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IV–V The author collected the data and participated in the data interpretation and preparation of the manuscript.

ABBREVIATIONS

AAA	antiadrenal antibodies
Ab	antibody
ACTH	adrenocorticotropic hormone
AGRP	agouti-related protein
AITD	autoimmune thyroid disease
ANA	antinuclear antibodies
APS	autoimmune polyendocrine syndrome
ASIP	agouti signalling protein
BCL2	B-cell lymphoma 2
BH4	tetrahydrobiopterine
b-HLH-zip	basic/helix-loop-helix/leucine zipper
BSA	body surface area
cAMP	cyclic adenosine monophosphate
CD8	cluster of differentiation 8
cDNA	complementary deoxyribonuclein acid
CNS	central nervous system
CNV	copy number variation
CREB1	cAMP responsive element binding protein 1
Ct	cycle threshold
DCT	dopachrome tautomerase
DLQI	dermatology life quality index
DNA	deoxyribonuclein acid
ES-Q	Emotional State Questionnaire
F	female
GHQ	general health quality
HCS	healthy controls
HLA	human leukocyte antigen
HPRT-1	hypoxanthine phosphoribosyl-transferase-1
H_2O_2	hydrogen peroxide
HPA	hypothalamic-pituitary-adrenal axis
ICAM-1	intercellular adhesion molecule
IFN- γ	interferon-gamma
IgG	immunoglobulin G
IL-2	interleukin-2
LEF1	lymphoid enhancer-binding factor 1
LS	lesional vitiligo skin
M	male
MARK	mitogenactivated protein kinase
MCHR1	melanine concentrating hormone receptor 1
MCR	melanocortin receptor
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke syndrome
MGB	minor groove binder

MHC II	main human complex
MITF	microphthalmia-associated transcription factor
MSH	melanocyte-stimulating hormone
NLS	non-lesional vitiligo skin
PASI	psoriasis area and severity index
PBMCs	peripheral blood mononuclear cells
PCA	parietal cells antibodies
PDCD4	programmed cell death-4
PKA	protein kinase A
PI3K	phosphoinositide 3-kinase
p70(S6)K	70 kDa ribosomal protein S6 kinase
QoL	quality of life
QRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
RF	reumatoid factor
RNA	ribonuclein acid
SNP	single nucleotide polymorphism
SOX	sex-determining region of the Y chromosome-like box
Th	T-helper
TNF-α	tumor necrosis factor alfa
TPO-ab	thyroid peroxidase antibody
TRP1	tyrosinase-related protein-1
TYR	tyrosinase
TYRP1	tyrosinase-related protein-1
USF1	upstream transcription factor 1
UVR	ultraviolet radiation
VGICC	Vitiligo Global Issues Consensus Conference

1. INTRODUCTION

Vitiligo is a common acquired depigmentary disease characterized by white patches of the skin, hair and mucous membranes due to selective destruction of melanocytes (Lerner and Nordlund 1978). The prevalence of vitiligo is 0.5% to 1% in Europe and the United States, but ranges from 0.1% in China up to 8.8% in some regions of India (Taieb and Picardo 2007; Alikhan *et al.* 2011; Lu *et al.* 2007; Behl and Bathia 1972). Vitiligo affects both genders equally and in 50% of the cases the disease appears before the age of 20 (Nordlund and Majumder 1997). Up to now clinical types of vitiligo have not been uniformly understood and reliably classified. The simplest classification divides vitiligo into segmental and nonsegmental types, based on the clinical course and different distribution of the pattern (Koga 1977). The classification of vitiligo created by Hann and Nordlund is more complicated and divides vitiligo into localized and generalized types with several subtypes (Hann and Nordlund 2000d). In 2011 the classification of vitiligo was revised in Vitiligo Global Issue Consensus Conference (VGICC). By the consensus segmental vitiligo should be classified separately from all the other types of vitiligo; the term "vitiligo" can be used as an umbrella term for all non-segmental types of vitiligo, including "mixed vitiligo" with combined segmental and non-segmental types, which is considered as a subgroup of vitiligo (Ezzedine *et al.* 2012). The disease has a longlasting chronic course and its phenotype can change during the years (Liu *et al.* 2005; Zaima and Goga 2002). Most cases are sporadic, the incidences of familial vitiligo range from 10% in China up to 74% in India (Wang *et al.* 2013; Misri *et al.* 2009). Epidemiological studies have revealed that vitiligo is inherited in a non-Mendelian, multifactorial and polygenic pattern with incomplete penetrance (Alkhateeb *et al.* 2003; Sun *et al.* 2006). Vitiligo can associate with several other autoimmune diseases, including thyroid disease, diabetes, pernicious anemia, lupus, Addison's disease, rheumatoid arthritis, *alopecia areata*, psoriasis, asthma, chronic urticaria, inflammatory bowel disease (Dawber 1970; Cunliffe *et al.* 1968; Gould 1985; Alkhateeb *et al.* 2003; Zhang *et al.* 2009). The same diseases occur more frequently in patients' first-degree relatives (Alkhateeb *et al.* 2003; Laberge *et al.* 2005). Vitiligo can be present in all types of autoimmune polyendocrine syndromes (APS), but most frequently it is associated with thyroid autoimmune disease in APS type three (Betterle 2004, Amerio *et al.* 2006). Several autoantibodies (thyroid, gastric parietal cells, antiadrenal cortex, antinuclear) have been detected in the sera of the patients with vitiligo without clinical manifestation of another autoimmune disease during the years (Kahaly 2009; Daneshpazhooh *et al.* 2006; Farrokhi 2005; Alkhateeb *et al.* 2003; Hann *et al.* 1993).

Vitiligo does not cause notable physical impairment but affects human sense of well-being and self-esteem in a different way: it predisposes social isolation, depression, difficulties in sexual relationship (Mechri *et al.* 2006; Firooz *et al.* 2004, Wang *et al.* 2011; Porter *et al.* 1990). Dermatology life quality index (DLQI) of vitiligo varies from 4.4 in Indonesia to 17.1 in Saudi Arabia (Chan *et*

al. 2012; Al-Mubarak *et al.* 2011). Females and people with dark skin colour are more stigmatised (Linthorst *et al.* 2009; Ongenae *et al.* 2005b; Dolatshahi *et al.* 2008). Quality of life (QoL) issues that arise from the loss of the pigment are the following: choice of clothes, use of sunblocks, use of camouflage, avoidance of activities and negative reactions by other people (Ongenae *et al.* 2005a, b; Tanioka *et al.* 2010). Some studies have pointed out psychiatric comorbidity in vitiligo, the prevalence in Europe and in India stays between 25–35% (Picardi *et al.* 2001; Mattoo *et al.* 2002).

The exact pathogenesis of vitiligo is still unknown. Several pathophysiological theories have been proposed to explain the dysfunction or loss of melanocytes in the epidermis of vitiligo patients (Alikhan *et al.* 2011). These include an autoimmune mechanism, biochemical mechanism (auto-cytotoxicity and oxidative stress), neurohumoral mechanism and a decreased melanocyte survival hypothesis (Ongena *et al.* 2003; Schallrauter *et al.* 1999a; Moretti *et al.* 2002; Lee *et al.* 2005a). The convergence theory comprises all these mechanisms and states that autoimmunity, mutations, stress, accumulation of toxic compounds, altered cellular environments and impaired melanocyte migration all contribute to pathogenesis of vitiligo and it depends on the patient which mechanism dominates (Le Poole *et al.* 1993).

Dysfunction of the neural regulation has been shown in different autoimmune and inflammatory disorders. Dysregulation of the nervous system at a central or local level may damage melanocytes also in vitiligo. The importance of central neuroendocrine system emerges at the onset or aggravation of pigment loss during or after increased stress period in vitiligo up to 62.5% of the cases (Firooz *et al.* 2004). The melanocortin system is a part of the neuroendocrine system. It consists of five melanocortin receptors (MCR1-MCR5), four agonists of these receptors (α-, β-, γ- melanocyte-stimulating hormone and adrenocorticotropic hormone) and two antagonists of melanocortin receptors: agouti and agouti-related protein (Gantz and Fong 2003). The expressional level of the peptides of the melanocortin system is highest in the brain, but it involves also peripheral tissues, including the skin, where these peptides are secreted by the keratinocytes and melanocytes (Kono *et al.* 2001). The melanocortin system completes different functions in the skin associated with response to the stress, it regulates pigmentation, inflammation, exocrine secretion, analgesia, immunomodulation and temperature control (Gantz and Fong 2003; Slominski *et al.* 2000; Eves and Haycock 2010).

Vitiligo as a disease has received little scientific attention in the Nordic countries. There are no previous studies analyzing the mRNA ecxpression levels of the melanocortin system in vitiligo.

2. REVIEW OF THE LITERATURE

2.1. Epidemiology, clinical types, triggering factors and course of vitiligo

2.1.1. Epidemiology of vitiligo

Vitiligo is a common acquired depigmentary disease characterized by white patches of the skin, hair and mucous membranes due to selective destruction of melanocytes (Lerner and Nordlund 1978). The disease occurs with an estimated prevalence of 0.15–3.0% in Western Europe and in Turkey, around 1% in the United States, 2.6–4% in Mexico, 0.1–8.8% in India and 0.1–0.6% in China (Dawber 1968; Perrot 1973; Arican 2003; Taieb and Picardo 2007; Alikhan *et al.* 2011; Ruiz Maldonnado 1977; Canizares 1960; Handa and Kaur 1999; Koranne and Sachdeva 1988; Behl and Bathia 1972; Lu *et al.* 2007; Xu *et al.* 2002; Wang *et al.* 2013). The latest review based on published epidemiological studies has shown an estimated worldwide prevalence of vitiligo from 0.06% to 2.28% (Krüger and Schallreuter 2012). Gradually increasing age-specific prevalence has been demonstrated in vitiligo in China and Denmark: 0.1% in the age-group of 0 to 9 years, 0.9% in the age group of 60 to 69 years, 1.7% in the age-group of 70 years and over (Howitz *et al.* 1977; Wang *et al.* 2013). Most studies have demonstrated that vitiligo affects both sexes equally, except hospital-based studies where women have dominated (Alkhateeb *et al.* 2003; Boisseau-Garsaud *et al.* 2001). Such female superiority can be explained by the desire to look good and willingness to seek medical help in order to satisfy this desire. The loss of pigmentation can start at any age during lifetime and the onset age varies significantly between the studies in different regions of the world. The usual age of the onset of vitiligo is between 10 and 30 years and in half of the cases the disease starts before the age of 20 years, in 70–80% of the cases before the age of 30 years (Zhang *et al.* 2004; Liu *et al.* 2005; Nordlund and Lerner 1982). In Denmark the onset of vitiligo was observed most frequently between the ages of 40 and 60 years (Howitz *et al.* 1977). Positive family history influences the onset of vitiligo and in those cases the disease appears earlier than in sporadic cases (Ando *et al.* 1993; Laberge *et al.* 2005). A hospital-based study in Greece revealed the peak prevalence in females in the first decade of life and in males in the fifth decade. Vitiligo was significantly more prevalent in young women at the age up to 30 years and in middle-aged men at the age 31–60 years (Kyriakis *et al.* 2009).

2.1.2. Clinical types of vitiligo

There is no uniform understanding of vitiligo clinical types and exact pathophysiology of the disease remains unknown. Different approaches have been tried in classification of vitiligo but no etiopathogenetic classification as a gold standard has been worked out yet (Hercogova *et al.* 2012). The simplest

classification divides vitiligo into nonsegmental and segmental types based on different distribution pattern and clinical course of the disease (Koga 1977). A more complex clinical classification of vitiligo was created by Hann and Nordlund at the beginning of this century. According to the extension and pattern of pigment loss distribution, they differentiated the disease as localized, generalized and universal with several subtypes (Hann and Nordlund 2000d, Table 1).

Table 1. Clinical types and subtypes of vitiligo by Hann and Nordlund (2000).

Type of vitiligo	Subtype	Description
Localized vitiligo	Focal	One or more macules in one area, but not in a segmental distribution
	Segmental	One or more macules in one area in a segmental distribution
	Mucosal	Macules only in mucous membranes
Generalized vitiligo	Acrofacial	Macules on distal extremities and the face
	Vulgaris	Scattered macules with symmetrical distribution all over the body
	Mixed	Segmental and vulgaris, segmental and acrofacial, acrofacial and vulgaris
Universal vitiligo		Complete or nearly complete depigmentation

Localized clinical subtypes of vitiligo stay at minority in most clinical studies. Focal vitiligo is characterized by one or a few depigmented macules in a small area (10–15cm^2) without an obvious distribution pattern; it can be a subset of segmental or generalized vitiligo before the extension (Taieb and Picardo 2007). Focal vitiligo has been the first presentation of vitiligo from 27% to 70% of the cases in China and Nigeria (Zhang *et al.* 2009; Liu *et al.* 2005; Onunu and Kubeyinje 2003). VGICC suggests to call focal vitiligo as undertermined or unclassified type of vitiligo until more definitive classification can be made on clinical grounds, generally after 1–2 years of follow-up (Ezzedine *et al.* 2012). Mucosal subtype describes isolated depigmentation of the lips, oral or genital mucosa. It is quite rare and more characteristic, up to 10% of the cases, for individuals with dark skin phototype in India (Dave *et al.* 2002). Cases of vitiligo with long-lasting focal lesions or of pure mucosal depigmentation may remain simply "unclassifiable" vitiligo (Ezzedine *et al.* 2012). Segmental vitiligo is defined as an "acquired chronic pigmentation disorder characterized by white patches in unilateral distribution that may totally or partially match a dermatome, but not necessarily" (Taieb and Picardo 2007). The occurrence of this subtype of vitiligo varies also between the studies from 2.5% to 27.9% and is more characteristic of childhood vitiligo (Wang *et al.* 2013; Koga and Tango 1988; Halder *et al.* 1987; Jaisankar *et al.* 1992). Several patterns of segmental vitiligo in the facial region have been described in Korea (Hann *et al.* 2000a**;** Kim *et al.* 2011). Different clinical phenotypes of segmental vitiligo like unila-

teral segmental, bilateral segmental, Blaschkoid, mixed segmental with generalized subtypes were described lately in Belgium and India (van Geel *et al.* 2011a; Khaitan *et al.* 2012).

Generalized vitiligo is the most common type of vitiligo in adults and *vitiligo vulgaris* has been the most frequent clinical pattern in many published papers, comprising 39–83% of the subjects (Handa and Kaur 1999; Dogra *et al.* 2005; Liu *et al.* 2005; Mason and Gawkrodger 2005). By the definition of Vitiligo European Task Force *vitiligo vulgaris* is an acquired chronic pigmentation disorder characterized by white patches, often symmetrical distribution, which usually increase in size with time, corresponding to a substantial loss of functioning epidermal and sometimes hair follicle melanocytes (Taieb and Picardo 2007). The sites of predilection for this type are hands, wrists, knees, elbows, axilla, groin, neck, head and body orifices (Gawkrodger *et al.* 2008; Kovacs 1998). Acrofacial vitiligo involves distal parts of the limbs and circumferential pattern of the face orifices. This clinical subtype of vitiligo comprises 3–12% in clinical studies (Handa and Kaur 1999; Wang *et al.* 2013; Zhang *et al.* 2009). Acrofacial vitiligo has been reported between 17% and 35% of the subjects in India but a similar pattern called lip-tip vitiligo (vitiligo on the lips and distal parts of the fingers) was described additionally in 7% of the cases as well (Dave *et al.* 2002; Martis *et al.* 2002). The mixed type of vitiligo is a new and quite confusing entity, as it comprises different clinical types of vitiligo at the same time on the patients body; the number of such cases reported in the literature is too small to understand the real nature of this type (Mulekar *et al.* 2006; van Geel *et al.* 2011a). In universal type nearly complete (BSA > 80%) or complete depigmentation of the skin appears. This type presents from 0.5% to 18% of the cases in vitiligo (Dogra *et al.* 2005; Wang *et al.* 2013). VGICC classification recommends to use the term "vitiligo" further as an umbrella term for all non-segmental forms of vitiligo including "mixed vitiligo", in which segmental and non-segmental vitiligo are combined and it is considered now as a subtype of vitiligo (Ezzedine *et al.* 2012, Table 2).

Table 2. Vitiligo Global Issues Consensus Conference classification (2012).

Type of vitiligo	Subtypes
Nonsegmental vitiligo	Acrofacial
	Mucosal (more than one site)
	Generalized
	Universal
	Mixed
	Rare subtypes
Segmental vitiligo	Uni-, bi- or plurisegmental
Undetermined/unclassified vitiligo	Focal
	Mucosal (only one site)

Some studies have found correlation between the disease onset and different clinical types in vitiligo. Segmental vitiligo has been more characteristic of children and young people (median age at onset 12–16 years) and was reported only 0–4.5% in adults (Khaitan *et al.* 2012; Liu *et al.* 2005; Berti *et al.* 2011; Dogra *et al.* 2005; Mason and Gawkrodger 2005).

By classical morphology of vitiligo the lesions are discrete, uniformly milky-white macules with round, oval or irregular shape in size from millimeters to many centimeters, surrounded by normal or hyperpigmented skin (Ortonne 2008). The disease is asymptomatic, but in some cases itching or burning sensation can present (Behl and Bhatia 1972). Vitiligo prefers the sites of friction that are normally hyperpigmented like face, dorsal surface of the hands, axilla, nipples, sacrum, ingvinal and anogenital regions and the surface of joints. Leukotrichia develops in 3.7–47.3% of the cases of vitiligo (Handa and Kaur 1999; Dogra *et al.* 2005; Akrem *et al.* 2008). Mucosal involvement has been reported between 7.4–74% in generalized types of vitiligo (Onunu *et al.* 2003; Hann *et al.* 1997).

There are some rare morphological variations of vitiligo: vitiligo ponctue', tri-, quadri- and pentachrome vitiligo, blue vitiligo and inflammatory vitiligo (Ortonne 2008; Halder and Taliaferro 2008). Vitiligo ponctue' shows discrete and confetti-like depigmented macules on normal or hyperpigmented skin (Ortonne 2008). Trichrome vitiligo is recognized by the presence of a narrow to broad intermediate colour zone between a vitiligo macule and normal pigmented surrounding (Hann *et al.* 2000b). Quadrichrome vitiligo shows four colours and macular perifollicular or marginal hyperpigmentation in the lesion and it usually occurs in darker skin phenotypes (Behl *et al.* 2003). Pentachrome vitiligo has been described in black-skinned people with variation of five colours: white, tan, brown, blue-gray hyperpigmentation and the normal skin (Fargnoli and Bolognja 1995; Zhang and Zhu 2013). Blue vitiligo has a blue-gray hue as a mark of postinflammatory hypermelanosis (Ivker *et al.* 1994). Inflammatory vitiligo could be caused by an agressive therapy and it has an erythematous, raised border in a vitiligo macule with frequent itching or burning (Ortonne *et al.* 1979).

2.1.3. Triggering factors and course of vitiligo

In clinical studies 10–76% of the patients with generalized vitiligo can name the factors that have preceded the onset or exacerbation of the disease: skin injury, emotional stress, sunburn, hormonal changes (Behl and Bhatia 1972; Koo *et al.* 1996; Verma 2009; Manolache and Benea 2007). These factors were not associated with segmental type of vitiligo (Hann and Lee 1996; Khaitan *et al.* 2012). Psychological stress is the most frequently reported triggering factor in vitiligo, experienced by 47–65% of the patients before the onset of pigment loss (Boisseau-Garsaud *et al.* 2001; Firooz *et al.* 2004; Agarwal 1998; Manolache and Benea 2007). Very often pigment loss starts after skin trauma and prefers then the sites of friction, scratches or scars; such a development is known as the Koebner

phenomenon, originally described in psoriasis. The Koebner phenomenon has been observed in 5–62% of the cases in vitiligo and it is associated with the active stage of the disease and extensive pigment loss (Hann *et al.* 1997; Barona *et al.* 1995; van Geel *et al.* 2012a). Sunburn has been recorded in up to 29% of those cases when the external factor was named, but suspected chemicals are seldom confirmed to be the triggers in vitiligo (Vrijman *et al.* 2013).

The course of vitiligo is unpredictable and depends on the clinical type of the disease. Generally vitiligo begins insidiously in sun-exposed areas and has a slowly progressive lifelong course with partial spontaneous repigmentation in some of the lesions (Alikhan *et al.* 2011). Several studies have demonstrated a slow course in vitiligo with pigment loss under 20% of body surface area (BSA) up to 90% of the cases with median disease duration 4–6 years (Daneshpazhooh *et al.* 2006; Handa and Kaur 1999). BSA under 5% was shown in 78% of the cases of vitiligo in China with a median disease duration 18 months (Zhang *et al.* 2009). Segmental vitiligo has revealed a rapid onset in Japan with median progression during 0.7 years over the affected dermatomal area, after that its activity will cease and the disease stays stable for the rest of the patient's life (Koga and Tango 1988). In clinical studies progression is defined as an active stage of the disease characterized by the increase of the size or number of depigmented macules during three months prior to examination. Progression has been reported between 70% and 90% of the cases in generalized vitiligo (Dave *et al.* 2002; Chun and Hann 1997). The clinical phenotype of vitiligo can change during the progression of the disease (Liu *et al.* 2005; Zaima and Koga 2002). Some clinical characteristics like family history of vitiligo, nonsegmental type, long duration of the disease, the Koebner phenomenon and mucosal involvement have been associated with progression of vitiligo in Korea and India (Hann *et al.* 1997; Dave *et al.* 2002). These authors found also correlation between the initial site on posterior trunk, hands or feet and the disease progression in vitiligo (Hann *et al.* 1997). Vitiligo on the hands has been associated with disease progression to the face (Chun and Hann 1997). The presence of halo nevi and leukotrichia have been the factors associated with progression of segmental type to mixed type in vitiligo (Ezzedine *et al.* 2012). Spontaneous repigmentation has been also described in vitiligo, usually it is partial, nonstable and occurs during summer-time in sun-exposed sites.

2.2. Associated diseases, the presence of autoimmune polyendocrine syndrome (APS) and autoantibodies in vitiligo

2.2.1. Associated diseases in vitiligo

Based on autoimmune hypothesis in pathogenesis of vitiligo, there are numerous studies focused on associated diseases in vitiligo patients and also in their families. At present there is a long list of different concomitant disorders and syndromes described in vitiligo, some of them are more common and some are less reported (Alikhan *et al.* 2011, Table 3).

Table 3. Disorders and syndromes associated with vitiligo (presented in alphabetical order).

