The effect of balneophototherapy in the Blue Lagoon in Iceland on psoriasis compared with phototherapy alone

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Thesis for the degree of Philosophiae Doctor

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Áhrif Bláa Lóns meðferðar á psoriasis miðað við hefðbundna UVB ljósameðferð

Jenna Huld Eysteinsdóttir

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Ágrip

Sóri (e. psoriasis) er langvinnur ónæmismiðlaður bólgusjúkdómur í húð með veruleg neikvæð áhrif á lífsgæði sjúklinga. Ljósameðferð með útfjólublá aum B geislum (UVB) er þekkt sem áhrifarík meðferð við sóra og fyrri rannsóknir benda til þess að samsett meðferð með böðun í Bláa Lóninu fyrir ljósameðferð auki ennfremur á áhrif ljósameðferðarinnar, en þetta hefur ekki verið sannað með óyggjandi hætti.

Markmið rannsóknarinnar var með meta klínísk, sálfélagsleg, vefjafræðileg og ónæmisfræðileg áhrif samsettrar meðferðar í Bláa Lóninu (BL) samanborið við hefðbundna UVB ljósameðferð á psoriasis.

Í byrjun rannsóknartímabils var framkvæmd forrannsókn með 12 sóra sjúklingum til að meta hugsanleg ónæmisfræðileg áhrif samsettrar meðferðar í BL samanborið við hefðbundna UVB ljósameðferð. Ennfremur var tilgangurinn sá að meta rannsóknaraðferðir fyrir fyrirhugaða framskyggnu samanburðarrannsóknina. Þegar forrannsókninni var lokið, var slembröðuðu framskyggnu samanburðarrannsókninni með 68 sóra sjúklingum ýtt úr vör. Tilgangurinn var að meta klínísk, sálfélagsleg, vefjafræðileg og ónæmisfræðileg áhrif samsettrar meðferðar í BL samanborið við hefðbundna UVB ljósameðferð á psoriasis með því að sjúklingunum var slembiraðað í þrjá meðferðarhópa: 1) Göngudeildarmeðferð í BL, 2) Innlagnarhópur í BL, og 3) UVB ljósameðferð á göngudeild. Allar meðferðirnar voru í 6 vikur. Að lokum var hið vefjafræðilega sóra skor, Trozak skor, metið með því að bera það skor saman við aðra mælikvarða sem eru yfirleitt notaðir í rannsóknum á sóra. Trozak skorið var notað við gerð þessarar doktorsritgerðar sem viðbótar mælikvarði á árangur meðferðar.

Húðmerktar (CLA) T eitilfrumur sem tjá húðsækna viðtaka í blóði sórasjúklinga fækkaði töluvert við samsetta meðferð í BL í forrannsókninni, eða þeim sem tjáðu CCR4 fækkuðu um 68% eftir 3ja vikna meðferð, þeim sem tjáðu CD103 um 74%, þeim sem tjáðu CCR10 um 65%, þeim sem tjáðu bæði CCR4/CCR10 um 80% og þeim sem tjáðu bæði CD103/CCR4 um 100% ($p<0.001$ fyrir allar mælingar). Í slembiröðuðu framskyggnu samanburðarrannsókninni náðu mun fleiri sjúklingar PASI 75 eða PASI 90 eftir 6 vikna samsetta meðferð í BL samanborið við hefðbundna UVB ljósameðferð: göngudeildarmeðferð í BL (68.1% og 18.2%), innlagnarhópur í BL (73.1% og 42.3%) og UVB ljósameðferðarhópur (16.7% og 0%; $p<0.05$ í öllum samanburðum). Samsett meðferð í BL hafði einnig betri sálfélagsleg áhrif á sjúklingana og leiddi til betri vefjafræðilegs bata með góðri fylgni við klíníska

bata, ásamt því að færri meðferðarskipti þurfti til að ná PASI 75 (14.7±4.2 fyrir göngudeild BL, 17.9±10.0 fyrir innlögn BL og 25.0±6.6 fyrir UVB ljósameðferð; p<0.001 þegar göngudeild BL var borin saman við UVB hópinn og p<0.01 þegar innlögn BL var borin saman við UVB hópinn). Þetta leiddi til minni UVB geislunar og lengri batatíma eftir að meðferð lauk. Varðandi ónæmisfræðileg áhrif samsettrar meðferðar í BL þá var enginn munur miðað við hefðbundna UVB ljósameðferð, þannig að allir meðferðarhóparnir voru skoðaðir saman. Th17 ($CD4^+CD45RO^+IL\text{-}23R^+$ T frumur) og Tc17 ($CD8^+CD45RO^+IL\text{-}23R^+$ T frumur) fækkuðu um meira en 60% eftir aðeins tveggja vikna meðferð (Th17 frá 12.25±7.44% til 3.64±5.51%, p<0.001, og Tc17 frá 15.37±6.37% til 5.89±4.61%, *p<0.001*) með góðri fylgni við klíníska og vefjafræðilegan bata (*p<0.01).* Húðsæknar T17 (IL-17 framleiðandi CLA^+ $CD4^+/CD8^+$ T frumur) og T22 (IL-22 framleiðandi CLA^+ $CD4^+/CD8^+$ T frumur) í blóðinu fækkuðu verulega við meðferð (*p<0.05*), ásamt $CD4^+/CD8^+$ húðsæknum T frumum sem tjá húðfesti viðtakann CD103 (*p<0.05*). Ennfremur sást fækkun á húðsækni viðtakanum CCL17 í sermi og IL-17 tjáningu í leðurhúð sjúklinganna með meðferð í góðri fylgni við klíníska bata (*p<0.05*). Ónæmisfræðilegar litanir á húðsýnum sýndu töluverða fækkun á $CD3^+$, $CD4^+$ og $CD8^+$ T frumum (*p<0.01*) sem fylgdi vel vefjafræðilegum bata (*p<0.05).* Áhugavert var að meðferðirnar virtust ekki hafa svo mikil áhrif á Tc1/Th1 eitilfrumur í blóði. Jafnframt kom í ljós að vefjafræðilegt Trozak skor minnkaði hægt og þétt úr 10.3 fyrir meðferð niður í 5.1 eftir 2ja vikna meðferð og niður í 3.2 eftir 6 vikur (*p*<0.0001). Þessi minnkun var í góðri fylgni við klíníska bata (r=0.49, *p*<0.0001), sálfélagsfræðilegan bata (r=0.61, *p*<0.01) og minnkun á þykkt yfirhúðarinnar í húðsýnunum (*p*<0.001). Þykkt yfirhúðarinnar hafði góða fylgni við Trozak (r=0.68, *p*<0.0001) en ekki við klíníska skorið PASI.

Þessar niðurstöður staðfesta að samsett meðferð í BL er áhrifaríkari meðferð en einungis hefðbundin UVB ljósameðferð ein og sér, bæði klínískt, vefjafræðilega og einnig varðandi betri lífsgæði sóra sjúklinga. Þegar ónæmisfræðileg áhrif meðferðanna voru skoðuð, fannst aftur á móti enginn marktækur munur á milli hinna mismunandi meðferðarúrræða. Ekki hefur áður verið sýnt fram á að húðsækni viðtakarnir CCR4, CCR10 og húðfesti viðtakinn CD103 nær hverfi í blóði sóra sjúklinga eftir. Að lokum leggjum við til notkun Trozak skors sem viðbót við klínísk skor í klínískum rannsóknum á sóra, þar sem það hafði góða fylgni við bæði lífsgæða og klínísk skor.

Lykilorð: Sóri, Bláa Lónið, IL-17, IL-22, mælingar á alvarleika sjúkdóms

Abstract

Psoriasis is a chronic immune-mediated inflammatory skin disease with profound effects on psychosocial wellbeing. Narrowband UVB phototherapy is a known effective treatment for chronic plaque psoriasis and previous studies suggest an additional beneficial effect of balneophototherapy (BPT) at the Blue Lagoon (BL) (bathing in geothermal seawater followed by UVB therapy), but the scientific rationale for this empirical observation remaines elusive.

The aim of the study was to evaluate the clinical, psychosocial, histological, and immunological effects of BPT at the BL has on plaque psoriasis compared with UVB phototherapy alone.

Initially, a pilot study was conducted where 12 psoriasis patients were enrolled to evaluate the potential immunological effect of inpatient BPT at the BL. This was compared with UVB phototherapy was explored, as well as validating methods to use in the prospective randomized controlled trial. Secondly, a prospective randomized controlled trial was conducted where 68 psoriasis patients were enrolled into three different treatment groups to evaluate the clinical, histological, psychosocial of BPT at the BL compared with UVB phototherapy. These groups were as follows: 1) outpatient BPT at the BL (GSW group), 2) Inpatient BPT at the BL (IT-GSW group), and 3) UVB monotherapy (UVB group). All treatments were given for 6 weeks. Thirdly, the histological score of Trozak used as an additional secondary outcome in this thesis was evaluated by comparing it with other psoriasis outcome measures.

Circulating skin homing (CLA) T cells expressing skin homing markers were significantly reduced with BPT at the BL in the pilot study. The reduction was 68% for CCR4 ($p<0.001$), 74% for CD103 ($p<0.001$), 65% for CCR10 ($p<0.001$), 80% for skin homing T cells co-expressing CCR4/CCR10 ($p<0.001$), to no detection (100%) for skin hominig T cells co-expressing CD103/CCR4 ($p<0.001$) after 3 weeks of treatment. In the randomized controlled trial, the percentage of patients who achieved PASI 75 and PASI 90 after 6 weeks of treatment was significantly greater for both BPT regimens, bathing in geothermal seawater three times a week (GSW group; 68.1% and 18.2%) and intensive treatment with geothermal seawater (IT-GSW; 73.1% and 42.3%) compared with UVB monotherapy (UVB group; 16.7% and 0%; $p<0.05$ in all comparison). An improvement in quality of life and histological score paralleled with clinical improvement, and patient treated with BPT required fewer treatment sessions to attain PASI 75 (14.7±4.2 for GSW group, 17.9±10.0 for IT-GSW group and 25.0±6.6 for UVB group; $p<0.001$ GSW group vs UVB group and $p<0.01$ IT-GSW group vs UVB group) and consequently less UVB radiation and achieved longer remission time. Regarding the immunological effect of the treatment groups, no significant difference was

found, so all treatment groups were analysed together to see the difference before and after all treatment regimens. Circulating Th17 ($CD4^+CD45RO^+IL\text{-}23R^+$ T cells) and Tc17 ($CD8^+CD45RO^+IL\text{-}23R^+$ T cells) reduced by more than 60% after only two weeks of treatment (Th17 from 12.25±7.44% to 3.64±5.51%, p<0.001, and Tc17 from 15.37±6.37% to 5.89±4.61%, *p<0.001*) in correlation with both clinical and histological improvement (*p<0.01).* In addition, circulating skin homing peripheral blood T17 (CLA^+ $CD4^+/CD8^+$ T cells producing IL-17) and T22 (CLA^+ $CD4^+/CD8^+$ T cells producing IL-22) reduced significantly with treatment (*p<0.05*), as well as $CD4^+/CD8^+$ skin homing T cells expressing the skin resident marker CD103 (*p<0.05*). Furthermore, the reduction of the skin homing chemokine CCL17 in serum and dermal immunohistochemistry analyses of IL-17 correlated with clinical improvement as measured by PASI (*p<0.05*). Immunohistochemical analysis showed significant depletion of $CD3^+$, $CD4^+$ and $CD8^+$ in the skin (*p<0.01*) in correlation with histological improvement (*p<0.05).* Interestingly, the above treatment protocols did not have any effect upon peripheral blood effector Th1/Tc1 or Th2/Tc2 T cells. Finally, the Trozak histological score was significantly reduced from 10.3 before treatment to 5.1 after two weeks and 3.2 after 6 weeks (p<0.0001) with strong correlation with the reduction in PASI (r=0.49, p<0.0001), DLQI (r=0.61, p<0.01) and Epidermal Thickness (ET) (p<0.001). ET correlated strongly with Trozak score (r=0.68, p<0.0001) but not with PASI.

These findings collectively suggest a superior clinical and psychosocial effect of BPT at the BL compared with UVB phototherapy alone in psoriasis. No significant difference between these psoriasis treatments was observed regarding the impact on immune pathways underlying psoriasis. Interestingly, we observed marked reduction of skin homing markers CCR4, CCR10 and skin resident marker CD103 with treatment in the peripheral blood of psoriasis patients. This has not been shown before. In addition, we propose that the Trozak histological assessment could be a useful additional objective measure of disease severity in combination with clinical severity and quality of life scores to improve the quality of psoriasis clinical trials.

Keywords: Psoriasis, balneophototherapy, IL-17, IL-22, outcome measure

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Contents

List of abbreviations

AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Antigen presenting cell(s)
BL	Blue Lagoon in Iceland
CCL	Cysteine-Cysteine chemokine ligand
CCR	Cysteine-Cysteine chemokine receptor
CLA	Cutaneous lymphocyte-associated antigen
cm	Centimetres
CV	Cardiovascular disease
CXL	Cysteine-X-Cysteine chemokine ligand
CXCR	Cysteine-X-Cysteine chemokine receptor
DCs	Dendritic cells
DLQI	Dermatology life quality index
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ET	Epidermal thickness
FACS	Fluorescence activated cell sorting
GPP	Generalised pustular psoriasis
GSW	Geothermal seawater
H&E	Haemotoxylin and eosin
HLA	Human leukocyte antigen
IF	Immunofluorescence
IHC	Immunohistochemistry
IFN-γ	Interferon gamma
IL	Interleukin
KCs	Keratinocytes
LC	Langerhans' cells (subset of DCs in the epidermis)
LPP	Localised pustular psoriasis
LS-PGA	Lattice System Physicians Global Assessment
m	Metres
mm	Millimetres
mDCs	Myeloid dendritic cells (subset of DCs in the dermis)
µm	Micrometres
MHC	Major histocompatibility complex
N or **n**	Total number
UVB	Narrowband Ultraviolet B phototherapy
OCT	Optimal cutting temperature
P	P value
PASI	Psoriasis area and severity index
PBMCs	Peripheral blood mononuclear cells

pDC	Plasmacytoid dendritic cells (subset of DCs in the dermis)
PPP	Palmoplantar pustulosis
PsA	Psoriatic arthritis
PSORS	Psoriasis susceptibility gene
PUVA	Psoralen plus ultraviolet light A
QoL	Quality of life
r	Pearson's Correlations Coefficient test
RCT	Randomised controlled trial(s)
RNA	Ribonucleic acid
SD	Standard deviation
T1	Type 1 T cells (both helper and cytotoxic)
T17	Type 17 T cells (both helper and cytotoxic)
T22	Type 22 T cells (both helper and cytotoxic)
Tc	T cytotoxic cells
Th	T helper cells
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
Treg	T regulatory cells
Trm	Tissue resident memory T cells
UVA	Ultraviolet A
UVB	Ultraviolet B
vs	Versus

List of figures

List of tables

List of original papers

This thesis is based on the following two original publications and two original manuscripts, which are referred to in the text by their Roman numerals (I-IV) as needed:

I. Eysteinsdottir JH, Sigurgeirsson B, Olafsson JH, Fridriksson Th, Agnarsson BA, Davidsson S, Ludviksson BR. The role of Th17/Tc17 peripheral blood T cells in psoriasis and their positive therapeutic response. *Scandinavian Journal of Immunology.* 2013 Dec;78(6):529-37

II. Eysteinsdottir JH, Olafsson JH, Agnarsson BA, Ludviksson BR, Sigurgeirsson B. Psoriasis treatment: faster and long-standing results after bathing in geothermal seawater. A randomized trial of three UVB phototherapy regimens. *Photodermatology, Photoimmunology and Photomedicine.* 2014 Feb;30(1):25-34.

III. Eysteindottir JH, Sigurgrímsdottir H, Einarsdottir HK, Freysdottir J, Björnsdottir EÖ, Agnarsson BA, Olafsson JH, Sigurgeirsson B, Ludviksson BR. Effective treatment with balneophototherapy and narrowband UVB monotherapy reduces skin homing Th17/Tc17 and Th22/Tc22 effector cells in peripheral blood in patients with psoriasis. Submitted for publication.

IV. Eysteinsdottir JH, Olafsson JH, Agnarsson BA, Jonasdottir S, Sigurgeirsson B. Trozak histological assessment score as an additional objective psoriasis assessment tool in clinical trials Submitted for publication.

Declaration of contribution

I participated in design of the study and formulation of the research questions together with Bárður Sigurgeirsson, Jón Hjaltalín Ólafsson, Björn Rúnar Lúðvíksson and Bjarni A. Agnarsson. Together with my supervisor, I was responsible for communicating with the Icelandic authorities, the National Bioethics committee, Data Protection Authority and the Ethics Committee of Landspitali-The National University Hospital of Iceland. I had a leading role in recruitment and screening of the study subjects, with the assistance of dermatologists Bárður Sigurgeirsson and Jón Hjaltalín Ólafsson. I was responsible for all the follow-up visits. I did the clinical examinations and severity scoring with essential guidance from Bárður Sigurgeirsson and Jón Hjaltalín Ólafsson. I back translated and presented the questionnaire used in this thesis, Dermatology Quality of Life Index (DLQI), with the help of Bárður Sigurgeirsson and Jón Hjaltalín Ólafsson.

I drew all the blood samples and took skin samples, performed the isolation of lymphocytes, in vitro cell culturing and stimulation of the T cells and fluorescence activated cell-sorting (FACS) analysis with essential assistance of Þór Friðriksson (Paper I) and Inga Skaftadóttir at the department of Immunology of Landspitali. Serum cytokine measurement was done by Þór Friðriksson (Paper I) and Hildur Sigurgrímsdóttir at the department of Immunology of Landspitali (Paper III). Bjarni A. Agnarsson and Sigurrós Jónasdóttir at the department of Pathology, Landspitali prepared all the formalin-fixed skin biopsies and performed the H&E and Ki-67 staining. Bjarni A. Agnarsson encrypted the skin biopsies and I examined them in a blinded fashion for the histological Trozak score. Guðmundur Bergsson, at the Immunology Department of Landspítali performed the immunohistochemical staining on the frozen skin biopsies (Paper III and IV) and Hildur Sigurgrímsdóttir performed the immunofluorescence staining (Paper III). I did all statistical analysis with assistance from Gunnar Stefánsson and his students at the Science Institute of the University of Iceland.

I prepared and interpreted the results presented in this thesis with the help of my supervisor and co-authors. I prepared all the manuscripts on which the thesis is based with critical revision of the final versions of the manuscripts from all authors. I have submitted abstracts and prepared and presented 4 posters and 2 oral presentations at international meeti

1 Introduction

In this chapter, a literature review of the background of this thesis is presented. In the first part of the chapter, an overview of the clinical, histological and pathogenesis of psoriasis is provided. Subsequently, a comprehensive discussion of the current knowledge in immunopathogenesis of psoriasis. A review of the literature, pertaining to climatotherapy of psoriasis, in particular balneotherapy in the Blue Lagoon (BL) in Iceland follows, laying the foundations for the aims and objectives underlying this thesis.

1.1 Psoriasis

"Psoriasis is an antidote for dermatologists' ego"

Paul E. Bechet (reviewed in Bechet, 1936)

1.2 Epidemiology of psoriasis

Psoriasis is a chronic, immune-mediated inflammatory skin disease of undefined aetiology. Psoriasis may occur at any age, from birth to senility, and men and women are equally affected.[1] It is widely distributed throughout the world and estimates of the prevalence vary globally with a prevalence that ranges from 0.91 to 8.5% in adult patients[2-4] and 0 to 2.1% in children.[5] General population-based surveys show higher prevalence in Caucasians (1.6% in the United Kingdom, 2.2% in the United states, 2.8% in the Faroe Islands, 2% in Sweden, 1.17-1.43% in Spain, 4.8% in Norway) compared with non-Caucasian (0.19 percent in Egypt, 0.23 percent in Taiwan, zero in the Indian population of Latin America, 0.44 percent in Sri-Lanka, and 0.123 to 0.35 percent in China).[4] Psoriasis exhibits a bimodal distribution with a peak between 15 and 20 years of age and another peak between 55 and 60 years.[6] On the basis of the bimodal distribution of the age of onset and inheritance, it has been classified into early-onset psoriasis (EOP) type I psoriasis and late-onset psoriasis (LOP) or type II psoriasis. Presentation of EOP (approximately 65% of the psoriasis population) is characterised by onset below the age of 40, a positive family history of psoriasis, a higher incidence of a guttate phenotype, more frequent exacerbations and often relapses and a more aggressive clinical course of the

disease than LOP. Late onset psoriasis, with first symptoms usually after the age of 50, presents with a milder and more stable disease course and mainly affects female patients.[3,6]

1.3 Clinical phenotypes of psoriasis

The most commonly used diagnostic method used in dermatology to diagnose psoriasis has been clinical pattern recognition. In unclear cases, histological analysis of psoriasis plaques may sometimes be useful but it is not a substitute for clinical examination nor it is routinely required.[7] There are several psoriasis phenotypes which vary in morphology, distribution, course and severity, where each form can coexist or interchange with other forms. [7,8] Consensus is still lacking on how subtyping should be performed, but here it is presented according to the International Psoriasis Council classification:[8]

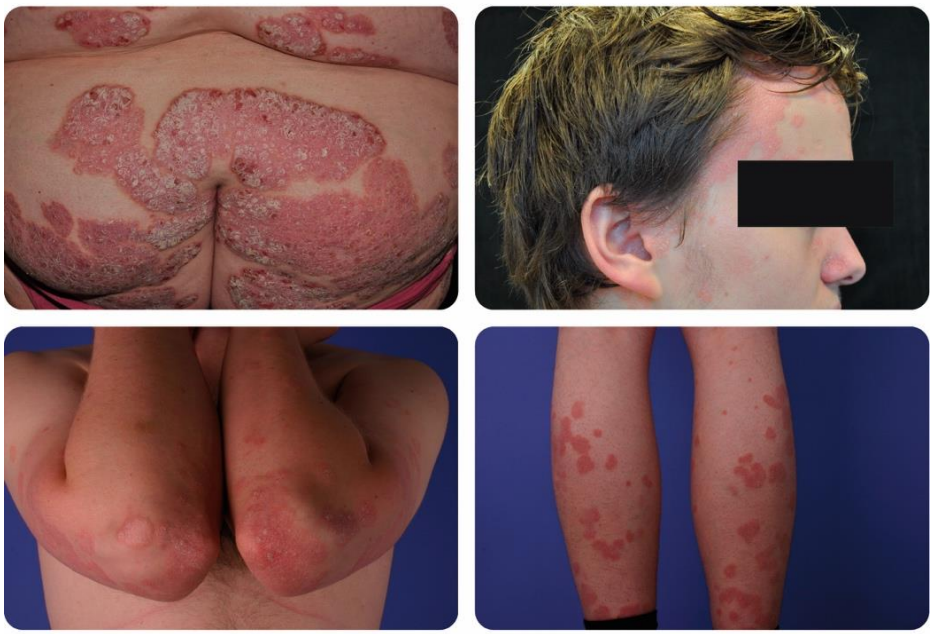

Figure 1. Chronic plaque psoriasis

Photographs were obtained from subjects of this study, and reproduced with permission

1.3.1 Chronic plaque psoriasis

The most common phenotype of psoriasis, chronic plaque psoriasis or psoriasis vulgaris, affects 80% to 90% of psoriasis patients. Chronic plaque psoriasis (*Figure 1*) is characterized by red, scaly, well-demarcated discoid lesions varying in size (from 0.5 cm in diameter to large confluent areas), thickness (from thin ≤0.75 mm to thick plaques >0.75 mm in elevation) and disturbution. The areas that are affected the most are the scalp, trunk and extensor surfaces of the elbows and knees. Other predilection sites include hands, fet, nails and the intertriginous ares (groins, axilla, umbilicus, crena ani, retroauricular folds). Based on the location of the plaques, psoriasis is divided into the following subtypes (localised forms of plaque psoriasis):

- *Flexural or intertriginous or inverse psoriasis* with thin plaques characterised by an oozing, red inflammation without scaling, located on the inframammary folds, axilla, intergluteal cleft, inguinal and genital region.[9]
- *Seborrhoeic-like psoriasis or sebopsoriasis* shares similar clinical and distributional features with seborrhoeic dermatitis, such as scalp, eyebrows, nasolabial creases, ears, sternum and between the shoulder blades. Young people are more frequently affected by this form of psoriasis.[10]
- *Scalp psoriasis* is usually confined within the hairline region and pre- and post-auricular areas (*Figure 1*). The scalp is usually the first part of the body which is affected, whilst 80% of plaque psoriasis patients have scalp involvement.[11]
- *Palmar/plantar psoriasis* is represented on weight-bearing areas of the soles and the center of the palms by symmetrically distributed red scaly plaques, usually with significant involvement of the nails. Skin fissures of the fingertips and heels are frequent.[12]
- *Nail psoriasis*; nail manifestations are common in psoriasis, occurring in 40-50% of plaque psoriasis and in 80% of patients with coexisting psoriasis arthritis.[13] These include both the nail matrix (pits, leukonychia, red spots in the lunula) and the nail bed (onycholysis, hyperkeratosis, salmon patches, splinter hemorrhage).[12]

There are some clinical signs that assist with the clinical diagnosis of plaque psoriasis as the *Woronoff´s ring* or the blanched halo in the periphery of the plaques[14], *Auspitz´s sign* or the punctuate bleeding after scraping the scale off plaques and the *Koebner phenomenon* which describes the *de novo*

appearance of plaques upon injury of the skin and has been linked to severe, early onset, plaque psoriasis.[8]

1.3.2 Guttate psoriasis

Guttate psoriasis (guttate derives from the Latin word “gutta”=drop) is the second most common clinical phenotype of psoriasis with 2% of psoriasis patients affected.[15] The clinical presentatioin is most often an acute eruption of numerous "teardrop“ skin lesions (2-10 mm) and small plaques (<1cm in diameter). Guttate psoriasis usually affects the trunk and proximal extremities. It is associated with a preceding throat infection with beta (β)-haemolytic streptococci by 2-3 weeks and affects younger patients than chronic plaque psoriasis, children, teenagers and young adults.[16] The prognosis of guttate psoriasis is typically good where it generally resolves spontaneously within 3-4 months of onset, but in 33-68% of cases it progesses to plaque psoriasis.[17]

1.3.3 Pustular psoriasis

Pustular psoriasis is characterized with extensive development of sterile pustules on the skin´s surface and includes two subtypes: generalised pustular psoriasis (GPP) and localised pustular psoriasis (LPP).[12] GPP is a rare and serious skin disorder that presents with flares of widespread sterile pustules on a background of red and tender skin. It is also known as acute generalised pustular psoriasis of von Zumbusch. It can be triggered by sudden withdrawal of corticosteroids, drugs (lithium, aspirin and some beta-blockers) and infection. Currently GPP is considered distinct entity from plaque psoriasis with different immune activation, but may occur concomitantly or evolve into plaque psoriasis.[18] Patients with GPP may experience systemic organ involvement and is therefore potentially life threatening.[19] LPP is divided in two subtypes: 1) Acrodermatitis continua of Hallopeau, characterised by pustular eruptions beginning in the tips of fingers and toes (digits) after local skin injury. Nails are usually affected; 2) Palmoplantar pustulosis (PPP) which is a chronic pustuluar conditions with hyperkeratosis on the palms and soles which predominates in females and older age groups (5^{th} to 6^{th} decade of life). Although PPP can coexist with plaque psoriasis in 20% of cases it does not share the same genetic, epidemiological and clinical characterstics.[20] Smoking and the use of new biologic agents such as TNF-α inhibitors have been linked to the onset of PPP.[21]

1.3.4 Erythrodermic psoriasis

Erythrodermic psoriasis is a severe type of psoriasis that can be fatal. It usually occurs in the setting of known worsening or unstable psoriasis but may uncommonly be the first presentation of psoriasis.[8] First described by von Hebra in 1868, it presents with generalized erythema, fine scaling, involving more than 90% of the body surface and common mediators are infections, drugs and sudden withdrawal from oral steroids.[22]

1.3.5 Eczematous psoriasis

The distinction between psoriasis and eczema can be difficult where eczema can be a part of the clincal spectrum of psoriasis or it can affect the already estrablished plaque psoriasis. Therefore, some clinicians use the term eczematous psoriasis in such cases.[23]

1.3.6 Photosensitive psoriasis

Although most psoriasis patients experience sunlight helpful, about 5-20% of psoriasis patients have photosensitive psoriasis where the exposure to sunlight provokes a photo-distributed psoriasiform rash on the sun-exposed areas such as the face, neck, hands and forearms. Patients with fair skin of phototype I or II seems to be of major risk for developing photosensitive psoriasis and it is linked to positive family history, early onset of disease and female predisposition.[24]

1.4 Psoriasis associated comorbidities

Psoriasis is associated with a range of risk factors and several comorbidities involving nearly every organ system, suggesting that the underlying pathogenesis of the disease is more than “skin deep”. [25] Increased prevalence of psoriatic arthritis (PsA), non-melanoma skin cancer, melanoma, lymphoma, obesity, type 2 diabetes, metabolic syndrome, cardiovascular (CV) disease, autoimmune disease, psychiatric illness, liver disease, chronic obstructive pulmonary disease, sleep apnoea, smoking and alcohol abuse have been reported in psoriasis patients.[25-29] The main hypotheses are that psoriasis patients share a genetic predisposition to develop these comorbidities or that their increased systemic inflammatory status raise the risk.[30] The most commonly reported comorbidities of these above are psychological problems and CV diseases.[29] Furthermore, it has been reported that patients with severe

psoriasis have a significantly reduced life expectancy compared with the general population and patients with mild psoriasis, where the most common cause of death is CV disease.[31]

The relationship between psoriasis and its comorbidities is complex and statistical associations do not necessarily prove causality.[28] For example, a recent cohort study with 48,523 psoriasis patients and 208,187 controls concluded that "neither psoriasis nor severe psoriasis were associated with the short term risk of major CV events after adjusting for known cardiovascular risk factors".[32] In addition, the association is most often presented in terms of relative risk which could overestimate the effects of exposure. However, PsA has a much stronger pathophysiological/genetic association with psoriasis than their associated co-morbidities where it is considered a T cell driven inflammatory disease like psoriasis.[33] PsA usually develops several years after onset of skin disease with a very variable estimated prevalence, from 7.7% to 73%.[27,34,35] Patients with PsA have more severe skin symptoms and a lower quality of life compared with psoriasis patients without arthritis.[27] Depression and other psychiatric sympotms are associated with reduced quality of life in psoriasis patients and suicidal thinking is reported in 5.5% to 9.7% of psoriasis patients.[36,37] In addition, addictive behaviours like alcohol consumption and smoking are more prevalent in psoriasis patients than in the general population, indicating the emotional burden of having a stigmatizing disease.[38-42]

1.5 Histological features of psoriasis

The skin is the largest organ of the human body and has a protective and barrier function role against heat, sunlight, trauma and infections. It consists of three layers; the epidermis (the outermost, avascular layer of the skin), the dermis and subcutis (subcutaneous layer or hypodermis); (*Figure 2A*). Keratinocytes are the main cell types of the epidermis with a normal turnover time of approximately 30 days. In psoriasis the mitotic rate of the basal keratinocytes is increased causing thickening of the epidermis (*Figure 2B)*. The psoriasis scale is the result of this hyperproliferative epidermis with premature keratinocytes and incomplete cornification with retention of nuclei within the cells of the stratum corneum (parakeratosis). The redness of the lesions is due to increased dermal angiogenesis with tortuous and dilated capillaries that reach the skin surface.[43]

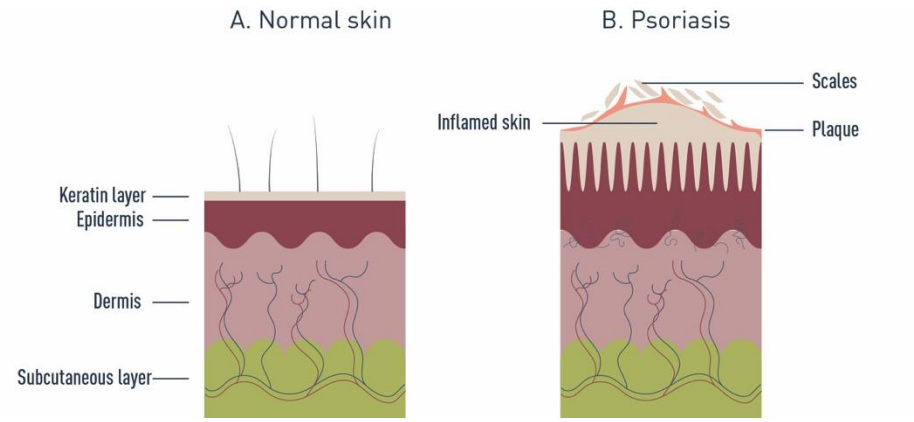

Figure 2. Schematic comparison of the normal skin and psoriasis skin

The Normal skin (A) can be divided into three main layers; the epidermis, the dermis and the subcutaneous layer. The epidermis includes five main layers where the outermost layer of the epidermis (stratum corneum) consists of dead keratin-filled keratinocytes which shed daily. In psoriasis (B) the inflammation in the skin causes thickening of the epidermis and dermis, as well as intense shedding of dead keratinocytes. The microvasculature of the dermis becomes dilated and tortuous, which accounts for the characteristic erythema of psoriasis.

Histological sections of psoriasis, stained with haematoxylin and eosin (H&E), show a characteristic psoriasiform pattern; abnormal hyperproliferation and differentiation of keratinocytes, resulting in diffuse hyperplasia (acanthosis), with regular elongation and club-shaped rete ridges, thickening of the cornified epidermal layer, atypical presence of nucleus-containing keratinocytes in the top layer of the epidermis (parakeratosis), increased mitotic rate of the basal keratinocytes (suprabasal mitosis), absence of the granular layer (hypogranulosis) and elongation of the dermal papillae (*Figure 3*).[44] In addition, a dense lymphocytic infiltrate is present in the epidermis and dermis (perivascular infiltration) composed of dendritic cells and CD4+ Th cells within the upper papillary dermis, and neutrophils and CD8+ Th cells within theepidermis where neutrophilic granulocytes form characteristic Munro's microabscesses and spongiform pustules.[45]

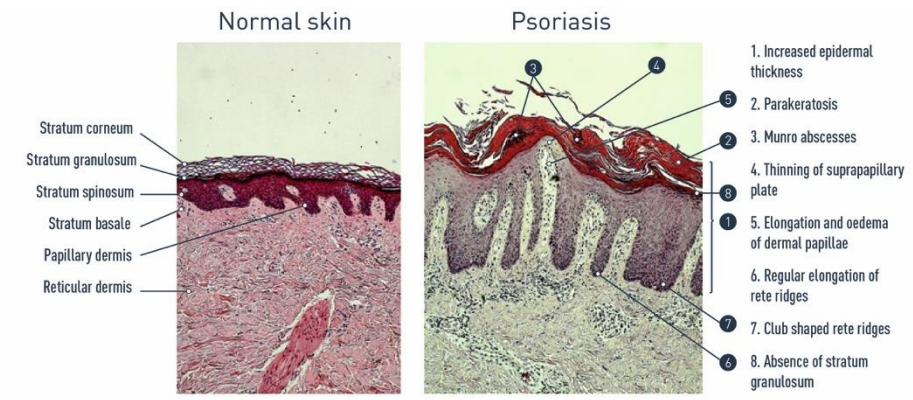

Figure 3. Histopathological changes of normal skin and psoriasis skin

Histopathological changes in normal skin and psoriasis skin seen in haematoxylin and eosin (H&E) staining. Histological changes common in chronic plaque psoriasis are; 1) increased epidermal thickness, 2) parakeratosis, 3) Munro microabscesses, 4) thinning of suprapapillary plate, 5) regular elongation of the rete ridges, 6) elongation and oedema of the dermal papillae, 7) club shaped rete ridges, 8) loss of stratum granulosum, 9) neutrophils in dermal papillae sometimes invading epidermis, mitosis above stratum basale and spongiform pustules. Pictures were obtained from subjects of this study, and reproduced with permission.

1.6 Pathogenesis of psoriasis

1.6.1 Genetics

Psoriasis is a multifactorial and genetically complex disease where multiple genetic determinants may be needed to generate the disease under specific environmental conditions.[46] A crucial epidemiological work leading to major insights into the genetics of psoriasis was performed by a Danish dermatologist, Gunnar Lomholt, published in 1963. He visited many housholds on the Faroe Islands and examined 10 000 inhabitants where he made the key observation that the prevalence of psoriasis was much greater among first-degree relatives than in unrelated individuals.[47] Subsequently, several studies in twins have demonstrated strong genetic basis in psoriasis, with concordance of 73% and 20% in identical and nonidentical twins in one study, respectively.[48] In the 1990s nine genetic regions that potentially harboured susceptibility genes were identified, including the psoriasis susceptibility locus PSORS1~PSORS9 located within the major histocompatibility complex (MHC).[49] PSORS1 is the most consistently reported genetic region with the strongest association with psoriasis[50] and fine mapping of PSORS1 revealed

that HLA-Cw*06:02 is the main susceptibility loci for psoriasis, particularly in Caucasian populations.[51]

Eventhough over 60 susceptibility loci associated with psoriasis vulgaris have been identified in recent years with genome-wide association studies (GWAS), it is estimated to explain only about 30% of the total genetic contribution to psoriasis.[52] While these genetic loci identified do not directly identify the causative mutations, they are of great biological interest where they reveal a tendency to cluster broadly around three key pathways who are important in the pathogenesis of psoriasis:[53] 1) the epidermal barrier; 2) innate immune pathways, including antimicrobial interferon signalling; and 3)adaptive immune pathways with T helper cell (Th)17 differentiation. Genetic research has provided major insights into the biology and natural history of psoriasis and has suggested new targets for drug discovery.

1.6.2 Environmental triggers

Psoriasis can be provoked or exacerbated by a variety of different environmental risk factors, including infections (streptococcal pharyngitis, human immunodeficiency virus-HIV infection),[54,55] physical and psychological stressors (physical trauma, major life events, crises, daily stress),[56-58] weather changes (humidity, cold),[59] lifestyle (smoking, alcohol),[38,60] hormonal changes (pregnancy, pospartum period, menopause)[61] and drugs (beta-blockers, lithium, anti-malarial).[62] How these exogenous factors trigger or exacerbate psoriasis still remains unclear but it is likely that psoriasis patients inherit only a predisposition to the disease, that requires an exogenous stimulus to express its phenotype.[63]

1.6.3 Immunopathogenesis of psoriasis

Psoriasis is an inflammatory skin disease mediated by both the innate and adaptive immune systems, but with a key responses of normal skin cells and associated cytokines in both emergence and maintenance of psoriatic lesion. Healthy skin is an effective state of immune tolerance with constitutive or inducible immune pathways which become abnormally activated and amplificated in psoriasis.

1.6.3.1 Steady-state immunity in healthy skin

As illustrated in *Figure 4A*, the epidermis is formed by slowly differentiating keratinocytes that lose their nucleus as basal keratinocytes transition to corneocytes which, with many layers of neutral lipids, form an effective water-impermaeable barrier in the skin. The epidermis has a key role in innate immunity where it contains granular keratinocytes that express several important molecules in innate immunity, such as the antimicrobial peptides (AMPs), S100A7 (psoriasin), S100A8 (calgranulin A), S100A9 (calgranulin B), lipocalin 2, β-defensin, cathelicidin (LL-37)[64,65]. Furthermore, the skin contains populations of dendritic cells (DCs) that are antigen-presenting cells (APCs) that are crucial for activation of the adaptive immune system and thereby, linking the innate and adaptive immune systems together. During steady-state there are three main DC populations in the skin: epidermal Langerhans cells (LCs), resident dermal myeloid DCs (mDCs) and dermal plasmacytoid DCs (pDCs)[66].

Over the past decade, it has been known that T cells are present in normal human skin in nearly twice the number compared with the entire circulation[67], and may comprise a skin specific immune system[68-71]. These T cells lodged in the skin are all $CD45RO^+$ memory cells, co-expressing the skin homing addressins cutaneous lymphocyte antigen (CLA) and CCR10, have potent effector functions, a diverse T cell repertoire and consequently have the potential to confer both tolerance and immunity depending on the local microenvironment[67,72,73]. They are termed as tissue-resident memory T cells (T_{RM} cells) and express the adhesion and migratory molecule CD69 and a subset also express CD103[74-76]. T_{RM} has been predominantly described for memory $CD8^+$ T cells, but it is now clear that $CD4^+$ and other T cells can establish residence in non-lymphoid tissues[75]. However, in contrast to $CD8^+$ T_{RM}, $CD4^+$ T_{RM} are low or negative for CD103 and appear to retain the capacity to recirculate [74,75]. Keratinocytes mediate memory T cell recruitment to the skin for immune surveillance by constitutively synthesize CCL27 (CTACK), which is the major chemokine that attracts $CCR10^+CLA^+$ skin homing T cells[77,78]. About 10-15% of the circulating T cell pool are destined for skin homing/protective immunity through the expression of CLA. Hence, healthy skin is inhabited by abundant resident T cells and constantly patrolled by additional recirculating T cells to maintain steady-state cutaneous immunity or a state of tolerance[79,80] (Figure 4A).

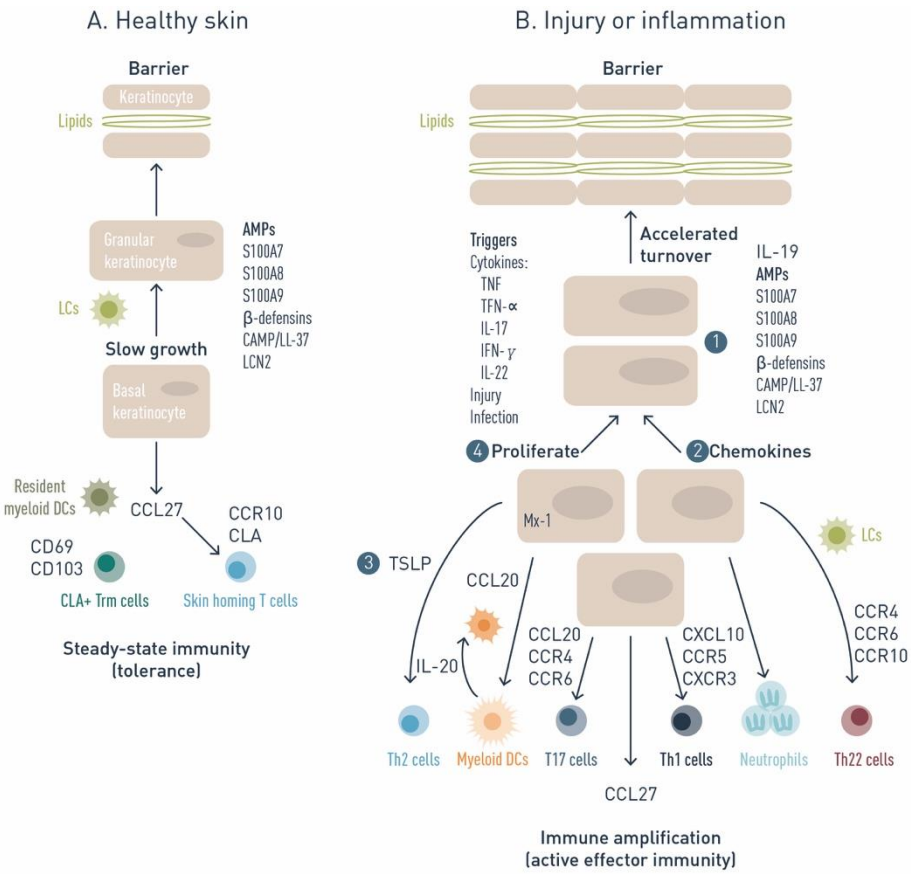

Figure 4. Steady-state immunity in healthy skin

A) The epidermis is composed of slowly differentiating keratinocytes and lipids that form an effective water-impermeable barrier. In the granular layer where the nucleus of keratinocytes is lost, antimicrobial peptides (AMPs) may be stored; S100A7, S100A8, S100A9, β-defensins, cathelicidin (CAMP/LL-37), and lipocalin 2 (LCN2). The epidermis contains immature antigen-presenting cells, Langerans cells (LCs), and the dermis resident myeloid dendritic cells (mDCs). Although there are non-recirculating cutaneous lymphocyte antigen (CLA)+CD69+CD103+ resident memory T cells (Trm cells) in the skin, keratinocytes constitutively synthesize CCL27 (CTACK) to attract skin homing T cells into the skin for immune surveillance and maintain steady-state cutaneous immunity. B) Keratinocytes can also participate in innate or adaptive immune responses to triggers such as injury or infection by: 1) increasing synthesis of innate effector molecules such as AMPs; 2) producing high-levels of the chemokines (CCL20/CXCL10/CXCR3) that attracts DCs and T1/ T17 cells; 3) synthesize thymic stromal lymphopoietin (TSLP) initiating Th2 immune response in the skin; and 4) proliferate in response to IL-22 to accelerate loss of surface keratinocytes and eliminate pathogens.

As mentioned before, activated myeloid dendritic cells (DCs) are crucial for linking the innate and adaptive immune systems together[66]. The activated DCs migrate to the nearest draining lymph node, present their antigens to naïve T cells that can thereby be activated to become effector and memory T cells; IL-12 cytokine response drives the development of IFN-γ/TNF-α producing type 1 helper (Th1) and cytotoxic (Tc1) T cells or T1 cells (Th1/Tc1), and IL-23 drives the develoment of IL-17 producing T17 cells (type 17 helper (Th17) and cytotoxic (Tc17) T cells, and IL-17 producing γδ T cells) as well as IL-22 producing T22 cells (T helper (Th22) and cytotoxic (Tc22) cells); and IL-4 drives the development of Th2 lineage cells[81,82]. Normally, an exogenous antigen (such as bacteria) are processed with MHC class II molecules for presentation with other costimulatory molecules to $CD4^+$ T cells, and endogenous antigens (such as viral particles) are processed with MHC class I for presentation to $CD8^+$ cytotoxic T cells[83]. Microenvironmental factors also dermine which tissue homing receptors the T cells express as activated T effector cells; T1 dominantly express the cysteine-cysteine chemokine receptor (CCR) 5 and cysteine-X-cysteine chemokine receptor (CXCR) 3 that recruit more T1 effector cells to the site of inflammation; T17 dominantly express CCR6 and CCR4, consequently recruiting more T17 cells and neutrophils [82,84,85], and T22 cells CCR4, CCR6 and CCR10[85,86].

