range 18–89 years, mean age of onset of psoriasis 29.7 years) were enrolled at the Department of Dermatology, University of Tartu. Patients with plaque psoriasis were divided into subgroups according to the age of disease onset and family history of psoriasis. In total, 179 patients had early-onset disease (onset before the age of 40), and 75 patients had late-onset disease (onset at the age of 40 or later). Patients were considered to have familial psoriasis if they had at least one first- or second-degree relative with psoriasis (n = 101), otherwise they were considered to have sporadic disease (n = 153). The control group consisted of 224 healthy unrelated Caucasians (110 women, 114 men, age range 18–71 years) without a personal or family history of psoriasis. These individuals were recruited at the University of Tartu from among medical students, health-care personnel, and patients who had visited the dermatological outpatient clinic with mild expression of either facial teleangiectasis or skin tags.

Further association analysis of chr 6q22–24

Unrelated Caucasian patients living in Estonia with an unequivocal clinical diagnosis of psoriasis vulgaris (n= 548; 252 female, 296 male, age range 18–89 years) were enrolled at the Department of Dermatology, University of Tartu, Estonia.[79,82–84]

The control group consisted of 328 healthy unrelated Caucasians (182 female, 146 male, and age range 18–71 years) without a personal or family history of psoriasis. These individuals were recruited at the University of Tartu from among medical students, health care personnel, and patients who had visited the dermatological outpatient clinic with mild expression of either facial telangiectasia or skin tags as described earlier.[79,82–84]

Association analysis of *IL19*, *IL20*, and *IL24* genes in palmoplantar pustulosis

Unrelated Caucasian patients living in Estonia with a clear clinical diagnosis of palmoplantar pustulosis (PPP) (n = 43; 35 female, 8 male, age range 26–75 years, mean age of onset of PPP 47.8 years) were enrolled at the Department of Dermatology, University of Tartu, Estonia. The diagnosis of PPP was made on the basis of the following criteria: (i) symmetrical erythema and infiltration with pustules and scaling on the palms and/or soles; (ii) no lesions of eczema or psoriasis on any other part of the body. The control subjects were 149 healthy unrelated Caucasians (92 female, 57 male, age range 18–71 years) without a personal or family history of psoriasis, recruited at the University of Tartu from among medical students, healthcare personnel and patients who had visited the dermatological outpatient clinic with mild facial telangiectasia or skin tags.

Gene expression study of IL10 family genes in vitiligo skin biopsies, peripheral blood mononuclear cells and sera

All patients and control subjects of the study were Caucasians living in Estonia. Unrelated patients with vitiligo from the Department of Dermatology, University of Tartu, were included in the study. The main characteristics of participants and patients with vitiligo are shown in Table 2. The diagnosis of vitiligo was based on such clinical signs as characteristic skin depigmentation with typical localization and white color on the skin lesions under Wood's lamp. Patients were classified to have active vitiligo if new areas of depigmentation were observed during the previous three months (n = 12 in the biopsy group, n = 15 in the PBMC group) and stable vitiligo if no new depigmentation or enlargement of depigmentation had been observed for more than three months (n = 3 in the biopsy group, n = 5 in the PBMC group). Some of the patients had additional autoimmune diseases in addition to vitiligo: five of the patients had autoimmune thyroiditis, one of the patients with autoimmune thyroiditis also had multiple sclerosis and diabetes, one of the patients with vitiligo had pernicious anemia, and halo naevi occurred in five patients. Mean age at onset of vitiligo was 32.7 years in the skin biopsy study group and 24.5 years in the PBMC study group. Healthy volunteers, with no positive family history of vitiligo and other chronic dermatoses, served as a control group. The control subjects were recruited from medical students, health care personnel and patients who had visited the dermatological outpatient clinic with either facial telangiectases or skin tags. All the subjects enrolled in this study gave written informed consent for the study as indicated in the approval note by the Ethics Review Committee on Human Research of the University of Tartu.

Genotyping

Preparation of genomic DNA

Peripheral blood was obtained from the patients and control subjects. Genomic DNA was extracted from whole blood according to a standard procedure where first the cells are lysed, then the nuclei are lysed and lastly DNA is precipitated from the solution by ethanol. The precipitate was washed with 70% ethanol and dissolved in Tris/EDTA.

Genotyping with tetra-primer ARMS-PCR

SNPs for *IL20RA* and *IL20RB* association study were chosen from dbSNP database (www.ncbi.nlm.nih.gov/SNP/) according to the following criteria: 1) minor allele frequency >10% based on suggestion that loci with minor allele frequency <10% have significantly lower power to detect weak genotypic risk ratios;[85] 2) distance between adjacent SNPs at least 2000 bp to evenly cover the genomic regions. Selected SNPs of *IL20RA* (rs1184860, rs1167846, rs1167849, and rs276504) and *IL20RB* (rs835634, rs10935213, rs747842 and rs108858) genes were in Hardy-Weinberg equilibrium in group of patients with psoriasis and in group of healthy controls (Figure 1 and Figure 2).

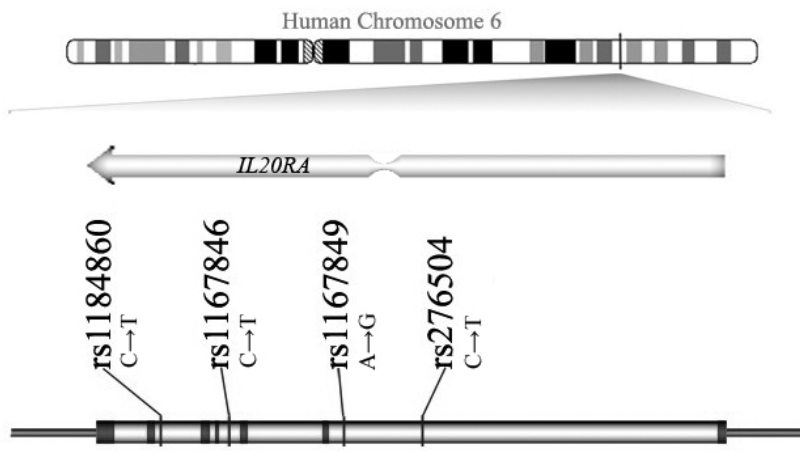

Figure 1. Genomic localization of the single nucleotide polymorphisms (SNPs) in the *IL20RA* gene used in the current study. Relative positions of selected SNPs are represented on the illustration by their cluster ID numbers in Public Polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/) with allelic substitutions included. Arrow indicates the direction of transcription of the *IL20RA* gene with narrowness of the arrow representing a recombination hotspot. Coding region of a gene is represented as box on a narrow bar with dark bands representing exons.

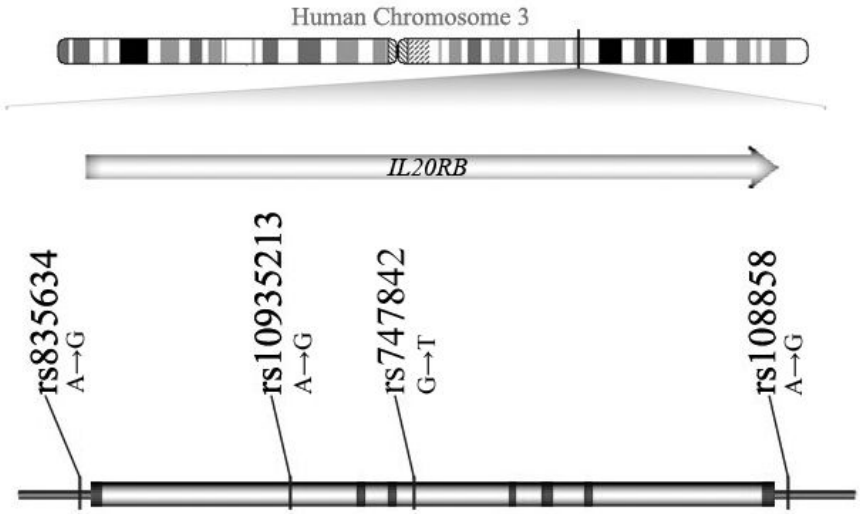

Figure 2. Genomic localization of the single nucleotide polymorphisms (SNPs) in the *IL20RB* gene used in the current study. Relative positions of selected SNPs are represented on the illustration by their cluster ID numbers in Public Polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/) with allelic substitutions included. Arrow indicates the direction of transcription of the *IL20RB* gene, whereas coding region of a gene is represented as box on a narrow bar with dark bands representing exons.

SNPs of the *IL20RA* and *IL20RB* genes were analyzed by the tetra-primer Amplification Refractory Mutation System (ARMS)-PCR method as previously described.[15,79,82,83] For each SNP, a set of four primers was designed using the online program available at http://cedar.genetics.soton.ac.uk/public_html/primer1.html. Each PCR reaction was carried out in a total volume of 10 μL, containing 100 ng of template DNA, 20 pmol of each inner primer, 2 pmol of each outer primer, 0.2 mM deoxyribonucleotide triphosphate, 1x reaction buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 0.1 mg/mL Tartrazine, 1% Sucrose (Naxo, Tartu, Estonia)), 0.5 U Smart-Taq DNA polymerase (Naxo, Tartu, Estonia). Sequences and melting temperatures of primers, annealing temperatures for specific PCR reactions and optimized concentrations of Mg^{2+} used for genotyping of SNPs are shown in Table 1. The direct sequencing of incidental DNA samples was performed using ABI Genetic Analyzer 310 (Applied Biosystems, Foster City, CA, USA) for verification of the tetra-primer ARMS-PCR results and validation of the polymorphisms. The results of the tetra-primer ARMS-PCR were completely consistent with the results of direct sequencing.

Table 1. Sequences and other details of tetra-primer ARMS-PCR primers used for genotyping of SNPs of *IL20RA* and *IL20RB* genes.

Genetic polymorphism	Primers (5′-3′)	Tm	Annealing temperature	Mg^{2+}	Amplicon size
rs1184860 T/C	Forward inner primer (C allele): TTT TAA TGT GAG TAA AGA AAT GAC AGC GC Reverse inner primer (T allele): TTT TTG GGT ATG TTT TAG GCA TCT TGA TAA Forward outer primer: TTT ATA GTA GAG ATG GGG TTT TGC CAT G Reverse outer primer: AAA ATT GCT TTG TTT CCT TAT GAC AGC A	65°C 65°C 65°C 65°C	60°C	2.5 mM	289 bp – control 193 bp – T allele 155 bp – C allele
rs1167846 C/T	Forward inner primer (C allele): CAG TCA TTC AAC TCA TAT TTA TTG GGG GC Reverse inner primer (T allele): AGA GGA ACA CAA TTC AAC CCA TAA TCA Forward outer primer: TAC TCT GGT TTA TGT TTA GTT GCC GAG A Reverse outer primer: CCA CCT GAC TTC AGT ATG ATC TCA TGT T	64°C 64°C 64°C 64°C	60°C	2.5 mM	376 bp – control 244 bp – T allele 188 bp – C allele
rs1167849 G/A	Forward inner primer (A allele): CAT TTA GGT AAG TGG GAA ATG CTC CAA A Reverse inner primer (G allele): TAT AAT CTT TTC TTC CCA CAA CAC TGT CCC Forward outer primer: AGA AAG AGC TCA GGA ATT ATT CGC TCA G Reverse outer primer: AAC TAT GAA CAG TTC CAC CAG GAA AAG C	66°C 66°C 66°C 66°C	60°C	2.5 mM	356 bp – control 236 bp – G allele 178 bp – A allele