More commonly associated disorders	Addison's disease, alopecia areata, atopic dermatitis, autoimmune thyroid disease, chronic urticaria, diabetes mellitus, halo nevi, hypoacusis, ichthyosis, ocular abnormalities, pernicious anaemia, psoriasis, rheumatoid arthritis
Less commonly associated disorders	Acrokeratosis paraneoplastica Bazex, Alezzandrini syndrome, APS1, asthma, ataxia-teleangiectasia, deafness, dysgammaglobulinaemia, inflammatory bowel disease, Kabuki syndrome, Kaposi sarcoma, melanoma, MELAS syndrome, morphea, multiple sclerosis, myasthenia gravis, nonmelanoma skin cancer, pemphigus vulgaris, sarcoidosis, Schmidt syndrome, systemic lupus erythematosus, Turner syndrome, twenty-nail dystrophy, Vogt-Koyanagi-Harada syndrome

Concomitant autoimmune diseases have been reported in 2% of the cases in Nigeria and India, 20% in USA and Japan, 55% in Turkey in vitiligo (Onunu and Kubeyinje 2003; Poojary 2011; Alhateeb *et al.* 2003; Narita *et al.* 2011; Akay *et al.* 2010). Patients with familial vitiligo have demonstrated high prevalence of autoimmune diseases in China (12%) and in the USA (37%) (Zhang *et al.* 2009; Laberge *et al.* 2005). In vitiligo autoimmune association has been more frequent in females, in generalized type of vitiligo and in acrofacial location (Amerio *et al.* 2010; Barona *et al.* 1995; Klisnick *et al.* 1998). The increase of incidences of autoimmune thyroiditis, pernicious anemia, Addison's disease, systemic lupus erythematosus and inflammatory bowel disease in vitiligo have been described in the Caucasian population. The increase was not found in the frequencies of *alopecia areata*, multiple sclerosis, *myasthenia gravis*, psoriasis, rheumatoid arthritis, scleroderma and Sjogren's syndrome (Alkhateeb *et al.* 2003). In Japan autoimmune thyroid disease (12%) and *alopecia areata* (5.3%) have been the most often described autoimmune diseases

of the patients with generalized vitiligo (Narita *et al.* 2011). In China the prevalence of associated disorders in vitiligo has been investigated and compared with the prevalence of these disorders in general population in two large-scale studies with subject numbers 3742 and 6516 (Liu *et al.* 2005; Zhang *et al.* 2009). Both studies showed lower to equal prevalence of hyper- and hypothyroidism (0.8–1.3% *vs* 1.2% and 0.6–1.1% *vs* 1.0%, respectively) and higher prevalence of rheumatoid arthritis (2.2% *vs* 0.3%), chronic urticaria (0.9% *vs* 0.1%), *alopecia areata* (0.3–0.9% *vs* 0.09%) in vitiligo compared with the prevalence in general population. The prevalence of psoriasis (0.3% *vs* 0.1%) and *ichtyosis* (0.3% *vs* 0.1%) was also increased in vitiligo compared with the prevalence in general population in China (Zhang *et al.* 2009). In Germany the higher prevalence of autoimmune thyroid disease (7.8%) and the higher presence of thyroid antibodies was demonstrated in vitiligo, but random prevalence of diabetes mellitus, *alopecia areata*, psoriasis, pernicious anaemia and atopic eczema was shown compared with the control population. No eye or inner-ear involvement in vitiligo was found (Schallreuter *et al.* 1994). In spite of that, some studies have demonstrated hypoacusis in up to 20% of the cases and ocular abnormalities like uveitis, iris and retinal hypopigmentation in up to 40% of the cases of vitiligo (Hong *et al.* 2009; Gopal *et al.* 2007; Cowan *et al.* 1986). These changes are usually mild and do not cause noticeable visual or hearing impairment for the patients. The primary reports from India and Nigeria revealed low incidence of thyroid disease (0.5–0.6%), but the cases could be underdiagnosed as they screened only subjects with clinical features (Handa and Kaur 1999; Onunu and Kubeyinje 2003). Later a higher prevalence of hypothyroidism (12%), anemia (20%), diabetes mellitus (16%) and *alopecia areata* (7.4%) was reported in a comparative study also in India (Gopal *et al.* 2007). A hospital-based study from India demonstrated co-occurrence of vitiligo with skin associated autoimmune diseases like *morphoea, alopecia areata, discoid lupus erythematosus*, and *pemphigus erythematosus* (Poojary 2011). Halo nevi are described in up to 31% of the cases in vitiligo, they are more characteristic of children and young adults (van Geel et al. 2011c; Barona *et al.* 1995). In addition, familial vitiligo probands have demonstrated also higher prevalence of diabetes mellitus (3.3% *vs* 0.7%) and asthma (0.8% *vs* 0.4%) (Liu *et al.* 2005; Zhang *et al.* 2009). In the USA the members of "multiplex" vitiligo families had elevated frequencies of autoimmune thyroid disease (21.4% *vs* 1.9%), rheumatoid arthritis (3.8% *vs* 0.9%), psoriasis (5.3% *vs* 1%), adult-onset insulin-dependent diabetes (3.8% *vs* 0.6%), pernicious anemia (2.3% *vs* 0.2%) and Addison's disease (0.3% *vs* 0.005%), but not alopecia areata (2.8% *vs* 1.8%), than in general population (Laberge *et al.* 2005).

In most studies familial cases of vitiligo stay between 8% and 36%, but cases have been varied from 3.4% to 74% in India (Handa and Kaur 1999; Gopal *et al.* 2007; Poojary 2011; Misri *et al.* 2009). In the USA the frequency of vitiligo among siblings of nonselected vitiligo probands is 6.1% and the frequency of vitiligo among siblings of familial vitiligo probands is 38.9%; that shows clearly the heritable risk of non-Mendelian pattern in vitiligo (Laberge *et*

al. 2005). Family history has been more characteristic of generalized vitiligo, but in 11.5% of the cases of familial segmental vitiligo the clinical type of the relative was also the segmental vitiligo in Korea (Hann and Lee 1996). Autoimmune diseases of close relatives of vitiligo have been reported in up to 46% of the cases in Italy (Amerio *et al.* 2010). Very high prevalence of autoimmune diseases among the siblings of vitiligo probands with other autoimmune diseases (41%) compared with the prevalence among the siblings of probands with only vitiligo (14%) has been demonstrated in the USA (Laberge *et al.* 2005). In the USA the elevation of autoimmune thyroiditis, pernicious anemia, Addison's disease, systemic lupus erythematosus and inflammatory bowel disease has been demonstrated in the first-degree relatives in vitiligo (Alhateeb *et al.* 2003). In familial cases of vitiligo the first-degree relatives have shown higher prevalence of chronic urticaria (0.6% *vs* 0.1%), rheumatoid arthritis (0.6% *vs* 0.3%) and psoriasis (0.2% *vs* 0.1%) in China (Zhang *et al.* 2009).

2.2.2. The presence of APS and autoantibodies in vitiligo

Vitiligo can be part of all types of autoimmune polyendocrine syndromes (APS), but most often it occurs with autoimmune thyroid disease (Hashimoto's thyroiditis and Graves' disease) in APS-3. A systematic review of published papers shows a median prevalence of 14.3% and a relative risk 2.5 of autoimmune thyroid disease, 20.8% and 5.2 of the presence of thyroid-specific autoantibodies in patients with vitiligo compared with nonvitiligo subjects, and the risk seems to increase with age (Vrijman *et al.* 2012). Autoimmune thyroid disease has been the most frequently reported concomitant disease in children (5–24%) and up to 34% in adults with vitiligo (Pagovich *et al.* 2008; Kurtev and Dourmishev 2004; Mason and Gawkrodger 2005). Usually vitiligo is the first disease of APS-3 and precedes autoimmune thyroid disease by 4–35 years (Zettinig *et al.* 2003; Betterle and Zanchetta 2003; Amerio *et al.* 2006, 2010). Thyroid antibodies have been detected in 18–50% of the cases of vitiligo in comparative studies of children and adults (Kurtev and Dourmishev 2004; Hegedus *et al.* 1994; Daneshpazhooh *et al.* 2006). Vitiligo occurs in 4.5–20% of the cases in APS-2 and 0–25% of the cases in APS-1 (Papadopoulos *et al.* 1990; Dittmar and Kahaly 2003; Perniola 2000; Myhere 2001). A study from Germany has pointed out the most often combined autoimmune diseases in patients with APS: first type diabetes and autoimmune thyroid disease in 41%, AITD and Addison's disease in 15%, first type diabetes and vitiligo in 10% and AITD and vitiligo also in 10% of the cases (Kahaly 2009). Thyroid autoimmune disease, autoimmune gastritis and *alopecia areata* have been the most common autoimmune diseases associated with vitiligo in cases of APS-3 in Italy (Amerio *et al.* 2010). All other endocrine and nonendocrine autoimmune diseases combined with vitiligo not classified under the first three types of APS, like *alopecia areata*, rheumatoid arthritis, pernicious anemia, type 1 diabetes, bullous pemphigoid, can now be classified under APS type 4 (Betterle and Zanchetta 2003; Amerio *et al.* 2010).

Autoantibodies have been detected in the range from 2% to 70% in different studies in vitiligo (Barona *et al.* 1995; Bystryn 1989). In addition to thyroid antibodies, there are several other antibodies detected in the sera of patients with vitiligo with widely variable results. Long follow-up of patients with APS has confirmed that "silent" autoantibodies may precede clinical manifestation of the disease 3–30 years and they are predictive for the development of autoimmune disorder in the future (Dittmar and Kahaly 2003). Antimelanocyte antibodies were detected in 31% of the cases and RF in 11% of the cases in vitiligo and the difference was statistically important compared with the controls in Iran (Farrokhi *et al.* 2005). In Germany the difference of antibodies against cell surface antigens of melanocytes was not shown between vitiligo and controls (Schallreuter *et al.* 1994).

2.3. Quality of life and emotional state in vitiligo

Pigment loss is not merely a cosmetic problem as it influences psychological and social well-being and impairs the quality of life. Patients with vitiligo have reported that healthcare professionals do not take their condition seriously (Ongenae *et al.* 2004). A survey among dermatologists in the Netherlands brought out that only 16% of the specialists had used treatment in vitiligo, most of them had simply provided information about the disease (Njoo *et al.* 1999). QoL studies allow us to better understand psychological and disease-related problems in vitiligo nowadays. More than a half of the respondents of the Vitiligo Society in the United Kingdom stated that vitiligo had moderately or severely affected their QoL (Talsania *et al.* 2010). Vitiligo does not cause notable physical impairment but affects human self-esteem in different ways: predisposes social isolation, depression, difficulties in sexual relationship and suitability for marriage (Mechri *et al.* 2006; Firooz *et al.* 2004; Wang *et al.* 2011; Porter *et al.* 1990). People with dark skin colour are more stigmatised (Linthorst *et al.* 2009; Dolatshahi *et al.* 2008). QoL issues that arise from the loss of the pigment are the following: choice of clothes, use of sunblocks, use of camouflage, avoidance of activities; and negative reactions of others (Ongenae *et al.* 2005a,b; Tanioka *et al.* 2010; Porter 2000). Some studies have stressed psychiatric comorbidity in patients with vitiligo with the prevalence of 25–35% in Europe and in India (Kent and al-Abadie 1996; Picardi *et al.* 2000; Mattoo *et al.* 2001, 2002). Adjustment disorders have been revealed in more than half of the vitiligo cases in India, but it does not correlate well with the severity or extension of depigmentation (Mattoo *et al.* 2001, 2002).

Many studies have used Dermatology Life Quality Index (DLQI) questionnaire for measuring the impact of QoL in vitiligo. DLQI was worked out by Finley and Khan in 1994 for assessment of QoL in patients with cutaneous diseases (Finlay and Khan 1994). DLQI has given widely variable results in vitiligo. DLQI has shown small effect on patients' life in Indonesia (4.4), U.K (4.8) and Belgium (4.95) and moderate effect in Japan (5.9), Germany (7.0),

Iran (7.1–8.2), France (7.2) and China (8.4) (Chan *et al.* 2012; Kent and al-Abadie 1996; Ongenae *et al.* 2005b; Tanioka *et al.* 2010; Radtke *et al.* 2009; Dolatshahi *et al.* 2008; Aghaei *et al.* 2004; Mashayekhi *et al.* 2010; Kostopoulou *et al.* 2009; Wang *et al.* 2011). According to DLQI, the impact of vitiligo on QoL has been very large in India (10.7) and Saudi Arabia (14.7–17.1) (Parsad *et al.* 2003; Al Robaee 2007; Al-Mubarak *et al.* 2011). Higher DLQI scores are associated with darker skin as the contrast of skin colour in dark-skinned people attracts more unwanted attention, which is emotionally disturbing and displeasing. A survey of Malaysian vitiligo patients has shown that the mean DLQI was not associated with gender or age or disease duration or family history of vitiligo (Wong and Baba 2012). Several studies have reported lower QoL in women, as they are more emotional and more sensitive about their appearance (Radtke *et al.* 2009; Mashayekhi *et al.* 2010; Belhadjali *et al.* 2007; Borimnejad *et al.* 2006). Comparative studies have pointed out that subjects with vitiligo are more disturbed in symptoms and feelings, leisure and daily activities (Wang *et al.* 2011; Ongenae *et al.* 2005b). Vitiligo has no impact on such activities like going to school or work, as pigment loss does not cause physical disability (Wang *et al.* 2011; Ongenae *et al*. 2005b; Radtke *et al.* 2009; Wong and Baba 2012). It affirms that subjects with vitiligo are embarrassed and do not feel free in dressing and spending time with other people as they have to choose clothes to hide skin imperfection. This group of patients (40%) belongs to the group of "poor adjustment" by the Porter, they have lower self-esteem and they experience difficulties in coping well with vitiligo (Porter *et al.* 1978, 1979). Most studies have emphasized the association between disease extension and lower QoL (Wang *et al.* 2011; Dolatshahi *et al.* 2008; Ongenae *et al.* 2005b; Parsad *et al.* 2003; Belhadjali *et al.* 2007; Ingordo *et al.* 2012). Vitiligo on uncovered areas like the face and hands has a serious negative impact on QoL, as stated by several investigators (Aghaei *et al.* 2004; Wong and Baba 2012; Ingordo *et al.* 2012). Camouflage has decreased the mean DLQI score in women with vitiligo by 1–1.5 score-points and is highly suggested for those who have pigment loss on uncovered areas (Tanioka *et al.* 2010; Ongenae *et al.* 2005a).

2.4. Etiopathogenesis of vitiligo. Neuroendocrine dysregulation in vitiligo

2.4.1. Etiopathogenesis of vitiligo

The exact etiology and pathogenesis of vitiligo is still unknown. Scientists do not know the real cause of damage of melanocytes and their disappearance from affected skin as has been shown in immunohistological and ultrastructural studies. Experimental studies have supported several pathways how melanocytes can disappear: an apoptotic process, a necrotic process and melanocytorrhagy (van den Wijngaard *et al.* 2000a; Gauthier *et al.* 2003a,b; Le Poole

et al. 2004). According to the theory of melanocytorrhagy, melanocytes are weakly attached and a minor friction can induce upward migration and their loss (Gauthier *et al.* 2003a,b). There is also evidence that melanocytes are never completely absent in the lesional epidermis of vitiligo and they are able to recover their functionality even after long duration of the disease (Tobin *et al.* 2000). Several pathophysiological hypotheses have been proposed to explain the dysfunction and/or loss of melanocytes in epidermis in vitiligo (Alikhan *et al.* 2011). These include an autoimmune hypothesis, biochemical hypothesis (auto-cytotoxicity and oxidative stress) and decreased melanocyte survival hypothesis (Ongena *et al.* 2003; Schallrauter *et al.* 1999a; Moretti *et al.* 2002; Lee *et al.* 2005a).

Studies have confirmed the increase of autoimmune thyroid disease and several other autoimmune diseases in vitiligo probands and their close relatives, indicating the shared genetic etiologic links between vitiligo and these disorders (Alhateeb *et al.* 2003; Laberge *et al.* 2005; Zhang *et al.* 2009). Cellular immunity is altered in vitiligo and it can be combined with a humoral response (Passeron and Ortonne 2005; Alikhan *et al.* 2011). Immunohistological studies have shown an increase of $CD8^+$ T cells and an increase of CD8/CD4 ratio of T cells in the perilesional skin in vitiligo (Le Poole *et al.* 1996; Lili *et al.* 2012). $CD8^+$ T cells express the skin-homing cutaneous lymphocyte antigen, cytotoxic proteins perforin and granzyme B, IL-2 (CD25), MHC II (HLA-DR), and they secrete IFN-γ, which increases ICAM-1 expression and promotes T cell migration to the skin (van den Wijngaard *et al.* 2000b; Sharquie *et al.* 2004; Badri *et al.* 1993; Wankowicz-Kalinska *et al.* 2003). Regulatory T cells that modulate Th1 and Th17 response are reduced in vitiligo skin and their function is impaired, that allows activation of cytotoxic T cells (Klarquist *et al.* 2010; Lili *et al.* 2012). Experimental studies in Smyth line chickens, an animal model of vitiligo, have shown melanocytes' death via apoptosis induced by cytotoxic T lymphocytes (Wang and Erf 2004). Recently a mouse model with a phenotype of vitiligo has been developed by using melanocyte-specific $CD8^+$ T cells (Harris *et al.* 2012).

Auto-cytotoxic mechanism encompasses metabolic deregulation that can lead to toxic damage of the melanocytes (Hann and Chun 2000b). These toxic metabolites, derived from the environment (phenols or quinones) or produced as byproducts of altered melanin synthesis pathway, accumulate and damage melanocytes of genetically susceptible individuals (Schallreuter *et al.* 1994b). Increased level of 6-tetrahydrobiopterine (6BH4), a cofactor of phenylalanine hydroxylase, leads to an accumulation of byproducts 7BH4 and H_2O_2. Accumulation of H_2O_2 in turn influences the increase of 6-biopterine that is cytotoxic for cells at high concentration (Schallreuter *et al.* 1999a; Schallreuter *et al.* 2001). The disturbed biosynthesis of catecholamines has been under research in vitiligo as the patients with segmental vitiligo have shown the increase of catecholamines and their metabolites in the sera and urine (Morrone *et al.* 1992; Cucci *et al.* 2000, 2003). The high level of catecholamines may be directly cytotoxic to the melanocytes via oxidative stress caused by increased level of

dopamine, with the result of the apoptosis of melanocytes (Chu *et al.* 2006). Decreased level of catalase enzyme and increased level of H_2O_2 in the skin, but also defective calcium uptake can alter oxidative balance in vitiligo (Schallreuter *et al.* 1999a). Melanocytes at the margin of lesional skin have shown high sensitivity to oxidative stress in vitiligo (Koca *et al.* 2004; Jimbov *et al.* 2001). There is growing evidence that stressed melanocytes can initiate an immune response. The first proof of this hypothesis was the activation of dendritic cells after melanocytes damage by 4-tertiary butyl phenol exposure (Kroll *et al.* 2005). Phenols can activate the unfolded protein response in melanocytes that leads to upregulation of IL-6 and IL-8 (Toosi *et al.* 2012). This upregulation could be the missing link between oxidative stress and immune response in vitiligo as it can reduce the modulation of regulatory T cells (Passeron and Ortonne 2012). Another experimental study has found that subtoxic levels of H_2O_2 could also stimulate secretion of IL-6 by cultured epidermal melanocytes and generate autoimmune response (Yao *et al.* 2012).

The theory of decreased melanocyte survival hypothesis says that vitiligo can be caused by the abnormality of melanocytes or of surrounding keratinocytes-producing factors necessary for the survival and functioning of the melanocytes (Ongena *et al.* 2003; Moretti *et al.* 2002). The paucity of keratinocyte-derived stem cell factor influences also the synthesis of melanin and predisposes the apoptosis of melanocytes (Lee *et al.* 2005a). However, there exists a convergence theory that comprises the previously reported mechanisms and states that autoimmunity, mutations, stress, accumulation of toxic compounds, altered cellular environments and impaired melanocyte migration all contribute to pathogenesis of vitiligo and it depends on the patient which mechanism dominates (Le Poole *et al*. 1993).

2.4.2. Neuroendocrine dysregulation in vitiligo

Dysfunction of the neural regulation has been shown in different autoimmune and inflammatory disorders. Dysregulation of the nervous system at central or local level may damage melanocytes also in vitiligo. The importance of central neuroendocrine system emerges at the onset or aggravation of pigment loss during or after increased stress period in vitiligo up to 62.5% of the cases (Firooz *et al.* 2004).

The melanocortin system is a part of the neuroendocrine system that acts as a coordinator and executor of responses to stress. Melanocortin system consists of five melanocortin receptors (MCR1–MCR5), four agonists of these receptors [(α-, β- and γ- melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH)] and two antagonists of melanocortin receptors: agouti (ASIP) and agouti-related protein (AGRP) (Gantz and Fong 2003). The components of the melanocortin system and their interactions are shown in Figure 1.

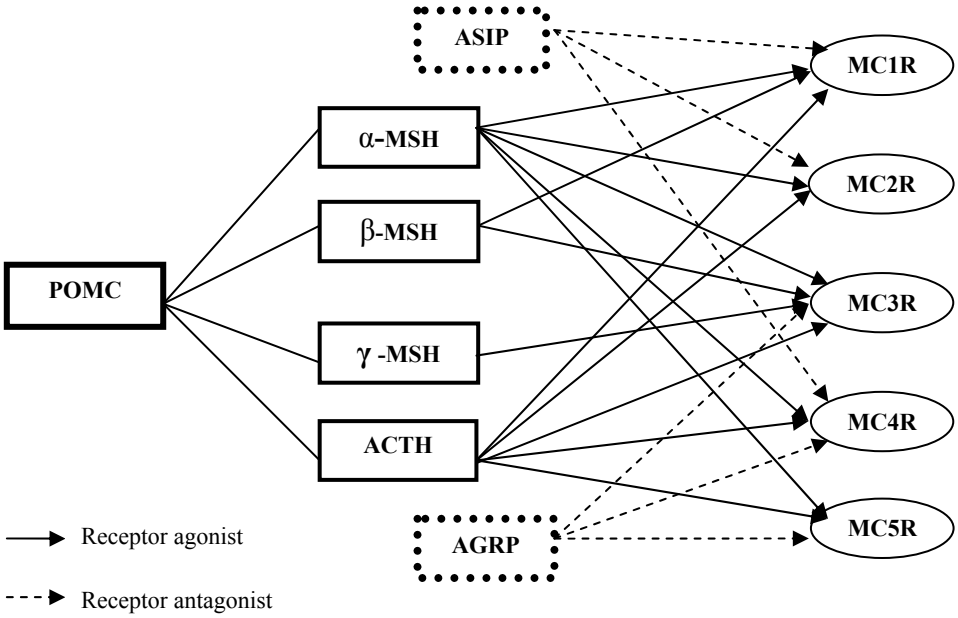

Figure 1. The components of the melanocortin system: POMC, proopiomelanocortin; ASIP, agouti signalling protein; α-MSH, alpha-melanocyte-stimulating hormone; b-MSH, beta-melanocyte-stimulating hormone; γ-MSH, gamma-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; AGRP, agouti-related protein; MC1R, melanocortin receptor 1; MC2R, melanocortin receptor 2; MC3R, melanocortin receptor 3; MC4R, melanocortin receptor 4; MC5R, melanocortin receptor 5.