As illustrated in Figure 4B, keratinocytes can also participate in innate or adaptive immune responses to triggers such as injury/infection by: 1) increasing synthesis of innate effector molecules such as AMPs[81,82]; 2) producing high-levels of the chemokine CCL20 that attract DCs and $CCR6^+$ T17 cells in response to injury[87] and the chemokines CXCL10 and CXCL11, in response to IFN-γ activation, and CCL17 and CCL22 that are ligands for CCR4 central for skin homing T cells,[88,89] leading to recruitment of more T effector cells[90]; 3) synthesize thymic stromal lymphopoietin (TSLP) initiating Th2 immune response in the skin[91]; and 4) proliferate in response to IL-22 to accelerate loss of surface keratinocytes and eliminate pathogens[90,92,93] (Figure 4B).

1.6.3.2 Psoriasis as a T cell mediated disease

Until the early 1980s, psoriasis was thought to be mostly a primary keratinocyte abnormality[94]. However, the findings that T cell immunosuppressive medications, such as cyclosporine, were highly effective in psoriasis, pointed to a T cell mediated etiology for this disease[95]. For the past three decades, psoriasis has been regarded as a T cell mediated disease, first as an TNF-α/IFN-γ (T1) mediated disease[33] but most recently, as an IL-17 mediated disease where treatment with IL-17 inhibitors result in [96]complete remission of

the disease[97]. In addition, the observation that eventhough psoriatic skin lesions completely resolve with therapy, they often recur in the same locations once therapy is discontinued, has led to speculations that psoriasis may be a T_{RM} mediated disease[96,98].

1.6.3.3 Initiation phase of psoriasis

As mentioned before (Chapter 1.6.2), psoriasis can be triggered by many environmental factors, such as injury and trauma[57], streptococcal infection[55], medications[54,62], and by the Toll like receptor (TLR) 7 agonist imiquimod[99] (Figure 5A). The exaxt mechanisms in this induction phase is not yet understood because of the difficulty pinpointing the specific triggers in each case. Murine studies indicate that application of the TLR7/8 agonist imiquimod may induce psoriasisform dermatitis through the IL-23/IL-17 axis and activated DCs,[100] and other triggers such as injury to the skin might initiate the cascade by causing injured or stressed keratinocytes to produce various AMP such as LL37 which is overexpressed in psoriasis[101]. Gilliet and coworkers have developed a model to explain the initiation phase of psoriasis where the AMP LL37 forms pro-inflammatory complexes with self-DNA and self-RNA released from necrotic cells, thereby breaking the immunologic tolerance (Figure 5A)[102-104]. The LL37/self-DNA complexes bind to intracellular TLR9 and the LL37/self-RNA complexes to intracellular TLR7 on pDCs, which causes activation and production of type I interferons IFNα and IFNβ. LL-37/self-RNA complexes can also activate resident myeloid DCs to produce IL-12 and IL-23, key cytokines in psoriasis, through TLR8. Consequently, myeloid DCs can be activated by type 1 interferons as well as by the LL37/RNA complex, driving T cell activation and the production of psoriatic cytokines.

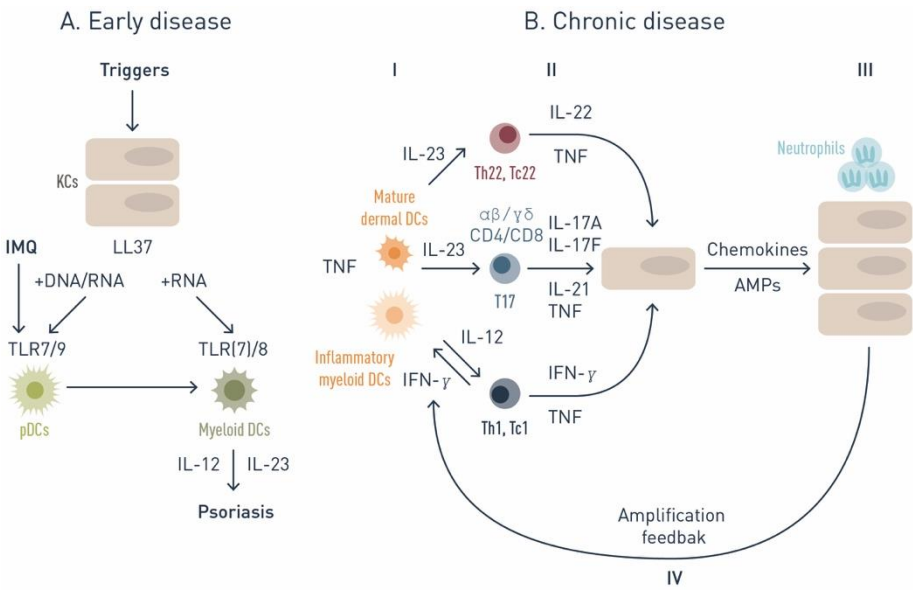

Figure 5. Pathways for initiation and maintenance of psoriasis

A) In early disease trigger factors can cause injured keratinocytes (KCs) in the epidermis, which in turn activate plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells through their Toll-like receptors (TLR7/8/9) and antimicrobial peptide LL37. B) The major pathogenic pathway in psoriasis occurs when (I) the activated mature dermal and inflammatory myeloid dendritic cells stimulate expansion of T cells in the psoriatic lesion with cytokines: IL-12 (Th1, Tc1), IL-23 (T17, Th22, Tc22). The activated T cells (II) produce cytokines that further stimulate the keratinocytes, IL-17 (T17), IFN-γ (Th1, Tc1), IL-22 (Th22, Tc22). Keratinocytes (III) augment further the immune response by producing chemokines and antimicrobial peptides (AMPs), thus a vicious inflammatory cycle is created (IV).

1.6.3.4 Maintenance phase of psoriasis

The next pathogenic immune pathway in psoriasis resulting in the chronic form of the disease appears to be mainly through activated mature dermal DCs and inflammatory mDCs in the skin (Figure 5B). Psoriasis inflammation induces dramatic changes in dermal DC population where inflammatory mDCs appears to be an additional population critical for disease pathogenesis[105]. These cells produce IL-12 and IL-23 which drive the activation and differentiation of naïve T cells to T1 (Th1/Tc1), T17 (Th17 and Tc17) and T22 (Th22/Tc22) effector cells. Furthermore, they produce IL20 that seems to induce epidermal

hyperplasia[106]. Consequently, activated inflammatory cells (T cells, DCs and keratinocytes) in psoriasis lesions produce abundant TNF and specialized T cell populations produce IFN-γ (T1 cells), IL-17 (T17 cells) and IL-22 (T22 cells), contributing to the cytokine milieu which further acts on keratinocytes[82,107]. Of these psoriatic cytokines, IL-17 seems to be the key cytokine in psoriasis immunopathogenesis[108-112]. Keratinocytes respond strongly to IL-17 by recruiting more inflammatory dendritic cells and IL-17 producing T cells through the chemokine CCL20 to the skin, increasing AMPs synthesis greatly, and upregulating mRNAs for a range of other inflammatory products (cytokines and chemokines). This creates a self-amplifying loop in psoriatic lesions, where these inflammatory products act back on the DCs, T cells, and neutrophils so that chronic T cell activation persists[81,82]. In addition, IL-17 can synergize with other cytokines, such as TNF and IL-19, to drive the chronic inflammation[113] and proliferative response in keratinocytes[114]. Furthermore, TNF-α is an activator of IL-23 synthesis in DCs, and the clinical benefit seen with TNF-α blockade is linked to suppression of the IL-23/T17 axis, supporting the thesis that IL-17 is the central cytokine in psoriasis, not IL-12/IFN-γ axis[105].

1.6.3.5 Skin homing markers and localized disease memory in psoriasis

T cells co-expressing the skin homing markers CLA and CCR4 are found abundantly in both peripheral blood and skin of normal subjects[67,77,115]. However, CLA is highly expressed in psoriatic skin[116-118] and the frequency of circulating skin homing CLA^+CD8^+ T cells have shown a strong correlation with disease severity as measured with the PASI score, indicating a pathogenic role of skin homing CLA^+ T cells in psoriasis[119]. The role of CCR4 is more unclear in the pathogenesis of psoriasis, where some studies have shown overexpression of CCR4 in psoriatic skin[117] and others not[120]. CCR4 has traditionally been thought to be expressed primarily on Th2 cells but has now been shown to be expressed on Tregs, Th17 and to be essential for skin homing T cells expressing CLA[121,122]. In addition, studies have shown that the CCR4 ligands, CCL17 and CCL22, are overexpressed in psoriasis and other inflammatory skin diseases such as atopic dermatitis[88,123]. The skin homing marker CCR10 and CD103 are weakly expressed in the peripheral blood of normal subjects and nearly undetected in normal skin[77,124,125]. However, both CCR10 and CD103 are strongly expressed in psoriatic skin indicating involvement in the immunopathogenesis of psoriasis[77,118,124].

Psoriasis is known to relapse in previous affected sited, indicating a site-specific disease memory. As mentioned above in chapter 1.6.3.1, tissue resident memory T cells (T_{RM} cells) are lodged in the skin co-expressing the skin homing markers CLA and CCR10[67,72,73] and the adhesion and migratory molecule CD69 and a subset also express the skin resident marker CD103[74-76]. A site-specific T cell–driven disease memory in psoriasis within the skin has been proposed where a population of CD8 T cells expressing CLA, CCR6, CD103, and IL-23R is found in the epidermis of resolved psoriatic skin but not in normal skin[124,126,127]. It is thought to play a role in maintaining and driving the recurrent disease of psoriasis, presumably contributing to epidermal localization and/or retention of a specific T cell subset.[74,75]. To further support that, studies have shown that T cell associated genes remaine upregulated in resolved psoriatic skin[128] and populations of oligoclonal CD8 T cells are found in relapsing psoriatic skin after periods of complete remission[129]. In addition, recent study reported that CD4/CD8 T cells derived from resolved psoriasis lesions produce the key psoriatic cytokines, IL-17 and IL-22, by restimulation after years of treatment with biologics and remission[126].

1.7 Assessment tools for psoriasis

A large variety of assessment tools have been used to evaluate the severity of psoriasis, but there is a lack of standardization[130-132]. The evaluation of psoriasis severity is a multidimensional approach that considers several factors such as severity of skin lesions, symptoms and health-related quality of life. As there are no reliable biomarkers available to assess disease severity, clinical scores are used in clinical trials and daily practice to measure severity and treatment response. According to the literature, the Psoriasis Area and Severity Index (PASI)[133] seems to be the most valid and reproducible clinical severity score, and Dermatology Life Quality Index (DLQI)[134] for assessing the burden of plaque-type psoriasis on health-related quality of life[135,136].

Since clinical severity and quality of life scores are lacking in objectivity and have several limitations in measuring psoriasis severity, some studies have tried to use a more observer-independent methods such as biophysical methods[137-139], histological scores[45,105,140-144], measuring epidermal thickness (ET)[105,140,143,145-152], and immunohistochemical markers[105,138-142,145-154] from target lesions (Table 1). Biopsies are objective, however, my not provide a representative sampling where psoriasis does not resolve in a uniform fashion. Nevertheless, histological changes[142], decrease in epidermal thickness (ET)[137,147,150], and loss of Ki67 in biopsies[150,154] before and after psoriasis treatments have shown to correlate with clinical severity scores, such as PASI,

and predict outcome weeks to months later[146]. In an effort to develop a system of grading psoriasis severity in a biopsy from a target lesion, Trozak developed histologic grading system for chronic plaque psoriasis in 1994 or Trozak score (se Appendix S1)[45]. Eventhough it is the only histological grading system for psoriasis available today, it is not widely used in psoriasis research[137,155].

STUDY	YEAR	COUNTRY	OUTCOME MEASURES				MARKERS THAT CORRELATED WITH CLINICAL SEVERITY
			CLINICAL	QOL	HISTOLOGICAL MARKERS	OTHER MARKERS	
Papp et al	2015	Canada	PASI sPGA BSA	NO	ET IHC: Ki-67, CD3, CD11	NO	NR
Kim et al	2015	Korea	PSI	NO	Their own histological grading score (0-40)	NO	NO
Sofen et al	2014	U.S.A.	PASI	NO	ET IHC: CD3, CD11c, K16	Serum cytokines and chemokines	Serum IL-17A
Jesionek-Kupnicka et al	2013	Polland	PASI	NO	IHC: Ki-67	NO	Ki-67
Wada et al	2012	U.S.A.	PASI sPGA	NO	Their own histological grading score (1-3) IHC: CD3, CD11c, K16	RT-PCR Cytometry Cytokine production	Histological score
Wolberink et al	2012	The Netherlands	NR	NO	HC: CD1a, CD3, filaggrin, CD31, Ki-67, K16	Reflectance confocal microscopy of histological changes	NR
Soyland et al	2011	Norway	PASI	NO	ET IHC: CD1a, CD4, CD8, FOXP3	Immunofluorescence RT-PCR Cytometry	NO
Gamblicher et al	2011	Germany	PASI	NO	Their own histological grading score (0-4) IHC: CD1a, CD4, CD8, Involucrin, Ki-67	NO	NO
Johnson-Huang et al	2010	U.S.A.	PASI	NO	ET IHC: K16, CD1c and more	Immunofluorescence Flow cytometry RT-PCR	NO
Reddy et al	2010	U.S.A.	PASI PGA	NR	ET IHC: Ki-67, CD3	Serum cytokines and chemokines	ET, Ki-67 and CD3
Morsy et al	2009	Denmark	PASI SAPASI	DLQI PLSI	ET with optical coherence tomography Trozak score	NO	ET correlated with SAPASI, DLQI, PLSI and Trozak, not PASI
Kvist et al	2009	Denmark	TCS	NO	ET, Epidermal morphology IHC: CD3, CD4, CD8, CD45RO, K10, K16, Ki-67	NO	ET, Ki-67 and K16
Werner et al	2008	Brazil	PASI	NO	ET Their own histological grading score IHC: CD1a, Ki-67, K10, K16, K19	NO	NR
Zaba et al	2007	U.S.A.	PASI	NO	ET Their own histological response score IHC: Ki-67, K16, CD11c, CD3, CD163	Immunofluorescence Flow cytometry RT-PCR	
Lago et al	2006	Brazil	PASI	NO	ET, presence of Munro´s abscesses and/or pustulae of Kogoj IHC: K10, K14, K16	NO	NR
Ormerod et al	2005	U.K.	Their own score	NO	ET with ultrasound IHC: Ki-67, CD1a, CD4, CD8, CD68	Erythema measured with pulsed A-scan ultrasound	NR
Bovenschen et al	2005	The Netherlands	The SUM score PASI	NO	IHC: Ki-67, CD4, CD8, CD45RO	NO	Epidermal T-cell markers correlated with the SUM score
Elias et al	2003	U.S.A.	PASI	NO	Their own histological grading score (0-4) ET IHC: CD1a	NO	NR
Krueger et al	1995	U.S.A.	NR	NO	ET IHC: Ki-67, K16, filaggrin, CD3, CD4, CD8, CD25, IGF-1R, α3-integrin	Cell culture analaysis	NR

NR, not reported; PASI, Psoriasis Area and Severity Index; SAPASI, self-administered PASI; sPGA, static Physician's Global Assessment; TCS, Total Clinical Score; PSS, Psoriasis Severity Scale; QOL, quality of life; DLQI, Dermatology Quality of Life Index; PLSI, psoriasis life stress inventory index; ET, Epidermal Thickness; IHC, immunohistochemistry; CD1a, Langerhans cells; CD3, a marker of T cells; CD4, a marker of T helper cells; CD8, a marker of cytotoxic T cells; CD45RO, a marker of memory T cells; CD11c, a marker of dendritic cells; CD161, a marker of neutrophils; CD163, a marker of macrophages; K10, keratin 10 (keratinocyte differentiation marker); K16, keratin 16 and Ki-67 (keratinocyte proliferation markers)

Table 1. Published studies that uses histological changes or markers an assessment tool for psoriasis

1.8 Management of psoriasis

The management of psoriasis is based on disease severity with a step-wise approach (*Figure 6*). The European consensus states the definition of disease severity and treatment goals for psoriasis; 1) mild psoriasis is defined as BSA ≤10 or PASI ≤10 and DLQI ≤10, 2) moderate to severe psoriasis is defined as BSA >10 or PASI >10 and DLQI >10.[156] Treatment goals (assessed after 10-16 weeks) are a reduction of PASI ≥75% and DLQI 0 or 1.[157] The recommended treatment for mild psoriasis is a topical therapy (corticosteroids, calcipotriol, tazarotene, tar, anthralin and keratolytics) and move to phototherapy (broadband (290 – 320 nm) ultraviolet light B (UVB), narrowband (311 nm) UVB, psoralen plus ultraviolet light A (PUVA) and climatotherapy) or systemic treatment (retinoids (acitretin), methotrexate, cyclosporine and fumaric esters) in refractory cases. For moderate to severe psoriasis, phototherapy or systemic therapies are recommended.[157,158] Over the past two decades, new insight into the immunopathogenesis of psoriasis have resulted in various biologic medications administered to severe psoriasis patients, or the T cell modulating agents (alefacept, efalizumab), inhibitors of TNF-α (adalimumab, etanercept, and infliximab), the inhibitor of IL-12 and IL-23 (ustekinumab)[159] and the newest and most promising one, IL-17 antagonists (secukinumab, ixekizumab and brodalumab).[160]

A detailed discussion of treatment options is out of the scope of this thesis, but here I discuss phototherapy emphasizing on climatotherapy and BPT at the BL in Iceland.

1.8.1 Phototherapy

Ultraviolet (UV) radiation has been an effective treatment for psoriasis, as well as other inflammatory skin diseases, for over 90 years. First it was used as a daily broad-band source (290-320 nm) combined with topical tar, known as the "Goeckerman" regimen[161]. The most common form of phototherapy used for psoriasis treatment today is narrow-band Ultraviolet B (UVB) (311±2 nm) treatment and Ultraviolet A radiation (320-400nm) in combination with psoralen (PUVA). Narrow-band UVB phototherapy has replaced the former use of broad-band UVB (290-320 nm) since narrow-band UVB is more effective of clearing of psoriasis.[162] UVB radiation is mostly absorbed by the epidermis, but the molecular mechanism of action is not fully understood. Locally, phototherapy has many immunosuppressive effects. It leads to depletion of T cells and DCs in the skin, probably by inducing apoptosis and decrease recruitment of T cells from the blood due to lower expression of the required adhesion and homing molecules [163-167]. Furthermore, UVB treatment leads to

a reduction of keratinocyte proliferation[163] and T cells display a functional shift toward suppressed IFN-γ and increased IL-4 and IL-10 production, that is toward anti-inflammatory response[164,168,169]. In addition, more recent studies show that UVB therapy specifically targets IFN-γ producing Th1 cells and T17 signaling pathways in psoriatic skin, which are critical in the pathogenesis of psoriasis[145,170]. Interestingly, Rácz *et al* showed downregulation of the Th17 and IFN signaling pathways, as well as epidermal differentiation, in both lesional and nonlesional psoriatic skin[170]. It is most likely that a combination of depletion and altered function of broad range of important molecular pathways in psoriasis is key to the effectiveness of UVB therapy.

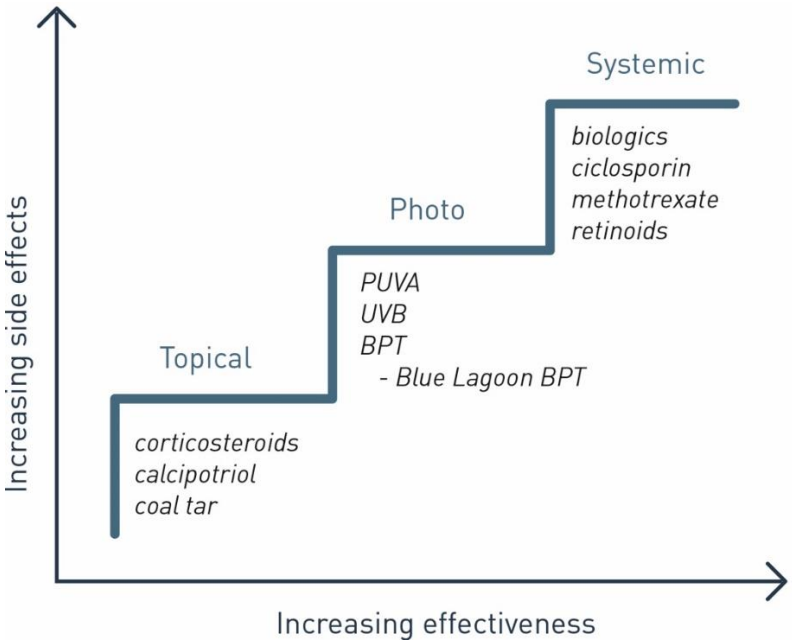

Figure 6.Treatment options for psoriasis

In general, there are three treatment options in psoriasis in a stepwise approach; 1) Topical treatment-skin lotions, ointments, creams, and shampoos; 2) phototherapy based treatments where the skin is exposed to ultraviolet light; and 3) systemic and biological therapies. PUVA, Photochemotherapy; UVB, Ultraviolet B phototherapy; BPT, Balneophototherapy.

1.8.1.1 Climatotherapy of psoriasis

Climatotherapy has been used as a treatment for inflammatory dermatosis such as psoriasis and atopic dermatitis for thousands of years.[171] It is the oldest form of phototherapy. It includes a treatment that utilizes the atmosphere, temperature, humidity, barometric pressure and sunlight.[171,172]

1.8.1.1.1 Thalassotherapy

There are different climatotherapeutic methods and the most known is the thalassotherapy of psoriasis at the Dead Sea[173] and the Black Sea coasts,[171,174] where patients bathe in sea water and apply mud, algae and sand on the skin. Afterwards they bathe in the sun, sometimes for several hours.[175]

1.8.1.1.2 Balneotherapy

Balneotherapy is referred to as an alternative climatotherapeutic method based on the immersion of the patient in mineral water baths or pools.[171] Examples of unique and special places are the Kangal hot spring in Turkey,[176] Comano spa in Italy,[177] Salies de Béarn in France,[178] and the Blue Lagoon in Iceland.[179] Mineral waters are natural solutions formed under specific geological conditions and have three fundamental characteristics: spring origin, bacteriologically pure, and therapeutic potential.[180] They are generally rich in sulphur, calcium and magnesium.[172,177] The exact mechanisms behind the effect of mineral waters on inflammatory skin diseases has not been fully elucidated, but probably incorporate thermal,[181] mechanical[179] and immunological effects.[177,182-185]

1.8.1.1.3 Balneophototherapy

Balneotherapy is frequently associated with phototherapy, where numerous studies[186-192] indicate an additional effect from the use of geothermal or salt water baths before UV exposure. Because balneotherapy is bound to specific geographic settings, regimens with synthetic salt solutions or saline spa water prior to UVB phototherapy (balneophototherapy;BPT) was established in rehabilitation centres in Germany about 40 years ago.[193] The spa water is salty (from 1% up to 30%) and some treatment modalities include a significant amount of magnesium, calcium and sulphate to mimic the Dead Sea

composition.[172,191] Today BPT is being practiced in many countries all over the world.[171,172]

1.8.1.1.4 The efficacy of climatotherapy in psoriasis

Empirical evidence indicates that thalassotherapy in the Dead Sea is a highly effective treatment for chronic plaque psoriasis.[175,189,194-199] However, it has yet to be accepted as a well-established treatment modality for psoriasis because randomized controlled trials are somewhat lacking to confirm these results.[200,201] The main therapeutic effect of treatment at the Dead Sea seems to be primarily attributed to the sunshine[175,189,197,202] and the increased incidence of photodamage[203] and non-melanotic skin cancer among psoriasis patients treated at the Dead Sea[204] has raised questions about long-term safety of the treatment.[202] Two randomized controlled studies have been done concerning pre-treatment with Dead Sea salts before UVB phototherapy where one study shows[191] that bathing in Dead Sea salt solution prior to UVB phototherapy is superior to UVB phototherapy alone, and the other study[205] found no additional benefit for Dead Sea salt solution soaks prior to UVB phototherapy. To our knowledge, no research has been done to compare Dead Sea salt solution prior to phototherapy with salt water or tap water. More randomized controlled trials are needed to confirm the empirical evidence.

It has been proposed that salt water may increase photosensitivity of the skin to UVB irradiation.[186,206,207] However, the data is conflicting where some randomized controlled trials have showed beneficial effect of salt water to enhance phototherapy[187,188,191,208] and other show no additional effect.[178,205,209] Three randomized controlled trials where no difference was found were comparative studies where bathing in saline spa water,[178] application of Dead Sea salt soaks[205] or salt water soaks[209] on the skin before phototherapy was examined. In addition, a recent systematic review and meta-analysis conducted by Bailey et al[201] where the efficacy of BPT was examined concluded that there was no significant difference of the therapeutic effect of BPT compared with UVB monotherapy.[178,187,188,193,205]

1.9 The Blue Lagoon in Iceland

The Reykjanes peninsula in Iceland is a part of the Mid-Atlantic Ridge, where the two continental plates meet. The geothermal area on the peninsula is called “Svartsengi” (Black Meadows) and consists mainly of porous volcanic lava, allowing seawater to enter deep into its aquifers. A geothermal power plant was built 1976 in Svartsengi where wells were drilled through the lava to depths

of up to 2000 m discharging a mixture of 2/3 seawater and 1/3 meteoric water with a temperature of about 240°C (here referred to as geothermal fluid). When the geothermal fluid from the deep geothermal reservoir interacts with the surrounding lava and the chemical composition changes. The magnesium concentration is reduced thousand fold to 1.41 mg/kg and the silica (SiO_2) increases roughly hundredfold because of high silica concentration in the surrounding lava, to 140 mg/kg compared with 1295 and 3 mg/kg in seawater, respectively.[210]

Most of the geothermal fluid left after it is used to produce electricity and to heat up freshwater is re-injected into the geothermal reservoir but some of it is discharged on the surface where it forms a geothermal lagoon, the BL. The BL contains about 6000 m^3 of geothermal fluid that is replenished every 40 h. The cooling of the water causes super saturation of silica, which then precipitates to form a white mud. This constant silica precipitation may add an extreme factor to the environment in the lagoon which is moderate in other aspects such as in temperature about 38°C, salinity of 2.5% and pH 7.5. The chemical composition of the fluid in the lagoon is given in *Table 2*.[210]

As the fluid in the lagoon is moderate in pH and temperature, it would be expected that the lagoon is high in microbial diversity. However, the BL has a unique microbial ecosystem of marine character where the microbial diversity is very low.[211,212] The dominating species comprise 85% of the randomly picked isolates: firstly, the coccoid blue-green microalgae *Cyanobacterium aponinum*, and secondly, the filamentous microalgae *Silicibacter lacuscaerulensis.*[212] The possible explanation for the low biodiversity is the salinity of 2.5% and the high silica content underlining the extreme characteristics of the environment in the lagoon. The ecosystem in the BL is annually stable where these two microalgae species show regular seasonal dominance shifts in the geothermal water. The *Cyanobacterium aponinum* uses light as the energy source, gradually dominating the ecosystem during the summer while *Silicibacter lacuscaerulensis* dominates in the autumn and winter.[211,212]

Due to high number of bathing guests every year and the lack of artificial disinfectants, it might be expected that human bacteria would be found in the water. Surprisingly, no human coliform bacteria or environmental bacteria have been isolated from the lagoon. The possible explanations are a short retention time of the geothermal water as well as the salinity and the continuous silica precipitation. Other unknown factors may be possible.[192,211,212]

pH/temp°C	7.7/24
SiO_2	137
Na	9280
K	1560
Ca	1450
Mg	1.41
CO_2	16.5
SO_4	38.6
H_2S	0.0
Cl	18500
F	0.14

Total dissolved solids mg/kg fluid: 31900

Table 2. The chemical composition (mg/kg of fluid) of the fluid in the Blue Lagoon

1.9.1 Psoriasis treatment at the Blue Lagoon

When the geothermal power plant was built in 1976 some individuals suffering from psoriasis noticed that they gradually improved when they rubbed the white silica mud onto their psoriasis plaques while bathing in the warm lagoon. In the following years, many psoriasis patients bathed in lagoon and claimed improvement of their psoriasis. The government of Iceland selected a committee to conduct a research to investigate these claims and to further analyse the usage of the area. Two preliminary studies[213,214] and two controlled studies[179,192] concerning the effect of bathing in the lagoon on psoriasis have shown that bathing in the lagoon has beneficial effects on chronic plaque psoriasis.

The first controlled study was done in 1992 and there were 28 psoriasis patients treated for 3 weeks in the lagoon.[179] The patients bathed three times a day for one hour at a time. The mean PASI score decreased from 16.1 to 8.1 and 5 of the 26 patients had an improvement rate of at least 75% (PASI 75). It was noticed that the scaling decreased very rapidly, and the lesions became thinner after only two weeks but thereafter the improvement slowed down. To take advantage of this early desquamation and thinning of the lesions that was observed, UVB treatment after bathing in the lagoon (geothermal seawater) was added to the treatment regimen. To establish the clinical effect of bathing in the lagoon a second study was conducted that included a control group, patients treated with UVB monotherapy.[192] Twenty three psoriasis patients were treated with bathing in the lagoon three times a day for one hour at a time

for four weeks and UVB treatment daily 5 times per week. The control group received UVB treatment 5 times per week for four weeks. The mean PASI score decreased from 20.3 to 2.8 or by 86% in 4 weeks in the combination treatment group, compared to 16.7 to 6.9 or by 59% in the control group. In other words, 20 of 21 patients had received more than 75% precent improvement of their original PASI (PASI 75) after 4 weeks of treatment in the combination group compared with only 4 of 17 patients in the control group. Mild adverse effects were noticed, mostly dry skin and itching or burning after bathing in the lagoon, which was easily remedied with moisturizing creams.

The studies above suggest an additional beneficial effect of bathing in the geothermal seawater (BPT) in the BL and the scientific rationale for this empirical observation remaines elusive. Some might suggest that bathing in hot baths could explain this beneficial effect as it has been proposed that salt water may increase photosensitivity of the skin to UVB irradiation[186,206,207]. There is no concrete answer to this question today since no randomized controlled studies have been done comparing bathing in regular hot baths before UVB therapy with bathing in the geothermal seawater before UVB therapy. However, hot baths are very common in Iceland as in many other contries, and no reports of improvement of psoriasis has been documented in comparison with the results of the first controlled study[179] by Olafsson *et al*. showing on average 50% improvement of the PASI score by only bathing in the geothermal seawater. In addition, the data indicating that salt water increases photosensitivity of skin is conflicting where some studies show that salt water enhances phototherapy[187,188,191,208] and other show no additional effect.[178,205,209] The additional effect can very unlikely be explained by the sunlight which plays a significant role in thalassotherapy at the Dead Sea[175] and Black Sea[171], where the natural sun is relatively weak on the northern latitude of Iceland and not reliable for treating psoriasis except for a short period of the summer.[215]

To the best of our knowledge, the geothermal microbial ecosystem in the BL is not found in other areas in the world,[211] and two studies indicate that the silica mud, the coccoid blue-grean microalgae *Cyanobacterium aponinum* and the filamentous microalgae *Silicibacter lacuscaerulensis* from the lagoon can explain at least some of the benefical clinical effect.[216,217] The first study shows that extracts from the silica mud and both of the microalgae mentioned above improve skin barrier function and prevent premature skin ageing.[216] The extracts improve the skin barrier by inducing transcriptional expression of genes which are required for keratinocyte differentiation, such as involucrin, loricrin and filaggrin, and prevented the skin from ageing by protected the skin against UVA radiation-induced matrix metalloproteinase-1 expression. The second study, recently reported by Gudmundsdottir *et al.*[217] (2015) further

supports biological activity of the coccoid blue-grean microalgae *Cyanobacterium aponinum.* Human monocyte-derived dendritic cell (DCs) were matured in the presence or absence of extracts from the microalgae *in vitro* and the DCs secreted higher levels of the anti-inflammatory cytokine IL-10 in the presence of the extracts and a reduced ratio of the IL-17^{+}RORγt^{+}/IL-10^{+}FoxP3^{+} in CD4^{+} T cells was observed, indicating anti-inflammatory effect of *Cyanobacterium aponinum*.

Eventhough all the clinical studies mentioned above suggest a beneficial effect of of bathing in the geothermal seawater at the BL, most of the data are based on case series or studies without randomization and control groups, making it difficult to draw concrete conclusions. In addition, no assessment on quality of life, remission time, immunological or histological response of bathing in geothermal seawater at the BL has been done. Therefore, randomized controlled study on the clinical, immunological, histological, and psychosocial effect of BPT at the BL (bathing in geothermal seawater followed by UVB phototherapy) was lacking, and that is the reason this study was designed.

2 Aims

The hypothesis upon which this thesis was based is the following: balneophototherapy (BPT) at the BL (bathing in geothermal seawater followed by UVB phototherapy) for chronic plaque psoriasis is superior to UVB phototherapy alone.

The overall aim of this thesis was to compare clinical, psychosocial, histological, and immunological effect of BPT at the BL on psoriasis, compared with UVB phototherapy alone.

First, we conducted a small pilot study to evaluate the potential immunological effect of BPT at the BL compared with UVB phototherapy alone, as well as validating methods to use in the prospective randomized controlled trial (Paper I).

Second, we aimed to evaluate, in a prospective randomized controlled trial (RCT), the clinical, histological and psychological effect of BPT at the BL compared with UVB phototherapy alone on chronic plaque psoriasis (Paper II).

Third, we aimed to understand the impact of BPT at the BL on inflammatory pathways underlying psoriasis and see if there were any difference compared with UVB therapy alone (Paper III).

Fourth, since the histological score of Trozak used in this thesis is not widely used in clinical trials, we aimed to evaluate it by comparing it with other psoriasis outcome measures (Paper IV).

3 Materials and methods

Two separate studies were conducted to further investigate and identify the potential beneficial effect of BPT at the BL compared with UVB therapy alone on chronic plaque psoriasis; a pilot study to explore potential immunological differences and validate methods to use in the larger study (Paper I), and a randomized controlled trial (RCT) to identify potential clinical, psychosocial, histological, and immunological differences between the study groups in both blood and skin (Paper II-IV). Both studies were approved by The National Bioethics Committee of Iceland (Reference number 08-010-S1 and 08-097-S1), the Data Protection Authority of Iceland and the Ethics Committee of Landspitali-The National University Hospital of Iceland. It was performed in compliance with the 1964 Declaration of Helsinki and its later amendments.

3.1 Subjects

3.1.1 Pilot study (Paper I)

Recruitment and screening of chronic plaque psoriasis patients for the pilot study took place from January to May 2008. After providing informed consent, twelve patients with chronic plaque psoriasis entered the study. All the patients were referred by dermatologists. Inclusion and exclusion criteria are listed in Table 3. Data were collected at the dermatology outpatient center at Landspitali-The National University Hospital of Iceland and the Blue Lagoon clinic (BL clinic). For randomisation, a random number table was used. Twelve patientes were enrolled to the study and randomly assigned, in a 1:1:1 ratio, to two therapeutic arms:

1. *Balneophototherapy at the BL:* Six patients received inpatient treatment at the BL clinic for two weeks, where the treatment included bathing in geothermal seawater two times daily for at least 1 hour combined with UVB phototherapy 5 days per week. After treatment at the BL clinic, patients used moisturizing creams for 6 weeks.
2. *UVB therapy:* Six patients received conventional UVB phototherapy three times per week for 8 weeks. The patients were instructed to use moisturizing creams during treatment.

The same type of Waldmann 7000 UVB cabins (Philips TL 100W/01, Philips, Villingen-Schwenningen, Germany) were used at the outpatient dermatology clinic at Landspitali-The National University Hospital of Iceland and at the BL clinic. The same UVB treatment protocol based on skin type was

used for all patients, with initial doses of 130–400 mJ/cm² with subsequent increases of 15–65 mJ/cm² after each treatment.

In addition, a small control group from three anonymous healthy blood donors from Landspitali-The National University Hospital of Iceland blood bank was added for the immunological analysis in this study.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Chronic plaque psoriasis of PASI ≥ 7 diagnosed by a dermatologist	Other psoriasis phenotypes (e.g. guttate, pustular, erythrodermic)
Age ≥ 18	Other skin diseases that could interfere with study evaluations
Non-responsive to topical treatment and candidates for phototherapy or systemic treatment	Unwillingness to stop psoriasis treatment at least 4 weeks prior to study entry
Signed informed consent	History of abnormal UVA sensitivity
	Pregnant or lactating women

Table 3. Inclusion and exclusion criteria

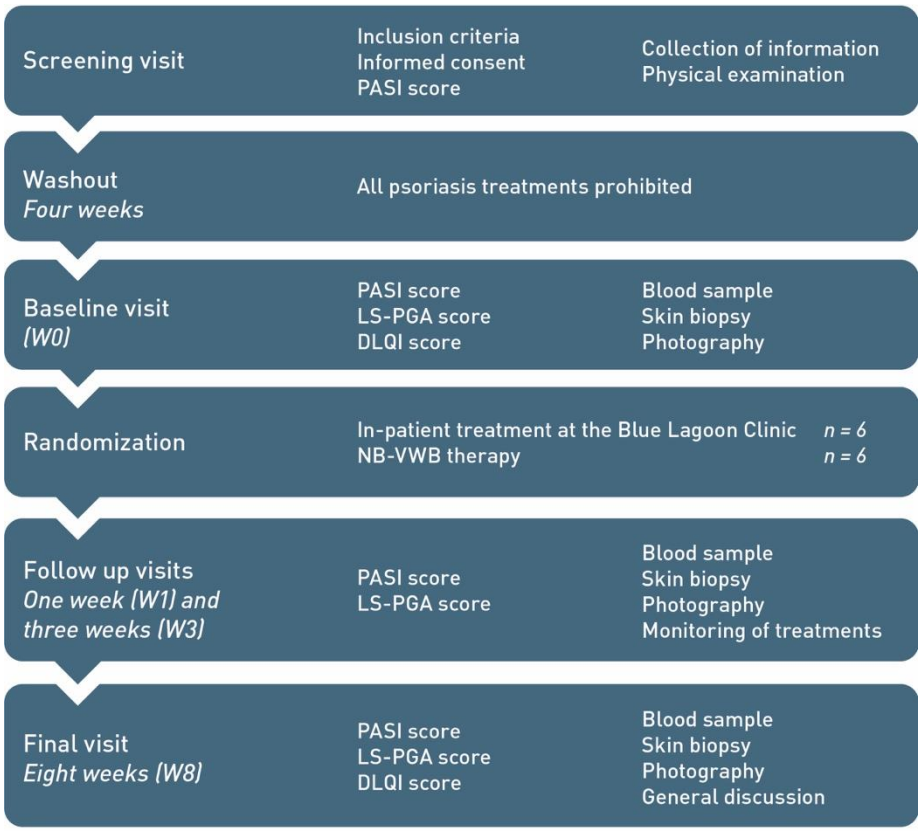

Figure 7. Flow chart of the study desing in the pilot study

PASI, Psoriasis Area and Severity Index; LS-PGA, The Lattice System Physician's Global Assessment; DLQI, Dermatology Quality of Life Index; UVB, narrowband ultra violet B phototherapy.

3.1.2 Randomized controlled trial (RCT; Paper II-IV)

Recruitment and screening of chronic plaque psoriasis patients for the open multi-arm randomized controlled trial (RCT) took place from September 2009 to August 2010 (Paper II-IV). One hundred and nineteen patients were screened. The majority of patients (80%) were referred by a dermatologist, but the rest responded to an advertisement in a newspaper. The diagnosis of psoriasis had been confirmed by a dermatologist in all cases. Inclusion and exclusion criteria are listed in Table 3. Sixyt-eight patients fulfilled all criteria and were enrolled in the study. All patients provided informed consent before participating in the study. Of the 51 patients that did not fulfill the criteria, 27 had a PASI score lower than 7, 6 had another psoriasis subtype and 10

patients were unwilling to stop ongoing psoriasis treatment. Data were collected at the dermatology outpatient center at Landspitali-The National University Hospital of Iceland and the BL clinic. A random number table was used for randomisation. Since there were so promising results from the pilot study (Paper I) regarding intensive BPT for two weeks at the BL, a third therapeutic arm was added to this study, Intensive BPT at the BL (IT-GSW; see results 4.2). In the effort of achieving a more long-standing result, a 4 week after-treatment was added to the protocol. Enrolled patients were randomly assigned, in a 1:1:1 ratio, to these three therapeutic arms *(Figure 8)*:

1. *Outpatient BPT at the BL (GSW):* Twenty-two patients received outpatient treatment at the BL clinic which included bathing in geothermal seawater for 1 h and UVB therapy immediately afterwards three times a week for 6 weeks. Patients were instructed to rub the silica mud from the lagoon on the skin while bathing and to use moisturizing cream (BL Mineral Intensive Cream) twice daily.
2. *Intensive BPT at the BL (IT-GSW):* Twenty-two patients received inpatient treatment at the BL clinic for 2 weeks. The treatment protocol consisted of bathing in geothermal seawater for 1 h two times a day and UVB therapy once daily immediately after the first bath six times/week. Patients were instructed to rub the silica mud from the lagoon on the skin while bathing and to use moisturizing creams twice daily (BL Mineral Intensive Cream). After the inpatient treatment at the BL clinic, patients were treated with a conventional outpatient UVB therapy three times a week for 4 weeks.
3. *Conventional narrowband UVB therapy (UVB):* Twenty-four patients received a regular, monitored UVB phototherapy three times weekly for 6 weeks. The treatment protocol consisted of shower immediately before the UVB treatment was given and moisturizing creams twice daily (Eucerin Original Healing lotion, Beiersdorf, Hamburg, Germany).

As for the pilot study, same UVB treatment protocol was used for all patients (see chapter 3.1.1).

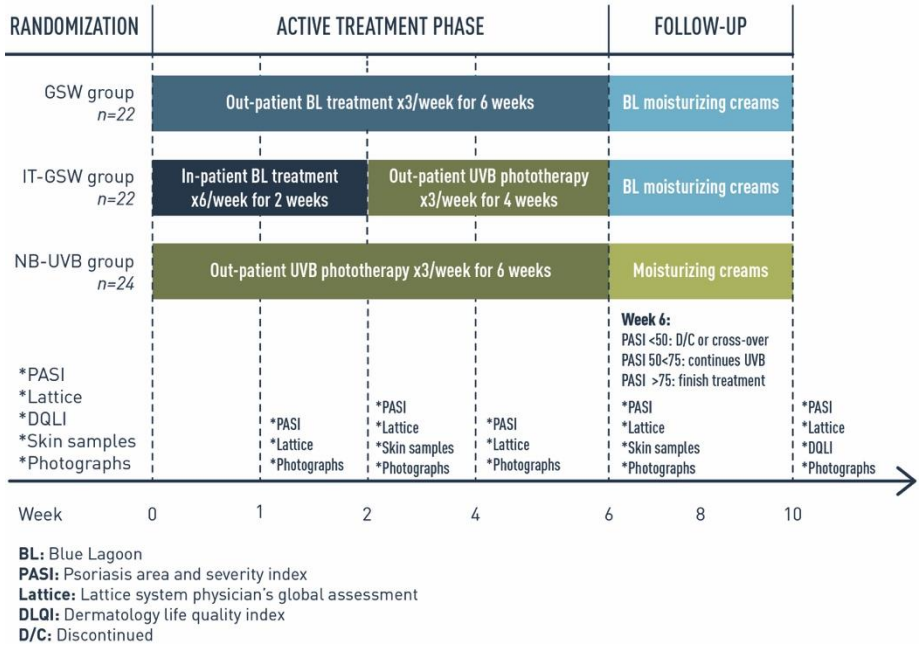

Figure 8. Flow chart of the randomized controlled trial (RCT)

PASI, Psoriasis Area and Severity Index; LS-PGA, The Lattice System Physician's Global Assessment; DLQI, Dermatology Quality of Life Index; UVB, narrowband ultra violet B phototherapy.

3.2 Visit schedule

3.2.1 Pilot study (Paper I)

Patients were enrolled in the study after a screening visit where detailed data was collected: demographics, psoriasis features and concomitant psoriasis treatments. The severity of psoriasis was assessed, and a brief physical examination performed. Patients were included after a 4-week washout period and then assessed at baseline (W0), one (W1), three (W3) and eight (W8) weeks after starting treatment. Disease severity was assessed by the same physician (J.H.E.) at each time point with PASI score and photographic documentation, and skin biopsies and blood samples for immunological measurements were obtained.

3.2.2 Randomized controlled trial (RCT; Paper II-IV)

Patients were enrolled in the RCT after a screening visit as for the patients in the pilot study (*Figure 9*). The baseline visit included clinical examination, assessment of psoriasis clinical severity using both PASI and LS-PGA scores, and health-related quality of life with DLQI score. Photographic documentation was done, and blood samples and skin biopsy were obtained from the first 7 patients enrolled in each study group. The thickest lesion on the extremities was selected as the target lesion for skin biopsy. The clinical follow-up was scheduled 1, 2, 4, 6 and 10 weeks after beginning the treatment. All patients were examined in the following order by the same physician (JHE): clinical examination, photographic documentation, PASI score and Lattice score determination. Blood and skin samples for immunological and histological measurements were collected again after 2 and 6 weeks of treatment. Follow-up DLQI assessment were collected at the 10-week follow-up. Finally, patients were followed up for 1 year with telephone interviews where they were asked if they had started another treatment for their psoriasis (topical treatment, phototherapy, or systemic therapy). The remission time was defiened as the number of days from the study treatment until retreatment.

3.3 Study end points (Paper II)

The primary endpoint in the RCT was the proportion of patients who achieved at least 75% reduction in PASI (PASI 75) after 6 weeks of treatment. Key secondary efficacy endpoints included: 1) The proportion of patients who achieved at least 90% reduction in PASI (PASI 90) at week 6; 2) The proportion of patients with a LS-PGA score of ‘clear of disease’ or ‘almost clear’ at week 6; 3) The change from baseline in Dermatology Life Quality Index (DLQI) at week 10; 4) Histological assessment at baseline compared with after 6 weeks of treatment; and 5) Remission time or the number of days until another psoriasis treatment was needed.

Patients who did not achieve at least a 50% reduction in PASI (PASI 50) from baseline at week 6 were defined as non-responders and either withdrawn from the study or invited to cross over to receive intensive BPT at the Blue Lagoon (*Figure 8*). Patients in all study groups who achieved a PASI 50 continued UVB therapy three times a week for 4 weeks or until attainment of PASI 75/PASI 90, and patients who achieved PASI 75 were invited to continue UVB therapy until attainment of PASI 90 (maximum 10 weeks/patient).