Genetic polymorphism	Primers (5′-3′)	Tm	Annealing temperature	Mg2+	Amplicon size
rs276504 T/C	Forward inner primer (C allele): AAC CAC TGG TGC ACA CTT CAG AAA TCA C Reverse inner primer (T allele): ACT GAA CGT TGT TTT GCA GCA CGT GCC A Forward outer primer: GGC CAA TGG CAG TGG ACA TCA AAA GAC T Reverse outer primer: CCC CTA GGC TAC CAA CCT GTA CTG CAT GC	69°C 75°C 72°C 72°C	65°C	2.6 mM	408 bp – control 275 bp – T allele 189 bp – C allele
rs835634 A/G	Forward inner primer (A allele): GAA GAG AAA AAT GGG AAC AAG ACT TGT A Reverse inner primer (G allele): TCA AAC GAT ATA CAA AGA TAT TCA TTC Forward outer primer: TTT GCA AAA AGA AAA ACT AAA AGA AGA T Reverse outer primer: TAT TCC ACT ATT GTA TTT GCA CAT TAG G	63°C 57°C 60°C 60°C	55°C	2.5 mM	285 bp – control 200 bp – A allele 140 bp – G allele
rs10935213 A/G	Forward inner primer (G allele): TCT AAC TAT AAT GAC CAT TTT ACA TTT TG Reverse inner primer (A allele): AGG AGG CTC ATG ATG TCC AGA GAT AT Forward outer primer: TGT AAT AGT CTT GGC TAG CAG GTT TT Reverse outer primer: TTT ATG TAT TAT CAG TAT CCC TAA GGG G	57°C 64°C 60°C 60°C	55°C	2.5 mM	354 bp – control 235 bp – A allele 174 bp – G allele

Genetic polymorphism	Primers (5´-3´)	Tm	Annealing temperature	Mg2+	Amplicon size
rs747842 T/G	Forward inner primer (G allele): CTC AGG AGC TGT TCT ATT CTC CTT TCC GTG Reverse inner primer (T allele): AGG GAA GGG CAC CAG GGT AGA GGG TA Forward outer primer: TTT CAC AAC AGA AGC TCT TCT GCC ACA TC Reverse outer primer: GAT TAT AGG AAT GAG CCA TCG CAC AGG G	70°C 71°C 70°C 70°C	65°C	2.5 mM	415 bp – control 270 bp – T allele 201 bp – G allele
rs108858 A/G	Forward inner primer (G allele): GCC AGG GTG GCC CTT TTT ATT TGA TG Reverse inner primer (T allele): CCT TTT TTC TGT TGG TAA AGT ACA GAA GTT Forward outer primer: TAT CCT TTG GAG CCT CTC TGG ACT TG Reverse outer primer: CTG TAA AGT GTT GAC AGT GTG TGC ACA C	71°C 62°C 66°C 66°C	60°C	2.5 mM	426 bp – control 162 bp – G allele 120 bp – A allele

Genotyping with SNPlex Genotyping System

SNPbrowser version 3.5 was used for SNP selection and SNPlex assay pool design. The selection process employed the LD Map database from Applied Biosystems, SNP selection function based on density, spacing criterion was 10 kb, minor allele frequency cut-off 5% and non-synonymous SNPs always included.

SNPlex Genotyping System utilizes pre-optimized universal assay reagent kits and a set of SNP-specific ligation probes allowing to genotype up to 48 SNPs in a single reaction. It is based on the oligonucleotide ligation/PCR assay (OLA/PCR) with a universal ZipChute probe detection for high-throughput SNP genotyping. Fluorescent-labelled ZipChute probes are hybridized to complementary ZipCode sequences that are part of genotype-specific amplicons. These ZipChute probes are eluted and detected by electrophoretic separation on a 3730 Genetic Analyzer. The GeneMapper 3.7 software was used for automated allele calling of possible SNPs in each DNA sample.[86]

Statistical and bioinformatical analysis of SNPs

Single-marker association analysis and multimarker haplotype association tests of individuals were performed using the Haploview program. Allele frequencies were investigated using the χ^2-test. To evaluate the deviation from the Hardy-Weinberg equilibrium, observed and expected genotype frequencies were compared by Fisher's exact test in the examined groups (cases and controls). For determining haplotype-based associations, an accelerated expectation-maximization (EM) algorithm similar to the partition/ligation method described by Qin et al. was used.[87] The haplotype association test was performed on the set of blocks selected based on the LD. Pairwise LD was estimated by a log-linear model and standardized D' characteristics were used to demonstrate the extent of disequilibrium. Haplotype analysis was performed only for the haplotypes with a frequency of ≥1% (association analysis of *IL20RA* and *IL20RB* genes in psoriasis) or ≥2% (association analysis of *IL19*, *IL20* and *IL24* genes in palmoplantar pustulosis). The significance level of the tests for declaring a probability value as significant was set to 0.05.

For *IL20RA* and *IL20RB* association analysis, to correct for multiple testing in comparing allele frequencies between the group of patients with psoriasis and the control group, the P-values (P_{nom}-value) were adjusted by permutation testing (P_{adj}-value). Altogether 1,000 permutations were performed for correction of multiple comparisons. In addition, in an exploratory analysis, we compared allele frequencies and haplotype frequencies of subgroups of psoriasis stratified according to age of onset and presence of a positive family history for psoriasis with healthy controls. The sequence of the *IL20RA* gene was analyzed with MatInspector version 7.4.8 (http://www.genomatix.de) to find potential binding sites for the transcription factors in the close proximity of

SNPs. The SNPInspector (http://www.genomatix.de) program was used to identify transcription factor-binding sites affected by SNPs investigated in our study.

mRNA expression measurement

Preparation of biopsies

Two skin biopsies (Ø 4 mm) were taken from each patient with vitiligo: one from the central part of involved skin and another from non sun-exposed uninvolved skin. One skin biopsy (Ø 4 mm) from non sun-exposed skin was taken from healthy control subjects. The non sun-exposed skin was defined as the skin never exposed to UVR previously and definitely not exposed to natural UVR in the last 12 months. Biopsies from uninvolved skin of vitiligo patients and healthy controls were taken from the lower abdomen. All probands had skin phototype II or III, Fitzpatrick classification. Biopsies were instantaneously snap-frozen in liquid nitrogen and stored at –80 °C until further use.

RNA isolation from biopsies

Total RNA was isolated from tissues using RNeasyFibrous Tissue Mini Kit (QIAGEN Sciences, MD, USA) following the protocol suggested by the manufacturer. For tissue homogenization, Ultra-Turrax T8 homogenizer (IKA Labortechnik, Germany) was used. RNA was dissolved in RNase free water and stored until further use at –80°C. Concentration of total RNA was measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA) and quality of the RNA sample was assessed by gel electrophoresis.

RNA extraction from peripheral blood mononuclear cells

Blood was collected from subjects between 08.00 and 12.00 h to limit the effect of circadian variation in cytokine production. PBMC were separated from other blood cells using BD Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), in which they were centrifuged at 1500 g for 30 min at 20 °C. Isolated mononuclear cells were washed twice with 6 mL phosphate-buffered saline and centrifuged at 190 g for 10 min at 20 °C. Half of the PBMC were cultivated in the presence of 0.01 mg/mL LPS for 12 h, and the other half was cultivated without LPS, using RPMI-1640 medium (includes 10% fetal calf serum and 1% penicillin/streptomycin). RNA was extracted from PBMC with the Trizol method according to the manufacturer's protocol (Invitrogen, San

Diego, CA, USA) and stored at –80 °C until cDNA synthesis. Concentration of total RNA was measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA) and quality of the RNA sample was assessed by gel electrophoresis.

cDNA synthesis

cDNA was synthesized using 250 ng (RNA from mononuclear cells) or 500 ng (RNA from biopsies) of total RNA, oligoT18 primers and Superscript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. The reaction mixtures were incubated at 65 °C for 5 min, at 0 °C for 1 min, at 50 °C for 90 min, at 70 °C for 5 min and finally stored at –80 °C.

Quantitative real-time polymerase chain reaction analysis

Gene expression levels were detected in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in 10 μL reaction volumes in four replicates. TaqMan-QRT-PCR method reactions were carried out using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Following TaqMan Assay-On-Demand FAM-labelled MGB-probe gene expression assay mixes (20×, Applied Biosystems, Foster City, CA, USA) were used for the genes of interest accordingly: Hs00174086 (*IL10*), Hs00155485 (*IL10RA*), Hs00175123 (*IL10RB*), Hs00604655 (longer *IL19* isoform 1), Hs00203540 (shorter *IL19* isoform 2), Hs00604657 (whole transcript of *IL19*), Hs00218888 (*IL20*), Hs00205346 (*IL20RA*), Hs00220924 (*IL22*), Hs00222035 (*IL22RA1*), and Hs00169533 (*IL24*). Assays for *IL20RB* and *IL22RA2* were not commercially available at the time of the experiment.

For the detection of *HPRT1* (hypoxanthine phosphoribosyl-transferase-1) expression level, gene-specific primers (*HPRT1* exon 6, 50-GACTTTGCTTT CCTTGGTCAGG- 30; *HPRT1* exon 7, 50-AGTCTGGCTTATATCCAACA CT TCG- 30; final concentrations 300 nM) and VIC-TAMRA-labelled probe (VIC-50-TTTCACCAGCAAGCTTGCGACCTTGA-30-TAMRA; final concentration 200 nM) were used.

For quantification of mRNA, we used comparative Ct method (ΔCt value), where the amount of target transcript was normalized according to the level of endogenous reference *HPRT-1*. Adjustment to normal distribution was tested by the Kolmogorov-Smirnov test. The distribution of measurements of gene expressions by the applied method did not follow a Gaussian distribution. Mann-Whitney U-test and Kruskal-Wallis test were used to test for differences between the groups using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA). Correlation analysis was used to investigate relations

between two parameters of one group. For measure of correlation, the Spearman rank correlation was applied. For all tests, a P value < 0.05 was considered significant.

Table 2. Characteristics of study participants of gene expression study of IL10 family genes in vitiligo.

	Total number of		Age range		Average age	
	Skin	**Blood**	**Skin**	**Blood**	**Skin**	**Blood**
Vitiligo	15	20	22–71	20–69	47.4	24.5
Male	3	8	37–65	20–62	55.7	35.5
Female	12	12	22–71	22–69	45.3	45.5
Healthy	18	34	21–67	21–69	33.9	35.5
Male	6	12	23–26	23–54	24.2	33.6
Female	12	22	21–67	21–69	38.8	36.8

Protein level measurement

ELISA analysis of IL10, IL20 and IL22 from sera was performed using Peprotech ELISA Development Kits (Peprotech, Rocky Hill, NJ, U.S.A.) according to the manufacturer's protocol. ELISA kits for the remaining products of investigated genes were not commercially available at the time of the experiment.

RESULTS

Association analysis of *IL20RA* and *IL20RB* genes in psoriasis

Allele frequencies of single SNPs had no statistical difference when the group of patients with psoriasis was compared to the group of controls (Table 3). However, SNPs rs1167846 and rs1167849 showed significant changes in allelic variance when the subgroup of familial psoriasis was compared to the control group (Table 4); minor allele frequencies of rs1167846 (18.8 vs. 25.9%; P = 0.05, OR 0.66, 95% CI 0.44–1.00) and rs1167849 (15.8 vs. 23%; P < 0.05, OR 0.63, 95% CI 0.41–0.98) were decreased in the subgroup of familial psoriasis.

Haplotype analysis of the *IL20RA* and *IL20RB* genes was performed according to the pairwise linkage disequilibrium pattern in the pooled data set. *IL20RA* SNPs rs1184860, rs1167846, and rs1167849 showed nearly complete linkage disequilibrium (| D' | 0.77–0.93); rs276504, located in the first intron of the IL20RA gene, showed weak linkage disequilibrium with the rest of the *IL20RA* SNPs and was therefore excluded from further haplotype analysis. Nearly complete linkage disequilibrium (| D' | 0.83–0.95) was found among all selected SNPs of *IL20RB* gene. Six common haplotypes of *IL20RA* locus (98.9% of all *IL20RA* haplotypes) and *IL20RB* locus (96.8% of all *IL20RB* haplotypes) with estimated frequencies ≥1% were found in the pooled group of study participants (Table 5).

Haplotype analysis revealed a significantly increased frequency of the *IL20RA* haplotype CGG in group of patients with psoriasis compared to the group of healthy controls (7.6 vs. 2.6%; P_{nom}< 0.001, P_{adj}< 0.01, OR 3.14, 95% CI 1.61–6.14), whereas haplotype TTG showed decreased frequency in the group of patients compared to the group of controls (0.9 vs. 4.4%; P_{nom}< 0.001, P_{adj}< 0.01, OR 0.20, 95% CI 0.07–0.55; Table 6). *IL20RA* haplotype CGG showed similar significant associations when the subgroups of psoriasis patients were compared with the control group (OR 3.02–3.49; Table 6). *IL20RB* haplotype AGTA showed decreased frequency in the group of patients, but the borderline significance did not withstand after correction for multiple testing (P_{nom}< 0.05, P_{adj} = 0.45, OR 0.44, 95% CI 0.20–0.97; Table 6); AGTA haplotype frequencies in the subgroups of psoriasis patients were insignificant (data not shown).

