In depth analyses of peripheral blood immune cell populations in patients with psoriasis - effect of biological treatment and alternative medicine

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Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

The following doctoral work was conducted in the period 2018 to 2023 at the Broegelmann Research Laboratory, Department of Clinical Science, Faculty of Medicine, University of Bergen, Norway. Throughout my work, I have been enrolled at the Bergen Research School of Inflammation. This project was guided by my main supervisor Professor Silke Appel and co-supervisors Professor Roland Jonsson and Associate Professor Silje Michelsen Solberg.

The flow cytometry experiments were performed at the Flow Cytometry Core Facility, Department of Clinical Science, University of Bergen.

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Abbreviation

APC	Antigen presenting cell
AHR	Aryl hydrocarbon receptor
BMI	Body mass index
CCL2	Chemokine (C-C motif) ligand 2
CD	Cluster of differentiation
cDC	Conventional dendritic cells
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCR3	C-X-C chemokine receptor type 3
DC	Dendritic cells
DLQI	Dermatological life quality index
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia
EPA	Eicosapentaenoic acid
FBXL19	F-box, leucine rich repeat protein 19 gene
FC	Flow cytometry
FCS	Fetal calf serum
HC	Healthy controls
HLA	Human leukocyte antigen
HRO	Herring roe oil
i.e.	Id est
IFN	Interferon
IFX	Infliximab
IL	Interleukin
IL12B	Interleukin 12 subunit beta gene
IL23A	Interleukin 23 subunit alpha gene

IL23R	Interleukin 23 receptor gene
ILC	Innate lymphoid cell
JAK	Janus kinase
KC	Keratinocyte
KLF4	KLF transcription factor 4 gene
LL37	Cathelicidin
MFI	Median fluorescence intensity
MI	Myocardial infarction
MPA	Monocyte-platelet aggregates
MTX	Methotrexate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFKBIA	NFKB inhibitor alpha gene
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
PASI	Psoriasis area and severity index
pDC	Plasmacytoid dendritic cells
PBMC	Peripheral blood mononuclear cells
PsA	Psoriatic arthritis
PsO	Psoriasis
PUVA	Psoralen and ultraviolet A
REL	REL proto-oncogene, NF-KB subunit gene
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RORγt	Retinoid-acid receptor-related orphan receptor gamma T
STAT	Signal transducer and activator of transcription
Tc	Cytotoxic T cell
TGF-β	Transforming growth factor beta
Th	Helper T cell
TNIP1	TNFAIP3 interacting protein 1 gene

TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFAIP1	Tumor necrosis factor, alpha induced protein 1 gene
TNFAIP3	Tumor necrosis factor, alpha induced protein 3 gene
Tregs	Regulatory T cell
Trm	Tissue-resident memory T cell
TYK	Tyrosine kinase
TYK2	Tyrosine kinase 2 gene
UV	Ultraviolet light

Abstract

Psoriasis is a chronic immune-mediated disease of the skin with systemic inflammation and accompanied comorbidities. Although the disease severity may vary over time, most patients with psoriasis suffer from mild to moderate disease. In many cases, local treatment will be sufficient to control the symptoms, but at the cost of several side effects. ω-3 poly-unsaturated fatty acids (PUFA) have shown promising results in clinical trials with mild-to-moderate psoriasis. Up to one-third of patients with psoriasis are affected with psoriatic arthritis (PsA). Targeted treatment with biologic therapies has transformed the management of both diseases. However, despite the success of therapy for some patients the existence of patients whose symptoms do not improve with applied treatment, highlight the needs for predictive biomarkers of response and more targeted treatment approach that should improve patient care and deliver substantial economic savings.

The general aim of this dissertation was to explore the systemic immune response through clinical and biological investigation and to identify disease-specific immune profiles and indicators in patients with psoriasis of different disease severity, treated with alternative medicine and biological treatment. In study I, we explored the impact of phospholipid bound docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contained in herring roe oil (HRO) on circulating immune cell activity and plasma cytokine levels in non-severe plaque psoriasis. The plasma concentrations of cytokines were measured by Luminex technology and circulatory immune cell activity was analyzed by multicolor flow cytometry. In study II, we employed flow cytometry to perform in-depth phenotyping of peripheral blood mononuclear cells (PBMCs) and examine their respective activity through the expression of cell surface markers in healthy controls and patients with moderate to severe plaque psoriasis/PsA on stable anti-tumor necrosis factor (TNF) treatment. In study III, we performed immune analyzes of dendritic cell (DC) populations in patients with moderate to severe plaque psoriasis/PsA, before and during therapy with the anti-TNF drugs infliximab (IFX), etanercept, the anti-IL-17A secukinumab, and the anti-IL12/IL-23 ustekinumab, to identify immune cell populations/subsets relative to clinical response. In all studies,

clinical and standard laboratory parameters were incorporated in the analyses. In study I, positive clinical outcome of ω-3 PUFAs in patients with psoriasis was possibly related to the decreased levels of CCL2 and increased levels of IFN-γ over time. Additionally, reduced activity due to lower expression of CD38 on $CD4^+$ and $CD8^+$ T cells, and $CD56^{bright}$ NK cells supported the beneficial effect of HRO supplementation. In study II, patients with psoriasis or/and PsA on stable biological treatment with infliximab (IFX) still retain imprint from the disease and have phenotypic peculiarity. The increase in active intermediate $CD14^+CD16^+$ monocytes reflected the preserved ongoing systemic inflammation. However, the beneficial effect of IFX treatment was reflected in reduced cytotoxicity of NK cells in both patient groups and $CD8^+$ T cells in patients with PsA. In study III, explored frequencies of DC populations and their subsets differed in patients compared to controls as well as patients with psoriasis compared to PsA, but mostly did not change upon treatment. However, the persisting low levels of pDC in peripheral blood in patients with PsA might relate to the presence of arthritis and should be further investigated. Sustained reduction of $CD5^+$ DC2 subset in secukinumab-treated patients could probably be related to the presence of PsA and lower treatment rensponsiveness.

Further studies of cytokines and peripheral blood mononuclear cells may be of great importance in the further stratification of patients for appropriate therapeutic interventions. More personalized treatment can improve quality of life and change the course of comorbidities.

Abstrakt

Psoriasis er en kronisk immun-mediert sykdom i huden med systemisk betennelse og ledsagende komorbiditeter. Selv om alvorlighetsgraden av sykdommen kan variere over tid, lider de fleste pasienter med psoriasis av mild til moderat sykdom. I mange tilfeller vil lokal behandling være tilstrekkelig for å kontrollere symptomene, men på bekostning av flere bivirkninger. ω-3 flerumettede fettsyrer (PUFA) har vist lovende resultater i kliniske studier med mild til moderat psoriasis. Opptil en tredjedel av pasienter med psoriasis er rammet av psoriasisartritt (PsA). Målrettet behandling med biologiske terapier har forvandlet behandlingen av begge sykdommene. Til tross for suksessen med terapi for noen pasienter, fremhever imidlertid eksistensen av pasienter hvis symptomer ikke blir bedre med anvendt behandling, behovet for prediktive biomarkører for respons og mer målrettet behandlingstilnærming som bør forbedre pasientbehandlingen og gi betydelige økonomiske besparelser.

I studie I undersøkte vi virkningen av fosfolipidbundet dokosaheksaensyre (DHA) og eikosapentaensyre (EPA) i silderognolje (HRO) på sirkulerende immuncelleaktivitet og plasmacytokinnivåer ved ikke-alvorlig plakkpsoriasis. Plasmakonsentrasjonene av cytokiner ble målt med Luminex-teknologi og sirkulatorisk immuncelleaktivitet ble analysert ved flerfarget flowcytometri. I studie II brukte vi flowcytometri for å utføre dyptgående fenotyping av perifere blodmononukleære celler (PBMC) og undersøke deres respektive aktivitet gjennom ekspresjon av celleoverflatemarkører i friske kontroller og pasienter med moderat til alvorlig plakkpsoriasis/PsA på stabil anti-tumornekrosefaktor (TNF) behandling. I studie III utførte vi immunanalyser av dendritiske cellepopulasjoner (DC) hos pasienter med moderat til alvorlig plakkpsoriasis/PsA, før og under behandling med anti-TNF-medisinene infliksimab (IFX), etanercept, anti-IL-17A secukinumab, og anti-IL12/IL-23 ustekinumab, for å identifisere immuncellepopulasjoner/undergrupper i forhold til klinisk respons. I alle studiene ble kliniske og standard laboratorieparametre inkorporert i analysene. I studie I var positivt klinisk utfall av ω-3 PUFA hos pasienter med psoriasis muligens relatert til de reduserte nivåene av CCL2 og økte nivåer av IFN-γ over tid. I tillegg støttet redusert aktivitet på grunn av lavere ekspresjon av CD38 på $CD4^+$ and $CD8^+$ T celler,

og $CD56^{bright}$ NK celler T-celler den gunstige effekten av HRO-tilskudd. I studie II beholder pasienter med psoriasis eller/og PsA på stabil biologisk behandling med infliksimab (IFX) fortsatt spor av sykdommen og har fenotypiske egenskaper. Økningen i aktive mellomliggende $CD14^{+}CD16^{+}$ monocytter reflekterte den bevarte pågående systemiske betennelsen. Den gunstige effekten av IFX-behandling ble imidlertid reflektert i redusert cytotoksisitet av NK-celler i begge pasientgruppene og $CD8^{+}$ T-celler hos pasienter med PsA. I studie III skilte utforskede frekvenser av DC-populasjoner og deres undergrupper seg hos pasienter sammenlignet med kontroller, så vel som pasienter med psoriasis sammenlignet med PsA, men endret seg stort sett ikke ved behandling. De vedvarende lave nivåene av pDC i perifertblod hos pasienter med PsA kan relateres til tilstedeværelsen av leddgikt og bør undersøkes videre. Vedvarende reduksjon av $CD5^{+}$ DC2 hos secukinumab-behandlede pasienter kan sannsynligvis være relatert til tilstedeværelsen av PsA og lavere behandlingsrespons.

Ytterligere studier av cytokiner og perifere mononukleære blodceller kan være av stor betydning i den videre stratifiseringen av pasienter for passende terapeutiske intervensjoner. Mer personlig behandling kan forbedre livskvaliteten og endre forløpet av komorbiditeter.

List of Publications

I **Petrovic A**, Bueide I, Tveit KS, Hallaråker H, Bjørndal B, Holmes TD, Davies R, Brokstad KA, Bergum B, Appel S. Herring roe oil in treatment of psoriasis – influence on immune cells and cytokine network.

- Manuscript

II **Petrovic A**, Samuelsen VM, Holmes TD, Sarkar I, Davies R, Bergum B, Jonsson R, Sandvik LF, Solberg SM, Appel S. Immune cell activity during anti-TNF treatment in patients with psoriasis and psoriatic arthritis.

- Manuscript

III **Petrovic A**, Ten Bergen LL, Solberg SM, Sarkar I, Bergum B, Davis R, Jonsson R, Appel S. Biological treatment in severe psoriasis – influence on peripheral blood dendritic cells.

- Manuscript

Contents

1. Introduction

1.1 Background psoriasis

Psoriasis is a complex, lifelong skin disease associated with other medical conditions including psoriatic arthritis (PsA), cardiometabolic diseases, chronic kidney disease, gastrointestinal diseases, and psychosocial disorders[1].

1.2 Epidemiology

The disease affects at least 125 million individuals worldwide making psoriasis a serious global problem[2]. The worldwide prevalence ranges from 2–4%, rising up to 9.7% in Scandinavian countries[3]. Disease is equally prevalent in men and women and can appear at any age[4]. Based on the genetic and immunological characteristics, psoriasis debut is bimodal, before the age of 40 - early onset (type I), and after the age of 40 - late onset (type II). Patients with early onset of disease generally have a family history with psoriasis in first-degree family members and more severe disease[5].