3.4 Assessment of clinical symptoms

3.4.1 Psoriasis Area and Severity Index (PASI)

PASI evaluates severity of the skin affected from the main three clinical signs of psoriasis: erythema, scaling and infiltration as as 0 (Absent), 1 (mild), 2 (moderate), 3 (severe) and 4 (very severe). The total body is divided into four sections: head (10% of the total body area), trunk (30%), upper limbs (20%) and lower limbs (40%), and for each area, the regional surface affected is calculate and estimated from 0 to 6. These four scores are then combined and expressed in numerical values from 0 to 72. PASI 75 and PASI 90 are a widely-used concept in clinical psoriasis trials, meaning more than 75% and 90% improvement in PASI from baseline to the primary endpoint, usually 12 to 16 weeks of treatment. Achieving a 75% improvement in the PASI is considered as a successful treatment.

However, the PASI score has limitations where it lacks sensitivity at the lower end of its range and certain sites (hands, nails, feet, face, genital organs) and symptoms (such as pruritus) are not included in the evaluation. In addition, quality of life impairment and comorbidities are not included in the score. Alternative severity scores such as the Lattice System Physician´s Global Assessment (LS-PGA) and Self-Administered Psoriasis Area Severity Index (SAPASI) has been invented to confront these problems. However, these scores are not as videly used as the PASI score and require better evaluation[130,136].

3.4.2 The Lattice System Physician´s Global Assessment

The Lattice System Physician's Global Assessment (LS-PGA)[218] consists of two steps. In the first step, the percentage of total body surface affected is evaluated using a 7-point scale. In the second step, average plaque thickness, erythema, and scale are evaluated using a 4-point scale. Then scores for percentage of total body surface and average plaque grade are combined in a lattice to determine a final rating from clear to very severe.

3.5 Assessment of psychosocial symptoms

The Dermatology Life Quality Index (DLQI) is a ten-item questionnaire evaluating the impact of dermatological disease on the quality of life of an affected person[134]. It consists of questions, scored 0 to 3, depending on how much impact the skin disease has on the patient´s life previous week: symptoms, embarrassment, shopping and home care, clothes, social and leisure, sport, work or study, close relationships, sex, treatment. It gan give a total score of 30 where higher score indicates poorer quality of life. The Icelandic version of the questionnaire was used, translated for this study using the translation-back translation method[219] (see Appendix S2).

3.6 Assessment of histological symptoms

In the pilot study (Paper I), a 3-mm punch biopsy was taken from each patient at baseline, after 1, 3 and 8 weeks. The biopsy was taken from the edge of the thickest lesion on the forearm, fixed immediately in formaldehyde and then transported to the department of Pathology where it was stained using H&E staining for histologic evaluation (see chapter 3.6.1 and 3.6.2) and immunohistochemistry (see chapter 3.7.3). In the RCT (Paper II-IV), two 3-mm punch biopsy were taken from a target lesion on the upper or lower extremity. One biopsy was fixed in formaldehyde as in Paper I, but the other one was stored in saline until transported to the Department of Immunology. There it was were frozen immediately in OCT compound and stored at -80ºC until required for immunofluorescence (see chapter 3.7.4; Figure 9).

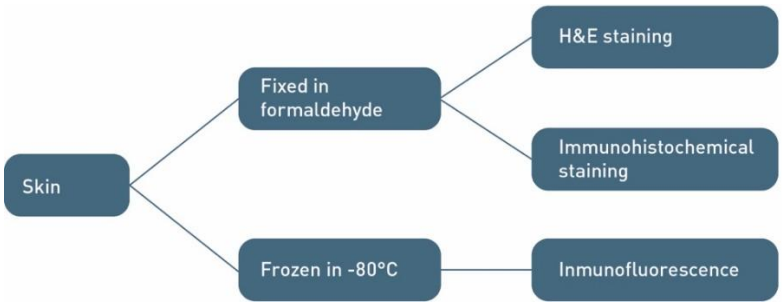

Figure 9. Processing of skin biopsies

H&E, Hematoxylin and eosin

3.6.1 Trozak histologic grading system (Trozak score)

The Trozak score is a histologic grading system for chronic plaque psoriasis developed by Trozak in 1994 (se Appendix S1)[45]. It comprises 10 different histomorphological features on H&E stained skin samples, each taking a score of 1, 2 or 3, depending on their histological specificity for psoriasis and relevance to disease activity. Findings such as perivascular dermal oedema, elongated rete ridged, perivascular mononuclear infiltrate in the upper dermis of papillae, focal absent granular layer and focal parakeratosis, that are not specific for psoriasis, are given a score of 1. On the other hand, thinning of the suprapapillary plate and the presence of Munro microabscesses and / or Kogoj pustules, which are more specific for psoriasis, take a value of 3. Findings there between, such as club shaped rete ridges, total absent granular layer, total parakeratosis, thinning of the suprapapillary plate and mitosis above the basal cell layer take a score of 2. Scores from the 10 variables are then added up to give a total score from 0 to 19.

3.6.2 Epidermal thickness (ET)

In Paper IV we used epidermal thickness (ET) to evaluate histological changes. ET is defined as the avarage distance in mm, between the base of stratum corneum and the tip of rete ridges, measured in different locations on H&E stained skin samples. ET was measured in three different locations using a calibrated microscope micrometer before treatment, after 2 and 6 weeks of treatment. The measurements were investigator-blinded and performed by the same investigator (BAA).

3.7 Immunological evaluation

3.7.1 Cell preparation, stimulation and flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized peripheral venous blood by gradient centrifugation with Ficoll-Paque PLUS (Healthcare, Uppsala, Sweden), collected at the interface, washed with HBSS medium (Gibco, Carlsbad, CA, USA) prior to staining with anti-human antibodies (Figure 11). PBMCs were stained with CD3, CD4, CLA, CD103 (all from Biolegend, San Diego, USA), CD8, CD45R0, CCR4 (all from BD Biosciences, San Jose, USA) and IL-23R (from R&D Systems, Abingdon, UK) monoclonal antibodies (mAbs) for T cell analysis. In Paper I the PBMCs were also stained with CD54 (BD Biosciences, San Jose, USA) and CCR10 (R&D Systems, Abingdon, UK) monoclonal antibodies (mAbs) for T cell analysis and

CD14, CD11c, TLR2 (all from Biolegend, San Diego, USA) and TLR6 (HyCult biotechnology, Uden, Netherlands) mAbs for monocyte analysis.

PBMC´s (1.0 X 10^6 cells/ml) were cultured for 16 hours in RPMI 1640 medium with penicillin-streptomycin (100 IU mL^{-1} and 0.1 mg mL^{-1}) (Gibco), in the presence of anti-CD3 (5 μg/ml), anti-CD28 (5.0 μg/ml) mAbs (Biolegend, San Diego, USA) and Brefeldin A (3.0 μg/ml) (eBioscience, San Diego, USA) at 37°C. In Paper I the T cells were first stained for cell surface receptors for CD4 and CD8, then fixed and permeabilized, and stained for intracellular cytokines with anti-human tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-17A (all from Biolegend, San Diego, USA) and IL-22 (R&D Systems, Abingdon, UK) mAbs in Paper I. In Paper III, the T cells were first stained for CD4 and CD8 as in Paper I except with the addition of the skin homing cell surface receptor CLA (Biolegend, San Diego, USA). The same antibodies were used for intracellular staining in Paper III as in Paper I. The cells were washed with phosphate-buffered saline (PBS) prior to fluorescence-activated cell sorting (FACS) analysis.

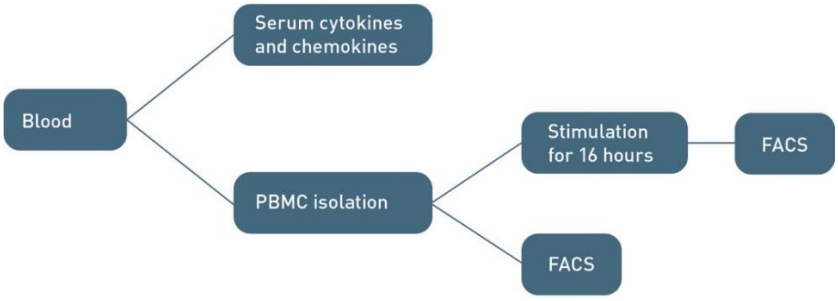

Figure 10. Processing of blood samples

PBMC, peripheral blood mononuclear cells; FACS, fluorescence-activated cell sorting

3.7.2 Cytokine and chemokine measurements in serum

The serum was frozen at -70 °C until used. In Paper I, the levels of IL-22, IL-17, IL-23, CCL20, IL-1β and TNF-α were determined by enzyme-linked immunosorbent assays (ELISAs), using commercially available kits (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. In Paper III chemokines (CCL17 and CXCL10) and cytokine (IL-19) were

measured using a magnetic Luminex assay (R&D systems) and analysed in Bio-Plex 200 system (Bio-Rad Laboratories, California, USA).

3.7.3 Immunohistochemistry

This part of the research (Paper III and IV) was done in collaboration with Guðmundur Bergsson, biochemist at the Immunology Department of Landspítali - The National University Hospital of Iceland and Bjarni A. Agnarsson, pathologist at the Pathological Department of Landspítali - The National University Hospital of Iceland. We used immunological markers to evaluate T cell infiltration (CD3, CD4, and CD8) in the skin and epidermal proliferation (Ki-67-positive keratinocytes). Ki-67 serves as a marker of proliferative activity in diseases with excessive cell proliferation like psoriasis and in neoplasms[220]. Sections were cut at 3µm, mounted on starfrost slides, and heated for one hour at 60°. They were heated in Envision-Flex Target-Retrieval Solution High pH (DM 828, Dako) for 25 minutes in a water bath after deparaffination. Immunohistochemical staining was performed in AutostainerLink 48 (Dako), and a two-step polymer method Envision™ Flex K8000 (Dako) was used. The four antibodies used were incubated for 30 minutes: polyclonal rabbit anti- human CD3 (Dako) 1:250, mouse monoclonal anti-human CD4 (Leica Novocastra) 1:25, monoclonal mouse anti-human CD8 (Dako) 1:100, and monoclonal-mouse anti-human Ki-67MIB1 (Dako) 1:200. Slides were developed with DAB reagent and counterstained with hematoxylin. All antibodies were diluted in Envision-Flex Antibody Diluent (DM830, Dako). The slides were evaluated using Leica Application Suite 3.5.0 and the cells were counted at 400x magnification.

3.7.3.1 Immunofluorescence

This part of the research (Paper III) was done in collaboration with Hildur Sigurgrímsdóttir, biochemist at the Immunology Department of Landspítali - The National University Hospital of Iceland. Frozen sections were cryosectioned (7µm) and double-stained by immunofluorescence for CD8 (two different antibodies used, one from BioLegend and one from Abcam) and either IL-17 or IL-22 (both from Abcam). All samples were blinded before staining. DyLight and Alexa Fluor secondary antibodies (Thermo Fisher Scientific Inc) were used. Imaging was done by a confocal microscope (Olympus FV1200). IL-17 and IL-22 staining was graded on the scale from 0 to 3 by three independent viewers in both epidermis and dermis. Only samples of good quality were included, which resulted in samples from three patients in the IT-GSW and UVB group and from four patients in the GSW group.

3.8 Expression of the data and statistics

Data analyses were performed using SigmaStat 3.1 (Systat Software, San Jose, CA, USA) in Paper I and GraphPad Prism software, version 5 (GraphPad Software Inc., USA) in Papers II-IV. P values of less than or equal to 0.05 were considered significant. In general, categorical variables were compared with two-sided Chi-Square or Fisher´s exact test as appropriate and continuous variables with analysis of variance (ANOVA) test for repeated measurments. Measurements expressed as mean ±SD. To evaluate the differences between treatment groups two-way repeated measures ANOVA was used. Mann-Whitney test was used when evaluating the differences between normal healthy controls (n=3) and patients, and measurements expressed as median (IQR). Efficacy data from all randomised patients were analysed on an intention-to-treat basis. Patients who discontinued study treatment due to unsatisfactory therapeutic effect or who did not follow the study treatment protocol were regarded as treatment failures. For analysis in such cases, missing values were replaced with the most recently available values for all efficacy variables (last observation carried forward) [221]. Correlation data were determined with Pearson´s correlation coefficient test.

In Paper II, the sample size calculation was based on the primary endpoint of PASI 75 after 6 weeks of treatment. The study was sufficiently powered to detect a difference of 20% between the combination treatment groups and the UVB group. Given these assumptions and taking into account the results of prior studies [179,192,213,222-224], a sample size of 15 patients per treatment group provided more than 99% power to detect at least one pair-wise treatment effect in the primary endpoint at an overall 5% level of significance. Patients who did not achieve more than a 50% reduction in PASI score and crossed over to intensive BPT group (IT-GSW), were included in efficacy summaries for IT-GSW group (Figure 12). The proportions of patients responding to treatment were determined with the two-sided Fisher´s exact test.

4 Results

4.1 Demographics and patients

All twelve patients in the pilot study (Paper I) finished the study. All enrolled patients in the RCT (Paper II-IV) patients had plaque psoriasis and no significant differences were between the study groups, except for baseline DLQI score that was significantly higher in the intensive BPT group at the BL (IT-GSW group) compared with the other groups ($p<0.05$; Table 4). Fifty-six patients completed the study of 68 patients enrolled (82.4%; Figure 11). Eleven patients were withdrawn from the study because of adverse events (1/68; 1.5%), protocol violations (9/68; 13.2%) and personal reasons (1/68; 1.5%). Five patients where withdrawn from their study group since they did not achieve a 50% reduction of the PASI score after 6 weeks of treatment (non-responders). Four of them were assigned to the IT-GSW group. One patient entered the cross-over IT-GSW group few days after withdrawal, two patients 2 weeks after withdrawal and one patient more than 4 weeks after withdrawal. Patients who achieved PASI 50/75 continued UVB therapy until attainment of PASI 75/90 (maximum 10 weeks/patient). Respectively, 30.8% (6/26) of the patients in the IT-GSW group, 31.8% (6/22) in the GSW group and 66.8% (16/24) in the UVB group continued UVB therapy three times/week (Figure 11).

CHARACTERISTICS	GSW n=22	IT-GSW n=24	UVB n=24	P VALUE †
Age - yr	41 (±10.8)	42.2 (±16)	37.9 (±14.4)	0.37
Male sex - no. (%)	12 (55)	12 (50)	15 (63)	0.82
Body mass index (BMI)	28 (±5)	28.6 (±5.4)	28.8 (±7.1)	0.96
Duration of psoriasis - yr	20 (±14)	16.4 (±11)	12.3 (±8.1)	0.09
Participants with psoriatic arthritis - no.(%)	4 (19)	3 (13)	5 (20)	0.71
Participants with nail psoriasis - no. (%)	10 (43)	12 (50)	9 (38)	0.61
Psoriasis area and severity index (PASI)	12.3 (±5.2)	11.6 (±6.2)	11.1 (±4.9)	0.22
Lattice system physician´s global asessment	Moderate to severe	Moderate to severe	Moderate to severe	1.00
Dermatology Life Quality Index score	7 (±4.2)	11.6 (±6.2)	7.3 (±5.1)	0.017*
Participants treated previously - no.(%)				
- Blue Lagoon	6 (27)	10 (42)	7 (29)	0.57
- Topical agent	21 (95)	23 (100)	21 (88)	1.00
- Phototherapy	21 (95)	19 (79)	16 (67)	0.51
- Systemic therapy	2 (1)	2 (8)	1 (4)	0.61
Smoking	6 (27)	8 (33)	4 (17)	0.77
Family history	18 (82)	12 (50)	14 (58)	0.35

Table 4. Baseline patient characteristics in the randomized controlled trial (RCT)

*Plus-minus values are means ±SD. P values are for the comparisons across all treatment groups and were calculated by means of analysis of variance (ANOVA) for continuous variables and the chi-square test for categorical variables. The PASI score range is from 0 to 72, with higher scores indicating more severe disease. The Lattice score range is from clear of disease to severe disease. Dermatology life quality index (DLQI) range is from 0 to 30, with higher scores indicating worse health-related quality of life. GSW, outpatient balneophototherapy (BPT) at the Blue Lagoon (BL) including bathing in geothermal seawater combined with UVB treatment; IT-GSW, intensive BPT at the BL indlucing bathing in geothermal seawater combined with UVB treatment; UVB, UVB treatment group. * = p<0.05.*

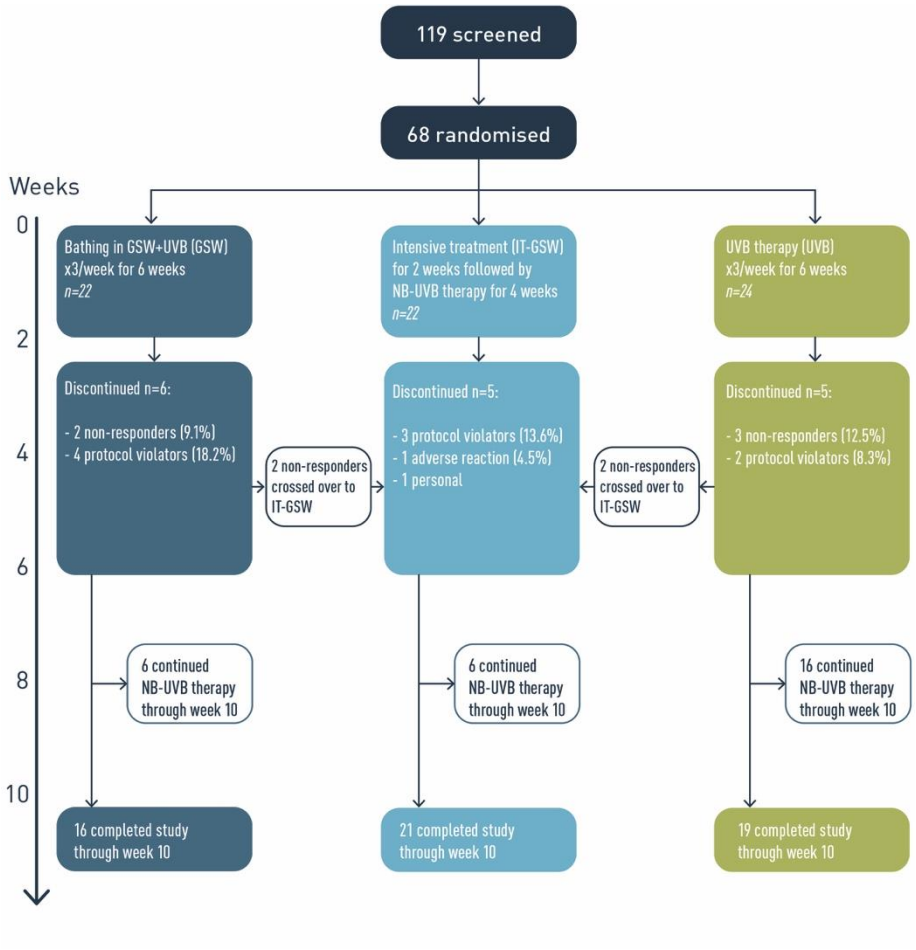

Figure 11. Study flow diagram in the randomized controlled trial (RCT)

Sixty-eight psoriasis patients enrolled in the study but 16 patients discontinued; 5 because of lack of efficacy (non-responders= <PASI 50 after 6 weeks of treatment), 1 because of adverse event, 9 because of protocol violation (received no treatment) and 1 because of personal reasons. 2 non-responders in the UVB group and 2 non-responders in the GSW group crossed over to the IT-GSW group.

4.2 Combined treatment at the Blue Lagoon is more effective than UVB therapy alone

In the pilot study (Paper I) with only 12 patients, inpatient BPT at the BL (bathing in geothermal seawater combined with UVB therapy) for two weeks was found to be more effective than UVB therapy for 8 weeks (Figure 12A and B). There was a significant difference in the reduction of the PASI score after

only one week of treatment (inpatient BPT at the BL 37.3%±10.3 vs. UVB treatment 18.3%±8.9, *P<0.05*) and even more after 3 weeks of treatment (67.3%±11.9 vs. 22.0%±12.0, *p<0.0001.* Figure 12C). However, as expected, patients treated at the BL started to get worse after 8 weeks because the inpatient treatment was only for two weeks with no after treatment (Inpatient BPT at the BL 54.5%±21.3 vs. UVB treatment 33.6%±11.8, *p>0.05*). Respectively, in the larger RCT (Paper II), the inpatient BPT group received after treatment that included outpatient UVB therapy three times per week for 4 weeks.

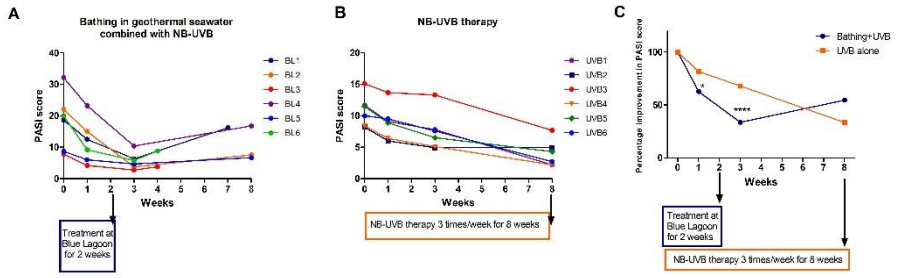

Figure 12. Clinical improvement in the pilot study

*Psoriasis Area and Severity Index (PASI) score in psoriasis patients treated with balneophototherapy (BPT) at the BL (A) and UVB therapy alone (B), as well as the median percentage improvement in PASI score with each treatment (C). All patients were examined before treatment (0), ant at 1 (W1), 3 (W) and 8 (W8) weeks of treatment. BL1-6, patients in the BPT group at the BL; UVB1-6, patients treated with UVB therapy alone. *p < 0.05, ****p < 0.0001.*

The RCT showed that the BPT groups at the BL were much more effective than UVB therapy alone after 6 weeks of treatment, where 20/26 patients (77.0%) of the patients in the IT-GSW group and 15/22 (68.2%) in the GSW group met the primary endpoint of a 75% reduction in the PASI score, compared with only 4/24 (16.7%) patients treated with UVB monotherapy (p<0.001 for GSW and IT-GSW vs. UVB; Table 5 and Figure 16A-B). In addition, PASI 90 was achieved by 42% of the patients in the IT-GSW group and 18% of the patients in the GSW group, compared with no patient in the UVB group (0%; p<0.05 for both comparisons; Table 5 and Figure 16D). The mean baseline PASI score was 12.3 (±5.2) in the outpatient treatment group at the BL (GSW group), 11.6 (±6.2) in the IT-GSW group and 11.1 (±4.9) in the

UVB therapy group (p=0.22). These findings were further supported by the Lattice clinical score, where higher percentages of patients in the GSW and IT-GSW groups received a lattice score of ´clear of disease´ or ´almost clear´ than in the UVB therapy group after 6 weeks of treatment (p<0.01 for GSW and p<<0.001 for IT-GSW vs. UVB; Table 5 and Figure 16C).

	PASI 75 6 weeks	PASI 75 10 weeks	PASI 90 6 weeks	PASI 90 10 weeks	DLQI score of 0 or 1 10 weeks	Attainment of Lattice score "Almost clear" or "Clear" at 6 weeks	Attainment of Lattice score "Almost clear" or "Clear" at 10 weeks	No. UVB treatments required for attaining PASI 75 (mean ± SD)	Total UVB dose required for attaining PASI 75 (mean ± SD) J/cm²	No. days required for attaining PASI 75 (mean ± SD)
GSW n=22	15 (68.1)***	13 (59.0)	4 (18.2)*	4 (18.2)	9 (40.9)*	14 (63.6)**	12 (55)*	14.7±4.2***	5.8±2.6***	35.5±10.4***
IT-GSW n=26	20 (77.0)***	17 (65.4)	11 (42)***	6 (23.1)	12 (46.2)*	17 (65)***	15(58)*	17.9±10.0**	8.3±5.9***	29.1±25.2***
UVB n=24	4 (17.0)	13 (54.2)	0 (0)	2 (8.3)	3 (12.5)	4 (17)	4 (17)	25.0±6.6	18.6±8.3	62.3±14.0

GSW, bathing in geothermal seawater + UVB; IT-GSW, intensive treatment in geothermal seawater + UVB; UVB, NB-UVB therapy alone; PASI, Psoriasis Area and Severity Index; DLQI, Dermatology Life Quality Index. Lattice score, Lattice system physician´s global assessment score. (%). *p<0.05, **p<0.01, ***p<0.001

Table 5. Response to treatment

*GSW, outpatient balneophototherapy (BPT) at the BL (bathing in geothermal seawater combined with UVB treatment); IT-GSW, intensive BPT at the BL (bathing in geothermal seawater combined with UVB treatment); UVB, UVB treatment alone; PASI, Psoriasis Area and Severity Index; Lattice, Lattice System Physician´s Global Assessment; DLQI, Dermatology Life Quality Index. *p<0.05, **p<0.01. ***p<0.001.*

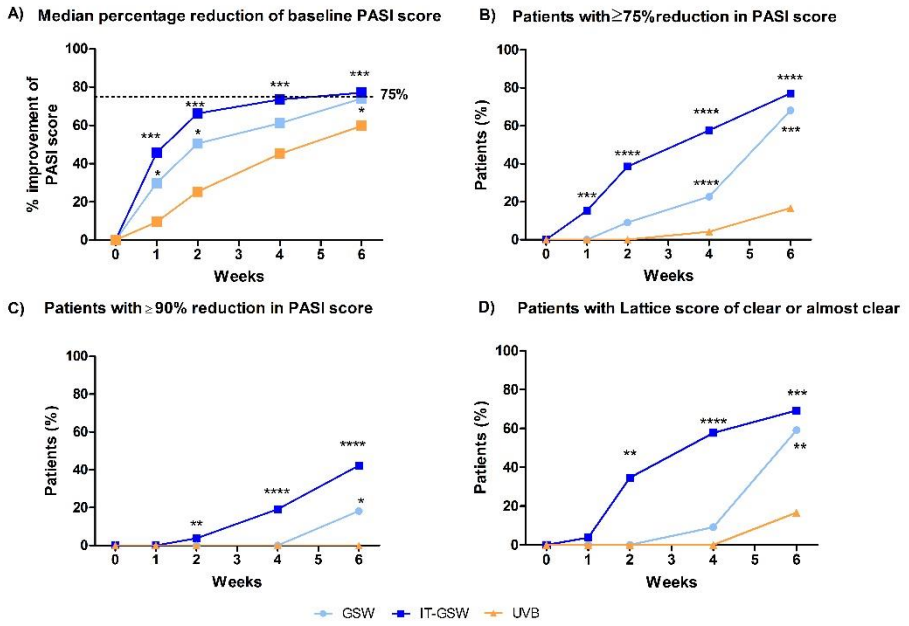

Figure 13. Clinical improvement in the randomized controlled trial (RCT)

*Median percentage reduction of baseline Psoriasis Area and Severity Index (PASI) score through week 6 using intention-to-treat population (A). Percentage of patients achieving 75% reduction from baseline PASI score (B). Percentage of patients achieving 90% reduction from baseline PASI score (C). Percentage of patients attaining Lattice System Physician´s Global Assessment score og ´clear´ or ´almost clear´(0 or 1; D). Last observation carried forward to week 6 for dropouts. GSW, outpatient balneophototherapy (BPT) at the BL; IT-GSW, intensive BPT at the BL; UVB, UVB monotherapy. *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001.*

4.3 Patients treated with balneophototherapy at the BL require less UVB-treatments

The time required to attain PASI 75 was significantly shorter for BPT groups at the BL than in the UVB therapy group, or 29.1 days for IT-GSW group, 35.5 days for GSW group, and 62.3 days for UVB group (p< 0.001 for both comparisons; Figure 16B-C). Respectivily, the number of UVB sessions (14.7±4.2 GSW vs. 17.2±10.0 IT-GSW vs. 25.0±6.6 UVB; p= 0.001 for GSW and IT-GSW vs. UVB; Table 5) and mean UVB dose required to attain PASI 75 was significantly less in the combined treatment groups compared with UVB

therapy (5.8±2.6 J/cm^2 GSW vs 8.3±5.9 J/cm^2 IT-GSW vs. 18.58±8.25 J/cm^2 UVB; $p<0.001$ for GSW and IT-GSW vs. UVB; Table 5). No statistical difference was found between the combination treatment groups ($p>0.05$). Furthermore, days until relapse was found significantly longer for IT-GSW BPT group than UVB therapy alone group, or 283 days compared with 140.9 days ($p<0.01$; Table 5). Despite the large difference in number of days until relapse between patients in the GSW group compared with the UVB group (246.1±161.0 vs. 140.9±165.3; $p=0.0796$), the difference did not reach statistical significance.

Patients who received BPT showed better response in areas poorly exposed to UVB radiation compared with patients treated with UVB therapy alone, for example the scalp. This was observed in 10 patients in the GSW group, 13 patients in the IT-GSW group and 12 patients in the UVB group. Furthermore, nine out of 19 patients (47.4%) who received UVB therapy alone had started another treatment after only one month, and most commonly for the scalp. In comparison, only one (1/21; 4.8%) of the patients in the IT-GSW group ($p=0.0028$) and 3 patients (3/16; 18.8%) in the GSW group ($p=0.15$) had started another treatment after one month.

4.4 Balneophototherapy at the Blue Lagoon improves quality of life

The percentage of patients achieving a DLQI score of 0 or 1 after 10 weeks was significantly higher in the BPT groups than in the UVB therapy group, or 40% in the GSW group and 46% in the IT-GSW group compared with 12% in the UVB group ($p<0.05$ for both comparison; Table 5).

4.5 Balneophototherapy at the Blue Lagoon improves histological changes in psoriatic skin

There was a significant reduction of the histological Trozak score in both treatment groups in the pilot study (Paper I) after three weeks of treatment, or a reduction from 10.3 ± 5.5 before treatment to 3.2 ± 4.4 after three weeks in the BPT group at the BL, and from 8.0 ± 4.6 before treatment to 3.7 ± 4.3 after three weeks in UVB treatment group; $p<0.05$). However, in the larger RCT (Paper III), there was a significant reduction of the Trozak score in both BPT groups after only 2 weeks of treatment, but not in the UVB only group: from 10.5 ± 4.7 to 3.3 ± 3.7 ($p<0.05$) for the GSW group and from 8.1 ± 2.4 to 3.0 ± 2.4 ($p<0.05$) for the IT-GSW group vs. from 10.0 ± 2.6 to 7.7 ± 1.6 ($p>0.05$)

UVB group. The histological changes improved even further after 6 weeks in the IT-GSW group (to 4.0 ± 3.7 ($p<0.01$), bot not in the GSW group and UVB group (Figure 14 a-c). When the treatment groups were compared with each other, there was a significant difference between the intenstive BPT group (IT-GSW) and UVB only group after two weeks (<0.05), but no significant difference was observed after 6 weeks.

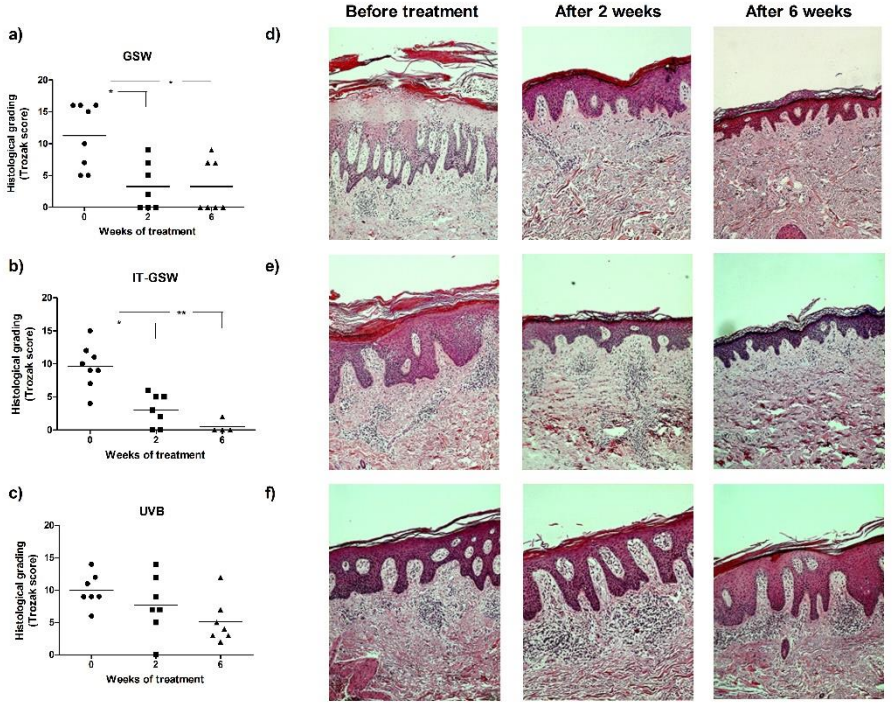

Figure 14. Histological assessment using Trozak´s score

*Histological Trozak score before and after 2 and 6 weeks of treatment. (a) Patients treated with outpatient balneophototherapy (BPT) at the BL (GSW). (b) Patients treated with intensive BPT at the BL (IT-GSW). (c) Patients treated with UVB therapy alone (UVB). (d-f) Representative photographs from one patient in each treatment group; (d) GSW, (e) IT-GSW and (f) UVB group. *p<0.05, **p<0.01.*

4.6 The immunological effect of balneophototherapy at the Blue Lagoon

4.6.1. Balneophototherapy at the Blue Lagoon and UVB monotherapy suppresses circulating skin homing and skin resident T effector cells

To see if adhesion molecules, chemokines, and their receptors in the peripheral blood of psoriasis patients respond to treatment, we evaluated intercellular adhesion molecule 1 (ICAM-1), E-selectin (CD62E), CD11c, the chemokine receptors CCR4 and CCR10 and αEβ7 integrin (CD103) on peripheral blood mononuclear cells before, during and after treatment in the pilot study (Paper I). The frequency of circulating CLA^+ T cells expressing CD103 (Median 5.7% vs. 1.5%; $p<0.05$), CCR10 (Median 5,1% vs. 1.7%; $p<0.05$), co-expressing CD103/CCR4 (Median 11.4% vs. 0.8%; $p<0.05$) and CCR4/CCR10 (Median 3.7% vs. 1.2%; $p<0.05$) were higher in psoriasis patients compared with healthy controls (Figure 15A-E). Circulating skin resident $CD103^+$ T effector cells ($r = 0.6036$; $p<0.05$) and skin homing CLA^+ T cells expressing CCR10 ($r = 0.7360$; $p <0.01$) correlated well with the clinical PASI score.

This increased frequency of circulating skin homing T effector cells expressing CD103, CCR4 and co-expressing CD103/CCR4 was significantly reduced with BPT at the BL, but not UVB therapy alone; 68-74% reduction of CLA+ T cells expressing CCR4 or CD103 ($p<0,001$; Figure 15A and B), no detection of CLA^+ T cells co-expressing CD103 and CCR4 ($p<0.05$; Figure 15C), and 80% reduction of CLA^+ T cells co-expressing CCR4 and CCR10 ($p<0.01$; Figure 15E) after three weeks (W3) of treatment (Figure 15A). However, both treatment groups achieved a significant reduction in CLA^+ T cells that expressed CCR10 (71% reduction vs. 44% reduction at W3; $p <0.001$ vs. $p<0.05$; Figure 3D) with a good correlation with PASI score ($r=0.63$, $p<0.0001$). An 80% reduction was also observed of circulating CLA^+ T cells that co-expressed CCR4 and CCR10 in the inpatient treatment group after three weeks of treatment (3.5 % before treatment and 0.7 % at W3; $p<0.01$; Figure 3E). No therapeutic changes were found regarding the expression of ICAM-1, CD62E, CD11c and other activation markers, such as CD25 and HLA-DR (data not shown).

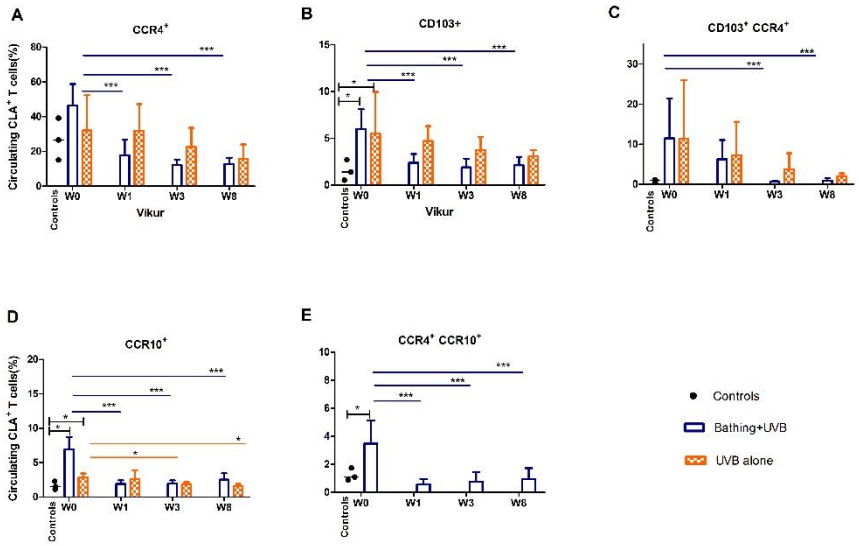

Figure 15. Reduction of circulating skin homing and skin resident T effector cells with treatment

*Circulating skin homing CLA+ T cells expressing CD103, CCR10 and CCR4/CCR10 are increased in psoriasis. The frequency of CLA+ T cells expressing CCR4 (A), CD103 (B), CCR10 (D), co-expressing CD103/CCR4 (C) and CCR4/CCR10 (E) from 3 healthy individuals (controls), 12 psoriasis patients; 6 treated with balneophototherapy at the BL (Bathing+UVB) and 6 treated with UVB phototherapy alone (UVB alone). All patients were studied before commencing treatment (W0), after 1 (W1), 3 (3W) and 8 (W8) weeks after treatment. Data expressed as mean ± SD, except controls expressed as scatter dot with median. *p < 0.05, **p < 0.01, ***p < 0.001.*

In the RCT (Paper III), the levels of circulating skin resident T effector as analysed further by adding markers for T helper (CD4) and cytotoxic (CD8) T effector cells; $CLA^+/CD4^+$ T cells expressing CD103 defined as skin resident Th cells and $CLA^+/CD8^+$ T cells expressing CD103 T cells defined as skin resident Tc cells (n=21). When all treatment groups were analysed together we observed a marked reduction of 30% after 6 weeks of treatment for both skin resident Th cells (from 12.59%±8.60 to 7.58%±8.07, $p<0.05$) and Tc cells (from 14.73±7.88% to 10.56±5.80%, $p<0.01$; Figure 16a), with positive correlation with clinical improvement or reduction of PASI score ($r=0.50$ and $r=0.48$, $p<0.0001$; Figure 16b). No correlation was found with the histological Trozak score. This reduction appeard to be bound to skin homing Th/Tc cells where $CD4^+/CLA^-$ T cells expressing CD103 and $CD8^+/CLA^-$ expressing

CD103 T cells were analysed, no reduction with treatment was found and no significant correlation with the PASI score. No significant difference was observed between the study groups (p=0.6 for $CLA^+/CD4^+$ T cells expressing CD103 and p=0.8 for $CLA^+/CD8^+$ T cells expressing CD103).

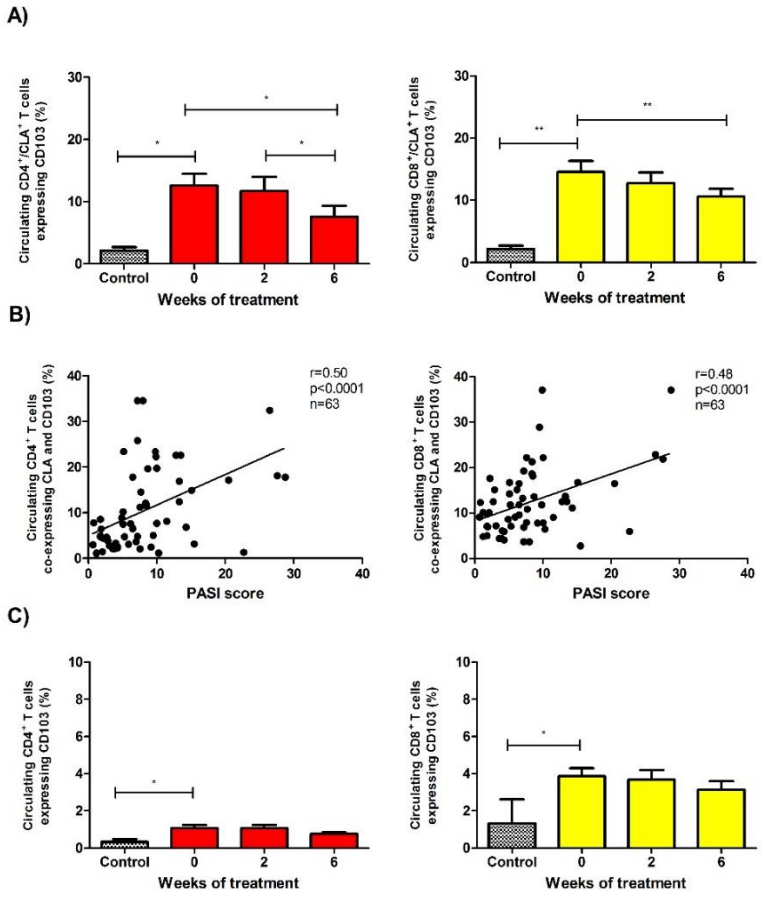

Figure 16. Reduction of circulating skin resident Th/Tc effector cells with treatment

*Bar columns showing values of healthy volunteers and psoriasis patients before treatment and 2 and 6 weeks after the induction of treatment of skin resident Th ($CD4^+CLA^+$ expressing CD103)/Tc ($CD8^+CLA^+$ expression CD103) cells (A), the correlations between PASI score and skin homing Th/Tc (B) and Th/Tc cells expressing the tissue-resident marker CD103 (c). The results are presented as mean±SD for controls (n=3) and psoriasis patients (n=21). Pearson's correlation coefficient (r) and p value are shown.*p < 0.05, **p < 0.01.*

4.6.2 Balneophototherapy at the Blue Lagoon and UVB monotherapy suppresses circulating Th17/Tc17 effector cells in correlation with both PASI and histological Trozak score

The expression profile of circulating T1(Th1/Tc1) and T17 (Th17/Tc17) effector cells in psoriasis patients and its clinical correlation was analysed in both the pilot study (Paper I) and the sub-study of 21 patients from the RCT (Paper III). T17 cells were analysed both as $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ circulating T cells expressing the IL-23 receptor (IL-23R) and CD4+/CD8+ cells expessing the cytokine IL-17 after stimulation (see 4.6.3).

When all treatment groups in the larger RCT (Paper III) were analysed together, pre-treatment levels of Th17 and Tc17 in psoriasis patients (Pre-treatment Th17 levels: 12.25±7.44% and Tc17 levels: 15.37±6.37%, n=21) was much higher than in the healthy controls (HC) (HC Th17 levels: 0.81±0.65%, $p<0.01$; HC Tc17 levels: 1.50±2.08%, $p<0.01$, n=3; Figure 17A and B). A marked reduction (81%) in frequency of circulating Th17 ($CD4^+$ T cells expressing IL-23R) was observed after only one week of inpatient treatment at the BL in the pilot study (Paper I; $p<0.001$, data not shown). A 53% reduction of the amount of IL-23R expressed (Mean fluorescence intensity; MFI) by these cells ($p<0.05$, data not shown) also reflected this reduction. In contrast, such immunological Th17 inflammatory response improvement was only detected after 8 weeks of UVB monotherapy ($p<0.05$, data not shown). However, when all the treatment groups were analaysed together in the larger RCT (Paper III), both frequency of Th17 and Tc17 significantly reduced by 70 and 61 percent after only 2 weeks of treatment (Th17 from 12.25±7.44% to 3.64±5.51%, $p<0.001$, and Tc17 from 15.37±6.37% to 5.89±4.61%, $p<0.001$; Figure 17 A and B). Furthermore, after 6 weeks of treatment both Th17 and Tc17 effector cell frequencies had been even further reduced by 90 and 76 percent (from 12.25±7.44% to 1.19±0.91%, $p<0.001$, and from 15.37±6.37% to 3.66±2.81%, $p<0.001$; Figure 17 A and B). This reduction of Th17 and Tc17 effector cells correlated with both clinical and histological outcome measure for each patient via the PASI and Trozak score ($p<0.002$; Figure 17A and B).