α-, β-, γ- MSH and ACTH are the posttranslational products of POMC prohormone gene. Posttranslational processing of the POMC prohormone is tissue specific (Pritchard *et al.* 2002). The expressional level of the peptides of the melanocortin system is highest in the brain, but it involves also peripheral tissues, including the skin (Kono *et al.* 2001; Pritchard *et al.* 2002). In the skin POMC mRNA has been detected in keratinocytes and melanocytes of the basal layer of epidermis and in pilosebaceous units (Kono *et al.* 2001). MC1R is the most highly expressed melanocortin receptor in melanocytes but expressed also in keratinocytes, fibroblasts, endothelial cells and antigen-presenting cells (Roberts *et al* 2006). MC1R is activated mainly by α-MSH and ACTH, the activation is weak by β- and γ- MSH. The main function of MC1R is the regulation of melanogenesis, skin and hair pigmentation (Gantz and Fong 2003). MC2R is expressed mainly in adrenal cortex and adipocytes, to a lesser extent in skin, including keratinocytes and melanocytes (Eves and Haycock 2010). This receptor is highly specific for ACTH and its main function is the regulation of steroidogenesis (Getting 2006). MC3R is expressed in gastrointestinal tract, kidneys, heart, placenta and is activated by all the melanocortins but especially by γ-MSH (Chhaljani 1996; Eves and Haycock 2010). There are no previous studies confirming the expression of MC3R in human skin (Eves and Haycock 2010).

This receptor is strongly involved in energy homeostasis (Gantz and Fong 2003). MC4R is predominantly expressed in CNS, to a lesser extent in dermal papilla cells. MC4R is activated mostly by α-MSH and ACTH. The binding of melanocortins with MC4R is similar to MC1R. MC4R regulates energy homeostasis and erectile function (Getting 2006; Bohm *et al.* 2006). MC5R is widespreadly expressed in many tissues, including adipocytes, sebaceous and sweat gland cells, skin mast cells (Chhaljani 1996; Slominski *et al.* 2000). MC5R has high affinity for α-MSH, lesser activated by ACTH and no affinity for γ- MSH (Gantz and Fong 2003). The main function of MC5R is the regulation of sebaceous gland secretion (Zhang *et al.* 2006). ASIP and AGRP are two endogenous paracrine signalling molecules with MCR subtype selectivity (Dinulescu and Cone, 2000; Rana 2003). ASIP is highly expressed in adipocytes but expression is also detected in many other tissues, including dermal papilla cells (Wilson *et al.* 1995). ASIP is high-affinity antagonist of α-MSH at MC1R that results in inhibition of cAMP mediated activation of melanogenesis (Slominski *et al.* 2000). ASIP is also a strong inhibitor of MC2R and MC4R (Dinulescu and Cone, 2000). AGRP is the second endogenous antagonist of melanocortin receptors that shares sequence homology with ASIP (Ollmann *et al.* 1997). AGRP is expressed mainly in the brain and adrenal tissue and it blocks the binding of α-MSH to MC3R and MC4R (Yang *et al.* 1999; Gantz and Fong 2003). The main function of AGRP is to control feeding and body weight (Adan and Gas 2003). Its action to melanogenesis in humans has not been verified yet. Melanocortin system is involved in determining skin and hair phenotypes, different skin inflammatory disorders and malignancies (Slominski *et al.* 1993; Sturm 2002). The studies have demonstrated a reduction in the level of the POMC peptide α-MSH both in the lesional skin and serum of vitiligo patients (Thody *et al.*1998; Pichler *et al.* 2006; Spencer *et al.* 2007). Low expression of α-MSH in the lesional skin of vitiligo patients has resulted from decreased expression of the peptide rather than a reduction in melanocyte numbers (Graham *et al.* 1999). Few studies have focused on analyzing connections between vitiligo and polymorphisms of genes of melanocortin system. Na *et al.* could not prove the association between variations of MC1R and ASIP genes and susceptibility to vitiligo in Korean patients (Na *et al.* 2003). Szell *et al.* demonstrated in the Hungarian vitiligo patients that Arg160Trp MC1R polymorphism might have protective effect against vitiligo (Szell *et al.* 2008). There are no previous studies analyzing the mRNA ecxpression levels of the melanocortin system in group of vitiligo patients.

Several different pathways are modulating the melanogenesis in humans. The most important pathway in human melanocytes through which signal from the melanocortin system reaches the melanogenesis enzymes tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1) and dopachrome tautomerase (DCT) is the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway, modulated by Wnt and mitogenactivated protein kinase (MAPK) pathways (deOliveira *et al.* 1996; Konda *et al.* 1994; Slominski *et al.* 2004). The melanocortin receptors activate adenylate cyclase giving rise to the intracellular cAMP

concentration and further activation of protein kinase A (Gantz and Fong 2003; Ao *et al.* 1998). Protein kinase A activates cAMP responsive element binding protein 1 (CREB1) through phosphorylation that increases the expression level of microphthalmia-associated transcription factor (MITF) (Sassone-Corsi 1998; Levy *et al.* 2006). MITF regulates positively the expression of TYR, TYRP1 and DCT increasing the transcription of these enzymes (Levy *et al.* 2006; Park *et al.* 2002; Yasumoto *et al.* 1997). MITF is an important gene of melanogenesis as it controls the differentiation of melanocytes. The studies have demonstrated that ectopic expression of MITF converts fibroblasts to the cells with melanocyte characteristics (Steingrimsson *et al.* 2004; Tachibana *et al.* 1996). MITF up-regulates the expression of the antiapoptotic factor B-cell lymphoma 2 (BCL2), the deletion of MITF in melanocytes results in an extensive apoptosis of these cells (McGill *et al.* 2002). Lymphoid enhancer-binding factor 1 (LEF1) is a transcription factor that participates in the Wnt signalling pathway (Eastman and Grosschedl 1999). LEF1 acts as a regulator of pigmentation in melanocytes, exerting its effects on MITF in two ways: it activates the transcription of the MITF gene and MITF can activate its own promoter together with LEF1 (Saito *et al.* 2002). The interaction of MITF and LEF1 also regulates the expression of DCT (Yasumoto *et al.* 2002). The upstream transcription factor 1 (USF1) belongs to the *basic/helix-loop-helix/ leucine zipper* (b-HLH-zip) family similarly with MITF (Corre and Galibert 2005). USF1 goes through phosphorylation by 38 kDa MAP kinase (p38) in melanocytes, binds to the promoter of TYR and activates its transcription regulating the pigmentation (Galibert *et al.* 2001). Promoter of DCT contains also the USF1 binding element (Schwahn *et al.* 2005). Phosphoinositide 3-kinase (PI3K) regulates the cycle, growth, differentiation and apoptosis of the cells (Garcia *et al.* 2006). PIK3K has an influence to melanogenesis by inhibiting the activation of p38 in melanocytes (Saha *et al.* 2006). Phosphoinositide 3-kinase/70 kDa ribosomal protein S6 kinase (PI3K/p70(S6)K) has coded by the gene RPS6KB1 and it participates in the regulation of translation, immune response and tissue reparation (Berven and Grouch 2000). Both kinases have an ambivalent effect on the melanogenesis pathways that depends on the concentration of the growth factors (Bohm *et al.* 1995). cAMP has an inhibitory effect on the PI3K/ p70(S6)K pathway (Busca *et al.* 1996).

3. AIMS OF THE STUDY

The general aim of this study was to obtain additional knowledge in vitiligo as the main pigmentary disorder in humans.

The specific aims of the study were:

1. To observe clinical aspects and the presence of autoantibodies in vitiligo.
2. To examine the impact of quality of life and emotional state in vitiligo and to compare these results with psoriasis patients and healthy volunteers.
3. To elucidate the regulative role of the melanocortin system in pathogenesis of vitiligo, assessing the expressional level of mRNA of melanocortin system peptides and their receptors but also the gene expression profile of the intracellular signalling pathways linking the melanocortin system with enzymes involved in melanogenesis in the skin in vitiligo and healthy controls.

4. SUBJECTS AND METHODS

4.1. Ethical considerations

The Ethics Review Committee on Human Research of the University of Tartu approved the study protocols and the informed consent forms. All participants signed the written informed consent.

4.2. Characteristics of the study participants

Adult vitiligo patients (≥18 years) across the country were asked to participate in the study, which was advertised in the newspaper. Patients with vitiligo were also recruited from among the attendants of the outpatient department of Dermatology Clinic at Tartu University Hospital. Subjects with psoriasis were collected mainly from the inpatient department of the clinic and control subjects were enrolled from among the health care personnel, students and attendants who had been consulted in the clinic with benign skin tumours. All the subjects who had participated in the study were Estonian-speaking Caucasians.

Table 2. Subjects' characteristics in the published papers.

Paper I	Vitiligo 155 (44 M, 111 F) Mean age 44.9 years, age range 18–82 years 141 tested for autoantibodies	
Paper II	Familial vitiligo 51 (15 M, 36 F) Mean age 41.7 years, age range 18–82 years Sporadic vitiligo 135 (42 M, 93 F) Mean age 45.5 years, age range 18–77 years 173 tested for autoantibodies	
Paper III	Vitiligo 54 (22 M, 32 F), mean age 36.6 years Mean disease duration 11.3 years Psoriasis 57 (27 M, 30 F), mean age 40.0 years Mean disease duration 18.6 years	Controls 57 (23 M, 34 F) Mean age 39.7 years
Paper IV	Vitiligo 31 (22 F, 9 M) Mean age 49.2 years, age range 22–75 years Type: focal 4 (F), segmental 1 (F), generalized 25 (17 F, 8 M), universal 1(M) Stage: active 22 (16F, 6M); stable 9 (6 F, 3 M) Mean disease duration: 19.2 years	Controls 24 (17 F, 7 M) Mean age 33.9 years Age range 21–67 years
Paper V	Vitiligo 39 (26 F,13 M) Mean age 49.4 years, age range 22–77 years Type: focal 7 (5 F, 2 M), segmental 1 (F), generalized 30 (20F, 10M), universal 1 (M) Stage: active 26 (18 F, 8 M), stable 13 (8 F, 5 M) Mean disease duration: 19.0 years	Controls (MITF-M, p38, PI3K, P70 (S6)K): 31 (22 F, 9 M) Mean age 38.1 years Age range 22–67 years Controls (CREB1, BCL2, LEF1, USF1): 18 (10 F, 8 M) Mean age 37.6 years Age range 22–66 years

4.3. Methods

4.3.1. Skin examination and disease status classification

The patients were examined at the Dermatology Department of Tartu University by an experienced dermatologist. The diagnosis of vitiligo was based on characteristic loss of skin pigmentation with typical localization and the examination under Wood's lamp. Wood's lamp is a device that helps better visualize pigment changes in the epidermis. The clinical types of vitiligo were classified as focal (one or a few macules in a nondermatomal distribution), segmental (unilateral segmental distribution), acrofacial (distal parts of the extremities and face), *vulgaris* (scattered over the body), universal (over 90% depigmentation). The palm method was used by calculating body surface area (BSA). The presence of leukotrichia, the Koebner phenomenon and halo nevi was noted. The evolution of vitiligo was considered active when new lesions appeared and the existing lesions had increased in size over the past 3 months. Vitiligo was considered stable when depigmentation had not increased during the last 3 months. Skin phototype, based on classification by Fitzpatrick, was determined (Fitzpatrick *et al.* 1967). Psoriasis area and severity index (PASI) was used in cases of psoriasis.

4.3.2. Data collection

Five different questionnaires were used for data collection in the study. The questionnaire about demographic and clinical data, including age, sex, nationality, skin phototype, site of onset of vitiligo, duration, the Koebner phenomenon, leukotrichia, mucous involvement, triggering factor, sunburn, mechanical trauma, concomitant disease, familial history of vitiligo, spontaneous repigmentation, previous treatment, diseases in the family, was completed by a dermatologist in cases of vitiligo. The same questionnaires without vitiligo-specific questions were used for healthy controls and psoriasis patients. Dermatology Life Quality Index (DLQI) questionnaire validated Estonian version was implemented to calculate the impact on quality of life of all the study subjects (Finley and Khan 1994). Ten items (Q1-2 symptoms and feelings, Q3-4 daily activities, Q5-6 leisure, Q7 work/school, Q8-9 personal relationships, Q10 treatment) were answered in DLQI questionnaire in a short time on four-point scale (0–3) with the sum of the score from 0 to 30. Emotional State Questionnaire (ES-Q) validated Estonian version was applied to assess the traits of depression and anxiety (Aluoja *et al.* 1999). ES-Q contains 28 items and is answered on a five-point scale (0–4). Eight items (sadness, loss of interest, inferiority, self-accusation, hopelessness about future, thought of suicide, feeling of loneliness and inability to be joyful) with cut-off score 12 was used for depressiveness; six items (fast irritation or getting angry, anxiety or fear, feeling of stress or inability to relax, too much worry about many things, physical restlessness and being very easily frightened) with cut-off score 12 for general anxiety; five items (sudden attacks of panic with palpitation, lack of air, feeling of fainting or other

frightening physical symptoms; fear to be alone away from home; fear of public places or streets; fear to faint among crowd; fear to be on a bus, tram, train or car) with cut-off scores 7 for panic disorder; two items (fear to be centre of attention, fear to communicate with strangers) with cut-off scores 4 for social phobia; four items (passivity or fatigue, decreased ability to concentrate or to pay attention, rest does not give strength; and fast tiredness) with cut-off scores 6 for asthenia; three items (difficulties in falling asleep, restlessness or fragmentary sleep; and awakening too early) with cut-off scores 5 for sleep disturbance.

4.3.3. The assessment of autoantibodies

14 ml blood was collected once for the detection of TPO-Ab, PCA, ANA, AAA and RF in the sera. The autoantibodies assessment was performed in the United Laboratories at Tartu University Hospital. ANA, PCA and AAA were determined by indirect immunofluorescence method, using rat liver as an antigenic substrate for ANA and mouse stomach as and antigenic substrate for the detection of PCA. AAA was detected on normal human adrenal tissue (Uibo *et al.* 1998; Betterle *et al.* 2007). Polyclonal rabbit anti-human IgG conjugated to fluorescein isothiocyanate (DAKO, Glostrup, Denmark) was used as a secondary antibody for the detection of all these antibodies. CLIA, Immulite 2000 (Siemens Medical Solutions Diagnostics) was used for the detection of TPO-Ab) and immunoturbidimetric assay, Cobas Integra 400 Plus (Roche Diagnostics GmbH) for the assessment of RF (Laulu *et al.* 2007).

4.3.4. Collection of skin samples

Two skin biopsies (Ø 4 mm) were obtained from each patient with vitiligo: one from the central part of involved skin and another from the non-sun-exposed uninvolved skin. One skin biopsy (Ø 4 mm) from nonsun-exposed skin was taken from healthy control subjects. The non-sun-exposed skin was defined as the skin never exposed to ultraviolet radiation (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 or inner side of the upper arm. Biopsies were instantaneously snap-frozen in liquid nitrogen and stored at -80 ºC until further use.

4.3.5. Complementary deoxyribonuclein acid (cDNA) synthesis

Total ribonuclein acid (RNA) was isolated from tissues using RNeasy Fibrous 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 ºC. For each RT-PCR reaction,

500 ng of total RNA were converted into cDNA. The reverse transcription reactions were performed with a reverse transcriptase (SuperScript III; Invitrogen Corp., Carlsbad, CA, USA) and poly (T18) oligonucleotides in accordance with the manufacturer's instruction. The reaction mixtures were incubated at 65 ºC for 5 min, at 0 ºC for 1 min, at 50 ºC for 90 min, at 70 ºC for 5 min and finally stored at -80 ºC.

4.3.6. Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR)

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 ml reaction volumes in four replicates. The expression levels of genes under the study were detected applying TaqMan-QRT-PCR method using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). For the detection of the expression levels of studied genes we used TaqMan Assay-On-Demand FAM-labelled MGB-probe gene expression assay mixes (20X, Applied Biosystems, Foster City, CA, USA).

The assay mixes used were Hs00361403_g1 (AGRP), Hs00181770_m1 (ASIP), Hs00252036_s1 (MC3R), Hs00271877_s1 (MC4R), Hs00271882_s1 (MC5R), Hs00167051_m1 (TRP1) and Hs00157244_m1 (DCT). Hs00165156_m1 (MITF), Hs00178872_m1 (PIK3CB), Hs00177357_m1 (RPS6KB1), Hs00176247_m1 (MAPK14), Hs00231713_m1 (CREB1), Hs00273038_m1 (USF1), Hs00608023_m1 (BCL2), Hs00212390_m1 (LEF1). For the detection of the expression levels of MC1R and MC2R, we used gene-specific primers (MC1R: forward 5'-TGCGGCT GCATCTTCAAG-3', reverse 5'- TGATGGCATTGCAGATGATGA-3'; MC2R: forward 5'-CTCGATCCCACACCAGGAA- 3', reverse 5'-TGTGATGGCCCC TTTCATGT-3') and MGB-labelled probe (MC1R: FAM-TTCAACCTCTTTCTC GCC-NFQ; MC2R: FAM-TCT CCACCCTCCCCAGA-NFQ). For the detection of hypoxanthine phosphoribosyl-transferase-1 (HPRT-1) expression level, gene-specific primers (HPRT-1 exon 6, 5'-GACTTTGCTT TCCTTGGTCAGG-3'; HPRT-1 exon 7, 5'-AGTCTGGCTTATATCCAACACTTCG-3'; final concentrations 300 nM) and VIC-TAMRA-labelled probe (VIC-5'-TTTCACCAG CAAGCTTGCGACCTTGA-3'- TAMRA; final concentration 200 nM) were used. The expression level of POMC was detected using qPCR Core Kit for SYBR Green I (Eurogentec, Seraing, Belgium) and gene-specific primers (forward 5'-CTACGGCGGTTTCATGACCT-3', reverse 5'-CCCTCACTCGCCCT TCTTG-3', final concentrations 100 nM). Gene expression analysis was performed at the Department of Physiology at Tartu University.

4.4. Statistical analysis

Standard chi-square analysis was used for finding differences in comparing study subgroups of vitiligo. In analyzing quality of life the data following normal distribution were parametrically tested by unpaired t-test and the data not following the normal distribution by Mann-Whitney t-test. For quantification of mRNA expression of genes of melanocortin system comparative cycle threshold (Ct) method (δCt value) was used, 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; therefore Mann-Whitney U-test and Kruskal-Wallis test were used to test for differences between the groups. Correlation analysis was used to investigate relations between two parameters of one group. For measure of correlation the Spearman rank correlation was applied.

For statistical analysis Microsoft Office 2008 Excel (Microsoft Corporate, Redmond, WA, USA) and GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA) were applied. P-values were calculated for each variable. Significance was defined as a probability value less than 0.05.

5. RESULTS

5.1. Clinical aspects and the presence of autoantibodies in vitiligo

Clinical aspects of vitiligo were observed in 155 patients, 44 males (28.4%) and 111 females (71.6%). The mean age of the patients was 44.9 ± 16.3 years (age range 18–82 years) and the mean age of vitiligo onset was 28.5 ± 17.2 years (age range 2–76 years). There were no statistically important differences in the onset of vitiligo and disease duration between the males and females (P = 0.733 and P = 0.295, respectively). The mean age of onset for males was 28.2 ± 15.5 years and for females 28.6 ± 17.9 years. The mean duration of vitiligo was 16.9 ± 13.5 years (0.5–58 years) for all patients, 16.2 ± 14.6 years for males and 17.2 ± 13.1 years for females. Age distribution of onset of vitiligo by the gender is shown in Figure 2.

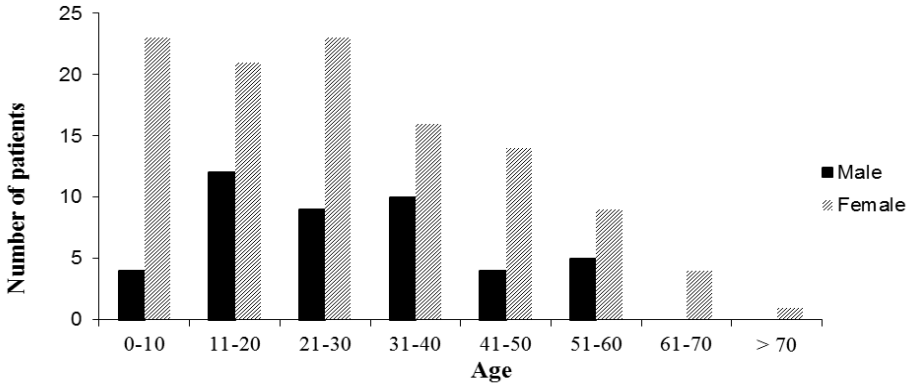

Figure 2. Distribution of the age of onset of females and males in vitiligo.

According to skin phototypes by Fitzpatrick, 51 subjects (32.9%) had skin phototype II, 102 subjects (65.8%) had skin phototype III and 2 subjects (1.3%) had skin phototype IV. *Vitiligo vulgaris* was the most common clinical type, observed in 126 cases (81.3%), followed by acrofacial, focal, segmental and universal vitiligo. The patients with segmental and universal vitiligo were affected earlier than those with other types of vitiligo. The comparison of the ages of onset in different subtypes of vitiligo revealed statistically important difference between segmental and acrofacial type ($P < 0.001$), segmental and focal type ($P < 0.05$), segmental and vulgar type ($P < 0.05$), universal and acrofacial type ($P < 0.05$) (Table 3).

Table 3. Mean ages of onset of vitiligo in the patients' groups with different clinical subtypes

Clinical subtype	Number (M/F)	(%)	Mean age (y) of the onset of vitiligo
Vulgaris	126 (30/96)	(81.3)	28.0
Universal	5 (2/3)	(3.2)	14.6
Acrofacial	12 (9/3)	(7.7)	44.2
Segmental	5 (1/4)	(3.2)	7.6
Focal	7 (2/5)	(4.5)	34.7
Total	155 (44/111)	(100)	28.5

Pigment loss started from the upper limb in 55 subjects (35.5%), followed by trunk/body folds in 50 (32.3%), lower limb in 27 (17.4%) and head/neck in 23 (14.8%) subjects. Twenty patients (12.9%) showed the Koebner phenomenon. At the time of examination the disease was active and progressed in 109 (70.3%) subjects, who confirmed appearance of new lesions or increase in the size of existing lesions within the past 3 months. 23 subjects (14.8 %) reported transformation of the initial clinical type of vitiligo. *Vitiligo vulgaris* was the initial clinical subtype in 113 subjects (72.9%) and five of them developed universal vitiligo. In 12 cases of focal vitiligo and 6 cases of acrofacial vitiligo the disease had transformed to *vitiligo vulgaris*. Segmental vitiligo did not transform to another subtype. Female gender was associated with increased risk of developing vulgar vitiligo ($P = 0.008$) and male gender of acrofacial vitiligo ($P = 0.0002$) (Table 3). In 82 cases (52.9%) up to 10% of body surface area was involved. BSA over 10% was associated with a higher risk of onset of vitiligo earlier, up to 20 years ($P = 0.026$). Pigment loss over 10% was statistically more prevalent in females comparing to males ($P = 0.006$). Leukotrichia was observed in 75 cases (48.4%) and mucosal involvement in 14 cases (9.0%). 54 patients (34.8%) had gone through a short-term treatment without any notable effect. During the course of vitiligo 43 subjects (27.7%) had noticed partial and unstable spontaneous repigmentation in some of their lesions.