1.3 Clinical presentation

Clinical heterogeneity of the disease is not only reflected in different presentations such as plaque, guttate, intertriginous, pustular, nail, erythrodermic psoriasis and PsA (Figure 1), but in highly variable morphology, distribution, symptoms (itch, burning), severity and simultaneously appearance of two or more clinical types [4, 6]. The most common form, chronic plaque, or psoriasis vulgaris, is present in 85-90% of patients with symmetrically distributed, sharply circumscribed red plaques covered with silver, non-adherent and dry scales (Figure 1A-C)[3, 6]. Plaques usually affect the extensor surfaces of legs and arms, lumbosacral and periumbilical area, scalp and retro-auricular regions[4, 6]. The skin lesions can gradually spread and affect more than 90% of skin surface developing into erythrodermic psoriasis, potentially life-threatening skin condition due to prominent, generalized erythema and exudative exfoliation (Figure 1E)[4, 6, 7].

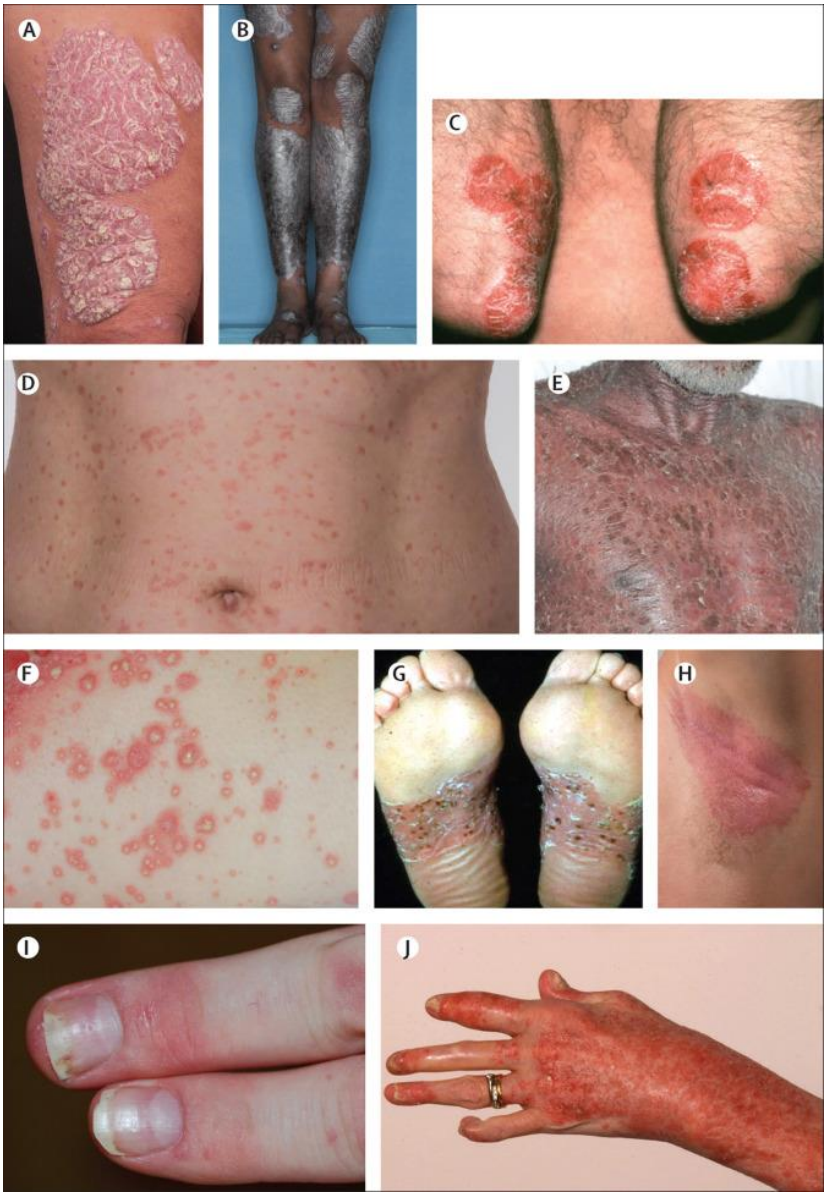

Figure 1. Various clinical presentations of psoriasis and its subtypes (A) A well demarcated, pink plaque on white skin. (B) On black skin, the plaques are grey. (C) Symmetry of plaques is characteristic. (D) Small centripetal papules in guttate psoriasis. (E) Erythroderma. (F) Generalised pustular psoriasis. (G) Palmoplantar pustulosis. (H) Flexural or inverse psoriasis with absence of scale. (I) Nail pitting and onycholysis. (J) Psoriatic arthritis with dactylitis and nail changes.

Reprint from the Lancet, Volume 397, Griffiths C. E. M. et al, Psoriasis, pages 1301-1315.[6]

Disease can appear as guttate psoriasis characterized by small tear-shaped papules, typically in children and young adults, most often after a previous streptococcal throat infection (Figure 1D)[6, 7]. In pustular psoriasis, sterile pustules can affect larger skin areas (generalized pustular psoriasis, von Zumbusch) or limited areas in palmoplantar psoriasis (Figure 1F, G)[4, 6, 7]. Inverse (flexural), red, shiny, well-demarcated plaques of psoriasis without scales appears in folds, especially axillary, inframammary, inguinal and, intergluteal (Figure 1H)[4, 6]. Psoriatic nail disease in form of small pits in the nail plate, distal or lateral onycholysis (Figure 1I), "oil spots" due to subungual hyperkeratosis is usually associated with palmoplantar pustulosis and vulgar psoriasis[4, 6]. Onycholysis doubles the risk of PsA, which is present in almost 30% of psoriasis patients as a chronic, progressive inflammatory, seronegative arthritis that leads to permanent erosions, joint destruction, and disability (Figure 1J)[8, 9]. PsA is usually lagging in onset behind the skin psoriasis by 10 years[10]. Usually, disease affects the distal interphalangeal joints asymmetrically, and occasionally axial structures. Other manifestations of PsA include enthesitis and dactylitis[10].

1.4 Assessment of disease severity

The severity of psoriasis can vary over time, although about 80% of patients have mild disease[11]. Psoriasis is usually divided into mild, moderate, or severe based on measurements made with common tools such as Psoriasis Area and Severity Index (PASI). It combines the severity of skin lesions (erythema (E), induration (I) and desquamation (D)) estimated on a scale from 0-4 (max) and percentage of affected area (A) over 4 body regions (head (h), upper extremities (u), trunk (t) and lower extremities (l)) transformed into a grade from 0 to 6 (0, < 10, 10–29, 30–49, 50–69, 70–89, 90–100% of involved area) into a unique score ranging from 0 to 72[12]. Given that the head and neck correspond to 10%, the upper extremities 20%, the trunk 30%, and the lower extremities 40% of the total body surface, the PASI score is calculated by the formula: PASI = 0.1 x (Eh + Ih +Dh) x Ah + 0.2 x (Ea + Ia + Da) x Aa + 0.3 x (Et + It + Dt) x At + 0.4 x (El +Il +Dl) x Al[12].

a

▾parameter
0 = none
1 = slight
2 = moderate
3 = striking
4 = exceptional striking

*area factor
1 = <10%
2 = 10-29%
3 = 30-49%
4 = 50-69%
5 = 70-89%
6 = 90-100%

	Head	Arms
Area	0,1 x _ (0-6)*	0,2 x _ (0-6)*
Erythema Induration Desquamation	_ (0-4) Ψ _ (0-4) Ψ _ (0-4) Ψ	_ (0-4) Ψ _ (0-4) Ψ _ (0-4) Ψ
	Trunk	**Legs**
Area	0,3 x _ (0-6)*	0,4 x _ (0-6)*
Erythema Induration Desquamation	_ (0-4) Ψ _ (0-4) Ψ _ (0-4) Ψ	_ (0-4) Ψ _ (0-4) Ψ _ (0-4) Ψ

b

Tunk
area factor = 2
E = 2
I = 2
D = 2

Arms
area factor = 3
E = 2
I = 2
D = 2

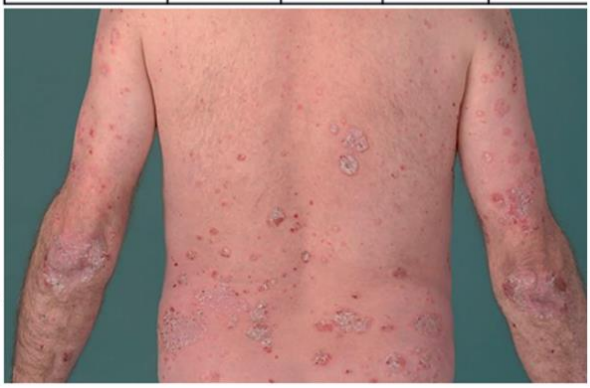

Figure 2. Assessment of disease severity in psoriasis vulgaris: PASI scheme and calculation; the neck is assessed together with the heat; buttocks are assessed with the legs (a). Example of a patient with plaque psoriasis (arms and trunk); the total sum of the PASI of this patient was 15.3 (b).
Reprint from the Clinical and Experimental rheumatology, Volume 33(5 Suppl 93), Oji V, Luger TA., The skin in psoriasis: assessment and challenges., pages S14-9.[14] *© 2015. With permission from Clinical and Experimental rheumatology.*

Scores above 10 are usually considered severe disease (Figure 2)[13, 14]. Treatment response in patients with psoriasis is often presented as a percentage reduction in PASI. PASI 75 indicates a 75% reduction in PASI score from baseline and demonstrate excellent improvement of the disease[13, 15]. However, according to today's expectations, that limit is moving towards PASI 90 or an absolute PASI score less than or equal to 3[16].

In routine daily clinical practice, Dermatology Life Quality Index (DLQI) is used as an assessment tool to evaluate the quality of life in patients with psoriasis[17]. The questionnaire is recommended even in people with minimal skin lesions[18]. It includes 10 questions that assess the impact of the disease on different aspects of patient's life[17]. Each question is estimated on a scale from 0-3 and the final score ranges from 0 - 30 (0-1 = no effect on patient's life, 2-5 = small effect on patient's life, 6-10 = moderate effect on patient's life, 11-20 = very large effect on patient's life, 21-30 = extremely large effect on patient's life)[19, 20]. Thus, the higher the score, the more quality of life is impaired.

Previous response to treatment, involvement of "special areas" (e.g., face, scalp, palms, soles, and genitalia) and the presence of arthritis and other comorbidities should also be considered when assessing disease severity[21, 22].

1.5 Histological feature

The histology of psoriatic lesions includes thickening of the epidermis due to increased keratinocyte (KC) proliferation of viable layer (acanthosis) and corneal layer (hyperkeratosis) with abnormal retention of the nuclei (parakeratosis), elongation of rete ridges, and reduction or absence of granular layer (hypogranulosis) (Figure 3)[6, 23, 24]. In the dermis, dilated and elongated dermal blood vessels reach into the tips of the dermal papillae (Figure 3B). Inflammatory cells migrate to the epidermis and dermis. Inflammatory infiltrate predominantly consists of T lymphocytes, but also of macrophages, dendritic cells (DC), mast cells, and neutrophils (Figure 3B, D)[23, 25].