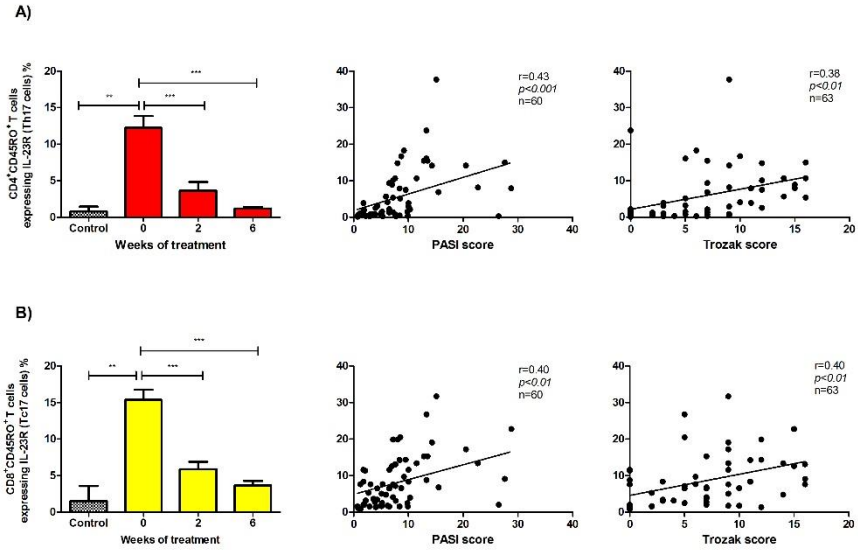

Figure 17. Circulating Th17 and Tc17 cells and their correlation with PASI and Trozak score

Bar columns showing values of Th17 ($CD4^+$ $CD45RO^+$ T cells expressing $IL\text{-}23R^+$) (A) and Tc17 ($CD8^+$ $CD45RO^+$ T cells expressing $IL\text{-}23R^+$) (B) of healthy volunteers and of psoriasis patients before treatment and 2 and 6 weeks. There was significant correlation between the reduction of peripheral Th17 (A) and Tc17 (B) and both clinical efficacy (PASI) and improvement in histological changes (Trozak score) with treatment. The results are presented as mean±SD; healthy controls (n=3) and psoriasis patients (n=21). Pearson's correlation coefficient (r) and p value are shown. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

4.6.3 Balneophototherapy at the Blue Lagoon and UVB monotherapy reduces cytokine secretion of peripheral blood Th17 and Th22/Tc22, but not Th1/Tc1, effector cells

In order to evaluate the biological response of peripheral key effector T cells in psoriasis, the frequency of CD4+/CD8+ cells expessing the cytokines IFN-γ/TNF-α (Th1/Tc1), IL-17 (Th17/Tc17), IL-22 (Th22/Tc22) and IL-4 (Th2) were analysed by flow cytometry after stimulation before and after treatment (Paper I and Paper III). When all the treatment groups were analysed together in the larger RCT study (Paper III), the frequency of Th17 (patients, 2.02% ± 0.76%; HC, 0.49% ± 0.28%), Th22 (patients, 1.44% ± 0.85%; HC, 0.37% ± 0.11%), IFNγ producing Th1 (patients, 5.44% ± 4.87%; HC, 0.37% ± 0.28%), TNF-α producing Th1 (patients, 14.42% ± 7.52%; HC, 0.81% ± 0.41%) and Th2 cells

(patients, 5.57% ± 4.15%; HC, 0.94% ± 0.23%) were significantly higher in psoriasis patients compared with healthy controls (HC) ($p<0.05$, Figure 18A). In addition, the frequency of IFNγ producing Tc1 (patients, 11.94% ± 9.56%; HC, 1.27% ± 0.96%) and TNF-α producing Tc1 (patients, 18.30% ± 10.50%; HC, 2.15% ± 0.65%) were significantly higher in psoriasis patients ($p<0.05$, Figure 18B). This difference between healthy controls and psoriasis patients for Tc17 and Tc22 was not observed in the larger RCT study (III), however, it was observed in the pilot study for both cell types; Tc17 cells 3.10% ± 1.05% and Tc22 4.28% ± 1.58% in psoriasis patients compared with 1.15% ± 0.55% and 0.90% ± 0.52% for healthy controls and Tc22 cells ($p<0.05$).

Successful treatment of psoriasis with either BPT at the BL or UVB monotherapy (Paper III) led to a 23% reduction of Th17 effector cells (pre-treatment, 2.02% ± 0.76%; after treatment, 1.55% ± 0.67%; $p<0.05$), and more than 33% reduction of both Th22 and Tc22 effector T cells after only 2 weeks of treatment (Th22 from 1.44% ± 0.85% to 0.94% ± 0.66%, $p<0.05$ and Tc22 from 1.18% ± 0.80% to 0.79% ± 0.51%, $p<0.05$; Figure 18A and 18B). Furthermore, this reduction was even more pronounced (>50%) after 6 weeks of treatment (Th22 from 1.44% ± 0.85% to 0.63% ± 0.40%, $p<0.001$, and Tc22 from 1.18% ± 0.80% to 0.60% ± 0.37%, $p<0.01$; Figure 18A and B). Furthermore, this reduction of Th17 and Th22/Tc22 effector cells had a positive correlation with the clinical PASI score (r= Pearson´s correlation coefficient: Th17, $r=0.34$, $p<0.01$; Th22, $r=0.56$, $p<0.0001$ and Tc22, $r=0.37$, $p<0.01$; Figure 18C and D). No such correlation was observed in the respect with other T-effector cell phenotypes associated with IFNγ, TNF-α or IL-4 except for IFNγ producing CD8+ T cells, Figure 18. Similar results were observed in the pilot study (Paper I; data not shown).

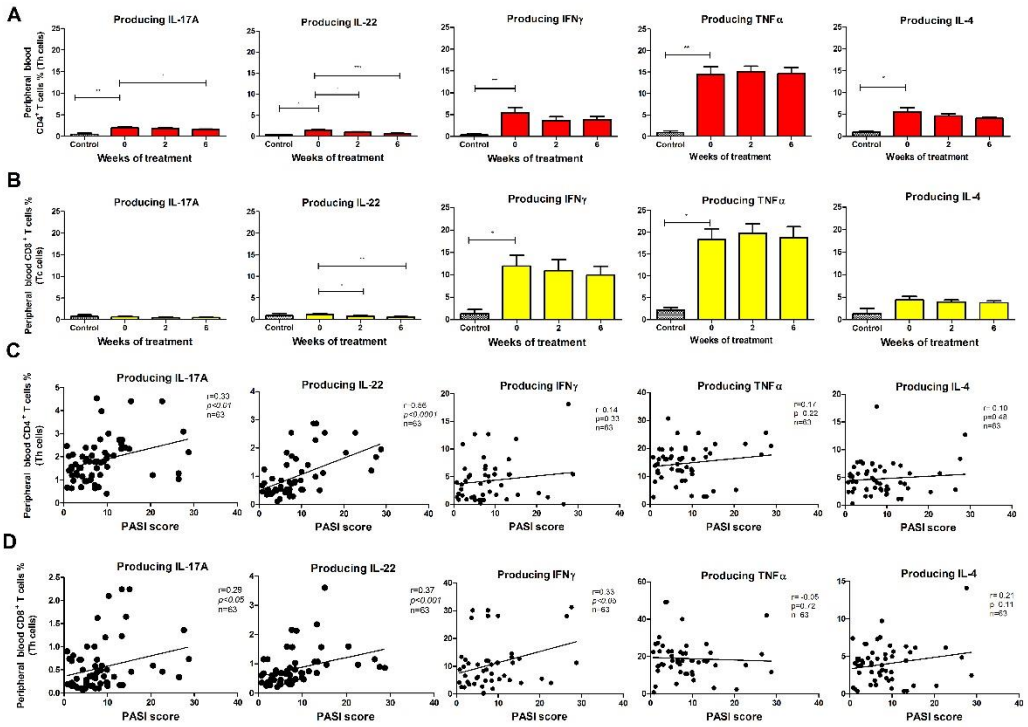

Figure 18. Effector T cell phenotype in the blood of psoriasis patients and correlation with PASI

*Bar columns showing values of healthy volunteers and psoriasis patients before treatment and 2 and 6 weeks after the induction of treatment of A) $CD4^+$ and B) $CD8^+$ T cells producing IL-17A, IL-22, IFNγ, TNF-α and IL-4, and their correlation with the clinical PASI score (C and D). The results are presented as mean±SD; healthy controls (n=3) and psoriasis patients (n=21). Pearson's correlation coefficient (r) and p value are shown. *p < 0.05, **p < 0.01.*

4.6.4 Balneophototherapy at the Blue Lagoon and UVB monotherapy suppresses circulating skin homing Th17/Tc17 and Th22/Tc22 effector cells

To see if this reduction of Th17/Tc17 and Th22/Tc22 cells in the circulation were skin specific we compared the relative frequencies of these cells in peripheral blood co-expressing the skin-homing receptor CLA by flow cytometry after intracellular cytokine staining in the larger RCT study (Paper III). The frequency of skin homing $CD4^+CLA^+$ T cells producing IL-17A (Patients = 6.83±3.54% vs HC = 2.17±2.64%, $p<0.05$; Figure 19A) and IL-22 (Patients = 1.44±0.85% vs. HC = 0.37±0.11%, $p<0.05$; Figure 19C) were significantly higher for patients compared with healthy controls. This was also observed for skin homing $CD8^+CLA^+$ T cells producing IL-22 (Patients = 4.16 ±2.23% vs HC = 1.27±0.07%, $p<0.05$; Figure 19D), but not for $CD8^+CLA^+$ T cells producing IL-17A (Figure 19B).

The frequency of circulating $CD4^+CLA^+$ IL-17A secreting T cells reduced by 35 percent following 6 weeks of BPT at the BL and UVB monotherapy (from 6.83±3.54% to 4.46±1.72%, $p<0.01$; Figure 19A) and $CD8^+CLA^+$ IL-17A secreting T cells by 42 percent (from 2.40±1.66% to 1.40±1.16%, $p<0.01$; Figure 19B). The frequency of skin homing CLA^+ marked $CD4^+/CD8^+$ T cells secreting IL-22 reduced even greater, or Th22 ($CD4^+CLA^+$ IL-22 secreting T cells) by 56% and Tc22 ($CD8^+CLA^+$ IL-22 secreting T cells) by 42% after 6 weeks of treatment (Skin homing Th22 from 3.83±2.10% to 1.68±1.03%, $p<0.001$; vs. skin homing Tc22 from 4.16±2.23% to 2.42±1.30%, $p<0.01$; Figure 19C and D).

The reduction of skin homing Tc22 effector cells had both a strong correlation with clinical (PASI score; $r=0.59$, $p<0.001$) and histological improvement (Trozak score; $r=0.32$, $p<0.05$). In addition, skin homing Tc17 ($r=0.45$, $p<0.001$; Figure 19B) and Th22 effector cells ($r=0.33$, $p<0.05$; Figure 19C) decreased in correlation with the clinical improvement (PASI score), but no correlation was found with histological improvement (Trozak score). Regarding Th1/Tc1 cytokines (IFNγ and TNF-α) and Th2 cytokines (IL-4) no difference was found with treatment (Data not shown). When the treatment groups were compared with each other, significant difference in the reduction of $CD4^+$ CLA^+ T cells producing IL-17A cells (skin homing Th17 cells) for both groups, and $CD8^+$ CLA^+ T cells producing IL-17A cells (skin homing Tc17 cells) for IT-GSW group, as compared with patients treated with UVB only (UVB group) was observed ($p<0.05$). However, this difference had disappeard after 6 weeks of treatment (Figure 20).

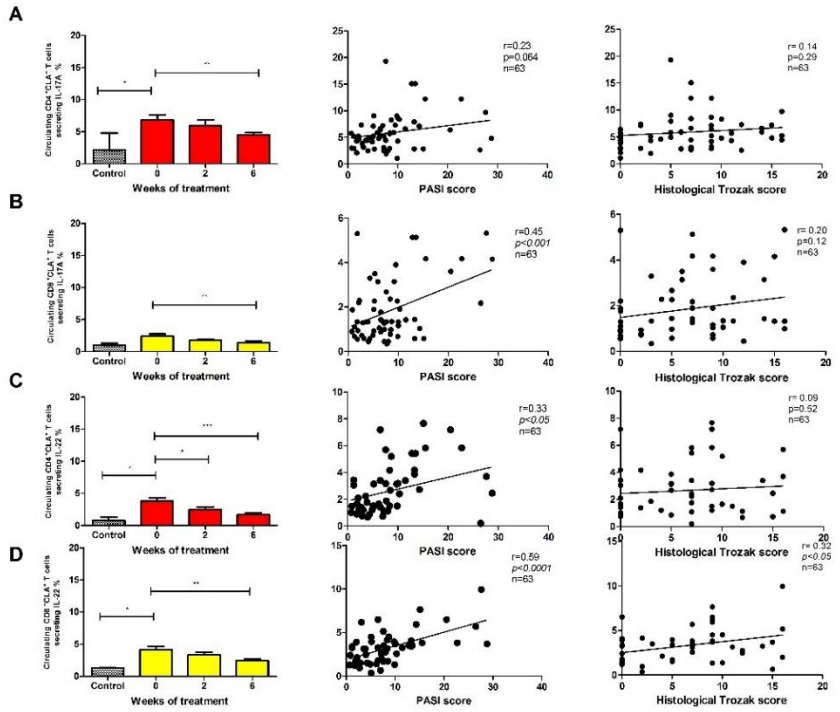

Figure 19. Circulating skin homing Th17/Tc17 and Th22/Tc22 effector cells and correlation with PASI and Trozak score

*Bar columns showing values of healthy volunteers and psoriasis patients before treatment and 2 and 6 weeks after the induction of treatment, their correlation with the clinical PASI score and histological Trozak score: A) Skin homing Th17 cells ($CD4^+CLA^+$ T cells producing IL-17A), B) skin homing Tc17 cells ($CD8^+CLA^+$ T cells producing IL-17A), C) skin homing Th22 cells ($CD4^+$ CLA^+ T cells producing IL-22) and D) skin homing Tc22 cells ($CD8^+$ CLA^+ T cells producing IL-22). The results are presented as mean±SD; healthy controls (n=3) and psoriasis patients (n=21). Pearson's correlation coefficient (r) and p value are shown. *p < 0.05, **p < 0.01, ***p < 0.001.*

4.6.5 Serum levels of the chemokines CCL17 and CXCL10, as well as IL-19 correlate with clinical improvement

In consideration of these changes in circulating Th17/Tc17 and Th22/Tc22 effector cells with treatment, biomarkers in serum connected to the pathogenesis of psoriasis were analysed. In Paper I, no significant difference of the levels of IL-22, IL-17, IL-23, CCL20, IL-1β and TNF-α was observed with treatment (Data not shown). Inn the larger RCT study (Paper III), a strong correlation of the chemokine CCL17 to the clinical PASI score was observed

(r=0.36, p<0.005), but not to Trozak score. However, the chemokine CCL22 did not correlate to either PASI or Trozak score (data not shown). The Th1 associated chemokine CXCL10 had a positive correlation to the PASI score (r=0.33, p<0.05) and the cytokine IL-19 correlated both with PASI score (r=0.39, p<0.01) and the histological Trozak score (r=0.34, p<0.05). No difference was observed between the study groups (Data not shown).

4.6.6 Skin infiltrating T cells in psoriatic skin and correlation with PASI and Trozak score

Immunohistochemical staining for T cell activity (CD3/CD4/CD8) as well as more specific immunofluorescence staining for IL-17 and IL-22, along with CD8, was performed to see how these above cellular phenotypic changes in blood correlated with T cell driven pathology in psoriatic skin in the larger RCT (Paper III). When both treatment groups (IT-GSW and UVB only groups) were analysed together, a 67-70% reduction was observed of CD3 (from 129.2±71.19 to 42.35 ±45.78), CD4 (from 73.00±43.23 to 21.85±25.76), and CD8 (from 53.60±34.33 to 15.10±14.91) positive cells after 6 weeks of treatment (Figure 20A; p<0.01). This depletion of T cells in the skin correlated with the improvement of histological Trozak score ($CD3^+$ r=0.38; vs. $CD4^+$ r=0.40; p<0.05), but not with the clinical PASI score. As shown in Figure 20E, IL-17 staining was widely observed in psoriatic skin with prominent staining in keratinocytes in the lower layer of the epidermis and immune cells in the upper dermis. The treatment response with the regards to IL-17 staining was predominantly confined to dermis since no significant reduction in the grade of IL-17 staining was observed within the epidermis after 6 weeks of treatment (dermal IL-17 score: 1.62±0.61 to 1.34±0.83; p=0.19). A positive correlation between the IL-17 grade in dermis and clinical improvement as measured with PASI was observed (r=0.565, *p*<0.01; Figure 20B). Finally, only few CD8+ cells co-expressed IL-17 (Data not shown) and IL-22 was mostly localized to keratinocytes lining the basement membrane in a pattern like IL-17, but less widely expressed in psoriatic skin (Data not shown).

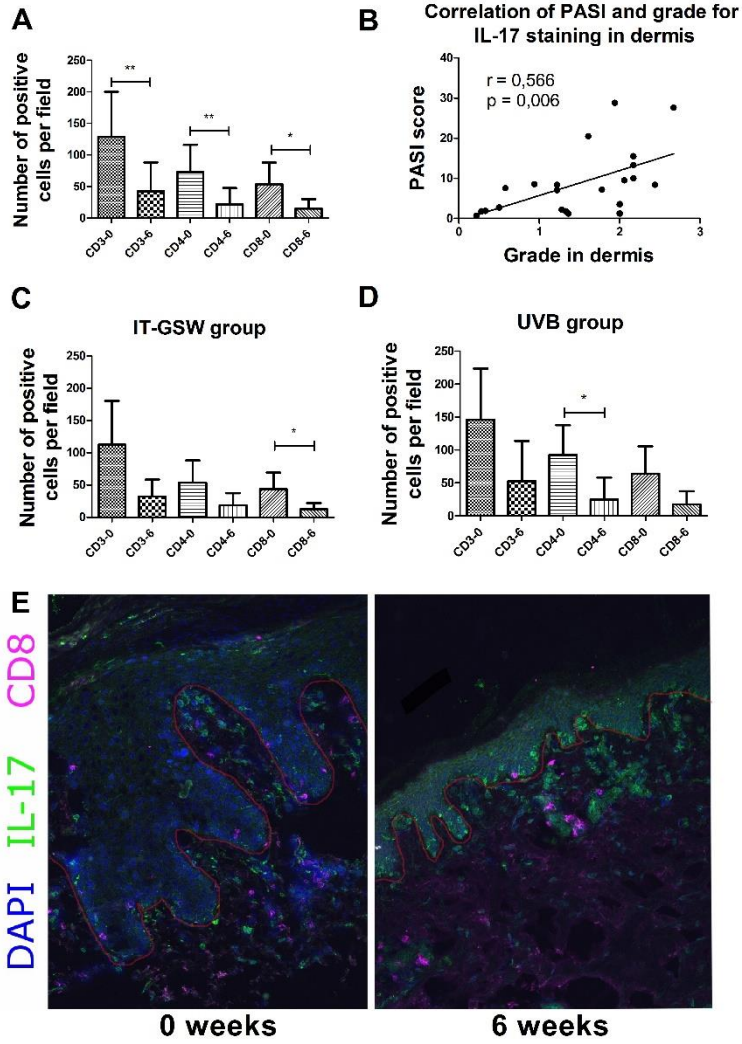

Figure 20. Immunohistochemistry on lesional skin from psoriasis patients before and after treatment

*Bar columns show the number of CD3, CD4 and CD8 positive immunoreactive cells in high powered field of vision of lesional psoriatic skin before treatment (0) and after 6 weeks (6). The intensive balneophototherapy group (IT-GSW group – fig. 6C) received an inpatient combination of bathing in geothermal seawater and UVB phototherapy and the control group received UVB monotherapy (UVB group – fig. 6D). All results are shown compiled (A). Dot plot shows the grade of IL-17 staining in the dermis and the correlation with PASI score (B). Pearson's correlation coefficient (r) and p value are shown. Representative three-colour immunofluoresence of $CD8^+$ (pink), $IL\text{-}17^+$ (green) cells with blue-stained nuclei in lesional plaque from psoriasis patient before treatment (0 weeks) and after 6 weeks of treatment (E). The red lines delineate the dermal-epidermal junction. The results are presented as mean±SD, *p<0.05 and **p<0.01.*

4.6.7 Difference between the treatment groups (Paper III)

The improvement in the parameters that significantly reduced with treatment in the larger RCT (Paper III) was in general significantly faster for the BPT groups (GSW and IT-GSW groups) compared with UVB only group, reaching statistical significance after only two weeks of treatment for the clinical PASI score, histological Trozak score, Th17 ($CD4^+CD45RO^+$ T cells expressing IL-23R), skin homing Th17 ($CD4^+$ CLA^+ T cells producing IL-17A cells) and skin homing Tc17 ($CD8^+$ CLA^+ T cells producing IL-17A) cells for IT-GSW group compared with UVB only group (Figure 21A). However, this difference had disappeared after 6 weeks of treatment except for the clinical PASI score (Figure 21B). No other significant difference in the parameters measured was observed when the treatment groups were compared with each other (Figure 20A and 20B).

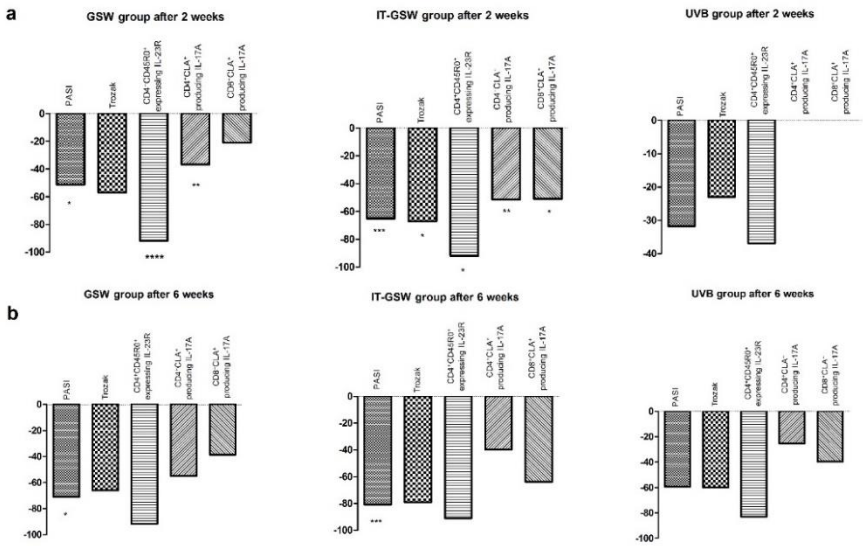

Figure 21. Comparison of all the treatment groups in the randomized controlled trial

*Column bar graphs showing median values of the percentage changes observed following treatment of psoriasis vulgaris with balneophototherapy (BPT) at the BL and UVB phototherapy (GSW group), intensive BPT (IT-GSW group) and UVB monotherapy (UVB group) 2 weeks (a) and 6 weeks after the induction of treatment (b). The results are presented as median (IQR), *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.*

4.7 Trozak score correlates with PASI, LS-PGA, epidermal thickness and Ki-67 staining

The histological grading system of Trozak (Trozak score) was used as a secondary outcome in both pilot study (Paper I) and randomized controlled trial (Paper III). Because it is not widely used in psoriasis research today, its correlation with other psoriasis outcome measures used in these studies was validated in Paper IV. The results of the Trozak has been reported previously (see chapter 4.5 and Figure 6), as well as for the PASI score (see chapter 4.2), LS-PGA (see chapter 4.2), DLQI score (see chapter 4.4) and CD3/CD4/CD8 immunohistochemical staining in the skin (se chapter 4.6.6; Table 6). However, in a sub-study from the larger RCT, epidermal thickness (ET) was measured and Ki-67 antigen staining performed to compare with Trozak score.

When all treatment groups were analysed together, ET significantly decreased from 397,4 µm on average to 277 µm after only two weeks of treatment ($p<0.01$) and to 246,5 µm after six weeks of treatment ($p<0.001$, Table 6). No significant difference of epidermal cells stained positive for Ki-67 antigen expression was found after two weeks of treatment but after six weeks the expression decreased significantly from 65.3 to 40.3 positive cells per field ($p<0.01$; Table 6).

	BEFORE TREATMENT (n=21)	AFTER 2 WEEKS (n=21)	*p* VALUE	AFTER 6 WEEKS (n=21)	*p* VALUE	Correlation with PASI; *r*	*p* VALUE
Biopsy grading (Trozac score)	10,3	5,1	< 0,001	3,2	< 0,001	0,41	< 0,001
Epidermal thickness (µm)	397,4	277	< 0,01	246,5	< 0,001	0,13	ns
Ki-67 (positive cells/field)	65,3	53,4	ns	40,3	< 0,01	0,28	0,025
CD3 (positive cells/field)	134,8	x	x	47,8	< 0,01	0,27	ns
CD4 (positive cells/field)	74	x	x	22	< 0,001	0,16	ns
CD8 (positive cells/field)	56,6	x	x	17,9	< 0,01	0,36	ns
PASI score	12,9	7,1	< 0,001	4,7	< 0,001	x	x
LS-PGA score	5	3	< 0,001	2,3	< 0,001	0,88	< 0,0001
DLQI score	10	x	x	6,1**	< 0,01	0,48	< 0,01

Table 6. Correlation of all outcome measures used in the larger randomized controlled trial (RCT) with PASI score

*Mean values for all variables used in the study (Paper IV). * p value compared with before treatment. Statistically significant difference at p <0.05. **DLQI after 10 weeks, not 6 weeks. Pearson's correlation coefficient (r).*

The Trozak score correlated strongly with the PASI score (Pearson´s $r=0.49$, $p<0.0001$), LS-PGA score (Pearson´s $r=0.48$, $p<0.0001$) and ET (Pearson´s $r=0.68$, $p<0.0001$), but weakly with Ki-67 antigen expression in

lesional skin (Pearson´s r=0.28, p<0.05; Table 6 and Figure 22). In addition, the Trozak score correlated well with the quality of life DLQI score (Pearson´s r=0.61, p<0.01; Table 6). Interestingly, even though there was a significant correlation between ET and Trozak score, ET did not correlate with either of the clinical scores used in this study; the PASI score (Pearson´s r=0.13, p=0.28; Table 6) and the LS-PGA score (Pearson´s r=0.20, p=0.10). However, ET correlated significantly with Ki-67 antigen expression (Pearson´s r=0.58, p<0.0001) and Ki-67 antigen expression shows weak significant correlation with the PASI score (Pearson´s r=0.28, p=0.025; Table 6) and the LS-PGA (Pearson´s r=0.37, p<0.01). Finally, CD3, CD4 and CD8 expression did not correlate with the PASI score (Table 6) or other outcome measures (data not shown).

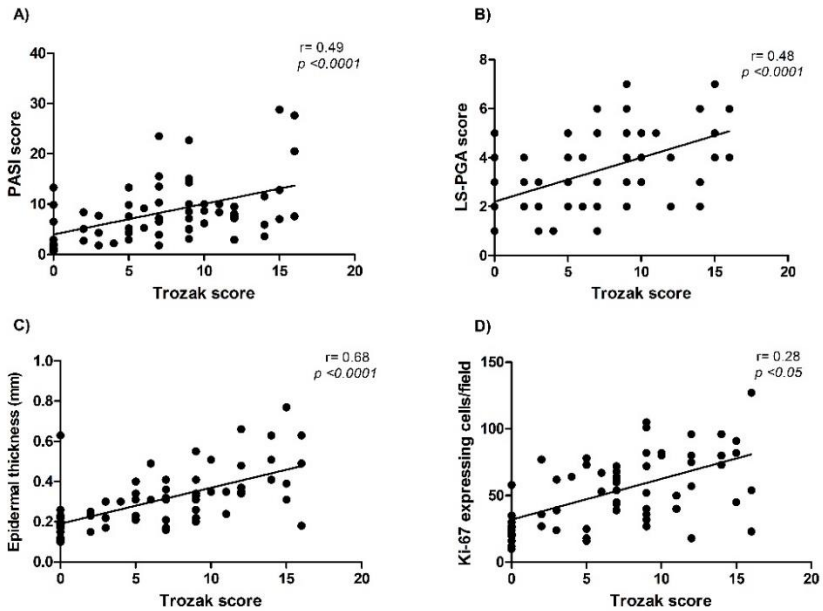

Figure 22. The correlation of clinical and histological outcome measures with Trozak score

Correlation of A) the Psoriasis Area and Severity Index (PASI), B) the Lattice System Physician Global Assessment (LS-PGA) score, C) epidermal thickness of lesional skin and D) Ki-67 antigen expression in the epidermis of lesional skin with Trozak's histological score. Pearson's correlation coefficient (r) and p value are shown.

5 Discussion

Psoriasis is a complex chronic inflammatory skin disease that has a profund effect on patient´s quality of life[25]. The disease is caused by a combination of genetic[50,51] and environmental factors[41,54], and has a range of disease phenotypes which differ in severity and response to treatment[8]. One treatment options for psoriasis patients in Iceland is balneophototherapy (BPT) at the BL. The treatment is combined by bathing in a geothermal seawater at the BL followed by UVB phototherapy. Two preliminary studies[213,214] and two controlled study[179,192] concerning the effect of bathing in the lagoon on psoriasis have shown that bathing in the lagoon has beneficial effects on chronic plaque psoriasis and is superior to UVB monotherapy[192]. However, these studies are without randomization and control groups, making it difficult to draw concrete conclusions. In addition, no other outcome measures than the Psoriasis Area and Severity Index (PASI score) were used in these studies. Although PASI is considered the gold standard in psoriasis research today, it has limitations such as low responsiveness in mild disease and certain sites (hands, nails, feet, face, genital organs), symptoms (such as pruritus), and the quality of life impairment are not evaluated[130,132,136]. Where psoriasis has a major impact on quality of life (QOL)[225], which is not necessarily in proportion to clinical severity [226], current clinical trials complement the PASI with quality of life score (DLQI) as well as other outcome measures in an effort to yield a more comprehensive view of the efficacy of psoriasis treatments[131,227].

The overall aim of the work presented in this thesis was to perform a careful and thorough examination of the clinical, psychosocial, histological, and immunological differences between BPT at the BL in Iceland (bathing in geothermal seawater combined with UVB phototherapy) and conventional UVB phototherapy. Clinical, psychosocial, and histological differences were examined in a prospective randomized trial (Paper II) derived from clinical data of 68 chronic plaque psoriasis patients. The effect of these treatment regimens on immunological pathways in peripheral blood and skin, were examined both in a small pilot study with 12 chronic plaque psoriasis patients (Paper I) and in a sub-study of 21 chronic plaque psoriasis patients from the randomized controlled trial (Paper III). Finally, the histological Trozak score, that was used as a secondary outcome measure in this work, was validated by comparing it with other outcome measures commonly used in psoriasis research (Paper IV).

The results from the data generated in this thesis shows that BPT at the BL (bathing in geothermal seawater followed by UVB therapy) induces a faster clinical and histological improvement, produces longer remission time as well as permits lower UVB doses than UVB therapy alone in psoriasis. In addition, BPT at the BL and UVB monotherapy suppresses primarily the

T17/T22 inflammatory pathway in the blood of psoriasis patients, not T1 pathway, and suppresses recruitment of T cells to lesional psoriatic skin. Furthermore, the treatments induce a site-specific depletion of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in lesional psoriatic skin in correlation with histological improvement, and depletion of IL-17 in the dermis in correlation with clinical improvement. Finally, the histological grading score of Trozak used in this thesis appears to be well applicable as an addiational objective outcome measure in psoriasis research where it correlated strongly with clinical severity scores, quality of life score (DLQI) and other histological markers commonly used in psoriasis research.

5.1 The pilot study gave important clues for the randomized controlled trial

In the first study (Paper I) the aim was to evaluate the potential immunological effect of BPT at the BL compared with UVB monotherapy and evaluate methods for the prospective randomized controlled trial (Paper II). The pilot study gave important information:

Firstly, a intensive inpatient balnophototherapy at the BL, which is a common treatment regimen at the BL, had never been studied before, where the previous controlled trial comparing BPT at the BL with conventional UVB therapy in psoriasis patients received inpatient BPT at the BL for 4 weeks[192]. The inpatient BPT in this study included bathing for one hour in the lagoon two times daily followed by UVB therapy one time daily 5 days per week for two weeks. The control group received conventional UVB therapy three times daily for 4 weeks. The results showed that intensive BPT at the BL was more effective than UVB alone, where 37% improvement of PASI was observed after only one week in the BL group compared with 18.3% in the UVB alone group. This improvement was even more significant after 3 weeks, or 67.3% improvement of PASI in the BL group compared with 22% in the UVB alone group. However, patients treated at the BL only received treatment for 2 weeks and patients treated with conventional UVB therapy for 4 weeks, and consequently this difference in clinical improvement disappeard after 8 weeks. Because of this marked clinical improvement of this novel intenstive BPT group it was decided to include this treatment regimen in the larger randomized controlled trial and add after treatment of conventional UVB therapy three times a week for 4 weeks (total 6 weeks).

Secondly, the use of the histological Trozak score[45] as an additional blinded objective outcome measure for psoriasis gave promising result in the pilot study where it significantly reduced after 3 weeks of treatment in both

study groups concomitatnt with PASI reduction. The use of the PASI score is the golden standard for psoriasis research to evaluate the clinical severity of psoriasis[136] and DLQI score for health-related quality of life[135]. However, these scores are lacking in objectivity and have several limitations leading to the additional use of more observer-independent methods to improve the quality of clinical research[137-139,141-144]. Biopsies are attractive because they are objective. Trozak score has not been widely used in psoriasis research[137,155,228,229] but takes into account 10 different histological features of psoriasis[45] and has good correlation with epidermal thickness measurements in psoriasis[137]. Since this study and upcoming randomized controlled trial were unblinded to the primary investigator, it was decided to improve the quality of the work with an additional blinded outcome measure. The Trozak score confirmed the clinical findings in the pilot study (Paper I) and respectively, it was decided to use the histological Trozak score further in the randomized controlled trial (Paper II-IV). The use of Trozak score in psoriasis research is discussed more detailed in chapter 5.4.

Thirdly, the results from the pilot study (Paper I) indicated that BPT at the BL and UVB monotherapy suppresses primarily the T17/T22 inflammatory pathways in the blood of psoriasis patients and the recruitment of circulating skin homing T cells to the lesional psoriatic skin. However, no therapeutic changes were found regarding the expression of adhesion molecules ICAM-1 and CD62E or other activation markers such as CD25 and HLA-DR. Consequently, the T17/T22 and T1 inflammatory pathway as well as skin homing T cells in the peripheral blood of psoriasis patients were studied in more detaile in a sub-study from the randomized controlled trial (Paper III). The immunological results from the pilot study (Paper I) and randomized controlled trial (Paper III) are discussed together in chapter 5.3.

5.2 Balneophototherapy at the Blue Lagoon is superior to UVB monotherapy

Although previous studies have reported beneficial effect of BPT at the BL[179,192,213,222-224], this is the first prospective randomized controlled trial on the effect of BPT at the BL compared with UVB phototherapy alone. BPT at the BL showed faster clinical and histological improvement as well as reduction in the quality of life score (DLQI), compared with UVB therapy alone (Paper II). Furthermore, BPT at the BL resultet in reduced total UVB dose needed for 75% improvement of the PASI score and a longer remission.

BPT at the BL had a rapid onset of action, as evidenced by significant improvements in PASI score occurring after only one week of treatment, and

by the significantly higher percentage of patients achieving PASI 75 or Lattice score of clear or almost clear as early as at week one. Furthermore, 42% of patients treated with intensive BPT at the BL (IT-GSW group) achieved PASI 90 after only 6 weeks of treatment. In contrast, only 17% of the patients treated with UVB monotherapy had reached PASI 75 at that time. Consequently, most of the UVB monotherapy patients continued UVB therapy until week 10. However, most of the patients in the BPT groups had reached PASI 75 or PASI 90 after 6 weeks and discontinued treatment. Respectively, the mean total cumulative dose of UVB, the number of exposures and the time required to achieve at least PASI 75 was significantly lower in both BPT groups compared with the UVB monotherapy group ($p<0.001$). The clinical improvement correlated well with the improvement in the quality of life (DQLI) and the blinded histological Trozak´s score. BPT at the BL almost completely reversed the histopathological changes of psoriasis as measured by Trozak´s score after only two weeks of treatment.

After six months, only 30% of patients in both BPT groups had started another therapy or relapsed, compared with 56% of patients treated with UVB monotherapy. The most common reason for starting another treatment was because of poor result in the scalp and intertrigionus areas that are poorly exposed during UVB therapy. This could, at least in part, explain why the efficacy was lower in the UVB monotherapy group. Other possibility is that ingredients in the geothermal seawater at the BL have additative immunosuppressive effects and healing effect on psoriasis. To support that, studies have shown that only bathing in the geothermal seawater has beneficial effect on psoriasis[179,213]. Furthermore, more recent studies using extracts from the silica mud and the blue-green algae *Cyanobacteria aponinum* and *Silicabacter lacuscaerulensis* from the lagoon indicate immunosuppressive effect of these extracts[216,217].

To our knowledge there are no clinical studies that have shown that bathing in tap water or hot baths may have a beneficial effect on psoriasis. However, there are some studies demonstrating that bathing in tap water before UVB exposure give a slightly better results than UVB monotherapy[208] and that salt water may increase photosensitivity of the skin to UVB irradiation[186,206,207]. Furthermore, there are indications that hot salt water baths alone have a minor therapeutic effect, usually not exceeding a 30% improvement of the PASI score [178,189]. The data is however conflicting where some studies show beneficial effect of salt water to enhance phototherapy[187,188,191,208] and other report no additional effect[178,205,209]. In this randomized controlled trial, the patients who were treated with UVB therapy alone took a shower immediately before the UVB treatment was given to make the treatment groups as comparable as possible.

Treatment options for psoriasis have increased considerably in recent years with many new thearpeutic agents, such as the biologic drugs that show excellent effect. However, they are expensive and can cause serious side effects. UVB therapy and BPT are important inexpensive treatment options in mild to moderate psoriasis with clinically beneficial effect and few side effects.

5.3 Balneophototherapy at the Blue Lagoon and UVB therapy suppresses T17/T22 inflammatory pathways and skin homing T cells in the peripheral blood of psoriasis patients

The aim of these two studies, the pilot study (Paper I) of 12 chronic plaque psoriasis patients and the sub-study from the randomized controlled trial of 21 chronic plaque psoriasis, was to evaluate the potential immunological effect of BPT at the BL compared with UVB phototherapy alone. As mentioned above, treatment regimens used in this thesis suppressed primarily the recruitment of T cells to lesional psoriatic skin and the T17/T22 inflammatory pathways in the blood of psoriasis patients and in general, no difference was observed between balnophototherapy and UVB therapy.

5.3.1 Skin homing and resident T cells in the peripheral blood

A site-specific T cell-driven disease memory in psoriasis has been proposed where memory T cells co-expressing the skin homing addressins cutaneous lymphocyte antigen (CLA) and CCR10 with diverse T cell repertoire are lodged in the skin, and have potent effector functions depending on the local microenvironment[67,72,73]. They are termed as tissue-resident memory T cells (T_{RM} cells), are primarly memory $CD8^+$ T cells expressing the adhesion and migratory molecule CD69 and subsets also expressing CD103[74-76]. However, $CD4^+$ T cells and other T-cells can establish residence in non-lymphoid tissues[75], but $CD4^+$ T_{RM} are different from $CD8^+$ T_{RM} where they are low or negative for CD103 and appear to retain the capacity to migrate[74,75]. T_{RM} is thought to play a role in maintaining and driving psoriasis, presumably contributing to epidermal localization and/or retention of a specific T cell subset[126].

We assessed in our pilot study (Paper I) whether the skin resident integrin CD103, skin homing markers CCR4 and CCR10, and CLA might be co-expressed by some T cells in the peripheral blood and if they respond to treatment. Untreated psoriasis patients were found to have increased

frequency of skin homing CLA^+ T cells expressing the skin resident marker CD103, skin homing marker CCR10, co-expressing CD103 and the skin homing marker CCR4, and co-expressing CCR4/CCR10 compared with healthy controls. In contrast, no difference was observed in the frequency of skin homing CLA^+ T cells expressing CCR4 between healthy controls and untreateted psoriasis patients. This is in an agreement with other studies showing that the skin homing marker CCR4 is abundantly expressed on circulating T cells with a skin-homing CLA^+ phenotype[115] in both normal subjects as well as psoriasis patients[117], and that CCR10 and CD103 are weakly expressed in peripheral blood of normal subjects[77,124].

Prior studies have reported that both the skin homing marker CCR10 and skin resident marker CD103 are present in the inflamed psoriatic skin but not normal skin, indicating their involvement in the immunopathogenesis of psoriasis[77,124]. Our findings further support the involvement of CCR10 and CD103 in psoriasis by showing significant reduction of circulating skin homing T effector cells expressing CD103 and CCR10 with treatment. Furthermore, our results from the RCT (Paper III), where we added CD4 and CD8 markers to the analysis, indicate that only a small subset of $CD8^+$ and $CD4^+$ T lymphocytes in the peripheral blood express CD103 in psoriasis patients in correlation with previous studies[124,127]. A marked reduction of the frequency of circulating skin resident Th and Tc cells concomitant with clinical efficacy in the RCT (Paper III) confirmed our previous results from the pilot study (Paper I). Interestingly, when $CD4^+CLA^-CD103^+$ and $CD8^+CLA^-CD103^+$ T cells were analyzed, no reduction with treatment was observed, indicating that the reduction was limited to skin homing $CD4^+/CD8^+$ cells expressing the integrin CD103. In addition, our results from both the pilot study (Paper I) and RCT(Paper III) indicate that the skin homing marker CCR4 has a potential role in the pathogenesis of psoriasis when co-expressed with the skin resident marker CD103, where there was no detection of skin homing T cells co-expressing CCR4 and CD103 in the peripheral blood after 3 weeks of inpatient BPT at the BL, and CCL17, ligand for CCR4, showed a high correlation to the PASI score and a reduction with treatment.

This is, to the best of our knowledge, the first studies that shows the response of the chemokine receptors CCR4 and CCR10 and the skin resident marker CD103, to UVB phototherapy in the peripheral blood of psoriasis patients. *Ekman et al* measured the chemokine receptors CXCL9/CXCL10 (Th1), CCL17/CCL22 (Th2) and CCL20 (Th17) in the peripheral blood of psoriasis patients before and after UVB therapy and they found no difference with treatment[123]. In summary, the data from these studies suggests that BPT at the BL and UVB phototherapy suppresses predominantly skin-homing tissue retaining T cells expressing the chemokine receptors CCR4 and CCR10 in the

peripheral blood of psoriasis patients, thus implicating the role of directing $CCR4^+/CCR10^+$ and $CD103^+$ subset of skin-homing T cells (CLA^+) into psoriasis plaques during active stage of the disease. Skin homing (CLA^+) T cells and T cells only expressing $CD103^+$ T cells did not change significantly with treatment, as well as adhesion molecules and activation markers analysed in this study, demonstrating that these findings are not only explained by the significant overall decrease in the inflammatory state of the patients following the treatment protocols.

5.3.2 T17 and T22 effector cells might be the ultimate effector cells in psoriasis

The results from both the pilot study (Paper I) and RCT (Paper III) highlight the critical role of T17 effector cells in the pathogenesis of psoriasis and is in agreement with previous reports[108,229]. We observed a strong correlation of the reduction of circulating Th17/Tc17 with both clinical efficacy score and histological Trozak score. Furthermore, a significant resolution of skin homing (CLA^+) Th17/Tc17 cells in the peripheral blood with treatment was observed where the reduction of skin homing Tc17 correlated well with clinical improvement, but not skin homing Th17 cells. IL-17 drives the production of proinflammatory cytokines and chemokines by keratinocytes and consequently activates leukocytes and recruits them into the skin[110]. In addition, our data demonstrates that circulating skin homing Th22/Tc22 cells reduced significantly with treatment in correlation with clinical improvement. IL-17 and IL-22 regulate distinct pathways where IL-17 is a strong inducer for synthesis of antimicrobial peptides in keratinocytes. IL-22 is also linked to keratinocyte activation and epidermal achantosis, a prominent morphological feature in psoriasis [90,230]. Thus, these above findings suggest that IL-22 is another critical cytokine in the pathogenesis of psoriasis[231]. We observed prominent reduction of circulating skin homing Tc22 which correlated with both clinical and histological improvement. However, circulating Th22 and Tc22 did not correlate with the histological Trozak score and therefore raises a question if skin homing $CD8^+$ (Tc17 and Tc22) play more significant role in the pathogenesis of psoriasis compared with skin homing $CD4^+$ (Th17 and Th22).

When IL-17 and IL-22 were analysed further with immunofluorescence staining of skin biopsies before and after treatment, a staining of both IL-17 and IL-22 in keratinocytes in the lower epidermis was observed, probably derived from cytokines bound to their receptor[90], and in lymphoid aggregates in dermis. One study has reported that keratinocytes can produce IL-17 themselves[232], but the consencus today is that eventough various immune cells can produce IL-17A and IL-22, keratinocytes do not[233]. A significant

depletion of IL-17 was not observed in the psoriatic skin with treatment as for the circulation. This contradicts previous reports that show that IL-17 and IL-22 mRNA in the skin normalizes with psoriasis treatment as cyclosporine[110], UVB phototherapy[145] and strongly correlates with histological improvement. This difference can be explained by different methods used or that we took skin biopsies after only 6 weeks of treatment and it takes longer time to see significant change in the skin with immunofluorescence staining. These studies that show normalization of IL-17 and IL-22 mRNA in the skin use real-time PCR and include only histologically normalized psoriasis plaques into their analysis [145], and use gene expression profiling[170]. We did not use any exclusion classification in our study so all plaques were included in our analysis and we use immunofluorescence staining. Furthermore, we observed a strong correlation between both clinical and histological improvement and the level of IL-19 in serum, but the cytokine IL-19 produced by keratinocytes has been shown to have a very strong relationship to the severity of psoriasis [114].

Eventhough IL-17 has been in the focus of many studies on the pathogenesis of psoriasis, it is generally regarded as a T cell-mediated immune disease with a mixed Th1/Th17 cytokine environment [81,82]. Psoriasis lesions contain elevated levels of IFN-γ and it has been shown to drive inflammation in psoriatic skin [234]. However, with the failure of an IFN-γ-targeted therapy, a central or critical role for IFN-γ in psoriasis has been cast into doubt[235]. IFN-γ most likely contributes to the cytokine storm in psoriasis by aiding other cytokines, in particular, IL-17A, which drives IL-1/IL-23 production to augment Th17 inflammatory pathway[82]. This is in an agreement with our results where we found no significant reduction of the T1 cytokines IFNγ and with treatment and no correlation with the clinical improvement, except for the skin homing CD8+ IFN-γ T cells. However, we observed a correlation between the Th1 ligand, CXCL10, in serum and clinical improvement. Nevertheless, our data indicates the impact of successful psoriasis treatment was much less on T1 inflammatory pathway in the peripheral blood of psoriasis patients, than the T17/T22 inflammatory pathways. Interestingly, the reduction of circulating skin homing $CD8^+CLA^+$ T cells producing all the cytokines measured in this thesis (IL-17A, IL-22, IFNγ, TNF-α and IL-4) correlated with clinical improvement (data not shown), but not skin homing $CD4^+CLA^+$ T cells except for skin homing $CD4^+CLA^+$ T cells producing IL-22. This could indicate that skin homing $CD8^+$ (Tc17/Tc22/Tc1) might be the ultimate effector cells in psoriasis.

Finally, in an agreement with other studies[163,164], a resolution of inflammatory $CD3^+/CD4^+/CD8^+$ T effector cells in psoriatic skin with successful BPT and UVB therapy was observed. Interestingly, the resolution of $CD3^+$, $CD4^+$ and $CD8^+$ cells in psoriatic skin with treatment correlated with the histological Trozak score but not with the clinical severity score. It could be

explained by the small number of patients (*n*=10) where immunohistochemical analysis was performed.