60 subjects (38.7%) named the factors that had brought on their vitiligo initially or worsened it. Both psychological stress and mechanical trauma were reported in 15 cases, followed by hormonal changes in 13 cases, sunburn in 6 cases, UVR or radiation in 6 cases, skin irritation in three cases, drug intake in three cases, and another disease in one case. The presence of triggering factor was statistically significantly associated with female gender ($P = 0.003$). Additionally, the subjects with pigment loss over 10% of BSA associated onset and exacerbation of the disease statistically more often with the triggering factor ($P = 0.0001$). 79 subjects (51.0%) had suffered from severe sunburn during their

life without significant difference between genders, but the reported mechanical traumas were statistically more often associated with male gender (P = 0.041).

In all, 104 subjects (67.1%) self-reported a concomitant disease and 57 of those (36.7%) had one or more diseases of autoimmune origin. Halo nevi were seen in 24 cases (15.5%); all but three had vitiligo vulgaris. In 9 cases (5.8%) halo nevi had anteceded to vitiligo. Personal history of thyroid disease was reported by 26 subjects (16.8%) and in 20 of those autoimmune thyreoiditis had been diagnosed. Other coexisting diseases are shown in Table 4. Female gender was associated with the increased risk of presence of concomitant autoimmune disease (P = 0.011) and autoimmune disease in the family (P = 0.042). Family history of vitiligo was positive in 40 cases (26.0%), thyroid disease in 32 (20.8%), diabetes in 21 (13.6%), psoriasis in 13 (8.4%), rheumatoid arthritis in 7 (4.5%) and hypertonia in 53 (34.4%) (Table 4).

Table 4. Vitiligo associated diseases.

Associated diseases	Number (%) of vitiligo subjects with the disease	Number (%) of vitiligo subjects whose relative has the disease
Halo nevus	24 (15.5)	2 (1.3)
Thyroid disease	28 (16.8)	32 (20.8)
Diabetes	6 (3.9)	21 (13.6)
Rheumatoid arthritis	6 (3.9)	7 (4.5)
Psoriasis	7 (4.5)	13 (8.4)
Gastroduodenal ulcer	2 (1.3)	0
Pernicious anemia	2 (1.3)	0
Alopecia areata	2 (1.3)	2 (1.3)
Ankylosing spondylitis	2 (1.3)	0
Multiplex sclerosis	1 (0.6)	0
Sclerodermia	1 (0.6)	1 (0.6)
Neurofibromatosis	1 (0.6)	0
Addison disease	0	1 (0.6)
Urticaria	8 (5.2)	0
Fast type allergy (hair, dust, pollen)	7 (4.5)	1 (0.6)
Asthma	5 (3.2)	3 (1.9)
Atopic dermatitis	2 (1.3)	0
Drug allergy	14 (9.0)	0
Oncological, hematological disease	8 (5.2)	3 (1.9)
Hypertonia	24 (15.5)	53 (34.4)

The levels of certain autoantibodies (TPO-Ab, PCA, ANA, AAA and RF) were measured in 141 subjects and the test proved positive in 70 cases (49.6%). Detected antibodies are shown in Table 5. Females showed statistically more positive blood tests of autoantibodies (P = 0.045), especially of TPO-Ab (P = 0.013) as compared to males.

Table 5. The presence of autoantibodies in the blood

Autoantibodies type	Number of vitiligo subjects, (M/F)	% of vitiligo subjects
TPO-Ab	52 (9/43)	36.9
PCA	20 (4/16)	14.2
AAA	4 (1/3)	2.8
ANA	4 (1/3)	2.8
RF	11 (4/7)	7.8

According to the newest classification of APS by Betterle, several APS types were discovered and are shown in Table 6. APS cases were statistically more often discovered in females comparing to males (P = 0.007).

Table 6. APS types in vitiligo.

Type of APS	Associated disease	No of subjects
APS-3 C	Autoimmune thyroiditis	13
APS-3 C (potential)	Positive TPO-Ab	35
APS-3 C + A	Autoimmune thyroiditis, multiplex sclerosis, diabetes	1
APS-3 C + D	Autoimmune thyroiditis, rheumatoid arthritis	3
APS-4	Alopecia areata	2

Differences between familial and sporadic cases of vitiligo were explored in 186 patients, 51 of them were familial cases (15 M, 36 F, average age 41.7 ± 15.6 years) and 135 sporadic ones (42 M, 93 F; average age 45.5 ± 16.3 years). In 173 of those the levels of TPO-Ab, PCA, ANA, AAA and RF were measured. In 67% of the familial cases, the subject had one relative with vitiligo, in 33% two or more. The average age of the onset of vitiligo was 24.8 years ± 17.5 (age range 3–76 years) in the familial group and 30.0 ± 16.5 years (age range 2–69 years) in the sporadic cases (Figure 3).

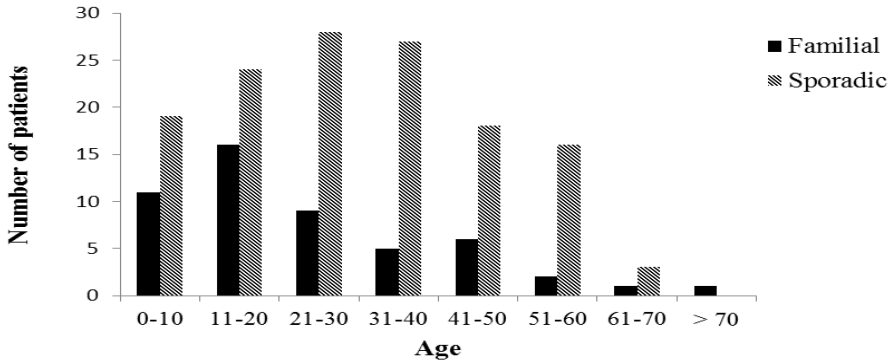

Figure 3. Distribution of ages of the onset in familial (n=51) and sporadic (n=135) cases of vitiligo.

The risk of the onset of the disease at the age up to 20 years was higher in the familial group (P = 0.008, OR 2.407, 95%CI 1.246–4.649). The patients in the familial group showed more widespread depigmentation compared with sporadic cases (BSA > 10%: P = 0.004, OR 2.606, 95% CI 1.341–5.064 and BSA > 50%: P = 0.001, OR 3.856, 95%CI 1.597–9.310; respectively). The distribution of the clinical subtypes of vitiligo was similar between the familial and sporadic vitiligo groups, as shown in Table 7. However, clinical subtype of *vitiligo vulgaris* at the onset of the disease was significantly associated with familial vitiligo (P = 0.008, OR 2.966, 95%CI 1.289–6.821).

Table 7. Comparison of clinical subtypes of onset and at the time of examination in the groups of familial and sporadic vitiligo.

	Number of the subjects (%)		P value
Clinical subtype at the onset	Familial vitiligo	Sporadic vitiligo	
Vitiligo vulgaris	43 (84%)	87 (65%)	0.008*
Focal vitiligo	5 (10%)	26 (19%)	0.23
Segmental vitiligo	1 (2%)	4 (3%)	0.706
Acrofacial vitiligo	2 (4%)	18 (13%)	0.065
Clinical subtype at the examination			
Vitiligo vulgaris	46 (90%)	107 (79%)	0.082
Focal vitiligo	0 (0%)	9 (7%)	0
Segmental vitiligo	1 (2%)	5 (4%)	0.548
Acrofacial vitiligo	1 (2%)	11 (8%)	0.125
Universal vitiligo	3 (6%)	3 (2%)	0.207

* P value < 0.05

The patients with family history of vitiligo had darker skin types (types III–IV), (P = 0.045, OR 2.139, 95% CI 1.007–4.543). Familial vitiligo patients with widespread eruption (BSA > 50%) had higher risk for leukotrichia, mucosal depigmentation and the presence of triggering factors. Family history of vitiligo was not associated with concomitant autoimmune diseases. Thyroid disease was the most common autoimmune disease and 61 TPO positive subjects were found in the whole vitiligo group. Moreover, thyroid disease among relatives was reported higher in familial group comparing with sporadic cases (P = 0.03, OR 2.200, 95% CI 1.064–4.548). Nevertheless, when the frequency of concomitant diseases, autoimmune diseases and positive autoantibody findings between familial and sporadic vitiligo patients were compared, statistically significant differences between the groups were not found. In sporadic cases, female gender and disease duration of 10 years and longer were a risk factor for more extensive depigmentation (BSA > 10%: P = 0.001, OR 3.984, 95% CI 1.668–9.520 and P = 0.001, OR 3.560, 95% CI 1.681–7.539; respectively). Very extensive depigmentation, BSA > 50%, was associated with reported triggering factors (mechanical injury, psychological stress, hormonal changes, sunburn, radiation, skin irritation, drug intake and another disease) both in familial (P = 0.0005, OR 10.560, 95% CI 2.451–45.497) and sporadic (P = 0.004, OR 7.630, 95% CI 1.580–36.858) cases. BSA > 50% was also a risk factor for mucosal involvement both in familial (P = 0.01, OR 8.000, 95% CI 1.261–50.772) and sporadic (P = 0.004, OR 7.375, CI 1.550–35.096) groups. The association between widespread depigmentation (BSA > 50%) and leukotrichia was witnessed in both groups, but statistical difference was significant in familial group (P = 0.0001; OR 26.923, 95% CI 3.178–228.060). The patients with sporadic vitiligo with BSA > 50% had higher risk for serological autoantibody findings (P = 0.03, OR 4.941, 95%CI 1.005–24.300), especially the presence of PCA and ANA antibodies (P = 0.04; OR 4.457; 95%CI 0.994–19.978 and P = 0.0002; OR 28.250; 95%CI 2.307–345.989 respectively) (Table 8).

Table 8. The association of widespread pigment loss (BSA > 50%) with a triggering factor, leukotrichia, mucosal involvement and autoantibodies positivity in the groups of familial and sporadic vitiligo.

BSA > 50%	Familial vitiligo			Sporadic vitiligo		
	Number of the patients	P	OR	Number of the patients	P	OR
Triggering factor	8	0.001	10.560	9	0.004	7,630
Leukotrichia	12	0.001	23.077	8	0.079	3.238
Mucosal involvement	4	0.014	8.000	3	0.004	7.375
TPO-Ab	6	0.302	2.000	4	0.712	1.282
PCA	1	0.371	0.377	3	0.04	4.457
ANA	0			2	0.0002	28.250
AAA	0			1	0.317	3.0556

5.2. Quality of life and emotional state in vitiligo compared with psoriasis and healthy volunteers

The study group consisted of 54 subjects with vitiligo (32 F, 22 M; mean age 36.6 ± 15.0; mean disease duration 11.3 ± 9.8 years), 57 subjects with psoriasis (30 F, 27 M; mean age 40.0 ± 13.3; mean disease duration 18.6 ± 12.7 years) and 57 unaffected controls (34 F, 23 M; mean age 39.7 ± 12.8 years). All the subjects were Caucasians with skin phototype I–IV (vitiligo: 17-II, 36-III, 1-IV; psoriasis: 1-I, 23-II, 33-III; healthy controls: 28-II, 28-III, 1-IV). The total mean DLQI score in vitiligo was 4.7 (ranges 0–22), which is statistically significantly higher from 0.6 (ranges 0–5) in healthy controls ($P < 0.001$) and statistically significantly lower from 13.1 (ranges 1–30) in psoriasis ($P < 0.001$). Based on the interpretation of the results of DLQI scale, no impairment of QoL was found in nine (16.7%) cases of vitiligo and in four (7%) cases of psoriasis, small impairment of QoL in 29 (53.7%) and in 10 (17.5%), moderate impairment of QoL in 12 (22.2%) and in 11 (19.0%), large impairment of QoL in three (5.6%) and in 20 (30%), a very large impairment of QoL in one (1.9 %) and in 12 (21%), respectively (Figure 4).

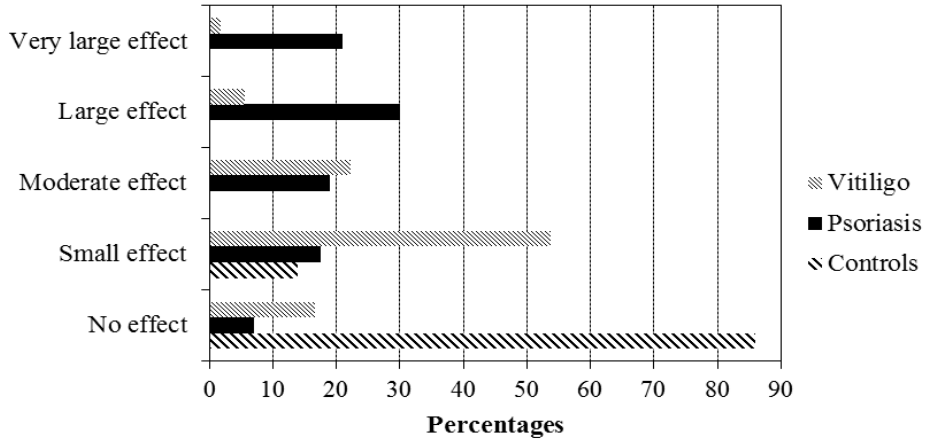

Figure 4. The percentage of the subjects with vitiligo, psoriasis and healthy controls who answered the DLQI questionnaire on the scale from "no effect" to "very large effect".

Vitiligo has highly significant impact on patients' QoL on the scale of symptoms and feelings ($P < 0.001$), daily activities ($P < 0.001$), leisure ($P < 0.001$), but also treatment ($P < 0.01$) and personal relationships ($P < 0.05$), compared with the healthy volunteers. The subjects with psoriasis showed higher mean score in every evaluated DLQI item, compared with healthy controls and with vitiligo, and all these differences were statistically significant, as shown in Figure 5.

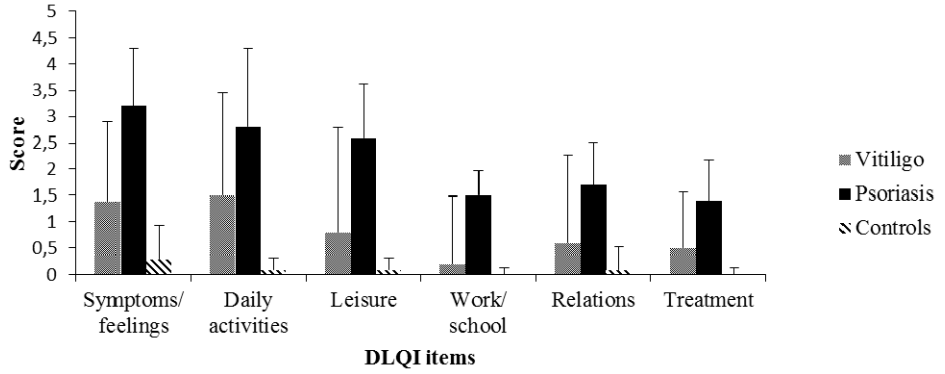

Figure 5. The data of mean scores on DLQI items scale in the group of vitiligo, the group of psoriasis and the group of healthy controls.

The highest individual mean scores in vitiligo were obtained in Q4 (clothing 1.07), Q2 (feelings 0.98) and Q5 (leisure 0.63). Female gender was associated with higher DLQI score in symptoms/feelings ($P = 0.003$) and male gender in relations ($P = 0.040$). In vitiligo the total DLQI was associated with disease activity ($P = 0.006$), disease extension (BSA $\leq$ 10% *vs* > 10%; $P = 0.005$), depigmentation of the hands ($P = 0.008$); and earlier disease onset ($\leq$ 20 *vs* > 20 years; $P = 0.040$). The total DLQI was not influenced by the gender, age, disease duration, pigment loss on other parts of the body, previous treatment, family history of vitiligo and concomitant diseases in the subjects with vitiligo. The total DLQI mean score was the highest (6.1) in the age group of 40–49 years. In psoriasis DLQI was associated with the severity of the disease (PASI $\leq$ 10 *vs* $\geq$20; $P = 0.027$) and concomitant arthritis ($P = 0.019$). Nail involvement did not show statistically significant effect ($P = 0.062$) on QoL. In the subjects with psoriasis DLQI was not associated with the gender, age, disease duration and family history of psoriasis.

The analysis of ES-Q item scale did not reveal any statistically significant differences on the mean score of depression, general or social anxiety, panic, asthenia and insomnia in vitiligo compared with healthy controls shown in Figure 6. The subjects with psoriasis showed statistically higher scores on ES-Q item scale in depression ($P < 0.05$), general anxiety ($P < 0.01$) and asthenia ($P < 0.05$), compared with vitiligo. The comparison between psoriasis and healthy controls accentuated statistically important difference in every item: depression ($P < 0.001$), general anxiety ($P < 0.001$) and insomnia ($P < 0.001$) but also panic ($P < 0.01$), asthenia ($P < 0.01$) and social anxiety ($P < 0.05$) (Figure 6).

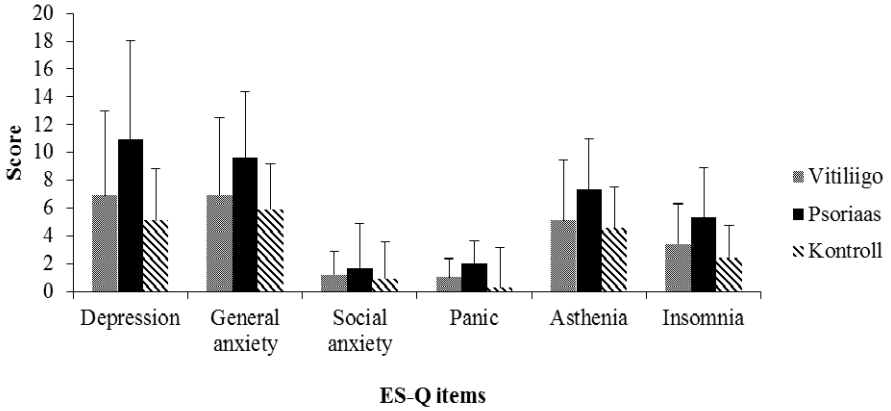

Figure 6. The data of mean scores on Emocional State Questionnaire (ES- Q) items scale in the group of vitiligo, the group of psoriasis and the group of healthy controls.

In spite of that, cut-off score of depression was found in 20% and general anxiety in 22% of the subjects with vitiligo, respectively. The subjects with depression symptoms scored higher on DLQI scale compared with undepressed vitiligo subjects (7.18 *vs* 4.19, $P < 0.05$) and had got vitiligo earlier in their life (mean age 18.1 *vs* 27.1). Even more, based on the cut-off score, 41% of the cases of vitiligo had asthenia with fatigue, 30% had sleep disturbances, 7% had social phobia and 2% had symptoms of panic disorder.

5.3. Gene expression analysis of the melanocortin system in vitiligo

Gene expression levels of POMC, the five melanocortin receptors (MC1R-MC5R) and endogenous melanocortin receptor antagonists (ASIP and AGRP) were measured by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) in punch biopsies from the lesional and non-lesional skin of the vitiligo patients (n = 31) and from the non-sun-exposed skin of healthy subjects (n = 24). The levels of two genes encoding enzymes concerned with melanogenesis – tyrosinase-related protein-1 (TRP1) and dopachrome tautomerase (DCT) – were measured.

The presence of mRNA expression of examined genes in the skin of healthy controls and vitiligo patients became evident in QRT-PCR with a relatively large number (27–36) of amplification cycles. In the skin samples, MC1R demonstrated the highest expression (amplification after 27 cycles), whereas the levels of MC2R, MC3R, MC4R and MC5R mRNAs were low (amplification after 32–36 cycles) (Figure 7, Kingo *et al.* 2007). POMC mRNA was detected at 28–32 cycles, ASIP and AGRP became evident at 34–36 cycles. TRP1 and DCT mRNAs were detected at 28–30 cycles.

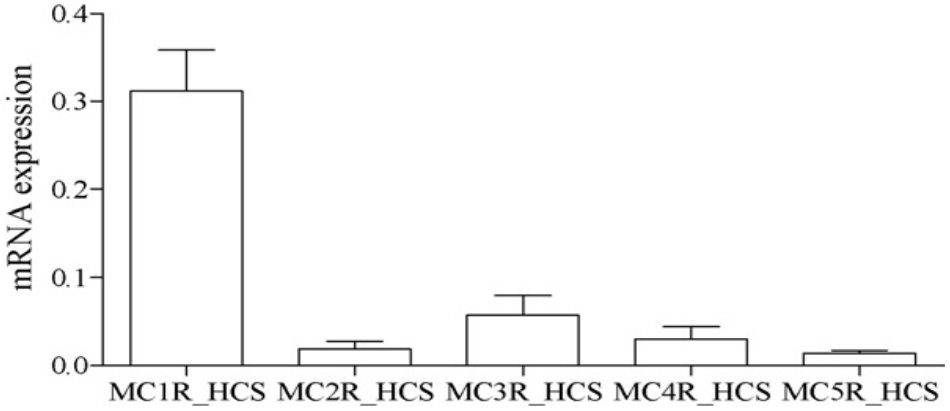

Figure 7. MC1R, MC2R, MC3R, MC4R and MC5R mRNA levels (relative to housekeeping gene HPRT mRNA level) in the skin from healthy controls (HCS).

Statistically significant differences in the expression of MC1R and MC4R between the healthy controls and vitiligo patients were found. The expression of MC1R mRNA was 1.6 fold higher in the non-lesional skin of vitiligo patients when compared to the healthy subjects ($p < 0.01$) (Fig. 8a, Kingo *et al.* 2007). The expressional level of MC4R in the non-lesional skin of vitiligo was 1.9 fold higher than in the healthy skin ($p < 0.01$) (Fig. 8b, Kingo *et al.* 2007). In the lesional skin 2.1 fold decrease of MC1R ($p < 0.0001$) and 2.5 fold decrease of MC4R ($p < 0.01$) expressions were detected when compared to the non-lesional skin samples from the patients with vitiligo (Fig. 8a,b, Kingo *et al.* 2007). The mRNA expression levels of the three other melanocortin receptors (MC2R, MC3R and MC5R) were also increased in the unaffected skin and decreased in the lesional skin of the vitiligo patients but these differences were not statistically significant (data not shown).