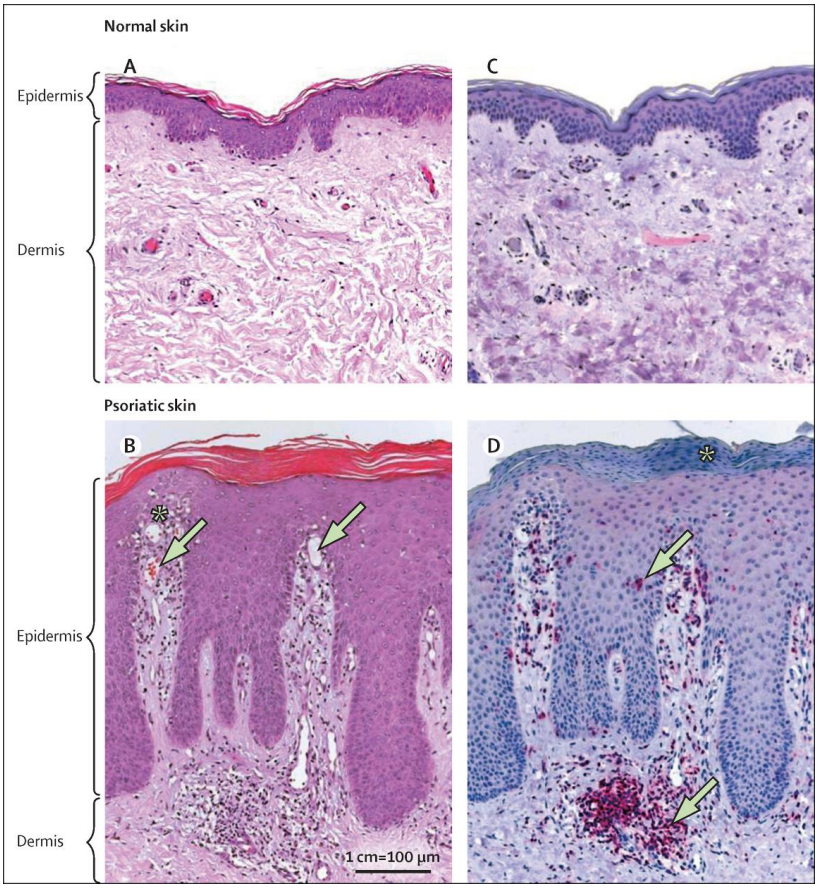

Figure 3. Histopathological features of psoriasis. Within the typical plaque, psoriatic epidermis shows marked epidermal acanthosis, hyperkeratosis, and elongation of rete ridges (A, normal skin and B, lesional psoriatic skin; stained with haematoxylin and eosin). Dilated and contorted dermal blood vessels reach into the tips of the dermal papillae (B, arrows). A mixed inflammatory infiltrate with neutrophils accumulating within the epidermis is noted (B, asterisk). By contrast with normal skin (C), immunohistochemical detection of CD3 reveals many T cells in the dermis and epidermis of lesional psoriatic skin (D, arrows). Cell nuclei present in the cornified layer of the epidermis are also characteristic for lesional psoriatic skin (D, asterisk).
Reprint from the Lancet, Volume 386, Boehncke W.H. et al, Psoriasis, pages 683-94.[23] *© 2015. With permission from Elsevier.*

Memory $CD8^+$ T cells (Tc) represent the majority of epidermal T cells infiltrate, adjacent to DC[26]. Sometimes neutrophil clusters in stratum corneum and/or stratum spinosum of the epidermis form the microabscesses of Munro and/or Kogoj spongiform

micropustules[25, 27]. Dermal infiltrates in lesional skin are mainly composed of T helper (Th)1 and Th17 lymphocytes but also of DC, innate lymphoid cells (ILC3s), γδ T, natural killer (NK) and natural killer T (NKT) cells [26, 28, 29].

1.6 Risk and triggering factors

It is not known what exactly causes psoriasis, we do know the immune system and genetics play key roles. Anyone can develop psoriasis, but family history, environmental and behavioral factors increase the risk of developing disease[30]. In genetically predisposed individuals, a triggering event may cause a change in the immune system and induce psoriasis[23]. Physical skin trauma, ultraviolet (UV) exposure, a bug bite or contact allergens and irritants can trigger psoriasis lesions on previously unaffected skin through Koebner phenomenon[31, 32]. Throat infections caused by *Streptococcus pyogenes* are associated with the onset of guttate psoriasis and exacerbation of plaque psoriasis via molecular mimicry between surface M-protein from group A β-hemolytic streptococci and keratin 17 (Figure 4)[33-36]. Certain medications including high blood pressure drugs (β blockers), lithium, antimalarial, non-steroidal anti-inflammatory drugs, as well as rapid withdrawal of oral or injected corticosteroids trigger or aggravate psoriasis[23]. Behavioral factors such as tobacco smoking and exposure to secondhand smoke, heavy alcohol consumption, weight gain, obesity and stress are risk factors for developing the disease[6].

1.7 Genetics

Inheritance is the main risk determinant for psoriasis occurrence[30, 37]. The risk of psoriasis is approximately 40% if both parents are affected, 14% if one parent is affected, and 6% if a sibling is affected[38]. Data from genome-wide association studies (GWAS) identified more than 80 psoriasis susceptibility loci, explaining about 28% of disease heritability[37, 39, 40]. These risk variants are involved in different processes including inflammation, antigen presentation, epidermal biology, cell signaling and transcriptional regulation [41]. One of the strongest and most prominent risk gene for early onset, more severe and guttate psoriasis is human leukocyte antigen

(HLA)-C*06:02 that accounts for up to 50% of disease heritability [42, 43]. Furthermore, HLA -C*06:02 as in charge for antigen presentation to $CD8^+$ T cells links psoriasis genetics to proposed autoantigens[44]. Through HLA-Cw*0602, $CD8^+$ T cells may be engaged in pathogenic crosstalk with KC and this process is the central driver of inflammatory activity in chronic plaque psoriasis[45].This gene is not associated with late onset disease, PsA, and pustular psoriasis[44]. The status of HLA-C*06:02 could offer substantial clinical benefit when selecting treatments for severe psoriasis[46]. Other susceptibility loci, including *IL23A, IL12B, IL23R, TYK2, TNIP1, TNFAIP1, TRAF31P1, REL, FBXL19, NFkB1A*, *KLF4, ERAP1,* and *ERAP2*, have a minor genetic effect, but they clearly link psoriasis to the cytokines interleukin (IL)-23, tumor necrosis factor (TNF), IL-17A as well as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription (STAT)3 signaling pathways, and antigen processing and presentation[37, 44, 47, 48].

1.8 Immunopathogenesis and inflammation in psoriasis

In the past decades, clinical and experimental evidence have demonstrated that psoriasis is an immune-mediated inflammatory disease with autoimmune background that occurs in genetically susceptible individuals[32, 49, 50]. The role of the immune cells is supported by the fact that psoriasis improves after bone marrow transplantation as well as after the administration of immunosuppressive drugs such as cyclosporine and methotrexate[51-53]. It has been shown that various clinical presentations of psoriasis occur due to the activation of different arms of the immune system. The adaptive immune system and autoimmunity is dominant in chronic plaque psoriasis while the innate immune system and autoinflammation is dominant in generalized pustular psoriasis[41]. Recently, autoantibodies including auto-keratin 17, auto-LL-37, auto-small nuclear ribonuclear protein (RNP), auto-4 cytoplasmic RNP and auto-integrins antibodies have been observed in patients with psoriasis and PsA[54-57]. However, their exact role in inflammation in psoriasis and psoriatic arthritis has not yet been elucidated.

1.8.1 Initiation of psoriatic lesions

Distinct autoantigens have been identified to trigger activation of antigen-specific T cells including cationic antimicrobial peptide cathelicidin (LL37), disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5 (ADAMTSL5), keratin 17, and phospholipase A2 group IVD (PLA2G4D) (Figure 4)[35, 36, 58-61].

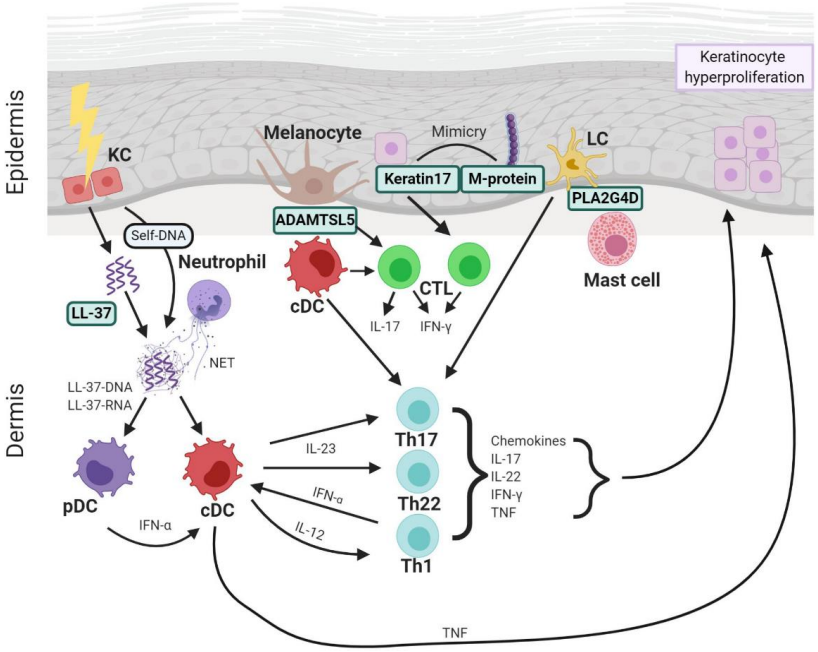

Figure 4. Autoantigens and their potential role in the development of psoriatic skin. As a result of environmental stimuli (e.g., infection or skin trauma), damaged keratinocytes release LL-37 in genetically susceptible individuals. The antimicrobial peptide LL-37 forms complexes with self-DNA/RNA originating from NETs. LL-37-DNA complexes initiate IFN-α release from pDCs, which in turn activates cDCs. Activation of cDC triggers the expression of TNF, IL-23 and IL-12 inducing Th17, Th22 and Th1 cell subsets, resulting in the production of pro-inflammatory cytokines. Alternatively, ADAMTSL5 in melanocytes results in the activation of intraepidermal CD8+ CTL and increased amounts of IFN-γ and IL-17. Moreover, CTL reactive to the surface M protein from streptococci may recognize keratin 17 via molecular mimicry resulting in IFN-γ production. Finally, psoriatic mast cells are a major source of PLA2G4D that generates neolipid antigens recognized by Langerhans cells, which in response activate lipid-specific T cells. ADAMTSL5 disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5, cDC conventional dendritic cell, CTL cytotoxic T cell, IL interleukin, IFN interferon, KC keratinocyte, LC Langerhans cell, NET

neutrophil extracellular trap, pDC plasmacytoid dendritic cell, PLA2G4D phospholipase A2 group IVD, Th T helper lymphocyte, TNF tumour necrosis factor.
Reprint from the Scandinavian Journal of Immunology, Volume 92(4):e12945, ten Bergen L.L. et al.[36] © 2020. With permission from John Wiley and Sons.

In response to skin trauma or infections, LL37 is produced by KCs and various immune cells (neutrophils, antigen presenting cells (APC), and mast cells)[58, 62]. Simultaneously, extracellular self-DNA and self-RNA released from neutrophil extracellular traps (NET) after skin injury, together with LL37, form aggregates that are taken up by plasmacytoid DC (pDC) via toll-like receptors (TLR) 9 and TLR7[63, 64]. pDC activation is followed by activation of conventional DC (cDC) through the released interferon alpha (IFN-α)[64, 65]. In addition, cDC can be activated via binding of RNA-LL37 aggregates to TLR7/TLR8[64]. Activated cDC produce proinflammatory cytokines TNF, IL-23 and IL-12 which further affect cell activation and differentiation as part of the pathophysiological process in psoriasis[64].