5.3.3 No qualitative difference between the immunological effect of balneophototherapy on psoriasis compared with UVB monotherapy

No qualitative difference regarding the immunological effect on psoriasis between BPT at the BL and UVB phototherapy was observed. In general, BPT at the BL induced a faster response, cinically, histologically and in the peripheral blood where a significant difference was found after 2 weeks of treatment for Th17 ($CD4^+CD45RO^+$ T cells expressing IL-23R), skin homing Th17 ($CD4^+$ CLA^+ T cells producing IL-17A cells) and skin homing Tc17 ($CD8^+$ CLA^+ T cells producing IL-17A). However, this difference had disappeard after 6 weeks of treatment. It is probable that BPT at the BL and UVB therapy induce similar effect on the inflammatory pathways underlying psoriasis. For example, *Furuhashi et al* observed a resolved Th17/Treg imbalance with PUVA and UVB therapy in psoriasis[236] and *Gudmundsdottir et al* found improved imbalance of Th17/Treg by human dendritic cells in vitro in the presence of the dominating blue-green algae *Cyanobacteria aponinum* from the BL[217]. Further studies on the immunomodulatory effects of the silica mud and microalgae in the BL would be interesting and specially on the expression of antimicrobial peptides and proteins (AMPs), that are located at the outermost skin layer.

5.4 Trozak score in psoriasis research

Following the hypothesis that an additional objective measure of psoriasis severity in research settings is needed to improve their quality, the use of the histological Trozak score of psoriasis was explored (Paper IV). The data from this study shows that Trozak score has a strong correlation with the gold standard outcome measure in psoriasis research, the PASI score, and the clinical LS-PGA score as well as health-related quality of life score DLQI. Furthermore, a correlation between epidermal thickness (ET) and Ki-67 staining in the target lesion was observed.

None of the current outcome measures used to evaluate the severity of psoriasis fulfils all of the validation criteria for the ideal severity score of high specificity and sensitivity and a low inter- and intra-observer variation[130]. The Psoriasis Area and Severity Index (PASI)[133] is considered as the gold standard to validate the clinical severity of psoriasis[130,132,136], but it has limitations such

as low responsiveness in mild disease and does not take into account the psychosocial impact of the disease[130,132,136]. Since psoriasis has a major impact on health-related quality of life[225] which is not necessarily in proportion to clinical severity[226], a patient-reported quality of life score (DLQI) is usually used in psoriasis research today to complement the PASI score. Two clinical scores were used in this thesis, both the gold standard PASI score and Lattice System Physician´s Global Assessment (LS-PGA), which has lower intra-observer and inter-observer variation compared with PASI[218]. No difference between these two scores were observed in this thesis, where all the patients achieving PASI75/PASI90 achieved a Lattice score of "clear" or "almost clear". Respectively, a very high correlation between these two scores was noted (Person´s $r=0.88$, $p<0.0001$). In an agreement with other studies, a weak correlation of DLQI with the PASI score was observed[132].

Histopathology has been suggested as a more observer-independent assessment tool than clinical assessment[237]. For example, target lesion severity score, supplemented by physician global assessment and quality of life measures, is the suggested psoriasis clinical outcome measures in mild diseases[131]. Eventhough the Trozak score is a complete grading system assessing psoriasis severity from histological changes, only a few researchers have used it in the past[137,155]. However, several studies use some form of histopathological assessment as a secondary outcome measure in combination with clinical score in psoriasis research, with no uniformity in the techniques used making comparison difficult[105,140-142,144]. Others use no scoring system, only a general histopathological examination which makes correlation with clinical severity of the disease and comparison with other studies even more difficult[147,148].

Epidermal thickness (ET) and Ki-67, a marker for epidermal proliferation, and immunohistochemical scoring of T cell activity in lesional skin before and after treatment are commonly used as a secondary outcome measure in psoriasis. However, the data concerning whether these findings correlate with clinical psoriasis scoring systems is somewhat conflicting, where some studies show correlation[147,150,153,154] and others do not[139,141,149,151,152]. Since there seemed be the most convincing data for ET and Ki-67 antigen expression[137,147,150,154], these markers were used in this study to compare with Trozak score. In an agreement with previous reports, a weak correlation between Ki-67 positive cells in lesional skin and PASI before and after treatment was observed. *Morsy et al* observed a correlation of the Trozak score and DLQI and ET. Interestingly, they did not observe any correlation between ET and PASI which is in an agreement with our results and others[137,151,152]. However, a strong correlation between PASI and the Trozak score was observed in this study indicating that the Trozak score reflects disease severity better than ET, both before and after therapy, possibly because Trozak score

takes into account 10 different histopathological features of psoriasis instead of only one as in ET measurements. It has been cast into doubt that ET is a suitable for psoriasis research do to the variety of ET between lesions sites and different persons[238,239].

Some researcher use immunological markers for T cell activity as an additional outcome measure of psoriasis[141,142,149,151,152], however there are only few studies that have found correlation of these markers with clinical severity scores[150,153]. No correlation between CD3, CD4 and CD8 positive T cells in the psoriatic skin and the clinical scores, PASI or LG-PSA, was observed in this study. Today there is much interest in monitoring the immune response in psoriasis with less invasive measurements in the blood. IL-17 measurements in serum are promising with good correlation with PASI[240], however, no reliable markers have been established yet.

In summary, we suggest the use of the histological Trozak score as an additional objective secondary outcome measure in psoriasis research settings where it correlates well with the PASI score which is the gold standard today.

5.5 Strengths and limitations of the study

This thesis adds important knowledge to psoriasis literature on the clinical effect of BPT at the BL in Iceland as well as the immunological effect of both BPT and UVB phototherapy on psoriasis. One of the strengths of this study is its desing as a prospective randomized controlled trial. Futhermore, there were no significant demographic differences between the study groups and controls, which further strengthen the study. Nevertheless, several weaknesses are acknowledged.

Both the pilot study and RCT was unblinded which limits this thesis. This observer bias was addressed, by having observer-blinded histological scoring in an effort to compensate the unblinded clinical scoring. A good correlation between clinical and histological improvement was observed in this thesis. In addition, patients were not blinded to BPT or UVB phototherapy and this might have influenced patient reported outcome in the health-related quality of life questionnaire (DLQI).

Finally, both of the study's cohort was relatively small, only 12 patients in the pilot study and 68 patients in the RCT.However, a power analysis was conducted prior to the study suggesting that 45 study participants would be required in the RCT in order to obtain significant results. In addition, Iceland is a small country and it can be a difficult task to recruit larger study cohort of psoriasis patients in a clinical study with several follow-up visits, biopsies, and blood samples.

6 Conclusions

The key aim of this thesis was to investigate the effect of BPT at the BL on plaque psoriasis compared with UVB monotherapy. The data herein demonstrate that BPT at the BL has superior clinical, psychosocial and histological effect compared with UVB monotherapy. Furthermore, it demonstrates that fewer UVB treatment sessions, and consequently lower cumulative UVB dose, are needed to acheive PASI75 with BPT at the BL, as well as a longer remission time is achived. In addition, it was demonstrated that there was no difference in the immunological effect of BPT and UVB monotherapy, where both treatments influenced the same immune pathways underlying psoriasis. Both treatments suppressed the T17/T22 immune pathways in the peripheral blood in correlation with clinical improvement, but not the T1 immune pathway. In particular, it was demonstrated that the skin homing markers CCR4 and CCR10, as well as the skin resident marker CD103, were suppressed on circulating skin homing (CLA) T cells in correlation with clinical improvement, that has not been shown before. In addition, these findings were further corroborated by the correlation of the circulating skin homing chemokine CCL17. Furthermore, these peripheral blood compartment respone was further strengthened by immunohistocehmical studies reflecting significant site-specific inflammatory resolution of CD3+, CD4+ and CD8+ T cells with treatment, and a correlation of dermal immunohistochemistry analysis of IL-17A with clinical improvement. Finally, we speculate that the histological score of Trozak used in this thesis is a potential objective assessment tool for evaluating psoriasis severity in combination with the gold standard clinical severity PASI score and DLQI score in research settings.

While the clinical effect of bathing in the geothermal seawater is confirmed in this thesis and we do not fully understand the biological basis for the additional effect of bathing in the geothermal seawater psoriasis. Possible future avenues of research would include basic studies on the probable immunomodulatory effect of the silica mud and the coccoid blue-grean microalgae *Cyanobacterium aponinum* and the filamentous microalgae *Silicibacter lacuscaerulensis* found in the BL. At present, there are ongoing studies trying to identify and isolate the agents responsible for the additional beneficial effects of bathing in geothermal seawater.

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Original publications

Paper I

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The Role of Th17/Tc17 Peripheral Blood T cells in Psoriasis and Their Positive Therapeutic Response

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Abstract

It is known that NB-UVB therapy can suppress a broad range of immune cells, but the additional effect of bathing in geothermal seawater still remains unclear. To study the influence of treatment on the expression of circulating immune cells contributing to the pathogenesis of psoriasis, six patients with psoriasis were treated with bathing in geothermal seawater two times daily combined with NB-UVB five times/week for 2 weeks and six patients were treated with NB-UVB therapy three times/week for 8 weeks. Disease severity (Psoriasis Area and Severity Index, PASI), chemokines, inflammatory cytokines, T cells and Toll-like receptors in the blood and skin samples were evaluated on enrolment (W0) and at 1 (W1), 3 (W3) and 8 (W8) weeks. Compared with healthy controls, psoriasis patients with active disease had significantly higher proportion of peripheral CLA+ T cells expressing CCR10 and CD103 and T cells with both Th1/Tc1 (CD4+/CD8+ IFN-γ+ or TNF-α+ cells) and Th17/Tc17 (CD4+CD45R0+IL-23R+, CD4+/CD8+ IL-17A+ or IL-22+ cells) phenotypes. Both treatments gave a significant clinical effect; however, bathing in geothermal seawater combined with NB-UVB therapy was more effective than NB-UVB therapy alone. This clinical improvement was reflected by a reduction in circulating CLA+ peripheral blood T cells and by a decreased Th1/Th17 and Tc1/Tc17 inflammatory response. These findings suggest that the inflammatory response in psoriasis is predominantly driven by both CD4+ and CD8+ skin-homing tissue retaining T cells of the Th17/Tc17 lineages.

Introduction

Bathing in geothermal seawater from the Blue Lagoon (BL) in Iceland has been reported to have a beneficial effect on psoriasis [1, 2]. Additional treatment with narrow-band ultraviolet (NB-UVB) phototherapy further increases the efficacy of the treatment [3–5]. The BL contains geothermal seawater originating from underground reservoirs filled with a mixture of fresh water and seawater. Sampling from the lagoon shows that no pathogenic bacteria thrive in this ecosystem [6]. The fluid in the lagoon has a high level of silica but is moderate in temperature (37 °C) and salinity (2.7%) [7]. Recent data indicate that both the silica mud and two microalgae species growing in the BL promote the integrity of the skin barrier and delay extrinsic skin ageing, thus indicating a biological activity in the lagoon [8].

Psoriasis is mediated by T cells that trigger keratinocytes to hyperproliferate and perpetuate the disease [9]. T helper (h)17 and Th1 cells and the cytokines produced by these cells are found in increased levels within psoriasis plaques [10] as well as in the circulation [11] and are thought to have an important role in psoriatic inflammation. The relationship between Th1 and Th17 cells is still unclear. The tissue-specific localization of T cells is thought to be guided by the skin-homing molecules such as cutaneous lymphocyte-associated antigen (CLA), various chemoattractants and their receptors, including chemokine receptors 4 (CCR4) and 10 (CCR10) [12]. In addition, adhesion molecules are thought to mediate T cell migration and retention in cutaneous tissue, such as the αE (CD103) β7 integrin that is overexpressed in psoriasis skin [13].

The main objective of this study was to evaluate the immunological therapeutic effect of two treatment protocols on psoriasis, focusing on the main inflammatory cytokines and effector T cell phenotypes known to be important for skin homing and tissue retention, thus potentially providing new insight into the immunopathogenesis of psoriasis. Our results confirm the role of Th1 and Th17 effector T cells in psoriasis. It also provides insight

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into the role of $CD8^+$ T cell secreting IFN-γ (Tc1) and IL-17 (Tc17) and $CLA^+/CD103^+$ effector T cells in its immunopathology.

Materials and methods

Patients and inclusion criteria. The Icelandic National Bioethics Committee (Nr. 08-010-S1) and the Icelandic Data Protection Authority approved the study. After providing informed consent, twelve patients with plaque psoriasis entered the study. They were assessed at baseline (W0), one (W1), three (W3) and eight (W8) weeks after starting treatment. Disease severity was assessed by the same physician (J.H.E.) at each time point with Psoriasis Area and Severity Index (PASI) [14] score and photographic documentation, and punch biopsies and blood samples were obtained.

Eligible patients were recruited to the study from January to May 2008. They were referred by dermatologists, and they were randomly assigned to two treatment groups. Patients were excluded if they had other forms of psoriasis, had other skin diseases or had received systemic psoriasis therapy, phototherapy or topical treatment within the previous 4 weeks. Of the 12 patients enrolled, six received inpatient treatment at the BL clinic for two weeks and 6 were treated with NB-UVB therapy three times weekly for 8 weeks. Psoriasis treatment at the BL clinic included bathing in geothermal seawater twice daily for at least 1 h combined with NB-UVB therapy 5 days per week for 2 weeks. After treatment at the BL clinic, patients used moisturizing creams for 6 weeks. The same type of Waldmann 7000 UVB cabins (Philips TL 100W/01, Philips, Villingen-Schwenningen, Germany) were used at the outpatient dermatology clinic at Landspitali University Hospital in Reykjavik and at the BL clinic. The same UVB treatment protocol was used for all patients based on skin type, with initial doses of 130–400 mJ/cm² with subsequent increases of 15–65 mJ/cm² after each treatment session [15]. Both groups were advised to use moisturizing creams daily. Patients who received combination treatment and NB-UVB therapy alone were comparable regarding age (mean: 36.7 years [range: 19–57] versus 33.7 years [range: 27–42]; $P = 0.41$), gender (five women/one man and five women/one man) and Psoriasis Area and Severity Index (PASI) [14] (18.2 [range: 7.8–32.2) versus 12.3 [range: 8.2–15.1]; $P = 0.19$). The only difference was that patients receiving combination treatment had a longer duration of the disease compared with patients receiving NB-UVB therapy (mean: 22.3 years [range: 6–36] versus 12.3 years [range: 5–23]; $P = 0.036$).

The control group consisted of 3 anonymous healthy blood donors from the Landspitali University Hospital (Reykjavik, Iceland) blood bank.

Cell preparation, stimulation and flow cytometry analysis. Heparinized peripheral venous blood was collected at each time point, and peripheral blood mononuclear cells (PBMC) were obtained by gradient centrifugation with Ficoll-Paque PLUS (Healthcare, Uppsala, Sweden), collected at the interface and washed with HBSS medium (Gibco, Carlsbad, CA, USA) prior to staining with such as anti-human CD3, CD4, CLA, CD103 (all from Biolegend, San Diego, CA, USA), CD8, CD45R0, CD54, CCR4 (all from BD Biosciences, San Jose, CA, USA), IL-23R and CCR10 (both from R&D Systems, Abingdon, UK) monoclonal antibodies (mAbs) for T cell analysis and CD14, CD11c, TLR2 (Biolegend) and TLR6 (HyCult Biotechnology, Uden, The Netherlands) mAbs for monocyte analysis.

The PBMC (1.0×10^6 cells/ml) were cultured for 16 h in RPMI 1640 medium with penicillin–streptomycin (100 IU/ml and 0.1 mg/ml) (Gibco), in the presence of anti-CD3 (5 μg/ml), anti-CD28 (5.0 μg/ml) mAbs (Biolegend) and brefeldin A (3.0 μg/ml) (eBioscience, San Diego, CA, USA) at 37 °C. The T cells were first stained for CD4 and CD8, then fixed and permeabilized and stained intracellularly with anti-human tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-17A (all from Biolegend) and IL-22 (R&D Systems) mAbs. The cells were washed with phosphate-buffered saline (PBS) prior to fluorescence-activated cell sorting (FACS) analysis.

Serum cytokine measurements. Serum samples were collected at each time point and frozen at −70 °C until used. At the end of the study period, the levels of IL-22, IL-17, IL-23, CCL20, IL-1β and TNF-α were determined by enzyme-linked immunosorbent assays (ELISAs), using commercially available kits (R&D Systems), according to the manufacturer's instructions.

Skin biopsies. A 3-mm punch biopsy was taken from the arm of each patient at every evaluation. The biopsy was taken from the edge of the thickest lesion on the forearm, then fixed in formaldehyde and stained using HE for histologic evaluation. Trozak's histological grading score [16] was used to evaluate the severity of the disease. The individual parameters were scored from 1 to 3, and a cumulative score between 0 and 19 was recorded for each biopsy. The observer was blinded (J.H.E).

Statistical analysis. Values are expressed as the mean ± 2 SD. To compare the treatment group with controls, we used the Mann–Whitney *U*-test. To evaluate the differences between before treatment, during and after treatment, the normality of each type of measurement was evaluated using a KS test based on the residuals from a simple linear model using patient and time as factors. In no case was normality close to being rejected ($P > 0.4$ in all cases). Hence, one-way repeated-measures ANOVA was used. However, to evaluate the differences between the two treatment groups, two-way repeated-measures ANOVA was used. Three patients who received combined treatment were not evaluated at week 8 because they had started another psoriasis treatment due to exacerbations: two of

those patients at week 4 (Fig. 1A; BL3 and BL6) and one patient at week 7 (Fig. 1A; BL1). For these patients, PASI evaluation was made at the time point their study participation was terminated, and they were not included in the analysis at week 8. All measurements were taken using SIGMASTAT 3.1 (Systat Software, San Jose, CA, USA). A P-value ≤ 0.05 was considered statistically significant.

Results

Clinical evaluation

In order to evaluate whether clinical improvement of psoriasis following bathing in geothermal seawater combined with NB-UVB and NB-UVB alone is preceded by changes in systemic inflammatory markers, the clinical efficacy of each treatment regimen was evaluated first. As shown in Fig. 1C, both treatment regimens demonstrated significant clinical improvements. Furthermore, the data suggested that patients receiving combined treatment demonstrated better clinical response, measured by the PASI score, than patients treated only with NB-UVB. This was seen both after one week (% improvement: combined treatment 37.3 ± 10.3 versus NB-UVB treatment 18.3 ± 8.9, $P < 0.05$) and after three weeks (67.3 ± 11.9 versus 22.0 ± 12.0, $P < 0.0001$). However, this was not the main aim of the study, and larger cohort and another control group would be needed to fully address this interesting observation.

Interestingly, bathing in the Blue Lagoon immediately following skin punch biopsy resulted in no infections and only minor skin irritation resolving in few days. In addition, the above clinical findings were confirmed by the histological Trozak's score where patients in both treatment groups showed a significant histological improvement at week 3 (Trozak's score: BL treatment = 10.3 ± 5.5 versus NB-UVB treatment = 8.0 ± 4.6; Fig. 2).

Immunological evaluation

Skin homing and positive correlation with PASI score

To understand the role of adhesion molecules, chemokines and their receptors in cutaneous lymphocyte homing in patients with psoriasis, we evaluated intercellular adhesion molecule 1 (ICAM-1), E-selectin (CD62E), CD11c, two chemokine receptors (CCR4 and CCR10) and $\alpha E\beta 7$ integrin (CD103) on peripheral blood mononuclear cells before, during and after each treatment regimen. In the active stage of the disease (W0) and compared with healthy control, patients with psoriasis had higher percentage of circulating CLA+ T cells expressing CD103 (median 5.7 versus 1.5%; $P < 0.05$), CCR10 (median 5, 1 versus 1.7%; $P < 0.05$) and co-expressing CD103/CCR4 (median 11.4 versus 0.8%; $P < 0.05$) and CCR4/CCR10 (median 3.7 versus 1.2%; $P < 0.05$) (Fig. 3A). In addition, a positive correlation between PASI and circulating CD103+ T cells ($r = 0.6036$; $P < 0.05$) and CLA+ T cells expressing

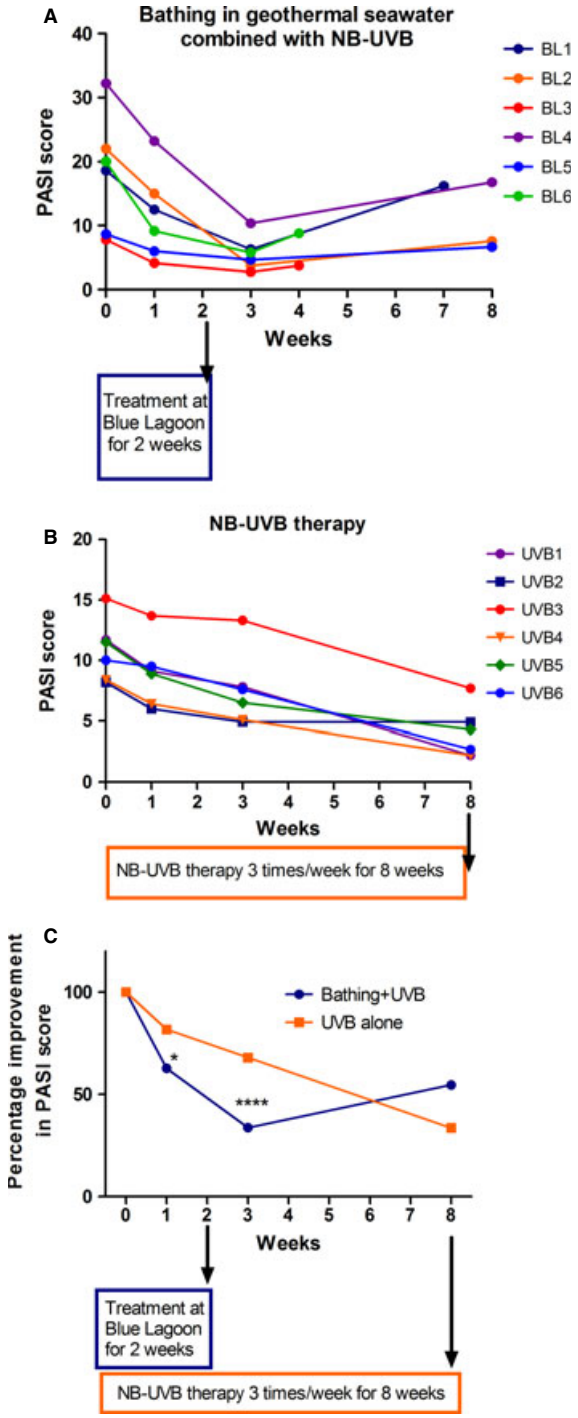

Figure 1 XY plots showing Psoriasis Area and Severity Index (PASI) score in psoriasis patients treated with bathing in geothermal seawater combined with NB-UVB therapy (A) and NB-UVB therapy alone (B), as well as the median percentage improvement in PASI score with each treatment (C). All patients were examined before treatment (0), and at 1 (W1), 3 (W3) and 8 (W8) weeks of treatment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

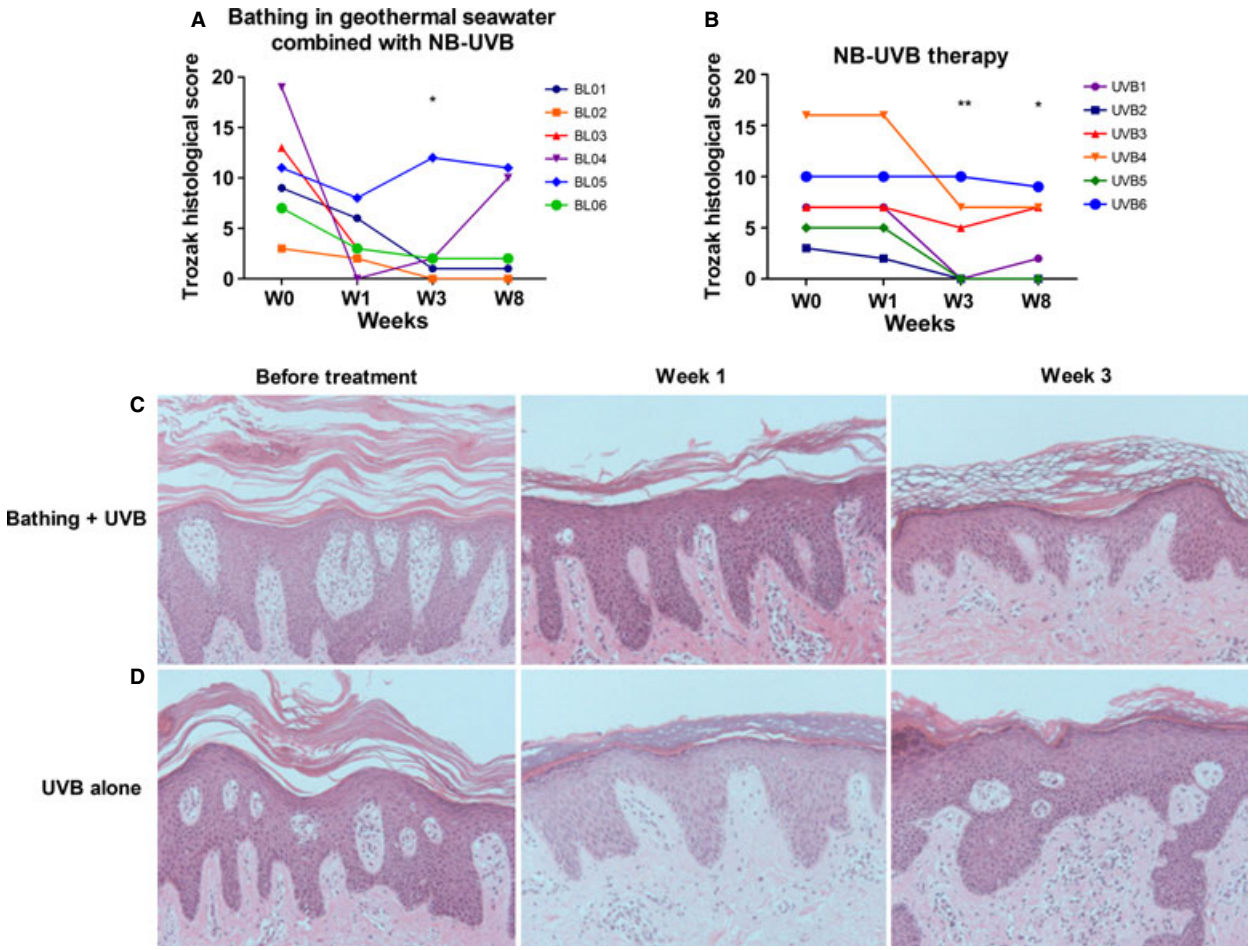

Figure 2 Histological assessment using Trozak´s grading system before, after 1, 3 and 8 weeks of treatment. Psoriasis patients who received bathing in geothermal water combined with UVB therapy (A) and NB-UVB therapy alone (B). Representative photographs from one patients who received bathing + UVB (C) and NB-UVB therapy alone (D). *$P < 0.05$, **$P < 0.01$.

CCR10 ($r = 0.7360$; $P < 0.01$) was similarly observed. No therapeutic changes were found regarding the expression of ICAM-1, CD62E, CD11c and other activation markers, such as CD25 and HLA-DR (data not shown).

In addition, patients receiving combined treatment had a significant reduction in CLA+ T cells expressing CCR4 or CD103 (68–74% reduction at W3, $P < 0.001$), while patients treated with NB-UVB alone did not (Fig. 3A). Furthermore, this reduction in CLA+CCR4+ T cells was predominantly confined to those who also expressed the CD103 integrin. Thus, no CLA+ T cells that co-expressed CD103 and CCR4 were detected in the circulation after 3 weeks (W3) in patients receiving combined treatment ($P < 0.05$; Fig. 3A). Both treatment groups achieved a significant reduction in CLA+ T cells that expressed CCR10 (71% reduction versus 44% reduction at W3; $P < 0.001$ versus $P < 0.05$; Fig. 3A). A marked reduction was also observed of circulating CLA+ T cells that co-expressed CCR4 and CCR10 in the combined treatment group (3.5% before treatment and 0.7% at W3; 80% reduction; $P < 0.01$; Fig. 3A).

Thus, the increased proportion of skin-homing T cells expressing CD103 and the chemokine receptors CCR4 and CCR10 was significantly reduced following clinical and histological improvements of psoriasis.

Effector T cell phenotype and its clinical correlation in psoriasis

To investigate the expression profile of circulating Th1/Tc1 and Th17/Tc17 cells in patients with psoriasis and its clinical correlation, their phenotypes were investigated amongst both CD4+/CD45RO+ and CD8+/CD45RO+ T cells. As expected in the active stage of the disease, patients with psoriasis had higher percentage of circulating CD4+ T cells expressing IFN-γ, TNF-α, IL-22 and IL-17 as compared with healthy controls (median 5.93 versus 2.06%, 9.08 versus 0.73%, 3.19 versus 0.33% and 4.78 versus 0.42%, respectively, $P < 0.05$ for all four subsets;

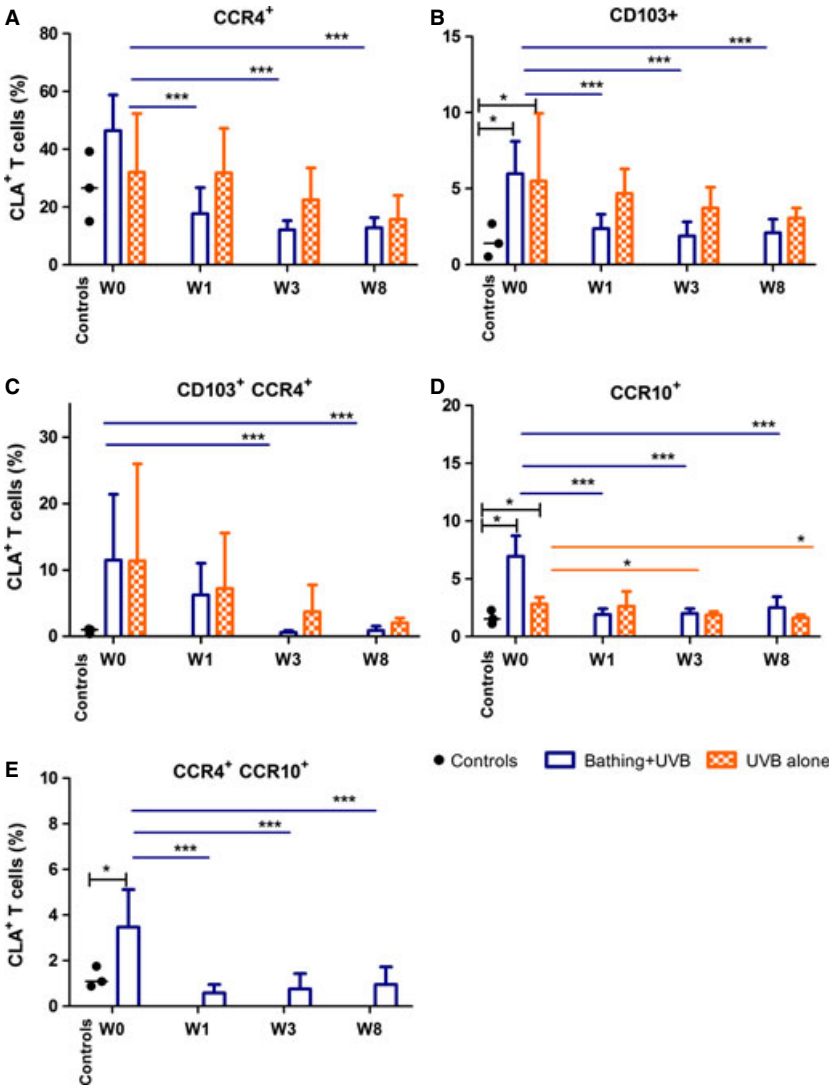

Figure 3 Circulating CLA+ T cells expressing CD103, CCR10 and CCR4/CCR10 are increased in psoriasis. The percentage of CLA+ T cells expressing CCR4 (A), CD103 (B), CCR10 (D) and co-expressing CCR4/CD103 (C) and CCR4/CCR10 among unstimulated cells from 3 healthy individuals (controls), 12 psoriasis patients; 6 treated with bating in geothermal seawater combined NB-UVB treatment (blue bars). All patients were studied before commencing treatment (W0), during treatment (W1), and at 3 (W3) and 8 (W8) weeks after treatment. Data expressed as mean ± SD, except controls expressed as scatter dot with median. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 4A). Furthermore, this was also observed for the CD8+ phenotype expressing IFN-γ, IL-22 and IL-17 (median 6.93 versus 2.37%, 2.39 versus 0.81% and 2.22 versus 0.89%, respectively, $P < 0.05$ for all three subsets; Fig. 5A).

When evaluating the clinical efficacy with its corresponding immunological profile, patients receiving combined treatment showed a marked reduction (81%) in circulating Th17 (IL-23R+CD4+ T cells) after only one week of treatment (Fig. 4A). This was also reflected by a 53% reduction in the amount of IL-23R expressed (MFI) by these cells ($P < 0.05$, data not shown) and their IL-17/IL-22 cytokine secretion. In contrast, such immunological Th17 inflammatory response improvement was only detected after 8 weeks of NB-UVB treatment (4a). Furthermore, both of the treatment protocols resulted in a significant reduction in Tc17 T cells (producing IL-17 and IL-22; Fig. 5A). Finally, a similar reduction was also noted for the Th1 and Tc1 phenotype (IFN-γ and TNF-α production, Figs. 4A and 5A, $P < 0.05$).

Discussion

The role of skin-homing, Th1 and Th17 immune response in the immunopathology of psoriasis is demonstrated in this study. In addition, the importance of Tc1 and Tc17 immune response is also suggested. Finally, NB-UVB therapy induced excellent clinical improvement preceded by a reduction in these above systemic inflammatory markers, strongly suggesting that immune modulation

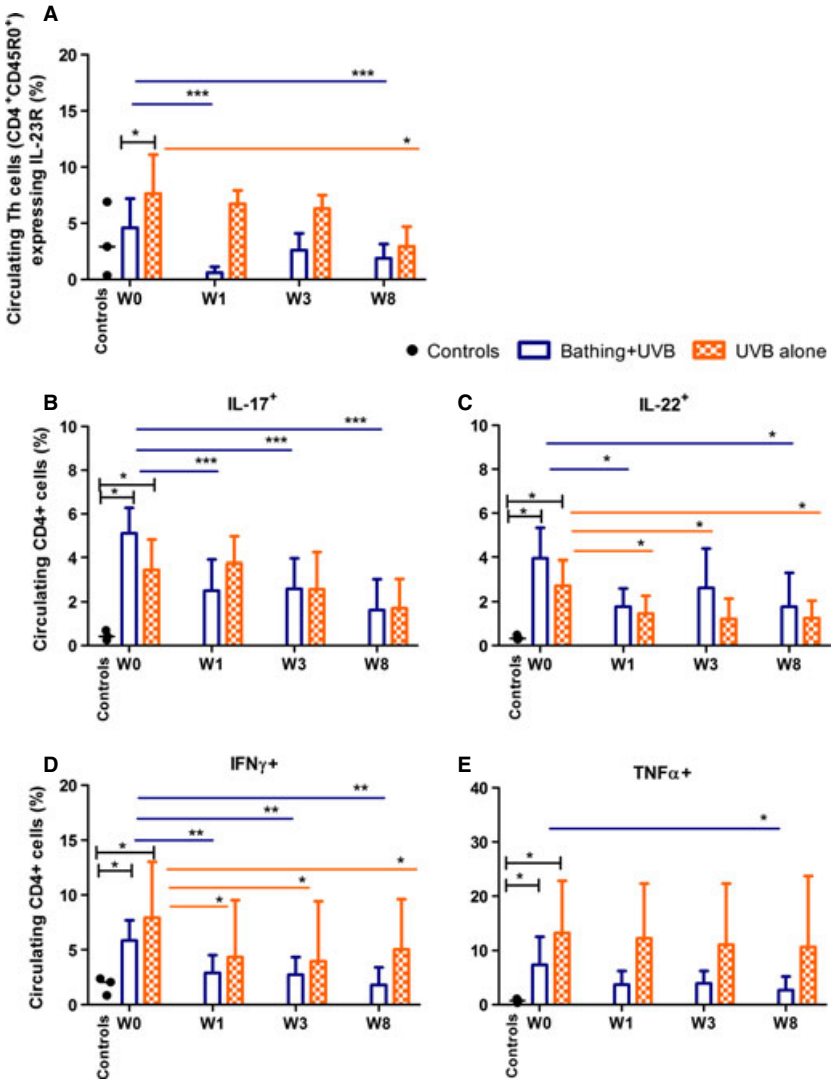

Figure 4 Decreases of the percentage of circulating Th17 and Th1 over time in 12 psoriasis patients, either receiving bathing in geothermal seawater combined with NB-UVB treatment or NB-UVB treatment alone. (A) The percentage of circulating Th17 cells (CD4+CD45R0+IL-23R$^+$), (B) Il-17, (C) Il-22, (D) IFNγ and (E) TNFα producing CD4+ T cells in 3 healthy individuals (controls), 12 psoriasis patients; 6 treated with bathing in geothermal seawater combined NB-UVB treatment (blue bars) and 6 receiving NB-UVB therapy alone (orange bars). All patients were studied before commencing treatment (W0), during treatment (W1), and at 3(W3) and 8 (W8) weeks after treatment. Data expressed as mean $\pm$ SD, expect controls expressed as scatter dot plot with median. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

mediated the observed clinical effect. Furthermore, an improvement by histological assessment is clearly demonstrated substantially validating the observed clinical improvements by using 'Trozak's score' as a measure of treatment efficacy.

There is evidence suggesting that bathing in the geothermal seawater without NB-UVB treatment has a beneficial clinical effect [1, 2]. It has also been noted that the scaling of psoriasis lesions disappears quickly, and the lesions get thinner with less erythema, indicating that bathing in this geothermal seawater has a direct anti-inflammatory effect on psoriatic lesions [2]. Another study demonstrated the beneficial effects of bathing in geothermal seawater where NB-UVB treatment after bathing gave an additional clinical effect compared with NB-UVB treatment alone [5], thus supporting our observation that bathing in the geothermal seawater might provide some additional clinical effect that was further reflected by the reduction in potential pathogenic T cells in the peripheral blood.

Psychological stress has been reported to influence psoriasis severity [17]. Inpatient treatment at the BL clinic in a relaxed environment might reduce stress and thereby indirectly improve the psoriasis lesions in addition to the UVB-induced effects. Immunological studies show that psychological stress increases the numbers of various immunological cells in the peripheral blood of patients with psoriasis, including HLA-DR$^+$ T cells, and decreases

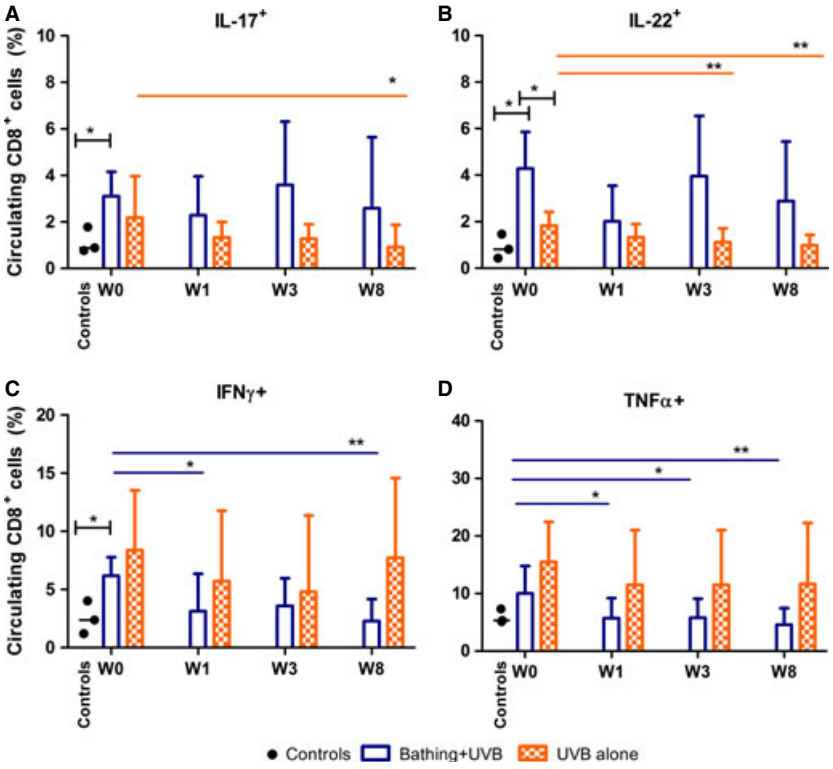

Figure 5 Decreases of the percentage of circulating Tc17 and Tc17 over time in 12 psoriasis patients, either receiving bathing in geothermal seawater combined with NB-UVB treatment or NB-UVB treatment alone. The percentage of circulating IL-17 (A), IL-22 (B), IFNγ (C) and TNFα (D) producing CD8+ T cells in 3 healthy individuals (controls), 12 psoriasis patients; 6 treated with bathing in geothermal seawater combined NBUVB treatment (blue bars) and 6 receiving NB-UVB therapy alone (orange bars). All patients were studied before commencing treatment (W0), during treatment (W1), and at 3 (W3) and 8 (W8) weeks after treatment. Data expressed as mean ± SD, except controls expressed as scatter dot plot with median. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the numbers of CD25$^+$ T cells [18]. However, in our study, the numbers of T cells expressing HLA-DR$^+$ and CD25$^+$ did not change significantly in the peripheral blood with both treatments, indicating that stress did not influence the outcome of our study.

The therapeutic properties of combined treatment with salt water baths and natural UV radiation (climatotherapy) and bathing in thermal water (spa therapy) have been known since ancient times [19, 20]. Today, it is being practised in many countries in the form of combination treatment of salt or thermal water baths and artificial UV radiation (balneotherapy) [21, 22]. Some studies indicate that the main therapeutic effect of climatotherapy at the Dead Sea can be attributed primarily to the sunshine and secondarily to the hypersaline seawater [21, 23]. Other studies show that balneotherapy with Dead Sea salt solution soaks in combination with NB-UVB therapy is superior to NB-UVB therapy alone [24, 25], which could be attributed to increased photosensitivity of the skin to UV radiation [26, 27]. We do not think that explains the results in our study for two reasons. As mentioned above, there are studies showing that bathing in the geothermal seawater without NB-UVB treatment has a beneficial clinical effect [1, 2]. In addition, the cumulative dose of NB-UVB therapy in this current study was only 10 treatment sessions for patients bathing in geothermal seawater combined with NB-UVB therapy compared with 24 sessions for patients treated with NB-UVB therapy alone.

However, the agents responsible for these beneficial effects of bathing in saline or thermal water have not been fully elucidated but most likely involve chemical [26, 28, 29], thermal [30], mechanical [2] and immunomodulatory effects [28, 31]. Furthermore, studies have shown that bathing in salt solutions has been associated with increased photosensitivity of the skin to UV radiation [26, 27]. Even though balneotherapy and spa therapy are widely used, the immune modulatory mechanisms are only partly understood. Few studies have shown immunomodulatory effects on epidermal Langerhans cells, inhibition of Th1 differentiation and cytokine production from keratinocytes [28, 31]. One recent study from Korea [32] showed that thermal spring water suppressed the expression of pro-inflammatory cytokines in human keratinocytes 'in vitro' as well as the differentiation of mouse CD4$^+$ T cells into Th1, Th2 and Th17 cells.

CCR4 has been found to be abundantly expressed on circulating T cells with a skin-homing CLA^+ phenotype [33] in normal subjects as well as in patients with psoriasis [34], which is consistent with our results. In contrast, CCR10 and CD103 are weakly expressed in the peripheral blood of normal subjects and nearly undetected in normal skin [35, 36]. In addition, CCR10 is expressed by a minority (approximately 30%) of circulating CLA^+ T cells [37]. However, both CCR10 and CD103 have been found in the inflamed psoriatic lesions [35, 36]. Their involvement in the immunopathogenesis of psoriasis is further suggested by our findings demonstrating the increased proportion of circulating skin-homing CLA^+ T cells co-expressing the tissue retention integrin CD103 and/or the chemokine receptors CCR4 and CCR10. More importantly, they had a positive correlation with the clinical improvements observed in the study, thus implicating the role of directing $CCR4^+/CCR10^+$ and $CD103^+$ subset of skin-homing T cells (CLA^+) into psoriasis plaques during the active stage of the disease. CLA+, CD103+ T cells, various adhesion molecules as well as activation markers did not change significantly during or after both treatment protocols. These findings demonstrate that the changes observed in our study are not only explained by the significant overall decrease in the inflammatory state of the patients following the treatment protocols. Today, it is known that CCR6 is a common chemokine receptor on Th17 T cells [38], but it is not included in our study. It is unfortunate, but at the time that our study was conducted, the role of CCR6 as a Th17 marker was being debated and unclear.

The immunopathogenesis of psoriasis has been connected to both Th1 and Th17 effector cells, and our observation that IL-17, IL-22 and IFNγ levels in the blood of patients with psoriasis returned to baseline with effective therapy supports this notion [9–11, 39]. Furthermore, the increased proportion of IL-17-/IL-22-producing $CD8^+$ T cells in the peripheral blood compared to healthy controls suggests their involvement in the immunopathogenesis of psoriasis, which has also been implicated by others [40]. In addition, the involvement of Tc17 cells in the immunopathogenesis was also evident by the positive correlation with individual clinical improvement measures. Similar to our findings, the therapeutic effectiveness of NB-UVB therapy has been associated with the corresponding Th1/Th17 pathway in psoriasis. In addition, in that study the role of innate immunity in psoriasis was suggested [41]. This has particularly been evaluated by the role of various Toll-like receptors in psoriasis. Thus, the expression of TLR2 has been found to be overexpressed in keratinocytes in psoriatic lesions [42], a finding also observed in our study with an increased expression of TLR2 on circulating monocytes ($CD14^+$) and dendritic cells ($CD11c^+$) in the peripheral blood of patients with psoriasis (data not shown). This study reflects the complexity behind the immunopathogenesis of psoriasis. It also reflects the following major confounding immunological elements. First, it confirms the importance of IFN-γ-, TNF-α-, IL-17- and IL-22-driven inflammatory response. Secondly, it suggests that these inflammatory cytokines are originating from both $CD4^+$ and $CD8^+$ T cells. Finally, this suggests that the inflammatory response is most likely predominantly driven by skin-homing tissue retaining T cells expressing the chemokine receptors CCR4 and CCR10.