A difference in POMC mRNA expression level, marginally satisfying the standard criteria of statistical significance ($p < 0.05$), was evident between the lesional and non-lesional skin of the vitiligo subjects (data not shown). The POMC mRNA expression was 1.9 fold lower in the depigmented skin compared with the uninvolved skin in the vitiligo group. No significant differences in ASIP and AGRP expressions were detected when comparing the study groups.

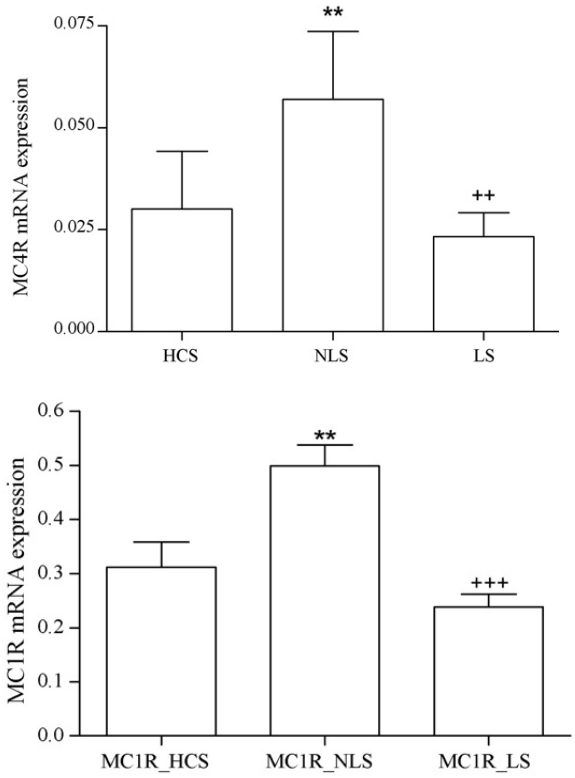

Figure 8. MC1R (a) and MC4R (b) mRNA levels (relative to housekeeping gene HPRT mRNA level) in the skin from healthy controls (HCS), the non-lesional vitiligo skin (NLS) and the lesional vitiligo skin (LS).
*Bars indicate mean _ S.E.M. **p<0.01 compared to the healthy controls; ++ p < 0.01, +++ p < 0.0001 compared to the non-lesional skin.*

Suppression of TRP1 and DCT expressions in the lesional skin compared to the non-lesional vitiligo skin and healthy controls was established. To be more exact, 6.8 fold decrease of TRP1 mRNA expression in the involved skin compared with the skin of healthy subjects ($p < 0.0001$) and 19.7 fold decrease of TRP1 in the lesional skin compared to the uninvolved skin in the vitiligo group ($p < 0.0001$), was detected (Fig. 9a, Kingo *et al.* 2007). The expression of DCT mRNA was 7.6 fold lower in the involved skin of vitiligo compared with the skin of healthy controls ($p < 0.0001$) and 12.9 fold lower in the lesional skin compared to the uninvolved skin in the vitiligo group ($p < 0.0001$) (Fig. 9b, Kingo *et al.* 2007). Contrarily, the expression of TRP1 mRNA was 2.9 fold higher in the unaffected skin of the vitiligo patients compared to the healthy controls ($p < 0.05$) (Fig. 9a, Kingo *et al.* 2007). The expression level of DCT in the non-lesional skin of the vitiligo patients showed also a tendency of increase when compared to the healthy subjects but this finding was not statistically significant ($p = 0.14$).

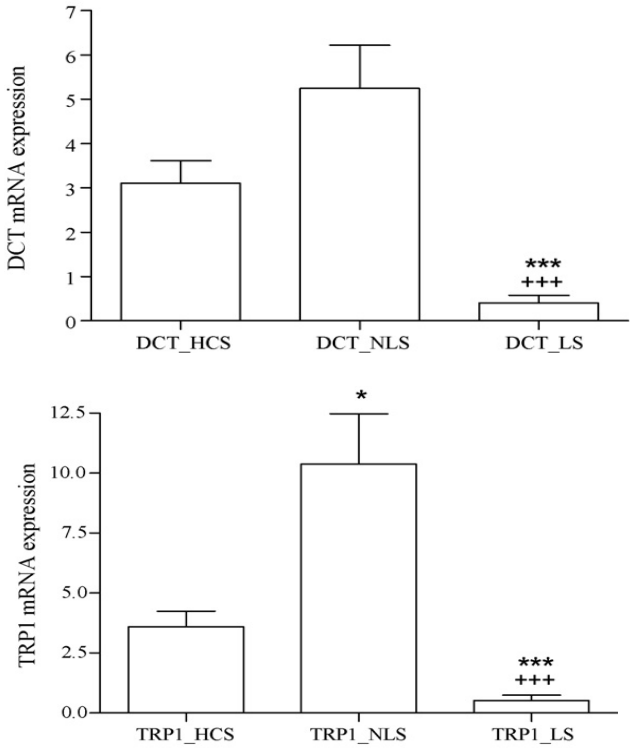

Figure 9. TRP1 (a) and DCT (b) mRNA levels (relative to housekeeping gene HPRT mRNA level) in the skin from the healthy controls (HCS), the non-lesional vitiligo skin (NLS) and the lesional vitiligo skin (LS).
*Bars indicate mean _ S.E.M. *p<0.05, ***p <0.0001 compared to the healthy control; +++ p <0.0001 compared to the non-lesional skin.*

Possible interactions between the expressions of the studied genes were examined by the Spearman rank correlation. In our previous study we had measured the expression of tyrosinase (TYR), an essential enzyme in melanin synthesis (Kingo *et al.* 2006). In addition to this, the correlations between the expressions of the genes of the melanocortin system and TYR were examined. TYR expression was elevated in the uninvolved vitiligo skin compared to the skin samples from controls, but the difference was not statistically significant. The data of the correlations between the expressions of the genes of the melanocortin system in the control group and patient group are presented in Tables 9–11.

Table 9. Results of the correlation analysis of the expression of studied genes of melanocortin system in the skin of healthy controls (Spearman rank)

	POMC	ASIP	AGRP	MC1R	MC2R	MC3R	MC4R	MC5R
POMC	1.00	0.32	0.05	0.1	0.07	0.54*	0.57**	0.41
ASIP		1.00	–0.01	0.73***	0.28	0.11	0.29	0.21
AGRP			1.00	–0.06	–0.27	–0.29	–0.21	–0.35
MC1R				1.00	0.43	0.02	0.01	0.13
MC2R					1.00	0.60*	0.60*	0.41
MC3R						1.00	0.79***	0.37
MC4R							1.00	0.36
MC5R								1.00

* p<0.05, * * p<0.01, * ** p<0.001

Table 10. Results of the correlation analysis of the expression of studied genes of melanocortin system in the uninvolved skin of vitiligo patients (Spearman rank)

	POMC	ASIP	AGRP	MC1R	MC2R	MC3R	MC4R	MC5R
POMC	1.00	0.42*	0.14	0.07	0.31	0.28	0.22	–0.12
ASIP		1.00	–0.04	0.27	–0.052	0.15	0.05	–0.35
AGRP			1.00	-0.09	0.02	0.27	0.11	0.28
MC1R				1.00	0.29	0.09	0.15	–0.21
MC2R					1.00	0.63***	0.54**	0.20
MC3R						1.00	0.79***	0.26
MC4R							1.00	0.51**
MC5R								1.00

* p<0.05, * * p<0.01, * ** p<0.001

Table 11. Results of the correlation analysis of the expression of studied genes of melanocortin system in the involved skin of vitiligo patients (Spearman rank)

	POMC	ASIP	AGRP	MC1R	MC2R	MC3R	MC4R	MC5R
POMC	1.00	0.16	–0.03	-0.15	0.29	0.24	0.27	0.26
ASIP		1.00	0.12	0.17	0.23	0.36	0.17	0.29
AGRP			1.00	0.36	0.24	0.16	0.29	–0.01
MC1R				1.00	0.30	0.17	0.45*	0.24
MC2R					1.00	0.44*	0.60**	0.28
MC3R						1.00	0.83***	0.76***
MC4R							1.00	0.62***
MC5R								1.00

* p<0.05, * * p<0.01, * ** p<0.001

Variables of the mRNA expression of different components of the melanocortin system were positively related. Among the expressions of TYR, TRP1 and DCT mRNAs, a strong positive correlation was noticed both in the skin of the healthy controls and in the skin of the vitiligo patients ($r > 0.70$; $p < 0.0001$) (Fig. 10a–c, Kingo *et al.* 2007).

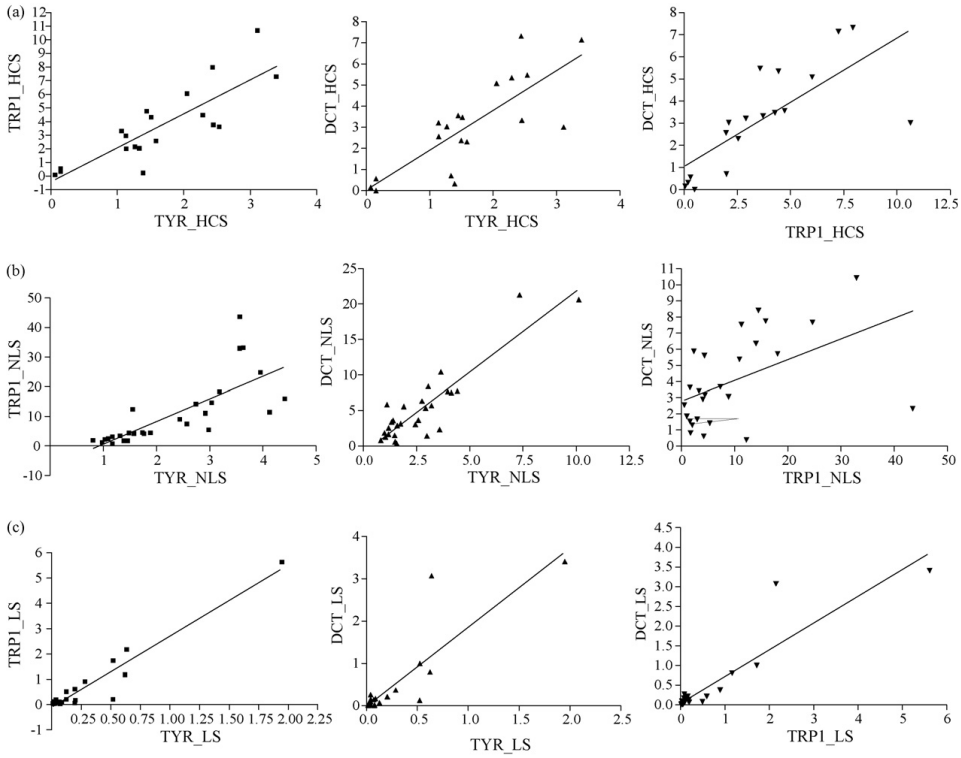

Figure 10.
(a) The relation of the levels of TYR (TYR_HCS), TRP1 (TRP1_HCS) and DCT (DCT_HCS) in the skin of the healthy subjects.
(b) The relation of the levels of TYR (TYR_NLS), TRP1 (TRP1_NLS) and DCT (DCT_NLS) in the non-lesional vitiligo skin.
(c) The relation of the levels of TYR (TYR_LS), TRP1 (TRP1_LS) and DCT (DCT_LS) in the lesional vitiligo skin.

Positive correlation was noted also between the levels of MC1R and TRP1 in the skin of healthy controls ($r = 0.47$; 95% CI 0.001–0.77; $p < 0.05$) (Fig. 11a, Kingo *et al.* 2007). Contrarily, no statistically significant correlation between variables of MC1R mRNA and the expressions of TYR, TRP1 and DCT mRNAs were noticed in the non-lesional and lesional vitiligo skin (Fig. 11 b,c; Kingo *et al.* 2007).

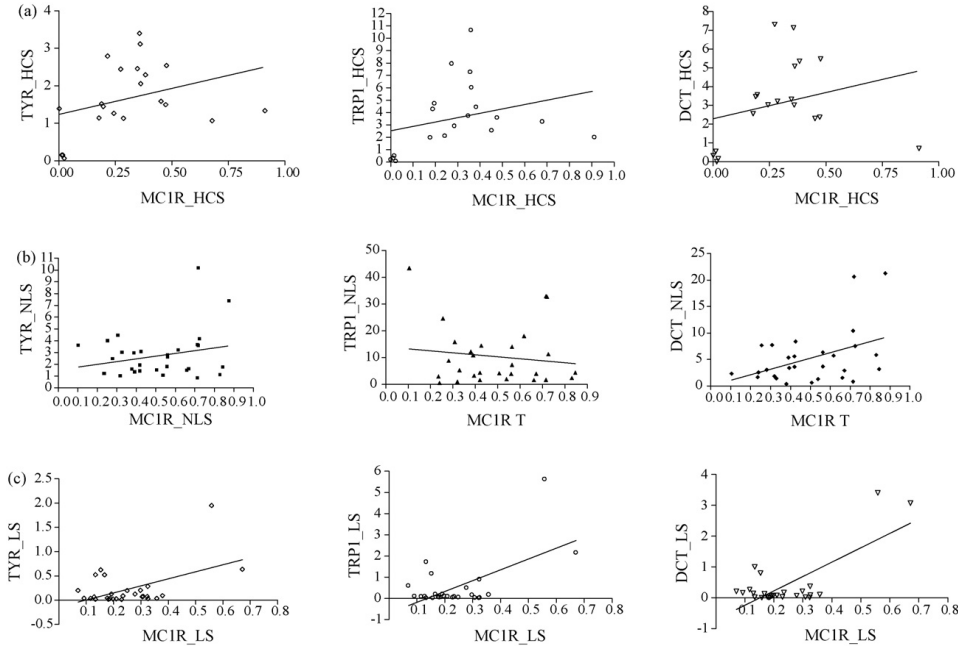

Figure 11.
(a) The relation of the levels of MC1R (MC1R_HCS) and TYR (TYR_HCS), TRP1 (TRP1_HCS) and DCT (DCT_HCS) in the skin of healthy controls.
(b) The relation of the levels of MC1R (MC1R_NLS) and TYR (TYR_NLS), TRP1 (TRP1_NLS) and DCT (DCT_NLS) in the non-lesional vitiligo skin.
(c) The relation of the levels of MC1R (MC1R_LS) and TYR (TYR_LS), TRP1 (TRP1_LS) and DCT (DCT_LS) in the lesional vitiligo skin.

Using QRT-PCR method, mRNA expression levels of eight genes related to signal transduction from the melanocortin system to melanogenesis enzymes were measured in the lesional and non-lesional skin of the vitiligo patients and in the skin of healthy control subjects. All the patients with vitiligo were analyzed as the one group. MITF mRNA expression in the lesional skin of the vitiligo patients was 3.3-fold lower compared with the non-lesional skin of the vitiligo patients ($p < 0.0001$) and 2.4-fold lower compared with the skin of healthy control subjects ($p = 0.0001$) (Fig.12a, Kingo *et al.* 2008). LEF1 expression level in the non-lesional skin of the vitiligo patients was 1.7-fold higher than in the lesional skin of the patients ($p < 0.0005$) (Fig.12b, Kingo *et al.* 2008). USF1 mRNA expression level in the non-lesional skin of the vitiligo patients was 1.6-fold higher compared with the skin of healthy control subjects ($p < 0.01$) (Fig.12c, Kingo *et al.* 2008). No statistically significant differences in CREB1 expression levels were found between the lesional and non-lesional skin of the vitiligo patients and between the skin of the vitiligo patients and healthy control subjects.

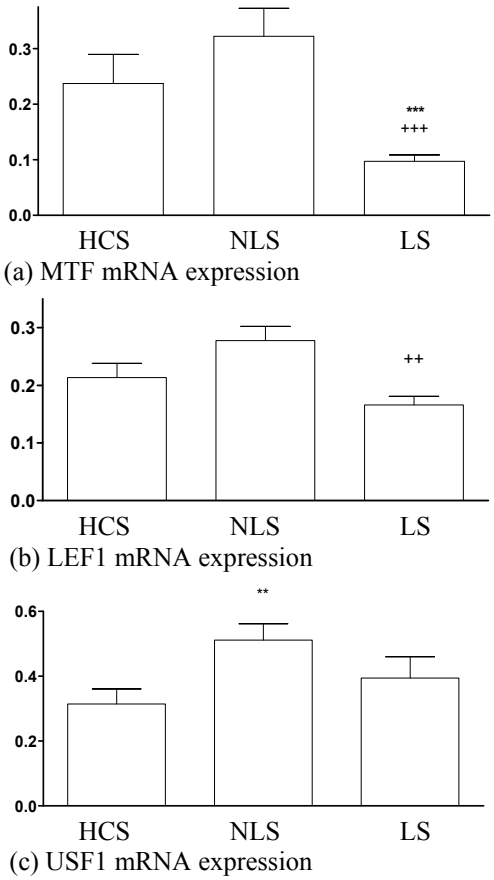

Figure 12. Gene expression levels of the MITF, LEF1, and USF1 (relative to house-keeping gene HPRT mRNA level) in the skin from healthy controls (HCS), the non-lesional vitiligo skin (NLS) and the lesional vitiligo skin (LS).
*Bars indicate mean _ S.E.M. *p<0.05, **p<0.01 and ***p <0.001 (compared to HCS sample); + p< 0.05, ++ p< 0.01 and +++ p<0.001 (compared to NLS sample).*

The expression of p38 mRNA was 1.6-fold lower in the lesional skin of the vitiligo patients compared with the skin of healthy control subjects ($p < 0.005$) (Fig.13a, Kingo *et al.* 2008). The expression of PI3KCB mRNA in the lesional skin of the vitiligo patients was 1.3-fold lower compared with the skin of healthy control subjects ($p = 0.01$) (Fig.13b, Kingo *et al.* 2008). The expression of RPS6KB1 mRNA in the lesional skin of the vitiligo patients was 1.3-fold lower compared with the skin of healthy control subjects ($p < 0.05$) (Fig.13c, Kingo *et al.* 2008). The expression level of BCL2 mRNA in the non-lesional skin of the vitiligo patients was 1.6-fold higher compared with the lesional skin of the patients ($p < 0.05$) (Fig.13d, Kingo *et al.* 2008). BCL2 expression in the non-lesional skin of the vitiligo patients was 2.3-fold higher when compared with the skin of healthy control subjects ($p < 0.05$) (Fig.13d, Kingo *et al.* 2008).

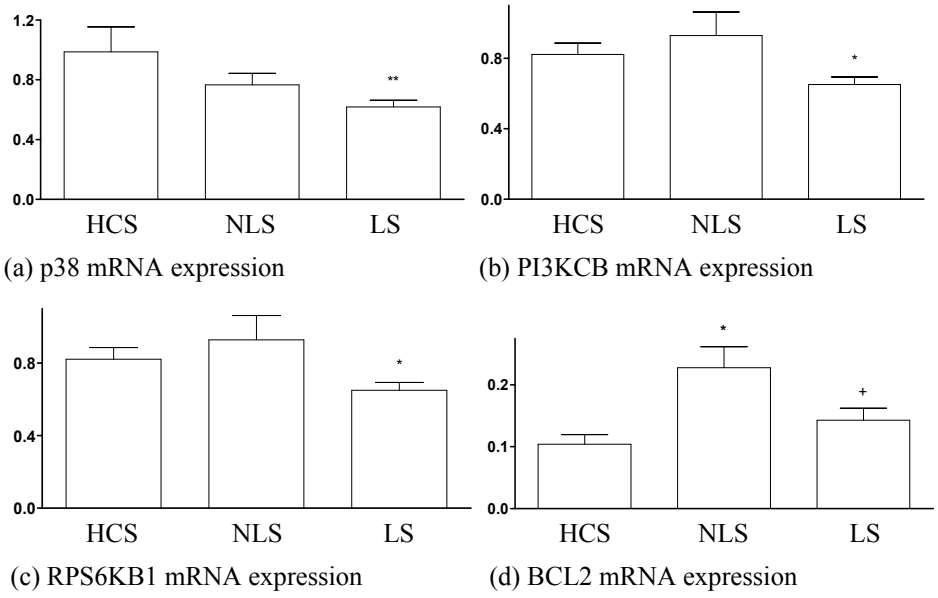

Figure 13. Gene expression level of the p38, PIK3CB, RPS6KB1 and BCL2 (relative to housekeeping gene HPRT mRNA level) in the skin from healthy controls (HCS), the non-lesional vitiligo skin (NLS) and the lesional vitiligo skin (LS).
*Bars indicate mean _ S.E.M. *p<0.05, **p<0.01 and ***p<0.001 (compared to HCS sample); + p< 0.05, ++ p< 0.01 and +++ p <0.001 (compared to NLS sample).*

Possible interactions between the expressions of the studied genes were examined by the Spearman rank correlation (Tables 12 and 13). In addition, the correlations between the expressions of the studied genes and the genes encoding enzymes of melanogenesis (TYR, TRP1 and DCT) were examined (Tables 12 and 13). In the present study statistically significant positive correlations were found between the expression levels of p38 and PI3KCB, and p38 and RPS6KB1 both in the skin of the vitiligo patients (respectively, $r = 0.75$, $p < 0.001$ and $r = 0.75$, $p < 0.001$) and in the skin of healthy control subjects (respectively, $r = 0.92$, $p < 0.001$ and $r = 0.82$, $p < 0.001$). The expression levels of MITF, PI3KCB and p38 were positively correlated with the mRNA levels of TYR, TRP1 and DCT in the skin of healthy control subjects ($p < 0.05$), but not in the skin of the vitiligo patients.