1.8.2 Innate and adaptive immune cells interplay and cytokine network

Along with activated DC, different immune cells including Th17, Tc17, ILC3, Th1, Tc1, Th22, Tc22 cells, and neutrophils are attracted to the skin (Figure 5)[3, 25]. Initially, in the lymph nodes, activated DC secrete various cytokines that influence the differentiation of naïve T cells into effector cells[3, 6, 66]. Hence, IL-12 and IFN-γ stimulate naïve $CD4^+$ T cells to differentiate into Th1 cells; IL-23, IL-1β and IL-6 into Th17 cells; and TNF and IL-6 into Th22 cells[67-69]. Finally, TGF-β together with IL-2, drives the differentiation of regulatory T cells (Tregs) that are responsible for blocking inflammation[70]. In the skin, these cells communicate between each other mainly through released cytokines (Th1 via TNF, IFN-γ, IL-2; Th17 via IL-17A/F, IL-22, IL-9; Th22 via IL-22, IL-13, TNF)[26, 71-74]. KCs hyperproliferation and release of antimicrobial peptides (AMP) and cytokines caused by IL-23, IL-17, IFN-γ, Il-22, and TNF causes recurrent activation of DC, neutrophils and T cells which in turn recruit new immune cells via chemoattractants CXCL1, CXCL2, CXCL8, CCL20, and maintain inflammation by forming self-sustaining feedforward loop[27, 32, 75, 76].

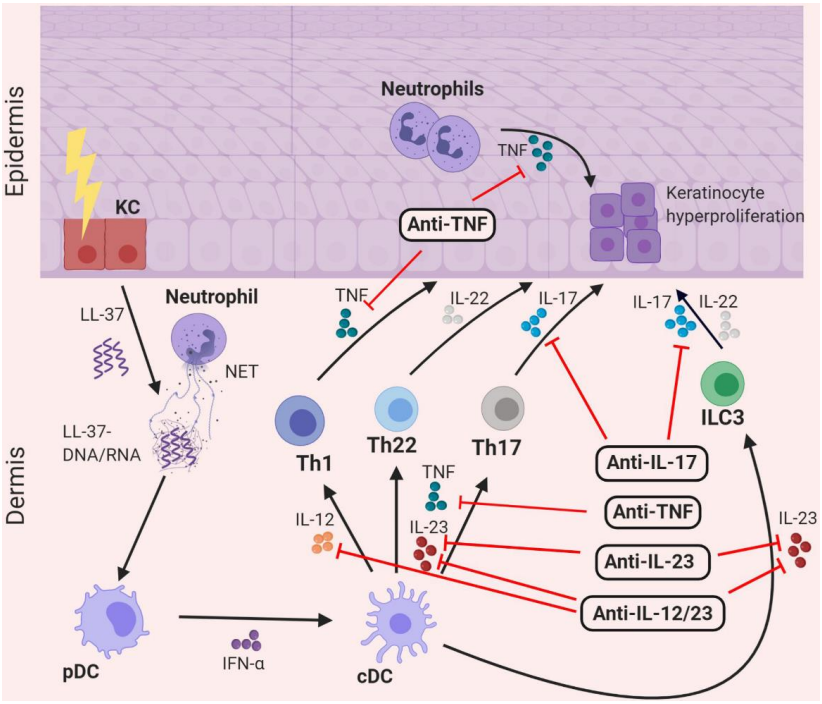

Figure 5. Biologic treatments targeting the TNF/IL-23/IL-17 axis in psoriatic skin. An initial trigger (eg biochemical stimuli, infections) induces cell damage, NET formation and increased production of antimicrobial peptides (eg LL-37). Self-nucleic acids and LL-37 complexes induce IFN type I production of activated pDC stimulating maturation of cDCs followed by production of IL-12, IL-23 and TNF. The proinflammatory cytokine IL-23 drives T cell differentiation and stimulates production of Th22/ILC3 (IL-22) and Th17/ILC3 (IL-17) cytokines, while IL-12 initiates Th1 differentiation and subsequent TNF secretion. The released cytokines stimulate the proliferation of keratinocytes with neutrophilic inflammatory infiltrate. The schematic depicts biologics targeting TNF, IL-12/23, IL-23 and IL-17 cytokines highlighting the central role of this signalling pathway in psoriasis. cDC, conventional dendritic cell; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; LL37, cathelicidin; NETs, neutrophil extracellular traps; pDC, plasmacytoid dendritic cells; Th, T helper lymphocyte; TNF, tumour necrosis factor.
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Moreover, IL-17A, IL-17F and IL-17C intensify the innate immune response by increasing the production of AMP and IL-26 and IL-29 cytokines with antiviral/antimicrobial effects making the skin barrier highly resistant to skin infections[77]. Other cell types including macrophages, ILC3, NK, NKT cells and γδ

T cells produce cytokines and contribute to the chronic inflammation in psoriasis[78-81]. In addition, disruption of Tregs function also contributes to the maintenance of inflammation and increased proliferation of pathogenic T cells[25, 82]. Instead of maintaining immune tolerance, these cells have reduced suppressive capacity induced by increased level of proinflammatory cytokines (IL-6 and IL-17) in psoriasis lesions[25, 70, 83-85]. The sharp demarcation of psoriasis lesions from clinically healthy skin, and characteristic recurrence of disease at previous sites of involvement, are features that can be explained by the presence of non-recirculating long-term persistent tissue-resident memory $CD8^+$ T cells (Trm) in the epithelium[86, 87]. This subset of T cells provides prompt protection against pathogens, but their aberrant activation as the result of response to autoantigens, can induce or aggravate psoriasis[58, 59, 87]. During treatment-induced remission at the local level, Tc17, Th22 and Trm represent site-specific disease memory[71, 73, 86, 88, 89]. These cells are reactivated when therapy is reduced or discontinued and restart skin inflammation at the original sites of involvement.

1.9 Systemic inflammation and comorbidities in psoriasis

Over the last few years, psoriasis has been acknowledged as a systemic inflammatory disease, rather than a single skin disease[90]. This is supported by the observation of a higher number of circulating lymphocytes and activation level of peripheral blood mononuclear cells (PBMC) in psoriasis[91-93]. Moreover, increased gene expression of transcription factors including T-bet, retinoid-acid receptor-related orphan receptor gamma t (RORγt,) and aryl hydrocarbon receptor (AHR), and cytokines involved in differentiation of Th1, Th17 and Th22 cells, respectively, and elevated levels of proinflammatory cytokines including TNF, IFN-γ, IL-6, IL-8, IL-12, IL-17A and IL-18 in the circulation contribute to this view[90, 94-97]. Radiological imaging, (18F)-fluorodeoxyglucose positron emission tomography/ computed tomography (FDG-PET/ CT) has shown that patients with moderate-to-severe psoriasis and psoriatic arthritis have subclinical inflammation in the liver, joints, and tendons, and significantly increased global arterial and subcutaneous inflammation, while patients

with mild psoriasis have a subclinical inflammation in the aorta[98-100]. Moreover, observational studies demonstrate that inflammation of aorta and carotid arteries measured by FDG-PET/CT has predictive value for major vascular events[101-103].

Other systemic, chronic, inflammatory diseases including cardiometabolic diseases (obesity, hypertension, type 2 diabetes and dyslipidemia), chronic kidney disease, inflammatory bowel disease (IBD), particularly Crohn's disease, psychosocial disorders, infection and malignancy represent comorbidities of psoriasis[1]. Patients with psoriasis have increased risk of obesity, hypertension, diabetes type 2 and dyslipidemia[1, 104-108]. Obese patients with psoriasis have increased volume of metabolically active fat depot defined as visceral adipose tissue (VAT)[109]. Dysfunction of immune cells with pro-atherogenic potential including classical monocytes, platelets, and low-density granulocytes together with increased VAT volume is associated with early signs of coronary plaque progression such as noncalcified burden (NCB) and vascular inflammation[99, 110]. VAT releases both pro-atherogenic and inflammatory cytokines which may upregulate bone marrow activity that can be assessed by FDG-PET/CT [110, 111]. In patients with psoriasis, the bone marrow FDG uptake participates partially in early atherosclerotic plaque formation, which can result in plaque rupture and subsequent CV events, all of which can be modulated by biological therapy[112]. According to meta-analysis and systematic review of 14 cohorts, patients with severe psoriasis have higher relative risk of cardiovascular disease (CVD) mortality (1.37), myocardial infarction (3.04), and stroke (1.59) compared to the general population[113]. Besides, cardiometabolic syndrome is a risk factor in developing psoriasis, and both conditions are associated with CVD[114, 115]. After adjusting for age, gender, diabetes, hypertension, smoking and hyperlipidemia, an additional risk of major adverse cardiovascular events has been observed in patients with severe psoriasis[116]. Compared to general populations, younger patients with severe psoriasis have more myocardial infarction (MI). Moreover, it has been observed that psoriasis is independently associated with MI in Japanese patients[117]. This finding suggests that psoriasis is an independent risk factor for CVD[117]. Better knowledge of the immunopathogenesis of both diseases

strengthens the view of a direct link between CVD and psoriasis[118]. Transcriptome analysis of both psoriatic skin and atherosclerotic plaque has shown two overlapping cytokines including IFN-γ and TNF involved in pathophysiology of both diseases. These proinflammatory cytokines together cause a stronger immune response of endothelial cells and atherosclerotic tissue, thus connecting these two conditions.[119] The role of IL-17A in atherosclerosis is controversial considering its pro-atherogenic and anti-atherogenic effect[118, 120-123]. High levels of IL-17 in atherosclerotic plaque and its influence on upregulation of proinflammatory cytokines suggest pro-atherogenic effect [124]. Furthermore, IL-17A affects neutrophil migration and infiltration into damaged endothelium, subsequent leukocyte recruitment, and foam cells formation, all of which lead to atherosclerosis[125-127]. On the contrary, upregulation of IL-17 due to the presence of anti-inflammatory substances and its role in plaque stabilization through collagen synthesis favor the idea of its anti-atherogenic effect[128].

Causal link between psoriasis and CVD can be partly explained by concept of "Psoriatic March"[129]. Findings of autoantigen-specific (i.e., LL37) Th/Tc cells and specific autoantibodies in circulation of most patients with moderate-to-severe plaque psoriasis indicate the autoimmune and systemic nature of the disease[55-58, 130-133]. In the "psoriatic march", the chronic state of inflammation appears to be a central mechanism in the pathophysiology of insulin resistance, visceral adiposity, hypertension and dyslipidemia[129]. Proinflammatory cytokines induce insulin resistance, which is followed by reduced nitric oxide (NO) production, vascular stiffness, and subsequent endothelial dysfunction. The cascade continues with the development of atherosclerosis and finally myocardial infarction or stroke (Figure 6)[126, 129].

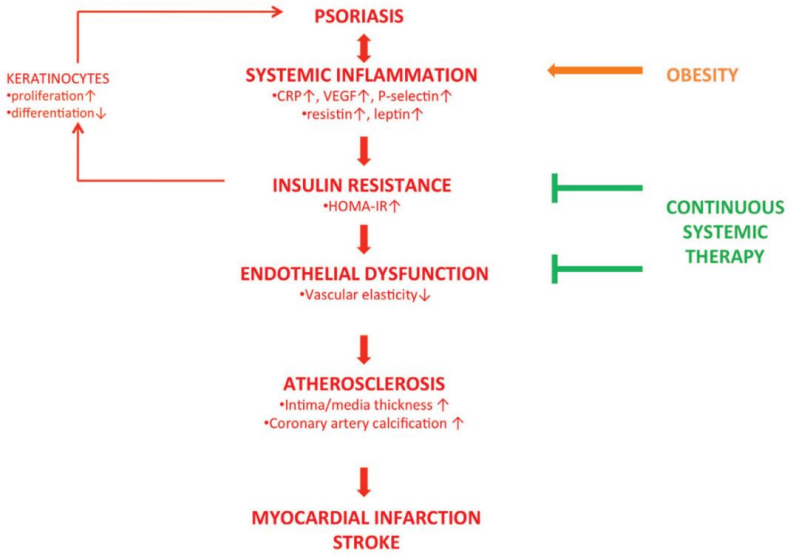

Figure 6. The concept of the 'psoriatic march'. It suggests a causal link between psoriasis as a systemic inflammatory condition and cardiovascular comorbidity, as systemic inflammation may cause insulin resistance, which in turn triggers endothelial cell dysfunction, subsequently leading to atherosclerosis and finally myocardial infarction or stroke (red, bold). This 'backbone' may be developed further by adding additional 'modules', such as a possible feedback of insulin resistance to epidermal homeostasis (red, fine). Obesity is a known risk factor for psoriasis and may induce the phenotype through systemic inflammation (orange, bold). Continuous effective systemic therapy may stop the 'psoriatic march' through interference with insulin resistance and endothelial dysfunction (green).
Reprint from Experimental Dermatology, Volume 20(4), Boehncke W.H. et al. Systemic Inflammation and Cardiovascular Comorbidity in Psoriasis Patients: Causes and Consequences, pages 303-7.[126] *© 2011. With permission from John Wiley and Sons.*

Hence, systemic inflammation demands systemic treatment that targets cytokine network involved in the pathogenesis of psoriasis and comorbidities including cardiometabolic syndrome.