MatInspector and SNPInspector were used to model effects of base exchanges to investigate whether the SNPs defining the psoriasis-associated haplotypes modify transcription factor (TF) binding sites. Results showed that two of the SNPs of *IL20RA* gene modify potential TF binding sites: rs1167846 C→T leads to loss of a paired box (PAX) 5 site and generates new site for POU class 2 homeobox 1; rs1184860 T→C leads to the loss of homeobox A9 TF site and generates new site for nuclear factor (erythroid-derived-2)-like-1 TF.

Table 3. Results of association analysis of the genes *IL20RA* and *IL20RB* in psoriasis patients are indicated. Allelic P-values were calculated using χ^2-test.

Gene	SNP ID	Chr Pos NCBI	Alleles	Minor allele frequency in controls (%)	Minor allele frequency in cases (%)	Allelic P-value	OR (95% CI)
IL20RA	rs1184860	137382297	T/C	28.3	32.4	0.19	1.20 (0.91–1.59)
	rs1167846	137385413	C/T	25.9	23.1	0.32	0.86 (0.64–1.16)
	rs1167849	137390718	G/A	23.0	21.8	0.65	0.93 (0.69–1.26)
	rs276504	137394212	T/C	35.0	36.7	0.60	1.07 (0.82–1.40)
IL20RB	rs835634	138159001	G/A	46.6	46.7	0.99	1.00 (0.78–1.29)
	rs10935213	138176157	A/G	41.6	43.1	0.64	1.06 (0.82–1.38)
	rs747842	138185819	T/G	40.8	44.9	0.20	1.18 (0.91–1.54)
	rs108858	138212583	A/G	33.0	32.3	0.82	0.97 (0.73–1.28)

Table 4. Results of association analysis of the *IL20RA* gene SNPs in psoriasis vulgaris. Allele frequencies of the four studied polymorphisms of the IL20RA gene were compared between the psoriasis patients and the controls, P-values for allelic association were calculated using a χ^2-test. *P-value < 0.05 comparing allelic frequencies between patients and controls.

	rs1184860			rs1167846			rs1167849			rs276504		
	Alleles (%)			Alleles (%)			Alleles (%)			Alleles (%)		
	T	C	P-value	C	T	P-value	G	A	P-value	T	C	P-value
Controls (n=224)	71.5	28.3	*	74.1	25.9	*	77.0	23.0	*	65.0	35.0	*
Early onset psoriasis (n=179)	68.2	31.8	*0.3177*	77.9	22.1	*0.2076*	79.3	20.7	*0.4291*	63.7	36.3	*0.7087*
Late onset psoriasis (n=75)	66.2	33.8	*0.2300*	74.7	25.3	*0.8921*	76.0	24.0	*0.8001*	63.3	36.7	*0.7193*
Familial psoriasis (n=101)	72.2	27.8	*0.8625*	81.2	18.8	*0.0494**	84.2	15.8	*0.0376**	66.8	33.2	*0.6413*
Sporadic psoriasis (n=153)	64.6	35.4	*0.0538*	74.2	25.8	*0.9529*	74.5	25.5	*0.4502*	61.4	38.6	*0.3157*

Table 5. Common haplotypes (estimated frequency ≥1%) formed by SNPs in the *IL20RA* and *IL20RB* genes.

IL20RA				*IL20RB*				
rs1184860	rs1167846	rs1167849	Frequencies of haplotype (%)	rs835634	rs10935213	rs747842	rs108858	Frequencies of haplotype (%)
T	C	G	66.4	A	G	G	A	38.1
C	T	A	17.9	G	A	T	G	30.3
C	C	G	5.3	G	A	T	A	20.4
C	T	G	3.4	A	A	G	A	3.3
C	C	A	3.4	A	G	T	A	2.9
T	T	G	2.5	A	A	T	A	1.8
Haplotype block of the IL20RA – 8 kb				*Haplotype block of the IL20RB – 36 kb*				

Table 6. Results of haplotype analysis of the *IL20RA* and *IL20RB* genes. The haplotype frequencies (%) and haplotypic ORs with their 95% CIs and P-values in plaque psoriasis patients compared to controls are indicated. Haplotype-phenotype associations were estimated using an expectation-maximization algorithm. Multiple testing was performed using permutation testing. *P-value < 0.05 haplotypic differences compared to the controls.

***IL20RA* haplotypes**	**Controls (n=224)**	**Plaque psoriasis patients (n=254)**	**P_{nom}-value**	**P_{adj}-value**	**OR (95% CI)**
TCG	66.8	66.1	0.81	1.00	0.97 (0.74–1.26)
CTA	18.2	17.6	0.81	1.00	0.96 (0.69–1.34)
CCG	2.6	7.6	0.0004*	0.007*	3.14 (1.61–6.14)
CTG	3.2	3.6	0.76	1.00	1.12 (0.56–2.2)
CCA	4.2	2.7	0.20	0.96	0.63 (0.31–1.28)
TTG	4.4	0.9	0.0006*	0.007*	0.20 (0.07–0.55)
***IL20RB* haplotypes**					
AGGA	35.9	40.0	0.20	0.96	1.19 (0.91–1.55)
GATG	30.5	30.1	0.89	1.00	0.98 (0.74–1.29)
GATA	20.2	20.5	0.91	1.00	1.02 (0.74–1.39)
AAGA	4.1	2.6	0.18	0.95	0.62 (0.30–1.26)
AGTA	4.1	1.9	0.04	0.45	0.44 (0.20–0.97)
AATA	1.8	1.7	0.91	1.00	0.94 (0.36–2.47)

Table 7. The *IL20RA* CCG and TTG haplotype frequencies (%) and haplotypic ORs with their 95% CIs and P-values in the subgroups of patients according to the age of onset, family history and severity of disease compared to controls are indicated.

Subgroups of psoriasis	**CCG (%)**	**P-value**	**OR (95% CI)**	**TTG (%)**	**P-value**	**OR (95% CI)**
Controls (n=224)	2.6			4.4		
Patients with early onset psoriasis (n=179)	7.6	0.0008	3.18 (1.57–6.45)	1.1	0.0052	0.24 (0.08–0.71)
Patients with late onset disease (n=75)	7.6	0.004	3.27 (1.40–7.65)	0.7	0.0295	0.15 (0.02–1.09)
Patients with familial psoriasis (n=101)	8.2	0.0009	3.49 (1.61–7.60)	0.7	0.0132	0.15 (0.03–0.84)
Patients with sporadic psoriasis (n=153)	7.2	0.0021	3.02 (1.45–6.30)	1.1	0.0097	0.24 (0.08–0.77)

Further association analysis of chr 6q22–24

From the initial set of 15 SNPs, the genotyping of rs3799487 and rs2797671 with the SNPlex platform failed; two SNPs (rs6570122 and rs14185994) deviated from the Hardy–Weinberg equilibrium (P-value < 0.01) in the group of healthy controls and were excluded from further analysis. The most prominent association occurred with SNP rs1342642 in the coding region of the *IL20RA* gene and remained significant after correction for multiple testing (Table 8). This SNP represents a C→T transition leading to a L (leucine) to F (phenylalanine) exchange at position 382 of the receptor chain. It is a relatively common SNP with C as ancestral allele and T as the minor allele. The T allele was less frequent in cases (25%) compared to controls (40.0%; OR 0.48; 95% CI 0.37–0.64) consistent with a protective effect.

For the LD and haplotype analyses we included four additional SNPs of the *IL20RA* gene (SNPs rs1184860, rs1167846, rs1167849 and rs276504) from our previous study.[84] These four *IL20RA* SNPs were not genotyped in the newly collected cases and controls; therefore we included for the LD and haplotype analyses only samples included in both studies (254 cases and 224 controls). LD analysis indicated the existence of four blocks at chr 6q22–24. Strong linkage disequilibrium (|D'| 0.51–0.93) existed between the polymorphisms rs1342642, rs1184860, rs1167846 and rs1167849 within the *IL20RA* gene. The rs276504 polymorphism, located in the first intron of the IL20RA gene was in strong linkage disequilibrium (|D'| 0.75–0.93) with SNPs rs276586 and rs276526 of the intergenic region between the *IL20RA* and *IL22RA2* genes. Additionally, there was linkage disequilibrium between SNPs rs276571 and rs719640 of the intergenic region between the *IL20RA* and *IL22RA2* genes and

between the SNP rs276466 of the *IL22RA2* gene. There was also complete linkage disequilibrium (| D' | 0.99) between the polymorphisms rs1343677 and rs2797665 of the intergenic region telomeric of the *IFNGR1* gene. Haplotype analysis revealed three haplotypes with potential association (Table 9). CTCG and CCCG of block 1 including rs1342642, rs1184860, rs1167846 and rs1167849 were more prevalent among patients than among control individuals (OR 1.99, 95% CI 1.48–2.66; and OR 4.02, 95% CI 1.73–9.33, respectively). The third haplotype was TTCG of the same block, which was less prevalent among cases than among controls (OR 0.10, 95% CI 0.05–0.20).

Table 8. Results of single marker association analysis. Allelic P-values were calculated using χ^2 test. Correction for multiple testing was done using 5,000 permutations.

SNP ID	Minor alleles	Minor allele frequency in controls	Minor allele frequency in cases	Allelic P_{nom}-value	Allelic P_{adj}-value	Minor allele OR (95% CI)
rs4896227	T	0.423	0.298	0.003	0.066	0.580 (0.403–0.832)
rs1342642	T	0.400	0.250	6.1E-7	0.0002	0.485 (0.367–0.636)
rs276586	C	0.491	0.451	0.267	0.998	0.851 (0.639–1.132)
rs276526	A	0.495	0.468	0.359	1.000	0.898 (0.715–1.129)
rs276571	A	0.495	0.498	0.910	1.000	1.014 (0.799–1.287)
rs719640	T	0.471	0.507	0.215	0.992	1.157 (0.919–1.456)
rs276466	G	0.185	0.183	0.910	1.000	0.983 (0.731–1.321)
rs7749054	G	0.127	0.144	0.411	1.000	1.152 (0.822–1.614)
rs1343677	C	0.374	0.438	0.026	0.465	1.305 (1.032–1.651)
rs2797665	T	0.202	0.210	0.776	1.000	1.054 (0.735–1.510)
rs12197182	A	0.126	0.129	0.887	1.000	1.026 (0.720–1.463)

Table 9. Results of haplotype analysis. The haplotype frequencies, P-values of haplotype associations and haplotypic ORs with 95% CIs in psoriasis patients compared with controls are indicated. Block 1 is formed by rs1342642, rs1184860, rs1167846, rs1167849; block 2 by rs276504, rs276586, rs276526; block 3 by rs276571, rs719640, rs276466 and block 4 by rs1343677, rs2797665.

	Haplo-type	Controls	Cases	P_{nom}-value	P_{adj}-value	OR (95% CI)
Block 1	CTCG	0.458	0.627	3.5E-6	0.0002	1.987 (1.484–2.38)
	TCTA	0.177	0.166	0.671	1.000	0.921 (0.630–1.347)
	TTCG	0.191	0.024	8.9E-16	0.00E0	0.103 (0.054–0.198)
	CCCG	0.022	0.081	5.0E-4	0.0128	4.018 (1.729–9.333)
	TCTG	0.031	0.031	0.976	1.0000	0.989 (0.428–2.253)
Block 2	TCA	0.453	0.415	0.183	0.910	0.856 (0.680–1.077)
	CAG	0.321	0.332	0.681	1.000	1.053 (0.825–1.344)
	TAG	0.168	0.185	0.434	1.000	1.127 (0.834–1.527)
Block 3	ACA	0.439	0.439	0.998	1.000	0.999 (0.794–1.258)
	GTA	0.345	0.375	0.292	0.991	1.137 (0.895–1.443)
	GTG	0.121	0.128	0.703	1.000	1.069 (0.755–1.513)
	ACG	0.047	0.054	0.621	1.000	1.144 (0.674–1.943)
Block 4	TG	0.623	0.562	0.034	0.363	0.776 (0.613–0.981)
	CG	0.184	0.227	0.072	0.607	1.304 (0.977–1.742)
	CT	0.190	0.210	0.376	0.999	1.139 (0.853–1.520)

Association analysis of *IL19*, *IL20*, and *IL24* genes in palmoplantar pustulosis

Fifteen single nucleotide polymorphisms, covering the locus of *IL19* (rs2243158, rs2243168, rs2073186, rs2243174, rs2243188, rs2243191, and rs2243193), *IL20* (rs2981572, rs2981573, rs2232360, and rs1518108) and *IL24* (rs3762344, rs1150253, rs1150256, and rs1150258) genes, were analysed on blood samples of 43 patients with PPP and 149 healthy control subjects. SNPs were chosen on the basis of a minor allele frequency above 5% as observed in earlier studies in a cohort of healthy Caucasians living in Estonia.