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Funding

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Conflict of interest

This study was conducted in collaboration with Blue Lagoon Ltd. and Landspitali University Hospital of Iceland.

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Paper II

ORIGINAL ARTICLE

Psoriasis treatment: faster and long-standing results after bathing in geothermal seawater. A randomized trial of three UVB phototherapy regimens

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Key words:
balneotherapy; Blue Lagoon; geothermal seawater; histological score; psoriasis; quality of life

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This study was primarily sponsored by the Icelandic Technology Development Fund and the Landspitali University Hospital Research Fund. The Blue Lagoon Ltd. offered the treatment and the ensuing expenses free of charge.

SUMMARY

Background

The combination of seawater baths and narrowband ultraviolet B (NB-UVB) is a known treatment for psoriasis. This study evaluates two treatment regimens that combine bathing in geothermal seawater and NB-UVB therapy in comparison with NB-UVB monotherapy.

Methods

Sixty-eight psoriasis patients were randomly assigned to outpatient bathing in geothermal seawater combined with NB-UVB therapy three times a week, intensive daily treatment involving bathing in geothermal seawater combined with NB-UVB therapy, or NB-UVB therapy alone three times a week; treatment period was 6 weeks. Disease severity [Psoriasis Area Severity Index (PASI) and Lattice System Physician's Global Assessment scores], quality of life (Dermatology Life Quality Index) and histological changes were evaluated before, during and after treatment. The primary end point was the proportion of patients who achieved PASI 75 at 6 weeks.

Results

At 6 weeks, the percentage of patients who achieved PASI 75 and PASI 90 was significantly greater for both regimens, bathing in geothermal seawater three times a week (68.1% and 18.2%, respectively) and intensive treatment with geothermal seawater (73.1% and 42.3%, respectively) than for NB-UVB monotherapy (16.7% and 0%, respectively) ($P < 0.05$ in all comparisons). Clinical improvement was paralleled by improvement in quality of life and histological score and a reduction in NB-UVB doses.

Conclusion

Bathing in geothermal seawater combined with NB-UVB therapy in psoriasis induces faster clinical and histological improvement, produces longer remission time and permits lower NB-UVB doses than UVB therapy alone.

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Water-based therapy involving natural thermal springs, hot springs, mineral water, or seawater is currently used to treat psoriasis patients in treatment centres all over Europe (1). Examples of unique places for water-based therapy are the Dead Sea in Israel, the Kangal hot spring in Turkey and the Blue Lagoon in Iceland. Climatotherapy, such as that received in the Dead Sea area, refers to a combination of sun exposure and bathing in seawater where an important role is attributed to natural UV radiation (2, 3). To simulate climatotherapy as linked to special geographic settings, a combined treatment with seawater baths and artificial UV irradation (balneophototherapy) was established. Both open prospective studies (4–6) and randomized controlled trials involving a large number of psoriasis patients show the superiority of balneophototherapy over UVB monotherapy (7, 8).

The Blue Lagoon in Iceland is a geothermal lagoon containing a mixture of seawater and freshwater that formed when warm saline fluid was discharged onto a lava field after a geothermal power plant was built in the area in 1976 (9). Open prospective studies show that bathing in this geothermal seawater for 3–4 weeks has beneficial effects on psoriasis (10–13), and combination treatment with narrowband ultraviolet B (NB-UVB) therapy further increases the efficacy (14, 15). There are three notable differences in psoriasis treatment at the Blue Lagoon compared with the usual balneotherapy or spa therapy. The chemical composition of the Blue Lagoon is different compared with other spas, with an extremely high concentration of silica (135–140 mg/kg), moderate salinity (2.7%) and no H_2S content (Table 1) (9). To the best of our knowledge, the dominant micro-organisms in the water, *Silicibacter lacuscaerulensis* and cyanobacteria (16, 17), are not found under similar conditions anywhere else in the world. Due to the northerly latitude of Iceland, the natural sun is relatively weak (18). Interestingly, no human pathogenic bacteria or fungi have been shown to thrive in the lagoon (19).

Table 1. Characteristics of the water in the Blue Lagoon

pH	7.70
Temperature (°C)	24
Dissolved solids (mg/kg water)	
SiO_2	137
Na^+	9280
K^+	1560
Ca^{2+}	1450
Mg^{2+}	1.41
CO_2	16.5
SO_4^{2-}	38.6
H_2S	0.0
Cl^-	18 500
F^-	0.14
Total	31 900

No randomized controlled trials have been conducted to assess the efficacy of bathing in geothermal seawater combined with NB-UVB therapy compared with traditional NB-UVB therapy. In addition, the histopathological and psychosocial effects of geothermal seawater baths combined with NB-UVB therapy have never been evaluated before. Here we present the results of a randomized controlled study evaluating the clinical, histopathological and psychosocial efficacy of NB-UVB therapy alone and two different treatment regimens including geothermal seawater baths followed by NB-UVB therapy.

MATERIALS AND METHODS

Study design and patients

This study was a randomized open multi-arm parallel study to evaluate NB-UVB therapy and two treatment regimens including bathing in geothermal seawater combined with NB-UVB therapy in patients with chronic plaque psoriasis. The Icelandic National Bioethics Committee (08-097-S1) and the Icelandic Data Protection Authority approved the study protocol. Patients provided written consent to participate in the study. Eligible patients were recruited to the study from September 2009 to May 2010 and followed up for 2 years. One hundred and nineteen patients were screened. The majority of the patients (80%) were referred by a dermatologist, but some responded to an advertisement in a newspaper. The diagnosis of psoriasis had been confirmed by a dermatologist in all cases. Key inclusion criteria were the following: (a) diagnosis of chronic plaque psoriasis; (b) Psoriasis Area and Severity Index (PASI) score (20) of 7 or higher; and (c) being unresponsive to topical treatment and being a candidate for phototherapy or systemic treatment. Patients with other forms of psoriasis (e.g. guttate, pustular or erythrodermic) or skin diseases that could interfere with study evaluations were excluded. All ongoing psoriasis treatment was stopped at least 4 weeks prior to inclusion in the study.

Of the 119 patients screened, 68 patients fulfilled all criteria and were enrolled in the study. All patients provided informed consent before participating in the study. Of the 51 excluded patients, 27 had a PASI score lower than 7, and 6 had another psoriasis subtype. Ten patients were excluded because of ongoing active treatment for psoriasis or psoriatic arthritis. Data were collected at the Department of Dermatology at the Landspitali University

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Hospital in Reykjavik and the Blue Lagoon Clinic. Blood and histological samples were processed and analysed at the Departments of Immunology and Pathology at the Landspitali University Hospital.

Treatment regimens

For randomisation of the patients, a random number table was used. Patients were randomly assigned, in a 1:1:1 ratio, to three therapeutic arms:

1 *Outpatient bathing in geothermal seawater and NB-UVB treatment (GSW).* The treatment included bathing in geothermal seawater for 1 h and NB-UVB therapy immediately afterwards three times a week for 6 weeks. Patients were advised to rub the silica mud from the lagoon on the skin while bathing and to use moisturizing cream (Blue Lagoon Mineral Intensive Cream), which contains mineral salts from the lagoon and no active ingredients, twice daily.

2 *Intensive treatment in geothermal seawater and NB-UVB therapy (IT-GSW).* This treatment protocol consisted of bathing in geothermal seawater for 1 h two times a day and NB-UVB therapy once daily immediately after the first bath six times/week for 2 weeks. Patients were advised to rub the silica mud from the lagoon on the skin while bathing and to use moisturizing creams twice daily (Blue Lagoon Mineral Intensive Cream). After 2 weeks, patients were treated with a conventional outpatient NB-UVB therapy three times a week for 4 weeks.

3 *Conventional narrowband UVB therapy (NB-UVB).* This group received a regular, monitored NB-UVB radiation therapy three times weekly for 6 weeks. Patients took a shower immediately before the UVB treatment was given and used moisturizing creams containing no active ingredients twice daily (Eucerin Original Healing lotion, Beiersdorf, Hamburg, Germany).

The same NB-UVB treatment protocol was used for all patients based on skin type, with initial doses of 130–400 mJ/cm^2 with subsequent increases of 15–65 mJ/cm^2 after each treatment session. The UVB source was a Waldmann 7001 cabin (Waldmann, Villingen-Schwenningen, Germany) with an NB-UVB lamp (TL01, Philips, Eindhoven, the Netherlands) with peak emission at 311 nm.

Efficacy end points

The primary objective of the study was to assess the efficacy of three different psoriasis regimens: conventional NB-UVB therapy and two different combination treatments involving bathing in geothermal seawater. The primary efficacy variable was the proportion of patients who achieved a reduction in PASI score (20) of at least 75% (PASI 75) after 6 weeks of treatment. Key secondary efficacy end points included (a) the proportion of patients with a reduction in PASI score of at least 90% (PASI 90) at week 6; (b) the proportion of patients with a Lattice System Physician's Global Assessment score (Lattice score) (21) of 'clear of disease' or 'almost clear' at week 6; (c) the change from baseline in Dermatology Life Quality Index (DLQI) (22) at week 10; (d) histological changes from baseline in skin biopsies at week 6; and e) the number of days with clearance or marked improvement of the disease (time to relapse).

The DLQI is a 10-item questionnaire that determines whether psoriasis affects patient-reported quality of life, with overall scores ranging from 0 (not at all) to 30 (very much) (22), and it was assessed at baseline and at 10 weeks. Trozak's histologic grading system for psoriasis (23) was used for histological blinded assessment of the skin biopsies, as it is a more observer-independent assessment tool. The presence of predetermined histopathological characteristics of psoriasis (regular elongation of rete ridges, club-shaped rete ridges, oedema and elongation of dermal papillae, perivascular infiltrate in the upper dermis, absent granular layer, parakeratosis, suprabasal mitosis, Munro's microabscesses and spongiform pustules) was scored in routine sections of biopsies. The individual parameters were scored from 1 to 3, and a cumulative score (0–19) was recorded for each biopsy specimen.

Disease activity was assessed at baseline and 1, 2, 4, 6 and 10 weeks after beginning the treatment. All patients were examined in the following order by the same physician (JHE): clinical examination, photographic documentation, PASI score determination, Lattice score determination and quality-of-life assessment with the DLQI. In addition, after these assessments at baseline, week 2 and week 6, a 4-mm punch biopsy from a target lesion was obtained from seven patients in each treatment group. The thickest lesion on the extremities was selected as the target lesion.

Patients who did not achieve at least a 50% reduction from baseline in PASI score at week 6 were either withdrawn from the study (non-responders) or invited to cross over to receive intensive combination treatment. Patients in all study groups who achieved a 50–75% reduction in PASI score continued NB-UVB therapy three times a week for 4 weeks or until attainment of PASI 75/PASI 90, and patients who achieved PASI 75 were invited to continue NB-UVB therapy until attainment of PASI 90 (maximum 10 weeks/patient).

Finally, to evaluate how long the effect of each treatment lasted, the number of days until relapse was calculated.

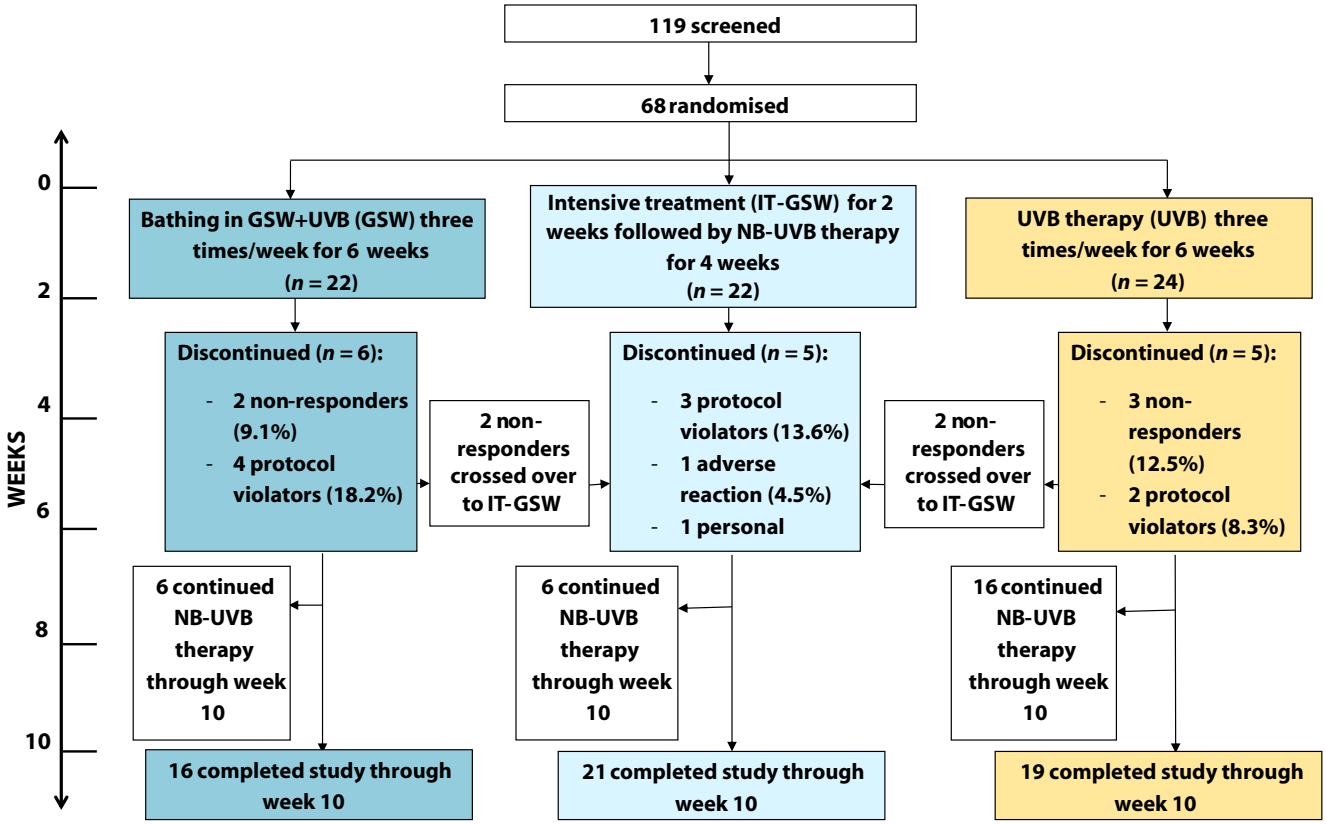

Fig. 1. Disposition of patients and reasons for discontinuation. Sixty-eight psoriasis patients enrolled in the study, but 16 patients discontinued: 5 because of lack of efficacy (Psoriasis Area Severity Index score still below 50 after 6 weeks of treatment), 1 because of an adverse event, 9 because of protocol violation (missing treatment) and 1 because of personal reasons. Two non-responders in the UVB group and 2 non-responders in the GSW group crossed over to the IT-GSW group. GSW, geothermal seawater.

Patients were followed up for 1 year with telephone interviews where they were asked if they had received retreatment with other antipsoriatic therapies (topical treatment, phototherapy or systemic therapy). We defined the number of days to relapse as the number of days from the study treatment until retreatment.

Statistical analysis

The sample size calculation was based on the primary end point of PASI 75 after 6 weeks of treatment. The study was sufficiently powered to detect a difference of 20% between the combination treatment groups and the UVB group. Given these assumptions and taking into account the results of prior studies (10–15), a sample size of 15 patients per treatment group provided more than 99% power to detect at least one pairwise treatment effect in the primary end point at an overall 5% level of significance.

Efficacy data from all randomised patients were analysed on an intention-to-treat basis. Patients who discontinued study treatment due to unsatisfactory therapeutic effect or who did not follow the study treatment protocol were regarded as treatment failures. For analysis in such cases, missing values were replaced with the most recently available values for all efficacy variables (last observation carried forward) (24). Patients who did not achieve more than a 50% reduction in PASI score and crossed over to receive IT-GSW were included in efficacy summaries for IT-GSW (Fig. 1). The proportions of patients responding to treatment were compared using the two-sided Fisher's exact test. Continuous response variables were compared with the use of analysis of variance (ANOVA). Also, we used Spearman's correlation coefficient to show the correlation between different parameters, including all visits. All statistical tests were two-sided and performed at an alpha level of 0.05.

Table 2. Baseline patient characteristics

Characteristics	GSW ($n = 22$)	IT-GSW ($n = 24$)	UVB ($n = 24$)	P value†
Age (years), mean ± SD	41 ± 10.8	42.2 ± 16	37.9 ± 14.4	0.37
Male, *n* (%)	12 (55)	12 (50)	15 (63)	0.82
Body mass index (kg/m^2), mean ± SD	28 ± 5	28.6 ± 5.4)	28.8 ± 7.1	0.96
Duration of psoriasis (years), mean ± SD	20 ± 14	16.4 ± 11	12.3 ± 8.1	0.09
Psoriatic arthritis, *n* (%)	4 (19)	3 (13)	5 (20)	0.71
Nail psoriasis, *n* (%)	10 (43)	12 (50)	9 (38)	0.61
PASI score, mean ± SD‡	12.3 ± 5.2	11.6 ± 6.2	11.1 ± 4.9	0.22
Lattice score§	Moderate to severe	Moderate to severe	Moderate to severe	1.00
DLQI score, mean ± SD¶	7 ± 4.2	11.6 ± 6.2	7.3 ± 5.1	0.017*
Treated previously, *n* (%)				
Blue Lagoon	6 (27)	10 (42)	7 (29)	0.57
Topical agent	21 (95)	23 (100)	21 (88)	1.00
Phototherapy	21 (95)	19 (79)	16 (67)	0.51
Systemic therapy	2 (1)	2 (8)	1 (4)	0.61
Smoking, *n* (%)	6 (27)	8 (33)	4 (17)	0.77
Family history, *n* (%)	18 (82)	12 (50)	14 (58)	0.35

*$P < 0.05$.
†For comparisons across all treatment groups; calculated by means of ANOVA for continuous variables and χ^2-test for categorical variables. ‡Possible scores range from 0 to 72, with higher scores indicating more severe disease.
§Possible scores range from 'clear of disease' to 'severe disease'.
¶Possible scores range from 0 to 30, with higher scores indicating worse health-related quality of life.
GSW, bathing in geothermal seawater combined with UVB treatment; IT-GSW, intensive treatment in geothermal seawater combined with UVB treatment; UVB, UVB treatment alone; PASI, Psoriasis Area Severity Index; Lattice, Lattice System Physician's Global Assessment; DLQI, Dermatology Life Quality Index.

RESULTS

Patients

All treatment groups were well balanced with respect to demographic characteristics (Table 2). The mean baseline PASI score was 12.3 ± 5.2 in the GSW group, 11.6 ± 6.2 in the IT-GSW group and 11.1 ± 4.9 in the UVB therapy group ($P = 0.22$). Of 68 patients, 56 completed the study (82.4%; Fig 1). Five patients were withdrawn as they did not achieve a 50% reduction in PASI score after 6 weeks of treatment (non-responders). Four of them were assigned to the IT-GSW group. One patient entered the cross-over IT-GSW group a few days after withdrawal, two patients 2 weeks after withdrawal and one patient more than 4 weeks after withdrawal. Other reasons for early termination included adverse events (1/68; 1.5%), protocol violations (9/68; 13.2%) and personal reasons (1/68; 1.5%).

Clinical efficacy

After 6 weeks of treatment, 20/26 patients (77.0%) of the patients in the IT-GSW group and 15/22 (68.2%) in the GSW group met the primary end point of a 75% reduction in PASI score, compared with only 4/24 (16.7%) patients treated with NB-UVB therapy alone ($P < 0.001$ for GSW and IT-GSW vs. NB-UVB) (Table 3, Fig. 2a and Fig. 2b). In addition, 11 out of 26 patients (42%) in the IT-GSW group and 4 out of 22 patients (18%) in the GSW group showed a 90% reduction in PASI score compared with no patients in the UVB group (0%; $P < 0.05$ for both comparisons; Table 3 and Fig. 2d).

According to the protocol, all the patients who achieved PASI 90 at week 6 discontinued active treatment, and patients who achieved PASI 75 were invited to continue NB-UVB therapy until attainment of PASI 90 (maximum 10 weeks/patient). Respectively, 6/26 (30.8%) of the patients in the IT-GSW group, 6/22 (31.8%) in the GSW group and 16/24 (66.8%) in the UVB group continued NB-UVB therapy three times a week until week 10 or until attainment of PASI 75/PASI 90 (Fig. 1). The time required for attaining a 75% reduction in PASI score was significantly shorter for both the IT-GSW group (29.1 ± 25.2 days) and the GSW group (35.5 ± 10.4 days) compared with the UVB group (62.3 ± 14.0 days) ($P < 0.001$ for both comparisons; Fig. 2b and Fig. 2c). No statistical difference was found between the combination treatment groups ($P > 0.05$). The number of NB-UVB sessions required for the patient to attain PASI 75 was also significantly less

Table 3. Response to treatment

	GSW ($n = 22$)	IT-GSW ($n = 26$)	UVB ($n = 24$)
PASI 75, *n* (%)			
6 weeks	15 (68.1)***	20 (77.0)***	4 (17.0)
10 weeks	13 (59.0)	17 (65.4)	13 (54.2)
PASI 90, *n* (%)			
6 weeks	4 (18.2)*	11 (42)***	0 (0)
10 weeks	4 (18.2)	6 (23.1)	2 (8.3)
Lattice score of 'clear'/'almost clear', *n* (%)			
6 weeks	14 (63.6)**	17 (65)***	4 (17)
10 weeks	12 (55)*	15 (58)*	4 (17)
DLQI score 0 or 1 at 10 weeks, *n* (%)	9 (40.9)*	12 (46.2)*	3 (12.5)
Treatment intensity needed to attain PASI 75, mean ± SD			
UVB treatment sessions (*n*)	14.7 ± 4.2***	17.9 ± 10.0**	25.0 ± 6.6
Total UVB dose (J/cm^2)	5.8 ± 2.6***	8.3 ± 5.9***	18.6 ± 8.3
Length of treatment (days)	35.5 ± 10.4***	29.1 ± 25.2***	62.3 ± 14.0

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.
GSW, bathing in geothermal seawater combined with UVB treatment; IT-GSW, intensive treatment in geothermal seawater combined with UVB treatment; UVB, UVB treatment alone; PASI, Psoriasis Area Severity Index; Lattice, Lattice System Physician's Global Assessment; DLQI, Dermatology Life Quality Index.

(GSW 14.7 ± 4.2, IT-GSW 17.2 ± 10.0, UVB 25.0 ± 6.6; $P = 0.001$ for GSW and IT-GSW vs. UVB; Table 3). Furthermore, the mean NB-UVB dose for achieving PASI 75 was 5.8 ± 2.6 J/cm^2 for the GSW group and 8.3 ± 5.9 J/cm^2 for the IT-GSW group compared with 18.58 ± 8.25 J/cm^2 for the UVB group ($P < 0.001$ for GSW and IT-GSW vs. UVB; Table 3).

When the Lattice score was examined, higher percentages of patients who received combination treatment (GSW and IT-GSW) had a Lattice score of 'clear of disease' or 'almost clear' compared with patients treated with NB-UVB therapy alone ($P < 0.01$ for GSW and $P < 0.001$ for IT-GSW vs. UVB) (Table 3; Fig 2c). No statistical difference was found between GSW and IT-GSW ($P > 0.05$) except in the percentages of patients with a 90% improvement in the PASI score at week 6 and in the PASI score results ($P < 0.05$). These clinical findings were also reflected by a significant correlation between the PASI score and Lattice score (Spearman's $r = 0.7318$, $P < 0.0001$).

Patients who received combined treatment showed better response in areas poorly exposed to NB-UVB radiation compared with patients treated with NB-UVB therapy alone. This was observed in 10 patients in the GSW group, 13 patients in the IT-GSW group and 12 patients in the UVB group, and these areas were noted to have cleared in 7 patients in the GSW group compared with only 2 patients treated with UVB alone.

Despite the large difference in number of days until relapse of psoriasis between patients in the GSW group and those in the UVB group (246.1 ± 161.0 vs. 140.9 ± 165.3; $P = 0.0796$), the difference did not reach statistical significance. However, the difference in number of days was statistically significant (283.9 ± 137.0 vs. 140.9 ± 165.3; $P < 0.0083$) in favour of the IT-GSW treatment group when this treatment was compared with NB-UVB therapy alone. Nine out of 19 patients (47.4%) who received NB-UVB therapy alone had started another treatment only 1 month after the last treatment session, compared with only one (1/21; 4.8%) of the patients in the IT-GSW group ($P = 0.0028$) and 3 out of 16 patients (18.8%) in the GSW group ($P = 0.15$).

Quality of life

Twelve patients out of 26 (46%) in the IT-GSW group and 9 patients of 22 (40%) in the GSW group achieved a DLQI score of 0 or 1 by week 10 compared with 3 patients out of 24 (12%) who were treated with NB-UVB therapy alone ($P < 0.05$ for both comparisons; Table 3).

Histological response to treatment

At baseline, typical histopathological characteristics of psoriasis were seen in all patients (hyperkeratosis, elongated rete ridges, perivascular mononuclear cell infiltrate

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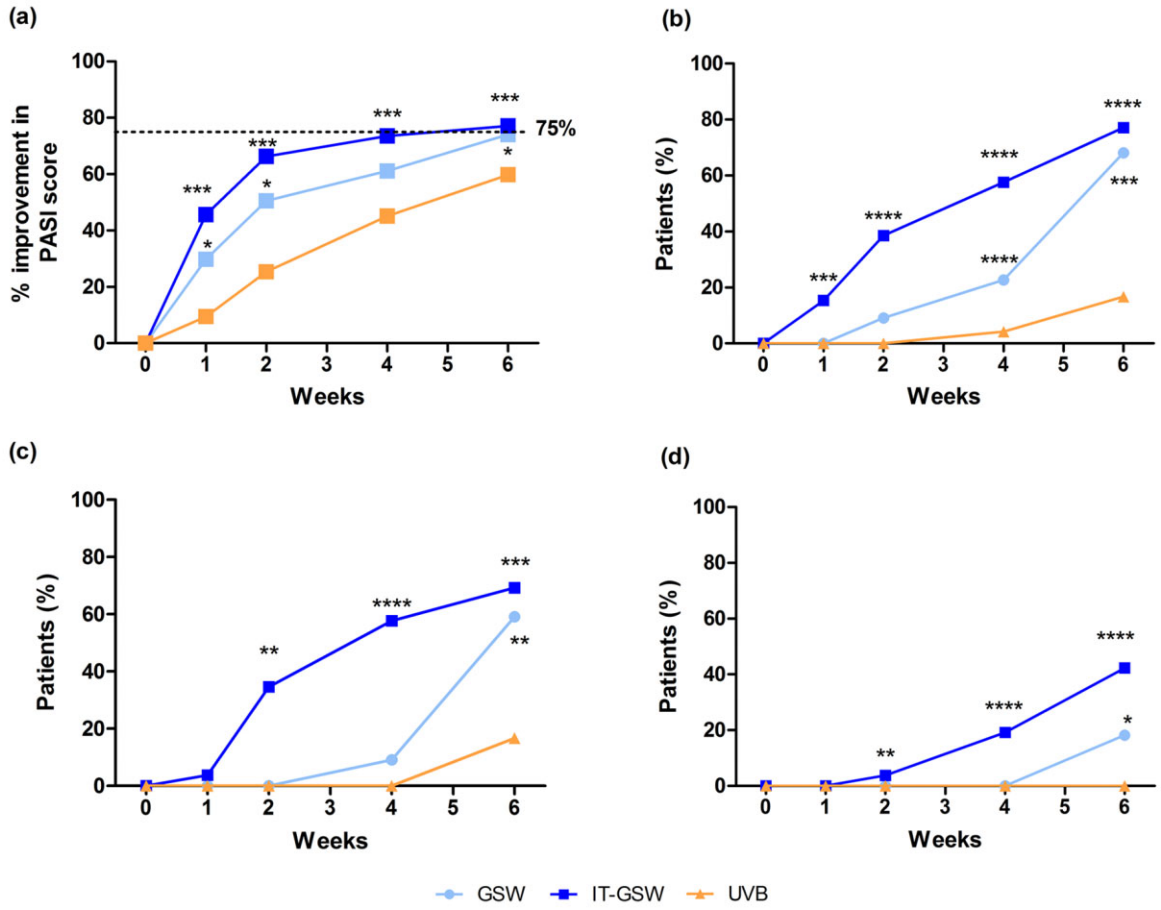

Fig. 2. Intention-to-treat analysis: proportion of patients achieving clinical responses from baseline through week 6 using intention-to treat population. (a) Median percentage reduction of baseline Psoriasis Area Severity Index (PASI) score through week 6. (b) Percentage of patients attaining 75% reduction from baseline PASI score. (c) Percentage of patients attaining Lattice System Physician's Global Assessment score of 'clear' or 'almost clear' (0 or 1). (d) Percentage of patients attaining 90% reduction from baseline PASI score. Last observation carried forward to week 6 for dropouts. GSW, outpatient bathing in geothermal seawater combined with UVB treatment; IT-GSW, intensive bathing in geothermal seawater combined with UVB treatment; UVB, UVB monotherapy. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

and Munro abscesses; see Fig 3). Patients in both combination treatment groups showed a significant decrease in psoriatic histological symptoms as measured by the Trozak score after only 2 weeks of treatment: from 10.5 ± 4.7 to 3.29 ± 3.7 ($P < 0.05$) for the GSW group and from 8.1 ± 2.4 to 3.0 ± 2.4 ($P < 0.05$) for the IT-GSW group (Fig 3). The histological symptoms were further reduced in biopsies from patients in the IT-GSW group after 6 weeks of treatment, to 0.5 ± 1.0 ($P < 0.01$, Fig 3). There was no significant difference in Trozak score in the GSW group between 2 and 6 weeks (Fig 3). No significant decrease was observed for patients treated with UVB therapy alone (10.0 ± 2.6 before treatment, 7.7 ± 1.6 after 2 weeks and 4.0 ± 3.7 after 6 weeks of treatment). In addition, no significant difference was observed when all the treatment groups were compared with each other. Trozak histological score was significantly correlated with both PASI score (Spearman's $r = 0.42$, $P < 0.001$) and Lattice score (Spearman's $r = 0.55$, $P < 0.0001$).

Safety

One treatment-related adverse event was reported, where one patient in the IT-GSW group developed an itchy papular eruption confined to the forearm, which was diagnosed as polymorphous light eruption. The patient was treated with topical steroids and excluded from the study. Two patients reported upper respiratory tract infections, one in the GSW group and one in the IT-GSW group. The most commonly reported adverse event was erythema at the biopsy site, which occurred in 4 (18%) patients in the GSW group, 5 (21%) in the IT-GSW group and 4 (17%) in

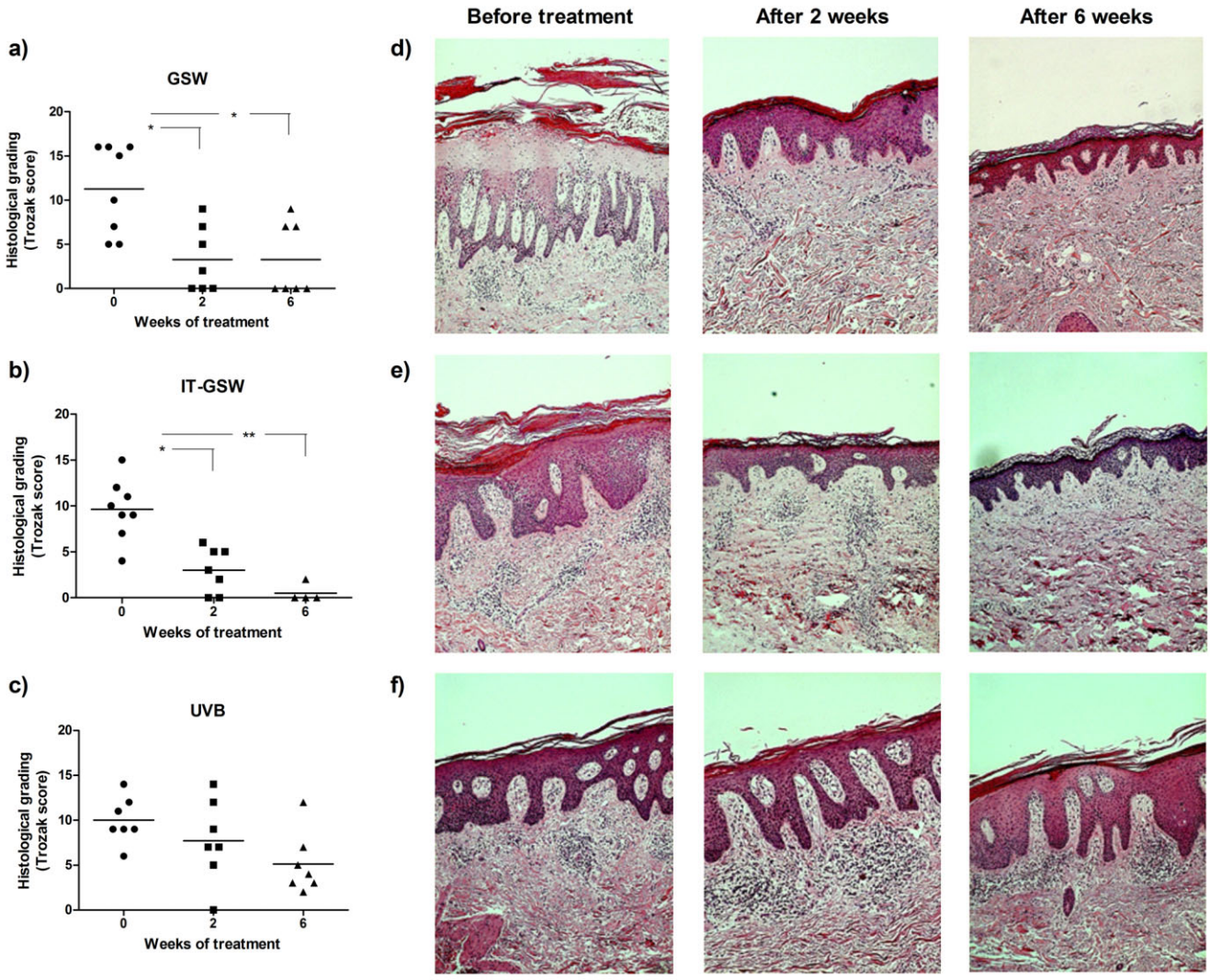

Fig. 3. Histological assessment using Trozak's grading system before and after 2 and 6 weeks of treatment. (a) Patients who underwent outpatient bathing in geothermal water combined with UVB therapy (GSW). (b) Patients who underwent inpatient bathing in geothermal water combined with UVB therapy (IT-GSW). (c) Patients who underwent UVB therapy alone (UVB). (d–f) Representative photographs from one patient each in the GSW group (d), IT-GSW group (e) and UVB group (f). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

the UVB therapy group. No serious adverse events were reported during the study period.

DISCUSSION

This study confirms previous reports (10–15) that the addition of UVB therapy to bathing in geothermal seawater is an effective treatment for psoriasis. NB-UVB therapy combined with bathing in geothermal seawater, compared with NB-UVB therapy alone, was associated with faster reduction in PASI score, Lattice score and Trozak histological score, as well as quality-of-life (DLQI) score. In addition, the combination treatments resulted in reduced total NB-UVB dose and a longer remission.

The combination of bathing in geothermal seawater and NB-UVB therapy had a rapid onset of action, as evidenced by significant reductions in PASI score occurring as early as week 1 and by the significantly higher percentage of patients achieving PASI 75 or Lattice score of 'clear' or 'almost clear' as early as week 1. Furthermore, 42% of patients who received intensive combination treatment achieved PASI 90 after only 6 weeks of treatment.

After 6 weeks, only 17% of the NB-UVB-treated patients had reached PASI 75. Therefore, most of these patients continued NB-UVB therapy until week 10. However, most of the patients in the combination treatment groups had reached PASI 75 or PASI 90 after 6 weeks and discontinued treatment. The mean total cumulative dose of NB-UVB,

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the number of exposures and the time required to achieve at least PASI 75 were significantly lower in both combination treatment groups compared with the UVB therapy group ($P < 0.001$).

The clinical improvement was paralleled by improvement in quality of life (DLQI score) and Trozak histological score. The blinded histopathological evaluation shows that combination treatment with bathing in geothermal seawater and NB-UVB therapy almost completely eliminated the characteristics of psoriasis as measured by Trozak score after only 2 weeks of treatment. The fact that the PASI evaluation was not blinded is a limitation of the study. However, the histological assessment was blinded, and it demonstrated a significant correlation with the clinical findings. The DLQI assessment was carried out at baseline and at week 10. The 10th-week evaluation point might not be optimal, but it was considered that too-frequent evaluation could lead to bias in the DLQI score.

After 6 months, only 30% of patients in either combination treatment group had relapsed (started another therapy), compared with 56% of patients treated with UVB monotherapy. The scalp and intertriginous areas are poorly exposed during NB-UVB therapy, and this could possibly explain why efficacy was lower in the UVB monotherapy group. It has been shown that the silica mud and the micro-organisms growing in the geothermal seawater are bioactive and can improve the skin barrier of normal skin and prevent premature skin aging (25). It is therefore possible that 'active ingredients' in the geothermal seawater have a healing effect on psoriasis.

Bathing in tap water or hot baths may have a beneficial effect on psoriasis, but to our knowledge, no clinical studies have shown this. One randomized controlled trial (8) demonstrates that bathing in tap water before UVB exposure is slightly better than UVB monotherapy. In the present study, the patients who were treated with UVB therapy alone took a shower immediately before the UVB treatment was given to make the treatment groups as comparable as possible. In addition, it is well documented that hot seawater baths alone have a minor therapeutic effect, usually not exceeding a 30% improvement in PASI score (5, 26).

We do not yet fully understand the biological basis for the efficacy of the geothermal seawater psoriasis treatment. At present, there are several ongoing studies trying to identify and isolate the agents responsible for the beneficial effects of bathing in geothermal seawater.

Treatment options for psoriasis have expanded considerably in recent years. Many new therapeutic agents, such as the biologic drugs, are expensive and can cause serious side effects. Despite the absence of long-term major therapeutic efficacy, UV radiation therapy combined with balneotherapy remains an inexpensive and clinically beneficial therapeutic option for psoriasis patients.

In conclusion, patients bathing in geothermal seawater before NB-UVB treatment need fewer sessions and lower cumulative doses compared with patients treated with NB-UVB monotherapy. In addition, combination treatment induces faster improvement in clinical and histological scores and a longer remission time after treatment as compared with NB-UVB therapy alone.

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Authors' contributions: The first author collected the data, which were maintained in a database at the Landspitali University Hospital of Iceland, and wrote the draft manuscript. BS and JHO participated in the design of the study and critically revised the manuscript. BAA supervised the histological examination and revised the manuscript. BRL revised the manuscript and participated in the design of the study. All authors had full access to the data and final responsibility for the decision to submit for publication.

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Paper III

Title page

Title

Effective treatment with balneophototherapy and narrowband UVB monotherapy reduces skin homing Th17/Tc17 and Th22/Tc22 effector cells in peripheral blood in patients with psoriasis

Running title

Narrowband UVB phototherapy reduces circulating T17/T22 cells in psoriasis patients

5-10 key words

Psoriasis, resolution of inflammation, T-cells, IL-17, IL-22, IFNγ

Abbreviations: PBMC, peripheral blood mononuclear cells; Th17, T-helper 17 cells; Tc17 cells, T cytotoxic 17 cells; PASI, psoriasis area and severity index.

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This study was conducted in collaboration with the Blue Lagoon Ltd and Landspítali - The National University Hospital of Iceland

Abstract

Introduction: Limited data exists regarding the molecular mechanisms underlying the efficacy of balneophototherapy (bathing in geothermal seawater combined with narrow-band (NB)-UVB phototherapy) and NB-UVB phototherapy in psoriasis. Here, we evaluate the impact of these treatments on the inflammatory pathways underlying psoriasis in both peripheral blood and lesional skin.

Materials and methods: A total of 68 patients with psoriasis were randomly assigned to two different balneophototherapy groups and one NB-UVB monotherapy group for six weeks. Disease severity (Psoriasis Area and Severity Index; PASI) was recorded, blood and skin samples obtained before, during and after treatment from 7 patients in each group. Histological score was used and T-cells were evaluated for relevant surface receptors and inflammatory cytokines in the blood and skin.

Results: Circulating Th17 ($CD4^+CD45RO^+IL\text{-}23R^+$ T cells) and Tc17 ($CD8^+CD45RO^+IL\text{-}23R^+$ T cells) reduced by more than 60% after only two weeks of treatment (Th17 from 12.25±7.44% to 3.64±5.51%, $p<0.001$, and Tc17 from 15.37±6.37% to 5.89±4.61%, $p<0.001$) in correlation with both PASI and histological score ($p<0.01$). Furthermore, circulating skin homing peripheral blood T17 (CLA^+ $CD4^+/CD8^+$ T cells producing IL-17) and T22 (CLA^+ $CD4^+/CD8^+$ T cells producing IL-22) reduced significantly with treatment ($p<0.05$), as well as $CD4^+/CD8^+$ skin resident T cells ($p<0.05$). Immunohistochemical analysis showed significant resolution of $CD3^+$, $CD4^+$ and $CD8^+$ in the skin ($p<0.01$) in correlation with histological score ($p<0.05$). Furthermore, the reduction of the skin homing chemokine CCL17 in serum and dermal immunohistochemistry analyses of IL-17 had a positive correlation with PASI score ($p<0.05$). Finally, the above treatment protocols did not have any effect upon peripheral blood effector Th1/Tc1 or Th2/Tc2 T-cells. When the treatment groups were compared with each other, there was no difference after 6 weeks of treatment.

Conclusion: Clinical improvement of psoriasis by balneophototherapy and NB-UVB phototherapy is linked to suppression of T17 and T22, but not Th1/Tc1, signaling pathways and the reduction of skin homing markers in the peripheral blood. Our results highlight the critical role of T17 effector cells in the pathogenesis of psoriasis and its associated skin homing T22 effector cells.

Introduction

Psoriasis is a chronic autoimmune inflammatory disease in the skin where the precise causal agents have not been identified. Genetic, environmental factors and the immune system have a major role in the development of psoriasis and it is thought that self-antigens or microbial antigens initiate a vicious circle of chronic autoimmune response (1). Its immunopathology is dominated by a Th17 and Th22 response that coinsides with the activation of inflammatory myeloid DCs (mDC) eventually leading to a complex interplay of inflammatory cytokines such as IFN-γ, TNFα, IL-12, IL-17, IL-22 and IL-23 (1-2). Furthermore, it has also been suggested that the above inflammatory response will drive keratinocytes into an inflammatory cascade of events involving proinflammatory cytokines (e.g., IL-1, IL-6, and TNF-α), chemokines and antimicrobial peptides (AMPs), eventually resulting in chronic psoriatic lesion (1-3). Respectively, the development of psoriasis is likely due to the coordinated efforts of several inflammatory cytokine pathways. These above discoveries have led to new and highly successful potent biological drugs aimed at key targets such as IL-23, TNFα, and IL-17 (4-6).

Ultraviolet-B (UVB) phototherapy has been an effective treatment for psoriasis, as well as other skin diseases, for decades. First, there was broad-band UVB phototherapy commonly combined with topical tar (Goeckerman regimen) (7), but for the past 35 years, it has been replaced by the more effective narrowband (NB) UVB phototherapy (8). Although NB-UVB phototherapy is one of the most efficacious treatment options for psoriasis, it´s mechanism of action is still not fully understood. However, researchers over the past two decades are beginning to define the mechanisms by which phototherapy improves psoriasis. Several studies describe depletion of Langerhans cells(9, 10) and T cells (11-13) in psoriatic skin in response to phototherapy, as well as apoptosis of T cells (14, 15). In addition, *Simon et al* and *Murphy et al* show that NB-UVB impairs *in vitro* antigen presentation by human Langerhans cells, converting them from immunogenic to tolerogenic(9, 16), and early studies describe up-regulation of anti-inflammatory cytokines IL-10(17, 18) and IL-4(19) in psoriatic skin after UVB treatment. More recent studies have found evidence of down-regulation of Th1/Th17 inflammatory axis in psoriatic skin(13, 19-21) with UVB treatment, and very few studies have observed suppression of Th1/Th17 cytokines and up-regulation of IL-10 in the circulation of psoriasis patients as well (22-24). Previous studies of circulating chemokine levels and their

response to NB-UVB therapy in psoriasis are very limited and show no reduction of skin homing chemokines with treatment (25).

Balneophototherapy is a combination treatment of bathing in salt or geothermal sea water before UVB phototherapy and is highly effective treatment for psoriasis (26, 27). Balneophototherapy at the Blue Lagoon (BL) in Iceland is based on bathing in a geothermal seawater originating from underground reservoirs formed after building a geothermal plant in the lava field in the Reykjanes peninsula combined with conventional NB-UVB phototherapy (28). The additional therapeutic effect of bathing in the BL on psoriasis is not known, but recent studies have indicate that it is probably based on thes unusual ecosystem in the geothermal seawater, inclucing the blue-green algae *Cyanobacteria aponinum* and *Silicabacter lacuscaerulensis,* and it's very high level of silica(29, 30).

Despite numerous studies have been published depicting either the histopathology or the peripheral blood compartment in psoriasis after NB-UVB phototherapy, limited amount of data exists regarding the impact of NB-UVB therapy in both blood and the skin simultaneously. Even more limited data exists regarding the effect of balneophototherapy on inflammatory pathways in the blood and skin in psoriasis patients. Thus, the main aim of this study was to simultaneously evaluate at both compartments the impact of NB-UVB therapy and balneophototherapy on inflammatory pathways underlying psoriasis, especially Th1/Th17 pathways, and to see if there was any difference between these two effective psoriasis treatments.

In this study, we demonstrate that circulating Th17 ($CD4^+CD45RO^+$ T-cells expressing IL-23R) and Tc17 ($CD8^+CD45RO^+$ T-cells expressing IL-23R) effector cells are reduced by more than 60% in psoriasis patients after only two weeks of treatment. In addition, such a peripheral blood biological inflammatory resolution had both a strong clinical and histophathological correlation (Clinical PASI score vs. histopathological Trozak score). Furthermore, such biological inflammatory resolution was largely confined to both CD4+ and CD8+ skin homing peripheral blood T17/T22 effector T-cells. Interestingly, no such correlation was associated with T1 effector cells that have also been implicated in its immunopathogenesis. Finally, these peripheral blood compartment and histopathology respones were further abrogated by immunohistocehmical studies reflecting significant site-specific inflammatory resolution of CD3+, CD4+ and CD8+ T-cells in the skin, and a correlation of dermal immunohistochemistry analysis of IL-17A with clinical improvement. In addition, these findings were further corroborated by the correlation of the circulating skin homing chemokine CCL17 and skin homing Th/Tc cells expressing the skin resident marker CD103 with treatment.