1.10 Treatment

Psoriasis is a life-long, systemic, immune-mediated inflammatory disease associated with several comorbidities with rare spontaneous improvement. Most patients require long-term treatment, and several factors should be considered in addition to the extent and clinical severity of the disease. These include psoriasis phenotype, previous

treatment response, involvement of “special areas” (e.g., face, scalp, palms, soles and genitals), presence of comorbidities, concomitant medications, conception plans, individual preferences and treatment goals[21, 134]. The need for treatment should be flexible as psoriasis evolves and patients’ needs change over time. Our improved understanding of the immunopathogenesis has led to the development of increasingly precise targeted therapies and treatment guidelines that help controlling the disease manifestations discussed in the following chapters[135]. Optimal management of psoriasis requires a patient’s full cooperation and active participation in treatment. Although psoriasis has a profound impact on patients' lives, adherence to psoriasis treatment remains suboptimal[136-138]. This highlights the need for new therapies and treatment strategies with the aim to improve compliance and add satisfaction with treatment to enhance the health-related quality of life for patients with psoriasis.

1.10.1 Topical treatment

Approximately 80% of patients with psoriasis have mild disease with 3% to 5% of affected body surface area. Treatment approach for psoriasis begins with evaluation of the presence of PsA, since the active arthritis may influence the treatment choice, which should be adequate for both psoriasis and PsA[139]. For patients with mild disease, topical treatment including topical steroids, vitamin D analogues calcipotriol, calcineurin inhibitors pimecrolimus, tacrolimus, topical retinoids tazarotene, adapalen, keratolytic salicylic acid and keratoplastic coal tar, remains the standard[11, 140]. They are usually applied either alone or in combination[141, 142]. Their regular application twice a week when lesions are quiescent is a proactive regimen during the maintenance period and reduces the risk of relapse[143].

1.10.2 Phototherapy

For patients requiring more than topical therapy and/or those wishing to avoid systemic drugs, phototherapy is an acceptable and effective treatment option. UV has local immunosuppressive effect through inhibition of epidermal hyperproliferation and angiogenesis, and selective reduction in skin T cells via apoptosis[144]. Particularly narrowband UVB (311 nm) is the most appropriate because up to 70% of patients with psoriasis reach PASI75 response[23, 145]. Furthermore, topical or systemic psoralen

together with UVA (PUVA 320-400 nm), affects the therapeutic response so that even 90% of patients reach a PASI75 response[145]. However, photochemotherapy is currently used less frequently in some parts of the world due to the cumulative risk of skin cancer, which is far superior to that caused by narrowband UVB[146].

1.10.3 Oral systemic treatment

Patients with psoriasis requiring systemic therapy have more than 10% of body surface area affected, and/or psoriasis at special sites, and/or non-response to topical treatment[6]. A widely used systemic drug for psoriasis treatment, methotrexate (MTX), is a folic acid antagonist which inhibits DNA synthesis, and cellular replication [147, 148]. A recent meta-analysis observed that 45% of patients achieve PASI75 after 3 or 4 months of treatment[149]. However, severe side effects including teratogenicity, bone marrow suppression, and hepatotoxicity, require proper patient selection and limit the use[134]. Fumaric acid esters are small molecules that inhibit maturation of DC, influence T cell recruitment, prevent the release of proinflammatory cytokines, and induce T cell apoptosis through the NF- κB inhibition[150, 151]. 64% of patients reach PASI50 response rate after 3 or 4 months of treatment, but the use is usually limited due to substantial side-effects, including flushing and diarrhoea[152]. A vitamin A analogue, acitretin is a synthetic retinoid that binds to nuclear retinoid receptors, normalizes gene transcription in KCs, returns KCs to normal proliferation and reduces proinflammatory cytokines, such as IL-6 and IFN-γ[153]. The drug is usually used in the treatment of erythrodermic and pustular psoriasis. The use of acitretin is limited in women in the reproductive period because of its teratogenicity, and pregnancy should be avoided for at least 2 years after the last dose[23, 134]. Cyclosporine (CyA), the first immunosuppressive drug that act selectively on T-cells is a systemic calcineurin inhibitor that reduces T cell activation through inhibition of IL-2 production[134, 154, 155]. 45-60% of patients treated with CyA achieve PASI75. Because of its rapid action and high efficacy, CyA is used for short-term treatment of patients with relapsing severe disease or as a bridge therapy to long-term treatments such as biologics or other oral medications[154]. It is not recommended to use CyA for more than 1 year due to side effects, including irreversible nephrotoxicity,

hypertension, and increased risk of infection[6]. The discovery of CyA changed the history of psoriasis treatment and the direction of future translational research to the more precise targeting of the immune system with the aim of more effective disease control[156]. The small molecule drug, apremilast, is indicated for the treatment of moderate–severe psoriasis and active psoriatic arthritis with a level of efficacy comparable to MTX. The drug is a phosphodiesterase-4 inhibitor that reduces expression of proinflammatory cytokines such as TNF-α, IL-23, IL-2, and IL-12 and increase anti-inflammatory cytokines such as IL-10[157]. The PASI75 response to apremilast is up to 40% after 4 months treatment[158]. Advantages of the drug include its oral administration, a favorable safety profile, and anti-inflammatory, rather than immunosuppressive effect[159]. The most common adverse effects are nausea, diarrhea, and weight loss[160]. The next small molecule, tofacitinib is an oral Janus kinase (JAK) inhibitor targeting JAK1 and JAK3 intracellular signaling pathways. It has been shown that up to 50% of patients treated with tofacitinib achieve PASI75 at week 12-16[161, 162]. JAK inhibitor is also effective in topical treatment and can be useful in treatment of psoriasis of the face and intertriginous areas[161]. Tyrosine Kinase 2 (TYK 2) inhibitor treatment has shown promising results. This intracellular signaling enzyme is involved in functional responses of IL-12, IL-23 and IFN receptors, so inhibition of this target affects the key cytokine pathways in psoriasis[163]. Up to 75% of patients reach a PASI75 rate response after 3 months of oral treatment with 12 mg TYK2 inhibitor[163]. However, recent analysis has shown a potential effect of therapeutic TYK2 inhibition on the risk of lung cancer and non-Hodgkin lymphoma, which is important implications for future evaluation of the safety of these inhibitors in development[164].

1.10.4 Biological treatment

Biologics are classified as peptides or proteins in the shape of monoclonal antibodies, growth factors or cytokines manufactured by living organisms or derived *in vitro* by recombinant gene expression methods[165]. In psoriasis, biologics specifically target the TNF/IL/23/IL-17 axis[166]. Compared to conventional systemic therapies, treatment with biologics results in a significant proportion of patients with moderate-

to-severe psoriasis achieving PASI75 and PASI90 response rate, highlighting the underlying role of the cytokine axis in the disease (Figure 5)[3, 167]. Although currently classified as immunosuppressants, it is obvious that these drugs cannot be divided as suppressors or stimulators, precisely because of their specific targeted function in the immune system[6]. Over the last 20 years, enormous effort has been made to establish current treatment options for psoriasis and PsA including 14 biologics in four different classes that antagonize TNF (anti-TNF), the p40 subunit of both IL-12 and IL-23 (anti-IL-12/IL-23p40), the p19 subunit of IL-23 (anti-IL-23p19), and IL-17 (anti-IL17) (Figure 7)[6].

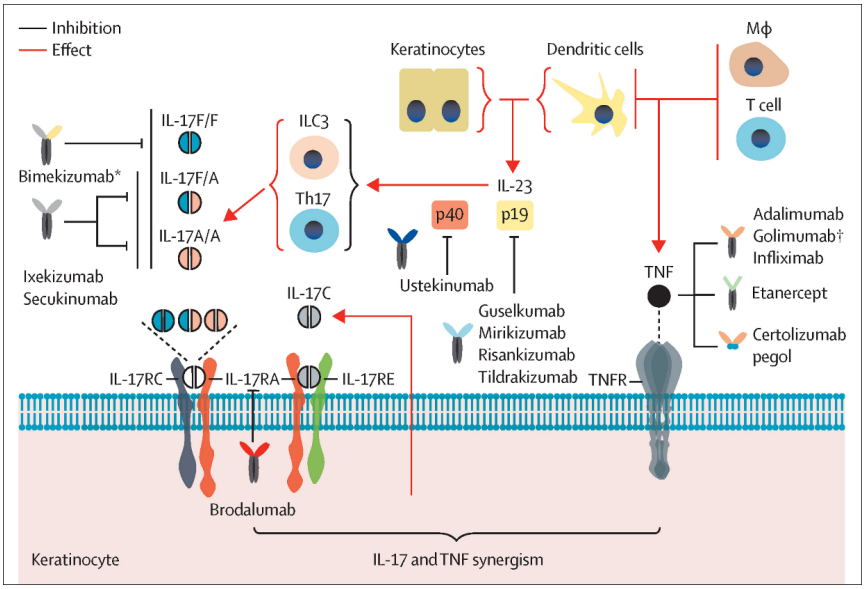

Figure 7. Biologics for the treatment of psoriasis and their targets. Currently, 11 biologics are approved for the treatment of psoriasis: four anti-TNF agents (adalimumab, infliximab, etanercept, and certolizumab pegol; a fifth, golimumab, is currently only approved for treatment of psoriatic arthritis), a single agent targeting the p40 subunit of IL-12 and IL-23 (ustekinumab), three biologics targeting the p19 subunit of IL-23 (guselkumab, risankizumab, tildrakizumab), two anti-IL17A agents (ixekizumab and secukinumab), and one biologic targeting the anti-IL17 receptor A (brodalumab). Two biologics are currently in late-phase clinical trials, including the p19 inhibitor mirikizumab and the bispecific anti-IL-17A and IL-17F agent bimekizumab. The figure shows the source of the key cytokine targets of biologics and their corresponding cytokine receptors. ILC=innate lymphoid cell. Mφ=macrophage. IL-17RC=IL-17 receptor C. Th17=helper T cells type 17. TNFR=TNF receptor. *Bimekizumab, the

bispecific anti-IL-17A and IL-17F agent, and mirikizumab, the p19 inhibitor, are not yet approved and are in phase 3 clinical trials. †Golimumab is currently only approved for treatment of psoriatic arthritis.
Reprint from the Lancet, Volume 397, Griffiths C. E. M. et al, Psoriasis, pages 1301-1315.[6] *© 2021 with permission from Elsevier*.