Genotype frequencies of these SNPs of patients with PPP and healthy control subjects had non-significant deviation from Hardy-Weinberg equilibrium. Fourteen of the SNPs had similar allelic frequencies in groups of patients and controls, whereas the minor allele G of *IL20* 1380 A→G (rs2981573) polymorphism was less frequent in patients with PPP compared with the group of healthy patients (allele frequency 0.14 vs. 0.24; $P < 0.05$).

Haplotype analysis of the *IL19*, *IL20*, and *IL24* genes was performed according to the pairwise linkage disequilibrium pattern observed within each of these genes. Strong linkage disequilibrium was found between all studied polymorphisms of *IL19* and *IL24* genes ($|D'|$ 0.77–0.98). High degrees of linkage disequilibrium were also found between *IL20* SNPs –1053 T→G

(rs2981572), 1380 A→G (rs2981573), and 1462 A→G (rs2232360). We excluded IL20 SNP 3978 T→C (rs1518108) from the analysis due to breakdown of linkage disequilibrium between this and the other three SNPs of the *IL20* gene (| D' | 0.02–0.16).

Three common *IL19* haplotypes (91% of all *IL19* haplotypes), four common IL20 haplotypes (98.7% of all *IL20* haplotypes), and three common *IL24* haplotypes (96.7% of all *IL24* haplotypes) were identified within the study groups (Table 10).

Among *IL19* haplotypes lower frequency of GATGATA was observed in patients with PPP compared with control subjects (0.062 vs. 0.138), but the difference had only borderline significance (P = 0.057, OR 0.41, 95% CI 0.16–1.05; Table 11). Among *IL20* haplotypes analysis revealed a significantly higher frequency of GAA haplotype in patients with PPP compared with healthy controls (0.166 vs. 0.077; P < 0.05, OR 2.39, 95% CI 1.17–4.86; Table 11), whereas GGG haplotype was less frequent in patients with PPP compared with control group (0.115 vs. 0.214; P < 0.05, OR 0.48, 95% CI 0.23–0.98). Frequencies of IL24 common haplotypes were similar in the studied groups.

Extended pairwise linkage disequilibrium pattern analysis of IL19, IL20, and IL24 haplotypes revealed two haplotype blocks with a recombination site between IL20 SNPs rs2232360 and rs151808 (Table 12). Similar haplotype blocks were also identified in an earlier study including patients with psoriasis vulgaris.[83] Four common haplotypes in block 1 account for 87% and five common haplotypes in block 2 account for 95% of all haplotypes in the respective blocks.

Extended haplotype of IL19 and IL20 genes, GACACCGGAA, showed increased frequency in the group of patients compared with the control group (0.129 vs. 0.060; P < 0.05, OR 2.31, 95% CI 1.05–5.10; Table 13). In extended haplotypes of IL20 and IL24 genes haplotype CAAAC was less frequent in patients (0.012 vs. 0.097; P < 0.01, OR 0.12, 95% CI 0.02–0.82) and CGGGT haplotype was more common in patients (0.395 vs. 0.280; P < 0.05, OR 1.68, 95% CI 1.02–2.78) compared to the controls.

Table 10. Common haplotypes with a frequency ≥2%, formed by SNPs in the *IL19*, *IL20* and *IL24* genes in pooled samples. The extent of LD between the *IL19*, *IL20* and *IL24* genes, expressed in terms of Hedrick's multiallelic D' characteristic is demonstrated. *Nonsynonymous polymorphisms.

IL19								***IL20***				***IL24***				
rs2243158	rs2243158	rs2073186	rs2243174	rs2243188	rs2243191*	rs2243193	Frequencies of haplotype (%)	rs2981572	rs2982573	rs2232360	Frequencies of haplotype (%)	rs3762344	rs1150253	rs1150256	rs1150258*	Frequencies of haplotype (%)
G	A	C	A	C	C	G	72.4	T	A	A	67.5	A	A	A	C	47.2
G	A	T	G	A	T	A	12.1	G	G	G	19.2	G	G	G	T	41.5
C	T	T	G	A	T	A	6.5	G	A	A	9.7	G	A	G	T	8.0
								T	G	G	2.3					
								D'0.92			*D' 0.07*					

Table 11. Results of haplotype analysis of the *IL19*, *IL20* and *IL24* genes. The haplotype frequencies (%) and haplotypic ORs with their 95% CI and P-values in palmoplantar pustulosis (PPP) patients compared with controls are indicated. Haplotype-phenotype associations were estimated using an expectation-maximization (EM) algorithm. *P-value < 0.05 haplotypic differences compared with the controls.

***IL19* haplotypes**	**Controls (n=149)**	**PPP patients (n=43)**	**P-value**	**OR (95% CI)**
GACACCG	70.5	79.1	0.116	1.58 (0.89–2.82)
GATGATA	13.8	6.2	0.057	0.41 (0.16–1.05)
CTTGATA	6.7	5.8	0.766	0.86 (0.31–2.36)
***IL20* haplotypes**				
TAA	67.3	68.3	0.868	1.05 (0.62–1.75)
GGG	21.4	11.5	0.039*	0.48 (0.23–0.98)
GAA	7.7	16.6	0.014*	2.39 (1.17–4.86)
TGG	2.2	2.5	0.894	1.10 (0.23–5.29)
***IL24* haplotypes**				
AAAC	48.5	42.9	0.357	0.80 (0.49–1.30)
GGGT	39.4	48.7	0.125	1.46 (0.90–2.37)
GAGT	8.2	7.1	0.744	0.85 (0.34–2.14)

Table 12. Common extended haplotypes with a frequency ≥ 2%, formed by SNPs in the *IL19*, *IL20* and *IL24* genes in pooled samples are indicated. The extent of LD between the formed haplotype blocks in a region of the *IL19* subfamily genes is expressed in terms of Hedrick's multiallelic D' characteristic.

***IL19*/*20* extended haplotypes**											***IL20*/*IL24* extended haplotypes**					
rs2243158	rs2243158	rs2073186	rs2243174	rs2243188	rs2243191	rs2243193	rs2981572	rs2982573	rs2232360	Frequencies of haplotype (%)	rs1518108	rs3762344	rs1150253	rs1150256	rs1150258	Frequencies of haplotype (%)
G	A	C	A	C	C	G	T	A	A	64.3	T	A	A	A	C	39.4
G	A	T	G	A	T	A	G	G	G	9.3	C	G	G	G	T	30.6
G	A	C	A	C	C	G	G	A	A	7.5	T	G	G	G	T	10.9
C	T	T	G	A	T	A	G	G	G	5.6	C	A	A	A	C	7.8
											C	G	A	G	T	6.5
D' 0.20																

Table 13. Results of extended haplotype analysis of the genes of the *IL19* subfamily in patients with PPP. The haplotype frequencies (%) and haplotypic ORs with their 95% CIs and P-values in PPP patients compared with controls are indicated. Haplotype frequencies and haplotypephenotype associations were estimated using an expectation-maximization (EM) algorithm. *P-value < 0.05 haplotypic differences compared with the controls.

***IL19*/*IL20* extended haplotypes**	**Controls (n=149)**	**PPP patients (n=43)**	**P-value**	**OR (95% CI)**
GACACCGTAA	64.3	65.0	0.904	1.03 (0.62–1.70)
GATGATAGGG	10.7	4.6	0.087	0.41 (0.14–1.19)
GACACCGGAA	6.0	12.9	0.035*	2.31 (1.05–5.10)
CTTGATAGGG	5.6	5.8	0.944	1.05 (0.37–2.94)
***IL20*/*IL24* extended haplotypes**				
TAAAC	38.7	41.6	0.629	1.13 (0.69–1.84)
CGGGT	28.0	39.5	0.041*	1.68 (1.02–2.78)
TGGGT	11.4	9.2	0.556	0.78 (0.35–1.77)
CAAAC	9.7	1.2	0.010*	0.12 (0.02–0.82)
CGAGT	6.7	5.8	0.766	0.86 (0.31–2.36)

Gene expression of IL10 family genes in vitiligo skin biopsies, peripheral blood mononuclear cells and sera

Gene expression in skin biopsies

mRNA expression of the studied cytokines was not detectable in skin biopsies. We measured expression levels of genes encoding cytokine receptor subunits (*IL10RA*, *IL10RB*, *IL20RA* and *IL22RA1*) and for statistical analyses the expression values were normalized against sample *HPRT1* values.

IL22RA1 expression levels were not statistically different between involved and uninvolved vitiligo skin and the control skin (data not shown). *IL10RA* expression of involved and uninvolved skin showed higher values compared with the control skin, but the expression levels deviated highly within the three groups and the differences between the groups did not reach statistical significance (data not shown). *IL10RB* expression levels were uniform in all sample groups comparing controls with involved and uninvolved skin and all subgroups (data not shown). *IL20RA* mRNA expression (Figure 3) was decreased in uninvolved skin to 0.78 fold compared with the control group ($P < 0.05$) and to 0.74 fold in the involved skin ($P < 0.01$). *IL20RA* levels were higher in the active form compared with the stable form of vitiligo – in involved skin a 1.6 fold increase ($P < 0.05$) and in uninvolved skin a 1.8 fold increase ($P < 0.05$).

Gene expression in peripheral blood mononuclear cells

In the gene expression analysis of PBMC we determined mRNA levels of *IL10*, *IL10RA*, *IL10RB*, *IL19* isoform 2, *IL19*, *IL20*, *IL22* and *IL24*. Expression levels of *IL19* isoform 1, *IL22*, *IL20RA* and *IL22RA1* remained below detection limits of particular assays.

A significant 5.5 fold *IL22* expression elevation ($P < 0.01$) was observed in patients with vitiligo compared with healthy control subjects (Figure 4). LPS stimulation caused a 1.8 fold elevation ($P < 0.05$) of *IL22* expression level in the control group. Samples from the active form of vitiligo had a 6.1 times higher ($P < 0.01$) *IL22* level compared with nonstimulated healthy control samples. Active form samples had a insignificant trend of higher level of *IL22* expression compared with the stable vitiligo form in nonstimulated groups, and this difference became statistically significant after LPS stimulation, as samples of active vitiligo had a 7.3 fold higher ($P < 0.05$) *IL22* expression compared with samples of the stable form.

Gene expression levels of *IL10* did not show any significant differences when controls were compared with patients with vitiligo. LPS stimulation increased the *IL10* expression levels to 1.1 fold compared with nonstimulated samples, both in groups of healthy controls and in patients with vitiligo, but the increase was not statistically significant (data not shown).

Although *IL19* isoform 1-specific signals did not surpass the detection limit, we were able to measure *IL19* expression levels with an *IL19* isoform 2-specific assay and an isoform-nonspecific assay. While *IL19* isoform 2 appeared to have 1.1 fold elevation ($P < 0.05$) in the group of patients with vitiligo compared with healthy controls, *IL19* isoform-nonspecific results did not confirm such a statistically significant finding. A statistically significant change in the vitiligo group was mainly contributed by the subgroup of active vitiligo, in which the elevation was 1.2 fold ($P < 0.05$) compared with the healthy control group. LPS stimulation, as with *IL10*, raised the expression levels of *IL19* in the healthy control group and the vitiligo group to 1.1 fold compared with nonstimulated samples, but the change was not statistically significant. A statistically significant elevation of *IL19* isoform 2 was not observed following LPS stimulation (data not shown).