No difference on the impact of balneophototherapy compared with NB-UVB therapy on the immune pathways underlying psoriasis was found.

Materials and methods

Patients

Patients were 18 years of age or older with mild-moderate to severe plaque psoriasis with a psoriasis area and severity index score (PASI score)(31) of 7 or higher. Sixty-eight patients with psoriasis were enrolled in the study and randomized into three therapeutic arms: 1) Out-patient treatment for six weeks in the BL, geothermal seawater group (GSW group (*n*=22)), 2) In-patient treatment for two weeks in the BL followed by maintenance UVB phototherapy for 4 weeks, intensive geothermal seawater group (IT-GSW group (*n*=23)), 3) Out-patient UVB phototherapy for six weeks (UVB group (*n*=24)). Disease severity (PASI) was recorded, blood samples and four-mm punch biopsies of target skin lesion were obtained from seven patients in each group before, 2 and 6 weeks after starting treatment. The target lesion was located either in arms, legs or trunk.

Cell preparation, stimulation and flow cytometry analysis

Heparinized peripheral venous blood was collected at each time point and peripheral blood mononuclear cells (PBMC) obtained by gradient centrifugation with Ficoll-Paque PLUS (Healthcare, Uppsala, Sweden), collected at the interface, washed with HBSS medium (Gibco, Carlsbad, CA, USA) prior to staining with anti-human CD3, CD4, CLA, CD103 (all from Biolegend, San Diego, USA), CD8, CD45R0 and IL-23R (R&D Systems, Abingdon, UK) monoclonal antibodies (mAbs) for T cell analysis. To evaluate cytokine secretion potential, the PBMCs (1.0 X 10^6 cells/ml) were cultured for 16 hours in RPMI 1640 medium with penicillin-streptomycin (100 IU mL^{-1} and 0.1 mg mL^{-1}) (Gibco), in the presence of anti-CD3 (5 μg/ml), anti-CD28 (5.0 μg/ml) mAbs (Biolegend, San Diego, USA) and Brefeldin A (3.0 μg/ml) (eBioscience, San Diego, USA) at 37°C. The T-cells that express CD4+/CD8+/CLA+ and produce interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), IL-17A (all from Biolegend, San Diego, USA) and IL-22 (R&D Systems, Abingdon, UK) after 16-hour stimulation in the presence of anti-CD3 and anti-CD28 were evaluated with flow cytometry (FACS).

Cytokine and chemokine measurements in serum

Serum samples were collected at all timepoints. Chemokines (CCL17 and CXCL10) and cytokine (IL-19) were measured using a magnetic Luminex assay (R&D systems) and analysed in Bio-Plex 200 system (Bio-Rad Laboratories, California, USA).

Histological assessment

Biopsies from target skin lesion were stained with haematoxylin and eosin and grading of the histological changes was done according to the histological grading system for assessing psoriasis severity by Trozak (32). Histopathological changes are scored from 1 to 3; parakeratosis, suprabasal mitosis, regular elongation of rete ridges, club shaped rete ridges, absent granular layer, oedema and elongation of dermal papillae, perivascular infiltrate in the upper dermis, munro microabscess and spongiform pustule. The cumulative score (0-27) was recorded for each biopsy.

Immunohistochemistry

Four-mm punch biopsies of target skin lesion were frozen immediately in OCT compound and stored at -80ºC until required. Frozen sections were cryosectioned (7µm) and stained by immunohistochemistry for CD3 (BioLegend), CD4 (Cayman Chemical) and CD8 (Abcam). All samples were blinded before staining. CD3, CD4 and CD8 positive cells were counted in 3 randomly chosen fields in 20x magnification. Only samples of good quality were included, or from five patients in the IT-GSW group and five patients in the UVB group.

Immunofluoresence

Frozen sections were cryosectioned (7µm) and double-stained by immunofluorescence for CD8 (two different antibodies used, one from BioLegend and one from Abcam) and either IL-17 or IL-22 (both from Abcam). All samples were blinded before staining. DyLight and Alexa Fluor secondary antibodies (Thermo Fisher Scientific Inc) were used. Imaging was done by a confocal microscope (Olympus FV1200). IL-17 and IL-22 staining was graded on the scale from 0 to 3 by three independent viewers in both epidermis and dermis. Only samples of good quality were included, which resulted in samples from three patients in the IT-GSW and UVB group and from four patients in the GSW group.

Statistical analysis

When evaluating the differences between normal healthy controls (n=3) and patients we used the Mann-Whitney test. Measurements are expressed as median (IQR). To evaluate the differences between active stage of disease and after two and six weeks of treatment, we used the repeated measures ANOVA and to evaluate the difference between treatment groups we used two-way ANOVA (n=20). Measurements are expressed as mean ±SD. $P<0.05$ was considered as statistically significant. The correlation analysis was performed by calculating the Pearson correlation coefficient. A p value of less than 0.05 was considered significant.

Results

The clinical severity of Psoriasis correlates with the reduction of both Th17 and Tc17 peripheral blood effector T-cells.

As expected all treatment protocols demonstrated improved clinical and histological outcomes based on PASI score (PASI score, n=21: Pre-treatment 13.06±6.52 vs. Post-treatment 4.88±5.64, $p<0.001$; Figure 1a) and Trozak score (Trozak score, n=21: Pre-treatment 10.3±3.7 vs. Post-treatment 3.2±3.3, $p<0.001$; Figure 1b). To evaluate peripheral blood Th17/Tc17 levels and correlate it with the above outcome measures, Th17 effector T-cells were defined as $CD4^+CD45RO^+$ T-cells expressing IL-$23R^+$ and Tc17 effector T-cells as $CD8^+CD45RO^+$ T-cells expressing IL-$23R^+$ by FACS analysis. As shown in Figure 1c and 1d both Th17 and Tc17 pre-treatment levels in psoriasis patients (Pre-treatment Th17 levels: 12.25±7.44% and Tc17 levels: 15.37±6.37%, n=21) was much higher than in the healthy controls (HC) (HC Th17 levels: 0.81±0.65%, $p<0.01$; HC Tc17 levels: 1.50±2.08%, $p<0.01$, n=3). Th17 and Tc17 levels were already significantly reduced by 70 and 61 percent after only 2 weeks of treatment (Th17 from 12.25±7.44% to 3.64±5.51%, $p<0.001$, and Tc17 from 15.37±6.37% to 5.89±4.61%, $p<0.001$; Figure 1B). In addition, after 6 weeks of treatment both Th17 and Tc17 effector T-cell levels had been even further reduced by 90 and 76 percent (from 12.25±7.44% to 1.19±0.91%, $p<0.001$, and from 15.37±6.37% to 3.66±2.81%, $p<0.001$; Figure 1c and 1d). Finally, both the Th17 and Tc17 peripheral blood levels had a significant positive correlation with the corresponding clinical and histological outcome measure for each patient via the PASI and Trozak score ($p<0.002$; Figure 1c and d).

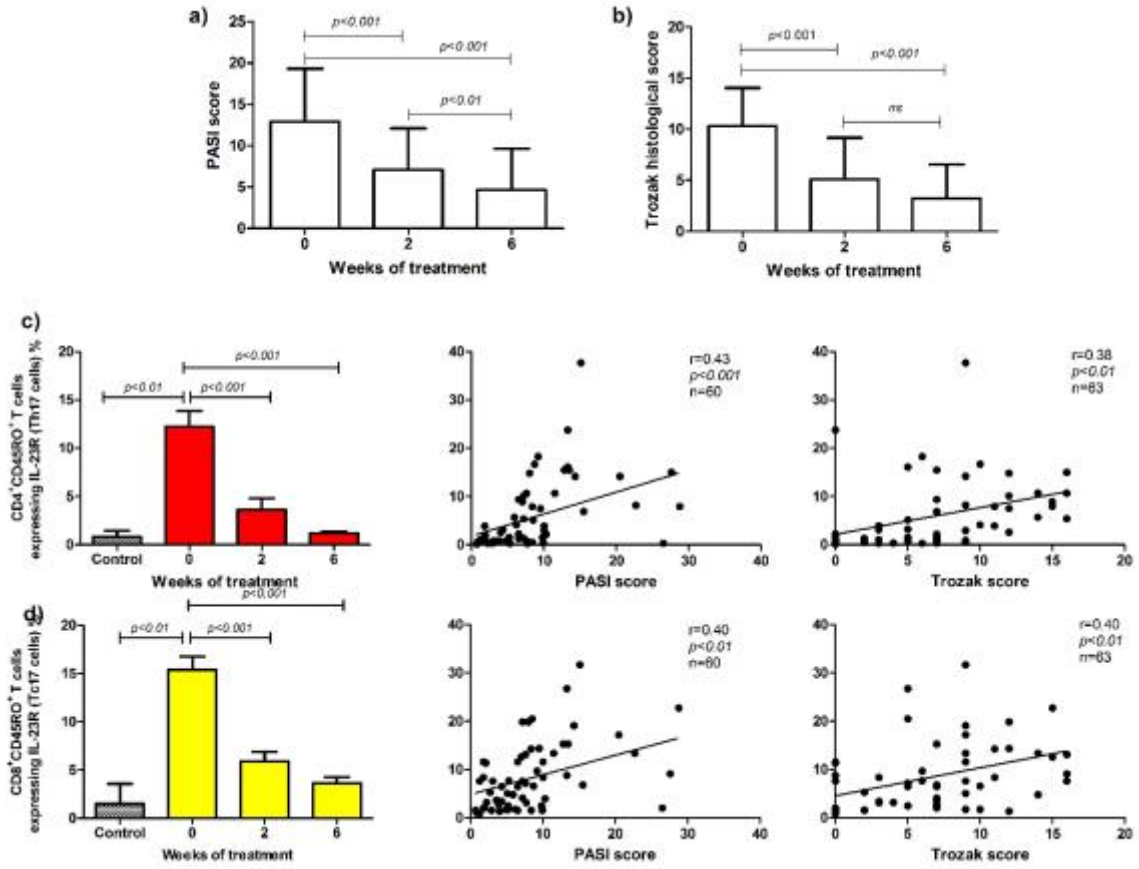

Figure 1. Clinical efficacy (a) and histological changes in lesional skin (b) improved in all cases with treatment. Bar columns showing values of Th17 ($CD4^+$ $CD45RO^+$ T-cells expressing IL-$23R^+$) (c) and Tc17 ($CD8^+$ $CD45RO^+$ T-cells expressing IL-$23R^+$) (d) of healthy volunteers and of psoriasis patients before treatment and 2 and 6 weeks. There was significant correlation between the reduction of peripheral Th17 (c) and Tc17 (d) and both clinical efficacy (PASI) and improvement in histological changes (Trozak score) with treatment. The results are presented as mean±SD; healthy controls (n=3) and psoriasis patients (n=21).

The reduction of cytokine secretion of peripheral blood T-cells in psoriasis is confined to IL-17A producing CD4+ T cells and IL-22 of both CD4+ and CD8+ T-cells, and has a positive correlation with PASI.

In order to evaluate the biological response of peripheral blood key effector CD4+ and CD8+ T-cells in psoriasis, the frequencies of Th17/Tc17 (IL-17A measured), Th22/Tc22 (IL-22), Th1/Tc1 (IFNγ and TNFα) and Th2 cytokine profile (IL-4) where analysed before and after treatment by flow cytometry after intracellular cytokine staining. As shown in Fig.2 the percentage of Th17 (patients, 2.02% ± 0.76%; HC, 0.49% ± 0.28%), Th22 (patients, 1.44% ±

0.85%; HC, 0.37% ± 0.11%), IFNγ producing Th1 (patients, 5.44% ± 4.87%; HC, 0.37% ± 0.28%), TNFα producing Th1 (patients, 14.42% ± 7.52%; HC, 0.81% ± 0.41%) and Th2 cells (patients, 5.57% ± 4.15%; HC, 0.94% ± 0.23%) were significantly higher in psoriasis patients compared with healthy controls (HC) ($p<0.05$, Fig.2a).

In addition, successful treatment of psoriasis led to a 23% reduction of Th17 effector cells (pre-treatment, 2.02% ± 0.76%; after treatment, 1.55% ± 0.67%; $p<0.05$), and more than 33% reduction of both Th22 and Tc22 effector T-cells after only 2 weeks of treatment (Th22 from 1.44% ± 0.85% to 0.94% ± 0.66%, $p<0.05$ and Tc22 from 1.18% ± 0.80% to 0.79% ± 0.51%, $p<0.05$; Figure 2a and b). Finally, this was even more pronounced (>50%) after 6 weeks of treatment (Th22 from 1.44% ± 0.85% to 0.63% ± 0.40%, $p<0.001$, and Tc22 from 1.18% ± 0.80% to 0.60% ± 0.37%, $p<0.01$; Figure 2a and b). Furthermore, the above findings had a positive correlation with the clinical PASI score (r= Pearson´s correlation coefficient: Th17, $r=0.34$, $p<0.01$; Th22, $r=0.56$, $p<0.0001$ and Tc22, $r=0.37$, $p<0.01$; Fig.2c and d). However, no such above correlation was found in the respect with other T-effector cell phenotypes associated with IFNγ, TNFα or IL-4 except for IFNγ producing CD8+ T-cells, Fig.2.

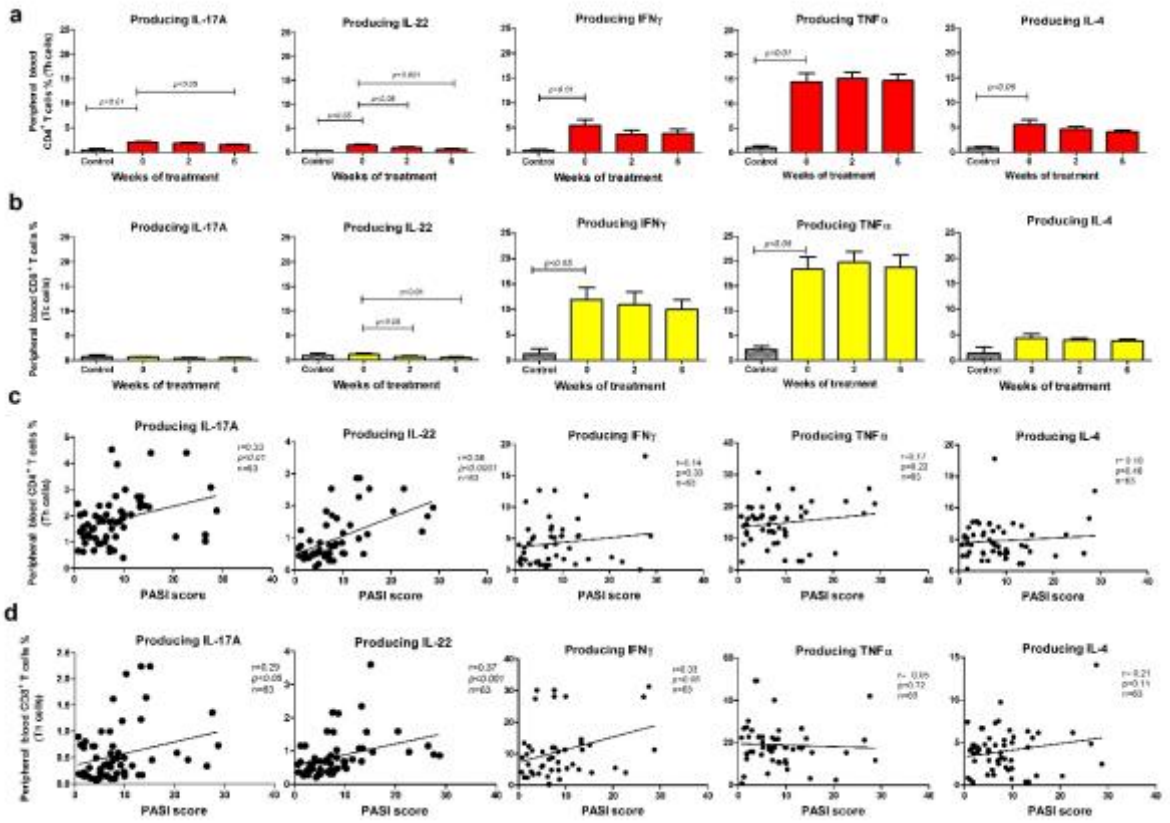

Figure 2. Bar columns showing values of healthy volunteers and psoriasis patients before treatment and 2 and 6 weeks after the induction of treatment of a) $CD4^+$ and b) $CD8^+$ T-cells producing IL-17A, IL-22, IFNγ, TNFα and IL-4, and their correlation with the clinical PASI score (c and d). The results are presented as mean±SD; healthy controls (*n*=3) and psoriasis patients (*n*=21).

The correlation of skin homing $CD8^+$ and CD4+ IL-17A or IL-22 producing effector T-cells with PASI in psoriasis.

To see if this reduction of Th17/Tc17 and Th22/Tc22 cells in the circulation were skin specific we compared the relative frequencies of these cells in peripheral blood co-expressing the skin-homing receptor CLA. Skin homing Th (CD4+CLA+) and Tc (CD8+CLA+) cells in the peripheral blood of psoriasis patients and healthy controls were analysed by flow cytometry after intracellular cytokine staining. Compared with healthy controls circulating levels of peripheral blood $CD4^+CLA^+$ T-cells producing IL-17A (Psoriasis = 6.83±3.54% vs HC = 2.17±2.64%, $p<0.05$; Figure 3a) and IL-22 (Psoriasis = 1.44±0.85% vs. HC = 0.37±0.11%, $p<0.05$; Figure 3c) in the patients were significantly higher.

When the levels of skin homing CLA^+ marked $CD4^+/CD8^+$ T-cells secreting IL-17A were analysed before and after treatment, circulating $CD4^+CLA^+$ IL-17A secreting T-cells had been reduced by 35 percent following 6 weeks of treatment (from 6.83±3.54% to 4.46±1.72%, $p<0.01$; Figure 3a) and $CD8^+CLA^+$ IL-17A secreting T-cells by 42 percent (from 2.40±1.66% to 1.40±1.16%, $p<0.01$; Figure 3b). An even greater reduction occurred when skin homing CLA^+ marked $CD4^+/CD8^+$ T-cells secreting IL-22 were analysed, or 56% reduction for Th22 ($CD4^+CLA^+$ IL-22 secreting T-cells) and 42% for Tc22 ($CD8^+CLA^+$ IL-22 secreting T-cells) after 6 weeks of treatment (Skin homing Th22 from 3.83±2.10% to 1.68±1.03%, $p<0.001$; vs. skin homing Tc22 from 4.16±2.23% to 2.42±1.30%, $p<0.01$; Figure 3c and d).

Skin homing $CD8^+CLA^+$ T-cells producing IL-17A ($r=0.45$, $p<0.001$; Figure 3b) and $CD4^+CLA^+$ T-cells producing IL-22 ($r=0.33$, $p<0.05$; Figure 3c) decreased in correlation with the clinical improvement (PASI score), but no correlation was found with histological improvement (Trozak score). Interestingly, $CD8^+CLA^+$ T-cells secreting IL-22 had both a strong correlation with the PASI score ($r=0.59$, $p<0.001$) and a correlated with the Trozak score ($r=0.32$, $p<0.05$). Regarding other cytokines measured, the levels of IFNγ and TNFα producing $CD4^+/CD8^+$ T-cells and skin homing $CD4^+/CD8^+$ T-cells were higher compared with healthy

volunteers but no difference was found with treatment (Data not shown). No difference was observed for IL-4 producing $CD4^+/CD8^+$ T-cells and skin homing $CD4^+/CD8^+$ T-cells (Data not shown).

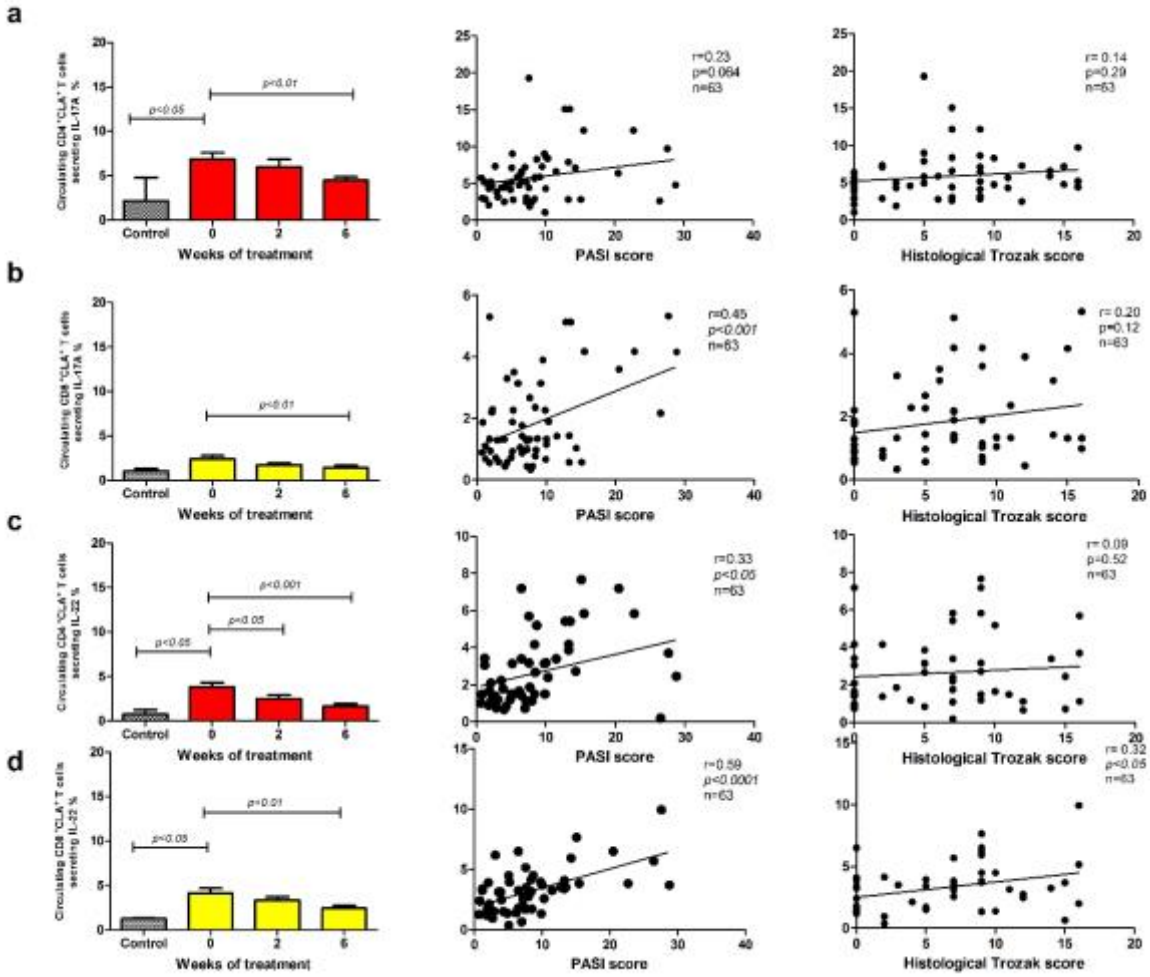

Figure 3. Bar columns showing values of healthy volunteers and psoriasis patients before treatment and 2 and 6 weeks after the induction of treatment, their correlation with the clinical PASI score and histological Trozak score: a) Skin homing Th17 cells ($CD4^+CLA^+$ T-cells producing IL-17A), b) skin homing Tc17 cells ($CD8^+CLA^+$ T-cells producing IL-17A), c) skin homing Th22 cells ($CD4^+$ CLA^+ T-cells producing IL-22) and d) skin homing Tc22 cells ($CD8^+$ CLA^+ T-cells producing IL-22). The results are presented as mean±SD; healthy controls (n=3) and psoriasis patients (n=21).

UVB phototherapy treatment regimens induces a decrease in circulating skin resident effector T-cells concomitant with clinical efficacy

To assess differences in the levels of circulating skin resident T effector cells with psoriasis treatment, we compared the level of the population $CLA^+/CD4^+$ T-cells expressing CD103 as skin resident Th cells and $CLA^+/CD8^+$ T-cells expressing CD103 T-cells as skin resident Tc cells (n=21). A marked reduction of 30% was observed after 6 weeks of treatment for both skin resident Th cells (from 12.59%±8.60 to 7.58%±8.07, $p<0.05$) and Tc cells (from 14.73±7.88% to 10.56±5.80%, $p<0.01$; Figure 4a). The reduction correlated strongly with the reduction of PASI score ($r=0.50$ and $r=0.48$, $p<0.0001$; Figure 4b) but no significant correlation was found with the histological Trozak score. Interestingly, when $CD4^+/CLA^-$ T-cells expressing CD103 and $CD8^+/CLA^-$ expressing CD103 T-cells were analysed, no reduction with treatment was found and no significant correlation with the PASI score.

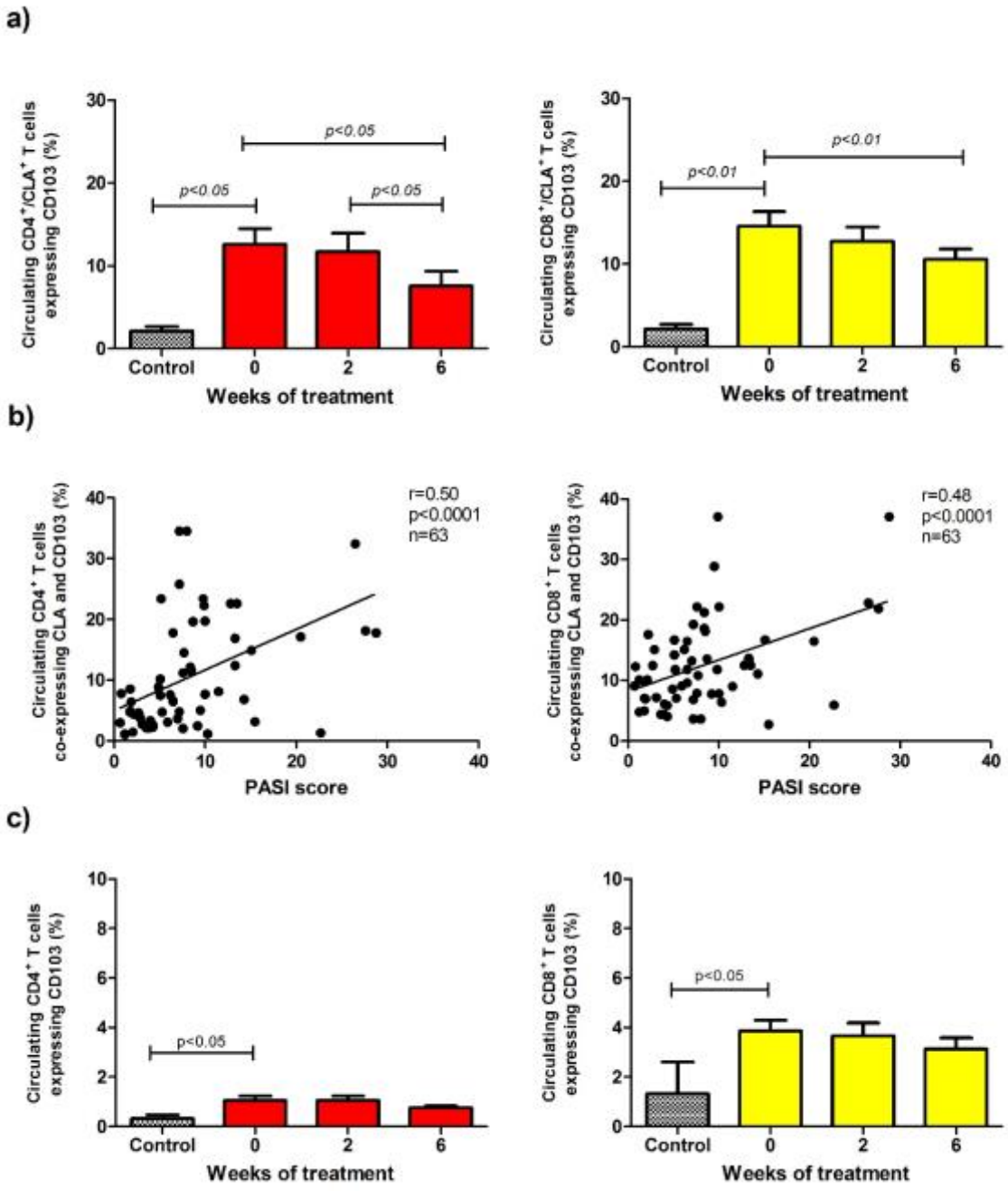

Figure 4. Bar columns showing values of healthy volunteers and psoriasis patients before treatment and 2 and 6 weeks after the induction of treatment of skin resident Th ($CD4^+CLA^+$ expressing CD103)/Tc ($CD8^+CLA^+$ expression CD103) cells (a), the correlations between PASI score and skin homing Th/Tc (b) and Th/Tc cells expressing the tissue-resident marker CD103 (c). The results are presented as mean±SD for controls (n=3) and psoriasis patients (n=21).

Serum levels of CCL17, CXCL10 and IL-19 correlate to the PASI score

In consideration of these changes in cellular composition of the blood it was decided to measure biomarkers in serum connected to the pathogenesis of psoriasis. Chemokines produced by keratinocytes and other nearby cells recruit various effector cells on site. CCL17 and CCL22 are ligands for CCR4 which is central for skin homing T cells. CCL17 level in serum correlated strongly to the PASI score ($r=0.36$, $p<0.005$, figure 5a) but not to Trozak score while CCL22 did not correlate to either PASI or Trozak score (data not shown). CXCL10 is a Th1 associated chemokine and its serum levels had a positive correlation to the PASI score ($r=0.33$, $p<0.05$, figure 5b). In addition, IL-19 (previously associated with psoriasis) correlated both to PASI score ($r=0.39$, $p<0.01$, figure 5c) and to the Trozak score ($r=0.34$, $p<0.05$, figure 5d).

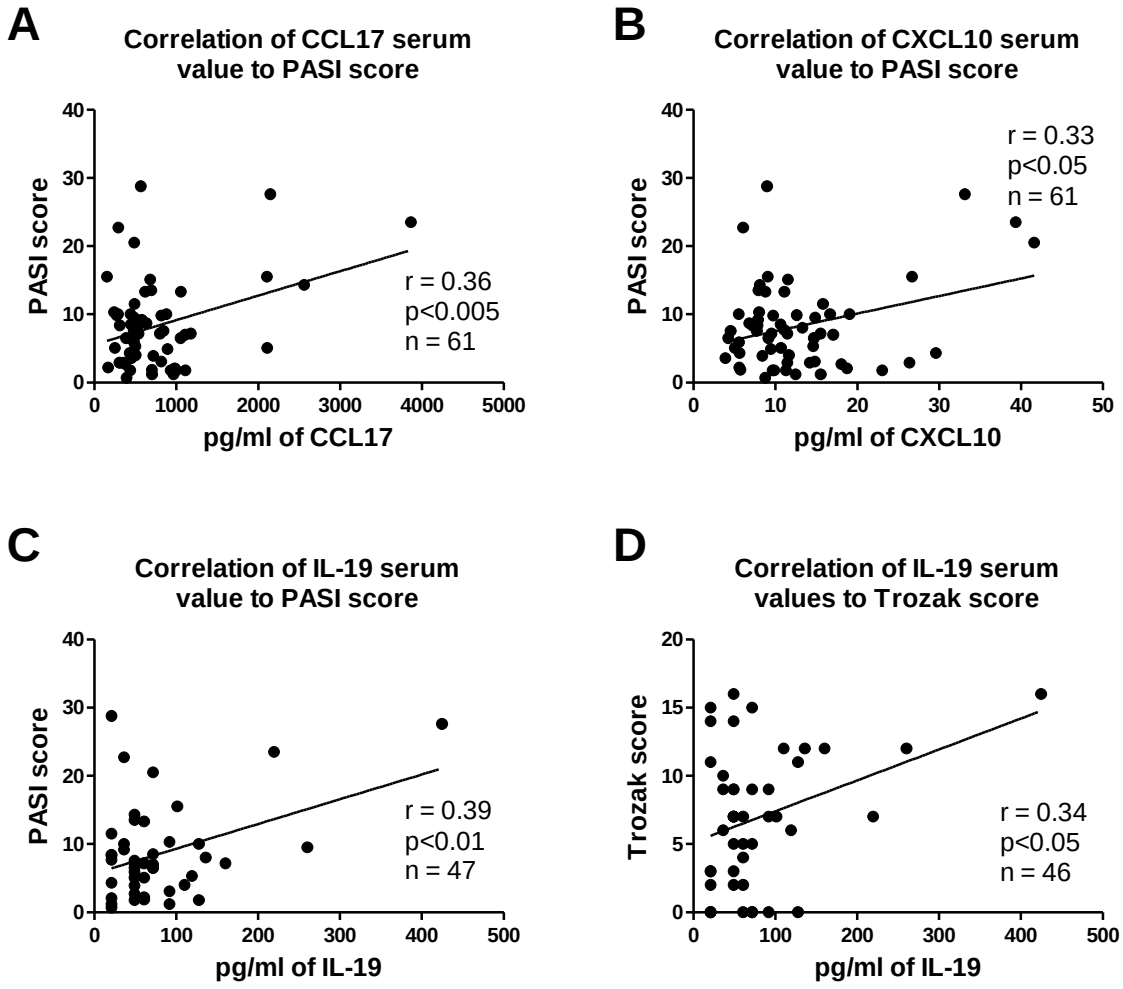

Figure 5.Plots showing the correlation between the PASI score and CCL17 (a), CXCL10 (b), IL-19 (c) and between the Trozak score and IL-19 (d). The results are from 21 psoriasis patients at 3 timepoints (n=63), serum values under the standard curve were excluded.

Histological grading of skin infiltrating T-cells in psoriasis and its severity association.

To evaluate the potential effects of balneophototherapy and NB-UVB phototherapy on the site specific reactions, immunohistochemical analyses were performed. There was a significant reduction of 67-70% for CD3 numbers in skin (from 129.2±71.19 to 42.35 ±45.78), CD4 (from 73.00±43.23 to 21.85±25.76) and CD8 (from 53.60±34.33 to 15.10±14.91) after 6 weeks of treatment (Figure 6A; $p<0.01$) with positive correlation with the histological Trozak score ($CD3^+$ $r=0.38$; vs. $CD4^+$ $r=0.40$; $p<0.05$). Interestingly, no correlation was found with the clinical PASI score.

IL-17A positive immunoreactive cells were present in the epidermis, throughout the dermal papillae and upper dermis in psoriatic skin

To see how these above cellular phenotypic changes in blood correlated with IL-17 driven pathology in the skin, we stained skin samples for IL-17 and IL-22, along with CD8. As shown in Figure 6e, IL-17 staining was widely observed in psoriatic skin with prominent staining in keratinocytes in the lower layer of the epidermis and immune cells in the upper dermis. The treatment response with the regards to IL-17 staining was predominantly confined to dermis since no significant reduction in the grade of IL-17 staining was observed within the epidermis after 6 weeks of treatment (dermal IL-17 score: 1.62±0.61 to 1.34±0.83; $p=0.19$). However, we observed a positive correlation between the IL-17 grade in dermis and PASI ($r=0.565$, $p<0.01$; Figure 6b). Finally, only few CD8+ cells co-expressed IL-17 (Data not shown). IL-22 was less widely expressed in psoriatic skin than IL-17 (Data not shown). When IL-22 staining was observed, it was mostly localized to keratinocytes lining the basement membrane, in a pattern like IL-17 (Data not shown).

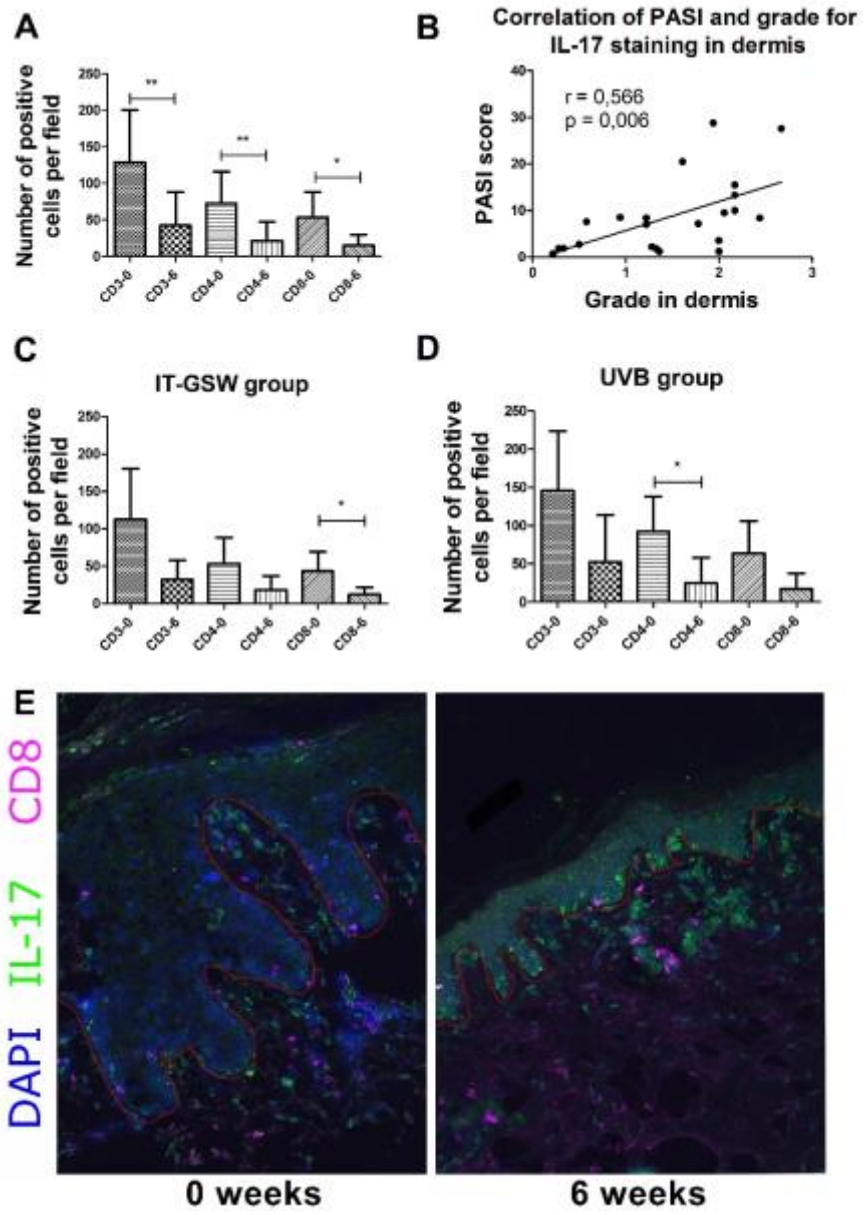

Figure 6. Immunohistochemistry on lesional skin from psoriasis patients before and after treatment. Bar columns show the number of CD3, CD4 and CD8 positive immunoreactive cells in high powered field of vision of lesional psoriatic skin before treatment (0) and after 6 weeks (6). The intensive group (IT-GSW group – fig. 6C) received an intensive combination of bathing in geothermal seawater and UVB phototherapy and the control group received UVB monotherapy (UVB group – fig. 6D). All results are shown compiled (A). Dot plot shows the grade of IL-17 staining in the dermis and the correlation with PASI score (B). Pearson's correlation coefficient (r) and p value are shown. Representative three-colour immunofluoresence of $CD8^+$ (pink), $IL\text{-}17^+$ (green) cells with blue-stained nuclei in lesional plaque from psoriasis patient before treatment (0 weeks) and after 6 weeks of treatment (E).

The red lines delineate the dermal-epidermal junction. The results are presented as mean±SD, *p<0.05 and **p<0.01.

Difference between balnophototherapy at the Blue Lagoon and NB-UVB monotherapy

Concerning the changes in the parameters that significantly reduced with treatment, we found that the improvement was in general significantly faster for the combination treatment groups (GSW and IT-GSW groups), reaching statistical significance after only two weeks of treatment in the clinical PASI score, $CD4^+CD45RO^+$ T-cells expressing IL-23R (Th17) and $CD4^+$ CLA^+ T-cells producing IL-17A cells (skin homing Th17 cells) for both groups, and in Trozak score and $CD8^+$ CLA^+ T-cells producing IL-17A cells (skin homing Tc17 cells) for IT-GSW group, as compared with patients treated with UVB only (UVB group) (Figure 7a). However, after 6 weeks of treatment this difference had disappeared except for the clinical PASI score (Figure 7b). No significance was observed between the treatment groups for $CD8^+CD45RO^+$ T-cells expressing IL-23R (Tc17), $CD4^+CLA^+$ T-cells producing IL22, $CD8^+CLA^+$ T-cells producing IL22, $CD4^+CLA^+$ T-cells expressing CD103 or $CD8^+CLA^+$ T-cells expressing CD103 (skin resident T effector cells) (Figure 7a and 7b).

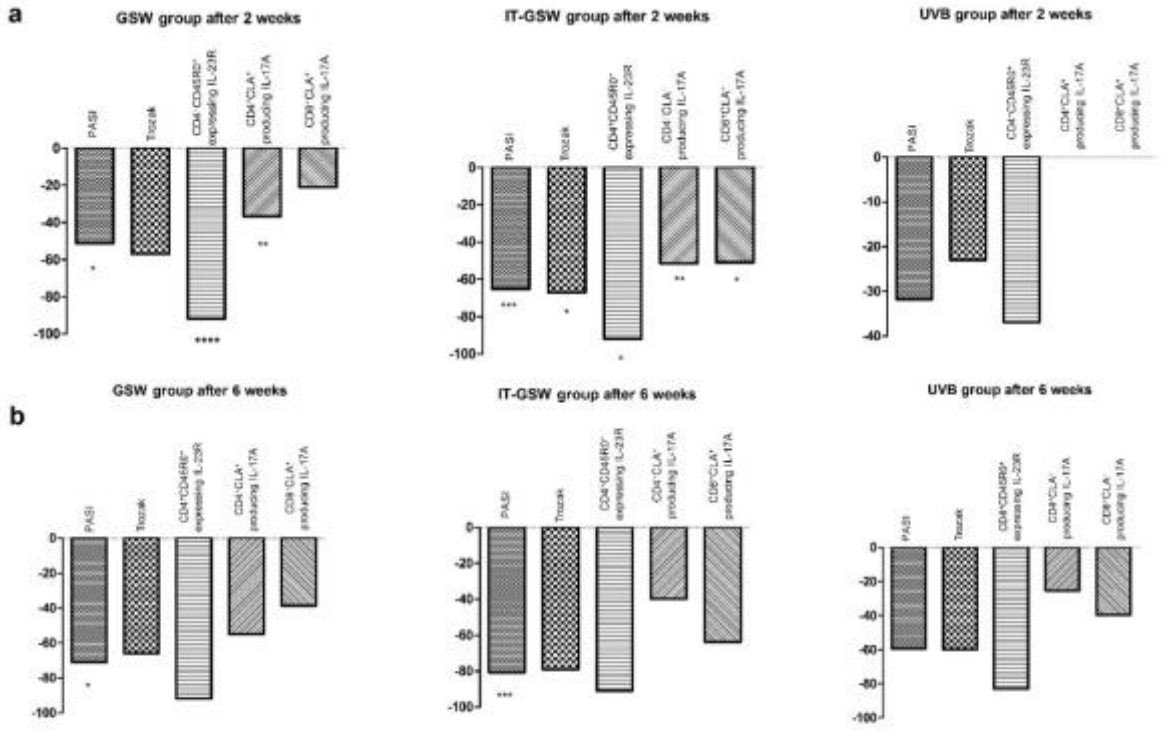

Figure 7. Column bar graphs showing median values of the percentage changes observed following treatment of psoriasis vulgaris with combination treatment of bathing in geothermal seawater and UVB phototherapy (GSW group), intensive combination treatment (IT-GSW group) and UVB monotherapy (UVB group) 2 weeks (a) and 6 weeks after the induction of treatment (b). The results are presented as median (IQR), *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Discussion

In this paper, we have focused on the effects of balneophototerapy and NB-UVB phototherapy at cellular and cytokine/inflammatory mediator levels to define the therapeutic associated inflammatory effects occurring in both blood and skin simultaneously. This study demonstrates rapid biological inflammatory reduction of circulating T17 effector cells, skin homing Th17/Tc17 and Th22/Tc22 cells, Th/Tc cells expressing the tissue retention integrin CD103 and T lymphocytes ($CD3^+$, $CD4^+$ and $CD8^+$) in the skin of psoriasis patients treated

with three different UVB regimens. This systemic and local reduction of inflammatory mediators correlated with clinical improvement as measured with the PASI score and the reduction of circulating Th17/Tc17, skin homing IL-22 producing $CD8^+$ cells and IL-19 in serum correlated well with histological improvement as measured with the Trozak score as well. No statistical significance was noted after 6 weeks of treatment between the treatment groups, however, the improvement was in general faster for the combination treatment groups (GSW and IT-GSW groups).

In agreement with previous reports (33, 34), our results highlight the critical role of T17 effector cells in the pathogenesis of psoriasis. We show a strong correlation of the reduction of circulating Th17/Tc17 with both clinical efficacy score and histological Trozak score, and a significant resolution of skin homing (CLA^+) Th17/Tc17 cells in the peripheral blood with treatment. The reduction of skin homing Tc17 correlated well with clinical improvement, however no such correlation was found regarding skin homing Th17 cells. IL-17 is a key mediator of psoriatic inflammation. IL-17 drives the production of proinflammatory cytokines and chemokines by keratinocytes, and consequently activates leukocytes and recruits them into the skin (35). In addition, the importance of IL-17 is further supported by the superior success of anti-IL-17 treatments(4, 36). IL-17 and IL-22 modulate distinct keratinocyte-response pathways where IL-17 is a strong inducer for synthesis of antimicrobial peptides in keratinocytes, and activation of Th22 cells results in increased production of IL-22 that is linked to keratinocyte activation and epidermal achantosis, a prominent morphological feature in psoriasis (37, 38). It has been suggested that IL-22 is another critical cytokine in the pathogenesis of psoriasis and as such is a target of drug development (39).