These drugs are more expensive than conventional systemic therapies, but the clinical efficacy is higher and risk of adverse events lower[168]. With the exception of infliximab (IFX), all biologics used for psoriasis are applied by subcutaneous injection[6]. Four anti-TNF agents including etanercept (Fc fragment of human IgG fusion protein of the receptor), infliximab (chimeric IgG1 monoclonal antibody (mAb)), adalimumab (human IgG1 mAb) and certolizumab (Fab fragment of humanized mAb) are approved for the treatment of moderate-to-severe psoriasis, while the fifth, golimumab (human $IgG_1\kappa$ mAB) is currently only approved for treatment of psoriatic arthritis. Certolizumab is the first choice for treatment during pregnancy and breastfeeding, as minimal placental transfer has been observed, with no increase in maternal or fetal adverse events[169, 170]. TNF inhibitors cause an indirect inhibition of IL-17 signaling via the regulation of IL-23 production from DC[166]. Moreover, they downregulate production of cytokines IL-8, IFN-γ-inducible protein-10 (CXCL10), MIP-3α (CCL20) and IL-1, that induce decreased infiltration of neutrophils, T cells, and DC[171]. TNF blockade interrupts the activation and maturation of DC, the subsequent activation of T lymphocyte and the production of cytokines, growth factors and chemokines such as ICAM1, whose expression is reduced on keratocytes, blocking the migration of leukocytes from the dermis to the epidermis[171]. Among TNF antagonists, IFX is the most effective followed by adalimumab and etanercept[172]. Etanercept is a human recombinant TNF receptor p75 protein that binds to TNF and lymphotoxin[173]. In IFX treated patients' immunogenicity occurs frequently, especially if MTX is not simultaneously given[174]. Anti-TNF treatment has an associated risk of serious infections, such as tuberculosis (TB), pneumonia, and cellulitis[175, 176]. Therefore, TB evaluation is mandatory before starting treatment with TNF blockers. More than 40% of IFX-treated patients with severe disease have significant weight gain, because of the involvement of TNF in body weight homeostasis[177]. Moreover, 2-5% of patients with psoriasis

treated with anti-TNF manifest “paradoxical psoriasis”, an overactive innate inflammatory response, driven by pDC-derived type I interferons[178]. Ustekinumab (human IgG1 mAb) binds to the p40 subunit common to IL-12 and IL-23 and blocks their interaction with the IL-12 receptor β1 subunit of the IL-12 and IL-23 receptor complexes on the surface of NK and T cells[179, 180]. It neutralize IL-12 mediated responses, including intracellular phosphorylation of STAT4, cell surface marker expression and IFNγ cytokine production, as well as IL-23-mediated responses, including intracellular STAT3 phosphorylation and IL-17A, IL-17F and IL-22 cytokines production[179]. The efficacy of ustekinumab in the treatment of psoriasis is better compared to etanercept, but not to anti-IL-17 therapeutics such as secukinumab, brodalumab and ixekizumab[181-184]. PASI75 response rates do not differ significantly between IFX and ustekinumab, but ustekinumab does not induce weight gain in contrast to IFX [185]. The drug is effective in treatment of Crohn’ disease, while secukinumab may exacerbate or induce IBD[186]. Finally, ustekinumab has a stable safety profile, like placebo and etanercept, but with significantly higher efficacy[187]. The humanized mAbs tildrakizumab, risankizumab and mirikizumab, and human mAb guselkumab are anti-IL-23p19 therapeutics[188-190]. 75% of patients achieved PASI90 with risankizumab, compared to less than 50% of patients treated with ustekinumab[189, 191]. In the NAVIGATE study, ustekinumab-treated patients benefited significantly from switching to guselkumab, suggesting that p19 neutralization leads to more potent inhibition of IL-23, highlighting its greater role compared to IL-12 in chronic psoriatic plaque maintenance[192]. In comparison with IL-17 blockers, targeting IL-23p19 seems to show similar or even better results[193, 194]. Long-term treatment with guselkumab has shown almost complete disease control compared to secukinumab[194]. Anti-IL-23p19 has a safety profile without increased rates of opportunistic infections, tuberculosis, mucocutaneous *Candida* infections, de novo onset or potential exacerbation of IBD or demyelinating disorders [195]. The most common adverse events are upper respiratory infections, nasopharyngitis, headache and arthralgias[196]. Two monoclonal antibodies directly targeting IL-17 are secukinumab and ixekizumab, while brodalumab targets the receptor subunit IL-17RA. Bispecific anti-IL-17A and IL-17F mAb bimekizumab is

the last biologic introduced in the treatment of psoriasis. Generally, anti-IL-17 induces the downregulation of keratinocytes chemokine CCL20, and reduces the migration of $CCR6^+$ cells such as DC and Th17 cells[197]. PASI75 and PASI90 responses were similar in patients treated with secukinumab, brodalumab and ixekizumab compared to ustekinumab[182-184, 198, 199]. 70% of patients treated with ixekizumab reaches a PASI90 compared to less than 50% in etanercept-treated[200]. Anti-IL-17 agents slightly increase the risk of opportunistic infections (mucocutaneous candidiasis) and the risk of initiation or worsening IBD[186].

Biosimilar is a biologic medical product manufactured as an almost identical copy of an existing "innovator" product. When the original product's patent expires, “copy versions” enter the market, thus making this class of drug more affordable. Many biosimilar versions of IFX, etanercept, and adalimumab are available, which, in addition to cost-saving in high-income countries, might allow patients in low-income and medium-income countries to have the opportunity to be treated[6]. Biosimilars have the same amino acid sequence as the originator biologic, but are not exact copies due to the complex structure and manufacturing process[201, 202]. Recent studies have shown that switching from originator to biosimilar had no significant impact on drug survival, and the safety profile was comparable[93, 203, 204].

1.10.5 Alternative medication, omega-3 fatty acids

The long-term use of topical medications and the variety of side effects lead to many patients with psoriasis being dissatisfied with their therapy, which contributes to poor medication adherence[205, 206]. Consequently, they become interested in other alternative treatment options that can be used alone or in combination with conventional medical treatment in the hope of improving it[140]. Fish and seafood contain high levels of omega-3 polyunsaturated fatty acids (ω3-PUFA) which have anti-inflammatory, vasodilatory and anti-aggregant properties[207]. Numerous mechanisms responsible for these effects are divided into four groups: 1) interference with the synthesis of arachidonic acid mediators (eicosanoids), 2) creation of specialized pro-resolving mediators (SPMs), 3) direct binding to cell-receptors, and 4) modification of plasma membrane fluidity and lipid raft organization (Figure 8)[208].

The long chain ω3-PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), interfere with the production of leukotrienes of lower chemotactic properties, thromboxanes without pro-aggregate activity, and partially active prostaglandins reducing vascular permeablity[209, 210].

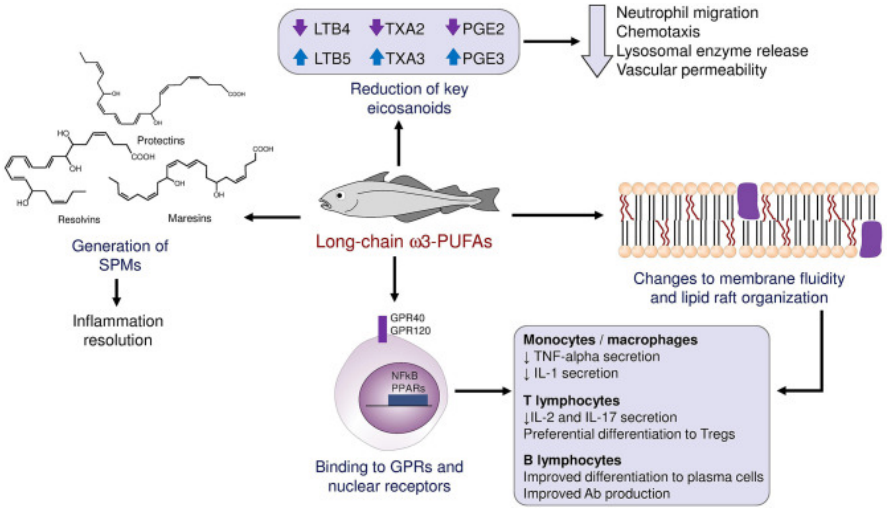

Figure 8. Schematic summary of the mechanisms through which ω3-PUFA influence the immune response and local inflammation.
Reprint with permission from Frontiers in Nutrition, Dietary Fish, Fish Nutrients, and Immune Function: A Review, Mendivil CO. et al.[208] *© 2021 Mendivil. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY).*

Through generation of SPMs (resolvins, maresins), EPA and DHA influence the inflammation resolution. Changes in plasma membrane, together with direct binding of SPMs to the fatty acid receptor GPR and nuclear receptors (NFkB, PPARs), reduce release of proinflammatory cytokines including TNF, IL-1, IL2, IL17 and IFNγ, and induce production of anti-inflammatory cytokines such as IL-4 and IL-10, recruit macrophages responsible for cleaning of cellular debris at the site of inflammation, and prevent trafficking of T cells and their differentiation into inflammatory subtypes[211-215]. Several clinical studies have shown that ω3-PUFAs are beneficial in psoriasis[216-219]. They reported improvement in the patients' erythema, scaling, itching, area involved and infiltration of the skin[219-222]. Reduction of PASI >50% and DLQI >70% but not visual analog scale (VAS) after 6 months of treatment

compared to control has been observed[223]. In our recent research, the significant reduction of PASI was observed only in patients with baseline PASI > 5.5[218]. However, several studies have reported no significant reduction in scaling, erythema, area affected, or thickness compared to control[224-227]. The conflicting results from fish oil studies could be due to study design, sample size, doses, and duration of the supplementation. Immunological studies performed in patients with psoriasis have shown a significant reduction in the influx of neutrophils into the lesional skin due to reduced LTB4 synthesis, but without proportional clinical improvement, and in addition, downregulation of CD25 expression on T cells with a consequent anti-inflammatory effect[228, 229]. Side effects of ω3-PUFAs supplements are usually mild and include unpleasant taste, bad breath, bad-smelling sweat, headache, and gastrointestinal symptoms such as heartburn, nausea, and diarrhea[230].

ω3-PUFAs as alternative medicine is not typically part of conventional medical care, but psoriasis patients with different clinical presentation including plaque, guttate and psoriasis arthritis, could benefit of it[208, 220, 221, 231]. Oral supplementation can be useful as monotherapy particularly to those who are either uninterested in the conventional approach, or as adjuvant for those receiving medical treatments including topical, oral-systemic, phototherapy and biologics[220-222, 226, 232]. Although larger, well-designed, controlled studies are needed to continue testing therapeutic efficacy and safety of ω3-PUFAs supplements, it has been found that most evidence supports their use for the treatment of psoriasis[233].

The severity of psoriasis can fluctuate over time, and symptoms of the disease are usually controlled with treatment, but not always completely. Some people may not respond at all (primary treatment failure) or may have an initial response that is later lost (secondary failure). It is difficult to know if the problem is disease relapse, loss of effect because of low blood concentrations of biologics and development of antidrug antibody, poor compliance, high body mass index (BMI), and gender differences, with women more likely to have a weaker initial response than men and more likely to lose biologic's therapeutic effect over time[234-237]. Patient adherence may be the largest barrier to treatment success with topical therapies; early follow-up (one week after

starting treatment) and good information about therapy can improve compliance[238]. During systemic treatment, particularly with biologics, the loss of efficacy or anti-drug antibody formation could be solved by increasing the dose and/or giving the concomitant immunosuppressive treatment or switching to another biologic. However, the risk of treatment failure is increased with the number of biologics a patient has received previously[239].

Outcome of the treatment in patients with psoriasis might be affected by many aspects, both genetic and non-genetic[240]. Additionally, patients can exhibit discordant responses for their different manifestations of psoriatic disease with introduction of one biologic resulting in sometimes dramatic improvements in skin lesions and little or no response in features of peripheral arthritis[241]. Treatment optimization and prediction of which patient will benefit from a certain treatment can be challenging and clinicians often use an individual's clinical features and history of previous treatment response as the best guide to treatment choice. The application of precision and stratified medicine is therefore needed, whereby psoriatic patients most likely to respond to different biologics can be identified. Stratification of patients could be supported by systemic biomarkers that reflect an individual's inflammatory signature[241]. The potential role of serum levels of cytokines and chemokines as predictors of treatment response has shown that patents with moderate to severe psoriasis treated with etanercept have significantly higher baseline IL-12 serum levels in responders compared to non-responders, while serum levels of IL-22 and vascular endothelial growth factor (VEGF) were not adequate to predict response to treatment with ustekinumab or TNF[241-243]. It has been shown that change of PASI correlates positively with fold change (follow-up/inclusion) of serum cytokines IL-2 and IL-12 in patients with psoriasis, before and 4 months after initiation of biological therapy. Moreover, an increase of IL-10, IL-5 and IL-15 at follow-up gives a higher chance of achieving PASI90[97]. Thus, cytokine profiling combined with deep profiling of immune cells can improve the stratification of patients and perhaps lead to the separation of patients into responders and non-responders.