IL20 expression levels did not show statistically significant differences between groups of patients with vitiligo and the control group (Figure 5). However, observing the tendencies, *IL20* expression in patients with vitiligo was about half of that in the control group. LPS stimulation slightly increased *IL20* mRNA level in the control group but not in the vitiligo group.

IL24 expression levels, which were rather uniform among different groups before stimulation, showed significant differences following stimulation by LPS. After stimulation, the *IL24* expression level of control samples was 1.6 fold higher compared with vitiligo samples ($P < 0.01$), as seen in Figure 6.

The significantly lower *IL24* expression level in vitiligo was mainly contributed by the stable form, which showed a 0.4 fold ($P < 0.05$) expression level, whereas the active form showed a 0.7 fold ($P < 0.05$) expression level compared with the stimulated control group.

Among the range of receptor subunit gene expressions that we studied, we were able to determine expression levels of *IL10RA* and *IL10RB*. Both *IL10RA* (Figure 7) and *IL10RB* (Figure 8) expression levels exhibited a significant 2.1 fold increase ($P < 0.001$) in patients with vitiligo compared with the healthy control group. No remarkable differences in expression level of *IL10RA* or *IL10RB* were observed between active and stable forms of vitiligo, while both of the subgroups followed a trend of significant increase compared with the group of healthy controls. *IL10RA* expression level was increased 2.0 fold ($P < 0.01$) in the stable form and 2.2 fold ($P < 0.001$) in the active form of vitiligo compared with the control group. *IL10RB* expression was increased 2.1 fold ($P > 0.05$) in the stable form and 2.1 fold ($P < 0.001$) in the active form of vitiligo compared with the control group. LPS stimulation caused a 1.9 fold decrease ($P < 0.01$) of *IL10RB* expression level in the vitiligo group but had no significant effect in the control group. The significant 2.1 fold elevation in expression level of both receptor subunits observed in the comparison of the nonstimulated vitiligo group with the control group was reduced by LPS stimulation. *IL10RA* expression level was 1.5 fold higher ($P < 0.05$) following LPS stimulation, and *IL10RB* expression level was reduced to the same level as that observed in the stimulated control group. *IL10RA* elevation in the active

form of vitiligo compared with the control group remained statistically significant after LPS stimulation, and *IL10RB* level in LPS-stimulated active vitiligo was 1.6 fold higher ($P < 0.01$) compared with stimulated healthy controls.

Protein level in serum

An elevated IL22 expression level in the PBMC of patients with vitiligo was supported by ELISA results showing a significant 2.4 fold ($P < 0.0001$) elevation of IL22 protein level in serum of patients with vitiligo compared with the control group. The subgroup of stable vitiligo showed 1.9 fold higher ($P < 0.05$) and the subgroup of active vitiligo a 2.5 fold higher ($P < 0.001$) concentration of IL22 protein in sera (Figure 9).

Serum IL10 protein level showed a tendency to decrease in patients with vitiligo compared with controls, but the results were not statistically significant (data not shown). Serum IL20 protein level also showed a tendency to decrease in patients with vitiligo (data not shown).

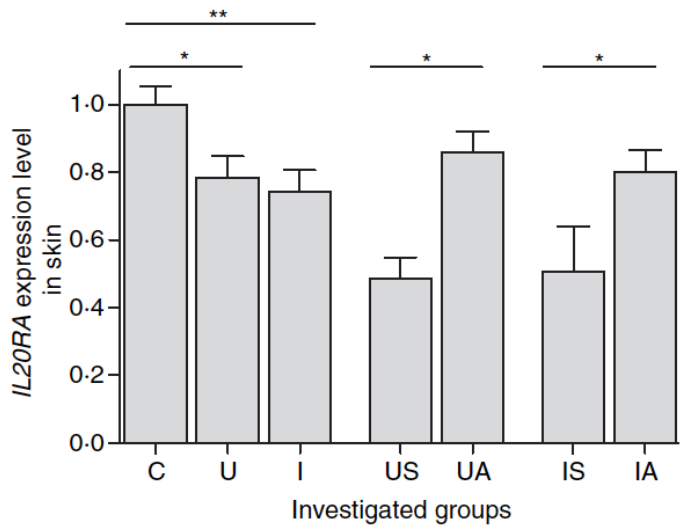

Figure 3. *IL20RA* expression levels in skin biopsies compared between groups of healthy control subjects (C) and uninvolved (U) and involved (I) skin of patients with vitiligo. Additionally, groups U and I were divided into subgroups of uninvolved skin of patients with the stable form of vitiligo (US), uninvolved skin of patients with the active form of vitiligo (UA), involved skin of patients with the stable form of vitiligo (IS) and involved skin of patients with the active form of vitiligo (IA). Results are displayed as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

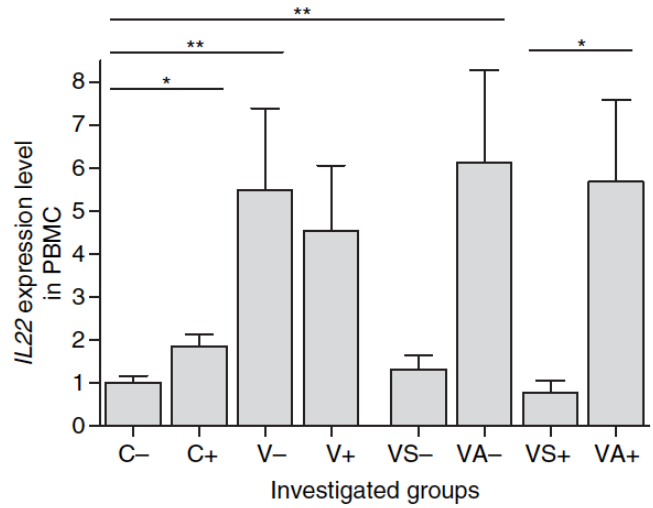

Figure 4. *IL22* expression levels detected from peripheral blood mononuclear cells (PBMC). Columns represent *IL22* mRNA levels in lipopolysaccharide-stimulated (+) and nonstimulated (–) samples of the control group (C) and the vitiligo (V) group, subdivided into stable (S) and active (A) vitiligo. Results are displayed as mean ± SEM. *P < 0.05, **P < 0.01.

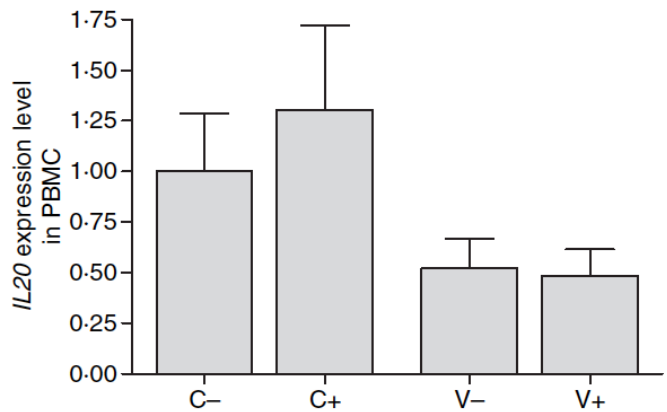

Figure 5. Mononuclear cells (PBMC). Columns represent *IL20* mRNA levels in stimulated (+) and nonstimulated (–) groups of healthy controls (C) and patients with vitiligo (V). Results are displayed as mean ± SEM.

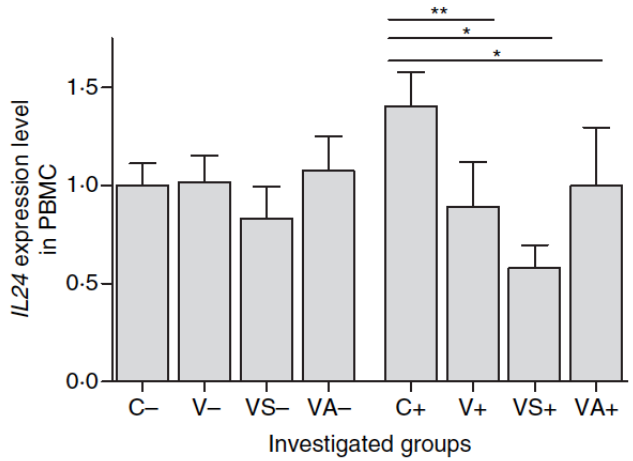

Figure 6. *IL24* expression levels detected from peripheral blood mononuclear cells (PBMC). Columns represent *IL24* mRNA levels in lipopolysaccharide-stimulated (+) and nonstimulated (–) samples of the control group (C) and the vitiligo (V) group, subdivided into stable (S) and active (A) vitiligo. Results are displayed as mean ± SEM. *P < 0.05, **P < 0.01.

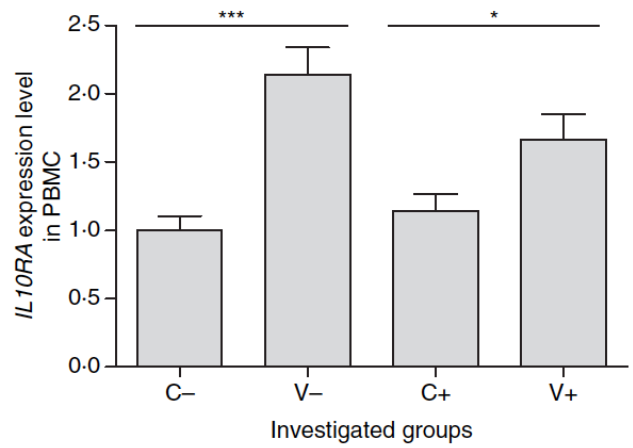

Figure 7. *IL10RA* expression levels in peripheral blood mononuclear cells (PBMC). Columns illustrate expression levels in lipopolysaccharide-stimulated (C+) and non-stimulated (C-) control group and stimulated (V+) and nonstimulated (V-) samples of patients with vitiligo. Results are displayed as mean ± SEM. *P < 0.05,***P < 0.001.

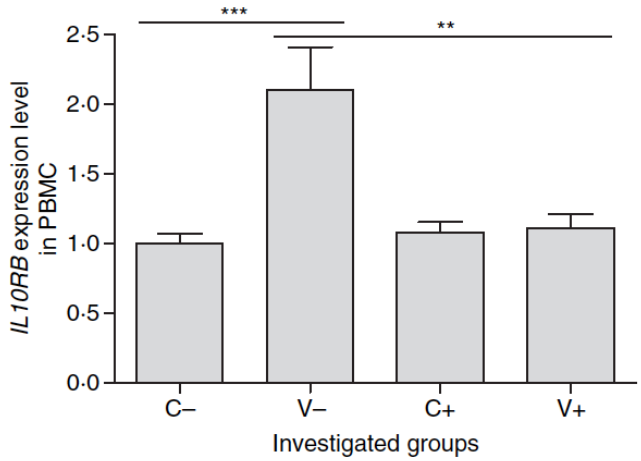

Figure 8. *IL10RB* expression levels in peripheral blood mononuclear cells (PBMC). Comparison of nonstimulated control group (C-) and patients with vitiligo (V-) with lipopolysaccharide-stimulated control group (C+) and patients with vitiligo (V+). Results are displayed as mean ± SEM. **P < 0.01, ***P < 0.001.

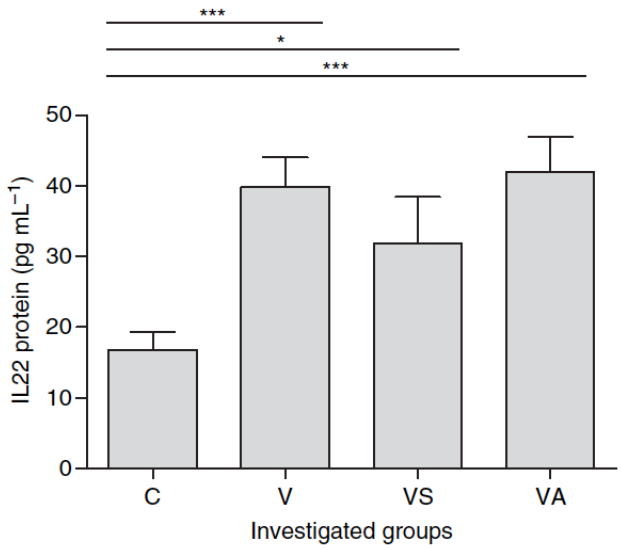

Figure 9. *IL22* protein levels in the sera of the control group (C), whole group of patients with vitiligo (V) and subgroups of stable (VS) and active vitiligo (VA) measured by enzyme-linked immunosorbent assay. Results are displayed as mean ± SEM. *P < 0.05, ***P < 0.001.