Table 12. Results of the correlation analysis of the expression of studied genes in the skin of healthy controls (Spearman rank)

	TYR	TYRP1	DCT	MITF	LEF1	USF1	p38	CREB1	PIK3CB	RPS6KB1	BCL2
TYR	1.00	0.80***	0.73***	0.50*	–0.14	0.60	0.57*	–0.71	0.52*	0.45	–0.50
TYRP1		1.00	0.82***	0.69**	0.54	0.60	0.76***	–0.14	0.75***	0.68**	–0.50
DCT			1.00	0.58*	0.31	–0.40	0.68**	–0.29	0.58*	0.51	–0.50
MITF				1.00	0.14	0.09	0.71***	0.07	0.66***	0.71***	0.03
LEF1					1.00	0.35	0.41	0.55*	0.24	0.58*	0.38
USF1						1.00	0.34	0.51	0.22	0.12	0.36
p38							1.00	0.33	0.92***	0.82***	0.45
CREB1								1.00	0.19	0.31	0.35
PIK3CB									1.00	0.85***	0.32
RPS6KB1										1.00	0.23
BCL2											1.00

*p < 0.05, **p < 0.01, ***p < 0.001

Table 13. Results of the correlation analysis of the expression of studied genes in the involved skin of vitiligo patients (Spearman rank)

	TYR	TYRP1	DCT	MITF	LEF1	USF1	p38	CREB1	PIK3CB	RPS6KB1	BCL2
TYR	1.00	0.74***	0.69***	–0.37	0.03	–0.30	–0.30	–0.33	–0.02	–0.47*	0.23
TYRP1		1.00	0.81***	–0.14	0.27	–0.11	–0.16	–0.30	0.11	–0.32	0.15
DCT			1.00	–0.12	0.13	–0.16	0.01	–0.32	0.34	–0.08	0.24
MITF				1.00	0.35	0.30	0.46**	0.43*	0.35*	0.35*	0.46
LEF1					1.00	0.57**	0.12	0.53**	0.04	0.03	0.62**
USF1						1.00	0.12	0.26	–0.04	–0.06	0.32
p38							1.00	0.22	0.63***	0.69***	0.19
CREB1								1.00	0.13	0.36	0.58**
PIK3CB									1.00	0.55***	0.12
RPS6KB1										1.00	0.01
BCL2											1.00

*p < 0.05, **p < 0.01, ***p < 0.001

6. DISCUSSION

6.1. Clinical aspects and the presence of autoantibodies in vitiligo

As only few studies have been conducted with adults suffering from vitiligo, it is difficult to compare these data with other studies. This could also be the reason why our study group showed older mean age of the subjects (44.9 years) and longer mean disease duration (16.9 years) compared with previous studies (Handa and Kaur 1999; Martis *et al.* 2002). We could not find statistically important differences in the onset of vitiligo and disease duration between males and females. In more than half of the cases vitiligo appeared at the age of 30, but in 15% of the cases late onset (50 plus) was observed, a higher indicator than reported earlier (Dogra *et al.* 2005). Up to now there has been no uniform understanding of clinical types or subtypes in vitiligo and the situation is complicated as the phenotype of the disease can change during the time. *Vitiligo vulgaris* was the most common clinical subtype in our study and this is in consent with many previous published papers (Handa and Kaur 1999; Dogra *et al.* 2005; Liu *et al.* 2005; Mason and Gawkrodger 2005). Recently the decision was made that the term "*vulgaris*" as the synonym for "common" conveys a negative connotation to patients and general public and therefore should not be used. VGICC classification renamed *vitiligo vulgaris* as generalized vitiligo that is now the main subtype of nonsegmental vitiligo (Ezzedine *et al.* 2012). In our study *vitiligo vulgaris* was statistically more prevalent in females and acrofacial vitiligo in males, the latter has not been mentioned earlier. Segmental, focal and universal subtypes were rare in this study (all together 11% of the cases). Focal vitiligo is called now undertermined or unclassified type of vitiligo until more definite type can be made on clinical description (Ezzedine *et al.* 2012). Some clinical patterns like localized mucosal subtype, mixed vitiligo and segmental subtype in the face region have been more characteristic for the patients in Asia and were not observed in our subjects (Hann and Lee 1996; Hann *et al.* 1997; Dave *et al.* 2002; Mulekar *et al.* 2006). Segmental vitiligo has been reported previously up to 4.5% of adults with vitiligo (Dogra *et al.* 2005; Mason and Gawkrodger 2005). Nowadays the word "segmental" has become a misnomer as lesions in Blaschko-linear distribution and lesions not following the commonly mentioned „dermatomal" distribution have also been described under this type (Schallreuter *et al.* 2007; Taieb *et al.* 2008; van Geel *et al.* 2011a). In our study the subjects with segmental and universal vitiligo were affected earlier than other subtypes of vitiligo. The segmental subtype was unisegmental and did not transform to another subtype. In most studies segmental vitiligo has been more characteristic for children and young adults with median age of onset 12–16 years, but unexpectedly late onset with median age of 41 years of this subtype was recently reported in China (Barona *et al.* 1995; Liu *et al.* 2005; Wang *et al.* 2013).

In our subject group disease had started from the upper limb more than in one-third of the cases - that is in agreement with other published papers -, in addition to the preferred localisation on lower limb and face region (Handa and Kaur 1999; Arycan *et al.* 2008; Akrem *et al.* 2008). Few studies have examined transformation of the clinical subtypes in vitiligo. In a Japanese study 59% of the focal vitiligo cases remained stable, 34% transformed to generalized and 7% to segmental type during the years from 0.5 to 8 (Zaima and Koga 2002). The survey of 3742 patients in China revealed that more than half of the focal vitiligo cases transformed into another clinical type during 3–5 years and 99% of all active cases constantly got worce (from focal to acrofacial, segmental, *vulgaris* or universal; from segmental or acrofacial to *vulgaris* or universal; from *vulgaris* to universal) (Liu *et al.* 2005). According to the self-reported initial location and description of pigment loss the clinical subtype of vitiligo was transformed only in 15% of the cases in our study. At the time of examination disease was active in 70% of the cases and this is in agreement with other studies (Dave *et al.* 2002; Chun and Hann 1997). Leucotrichia was observed almost in half of the cases, but the Koebner phenomenon and mucosal involvement were not common in our subjects. Leukotrichia occurs when melanocytes are damaged in the hairs inside depigmented area and it shows poor prognosis of repigmentation in vitiligo (Dutta and Mandal 1969). There has no unambigious method for assessment of Koebner phenomenon in vitiligo; this could be the reason why different studies have offered variable results of this phenomenon. A new classification of Koebner phenomenon takes into consideration historical, clinical and experimental aspects in assessment of this phenomenon (van Geel *et al.* 2011b). In the present study the Koebner phenomenon was assessed only on clinical basis and this was the reason why the number of the Koebner cases is low.

Pathogenetic models of vitiligo have implied the presence of external and internal predisposing factors in manifesting the pigment loss. In clinical studies 10–76% of the patients with vitiligo have named factors that had preceded the onset or exacerbation of the disease: emotional stress, skin injury, sunburn, hormonal changes (Behl and Bhatia 1972; Koo *et al.* 1996; Verma 2009; Manolache and Benea 2007). More than one-third of the subjects in this study could report predisposing or aggravating factors of vitiligo: psychological stress, mechanical trauma, hormonal changes, sunburn and UVR were among the most frequently mentioned. In this study the presence of self-reported predisposing factor was statistically significantly associated with female gender and more extensive pigment loss (BSA > 10%). Females with vitiligo have noticed the influence of hormonal factors on the course of the disease. There is the data that metabolites of estrogens can predispose oxidative stress via generating hydrogen peroxide and damage DNA of peripheral blood lymphocytes in vitiligo (Schallreuter *et al.* 2006). The exact mechanisms how all these factors influence the onset or course of vitiligo, is still obscure. In spite of the long mean duration of vitiligo, the subjects with minimal extension predominated, as more than a half of the cases of pigment loss did not exceed

10% of the BSA. It confirms a former opinion that vitiligo is a slowly progressing disease with a chronic and very long course. Our study revealed that extensive pigment loss (BSA > 10%) was associated with a higher risk of the onset of vitiligo at a more early age, up to 20 years, and it was statistically more frequently observed in females comparing to males.

Based on autoimmune hypothesis in the pathogenesis of vitiligo, studies have focused on finding associated diseases in vitiligo patients and also in their families, but the results have been variable in different populations. In our study with the mean age of 44.9 years, more than two-thirds of the subjects had a concomitant disease and more than one-third had a disease of autoimmune origin. The risk of presence of concomitant autoimmune disease was higher in females comparing to males and this is in consistent with previous studies (Amerio *et al.* 2010). Halo nevi were seen in 15.5% of the cases in this study. In published papers halo nevi have been associated with vitiligo in up to 31% of the cases, they are more characteristic for children and young adults but have been shown also in 3.8% of adults at the age 50 years and older (van Geel et al. 2011c; Dogra *et al.* 2005). The study of the patients who have vitiligo and halo nevi at the same time revealed halo nevi prior to the development of vitiligo in 61% of the patients with mean time interval 34 months (van Geel *et al.* 2011c). There is a belief that the occurrence of halo nevi may be an initiating factor of the pathogenesis of vitiligo. A recent study from Belgium supported the hypothesis that halo nevi can represent a distinct clinical condition: patients with only halo nevi had significantly less associated autoimmune diseases, were less likely to have a family history of vitiligo and were less likely to have the presence of the Koebner phenomenon compared with the patients of generalized vitiligo (Barona *et al*. 1995; van Geel *et al.* 2011c). The lack of association between halo nevi and other autoimmune diseases was also revealed in Italy (Berti *et al.* 2011).

Thyroid disease was the most often reported autoimmune disease in our subjects. Autoimmune thyroid disease has been reported from 5% to 24% of the children and up to 34% of the adults with vitiligo (Pagovich *et al*. 2008; Kurtev and Dourmishev 2004; Mason and Gawkrodger 2005). Studies from Europe, the USA and Asia (excluding China) have demonstrated a higher prevalence of thyroid autoimmune disease in patients with vitiligo compared with general population (Schallreuter *et al.* 1994; Alkhateeb *et al.* 2003; Narita *et al.* 2011). In China the prevalence of thyroid diseases has been low in general population and also in patients with vitiligo (Liu *et al*. 2005; Zhang *et al.* 2009). An elevated risk of thyroid disease among older women with nonsegmental vitiligo and in subjects with positive family history of thyroid disease has been demonstrated lately in the Netherlands (Kroon *et al.* 2012). In the present study high prevalence of thyroid disease (20.8%), diabetes (13.6%), psoriasis (8.4%), rheumatoid arthritis (4.5%) and Addison's disease (0.6%) was reported by the patients also in their families. Again, autoimmune disorders in the family were statistically more frequently reported by the females. The elevation of autoimmune thyroiditis, pernicious anemia, Addison's disease, systemic lupus

erythematosus has been demonstrated in the first-degree relatives with vitiligo in the Caucasian population, and rheumatoid arthritis, psoriasis and chronic urticaria in China (Alhateeb *et al.* 2003; Zhang *et al.* 2009). Family history of vitiligo was positive in 26% of our patients - that is in consent to most published papers where the percent remains between 8 and 36 (Handa and Kaur 1999; Gopal *et al.* 2007).

Autoantibodies have been demonstrated in the range from 2% to 70% in different studies in vitiligo (Barona *et al.* 1995; Bystryn 1989). Studies have revealed that the presence of circulating autoantibodies was correlated with a long duration of vitiligo and a later disease onset (Mandry *et al.* 1996; Betterle *et al.* 1985). As opposed to these findings, recent studies in Italy and India have highlighted the presence of autoantibodies to be correlated with a short duration of vitiligo (Ingordo *et al.* 2010; Pradhan *et al.* 2011). The presence of measured autoantibodies was established in half of the cases in our adult group and this is a high indicator. TPO-Ab was positive more than in one-third of the subjects and in 11% of the sera even two different antibodies were detected. The previous studies have demonstrated the presence of thyroid microsomal Ab from 3.0% to 4.4% of the adult population in Estonia and it is notably lower compared to our finding in vitiligo (Uibo *et al.* 1984, 1998; Metsküla *et al.* 2001). Thyroid antibodies have been demonstrated from 18% to 50% of the cases of vitiligo in comparative studies in children and adults (Kurtev and Dourmishev 2004; Hegedus *et al.* 1994; Daneshpazhooh *et al.* 2006). In the present study females showed statistically more often positive autoantibodies in the blood, especially positive TPO-test comparing to the males. The higher prevalence of TPO-Ab has been revealed in young women (18–35 years) with vitiligo in Iran (Daneshpazhooh *et al.* 2006). PCA was positive in 14% of the cases of vitiligo in our study and this is higher than 2.5% – 4.2% found in Estonian unselected adult population (Uibo *et al.* 1984, 1998; Metsküla *et al.* 2001). The published results of the previous studies concerning PCA in vitiligo compared to the controls are contradictory. Many studies have not shown the higher prevalence of PCA in vitiligo compared with the control population (Carmel 1992; Ottesen 1992; Schallreuter *et al.* 1994; Zettinig *et al.* 2003). Strong association between PCA and autoimmune gastritis in vitiligo has been verified in Italy (Betterle *et al* 1985; Amerio *et al.* 2010). The number of positive ANA cases in vitiligo did not differ from those observed from the unselected Estonian adults (1.8% – 7.4%) (Uibo *et al.* 1984, 998; Metsküla *et al.* 2001). Most studies have not shown the higher prevalence of ANA in vitiligo compared with the control population, except one study in Iran (7%) and Korea (12.4%) (Hann *et al.* 1993; Zettinig *et al.* 2003; Farrokhi 2005). The quantitative value of RF in the positive cases was quite low except one case with acute rheumatoid arthritis. Few studies have observed RF in vitiligo, the increase of RF in 11% of the cases in vitiligo compared with the controls has revealed in Iran (Farrokhi *et al.* 2005). According to the classification of APS by Betterle, 19 cases of APS (17 APS-3 and 2 APS-4) and 35 cases of potential APS-3 with only positive TPO-antibodies were discovered among 155 patients

with vitiligo and this is a high indicator. Again, females showed statistically more often positive APS cases comparing to the males. The subjects with only positive antibodies, among them four with AAA, should remain under observation for possible manifestation of autoimmune disease. Long follow-up of patients with APS has confirmed that "silent" Abs may precede clinical manifestation of the autoimmune disease many years and these antibodies are predictive for the development of autoimmune disorder in future (Dittmar and Kahaly 2003). Autoimmune adrenalitis may develop in 16–40% per year in the subjects with positive antiadrenal antibodies (Zosin 2004; Betterle and Volpato 1998; Muir *et al.* 1993). AAA have been stronger predictors for adrenal insuffiency in children than in adults, 100% *vs* 32% during the mean follow-up of six years (Coco *et al*. 2006).

We found several differences between familial and sporadic cases of vitiligo in the group of 186 subjects. The patients with family history of vitiligo showed a higher risk of earlier onset of the disease (≤ 20 years) and darker skin phototype (III–IV). The association between positive family history and the earlier onset of vitiligo has been shown in previous and confirmed in recent studies (Ando *et al*. 1993; Laberge *et al.* 2005; Misri *et al.* 2009; Alzolibani 2009). One study has found the association between skin phototype III–IV and positive family history in vitiligo (Alzolibani 2009). As opposed to the previous studies, our study shows that patients with familial vitiligo have a higher risk for widespread depigmentation and the association gets stronger in cases with BSA > 50% (Ando *et al*. 1993; Misri *et al.* 2009). In this study we verified that patients with family history of vitiligo have a higher risk for *vulgar vitiligo* at the onset of the disease. Similarly to other investigators, we could not find statistical correlation between positive family history of vitiligo and disease activity, areas involved, leukotrichia, mucosal involvement, the Koebner phenomenon and triggering factors (Misri *et al.* 2009; Alzolibani 2009; Laberge *et al.* 2005; Boisseau-Garsaud *et al.* 2001). Family history of vitiligo was not associated with the reported concomitant diseases, including autoimmune diseases and positive autoantibodies finding. Despite this, thyroid disease among relatives was reported higher in the familial group comparing with sporadic cases demonstrated earlier (Laberge *et al*. 2005). Our results stress the importance of female gender in vitiligo, as in sporadic cases female gender raises the risk for widespread depigmentation, not shown before. Widespread depigmentation (BSA > 10%) was also correlated with prolonged duration of vitiligo. We could not reveal any correlation between female gender and attendance of other autoimmune diseases (including thyroid disease) or antibodies positivity, as previous studies have found (Klisnick *et al.* 1998; Daneshpazhooh *et al.* 2006; Amerio *et al.* 2010). Patients with sporadic vitiligo and very extensive depigmentation (BSA > 50%) have also a higher risk for mucosal depigmentation, the presence of triggering factors, PCA and ANA antibodies in the sera. At the same time, patients with positive family history of vitiligo and very extensive depigmentation (BSA > 50%) have a higher risk for leukotrichia, mucosal depigmentation and the presence of triggering factors. This finding

supports overall agreement that external factors as well as concurrent autoimmune conditions predict the widespread eruption in vitiligo.

Our data do not allow to generalize the autoantibodies overall presence and to draw the comparative conclusions about autoantibodies increase in vitiligo as only certain autoantibodies were measured in the sera of the patients and control group was absent.

6.2. Quality of life and emotional state in vitiligo

Our case-control study in fair-skinned subjects demonstrated the disease-related small but close to moderate effect of QoL in vitiligo. The mean DLQI score 4.7 is significantly higher compared with healthy controls without impact on QoL (DLQI 0.6). Our mean DLQI score in vitiligo is in agreement with the studies from Belgium (DLQI 4.95), the U.K. (DLQI 4.8) and Indonesia (DLQI 4.4) (Kent and al-Abadie 1996; Ongenae *et al.* 2005b; Chan *et al.* 2012). The higher mean DLQI scores in vitiligo have been shown in Japan, Germany, Iran, France, China, India and Saudi Arabia (Tanioka *et al.* 2010; Radtke *et al.* 2009; Aghaei *et al.* 2004; Mashayekhi *et al.* 2010; Kostopoulou *et al.* 2009; Wang *et al.* 2011; Parsad *et al.* 2003; Al Robaee 2007; Al-Mubarak *et al.* 2011). Higher DLQI scores are associated with darker skin as the contrast of skin colour in dark-skinned people attracts more unwanted attention, which is emotionally disturbing and displeasing. In our vitiligo group the mean DLQI was not associated with the gender, age, disease duration and family history of vitiligo as reported in Malaysian vitiligo patients (Wong and Baba 2012). Studies have revealed lower QoL in women because they are more emotional and more sensitive about their appearance (Radtke *et al.* 2009; Belhadjali *et al.* 2007; Borimnejad *et al.* 2006). Our subjects with vitiligo were disturbed on every individual DLQI item compared with healthy volunteers, but more in symptoms/feelings, leisure and daily activities than shown previously (Wang *et al.* 2011; Ongenae *et al.* 2005b). In the group of vitiligo, women scored statistically higher in symptoms/feelings compared with men who were more impaired in sexual or other personal relations compared with women. The latter outcome is opposite to previous results but shows clearly that diverse skin colour can cause uncertainty in relations of both genders. Vitiligo has no impact on such activities like going to school or work, as pigment loss does not cause physical disability and this is in line with other studies (Wang *et al.* 2011; Ongenae *et al.* 2005b; Radtke *et al.* 2009; Wong and Baba 2012). In our vitiligo group the total DLQI was associated with disease progression, extension, depigmentation on the hands, and earlier disease onset. There are no reports in the literature confirming that impairment of QoL is associated with the active stage of the disease or the earlier disease onset. The progression of pigment loss is very disturbing for the patient, as there is no way to get the disease under control or to influence its lifelong course, and it undermines their confidence. The fact that those who have got the disease earlier in their life and have a

higher DLQI score, speaks clearly about difficulties in coping with vitiligo and disturbed emotional state (Ongenae *et al.* 2006). The subjects with depressiveness had lower quality of life compared with undepressed vitiligo subjects. Cognitive-behavioural therapy could benefit in vitiligo (Papadopoulos *et al.* 1999). Most studies have emphasized the association between disease's extension and lower QoL (Wang *et al.* 2011; Ingordo *et al.* 2012; Dolatshahi *et al.* 2008; Ongenae *et al.* 2005b; Parsad *et al.* 2003). Vitiligo on uncovered areas like the face and hands has demonstrated a serious negative impact on QoL (Aghaei *et al.* 2004; Ingordo *et al.* 2012). Camouflage has decreased the mean DLQI score in women with vitiligo by 1–1.5 score points and is highly suggested for those who have pigment loss on uncovered areas (Tanioka *et al.* 2010; Ongenae *et al.* 2005a). This may be the reason why our vitiligo group showed negative impact only in pigment loss on the hands, not on the face. Women have got used to covering faces with makeup but it is more inconvenient to use camouflage for other parts of the body.

The patients with psoriasis were more disabled and showed severe QoL impairment compared with vitiligo patients and healthy controls. Predictive clinical factors for low QoL were disease severity and concomitant arthritis. QoL was impaired in every DLQI item with two or three times higher mean score compared with vitiligo. The highest scores of DLQI individual scale in psoriasis were obtained in symptoms and feelings, daily activities and leisure. This profile is in agreement with previous comparative studies (Ongenae *et al.* 2005b; Radtke *et al.* 2009). In this study the interaction of disease and gender on DLQI was not observed in psoriasis, as stated earlier (Ongenae *et al.* 2005b). Based on the results of ES-Q, every fifth subject with vitiligo suffered from symptoms of depression and anxiety, every third had asthenia and sleep disturbances, but compared with healthy controls these differences did not show statistical significance. Studies have reported psychiatric morbidity in up to 35% of the subjects with vitiligo in Europe but the correlation with disease severity or extension has been weak (Mattoo *et al.* 2001, 2002). It confirms that other disease unrelated factors like trait of personality, racial variations, socio-cultural and socio-economical factors are affecting psychiatric morbidity (Finlay and Ryan 1996).

In our study the subjects with psoriasis were emotionally more disabled than the subjects with vitiligo: 42% had symptoms of depression, 33% had general anxiety and 65% had asthenia. The high number of the subjects with disturbed emotional state in psoriasis could be explained by the fact that the majority of the subjects were recruited from the inpatient department and they had either moderate or severe disease with arthritis. The comparison with healthy subjects revealed significant impairment of emotional state in every evaluated item in psoriasis. Psychiatric cormorbidity in psoriasis has been revealed in 24–53% of the patients and our results agree to this estimation (Mattoo *et al.* 2001; Sharma *et al.* 2001).

The limitation of this study is, that compared subjects groups were quite small to extrapolate the results. Most of the subjects with psoriasis were

recruited from the hospital population, which may cause bias in terms of the remarkably lower QoL in this group subjects.

6.3. Expressional differences of the genes of the melanocortin system and intracellular melanogenesis pathways in vitiligo

Systemic response to stress is mainly mediated by the hypothalamic-pituitary-adrenal axis (HPA) in the brain. HPA axis has an equivalent, expressed in the skin and it coordinates local response to stress. Stress response system in the skin contains the melanocortin system that is a part of the cutaneous HPA axis (Slominski *et al.* 2000). In the present study we analyzed the expression of the genes of melanocortin system in the skin biopsy samples of the patients with vitiligo. The analyzed genes of the melanocortin system in this study were: POMC, MC1R-MC5R, ASIP and AGRP.