1.11 Literature search

Literature searches were completed in January 2023.

2. Aims

The general aim of this thesis was to explore and potentially stratify patients with psoriasis based on their immune cell composition and cell activity, thereby predicting patients who will benefit from a certain treatment. Combined with cytokine profiling, these analyses might even provide guidance to responders/non-responders.

The specific aims were to:

Study I

- Investigate the inter-relation of plasma cytokine levels and severity of the disease, and to explore status of circulating immune cell activity in patients with non-severe psoriasis before and during herring roe oil supplementation.

Study II

- Perform in-depth phenotyping of PBMCs and examine their respective activity through the expression of cell surface markers in patients with moderate to severe psoriasis/psoriatic arthritis on stable anti-TNF treatment.

Study III

- Explore changes in frequencies of different peripheral blood DC populations/subsets of patients with severe plaque psoriasis, before and during treatment with different biologics, compared to age, sex and BMI matched HC, to identify potential DC subsets as biomarkers for clinical response.

3. Materials and methods

The present work was based on data from two different cohorts originating from one research center, the Department of Dermatology, Haukeland University Hospital, Bergen, Norway. Each clinical study and analysis method incorporated in this thesis had its limitations demanding careful considerations.

3.1 Materials

3.1.1 Study populations

Study I: Sixty-four patients diagnosed with psoriasis were enrolled into the 65-weeks long PSORAX 35 study from December 2017 to May 2019. The 26-week randomized, placebo-controlled, and double-blinded study was followed by a 39-week open-label study which was continued by 58 patients. Inclusion criteria were age > 18 years, stable psoriasis with PASI < 10, potential local anti-psoriatic maintenance treatment for more than two months before study start. Patients were supplemented with a soft-gel capsule containing active substance, omega-3 poly-unsaturated phospholipids (EPA and DHA, 1:3) or a control substance, coconut oil (caprylic acid and capric acid) respectively. All patient information during the regular visits was collected by dr. Kåre S. Tveit (Table 1). The study was approved by the regional ethical committee (2017/938). Written informed consent was obtained from all participants.

Study II and III: The second cohort (biobank) of 101 patients with moderate-to-severe psoriasis was organized by dr. Silje M. Solberg (SMS). Patients were included from April 2015 to September 2018, examined and their information was collected by two investigators SMS and dr. Lene F. Sandvik (Table 2 and 3). Inclusion criteria were age > 18 years, and prescription of biological medication. Nearly half of the patients were biologic-naïve, while the other half was previously treated with different biological drugs before inclusion in the studies.

The studies were approved by the regional ethical committee (2014/1489 and 2014/1373). Written informed consent was obtained from all participants.

Table 1. Characteristics of patients for cytokine levels analysis (n = 58) and immune cells investigation (n = 18) at inclusion and follow-up, approximately 12, 26, and 65 weeks after starting HRO treatment (Study I)

	Sex (M/ F)	Age	Onset of P < 40	Onset of P ≥ 40	AR	BMI	PASI w 0	PASI w 12	PASI w 26	PASI w 65
Patients for cytokine levels analysis (N=58)										
HRO	17/ 12	46.62 (13.42)	23 (n) 20.87 (9.20)	6 (n) 54.17 (14.63)	14	29.88 (5.88)	6.22 (1.90)	5.92 (2.74)	4.23 (2.49)	2.78 (1.8)
Control	18/ 11	52.07 (13.75)	24 (n) 21.37 (10.12)	5 (n) 49 (8.69)	14	28.83 (3.88)	5.99 (1.75)	5.48 (2.53)	5.48 (2.58)	2.96 (2.1)
Patients for immune cells investigation (N=18)										
HRO	5/5	45,64 (13,38)	17 (8.16)	/	/	29.95 (6.54)	5.75 (1.42)	6.15 (2.30)	5.19 (2.56)	3.36 (1.6)
Control	6/2	45.37 (11.03)	20.12 (9.85)	/	/	31.42 (4.76)	6.36 (1.75)	5.71 (2.18)	5.64 (2.83)	3.25 (1.9)

AR, arthritis; BMI, body mass index; PASI, Psoriasis Area Severity Index. Values are listed as mean (SD).

Table 2. Characteristics of patients (n=24) and helathy controls (n=32) included in the Study II.

Cohort	PsO	PsA	HC
Individual	16	8	32
Sex (M/F)	14/2	6/2	26/6
Age, years	53.4 (16.2)	49.9 (8.6)	48.3 (13.4)
Onset of P < 40	14	7	NA
Psoriasis duration, years	29.4 (11.1)	24.7 (8.5)	NA
Family history	10	5	NA
BMI, kg/m^2	27.9 (3.8)	28.9 (6)	26.6 (3.7)
CRP, mg/L	3.6	1.3	NA
PASI*	20.3 (10.7)	15.2 (4.4)	NA
PASI inclusion	1.9 (0.9)	1.4 (1.1)	NA
DLQI inclusion	1.1 (1.7)	0.6 (1.1)	NA
IFX** treatment (years)	4 (4.5)	5 (5.4)	NA
IFX, mg/kg	6.2 (1.4)	7.5 (1.3)	NA
IFX interval, weeks	7.2 (1.1)	7.1 (1.5)	NA
Trough level, µg/mL	12 (7.3)	10.5 (6.1)	NA
MTX, mg/week	11.9 (6.1)	11.6 (4)	NA
Prior biologics	10	5	NA

PsO, psoriasis patients; PsA, psoriatic arthritis; HC, healthy controls; BMI, body mass index; CRP, C-reactive protein; *, PASI before starting treatment of biologics, **, infliximab treatment before inclusion; MTX, methotrexate; NA, not applicable; Values are listed as mean (SD).

Table 3. Clinical characteristics of patients (N=38) and healthy controls (N=38). Follow-up was approximately 12 months after the initiation of biological treatment (Study III).

	All	**IFX**	**ETA**	**UST**	**SEC**	**HC**
N	38	17	4	7	10	38
PsO	23	14	4	4	1	NA
PsA	15	3	0	3	9	NA
Sex (M/F)	24/14	8/9	3/1	4/3	9/1	24/14
Age	43.3 (14.3)	42.3 (15.4)	27.2 (15.3)	45.6 (13.5)	50.4 (10)	43 (13.8)
Onset of P < 40	31	13	4	5	9	NA
Onset of P ≥ 40	7	4	0	2	1	NA
P/PsA duration	20.4 (12.3)	17.4 (10)	12.2 (2.9)	20.7 (11)	29.6 (12)	NA
BMI	30.5 (5.1)	31.6 (5.6)	28.3 (7.0)	28.2 (2.3)	32.5 (4.2)	27.8 (3.4)
PASI inclusion	9.7 (6.3)	8.9 (4.3)	9.6 (5.8)	12.5 (9.9)	9.4 (6.8)	NA
PASI follow up	2.5 (3.0)	1.4 (1.1)	2.7 (2.8)	3.6 (3.7)	3.7 (4.3)	NA
PASI %	72.8 (23)	80.2 (20.2)	74.5 (20)	70 (23.4)	61.8 (26.4)	NA
DLQI inclusion	13.6 (6.7)	14.3 (6.9)	16.2 (3.6)	11.4 (5.8)	13 (8.0)	NA
DLQI follow up	2.9 (3.2)	2.5 (2.5)	1.5 (1.3)	3 (2.2)	4.1 (5.0)	NA
DLQI change	75.1 (27.8)	79 (24.4)	91.3 (7.2)	67.5 (27.6)	67.3 (36.6)	NA
MTX	22	17	0	1	4	NA
Naïve to biologic	21	11	4	4	2	NA
Previously treated	17	6	0	3	8	NA

IFX, nfliximab; ETA, etanercept; UST, ustekinumab; SEC, secukinumab; PsO, psoriasis; PsA, psoriatic arthritis; BMI, body mass index; PASI, Psoriasis Area Severity Index; DLQI, Dermatology Life Quality Index; MTX, methotrexate; NA, not applicable; Values are listed as mean (SD).

3.1.2 Blood sampling, PBMC and plasma isolation and cryopreservation

All patient samples of PSORAX 35 study were collected at the Research Unit for Health Surveys, University of Bergen, and Helse Vest. The first blood sample was collected prior to starting treatment with active or control substance, while the other samples were taken at week 12, 26, and 65. Peripheral blood samples were collected in a Vacutainer® CPT™ tube (BD) and then processed by Karl Albert Brokstad at Broegelmann Research Laboratory. Plasma and PBMCs were isolated within one hour in a one-step process. Blood was centrifuged at 800g, 20 min, 23°C and two layers were used including upper layer designated as plasma and lower layer containing PBMC. The cells were washed, centrifuged, and cryopreserved in fetal calf serum (FCS) containing 10% dimethyl sulfoxide (DMSO) and stored at – 150°C. After collection, several 1ml aliquots of plasma samples were cryopreserved at –80°C until use.

Blood samples of the second cohort were collected at the Department of Dermatology, Haukeland University Hospital, and further processed and stored at the Broegelmann Research Laboratory. Peripheral blood from each patient was collected at the inclusion and after approximately 3 and 12 months in lithium-heparin tubes (BD 367526, Becton Dickinson Ltd., UK). PBMC were isolated by density gradient centrifugation at 800g, 20 min, 23°C with Lymphoprep (Axis-Shield Ltd., Scotland) and added to a mixture containing 42.5% freezing medium (Profreez^{TM} CDM), 50% serum free media (X-vivo-20^{TM}, Lonza, Basel, Switzerland), and 7,5% dimethyl-sulfoxide (DMSO). The PBMC at a concentration of 5 x 10^6/ml were cryopreserved in liquid nitrogen until usage.

Samples of age, sex, and BMI matched healthy controls (HC) were included in these studies from the Blood bank at the Haukeland University Hospital, taken at various time points during the year. The HC samples were treated, isolated, and stored the same way as patient samples. All samples were stored for no more than seven years before being processed.

3.1.3 Data collection and storage

Patients filled out DLQI questionaries themselves. Medical doctors registered patient data including age, gender, disease onset, BMI, and other relevant clinical and biochemical data. Evaluation of PASI was performed at every visit, most often by the same clinician. The collected data were stored, unidentifiable, on the Helse Vest's research server with the opportunity of patient identification through a digital key, according to regulations of the regional ethics committee.

3.2 Methods

3.2.1 Luminex® Technology

The Luminex® Technology is a bead-based multiplex immunoassay quantifying the presence of up to 80 different analytes from a single well exploiting a very small sample volume while saving time and reagents. This immunoassay is a multistep procedure. The pre-labeled beads with red and infrared dye in different concentrations, are coated with cytokine-specific antibody that bind distinct cytokine. This complex unit made of cytokine bounded to cytokine-specific antibody of the bead is detected with a secondary biotinylated detection antibody. Streptavidin coupled with a fluorochrome phycoerythrin (PE) binds biotinylated detection antibody. In the Luminex machine the beads are analyzed with double-laser flow cytometry. The distinct cytokine is detected with the red laser (635 nm) based on specific bead. The cytokine amount is directly proportional to fluorescence intensity of PE after excitation with a green laser (532 nm) (Figure 9)[244].