DISCUSSION

Association analysis of *IL20RA* and *IL20RB* genes in psoriasis

Data from animal models suggest that overexpression of *IL20* and/or altered signalling of IL20 and other members of the IL19 subfamily plays a central part in the initiation and maintenance of the epidermal changes associated with the development of a psoriasis-like phenotype.[88, 89] Similar effects of IL20 have been demonstrated in human epidermal cells,[20, 90] and *IL20RA and IL20RB* have been shown to be over-expressed in psoriatic lesions compared to healthy skin both at the mRNA and protein level.[91]

To the best of our knowledge, the associations of *IL20RA* and *IL20RB* polymorphisms with a chronic inflammatory disease have not been investigated before. With that in mind we analyzed the association of four SNPs in the *IL20RA* gene and four SNPs in the *IL20RB* gene with psoriasis vulgaris. We found no significant association of any of the investigated SNPs with the disease. In an exploratory subgroup analysis there was a weak association of two SNPs (rs1167846 and rs1167849) of the *IL20RA* gene with familial psoriasis in that the minor alleles of these SNPs were under-represented in cases compared to healthy controls, possibly suggesting a mild protective effect of the more frequent allele. However, it cannot be excluded that this finding is a false-positive result due to multiple testing.

LD analysis revealed strong LD over the 8421 bp and 53582 bp region of the *IL20RA* and *IL20RB* gene, respectively, and haplotype blocks for these genes could be established. For *IL20RA*, the block consists of rs1184860, rs1167846 and rs1167849, but not rs276504. For *IL20RB*, all four SNPs are in almost complete LD. Interestingly, association analysis of haplotypes revealed a significant association of the IL20RA CCG haplotype with psoriasis ($P_{adj} < 0.01$), whereas carriage of the *IL20RA* TTG haplotype seemed to have a protective effect ($P_{adj} < 0.01$). One *IL20RB* haplotype (AGTA) was associated with decreased risk for psoriasis, but this effect did not remain significant after correction for multiple testing. Therefore, our study suggests that polymorphisms in the *IL20RA* gene and possibly also the *IL20RB* gene may have potential roles in the development of psoriasis.

Previous studies have indicated the importance of *IL19*, *IL20* and *IL24* in the manifestation of psoriasis.[74,82,91,92] Psoriatic lesions are characterized by increased expression of *IL19* and *IL20*, which is normalized after treatment with cyclosporine or calcipotriol parallel to the clinical improvement of psoriatic lesions.[74,92] Although the data on the expression of IL20R types I and II in psoriasis are somewhat controversial it seems possible that changes in the expression and/or function of IL20-RI contribute to the changes of the IL19 cytokine system that are part of psoriasis pathophysiology.[91–93] Therefore, the present association study focused on the *IL20RA* and *IL20RB* genes.

The functional consequence of the observed association of the *IL20RA* CCG haplotype with psoriasis (OR 3.14) is currently under investigation. Bioinformatic modeling suggests that the C to T exchange in the case of SNP rs1167846 in the *IL20RA* gene causes the loss of a PAX5 site. PAX5 is a member of the PAX family of TFs that are important in the regulation of cell differentiation. Alterations in the expression of these TFs are thought to contribute to abnormal cell proliferation and malignant transformation.[94,95] Recently it was shown that PAX5 deletion induces the conversion of B lymphocytes to functional T lymphocytes,[96] indicating an additional role in the immune system that might be relevant in psoriasis. In the *IL20RB* gene SNP rs747842, leading to a loss of an interferon regulatory factor 4 (IRF4)-binding site, could be of particular interest, because the function of IRF4 is very closely related to the differentiation of T lymphocytes. For instance, IRF4 is important during IL12-induced Th1 cell differentiation and is also necessary for IL4-induced differentiation of Th2 cells and B cells.[97–99] Recent findings suggest that IRF4 is also involved in the development of Th17 cells[100] that are believed to be centrally involved in psoriasis.

The polymorphisms rs1167846 in the *IL20RA* gene and rs747842 in the *IL20RB* gene appear as interesting candidates for further studies. However, these hypotheses are currently speculative and require functional support. In addition, as there was a strong LD in the investigated genes, it is possible that other polymorphisms of these genes or of other genes in LD with the investigated haplotype might account for the observed association.

Although none of the investigated SNPs of the *IL20RA* and *IL20RB* genes were individually associated with psoriasis, we identified an *IL20RA* haplotype that seems to increase psoriasis susceptibility, and a second *IL20RA* haplotype that was associated with a protective effect. There is initial evidence that the identified *IL20RA* risk haplotype may be functionally relevant because it carries altered binding sites for TFs involved in cell differentiation and immunological functions, but the exact effects of the *IL20RA* polymorphisms on transcriptional activation and function warrant further studies.

In the consequent study we observed a significant association between a SNP in the *IL20RA* gene (rs1342642) and psoriasis. The functional consequence of the L382F amino acid exchange in the intracellular domain of the IL20Rα is presently unclear.

The haplotype-forming SNPs from the previous study (rs1184860, rs1167846, rs1167849) locate between rs1342642 and rs3799487 examined in the consequent study. SNPs rs1342642, rs1184860, rs1167846 and rs1167849 formed one haplotype block and three haplotypes from this block seemed to influence psoriasis susceptibility. Haplotypes CTCG and CCCG were associated with increased risk for psoriasis, while TTCG of the same block had a protective effect. It seems therefore possible that the effect of haplotypes described in our previous study is largely dependent on the effect of the non-synonymous SNP rs1342642 described here.

In conclusion, we observed a strong association between psoriasis and a polymorphism in the intracellular domain of *IL20RA* (L382F), a recently established candidate gene for psoriasis. While requiring replication, our data further support a role for the IL20 receptor in psoriasis.

Association analysis of *IL19*, *IL20* and *IL24* genes in palmoplantar pustulosis

The present report intended to collect the first information on the possible role of polymorphisms of genes belonging to the *IL19* family of cytokines as genetic factors in palmoplantar pustulosis. The study investigated 15 polymorphisms within the *IL19*, *IL20* and *IL24* genes including two nonsynonymous polymorphisms [IL19 43717 C→T (rs2243191) and IL24 3728 C→T (rs1150258)].

No associations between SNPs in the *IL19* and *IL24* genes and PPP were found, but the rare allele of the *IL20* 1380 A→G (rs2981573) SNP was less frequent in patients with PPP than in healthy control subjects. In the haplotype analyses performed, there was evidence that the *IL20* haplotype GAA is associated with an increased (OR 2.39, 95% CI 1.17–4.86) and the IL20 haplotype GGG with a decreased susceptibility for PPP (OR 0.48, 95% CI 0.23–0.98). In these haplotypes, the *IL20* 1380 A→G (rs2981573) SNP is in the middle position, however, it is not possible to discriminate whether the *IL20* SNP 1380 A→G (rs2981573) itself or another relevant variation in linkage disequilibrium with this polymorphism is responsible for associations of *IL20* haplotypes with PPP. Interestingly, the *IL20* haplotype GAA has also been found to be associated with psoriasis vulgaris,[79] suggesting that the *IL20* gene cluster might harbour a common genetic factor for PPP and psoriasis vulgaris. Further studies with large numbers of PPP subjects are necessary to verify this hypothesis.

Several studies have demonstrated the role of extended haplotypes in clusters of cytokine genes for complex traits, an approach based on the identification of blocks within gene clusters as derived from linkage disequilibrium analysis.[101 102] This study confirms the existence of two haplotype blocks with a recombination site in the 3' UTR region of the *IL20* gene within chromosome 1q32. The first haplotype block includes SNPs of the *IL19* and *IL20* genes. The second haplotype block includes SNP of the IL20 3' UTR region and several SNPs of the *IL24* gene. Similar haplotype blocks spanning the *IL19*, *IL20* and *IL24* genes have also been demonstrated in HCV infected patients among African-Americans and European-Americans.[103]

Extended haplotype analysis further supported the hypothesis that genes encoding the *IL19* subfamily of cytokines influence the risk of PPP. The extended *IL19*/*IL20* haplotype GACACCGGAA was associated with an increased susceptibility for PPP (OR 2.31, 95% CI 1.05–5.10), while carriers of the extended *IL20*/*IL24* haplotype CAAAC appeared to have a smaller risk for PPP (OR

0.12, 95% CI 0.02–0.82). A similar constellation has previously been observed in patients with psoriasis vulgaris compared with healthy individuals.[79 82 83] Interpretation has to be with caution because of the small sample size and preliminary character of this study, but it seems possible that the *IL19* gene cluster represents a susceptibility region shared by PPP and psoriasis vulgaris and is part of the genetic basis underlying the frequent co-existence of PPP and psoriasis vulgaris.

In the present study, SNPs with a minor allele frequency > 5% were used for haplotype reconstruction compared with the earlier study in psoriasis vulgaris where SNPs with a minor allele frequency < 10% were excluded.[79] This is a possible explanation why in the present study haplotype *IL19*/*IL20* GACACCG GAA contains two additional SNPs [IL19 35402 G→C (rs2243158) and IL19 37149 A→T (rs2243168)] compared with the *IL19*/*IL20* haplotype CACCGGAA in the previous study. The former longer version of the extended *IL19*/*IL20* haplotype was associated with PPP in the present study; the latter shorter version was associated with psoriasis vulgaris. To allow a better comparison of the findings, we also analysed the shorter version of the *IL19*/*IL20* haplotype CACCGGAA in the present study. The shorter haplotype was likewise associated with PPP (OR 2.21, 95% CI 1.01–4.83). At present, the functional consequence of the investigated polymorphisms is unclear. In an earlier study, the *IL19* rs2243191, *IL20* rs1400986, rs3024517 and rs2232360 polymorphisms and also two haplotypes in the *IL19*/*IL20* region had an effect on HCV clearance in African, but not European-American patients with HCV infection.[103] Although most of the SNPs selected in our study are distinct from those investigated in the above study, both studies suggest that carriage of *IL19*/*IL20* extended haplotypes may influence inflammatory responses.

In this study, we found the first evidence for an association of variations of the *IL19* gene family with PPP. Similar findings have previously been reported in psoriasis vulgaris. In contrast, genome-wide scans have so far failed to demonstrate linkage of psoriasis vulgaris to the region on chromosome 1q31–32, the locus that harbours the *IL19* gene family. However, because linkage analyses compared with case-control studies are less sensitive, this does not rule out the association of variations in this region with psoriasis vulgaris. Until now, no linkage studies have been conducted in PPP, probably because of the rarity and the late onset of the disease.

Clearly, the small sample size is also an important limitation of this study. Significant P values for the associations were in the range of 0.010–0.047. Because of the exploratory nature of the investigation, no correction for multiple comparisons was performed, and the presence of false positive results cannot be excluded.

In conclusion, our study provides initial evidence that polymorphisms of the genes encoding *IL19*, *IL20* and *IL24* may influence susceptibility to PPP. Genetic variations within the *IL19* gene cluster may affect both psoriasis vulgaris and PPP. The results obtained in this study encourage confirmation studies with larger sample sizes. It remains to be seen whether variations in the *IL19*, *IL20* and *IL24* genes confer susceptibility to other chronic inflammatory skin diseases.

Gene expression study of IL10 family genes in vitiligo skin biopsies, peripheral blood mononuclear cells and sera

Vitiligo is a pigmentation disorder, the cause of which is complex and not yet fully understood. By what is known so far and supported by several theories, melanocytes in the involved skin of patients with vitiligo are destroyed and thus the production of melanin is impaired. As demonstrated by Moretti et al., there is a significant change of epidermal cytokines in the involved skin of patients with vitiligo compared with the uninvolved skin and the skin of healthy controls, suggesting a possible involvement of epidermal cytokines in the pathogenesis of vitiligo.[49]

The main findings of the present study are the 5.5 times elevated *IL22* expression level in the nonstimulated PBMC of patients with vitiligo compared with healthy controls and the 2.4 fold higher *IL22* protein concentration in sera of the whole group of patients with vitiligo compared with healthy controls. The role of *IL22* has been established in several autoimmune conditions. *IL22* has been shown to be present in high quantities in blood samples and in colonic mucosa from patients with Crohn disease.[104,105] *IL22* is also expressed in rheumatoid arthritis synovial tissues.[106] In skin, *IL22* induces the expression of different pro-inflammatory molecules belonging to the S100 family of calcium-binding proteins.[107,108] *IL22* was shown to be expressed in skin samples of patients with psoriasis but not control donors. The recent finding that T helper 17 cells are a major source of *IL22* and the fact that *IL22* induces the expression of inflammatory genes in skin support the role of *IL22* in skin innate immunity.[109] For the same reason *IL22* might have a pro-inflammatory role in the pathogenesis of vitiligo.