Our data demonstrates that skin homing Th22/Tc22 cells in the peripheral blood compartment reduced significantly with treatment as well concomitant with PASI score. Interestingly, circulating Th22 and Tc22 did not correlate with the histological Trozak score but the reduction of circulating skin homing Tc22 correlated with both clinical and histological improvement. It raises a question if skin homing $CD8^+$ (Tc17 and Tc22) play more significant role in the pathogenesis of psoriasis compared with skin homing $CD4^+$ (Th17 and Th22), where skin homing Th17 had no correlation with clinical or histological improvement and skin homing Th22 no correlation with histological improvement.

Psoriasis is now generally regarded as a T cell-mediated immune disease with a mixed Th1/Th17 cytokine environment (1, 3). Psoriasis lesions have long been known to contain elevated levels of IFN-γ (40), and the frequency of circulating CD8+IFN-γ+ T cells in the blood of patients have been shown to correlate with disease severity (41). Although IFN-γ has been

shown to drive inflammation in skin, a central or critical role for IFN-γ in psoriasis has been cast into doubt with the failure of an IFN-γ-targeted therapy (42). However, IFN-γ likely contributes to the cytokine storm in psoriasis by aiding other cytokines, in particular, IL-17A, which drives IL-1/IL-23 production to augment Th17 responses (1). This is in consensus with our results where we found no significant reduction of IFNγ, TNFα or IL-4 producing $CD4^+$/$CD8^+$ T cells in the peripheral blood cells and no correlation with the clinical improvement, except for the skin homing CD8+ IFNγ T cells. Interestingly, the reduction of circulating skin homing $CD8^+CLA^+$ T-cells producing all the cytokines measured (IL-17A, IL-22, IFNγ, TNFα and IL-4) correlated with the reduction of PASI (data not shown) but not skin homing $CD4^+CLA^+$ T-cells except for skin homing $CD4^+CLA^+$ T-cells producing IL-22. This could indicate that skin homing $CD8^+$ (Tc17/Tc22/Tc1) play more significant role in the pathogenesis of psoriasis compared with skin homing $CD4^+$ (Th17/Th22/Tc1) as mentioned before.

Some studies indicate that UVB irradiation leads to resolution of inflammatory $CD3^+$/$CD4^+$/$CD8^+$ T effector cells in psoriatic skin (11, 12) as we report in our study, probably by inducing apoptosis (14, 15). Interestingly, we found a correlation between the histological Trozak score and the reduction of epidermal $CD3^+$, $CD4^+$ and $CD8^+$ cells in the skin with treatment but not with the clinical severity score. It could be explained by the small number of patients (n=10) where immunohistochemical analysis was performed. Numerous IL-17A-positive cells and less abundant IL-22-positive cells are localized in psoriasis lesions as showed by previous RT-PCR studies (13, 35), flow cytometry studies (35, 43) and immunohistochemistry studies (44, 45), where they can represent up to 30% of infiltrating T lymphocytes (46). IL-17A and IL-22 are produced by various immune cells but not by keratinocytes(47). However, there are some speculations that keratinocytes can produce IL-17 themselves(48), but the consencus today is that they do not. We observed staining of both IL-17 and IL-22 in keratinocytes in lower epidermis that is probably derived from cytokines bound to their receptor (38). Both cytokines were also expressed in lymphoid aggregates in dermis. IL-17 and IL-22 mRNA in the skin normalizes with psoriasis treatment as cyclosporine (35), narrow-band UVB (NB-UVB) phototherapy (13) and strongly correlates with histological improvement. However, we did not found a significant reduction of IL-17 in the skin by immunofluorescence staining as in the circulation. It can be explained by different methods used or that we took skin biopsies after only 6 weeks of treatment and it takes longer time to see significant change in the skin with immunofluorescence staining. These studies that have shown reduction of IL-17 in the skin after NB-UVB treatment are very different from our study.

Johnson-Huang et al evaluated IL-17 and IL-22 mRNA in the skin after 6 weeks of NB-UVB treatment and found strong correlation between decreased IL-17/IL-22 and histological improvement (13). However, they both use another method and they classified psoriasis plaques histologically into responsive and nonresponsive plaques where 8 were classified as normalized and 6 nonresponsive. We did not use any exclusion classification in our study so all plaques were included. *Rácz et al* performed gene expression profiling in human psoriatic skin irradiated with NB-UVB and revealed that the Th17 and IFN signlaing pathways (IL-17, INFγ) were suppressed (21). They took biopsies before treatment and than after the achievement of PASI50. Here again, a completely different method used and protocol.

Effective psoriasis treatment heals the skin without scarring, but typically psoriasis recurs in previously affected areas. A site-specific T cell–driven disease memory in psoriasis within the skin has been proposed where a population of CD8 T-cells expressing CLA, CCR6, CD103, and IL-23R is found in the epidermis of resolved psoriatic skin but not in normal skin(49-51). It is thought to play a role in maintaining and driving the recurrent disease of psoriasis, presumably contributing to epidermal localization and/or retention of a specific T cell subset. Our results indicate that only a small subset of CD8+ and CD4+ T lymphocytes in the peripheral blood express CD103 in psoriasis patients in correlation with previous studies (50, 51). We assessed in our previous study whether the skin resident integrin CD103 and CLA might be co-expressed by some T-cells and if they respond to treatment. CLA and CD103 was co-expressed by 5.92% (±3.2%) of freshly isolated PBMCs from psoriasis patients and by 1.39 % (median) of PBMCs from healthy donors. This observation is consistent with previous report (50). Furthermore, circulating CLA+ T-cells expressing CCR4 or CD103 reduced significantly with treatment and this reduction of CLA+CCR4+ T-cells was predominantly confined to those who also expressed the CD103 integrin(33). In our study, there was a decrease in circulating skin resident Th and Tc cells concomitant with clinical efficacy but not histological improvement. Interestingly, when $CD4^+CLA^-CD103^+$ and $CD8^+CLA^-CD103^+$ T-cells were analyzed, no reduction with treatment was observed, indicating that the reduction was limited to skin homing $CD4^+/CD8^+$ cells expressing the integrin CD103.

Our studies are, to the best of our knowledge, the first studies(33) that shows the response of the chemokine receptors CCR4 and CCR10 and the skin resident marker CD103, to NB-UVB phototherapy in the peripheral blood of psoriasis patients. *Ekman et al* measured the chemokine receptors CXCL9/CXCL10 (Th1), CCL17/CCL22 (Th2) and CCL20 (Th17) in the peripheral blood of psoriasis patients before and after NB-UVB therapy and they found no difference with treatment(25). We measured two CCR4 ligands, CCL17 and CCL22, in the

serum of participants. CCR4 has traditionally been thought to be expressed primarily on Th2 cells but has now been shown to be expressed on Tregs, Th17 and to be essential for skin homing T cells expressing CLA (52, 53). CCL17 showed a high correlation to the PASI score and a reduction with treatment while CCL22 showed no changes. Both chemokines are expressed by keratinocytes and endothelial cells (54, 55) and have previously been shown to be over-expressed in psoriasis and other diseases such as atopic dermatitis (25, 54). CXCL10 is a ligand for CXCR3 that is primarily expressed on Th1 cells. CXCL10 was measured as well and shows a correlation to the PASI score. The keratinocyte derived cytokine IL-19 has been shown to have a very strong relationship to the severity of psoriasis (56). Our results show a strong correlation between both PASI score and Trozak score to the level of IL-19 in serum.

We found no difference of the immunological effect on psoriasis between balnophototherapy and NB-UVB phototherapy, probably because their similar effect on the inflammatory pathways underlying psoriasis. For example, *Gudmundsdottir et al* observed immunomodulatory effects of the dominating blue-green algae *Cyanobacteria aponinum* from the Blue Lagoon *in vitro*, where secretion of the anti-inflammatory IL-10 cytokine by human dendritic cells was up-regulated and Th17/Treg imbalance improved (30). Interestingly, Furuhashi et al found similiar effect of PUVA and NB-UVB therapy, that is resolved Th17/Treg imbalance by reduced Th17 levels and increased Treg level in the blood of psoriasis patients (57). Further studies on the immunomodulatory effects of the bioactive molecules in the Blue Lagoon (the silica mud and microalgae), would be interesting and specially the impact on the outermost skin layer and consequently the expression of antimicrobial peptides and proteins (AMPs).

In conclusion, this study shows that balneophototherapy and NB-UVB monotherapy suppresses Th17/Tc17 and Th22/Tc22 inflammatory axis in the peripheral blood of psoriasis patients, concomitant with inflammatory resolution of CD3+, CD4+ and CD8+ skin resident T-cells, resulting in rapid improvement of the disease. No difference was found between the immunological effect of balneophototherapy and NB-UVB monotherapy on psoriasis. Combined, our data suggest that Th17/Tc17 and Th22/Tc22 effector cells have essential functions in the pathogenesis of psoriasis, not Th1/Tc1.

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Paper IV

Title page

Title

"Trozak histological assessment score as an additional objective psoriasis assessment tool in clinical trials."

Running head (not exceeding 50 characters)

Trozak histological assessment score of psoriasis vulgaris

Keywords:Psoriasis, outcome measure, histological score, quality of life

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Abstract

Background/Aims: Reliable assessment of severity in psoriasis is essential to document treatment responses in clinical research. Here we correlate the Trozak histological assessment score in chronic plaque psoriasis with Psoriasis Area Severity Index (PASI), histopathological markers commonly used in clinical trials and quality of life score (Dermatology Life Quality Index (DLQI)) in a substudy parallel to prospective randomized clinical trial.

Methods: Skin biopsies were collected from twenty-one patients. PASI and DLQI were evaluated at the same timepoints. To evaluate histopahtological severity of psoriasis, histological and immunohistochemical scoring methods such as histological grading score of Trozak, epidermal thickness (ET) and immunohistochemistry (CD3, CD4, CD8 and Ki-67) was used.

Results: Trozak histological score was significantly reduced from 10.3 before treatment to 5.1 after two weeks and 3.2 after 6 weeks ($p<0.0001$). This correlated strongly with the reduction in PASI (r=0.49, $p<0.0001$), DLQI (r=0.61, $p<0.01$) and Epidermal Thickness (ET) ($p<0.001$). ET correlated strongly with Trozak score (r=0.68, $p<0.0001$) but not with PASI.

Conclusion: We propose that the Trozak histological assessment could be an additional objective measure of disease severity in combination with clinical severity and quality of life scores to improve the quality of psoriasis clinical trials.

Introduction

Psoriasis is a chronic skin disease with a worldwide prevalence of around 2% [1] and high economic burden [2]. Reliable outcome measures of the disease severity are critical in clinical trials to measure the efficacy of an investigational treatment and very important in evidence-based medicine to provide comparisons among similarly designed trials. The ideal outcome measure to evaluate the severity of psoriasis should have a high specificity and sensitivity, a low inter- and intra-observer variation and take into account the psychosocial impact of the disease[3]. None of the currently available psoriasis score systems fulfils all of the validation criteria [4]. The Psoriasis Area and Severity Index (PASI)[5] is considered as gold standard to assess the clinical severity of psoriasis [4,6,7] but it has limitations such as low responsiveness in mild disease and low response distribution[4,6,7]. Another reliable clinical score is the "Lattice System Physician´s Global Assessment" (LS-PGA)[8] which has lower intra-observer and inter-observer variation than PASI [8]. Furthermore, psoriasis has a major impact on health-related quality of life (QOL) [9] which is not necessarily in proportion to clinical severity [10]. In an effort to yield a comprehensive view of the impact of psoriasis and to complement the PASI, a patient-reported quality of life score is often added to the outcome measures in clinical practice and clinical trials [11,12]. The most widely used measure for assessing quality of life related to psoriasis is the quality of life score Dermatology Life Quality Index (DLQI) [13].

Since clinical severity and quality of life scores are lacking in objectivity and have several limitations in measuring psoriasis severity, more observer-independent methods have been established such as biophysical methods[14-16], histological scores[17-23], measuring epidermal thickness (ET)[17,21,22,24-31], and immunohistochemical markers[15-18,20,22,24-33] from target lesions (Table 1). Biopsies are attractive because they are objective, however, their major limitation is that psoriasis does not resolve in a uniform fashion, and therefore biopsies may not provide a representative sampling of the lesions. Nevertheless, histological changes, decrease in epidermal thickness (ET), and loss of Ki67 in biopsies taken after 30 days of therapy will predict outcome weeks to months later[25].

Insert table 1

The histological assessment grading of psoriasis as suggested by Trozak [19] (Trozak score) includes 10 different histological features used in the diagnosis of psoriasis, not one like ET, and perhaps a more suitable histological assessment tool than ET. However, it has not been widely used in psoriasis research and therefore not validated [14,34-36]. In the Trozak score, each histological feature takes a score of 1, 2 or 3, depending on their histological specificity for psoriasis and relevance to disease activity. Features not specific for psoriasis are given a score of 1, such as elongated rete ridges and perivascular dermal oedema. On the other hand, thinning of the suprapapillary plate and the presence of Munro microabscesses and / or Kogoj pustules, which are more specific for psoriasis take a value of 3. Scores from the 10 variables are then added up to give a total score ranging from 0-19.

The aim of this study was to explore the potential use of the Trozak score as an additional objective assessment tool in psoriasis in research settings, in particular, comparing it with other outcome measures such as epidermal thickness (ET), DLQI and PASI. This study is a substudy of a larger randomized clinical trial that evaluated and compared three different UVB treatment regimens in patients with chronic plaque psoriasis[34], where the more objective histological Trozak score was used in a blinded fashion in an effort to improve the quality of the clinical trial.

Materials and methods

Patients

The Icelandic National Bioethics Committee and the Icelandic Data Protection Authority approved the study protocol. Patients provided written consent to participate in the study. Eligible patients were recruited to the study from September 2009 to May 2010 and followed up for 2 years. Skin biopsies were collected from 21 patients of total 68 patients included in the randomized clinical trial[34]. Key inclusion criteria were: (a) diagnosis of chronic plaque psoriasis, (b) Psoriasis Area and Severity Index score (PASI score)[5] of 7 or higher and c) patients who were non-responsive to topical treatment and were candidates for phototherapy or systemic treatment. Patients with other forms of psoriasis (e.g.

guttate, pustular or erythrodermic) or skin diseases that could interfere with study evaluations, were excluded. All on-going psoriasis treatment was stopped at least 4 weeks prior to inclusion in the study.

Treatment regimens

A total of 68 patients were randomly assigned to three different treatment regimens for 6 weeks; two composed of bathing in geothermal seawater followed by UVB therapy and one treatment regimen of UVB monotherapy. Clinical evaluation with PASI was performed at baseline and week 1, 2, 4, 6 and 10 weeks after beginning the treatment. Quality of life assessment with DLQI was assessed before treatment and after 10 weeks. For the substudy 4-mm punch biopsy from a target lesion was obtained at baseline, week 2 and 6 from the first 21 patients entering the clinical trial. The target lesion was selected as the thickest lesion on the extremities (either leg or arm) and the follow-up biopsies were obtained from the same localization. The clinical scoring and collection of samples were performed by the same dermatologist (the author JHE). The clinical characteristics of these 21 patients is summarized in table 2.

Insert table 2

Outcome measures

Histological assessment

Trozak score Trozak´s histologic grading system for psoriasis [19] was used for histological blinded assessment of the skin biopsies stained with haematoxylin and eosin. It comprises 10 different histomorphological features: elongated rete ridges, club-shaped rete ridges, oedema and elongation of dermal papillae, perivascular infiltrate in the upper dermis, absent granular layer, parakeratosis, thinning of the suprapapillary plate, suprabasal mitosis, the presence of Munro microabscesses and / or Kogoj pustules, each taking a score of 1, 2 or 3, depending on their histological specificity for psoriasis and

relevance to disease activity. The cumulative score (0-19) is recorded for each biopsy (Table 3). The scoring were investigator-blinded (blinded by co-author BAA) and performed by the same investigator (the author JHE) before treatment, after 2 and 6 weeks of treatment.

Insert Table 3

Epidermal thickness (ET). ET is defined as the avarage distance in mm, between the base of stratum corneum and the tip of rete ridges, measured in different locations. In this study ET was measured using a calibrated microscope micrometer in three different locations. All ET measurements were investigator-blinded and performed by the same investigator (BAA) before treatment, after 2 and 6 weeks of treatment.

Immunohistochemistry. The following markers were investigated: CD3, CD4 and CD8 to evaluate T cell infiltration in the skin, and Ki-67-positive keratinocytes to evaluate epidermal proliferation. Ki-67 serves as a marker of proliferative activity in neoplasms and other diseases with excessive cell proliferation such as psoriasis [37]. Sections were cut at 3µ, mounted on starfrost slides and heated for one hour at 60°. After deparaffination they were heated in Envision-Flex Target-Retrieval Solution High pH (DM 828, Dako) for 25 minutes in a water bath. Immunohistochemical staining was done in AutostainerLink 48 (Dako), and a two-step polymer method Envision ™ Flex K8000 (Dako) was used. All antibodies were incubated for 30 minutes. Slides were developed with DAB reagent and counterstained with hematoxylin. Four different antibodies were used: polyclonal rabbit anti- human CD3 (Dako) 1:250., mouse monoclonal anti-human CD4 (Leica Novocastra) 1:25, monoclonal mouse anti-human CD8 (Dako) 1:100, and monoclonal-mouse anti-human Ki-67MIB1 (Dako) 1:200. All antibodies were diluted in Envision-Flex Antibody Diluent (DM830, Dako). The slides were evaluated using Leica Application Suite 3.5.0 and the cells were counted at 400x magnification.

Clinical scores

Psoriasis Area and Severity Index score (PASI score) PASI is the most commonly used clinical score for assessing the clinical severity and extent of psoriasis and the current gold standard. It evaluates severity of the main three clinical signs of psoriasis: erythema, desquamation and infiltration from 0 to 4, weighted by the area of involvement. The whole body is divided into four regions (head, body, upper and lower extremitis separately) weighted according to its approximate percentage of the total body surface area. PASI is expressed in numerical values from 0 to 72 [5].

Lattice system physician´s global assessment score (LS-PGA) LS-PGA was also used as a clinical score to provide additional information. It quantifies psoriasis severity into eight descriptive categories from 'clear' to 'very severe' where it incorporates the involved body surface area (BSA) and the overall plaque morphology [8]. The BSA percentage involved is measured in categories of 0, 1-3, 4-9, 10-20, 21-29, 30-50 and 51-100%. LS-PGA score has been shown to correlate with PASI and studies have shown that the inter-observer variation is lower for LS-PGA compared with PASI [8,38].

The clinical scores were evaluated before treatment, after 2, 6 and 10 weeks by the same investigator (JHE).

Quality of life measures

As clinical assessments alone are not sufficient enough to evaluate psoriasis severity in clinical research [39], the quality of life (QoL) questionnaire Dermatology life quality index (DLQI) was used [13]. It is a 10-item questionnaire that determines whether psoriasis affects patient-reported QoL over the previous week, with overall scores ranging from 0 (not at all) to 30 (very much)[13]. These 10 questions cover 6 domains of health status; symptoms, feelings, daily activities, leisure, work or school,

relationships and side effects from therapeutic management. It was assessed at baseline and after 10 weeks.

Statistical analysis

Efficacy data from all randomised patients were analysed on an intention-to-treat basis. Patients who discontinued study treatment due to unsatisfactory therapeutic effect or who did not follow the study treatment protocol were regarded as treatment failures. For analysis in such cases, missing values were replaced with the most recently available values for all efficacy variables (last observation carried forward). The proportions of patients responding to treatment were compared using the two-sided Fisher´s exact test. Continuous response variables were compared with the use of analysis of variance (ANOVA). Also, we used Pearson´s correlation coefficient to show the correlation between different parameters including all visits. All statistical tests were two sided and performed at an alpha level of 0.05.

Results

Histological response to treatment

Trozak histological score

Untreated patients showed typical histopathological changes for psoriasis patients such as hyperkeratosis, elongated rete ridges, perivascular mononuclear cell infiltrate and Munro abscesses (see Fig.1). Patients showed significant decrease in histologic changes as measured by the Trozak score after only two weeks of treatment, or from 10.3±3.7 to 5.1±4.1 ($p<0.001$; Table 4 and Fig.1). The histological features were further reduced after 6 weeks of treatment or to 3.2±3.3 ($p<0.001$, Table 4). The Trozak score significantly correlated with the PASI score (Pearson´s $r=0.49$, $p<0.0001$, Table 4 and Fig. 2), LS-PGA score (Pearson´s $r=0.48$, $p<0.0001$), epidermal thickness (Pearson´s $r=0.68$, $p<0.0001$) and Ki-67

antigen expression in lesional skin (Pearson´s r=0.28, p<0.05)(Fig. 2). In addition, changes in the Trozak score correlated well with changes in DLQI score (Pearson´s r=0.61, p<0.01).

Insert Fig. 1-2 and Table 4

Epidermal thickness (ET)

ET of untreated lesional psoriasis skin was 397,4 µm on average when all participants were analysed together and significantly decreased to 277 µm after only two weeks of treatment (p<0.01) and to 246,5 µm after six weeks of treatment (p<0.001, Table 4 and Fig. 1). Interestingly, even though there was a significant correlation between ET and the histopathological Trozak score, it did not correlate with either of the clinical scores used in this study; the PASI score (Pearson´s r=0.13, p=0.28) and the LS-PGA score (Pearson´s r=0.20, p=0.10). However, ET correlated significantly with Ki-67 antigen expression (Pearson´s r=0.58, p<0.0001).

Immunohistochemical staining for CD3, CD4, CD8 and Ki-67 antigen expression

Before treatment 65.3 epidermal cells stained positive for Ki-67 antigen expression per field in lesional psoriatic skin when all participants were analysed together. No significant difference was found after two weeks of treatment but after six weeks the expression decreased significantly to 40.3 positive cells per field (p<0.01; Table 4 and Fig. 1). There was a significant difference in the amount of $CD3^+$, $CD4^+$ and $CD8^+$ positive epidermal T-lymphocytes before and after six weeks of treatment in lesional skin (p<0.01, Table 4). No immunohistochemical staining was performed for CD3, CD4 and CD8 on biopsies taken after two weeks of treatment, only after six weeks. CD3, CD4 and CD8 expression did not correlate with the PASI score, however, Ki-67 antigen expression shows weak significant correlation with the PASI score (Pearson´s r=0.28, p=0.025) and the LS-PGA (Pearson´s r=0.37, p<0.01). As mentioned before, Ki-67 expression shows weak correlation with the Trozak´s score (Pearson´s r=0.28,

p<0.05) and strong correlation with ET (Pearson´s r=0.58, p<0.0001). No significant difference was observed when the treatment groups were compared with each other.

Clinical efficacy

PASI score changed significantly after only two weeks of treatment from 12,9 to 7,1 (p<0,001) on average for all participants analysed together and after six weeks of treatment the PASI score has reduced to 4,7 (p<0,001, Table 4 and Fig.1). The LS-PGA score shows same reduction as the PASI and there is a high correlation between the two clincal scores (Pearson´s r=0.88, p<0.0001; Table 4). The PASI score showed strong correlation with the reduction of Trozak score (Pearson´s r=0.41, p<0.001) but no correlation with ET (Pearson´s r=0.13, p=0.28).

Quality of life assessment

DLQI baseline scores were significantly higher before the treatment compared with after 10 weeks for all treatment groups analysed together (Table 4). Five patients out of twenty one achieved a DLQI score of 0 or 1 by week 10. As mentioned before changes in the DLQI scores correlated well with changes in the clinical scores, the PASI score (Pearson´s r=0.48, p<0.01) and the LS-PGA score (Pearson´s r=0.46, p<0.01). The reduction of DLQI correlated well with the reduction of the Trozak score (r=0.61, p<0.01)

Discussion

The potential of the histological Trozak score of psoriasis has been explored, following the hypothesis that it may be an additional objective measure of disease severity in research settings. Although histological normalization in an index plaque does not always correlate with global clinical improvement here we show that the Trozak score has a strong correlation with two clinical severity scores, PASI and LS-PGA. In addition, the Trozak score correlated well with ET in lesional skin and has a weak but significant correlation with Ki-67 antigen expression in lesional skin and DLQI. Interestingly, we found weak correlation of DLQI with the PASI, which is used as an almost universal outcome measure in psoriasis trials. This finding is consistent with other studies [7].

Histopathology is not often used to quantify inflammatory skin disease. Nevertheless it has been suggested as a more observer-independent assessment tool than clinical assessment, as clinical severity scores may have innate limitations when used alone [3]. For example, it has been clearly established in clinical trials that the PASI is not sensitive to measure therapeutic response in those patients with low PASI score (<3)[8]. Target lesion severity score, supplemented by physician global assessment and quality of life measures, is the suggested current standard in those cases[12]. Few studies use histopathological assessment as a secondary outcome measure in combination with clinical score[14,17,18,20-23,26,27,34,35] but the data is conflicting because there is no uniformity in the techniques used and the assessment is often made at different time points which makes comparison difficult. The Trozak score is not widely used in psoriasis research today and only a few researchers have used it in their studies [14,34-36]. However, some previous studies have used a quantitative histological grading system as a severity assessment tool for psoriasis, which is similar to the Trozak score[17,18,20,22,23]. Others use no scoring system, only a general histopathological examination which makes correlation with clinical severity of the disease and comparison with other studies difficult[26,27].

The most commonly used histopathological outcome measures in clinical trials are ET, markers for epidermal proliferation (Ki-67) and immunohistochemical scoring of T cell activity in lesional skin before and after treatment (Table 1). There is conflicting data whether these findings correlate with

clinical psoriasis scoring systems, where some studies show correlation[26,29,32,33] and others do not[16,18,28,30,31]. Of these markers, there seems to be most convincing data for ET and Ki-67 antigen expression[14,26,29,33]. We found weak correlation between Ki-67 positive cells in lesional skin and PASI before and after treatment which is consistent with *Jesionek-Kupnicka et al*[33], *Kvist et al*[26] and *Reddy et al*[29]. *Morsy et al* found correlation of DLQI and Trozak score with ET, but surprisingly no correlation between ET and PASI which is in an agreement with our results [14]. They do not mention any correlation analysis of the Trozak score and PASI, however we showed strong correlation between PASI and Trozak score in our study [34]. In addition, we did not find any correlation between changes of ET in lesional skin and changes in PASI with treatment which is also consistent with previous studies [14,30,31]. This may indicate that the Trozak score reflects disease severity better than ET, both before and after therapy, possibly because it takes into consideration 10 different histological variables instead of one, making the score more sensitive.

These are only speculations because none of these methods, including ET, have been validated and therefore difficult to hypothesize which method is most sensitive to the degree of psoriatic change present at the time of biopsy. Some researcher that have validated ET in psoriasis patients before and after treatment, conclude that this measurement is not suitable for clinical studies do to variety of ET between lesions sites and different persons[40,41]. Our ET measurement showed thicker ET in untreated lesions than in treated lesions which was 397,4 µm before treatment and 246,5 µm after six weeks of treatment attributed to the normalization of the epidermis. These observations are in good agreement with previous studies that show ET of untreated lesions to be 266.7-352.5 µm and after treatment 312-131 µm[14,29,42].

Immunological markers for T cell activity are frequently used as an additional outcome measure of psoriasis[18,20,28,30,31], however there is not strong evidence that these markers are represantative of psoraisis activity where only few studies have found correlation with clinical severity scores[29,32]. Our result confirm these results from previous studies where we found no correlation between CD3, CD4 and CD8 positive T cells in the psoriatic skin and PASI or LG-PSA . Researchers are now more interested in monitoring the immune response in psoriasis with measurements in the blood which is of

course much less invasive than biopsy. However, though few markers are promising, like IL17 levels in the blood[43], no reliable markers have been established yet. Until such markers have been identified, we porpose the use of Trozak score as an additional objective outcome assessment in psoriasis research settings.

In conclusion, more observer-independent assessment tools could improve the quality of randomized controlled psoriasis trials (RCT) to assess psoriasis with more objectivity. Although the material is small the results indicate that the histological score of Trozak is a potential objective assessment tool for showing psoriasis severity in combination with the gold standard clinical severity PASI score and DLQI score in research settings.

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Declaration of interest

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Tables and Figures

Table 1. Published studies that uses histological changes or markers an assessment tool for psoriasis

STUDY	YEAR	COUNTRY	OUTCOME MEASURES				MARKERS THAT CORRELATED WITH CLINICAL SEVERITY
			CLINICAL	QOL	HISTOLOGICAL MARKERS	OTHER MARKERS	
Papp et al	2015	Canada	PASI sPGA BSA	NO	ET IHC: Ki-67, CD3, CD11	NO	NR
Kim et al	2015	Korea	PSI	NO	Their own histological grading score (0-40)	NO	NO
Sofen et al	2014	U.S.A.	PASI	NO	ET IHC: CD3, CD11c, K16	Serum cytokines and chemokines	Serum IL-17A
Jesionek-Kupnicka et al	2013	Poland	PASI	NO	IHC: Ki-67	NO	Ki-67
Wada et al	2012	U.S.A.	PASI sPGA	NO	Their own histological grading score (1-3) IHC: CD3, CD11c, K16	RT-PCR Cytometry Cytokine production	Histological score
Wolberink et al	2012	The Netherlands	NR	NO	HC: CD1a, CD3, filaggrin, CD31, Ki-67, K16	Reflectance confocal microscopy of histological changes	NR
Soyland et al	2011	Norway	PASI	NO	ET IHC: CD1a, CD4, CD8, FOXP3	Immunofluorescence RT-PCR Cytometry	NO
Gamblicher et al	2011	Germany	PASI	NO	Their own histological grading score (0-4) IHC: CD1a, CD4, CD8, Involucrin, Ki-67	NO	NO
Johnson-Huang et al	2010	U.S.A.	PASI	NO	ET IHC: K16, CD1c and more	Immunofluorescence Flow cytometry RT-PCR	NO
Reddy et al	2010	U.S.A.	PASI PGA	NR	ET IHC: Ki-67, CD3	Serum cytokines and chemokines	ET, Ki-67 and CD3
Morsy et al	2009	Denmark	PASI SAPASI	DLQI PLSI	ET with optical coherence tomography Trozak score	NO	ET correlated with SAPASI, DLQI, PLSI and Trozak, not PASI
Kvist et al	2009	Denmark	TCS	NO	ET, Epidermal morphology IHC: CD3, CD4, CD8, CD45RO, K10, K16, Ki-67	NO	ET, Ki-67 and K16
Werner et al	2008	Brazil	PASI	NO	ET Their own histological grading score IHC: CD1a, Ki-67, K10, K16, K19	NO	NR
Zaba et al	2007	U.S.A.	PASI	NO	ET Their own histological response score IHC: Ki-67, K16, CD11c, CD3, CD163	Immunofluorescence Flow cytometry RT-PCR	
Lago et al	2006	Brazil	PASI	NO	ET, presence of Munro's abscesses and/or pustulae of Kogoj IHC: K10, K14, K16	NO	NR
Ormerod et al	2005	U.K.	Their own score	NO	ET with ultrasound IHC: Ki-67, CD1a, CD4, CD8, CD68	Erythema measured with pulsed A-scan ultrasound	NR
Bovenschen et al	2005	The Netherlands	The SUM score PASI	NO	IHC: Ki-67, CD4, CD8, CD45RO	NO	Epidermal T-cell markers correlated with the SUM score
Elias et al	2003	U.S.A.	PASI	NO	Their own histological grading score (0-4) ET IHC: CD1a	NO	NR
Krueger et al	1995	U.S.A.	NR	NO	ET IHC: Ki-67, K16, filaggrin, CD3, CD4, CD8, CD25, IGF-1R, α3-integrin	Cell culture analysis	NR

NR, not reported; PASI, Psoriasis Area and Severity Index; SAPASI, self-administered PASI; sPGA, static Physician's Global Assessment; TCS, Total Clinical Score; PSS, Psoriasis Severity Scale; QOL, quality of life; DLQI, Dermatology Quality of Life Index; PLSI, psoriasis life stress inventory index; ET, Epidermal Thickness; IHC, immunohistochemistry; CD1a, Langerhans cells; CD3, a marker of T cells; CD4, a marker of T helper cells; CD8, a marker of cytotoxic T cells; CD45RO, a marker of memory T cells; CD11c, a marker of dendritic cells; CD16I, a marker of neutrophils; CD163, a marker of macrophages; K10, keratin 10 (keratinocyte differentiation marker); K16, keratin 16 and Ki-67 (keratinocyte proliferation markers)

Table 2. Clinical characteristics of patients included in the sub-study

Characteristics	GSW (N= 7)	IT-GSW (N=7)	UVB (N=7)	P Value †
Age - yr	46.1 (±10.8)	38.4 (±16)	37.9 (±14.4)	0.37
Male sex - no. (%)	3 (43%)	2 (29%)	3 (43%)	0.82
Body mass index (BMI)	32 (±5)	30.2 (±5.4)	28.8 (±7.1)	0.96
Duration of psoriasis - yr	23 (±14)	18.8 (±11)	12.3 (±8.1)	0.09
Participants with psoriatic arthritis - no.(%)	0 (0%)	1 (14%)	0 (0%)	0.71
Participants with nail psoriasis - no. (%)	3 (43%)	3 (43%)	1 (14%)	0.61
Psoriasis area and severity index (PASI)	13.8 (±5.2)	11.6 (±6.2)	11.1 (±4.9)	0.22
Lattice system physician´s global assessment	severe	severe	severe	1.00
Dermatology Life Quality Index score	7 (±4.2)	11.6 (±6.2)	8.3 (±5.1)	0.038*
Participants treated previously - no.(%)				
Blue Lagoon	5 (71%)	4 (57%)	2 (29%)	0.57
Topical agent	6 (86%)	7 (100%)	7 (100%)	1.00
Phototherapy	7 (100%)	7 (100%)	6 (86%)	0.51
Systemic therapy	1 (14%)	0 (0%)	0 (0%)	0.61
Smoking	3 (43%)	3 (43%)	2 (29%)	0.77
Family history	6 (86%)	4 (57%)	4 (57%)	0.35

Table 3. Trozak´s histologic grading system for psoriasis

Name of Study: _____

Slide Accession Number: _____

HISTOLOGIC GRADING SYSTEM FOR PSORIASIS

Microscopic Criteria		*Value/Criteria*	*Score*
1. Regular elongation of the rete ridge		1	
2. Club shaped rete ridges		2	
3. Elongation and edema of the dermal papillae		1	
4. Perivascular mononuclear infiltrate in the upper dermis of papillae		1	
5. Absent granular layer	a. focal	1	
	b. total	2	
6. Parakeratosis	a. focal	1	
	b. total	2	
7. Suprapapillary plate thinning		2	
8. Mitosis above basal cell layer		2	
9. Munro microabscesses		3	
10. Spongiform pustule		3	
	Score total:	19	
Epidermal Thickness			
Suprabasilar Mitosis Average per 8 HPF			

Comments: _____

_____

Investigators Signature _____ Date _____

Adapted from (Trozak, 1994) original paper.

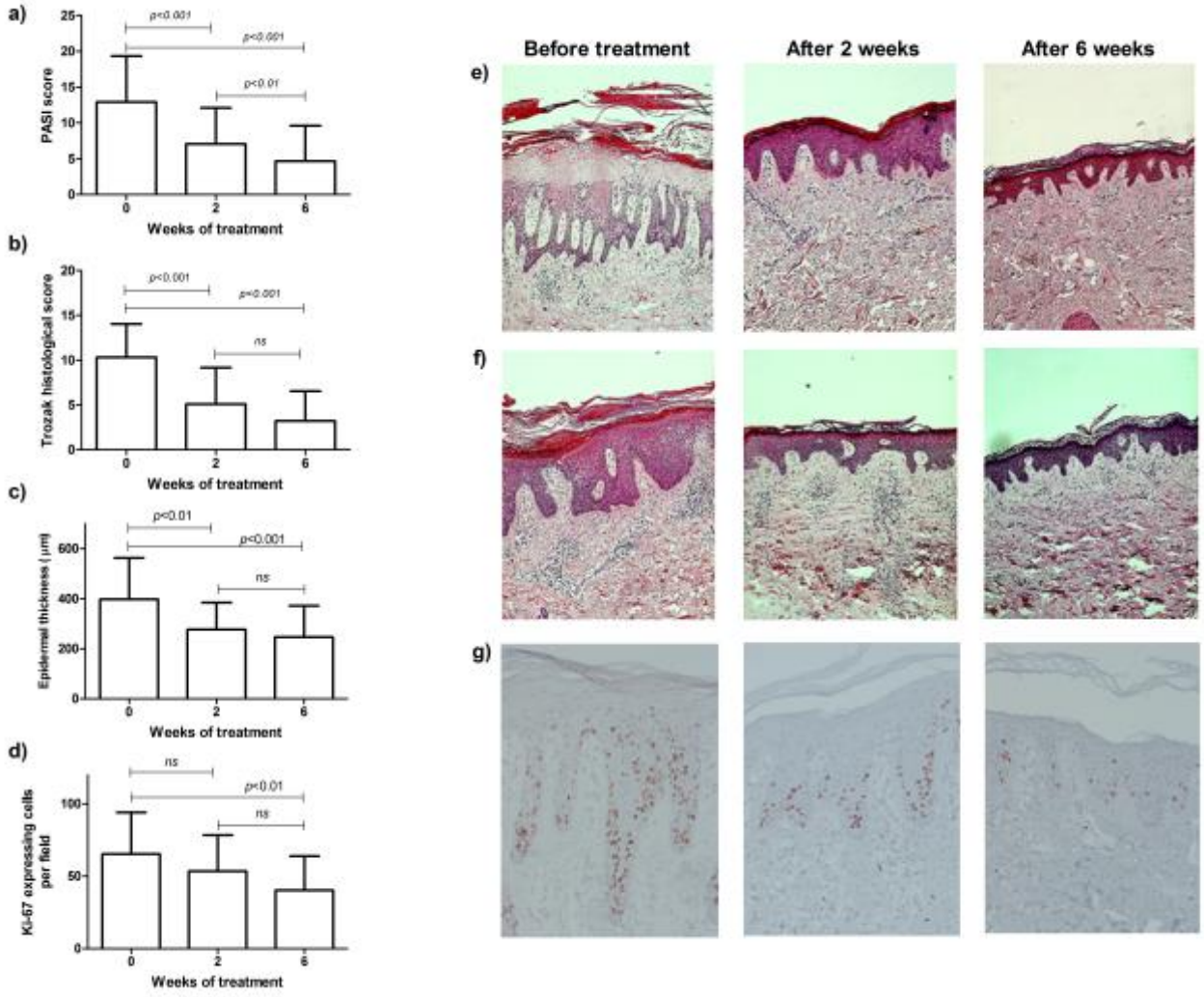

Figure 1: a) Patients showed significant decrease in psoriatic changes with treatment as measured by a) the PASI score, b) the Trozak score, c) epidermal thickness (ET) and d) Ki-67 expression in epidermis. Representative photographs from e) one patient in the GSW group and f) one patient from the IT-GSW group and g) Ki-67 expression with treatment. Data is representated as mean ±SD. *ns* = non significant.

	Before treatment (n =21)	After 2 weeks (n =21)	*p* value	After 6 weeks (n =21)	*p* value	Correlation with PASI; *r*	*p* value
Biopsy grading (Trozac score)	10,3	5,1	< 0,001	3,2	< 0,001	0,41	< 0,001
Epidermal thickness (µm)	397,4	277	< 0,01	246,5	< 0,001	0,13	ns
Ki-67 (positive cells/field)	65,3	53,4	ns	40,3	< 0,01	0,28	0,025
CD3 (positive cells/field)	134,8	x	x	47,8	< 0,01	0,27	ns
CD4 (positive cells/field)	74	x	x	22	< 0,001	0,16	ns
CD8 (positive cells/field)	56,6	x	x	17,9	< 0,01	0,36	ns
PASI score	12,9	7,1	< 0,001	4,7	< 0,001	x	x
LS-PGA score	5	3	< 0,001	2,3	< 0,001	0,88	< 0,0001
DLQI score	10	x	x	6,1**	< 0,01	0,48	< 0,01

Table 4. Mean values for all variables used in the study. * p value compared with before treatment. Statistically significant difference at p <0.05. **DLQI after 10 weeks, not 6 weeks. r: Pearson correlation coefficient.

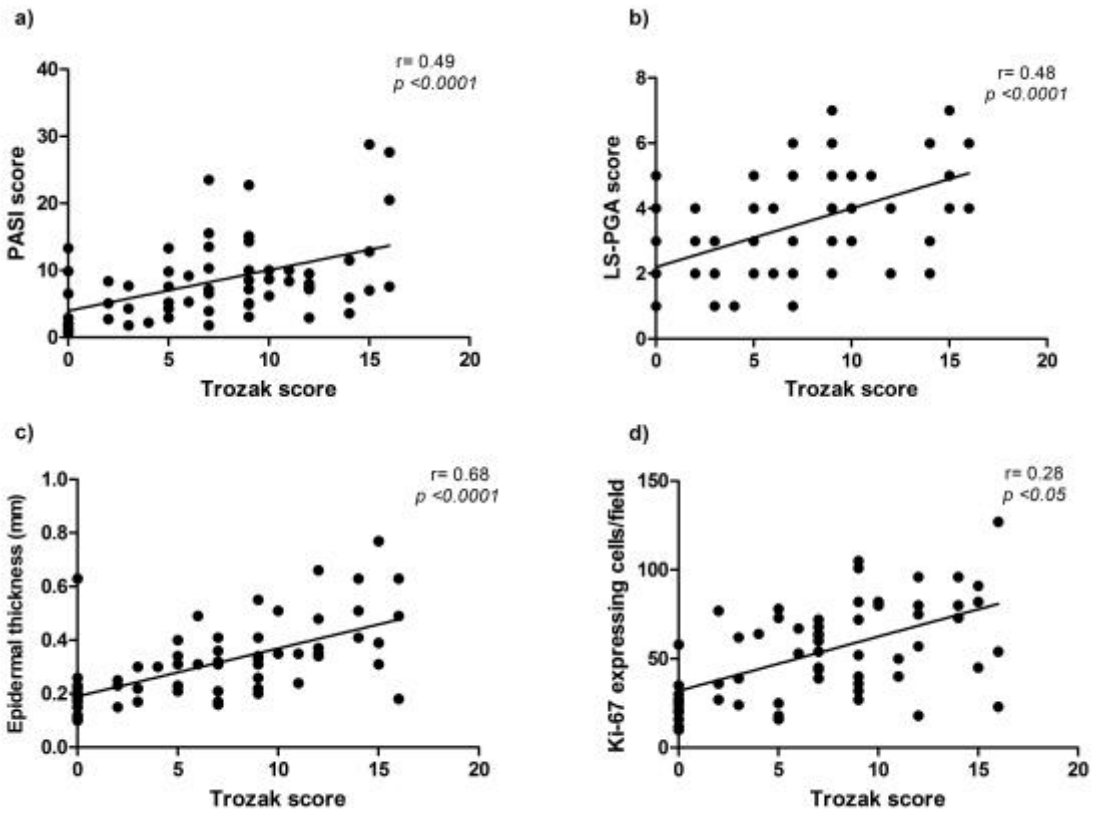

Figure 2: Correlation of a) the PASI score, b) the LS-PGA score, c) epidermal thickness of lesional skin and d) Ki-67 antigen expression in the epidermis of lesional skin with Trozak's histological score

7 Appendix

7.1 S1. Trozak´s histologic grading system for psoriasis

Name of study: _____

Slide accession number: _____

HISTOLOGIC GRADING SYSTEM FOR PSORIASIS

MICROSCOPIC CRITERIA		VALUE/CRITERIA	SCORE
Regular elongation of the rete ridge		1	
Club shaped rete ridges		2	
Elongation ad edema of the dermal papillae		1	
Perivascular mononuclear infiltrate in the upper dermis of papillae		1	
Absent granular layer	a. focal	1	
	b. total	2	
Parakeratosis	a. focal	1	
	b. total	2	
Suprapapillary plate thinning		2	
Mitosis above basal cell layer		2	
Munro microabscesses		3	
Spongiform pustule		3	
Score total:		**19**	

Epidermal thickness	
Suprabasilar mitosis average per 8 HPF	

Comments: _____

_____

Investigators signature: _____ Date: _____

Adapted from (Trozak, 1994) original paper.

7.2 S2. Icelandic translation of the Dermatology Quality of Life Index (DLQI)

LÍFSGÆÐAKÖNNUN FYRIR HÚÐSKJÚKDÓMA DLQI

BLUE LAGOON ICELAND

Markmið þessa spurningalista er að mæla hve mikil áhrif húðvandamál þitt hefur haft á líf þitt undanfarna viku. Merktu við einn reit við hverja spurningu.

1.	Undanfarna viku, hve mikill **kláði, eymsli, verkir** eða **stingir** hafa verið í húðinni?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki
2.	Undanfarna viku, hve mikið hefur þú verið **vandræðaleg(-ur)** eða **sjálfsmeðvituð(-aður)** vegna húðar þinnar?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki
3.	Undanfarna viku, hve mikið hindraði húðin að þú færir að **versla**, eða sinntir **heimilinu** eða **garðinum**?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki ☐ Á ekki við
4.	Undanfarna viku, hve mikið hefur húð þín haft áhrif á hvaða **fötum** þú klæðist?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki ☐ Á ekki við
5.	Undanfarna viku, hve mikið hefur húð þín haft áhrif á **félagslíf** þitt eða **tómstundir**?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki ☐ Á ekki við
6.	Undanfarna viku, hve mikið gerði húðin þér erfitt fyrir að stunda einhverja **íþrótt**?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki
7.	Undanfarnaviku, hefur húð þín komið í veg fyrir að þú stundir **vinnu** eða **nám**?	☐ Já ☐ Nei	
	Ef „nei", undanfarna viku, hve mikið hefur húð þín verið vandamál í **vinnu** eða **námi**?	☐ Mikið ☐ Dálítið ☐ Alls ekki	
8.	Undanfarna viku, hve mikið hefur húð þín skapað vandamál hjá þér og **maka** þínum eða einhverjum af **nánum vinum** eða **ættingjum**?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki ☐ Á ekki við
9.	Undanfarna viku, hversu miklum erfiðleikum hefur húð þín valdið þér í **kynlífi**?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki ☐ Á ekki við
10.	Undanfarna viku, hverju mikil vandamál hefur **meðferð** húðarinnar skapað þér, til dæmis með því að smita frá sér eða taka of langan tíma?	☐ Mjög mikið ☐ Mikið	☐ Dálítið ☐ Alls ekki ☐ Á ekki við

Gakktu úr skugga um að þú hafir svarað öllum spurningunum.
Takk fyrir.

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