Our results reconfirmed the expression of cutaneous stress response system in human skin, in spite the fact that there are no previous reports about the expression of MC3R mRNA in human skin (Eves and Haycock 2010). Further studies should discover the exact cell type capable to express MC3R in the skin. The expression profiles of the all five melanocortin receptors were similar between the study groups and this can indicate their functional relevance. A remarkable expressional difference between lesional and nonlesional skin of vitiligo patients ($p < 0.0001$) was found in the case of MC1R. MC1R showed the highest expression in skin samples but the rest of the MCRs had relatively low expression levels in the skin. The correlation analysis revealed a moderate up to strong positive relation between the levels of mRNA of the different melanocortin receptors. The similar expression profile of the all melanocortin receptors may be caused by high homology in sequences (Getting 2006). This kind of expression profile of the MCRs could also be explained by concomitant expression of these genes. Concomitant expression of MC1R and MC2R has been reported in human keratinocytes and epidermal melanocytes, MC1R and MC4R in human dermal papilla cells (Curry *et al.* 2001; Moustafa *et al.* 2002; Suzuki *et al.* 1996; Bohm *et al.* 2006). The role of the melanocortin system in the network of inflammation and pigmentation has been established in the skin but the role of markers of the melanocortin system in the pathogenesis of vitiligo is indistinct. In our study the expression of POMC mRNA was lower in the lesional skin compared with the non-lesional skin in the group of vitiligo ($p < 0.05$). At the same time we did not see the difference in the expression of POMC between the non-lesional skin from the vitiligo subjects and the skin from healthy controls. The mRNA expression levels of the MCRs, mediating the effects of POMC peptides, were decreased in the lesional skin compared to the uninvolved skin and increased in the unaffected skin of vitiligo patients compared to the healthy subjects. These differences were statistically significant ($p < 0.01$) in MC1R and MC4R. Decreased expression of the MC1R and MC4R

in the lesional skin was not surprising. Studies have shown the loss of functional melanocytes, a major cell type expressing MC1R, in vitiligo. The expression of MC4R has been very low and detected only in dermal papilla cells. The high homology in the sequences of MC1R and MC4R and similar regulatory regions can predispose comparable down-regulation of these receptors in lesional skin in vitiligo. Increased expression of MCRs in the nonlesional skin of the patients of vitiligo is not so well known. Skin biopsies were taken from the non-sun-exposed areas as UV radiation coud not influence the expression of the genes. The distribution of skin phototypes also did not differ between the study groups. Such over-expression of MCRs can indicate the existence of the compensation system to restore normal pigmentation in lesions of vitiligo. Lack of difference of POMC in the non-lesional skin of vitiligo and the control skin samples gives a hint that this over-expression is induced by systemic influences rather than local ones. TYR, TRP1 and DCT are involved in melanogenesis and in the biology of melanocytes. Down-regulation of the expression of TYR mRNA has been demonstrated in lesional skin in vitiligo (Machado *et al.* 2005). We have shown a statistically significant decrease of TYR expression in the lesional skin compared to the non-lesional skin of vitiligo and healthy controls (both $p < 0.0001$) in our previous study (Kingo *et al.* 2006). In addition to the previously stated, the expression of TRP1 mRNA by melanocytes has shown a significantly lower level of vitiligo compared to melanocytes of the controls (Jimbow *et al.* 2001). The expression of DCT mRNA has not been investigated in vitiligo. In the present study the expressions of TRP1 and DCT mRNAs were suppressed in the lesional skin of the vitiligo subjects that confirms the decreased melanin synthesis in vitiligo. Previous studies have demonstrated the absence or inactivity of melanocytes in the lesional area of vitiligo. As the values of mRNA were at a detectable level, it explanes that not all melanocytes are destroyed in the lesions.

In our study the genes involved in the melanin synthesis we up-regulated in the uninvolved skin samples of vitiligo patients compared to the samples from healthy controls. This difference was statistically significant in the case of TRP1. As the overexpression of melanocortin receptors was also detected in the same samples, it refers that the activation of enzyme genes in the non-lesional skin is a consequence of melanocortin stimulation. Our finding has a functional relevance. We suppose that the suppression of melanin production activates MCRs transcriptionally through negative feedback to restore normal pigmentation. Melanocortins have a wide spectrum of immunomodulatory and anti-inflammatory capacities (Luger *et al.* 2003; Wintzen and Gilchrest 1996; Teofoli *et al.* 1997). The previous studies have reported the increase of the expression of pro-inflammatory cytokines (IL-2, TNF-α, IL-6 and INF-γ) in lesional skin compared to uninvolved skin of vitiligo patients (Caixia *et al.* 1999; Grimes *et al.* 2004). Such up-regulation of the MCRs in the non-lesional skin may be an attempt to inhibit the production of proinflammatory factors and to suppress inflammatory response in the lesional skin of vitiligo patients. Positive correlation was also found between the levels of MC1R and TRP1 in

the skin of healthy controls but not in the skin of vitiligo patients. These findings indicate that significant changes in transcriptional regulation of the melanocortin and melanin synthesis system take place in the skin in vitiligo.

In the present study we determined the importance of genes of the signal transduction pathways between the melanocortin system and the melanogenesis enzymes in the pathogenesis of vitiligo. All investigated genes, except CREB1, had statistically significant differences in expression levels between the skin of the vitiligo patients and the control subjects. The expression levels of MITF ($p < 0.0001$) and LEF1 ($p < 0.0005$) in the lesional skin of vitiligo patients were lower comparing with the non-lesional skin of vitiligo patients. MITF expression in the lesional skin of vitiligo patients was lower when compared with the skin of healthy control subjects ($p = 0.0001$). The difference in MITF expression between the lesional and non-lesional skin of vitiligo patients has previously been published (Kitamura *et al.* 2004). To our knowledge, there are no expressional studies comparing the level of MITF mRNA in the skin of vitiligo patients with the level in the skin of healthy control subjects. In our study MITF and LEF1 both showed higher expression in the non-lesional skin of the vitiligo patients comparing with the skin of healthy control subjects but this finding was statistically non-significant. The expression profile tendencies of MITF and LEF1 are similar with the results published in our previous studies (Kingo *et al.* 2006, 2007). Statistically significant positive correlations between MITF and TYR ($r = 0.50$; $p < 0.05$), TRP1 ($r = 0.69$; $p < 0.01$) and DCT ($r = 0.58$; $p < 0.05$) expression levels in the skin of healthy control subjects but not in the skin of vitiligo patients let us assume that MITF's function of raising the transcription levels of melanogenesis enzymes is impaired in vitiligo. The expression level of p38 (MAPK14) was lower in the lesional skin of vitiligo patients when compared with the skin of healthy control subjects ($p < 0.005$). As p38 is an activator of CREB1 and USF1 proteins (Saha *et al*. 2006), therefore lowered p38 level in the skin of vitiligo patients may reduce the positive regulation of melanogenesis by CREB1 and USF1. The statistically significant positive correlations ($p < 0.001$) between the expression levels of p38 and PI3KCB and the levels of p38 and RPS6KB1 likely assume regulatory connections between the mRNA levels of these gene pairs. The finding of statistically significant positive correlations between p38 and TYR ($r = 0.57$; $p < 0.05$), TRP1 ($r = 0.76$; $p < 0.001$) and DCT ($r = 0.68$; $p < 0.01$) mRNA levels in the skin of healthy control subjects and the lack of these correlations in the skin of vitiligo patients may imply the impairment of positive regulation of melanogenesis by p38 in vitiligo. USF1 expression in the non-lesional skin of vitiligo patients was higher when compared with the skin of healthy control subjects ($p < 0.01$). As USF1 regulates the transcription of MC1R, TYR, and DCT (Galibert *et al.* 2001; Schwahn *et al.* 2005; Corre *et al.* 2004), it might be assumed that the elevated MC1R expression and the tendencies towards higher TYR and DCT mRNA levels in the non-lesional skin of vitiligo patients demonstrated in our previous studies could be the result of heightened USF1 expression (Kingo *et al.* 2006, 2007). However, we could not show statistically

significant Spearman correlations between the USF1 mRNA levels and the expression levels of MC1R, TYR and DCT. The expression of PIK3CB in the lesional skin of vitiligo patients was lower compared with the skin of healthy control subjects ($p = 0.01$). As we know PI3K has an antiapoptotic effect, thus the lowered level of PI3K in the lesional skin of the patients may cause increased susceptibility of skin cells to apoptosis (Martelli *et al.* 2006). Furthermore, the decreased level of PI3K may increase oxidative stress in the lesional skin of vitiligo patients, as the inhibition of PI3K results in reduction of the intracellular glutathione concentration (Ramos *et al.* 2005). These results agreed to the findings that the levels of apoptosis and oxidative stress are increased in the skin of vitiligo patients (Huang *et al.* 2002; Lee and Modlin 2005b; Koca *et al.* 2004; Schallreuter *et al.* 1999b). As to MITF and p38, the expression levels of PI3KCB correlated with the mRNA levels of melanogenesis enzymes in the skin of healthy control subjects (TYR: $r = 0.52$; $p < 0.05$; TRP1: $r = 0.75$; $p < 0.001$; DCT: $r = 0.58$; $p < 0.05$) but not in the skin of the vitiligo patients. Concequently, we can suppose that there are dys-functions in the pathways through which PI3K exerts positive effects on melanogenesis in the skin of vitiligo. The expression of RPS6KB1 (p70(S6)K) was decreased in the lesional skin of vitiligo patients compared with the skin of healthy control subjects ($p < 0.05$). As one of the most important functions of RPS6KB1 is to inactivate the translation inhibiting factor PDCD4 in ribosomes (Dorello *et al.* 2006), the decrease in RPS6KB1 expression may cause the inhibition of protein synthesis, reduction of cell growth and slowing of cell cycle in the skin of vitiligo patients. This support the data that melanocytes derived from the skin of vitiligo patients have growth defects in cell culture (Puri *et al.* 1987, 1989). There correlation between the mRNA levels of RPS6KB1 and TYR ($r = -0.47$; $p < 0.05$) in the lesional skin of vitiligo patients was negative. This correlation fits the data according to which the inhibition of p70(S6)K with rapamycin increases the expression of tyrosinase (Busca *et al.* 1996). The level of BCL2 expression was higher in the non-lesional skin of vitiligo patients compared with the skin of healthy control subjects ($p < 0.05$) and lower in the lesional skin of vitiligo patients compared with the non-lesional skin of patients ($p < 0.05$). Nevertheless, the study using flow cyto-metry, did not demonstrate significant difference in BCL2 expression between melanocytes from the non-lesional skin of vitiligo patients and melanocytes from the skin of healthy control subjects (van den Wijngaard *et al.* 2000a). We can not deny that the shown difference in our study is due to keratinocytes and this needs to be confirmed by further studies.

6.4. The future prospects

1. In the current study we observed the clinical aspects of vitiligo and our plans are to continue with these studies: to find associations between halo nevi and different subtypes of vitiligo at a molecular level, to continue with long term

follow-up of segmental subtype of vitiligo as the final course of this subtype is still obscure.

2. We hope to perform protein expression measurements in the skin and blood samples from vitiligo patients and control individuals. In case of blood, we hope to observe both PBMCs and blood sera. We would also like to perform immunohistochemical studies with skin and blood cells from vitiligo patients and controls. This way we could obtain additional information – namely, in which cells exactly our study objects are expressed in different conditions.
3. We also perform genetic variation (copy number variation) and single nucleotide polymorphism (SNP) analysis in candidate genes with the aim to explain the role of variations of established genes in vitiligo development.

7. CONCLUSIONS

1. We observed that *vitiligo vulgaris* is the most frequent clinical subtype of vitiligo in adults. The disease has a long-lasting course with a slow progression during the years. Female gender is associated with the extensive pigment loss, the occurrence of autoantibodies in the blood, autoimmune disease and APS.
2. We verified for the first time that the patients with familial vitiligo have a higher risk for vulgar subtype at the beginning of the disease. We also revealed that female gender increases the risk for more extensive depigmentation in sporadic cases of vitiligo, not shown before.
3. We demonstrated the disease-related small impairment of QoL in vitiligo. In vitiligo female gender is more influenced in symptoms/feelings and male gender in relations, the latter finding has not been revealed before. Lower QoL in vitiligo is associated with earlier disease onset, widespread pigment loss, disease progression and depigmentation on the hands.
4. We revealed that the expression of the genes of melanocortin system and the genes of the intracellular melanogenesis pathway is altered in vitiligo. The increase of the expression of these genes in the non-lesional skin among vitiligo patients has not been previously described. This up-regulation could function as a compensation to restore normal pigmentation in depigmented lesions of the skin.

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9. SUMMARY IN ESTONIAN

Vitiliigo: kliinilised aspektid, elukvaliteet ja melanokortiini süsteemi roll haiguse patogeneesis

Üldteoreetiline taust

Vitiliigo on omandatud, idiopaatiline haigus, mis melanotsüütide selektiivsest destruktsioonist põhjustatuna väljendub naha, karvade ja limaskestade koldelises või generaliseeritud depigmentatsioonis. Vitiliigosse haigestutakse enamasti lapse- ja noorukieas, aga haigus võib avalduda ka hilises eas, peale viiekümnendat eluaastat. Vitiliigot esineb 0,5–1,0% elanikkonnast Euroopas ja USA-s, 0,1–0,6% Hiinas ja kuni 8,8% Indias. Depigmentatsiooni ulatuse ja lokalisatsiooni alusel eristatakse mitmeid kliinilisi haigusvorme. Vitiliigo klassifitseerimisel pole päris ühtset seisukohta. Varasemast perioodist on levinud vitiliigo segmentaalne ja mittesegmentaalne jaotus, mille aluseks arvatakse olevat haiguse erinev patogenees. Detailsema klassifikatsiooni on 21.sajandi alguses esitanud Hann ja Nordlund, kes vastavalt depigmentatsiooni paigutusele ja ulatusele jaotavad vitiliigo lokaliseeritud, generaliseeritud ja universaalseks vormiks ning toovad välja nende vormide alavormid. Uuringud on näidanud, et haiguse fenotüüp võib aastate jooksul muutuda. 10–74% vitiliigo haigetest esineb antud haigus lähisugulastel. Vitiliigo esineb sageli koos teiste põletikuliste ja autoimmuunhaigustega (türeoidiit, pernitsioosne aneemia, reumatoidartriit, Addisoni tõbi, diabeet, erütematoosluupus, psoriaas, jt) ning haigete vereseerumist on leitud mitmeid autoantikehi [kilpnäärme koe vastased (anti-TPO), mao parietaalrakkude vastased (PCA), tuumavastased (ANA), neerupealise koore vastased (AAA)]. Vitiliigo võib olla autoimmuunse polüendokriinse sündroomi üheks väljenduseks, esinedes sagedamini koos kilpnäärme autoimmuunse kahjustusega.

Vitiliigosse haigestumine toob endaga kaasa mitmeid psühhosotsiaalseid probleeme. Varasemates uuringutes haiguse mõjust elukvaliteedile ja enesehinnangule on näidatud, et vitiliigo võib põhjustada sotsiaalset isolatsiooni, tugevat depressiooni (eriti naistel), mõjutada

inimese seksuaalsuhteid ja valmisolekut abieluks. Peamised elukvaliteediga seotud probleemid vitiliigo haigel on: riiete valik, päikesekaitset sisaldavate toodete ja varjava meigi kasutamine, aktiivse tegevuse piiramine, inimeste reageering haigusele ja emotsionaalsed probleemid. Uuringutes on dermatoloogia elukvaliteedi indeksi (DLQI) skoor vitiliigo haigetel olnud 4,4–17,1. DLQI tulemusi mõjutavad rassiline päritolu, nahavärvus, sugu ja kultuuriline taust.

Vitiliigo tekke põhjused pole tänaseni lõplikult selged. Melanotsüütide düsfunktsiooni ja degeneratsiooni põhjustavate patofüsioloogiliste mehhanismide kohta on esitatud mitmeid hüpoteese, milledest levinumad on immuun-, neuraalne-, biokeemiline- (oksüdatiivne stress, autotsütotoksiline) ja melanotsüütide lühenenud eluea hüpotees. Välja on pakutud vitiliigo konvergentsiteooria, mille kohaselt need nimetatud mehhanismid annavad nii eraldi kui ka

koos esinedes ühesuguse tagajärje: melanotsüütide isoleeritud kadumise nahast ning sõltub patsiendist, millise mehhanismi osatähtsus on kõige suurem. Neuraalse regulatsiooni düsfunktsiooni on näidatud erinevate autoimmuunsete ja põletikuliste haiguste korral, kuid seda on vitiliigo haigetel vähe uuritud. Neuroendokriinse süsteemi olulisusele vitiliigo patogeneesis viitab haiguse avaldumine või ägenemine seoses emotsionaalse stressiga. Melanokortiini süsteem on neuroendokriinse süsteemi üks osa, millesse kuuluvad viis melanokortiini retseptorit (MC1R-MC5R), nende retseptorite neli agonisti [α-, β- ja γ-melanotsüüte stimuleerivad hormoonid (MSH) ja adrenokortikotroopne hormoon (ACTH)] ning kaks antagonisti [(agouti-taoline valk (AGRP) ja agouti signaliseerimisvalk ehk ASIP)]. Melanokortiini süsteemi peptiidide ekspressioonitase on kõige kõrgem ajus, aga ekspressioon on olemas ka mitmete perifeersete kudede rakkudes, kaasa arvatud nahas, kus antud peptiide sekreteerivad keratinotsüüdid ja melanotsüüdid. Melanokortiini süsteem täidab nahas erinevaid stressivastusega seotud funktsioone, reguleerides pigmentatsiooni, põletikku, eksokriinset sekretsiooni, valutundlikkust ja kehatemperatuuri.

Uurimuse põhieesmärgid

1. Selgitada välja vitiliigo kliinilised aspektid ja autoantikehade esinemine haigete vereseerumis.
2. Uurida haigete emotsionaalset seisundit ja vitiliigo mõju elukvaliteedile ning võrrelda saadud tulemusi psoriaasihaigete ja tervete kontrollisikutega.
3. Melanokortiini süsteemi rolli selgitamine vitiliigo tekkemehhanismis, hinnates melanokortiini süsteemi peptiidide ning nende retseptorite mRNA ekspressioonitasemete erinevusi ning signaaliülekandes melanokortiini süsteemilt pigmentatsiooniensüümidele osalevate geenide ekspressioonitasemete erinevusi nahas vitiliigohaigetel ja tervetel kontrollisikutel.

Uuritavate grupid ja metoodika

Haiguse kliiniliste iseärasususte väljaselgitamiseks anketeerisime mõlemast soost täiskasvanud (18a. ja vanemad) vitiliigo haigeid, kes reageerisid ajalehes avaldatud uuringu üleskutsele või pöördusid SA TÜK nahahaiguste kliinikusse ambulatoorsele vastuvõtule. Uuritavaid teavitati suuliselt ja kirjalikult uuringu eesmärkidest ja meetoditest, mille järgselt nad allkirjastasid informeeritud nõusoleku uuringus osalemiseks. Uuringusse arvamise kriteeriumiks oli vitiliigo diagnoos, mis püstitati naha, limaskestade ja karvade vaatlusel loomulikus ja ultraviolettkiirte (Woodi lamp) valguses. Uuritavate kohta täideti ankeet, millesse märgiti vanus, sugu, rahvus, naha fototüüp, haigestumise aeg, haiguse kestus, depigmentatsiooni lokalisatsioon haigestumisel ja uuringu ajal, kliiniline alavorm haigestumisel ja uuringu ajal, haiguse aktiivsus, ulatus, Köbneri tunnus, haiguse kulg, haigestumist mõjutavad tegurid, kaasnevad haigused, vitiliigo ja teiste haiguste esinemine perekonnas, eelnev ravi, spontaanne repigmentatsioon. Uuringugrupp moodustus 155 haigest (44 M, 111 N; keskmine vanus 44,9 ± 16,3 a.; vanuse piirid 18–82 a.). Erinevusi perekondliku ja sporaadilise vitiliigo haigusjuhtude vahel võrdlesime 186 uuritaval, kelle seas

perekondliku vitiliigoga juhte oli 51 (15 M, 36 N; keskmine vanus 41,7 ± 15,6 a.; vanuse piirid 18–82 a.) ja sporaadilise vitiliigoga juhte 135 (42 M, 93 N; keskmine vanus 45,5 ± 16,3 a.; vanuse piirid 18–77 a.) ning antikehad olid määratud 173 uuritaval. Autoantikehade määramiseks paluti uuringugruppi kuuluvatel isikutel anda ühekordselt 14 ml veeniverd, millega nõustus 141 uuritavat. Autoantikehade (anti-TPO, PCA, ANA, AAA) ja reumatoidfaktori määramine vereseerumis toimus TÜK ühendlaboris kasutatavatel meetoditel. ANA, PCA ja AAA määrati kaudsel immunofluorestsentsmeetodil, antigeenina kasutati roti maksa (ANA), hiire magu (PCA) ja inimese neerupealise kudet (AAA). Sekundaarse antikehana kasutati fluorestseeruva ainega märgistatud küüliku polüklonaalset IgG. Anti-TPO määrati kemiluminestsents- ja RF immunoturbidimeetrilisel meetodil

Selgitamaks haiguse mõju elukvaliteedile, küsitlesime mõlemast soost täiskasvanuid (vanus ≥18a.) vitiliigoga 54 (22 M, 32 N; keskmine vanus 36,6 ± 15,0a., keskmine haiguse kestus 11,3 ± 9,8a.) ja psoriaasiga 57 (27 M, 30 N; keskmine vanus 40,0 ± 13.3a., keskmine haiguse kestus 18,6 ± 12,7a.) isikuid. Kontrollgrupi moodustasid mõlemast soost täiskasvanud 57 (23 M, 34 N, keskmine vanus 39,7 ± 12,8a.), kes ei põe vitiliigot ja teisi kroonilisi dermatoose, kellel puudub perekondlik vitiliigo anamnees. Kontrollgrupi isikud koguti eelnevalt healoomuliste nahamoodustistega kliinikus konsulteeritud isikute, meditsiiniüliõpilaste ja tervisehoolekande töötajate seast. Kõigi uuritavate küsitlemisel kasutati Eesti oludele kohaldatud dermatoloogia elukvaliteedi indeksi küsimustikku (Finley, 1994) ja üldkasutatavat emotsionaalse enesehindamise küsimustikku (EEK-2, Aluoja jt. 1999). Haiguse kliiniliste tunnuste iseloomustamiseks kasutati vitiliigo korral eelmises uuringus kirjeldatud ankeeti ja psoriaasi korral sarnast ankeeti (vanus, sugu, rahvus, naha fototüüp, haigestumise aeg, kestus, kliiniline vorm, haiguse ulatus, kulg, küünte ja liigeste haaratus, perekondlikkus, kaasnevad haigused ning haigused perekonnas).

Geenide ekspressioonitasemete uurimiseks nahas võtsime eelpoolnimetatud vitiliigo uuringugruppi kuuluvalt 31 ja 39 isikult lokaalanesteesias 4 mm stantsiga kaks nahabioptaati, ühe koldest ja teise näiliselt tervest nahast. Võrdlusandmete saamiseks võtsime 24, 31 ja 18 tervel kontrollgrupi isikul samal meetodil ühe nahatüki. Kontrollisikuteks olid tervisehoolekandetöötajad, meditsiiniüliõpilased ja nahahaiguste kliiniku ambulatoorsesse osakonda pöördunud isikud näo teleangiektaasiate ja healoomuliste nahanäsadega, kellel ei esinenud vitiliigot ja teisi kroonilisi dermatoose ning kellel puudus perekondlik vitiliigo anamnees. TÜ füsioloogia instituudis määrasime nahas TaqMan® QRT-PCR meetodil melanokortiini süsteemi peptiidide ja nende retseptorite mRNA ekspressiooni tasemed ja signaaliülekandes melanokortiini süsteemilt pigmentatsiooniensüümidele osalevate geenide ekspressiooni tasemed.