When the whole group of patients with vitiligo was divided according to disease activity, the active vitiligo samples seemed to be responsible for the increased expression of *IL22* observed in the whole group. While the nonstimulated PBMC of active vitiligo showed a significant 6.1 fold increase compared with healthy controls, the *IL22* expression level in the subgroup of stable vitiligo exhibited only a 1.3 fold increase. Therefore, the significant elevation of *IL22* is related to the period of progressive vitiligo, rather than the period of stable vitiligo in which *IL22* expression level resembles that of healthy controls. Similar to the results of mRNA expression profiles, the subgroup of stable vitiligo showed a lower concentration of *IL22* protein compared with the subgroup of active vitiligo. It is likely that in vitiligo during the active stage of destruction of melanocytes, inflammation is provoked by a pathway including *IL22*.

While LPS stimulation increased *IL22* mRNA expression level in controls, such an increase was not observed in patients with vitiligo where *IL22* expression level was already higher without stimulation. This observation suggests that in vitiligo the *IL22* expression has already been upregulated to a maximum

level and additional stimulation with LPS fails to increase the expression level. A similar finding has been described by Wolk et al., who showed that *IL22* induces expression of LPS binding protein to the concentrations known to neutralize LPS in healthy mice.[104] This could explain LPS unresponsiveness in patients with elevated *IL22* expression.

IL22 signalling uses a receptor complex of subunits *IL10RB* and *IL22RA1*. We measured *IL10RB* mRNA expression levels in skin biopsies and PBMC. Due to the low level of *IL22RA1* mRNA in PBMC the expression of *IL22RA1* was measured in skin only. No significant differences were found. In skin biopsies *IL10RB* expression level was uniform in all studied groups, but in PBMC the expression level was doubled in the vitiligo group compared with the controls. Elevated *IL10RB* level detected in the PBMC of patients with vitiligo suggests activated *IL22* signalling in vitiligo.

Interestingly, LPS stimulation lowered the *IL10RB* expression of vitiligo samples to the level of control samples. This is similar to the case of *IL22* expression levels. Further studies are needed to support and elucidate the phenomenon of *IL10RB* showing elevated expression in the PBMC of patients with vitiligo and being subject to suppression by LPS stimulation.

IL10RA had a remarkably similar expression profile to that of *IL10RB* in nonstimulated PBMC, with both of the subunits having 2.1 fold higher expression level in patients with vitiligo compared with the control group. Increased expression of both of the *IL10* receptor subunits in the PMBC of vitiligo suggest an important systemic role of *IL10* family cytokine signalling in the pathogenesis of vitiligo. There is also the possibility that *IL10RA* upregulation is caused by transcriptional co-regulation of functionally similar receptor subunits.

IL19, *IL20* and *IL24* did not show significant changes in gene expression profiles. With LPS stimulation the differences of *IL24* became significant. An elevated difference was mainly contributed by increased *IL24* expression in the control group PBMC in response to LPS stimulation, whereas the expression in the stimulated vitiligo group remained close to the level in nonstimulated cells. It would be interesting to investigate further why LPS stimulation, which has been shown to increase cytokine expression, fails to act in this way on *IL24* in the PBMC of the vitiligo group, while being able to increase *IL24* in the control group PBMC.

IL20RA showed a significant decrease in expression levels in the involved and uninvolved skin of patients with vitiligo compared with the control group. Expression level was lower in the stable form and higher in the active form of vitiligo, showing dependency on the progression stage of vitiligo. Whereas *IL19* and *IL20* can also bind to other receptor complexes, *IL24* can only signal over the *IL20RA /IL20RB* complex. Therefore the transduction of IL24 signalling has been downregulated by decreased expression of the receptor complex subunit IL20RA in vitiligo skin.

In conclusion, we report that IL22 is significantly associated with vitiligo, especially with the active stage of vitiligo, as shown by results of mRNA expression and supported by results of protein levels in sera. IL22 may provoke

inflammation, which leads to the destruction of melanocytes. However, the actual role of IL22 during pathogenesis of vitiligo remains to be better characterized. Signal transductions of other investigated cytokines seem to be regulated on the expression level of their receptor complex subunits.

CONCLUSION

- Studying the roles of allelic variations in *IL20RA* and *IL20RB* in predisposition to psoriasis, we found that one of the *IL20RA* haplotypes – CCG (rs1184860, rs1167846, rs1167849) – significantly increases psoriasis susceptibility (OR 3.14, 95% CI 1.61–6.14), and another *IL20RA* haplotype – TTG – was associated with a protective effect (OR 0.20, 95% CI 0.07–0.55). There is initial evidence that the *IL20RA* risk haplotype may have functional relevance due to carrying altered binding sites for TFs involved in cell differentiation and immunological functions.
- In the subsequent study of SNPs in the chromosomal region 6q22–24 we observed a strong association between psoriasis and SNP rs1342642 in the intracellular domain of *IL20RA* (L382F) – a recently established candidate gene for psoriasis. While requiring replication, our data further support a role for the IL20 receptor in psoriasis.
- We provided initial evidence that polymorphisms of the genes encoding *IL19*, *IL20* and *IL24* may influence susceptibility to PPP. Genetic variations within the *IL19* gene cluster may affect both psoriasis vulgaris and PPP. Despite the small group of patients the results are encouraging for carrying out studies with larger groups.
- We found IL22 to be significantly associated with vitiligo, especially with the active stage of vitiligo, as shown by the results of mRNA expression and supported by the results of protein levels in sera. Signal transductions of other investigated cytokines seem to be regulated on the expression level of their receptor complex subunits.
- Since IL22 appears to be a central cytokine mediating inflammatory responses in both psoriasis and vitiligo, our data supports a hypothesis that different autoimmune skin diseases, such as psoriasis and vitiligo, may have similar pathogenic mechanisms.

THE FUTURE PROSPECTS

There is initial evidence that the identified *IL20RA* risk haplotype may be functionally relevant, because it carries altered binding sites for TFs involved in cell differentiation and immunological functions, but the exact effects of the *IL20RA* polymorphisms on transcriptional activation and function need further studies.

The results obtained in the PPP study encourage further studies with larger sample sizes to confirm the affect of *IL19* gene cluster variations on both, psoriasis vulgaris and PPP. Gene expression studies of IL10 family cytokines are needed to evaluate their role in the pathogenesis of PPP.

While studying group of vitiligo patients we found that IL22 may provoke inflammation, which leads to the destruction of melanocytes, but the actual role of IL22 in the pathogenesis of vitiligo remains to be better characterized.

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SUMMARY IN ESTONIAN

Psoriaasi ja vitiliigo geneetika IL10 perekonna tsütokiinide aspektist

Üldteoreetiline taust

Psoriaas ja vitiliigo on kompleksse taustaga nahahaigused ning vaatamata rohketele uurimustele pole tänaseni veel selgust, miks mõnedel meist kujunevad sellised haigused. Psoriaasi peamisteks kliinilisteks tunnuseks on erineva suurusega selgelt piiritletud, hõbedase ketuga kaetud punetavad infiltreeritud naastud; vitiliigo tunnusteks on ebaregulaarse kujuga depigmenteeritud laigud nahal ja ka juustes. Vaatamata kliinilise pildi erinevustele leitakse neil haigustel ka palju ühist. Eksponeeritus keskkonnamõjudele ja geneetiline eelsoodumus võivad viia normaalse immuunvastuse ja geeniekspresiooni tasakaalust välja, mis võibki viia autoimmuunhaiguse kujunemiseni. Sarnaselt immuunrakkudele on ka keratinotsüüdid ise võimelised produtseerima tsütokiine, milledest mitmed on põletikulised ja seetõttu otseselt immuunvastust mõjutavate omadustega. Interleukiin(IL)-10 olulist rolli on näidatud mitmetes immuunreaktsioonides ning selle tsütokiini ebatavaline ekspressioon on ilmnenud mitmete autoimmuunhaiguste puhul. Erinevad uurimistööd kinnitavad, et IL10 ise ja IL10 perekonna tsütokiinid on seotud epidermise funktsioonidega ja on seostatavad psoriaasiga; ka vitiliigoga patsientide veres ja kahjustunud nahas on tõusnud põletikuliste tsütokiinide ekspressioon. Hiljutised uurimistööd on näidanud, et Th17 rakkude poolt produtseeritavate tsütokiinide, sealhulgas IL22, ekspressiooni tõus psoriaatilises nahas põhjustab antimikroobsete peptiidide ja kemokiinide tootmise ning IL22 vahendab immuunsüsteemi ja epiteelrakkude omavahelist signaliseerimist. Sellistele uurimustele tuginedes otsustasime uurida IL10 perekonna IL10 perekonna tsütokiinide ja nende retseptorite rolle nahka kahjustavate autoimmuunhaiguste psoriaasi ja vitiliigo patogeneesis.

Uurimuse põhieesmärgid

* Selgitada SNP-de ahelduvusanalüüsiga välja *IL20RA* ja *IL20RB* geenide seos psoriaasiga.
* Täiendavalt analüüsida *IL20RA*, *IL22RA2* ja *IFNGR1* geene hõlmava 6q22–24 kromosomaalse piirkonna SNP-de seost psoriaasiga.
* Lähtudes olulistest seostest naastulise psoriaasiga selgitada, kas sarnased seosed on *IL19*, *IL20* ja *IL24* geenide SNP-de puhul leitavad ka palmoplantaarse pustuloosi (psoriaasi mädavillilise lokaliseeritud vormi) puhul.
* Hinnata nahast ja perifeerse vere monotsüütidest leitavate IL10 perekonna tsütokiinide mRNA-de ja vereseerumist leitavate sama perekonna valkude võimalikku olulisust vitiliigo puhul.

Metoodika

IL10 perekonna tsütokiinide ja nende retseptorite võimalike seoste tuvastamiseks naastulise psoriaasi, vitiliigo ja palmoplantaarse pustuloosiga kasutati erinevaid meetodeid: SNP-de, mRNA ja valkude analüüs. Patsientide gruppidesse kuulusid suguluses mitteolevad naastulise psoriaasiga patsiendid, palmoplantaarse pustuloosiga patsiendid ja vitiliigoga patsiendid.

Leidmaks *IL20RA* ja *IL20RB* geenipiirkondade seoseid naastulise psoriaasiga, genotüpeeriti ARMS-PCR meetodiga 254 naastulise psoriaasiga patsiendi ja 224 kontrollisiku verest eraldatud DNA proovidel vastavate geenide SNP-de alleelsed variandid. *IL20RA*, *IL22RA2* ja *IFNGR1* geene hõlmava 6q22–24 piirkonna täiendaval analüüsil kasutati 548 naastulise psoriaasiga patsiendil ja 328 kontrollisikul SNP-de genotüpeerimiseks SNPlex meetodit. *IL19*, *IL20* ja *IL24* geenide alleelsete variantide seostamisel palmoplantaarse pustuloosiga genotüpeeriti 43 palmoplantaarse pustuloosiga patsienti ja 149 isikut kontrollgrupist.

Vitiliigo ja IL10 perekonna geenide ekspressioonitasemete seoste leidmiseks eraldati 20 vitiliigoga patsiendi perifeersetest mononukleaarsetest rakkudest ja 15 vitiliigoga patsiendi naha biopsiatest RNA, millelt sünteesitud cDNA analüüsiti TaqMan-QRT-PCR meetodiga. Ekspressioonide tulemusi patsientidelt võrreldi vastavate tulemustega kontrollgrupis (34 isiku perifeerse vere mononukleaarsed rakkud ja 18 isiku naha biopsiad). Uuritavate geenide ekspressioonide tasemed normaliseeriti samadest proovidest määratud koduhoidjageeni *HPRT1* ekspressiooni suhtes. Valgutasemete määramiseks vitiliigoga patsientide ja kontrollgrupi vereseerumites kasutati IL10-, IL20- ja IL22-spetsiifilisi ELISA analüüsikomplekte.

Tulemused

Psoriaasihaigete ja kontrollgrupi *IL20RA* ja *IL20RB* genotüüpide võrdlemisel ilmnes kahel SNP-l oluline seos psoriaasi perekondliku alavormiga: harvemate alleelide sagedus oli selle alavormi puhul langenud SNP-del rs1167846 (18.8 vs. 25.9%; P = 0,05, OR 0,66; 95% CI 0,44–1,00) ja rs1167849 (15,8% vs. 23%; P < 0,05, OR 0,63; 95% CI 0,41–0,98). SNPde ahelduvusanalüüsi põhjal moodustavad SNP-d rs1184860, rs1167846 ja rs1167849 tugevalt aheldunud *IL20RA* haplotüüpide ploki ($|D'|$ 0,77–0,93). *IL20RA* haplotüüp CGG näitas tugevat seost psoriaasiga, olles psoriaasihaigete grupis võrreldes kontrollgrupiga oluliselt sagedasem (7,6 vs. 2,6%; $P_{nom} < 0,001$, $P_{adj} < 0,01$; OR 3,14; 95% CI 1,61–6,14), samas kui haplotüüp TTG oli sagedasem kontrollisikute grupis (0,9 vs. 4,4%; $P_{nom} < 0,001$, $P_{adj} < 0,01$; OR 0,20; 95% CI 0,07–0,55). Transkriptsioonifaktorite seondumiskohtade mudelitest leidsime, et *IL20RA* SNP-de rs1167846 ja rs1184860 alleelsed variandid modifitseerivad transkriptsioonifaktorite võimalikke seondumiskohti. Genotüpeeritud *IL20RB* SNP-d olid kõik omavahel tugevalt aheldunud ($|D'|$ 0,83–0,95) ja moodustasid ühe haplotüüpide ploki, kuid *IL20RB* haplotüüpidel ei ilmnenud olulisi seoseid psoriaasiga.

Kromosomaalse piirkonna 6q22–24 genotüüpide täiendaval uurimisel leidsime, et *IL20RA* geeni kodeerivas alas asuva SNP rs1342642 harvemat alleeli T esines psoriaasihaigete grupis oluliselt harvemini kui kontrollgrupis (25% vs. 40.0%; OR 0,48; 95% CI 0,37–0,64). *IL20RA* geeni laiendatud haplotüübid CTCG ja CCCG (rs1342642, rs1184860, rs1167846 ja rs1167849) esinesid psoriaasihaigete grupis võrreldes kontrollgrupiga sagedamini (vastavalt OR 1,99; 95% CI 1,48–2,66 ja OR 4,02; 95% CI 1,73–9,33). Sama ploki haplotüüp TTCG esines sagedamini kontrollgrupis (OR 0.10; 95% CI 0,05–0,20).

Otsides *IL19*, *IL20* ja *IL24* genotüüpide seoseid palmoplantaarse pustuloosiga, leidsime, et *IL20* geeni SNP rs2981573 harvemat alleeli G esines palmoplantaarse pustuloosiga haigete grupis oluliselt harvemini kui kontrollgrupis (14% vs. 24%; $P < 0,05$). *IL20* haplotüübi GAA (rs2981572, rs2981573 ja rs2232360) esinemissagedus palmoplantaarse pustuloosiga haigetel oli oluliselt suurem võrreldes kontrollisikutega (1,66% vs. 7,7%; $P < 0,05$, OR 2,39; 95% CI 1,17–4,86); haplotüüp GGG oli aga sagedasem kontrollgrupis (11,5% vs. 21,4%; $P < 0.05$; OR 0,48; 95% CI 0,23–0,98). *IL19* ja *IL20* geenide laiendatud haplotüüp GACACCGGAA oli palmoplantaarse pustuloosiga patsientidel oluliselt sagedasem võrreldes kontrollidega (12,9% vs. 6%; $P < 0.05$; OR 2,31; 95% CI 1,05–5,10). Samuti oli patsientidel sagedasem *IL20* ja *IL24* laiendatud haplotüüp CGGGT (39,5% vs. 28%; $P < 0.05$; OR 1,68; 95% CI 1,02–2,78), kuid CAAAC haplotüüp näitas kaitsvat efekti, olles sagedasem kontrollisikutel (1,2 vs. 9,7; $P < 0;01$; OR 0.12; 95% CI 0,02–0,82).

Vitiligoga patsientidel ja kontrollgrupil läbi viidud IL10 perekonna interleukiinide ja nende retseptorite ekspressiooniuuring tuvastas, et vitiligoga patsientide kahjustumata nahas oli *IL20RA* mRNA 0,78 korda madalam ja kahjustunud nahas 0,74 korda madalam kontrollisikutel määratud tasemest. IL20RA tase oli oluliselt kõrgem vitiligo aktiivse vormi puhul, võrreldes stabiilse vormiga: vitiligopatsientide kahjustunud nahas 1,6 korda kõrgem tase, kahjustumata nahas 1,8 korda kõrgem tase (mõlemal $P < 0,05$). Võrreldes ekspressioonitasemeid vitiligohaigete ja kontrollisikute perifeerse vere mononukleaarsetest rakkudest, leidsime vitiligohaigete rakkudest 5,5 korda kõrgema *IL22* mRNA taseme võrreldes kontrollisikutega. mRNA taseme muutust toetas ka IL22 valgu taseme määramine vereseerumist, mis näitas vitiligogrupi puhul 2,4 korda kõrgemat IL22 valgu taset võrreldes kontrollgrupiga ($P < 0,0001$). Vitiligo aktiivse ja stabiilse vormi võrdluses näitasid stimuleeritud rakud aktiivse vormi puhul 7,3 korda kõrgemat *IL22* mRNA ekspressiooni kui stabiilse vormi rakud. *IL24* mRNA tase, mis stimuleerimata rakkude puhul oli vitiligo- ja kontrollgrupi vahel sarnane, oli peale stimuleerimist kontrollgrupis 1,6 korda kõrgem kui vitiligogrupis ($P < 0,01$).

Järeldused

IL20RA ja *IL20RB* geenide alleelsete variantide assotsiatsioonianalüüsil leidsime, et SNP-dest rs1184860, rs1167846 ja rs1167849 moodustuv haplotüüp CCG tõstab oluliselt psoriaasi riski (OR 3,14; 95% CI 1,61–6,14), samas kui haplotüüp TTG näitas kaitsvat mõju (OR 0,20; 95% CI 0,07–0,55). *IL20RA* riskihaplotüübiga kaasnevad muutused transkriptsioonifaktorite seondumiskohtades võivad mõjutada nende faktorite poolt kontrollitavat rakkude diferentseerumist ja immunoloogilisi funktsioone. SNP rs1342642 *IL20RA* geeni kodeerivas alas (L382F) näitas tugevat protektiivset efekti psoriaasi suhtes.

Tehes *IL19*, *IL20* ja *IL24* geenide assotsiatsiooniuuringu väikesel grupil palmoplantaarse pustuloosiga patsientidel, leidsime statistiliselt olulisi seoseid, mis lubavad oletada, et selle piirkonna alleelsed varieeruvused võivad mõjutada vastuvõtlikkust palmoplantaarsele pustuloosile. Esialgsete seoste kinnitamiseks on vajalik uuring suurema patsientide grupiga.

Ekspressioonianalüüsil leidsime, et IL22 on oluliselt seotud vitiliigo, eriti just aktiivse staadiumi avaldumisega. mRNA tulemusi kinnitas ka valgu taseme uuring vereseerumites. Kuna IL22 on tsentraalne põletikuline tsütokiin ka psoriaasi puhul, toetavad meie tulemused hüpoteesi, et erinevatel immunoloogilise taustaga nahahaigustel on sarnane patogeneetiline mehhanism.

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Publications

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2005–2010	Tartu ülikool, doktoriõpe, neuroteadused

Erialane teenistuskäik

2005–2009	Quattromed AS, teadur, tootmisjuht
2009–…	Icosagen AS, immunoanalüüsi labori juhataja

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1. Kõks S, Kingo K, **Rätsep R**, Karelson M, Silm H, Vasar E. Combined haplotype analysis of the interleukin-19 and -20 genes: relationship to plaque-type psoriasis. Genes Immun. 2004 Dec;5(8):662–7. PubMed PMID: 15496954.
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6. Kingo K, Aunin E, Karelson M, Philips MA, **Rätsep R**, Silm H, Vasar E, Soomets U, Kõks S. Gene expression analysis of melanocortin system in vitiligo. J Dermatol Sci. 2007 Nov;48(2):113–22. Epub 2007 Jul 25. PubMed PMID: 17651944.
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9. **Rätsep R**, Kingo K, Karelson M, Reimann E, Raud K, Silm H, Vasar E, Kõks S. Gene expression study of IL10 family genes in vitiligo skin biopsies, peripheral blood mononuclear cells and sera. Br J Dermatol. 2008 Dec;159(6):1275–81. Epub 2008 Aug 19. PubMed PMID: 18717682.
10. Kingo K, Mössner R, Traks T, **Rätsep R**, Raud K, Reimann E, Krüger U, Silm H, Vasar E, Reich K, Kõks S. Further association analysis of chr 6q22–24 suggests a role of IL-20RA polymorphisms in psoriasis. J Dermatol Sci. 2010 Jan;57(1):71–3. Epub 2009 Nov 17. PubMed PMID: 19926456.
11. Philips MA, Kingo K, Karelson M, **Rätsep R**, Aunin E, Reimann E, Reemann P, Porosaar O, Vikeså J, Nielsen FC, Vasar E, Silm H, Kõks S. Promoter polymorphism -119C/G in MYG1 (C12orf10) gene is related to vitiligo susceptibility and Arg4Gln affects mitochondrial entrance of Myg1. BMC Med Genet. 2010 Apr 8;11:56. PubMed PMID: 20377893; PubMed Central PMCID: PMC2856544.
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DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

1. **Sirli Raud.** Cholecystokinin$_2$ receptor deficient mice: changes in function of GABA-ergic system. Tartu, 2005.
2. **Kati Koido.** Single-nucleotide polymorphism profiling of 22 candidate genes in mood and anxiety disorders. Tartu, 2005.
3. **Dzhamilja Safiulina.** The studies of mitochondria in cultured cerebellar granule neurons: characterization of mitochondrial function, volume homeostasis and interaction with neurosteroids. Tartu, 2006.
4. **Tarmo Areda.** Behavioural and neurogenetic study of mechanisms related to cat odour induced anxiety in rodents. Tartu, 2006.
5. **Aleksei Nelovkov.** Behavioural and neurogenetic study of molecular mechanisms involved in regulation of exploratory behaviour in rodents. Tartu, 2006.
6. **Annika Vaarmann**. The studies on cystatin B deficient mice: neurochemical and behavioural alterations in animal model of progressive myoclonus epilepsy of Unverricht-Lundborg type. Tartu, 2007.
7. **Urho Abramov.** Sex and environmental factors determine the behavioural phenotype of mice lacking CCK_2 receptors: implications for the behavioural studies in transgenic lines. Tartu, 2008.
8. **Hendrik Luuk.** Distribution and behavioral effects of WFS1 protein in the central nervous system. Tartu, 2009.
9. **Anne Must.** Studies on molecular genetics of male completed suicide in Estonian population. Tartu, 2009.
10. **Kaido Kurrikoff**. Involvement of cholecystokinin in chronic pain mechanisms and endogenous antinociception. Tartu, 2009.
11. **Anu Aonurm-Helm.** Depression-like phenotype and altered intracellular signalling in neural cell adhesion molecule (NCAM)-deficient mice. Tartu, 2010.
12. **Silva Sütt.** Role of endocannabinoid system and *Wfs1* in regulation of emotional behaviour: behavioural, pharmacological and genetic studies. Tartu, 2010.
13. **Mari-Anne Philips.** Characterization of *Myg1* gene and protein: expression patterns, subcellular localization, gene deficient mouse and functional polymorphisms in human. Tartu, 2010.