Andmete analüüs vitiliigo haigete alagruppide võrdlemisel toimus hii-ruut testi ja Microsoft Office 2008 Excel (Microsoft Corporate, Redmond, WA, USA) tarkvara abil. Elukvaliteedi uuringus testisime normaaljaotusele vastavaid andmeid parameetriliselt paaritu t-testi teel ja normaaljaotusele mittevastavaid andmeid Mann-Whitney t-testi abil. Geenide ekspressiooni määramisel

kasutasime mRNA kvantifitseerimiseks võrdleva tsükliläve (Ct) meetodit (inglise keeles *comparative cycle threshold method*), sihtmärk-geeni ekspressioonitase normaliseeriti endogeense võrdlusgeeni HPRT1 suhtes. Geenide mRNA ekspressioonitasemeid iseloomustava parameetrina kasutasime $2^{-\delta Ct}$ väärtusi. Geenide ekspressioonitasemete gruppidevahelised erinevusted kordades leiti kui võrreldava grupi $2^{-\delta Ct}$ ja referentsiks valitud grupi $2^{-\delta Ct}$ suhe. Normaaljaotusele vastavust testisime Kolmogorov-Smirnov'i testi abil. Geenieksprессiooni tasemete mõõtmistulemuste jaotus selle meetodi põhjal ei vastanud Gaussi jaotusele. Gruppidevaheliste erinevuste testimiseks kasutasime Mann-Whitney U-testi ja Kruskal-Wallis'e testi. Ühe grupi kahe parameetri vaheliste suhete uurimiseks kasutasime korrelatsioonianalüüsi, korrelatsiooni tugevust hindasime Spearman'i korrelatsiooni meetodi abil. Andmetöötlus toimus GraphPad Prism 4 tarkvaraga (GraphPad Software, San Diego, CA, USA). Kõigi testide puhul lugesime statistiliselt oluliseks p väärtuse <0,05.

Tulemused

Vitiligo avaldus 30.eluaastaks enam kui pooltel juhtudest, 15%-l esines hiline (≥50a.) haigestumine. *Vitiligo vulgaris* oli levinum kliiniline alavorm nagu ka mujal maailmas, esinedes 81%-l haigetest. Kliiniliste alavormide võrdlemisel ilmnes statistiliselt oluline erinevus haigestumise vanuse osas segmentaalse ja akrofatsiaalse ($P < 0.001$), segmentaalse ja koldelise ($P < 0.05$), segmentaalse ja hariliku ($P < 0.05$), universaalse ja akrofatsiaalse ($P < 0.05$) alavormi korral. Segmentaalne ja universaalne vitiligo avaldusid juba lapseeas, kuid esinesid antud täiskasvanute grupis harva (6%). Pigmendi kadu algas sagedamini ülajäsemelt ja kehatüvelt, aja jooksul oli kliiniline vorm muutunud 15%-l. Köbneri tunnus esines 15%-l ja *leukotrichia* 48%-l haigetest. Enam kui pooltel vitiligo haigetest ei ületanud pigmendi kadu 10% nahapinnast ja haigus oli aktiivses faasis 70%-l. Pigmendi kadu esilekutsuvaid tegureid nimetas 39%, olulisemad olid stress, mehhaaniline trauma ja hormonaalsed nihked. Välise teguri olemasolu oli statistiliselt oluliselt seotud naissooga ($P = 0.003$) ja laialdasema (üle 10% nahapinnast) pigmendi kaoga ($P = 0.0001$). Kaasnev autoimmuunhaigus oli diagnoositud 37%-l, sagedamini kilpnäärme haigus (17%), haloga neevus (16%), psoriaas (5%), reumatoidartriit (4%). Vitiligo esines perekonnas 26%-l uuritavatest, kilpnäärme haigus 21%-l, psoriaas 8%-l, reumatoidartriit 5%-l, Addisoni tõbi 1%-l. Vereseerumis leidus autoantikehi 50%-l: TPO-ak 35%-l, PCA 12%-l, RF 8%-l, ANA 3.5%-l ja AAA 2.9%-l uuritavatest. Kogutud andmete alusel esines 19-l haigel APS ja 35-l juhul oli tegemist võimaliku APS-3 (seerumis TPO-ak, kliiniliselt uurimata). Meestega võrreldes esines naistel statistiliselt enam kaasnevat autoimmuunhaigust ($P = 0.011$), vereseerumi positiivsust autoantikehadele [($P = 0.045$), TPO-ak ($P = 0.013$)], APS ($P = 0.007$) ja autoimmuunhaigust perekonnas ($P = 0.042$).

Perekondliku ja sporaadilise vitiligo juhtude võrdlemisel selgus, et perekondliku vitiligo haigetel on suurenenud mitmed riskid: haigestuda 20.eluaastaks ($P = 0,008$; OR 2,407; 95%CI 1,246–4,649), ulatuslikumaks depigmentatsiooniks (BSA üle 10%: $P = 0,004$; OR 2,606; 95% CI 1,341–5,064 ja

BSA üle 50%: P = 0,001; OR 3,856; 95%CI 1,597–9,310), hariliku vitiliigo vormi tekkeks (P = 0,008; OR 2,966; 95%CI 1,289–6,821) ja kilpnäärmehaiguse esinemiseks perekonnas (P = 0,03; OR 2,200; 95% CI 1,064–4,548). Sporaadilistel juhtudel olid naissugu ja haiguse kestus ≥10a. riskiteguriteks ulatuslikuma depigmentatsiooni tekkel (BSA > 10%: P = 0,001; OR 3,984; 95% CI 1,668–9,520 ja P = 0,001; OR 3,560; 95% CI 1,681–7,539; vastavalt). Väga laialdane depigmentatsioon (BSA > 50%) suurendas mõlemas grupis riski pigmendi kaoks limaskestadel (perekondlik P = 0,01; OR 8,000; 95% CI 1,261–50,772; sporaadiline P = 0,004; OR 7,375; CI 1,550–35,096), vallandava teguri olemasoluks (perekondlik P = 0,0005; OR 10,560; 95% CI 2,451–45,497; sporaadiline P=0,004; OR 7,630; 95% CI 1,580–36,858) ja leukotrihhiaks, mis oli statistiliselt oluline perekondlike juhtude grupis (P = 0,0001; OR 26,923; 95% CI 3,178–228,060). Sporaadiliste vitiliigo juhtude korral suurendas laialdane depigmentatsioon (BSA > 50%) riski autoantikehade olemasolule vereseerumis (P = 0,03; OR 4,941; 95%CI 1,005–24,300), eriti PCA ja ANA osas (P = 0,04; OR 4,457; 95% CI 0,994–19,978 ja P = 0,0002; OR 28,250; 95%CI 2,307–345,989 vastavalt). Uuring näitas, et vitiliigo mõju elukvaliteedile on heledanahalistel täiskasvanutel väike (DLQI keskmine 4,7), see on oluliselt madalam psoriaasi mõjust elukvaliteedile (DLQI keskmine 13,1; P < 0,001), aga oluliselt kõrgem mõjust tervetel kontrollidel (DLQI keskmine 0,6; P < 0,001). Kontrollisikutega võrreldes avaldas vitiliigo DLQI skaalal statistiliselt olulist mõju sümptomitele ja tunnetele (P < 0,001), aktiivsele tegevusele (P < 0,001), vaba aja veetmisele (P < 0,001), aga ka ravimisega seotud toimingutele (P < 0,01) ja isiklikele suhetele (P < 0,05). Sugu oli seotud kõrgema skooriga sümptomite/tunnete skaalal naistel (P = 0,003) ja suhete skaalal meestel (P = 0,040). Vitiliigo korral oli DLQI seotud varase haigestumisega (≤ 20a. *vs* >20a.; P = 0,040), haiguse aktiivsusega (P = 0,006), depigmentatsiooni ulatusega (BSA ≤ 10% *vs* > 10%; P = 0,005) ja pigmendi kaoga labakätel (P = 0,008). Psoriaasi haigetel oli DLQI seotud lööbe ulatuse ja tugevuse indeksiga (PASI ≤ 10 *vs* ≥20; P = 0,027) ning kaasneva artriidiga (P = 0,019). Vitiliigo haigetel ei esinenud tervete kontrollidega võrreldes statistiliselt olulisi erinevusi emotsionaalse enesehinnangu kogu skaala (depressioon, üldine ja sotsiaalne ärevus, paanika, asteenia, unetus) keskmiste skooride osas. Vastavalt EEK-2 esines depressiivsus 20% vitiliigo haigetest, kellel oli ka mõju elukvaliteedile oluliselt suurem kui mittedepressiivsetel vitiliigo haigetel (DLQI 7,2 *vs* 4,2; P < 0.05). Vitiliigoga võrreldes avaldas psoriaas olulist mõju depressiooni (P<0.05), üldise ärevuse (P < 0.01) ja asteenia (P < 0.05) skaalal ning kontrollisikutega võrreldes skaala kõigi hinnatavate tunnuste osas.

Melanokortiini süsteemi peptiidide, nende retseptorite ja signaaliülekandes melanokortiini süsteemilt pigmentatsiooniensüümidele osalevate geenide mRNA ekspressioonitasemete hindamine nahas vitiliigo haigetel ja tervetel kontrollisikutel tõi välja mitmeid erinevusi. Selgus, et vitiliigo haigete depigmenteerunud nahas on POMC'i mRNA ekspressioon 1,9 korda madalam kui haigete visuaalselt kahjustamata nahas (p < 0,05). MC1R'i ekspressioonitase oli vitiliigo haigete kahjustamata nahas 1,6 korda kõrgem ekspressioonist kontroll-

isikute nahas ($p < 0,01$) ja vitiliigokoldes 2,1 korda madalam ekspressioonist haigete kahjustamata nahas ($p < 0,0001$). MC4R'i ekspressioon oli vitiliigo haigete kahjustamata nahas 1,9 korda kõrgenenud võrreldes kontrollisikute nahaga ($p < 0,01$) ja ekspressioon haiguskoldes langenud 2,5 korda võrreldes haigete kahjustamata nahaga ($p < 0,01$). Sama trend esines ka MC2R, MC3R ja MC5R puhul, kuid need erinevused ei olnud statistiliselt olulised. TYR'i ekspressioonitase vitiliigokoldes oli 14,0 korda madalam võrreldes kahjustamata nahaga ($p < 0,0001$) ja 8,5 korda madalam võrreldes tervete kontrollisikute nahaga ($p < 0,0001$), ekspressiooni tõus vitiliigo haigete kahjustamata nahas võrreldes kontrollisikute nahaga ei olnud statistiliselt oluline. TRP1 mRNA ekspressioon vitiliigokoldes oli 6,8 korda madalam ekspressioonist tervete kontrollisikute nahas ($p < 0,0001$) ja 19,7 korda madalam ekspressioonist vitiliigo haigete kahjustamata nahas ($p < 0,0001$), ekspressioon haigete kahjustamata nahas oli 2,9 korda kõrgem võrreldes tervete kontrollisikute nahaga ($p < 0,05$). DCT mRNA ekspressioon haiguskoldes oli 7,6 korda madalam ekspressioonist tervete kontrollisikute nahas ($p < 0,0001$) ja 12,9 korda madalam ekspressioonist vitiliigo haigete terves nahas ($p<0,0001$), ekspressiooni kõrgenemine haigete kahjustamata nahas võrreldes tervete kontrollisikute nahaga ei olnud statistiliselt oluline ($p = 0,14$). MITF-M'i mRNA ekspressioon haiguskoldes oli 3,3 korda madalam kui haigete kahjustamata nahas ($p < 0,0001$) ja 2,4 korda madalam kui tervete kontrollisikute nahas ($p = 0,0001$). LEF1 ekspressioon oli vitiliigo haigete kahjustamata nahas 1,7 korda kõrgem kui haiguskoldes ($p < 0,0005$). USF1 mRNA ekspressioonitase vitiliigo haigete terves nahas oli 1,6 korda kõrgem kui tervete kontrollisikute nahas ($p < 0,01$). CREB1 ekspressioonitasemetes ei olnud statistiliselt olulisi erinevusi vitiliigo haigete kahjustatud ja kahjustamata naha vahel, aga ka võrdluses tervete kontrollisikute nahaga. CREB1 ekspressioon oli statistiliselt mitteoluliselt kõrgenenud haigete kahjustamata nahas võrreldes kahjustatud naha ja kontrollisikute nahaga. p38 mRNA ekspressioon vitiliigokoldes oli 1,6 korda langenud võrreldes tervete kontrollisikute nahaga ($p < 0,005$). PIK3CB mRNA ekspressioon vitiliigokoldes oli 1,3 korda langenud võrreldes tervete kontrollisikute nahaga ($p = 0,01$). RPS6KB1 mRNA ekspressioon vitiliigokoldes oli 1,3 korda langenud võrreldes tervete kontrollisikute nahaga ($p < 0,05$). BCL2 mRNA ekspressioonitase vitiliigo haigete kahjustamata nahas oli 1,6 kõrgem kui haiguskoldes ($p < 0,05$) ja ekspressioon haigete kahjustamata nahas oli 2,3 kõrgem ekspressioonitasemest tervetel kontrollisikutel ($p < 0,05$). Spearmani korrelatsioonianalüüs näitas tugevat positiivset korrelatsiooni TYR, TRP1 ja DCT mRNA tasemete vahel nii vitiliigo haigete kahjustamata ja kahjustatud nahas kui ka tervete kontrollisikute nahas ($r > 0,70$; $p < 0,0001$). Positiivne korrelatsioon ilmnes tervete kontrollisikute nahas MC1R ja TRP1 tasemete vahel ($r = 0,47$; $p < 0,05$). Korrelatsioonid MC3R ja MC4R ($r > 0,79$), p38 ja PI3K ($r > 0,63$), PI3K ja p70(S6)K ($r > 0,55$) ning p38 ja p70(S6)K ($r > 0,69$) ekspressioonitasemete vahel olid statistiliselt olulised ($p < 0,001$) nii vitiliigohaigete nahas kui ka kontrollisikute nahas. Statistiliselt olulised ($p < 0,05$) olid ka korrelatsioonid MITF'i ja p38 ($r > 0,43$), MITF'i ja PIK3CB ($r >$

0,35) ning MITF'i ja p70(S6)K ($r > 0,51$) mRNA tasemete vahel nii vitiliigo-haigete nahas kui ka kontrollisikute nahas.

Antud töös esineb piiranguid. Saadud tulemused ei võimalda teha üldistusi autoantikehade esinemise kohta vitiliigo haigetel, kuna seerumis määrati ainult teatud autoantikehi ja puudus kontrollgrupp. Uuritavate arv elukvaliteedi uuringugruppides oli väike, mis ei luba teha kaugeleulatuvaid järeldusi. Oluliselt madalam elukvaliteet psoriaasi haigetel võib olla mõjutatud nende uuritavate värbamisest statsionaarsest osakonnast. Uurisime geenide ekspressiooni täisnaha koeproovides, mis ei võimalda täpsustada milliste rakkudega on ekspressiooni tõus seotud.

Järeldused

1. Uurimus selgitas välja, et v*itiligo vulgaris* on vitiliigo enamesinev kliiniline alavorm täiskasvanutel. Vitiliigo kulg on pikaajaline, haigus progresseerub aeglaselt aastate jooksul. Naissugu on seotud ulatuslikuma pigmendi kaoga, autoantikehade esinemisega vereseerumis, kaasneva autoimmuunhaiguse ja APS-ga.
2. Antud uurimus näitas esmakordselt, et perekondliku vitiliigoga haigetel on kõrgenenud risk *vitiligo vulgaris'*e väljakujunemiseks varases haigusjärgus. Leidsime, et naissugu on oluliseks riskiteguriks ulatusliku depigmentatsiooni tekkel sporaadilistel juhtudel, mida pole varem näidatud.
3. Leidsime, et vitiliigo mõju elukvaliteedile on täiskasvanutel väike. Elukvaliteedi indeksi analüüs näitas, et naissugu on enam mõjutatud sümptomite/tunnete ja meessugu isiklike suhete skaalal, viimast pole varem rõhutatud. Madalam elukvaliteet on vitiliigo haigetel seotud varasema haigestumisega, ulatusliku pigmendi kaoga, haiguse progresseerumisega, depigmentatsiooniga labakätel.
4. Selgitasime välja, et melanokortiini süsteemi geenide ja signaalülekandes melanokortiini süsteemilt pigmentatsiooniensüümidele osalevate geenide ekspressioon on vitiliigo korral muutunud. Melanokortiini süsteemi geenide ekspressioonitasemete tõusu vitiliigo haigete visuaalselt kahjustamata nahas ei ole varem näidatud ning see viitab kompensatoorse mehhanismi olemasolule taastamaks normaalset pigmentatsiooni haiguskolletes.

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PUBLICATIONS

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Research and development work:

The aim of scientific work is to identify network of genes and biomolecules which are involved in and malfunction of which leads to development vitiligo, psoriasis and atopic dermatitis. Main objectives of the experiments are determining effects of gene polymorphisms of Class II Helical Cytokines (HCII) and their receptors (HCRII) on vitiligo and psoriasis and identifying regulatory role of nervous system in development of these diseases, by determination and comparison of expression levels of neurotransmitters, neuropeptides, neurohormones and their receptors in healthy control group and patients.

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2. **Karelson M**, Silm H, Salum T, Kõks S, Kingo K. Differences between familial and sporadic cases of vitiligo. J Eur Acad Dermatol Venereol 2012; 26(7): 915–918.
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6. Douroudis K, Kingo K, **Karelson M**, Silm H, Reimann E, Traks T, Vasar E, Kõks S. The PRO2268 gene as a novel susceptibility locus for vitiligo. Acta Derm Venereol 2011; 91(2): 189–191.
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8. Kingo K, Reimann E, **Karelson,** M, Rätsep R, Raud K, Vasar E, Silm H, Kõks S. Association analysis of genes of the IL19 cluster and their receptors in vitiligo patients. Dermatol 2010; 221: 261–266.
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18. Silm H, **Karelson M**. Terbinafine: efficacy and tolerability in young children with tinea capitis due to *Microsporum canis*. J Eur Acad Dermatol Venereol 2002; 16(3): 228–230.

Research grants:
- PUT 177 “Molecular mechanisms of the patogenesis of vitiligo, the role of T-cells in the patogenesis of vitiligo”, 2013–2016.
- SF0180043s07 “Chronic dermatoses: pathogenetic mechanisms”, 2007–2012.
- ETF 6576 “The Interleukin-10 family cytokines genes polymorphisms in vitiligo”, 2006–2009.
- ETF 5712 “The Interleukin-10 family cytokines gene polymorphisms in psoriasis”, 2004–2007.
- SF0182128s02 ”Cutaneous and sexually transmitted diseases: pathogenetic mechanisms and epidemiology in Estonia”, 2002–2006.

Membership of professional organizations:
- Academy of European Dermatologists and venereologists, member
- Association of the Central and East Eoropean Dermatovenereologists, member
- Association of the Baltic Dermatovenereologists, member
- Estonian Union of Sexually Transmitted Infections, member
- Estonian Society for Dermatovenereologists, secretary 2000–2006, board member 2012–.

ELULOOKIRJELDUS

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Haridus:

1977–1980	Antsla Keskkool
1982–1990	Tartu Ülikool, arstiteaduskond, ravi eriala, arsti kutse 25.06.1990
1990–1991	TÜ nahahaiguste kliinik, internatuur 25.06.1991
1990–1994	TÜ nahahaiguste kliinik, dermatoveneroloogia kliiniline ordinatuur ja residentuur, dermatoveneroloogi kutse 28.09.1994
2009–2013	Tartu Ülikool, arstiteaduskond, nahahaiguste kliinik, doktorantuur

Keelteoskus:
Inglise keel kõnes ja kirjas
Vene keel kõnes ja kirjas

Teenistuskäik:

2004– …	Tartu Ülikool, nahahaiguste kliinik, vanemassistent
2000– ...	SA TÜKliinikum, nahahaiguste kliinik, osakonnajuhataja/vanemarst-õppejõud administratiivsetes kohustustes
1994–2004	Tartu Ülikool, nahahaiguste kliinik, assistent

Teaduslik ja arendustegevus:
Peamiseks tegevusvaldkonnaks teadustöös on olnud krooniliste dermatooside etioloogia, patogeneesi ja kliiniliste iseärasuste uurimine. Teadustöö eesmärkideks on avastada nihkeid biomolekulide ja geenide võrgustikus, mis on seotud vitiliigo, psoriaasi ja atoopilise dermatiidi avaldumisega. Eesmärkide püstitusest tulenevalt uurime tsütokiinide ja nende retseptorite geenide polümorfisme vitiliigo ja psoriaasi haigetel ning samuti uurime närvisüsteemi regulatiivset rolli nimetatud haiguste tekkes, määrates neurotransmitterite, neuropeptiidide ja neurohormoonide ekspressioonide tasemeid haigetel ja võrreldes saadud tulemusi tervete kontrollisikutega.

Publikatsioonid eelretsenseeritavates ajakirjades:

1. **Karelson M**, Silm H, Kingo K. Quality of Life and Emotional State in Vitiligo in an Estonian Sample: Comparison with Psoriasis and Healthy Controls. Acta Derm Venereol 2013; 93: 446–450.
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Osalus teadusprojektides:

– PUT 177 „Vitiliigo patogeneesi molekulaarsed mehhanismid, T-rakkude roll vitiliigo patogeneesis", 2013–2016.
– SF0180043s07 „Krooniliste dermatooside patogeneesi molekulaarsed mehhanismid", 2007–2012.
– ETF grant GARNH 6576 „Interleukiin 10 perekonna tsütokiinide geenide polümorfismide seosed vitiliigoga", 2006–2009.
– ETF grant GARNH 5712 „Interleukiin 10 perekonna tsütokiinide geenide polümorfismi seosed psoriaasiga", 2004–2007.
– SF0182128s02 „Naha- ja suguhaiguste patogeneesi molekulaarsed mehhanismid ja epidemioloogilised aspektid Eestis", 2002–2006.

Muu teaduslik organisatsiooniline ja erialane tegevus.

– Euroopa Dermato-Veneroloogide Akadeemia, liige
– Kesk- ja Ida-Euroopa Dermatoveneroloogide Assotsiatsioon, liige
– Balti Dermato-Veneroloogide Assotsiatsioon, liige
– Seksuaalsel Teel Levivate Infektsioonide Eesti Ühing, liige
– Eesti Naha- ja Suguhaiguste Arstide Selts, sekretär 2000–2006, juhatuse liige 2012–.

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