In study I, we used 96 well plate of which 16 wells were utilized for standards and 80 wells for patient samples. We included 57 patients (one of a total of 58 patient had no samples at multiple time points) with mild psoriasis and analyzed samples at inclusion, and after starting treatment with active or control substance at week 12, 26, and 65. In study I, we used a custom-designed Human Luminex Discovery Assay LXSAHM-20 and LXSAHM-01 to detect CCL2, CCL3, CCL4, CD25/sIL-2Rα, CXCL9, CXCL10, IFN-γ, IFN-γ R1, IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IL-17, IL-21, IL-33, TNF-α, VEGF, CCL5 and IL-23 in plasma. Data were acquired on a Luminex 100

System (Luminex Corp., Austin, TX, USA) and StarStation Software v.3.0 (Applied Cytometry System, Dinnington, UK).

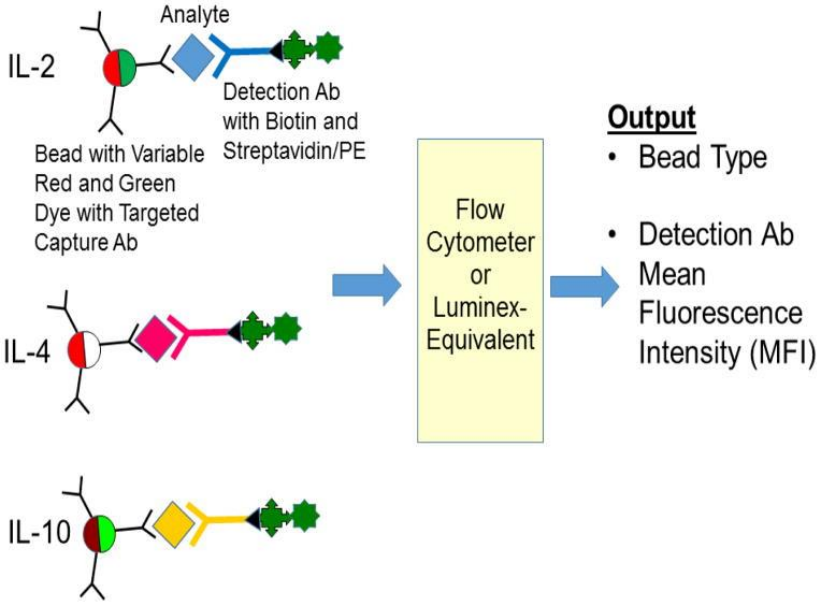

Figure 9. Overview of bead-based immunoassays. Different color-coded beads with dyes that fluoresce either red or green are used. The instrument measures the bead color intensity and the mean fluorescence intensity of the labeled detection antibody which is typically labeled with a streptavidin/phycoerythrin (PE) conjugate.
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3.2.2 Flow cytometry

Multicolor flow cytometry is a laser-based technology, which allows rapid, detailed analysis of cell populations at a single-cell level. Cells passed through a fluidic system are hydrodynamically separated from each other. Individual cells are hit by one or more laser beams, with the resulting scattering and fluorescent light. Scattering light, particularly the forward scatter (FSC) indicates the size of the cell, while the side scatter (SSC) points to the cell granularity. Fluorescent light is the result of the excited fluorochrome of specific wavelength, which is typically conjugated to the monoclonal antibody targeted to bind cell proteins. The emitted light is discriminated by optical filters before detection. Next, all light signals are detected by photomultiplier tube,

where they are amplified, and converted to voltages, which are changed into a digital signal allowing for data analysis on a PC. Multiple biomarkers targeted with different fluorochromes allow phenotyping of cell populations (Figure 10)[245].

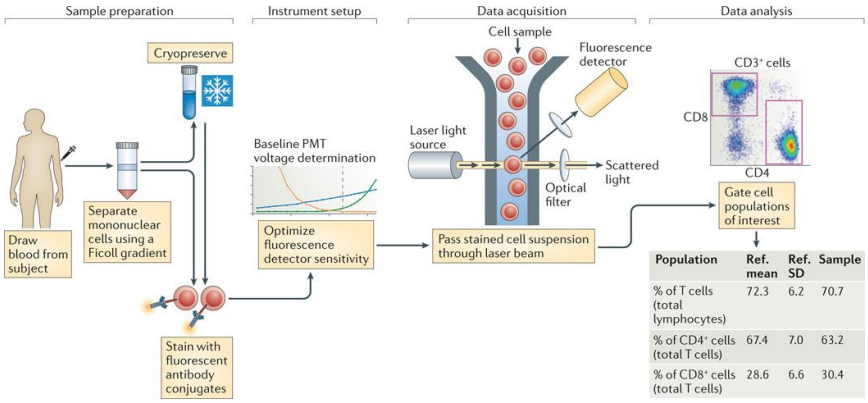

Figure 10. A typical flow cytometry experiment. Sample preparation from blood often involves Ficoll gradient separation of PBMCs, sometimes cryopreservation, before staining with fluorescent antibody conjugates. Instrument setup involves setting voltage gains for the photomultiplier tubes (PMTs) to achieve optimal sensitivity. Data acquisition involves passing the stained cells through a laser beam and recording the fluorescence emission from the bound antibody conjugates. This is followed by data analysis, in which cell populations of interest are defined and reported on. Ref., reference; SD, standard deviation.
Reprint with permission from Springer Nature, Nature Reviews Immunology, Standardizing immunophenotyping for the Human Immunology Project, Maecker, H.T. et al.[245] *© 2012. with permission from Springer Nature and Copyright Clearance Center.*

Unfortunately, each fluorochrome has an emission spectrum that can potentially overlap with another, so the number of fluorochromes in a single experiment is limited. However this shortcoming is typically corrected with color compensation, the mathematical calculation of the spill-over from one fluorochrome into other detector channels[246].

In study I and II we applied the twelve fluorochrome-conjugated antibodies in multicolor flow cytometry to phenotype and analyze the activity state of the main mononuclear immune cell types, T cells, monocytes, B cells, NK cells and NKT-cells. In study III, 18 fluorochrome-conjugated antibodies were used to discriminate DC populations and subsets in psoriasis patients. Cell subset definitions for study I and II are listed in Table 4, and for study III in Table 5.

3.2.3 Data processing and statistical analysis

In all three studies, initial visualization and analysis of data was done in FlowJo version 10.2 (Tree Star). Identification of immune cell populations and their subsets in flow cytometry experiment was based on light scatter characteristics, live-dead properties, and the relative expression of CD markers. Flow cytometry data was analyzed in FlowJo v10.6.1 (Tree Star, Ashland, OR, USA).

Flow cytometry data are complex multiparametric data at the single-cell level, and computational high-dimensional data reduction algorithms can be used for their analysis and visualization[247]. Uniform Manifold Approximation and Projection (UMAP) is a machine learning algorithm used for dimensionality reduction to visualize high parameter datasets in a two-dimensional space. It allows UMAP to preserve short run times as well as the local and global data structures[247]. In study II, the UMAP algorithm was used for visualization of isolated immune cell populations and subsets. Each sample was downsampled to 200,000 live cell events. For UMAP visualization all samples were concatenated together. Samples belonging to groups (HC, psoriasis, and psoriatic arthritis) were identified, and 200,000 random events from each group were clustered using a UMAP algorithm to visualize the live cells on a 2D plain.

To compare the data acquired in flow cytometry, non-parametric tests were used. In study I and III, the Mann-Whitney U test was used for independent, unpaired data, and Wilcoxon signed-rank test for paired data (between inclusion and follow-up). In study II Kruskal-Wallis test with an uncorrected Dunn's test was used in the comparison between the patients' groups and HC. Differences were considered statistically significant when $p < 0.05$. The flow cytometry experiments were of exploratory nature and hence no correction was made for multiple comparisons. For correlation analyses in study I the strength of correlations was defined by Spearman's rank order test. Degree of correlation was interpreted with r: 0.00-0.19 (very weak), 0.20-0.39 (weak), 0.40-0.59 (moderate), 0.60-0.79 (strong), 0.80-1.00 (very strong)[248].

Comparisons between investigated groups, and the production of associated graphs in all three studies were done using GraphPad Prism (Version 9.1.1 (225)). In study II volcano plots and heatmaps were generated in Microsoft Excel.

3.3 Legal and ethical aspects

The studies presented in this doctoral thesis, and the biobank were approved by the regional ethics committee (2017/938) and (2014/1489 and 2014/1373), respectively. Written informed consent was obtained from all participants. External funding sources did not influence planned methods, data analyses or presentation of the results.

4. Results

4.1 Study I

Psoriasis patients on herring roe oil (HRO) treatment have alterations in peripheral blood immune cells compared to placebo controls.

Since psoriasis patients receiving HRO (ω-3 PUFAs) had a significant reduction in mean PASI, we followed up these findings and analyzed the composition and phenotype of PBMC in selected patients before and 26 weeks after initiation of HRO treatment by flow cytometry. Exploring the activity state of immune cells, we found a significant decrease of $CD38^+$ T cells in HRO treated patients after 26 weeks, both $CD4^+$ and $CD8^+$ T cells, and particularly $CD38^+$ $CD4^+$ T_N cells, $CD4^+$ T_{CM} cells and $CD8^+$ T_N cells in patients treated with HRO. In addition, we observed a significantly higher percentage of $CD107a^+CD8^+$ T cells, and $CD107a^+$ $CD8^+$ T_{TD} cells in the control group compared to HRO treated patients. We next determined expression of activation markers on peripheral NK cells and found a significant reduction of $CD38^+$ $CD56^{bright}CD16^-$ NK cells after treatment with HRO. We then analyzed frequencies of the different monocyte populations. The frequency of non-classical and classical monocytes in the controls was significantly lower at week 26. Further analysis of activation markers on classical monocytes showed a significant decrease in expression of CD38 over time in both patient groups.

Furthermore, we studied plasma levels of proinflammatory and anti-inflammatory cytokines at four time points (inclusion, after 12, 26, and 65 weeks of treatment) in patients and found decreased levels of CCL2 over time from week 12 in both treatment groups and significant decrease at week 65. On the contrary, CCL5, CD25/sIL-2R, CXCL10, and IFN-γR1 levels increased at the end of the study period. Moreover, correlation analysis revealed moderate negative correlation between severity of the disease (PASI) at week 12 and FN-γ R1 plasma levels in HRO supplemented patients.

4.2 Study II

Patients with moderate-to-severe psoriasis on continuous stable therapy with infliximab displayed skewed immune cells frequencies in patients with psoriasis (PsO) and PsA

Multi-color flow cytometry was used to perform in-depth phenotyping of PBMCs and to examine cell distribution and the expression of cell surface markers that indicate cellular activation in PsO and PsA patients. We observed a significant decrease of NK cells and their subset $CD56^{dim}CD16^{+}$ NK cells in PsA compared to both HC and PsO. This decrease of the $CD56^{dim}CD16^{+}$ NK subset was further confirmed within NK cells with a corresponding increase in the frequency of $CD56^{bright}CD16^{-}$ NK cell subset when comparing HC to PsA. Compared to HC, PsO demonstrated shifts of the three B cell subsets with a decrease in transitional $CD27^{-}CD38^{high}$ B cells, while no shifts were apparent in PsA. Examination of the activity state of various immune cells showed notable increase expression of CD69 in monocytes in PsA as well as in both intermediate $CD14^{+}CD16^{+}$ and classical $CD14^{+}CD16^{-}$ monocytes compared to both HC and PsO. A skewed CD38 expression level in multiple immune cell subsets was the most notable feature in PsO, with increased expression in the $CD4^{+}$ T_{EM} $CD45RO^{+}CD27^{-}$, and T_{TD} $CD45RO^{-}CD27^{-}$ cells, as well as in intermediate $CD14^{+}CD16^{+}$ monocytes and NKT-like cells compared to HC. Interestingly both patient groups displayed reductions of CD38 in memory $CD27^{+}CD38^{+/-}$ B cells compared to HC. Although differences were small, a consistent decrease of CD107a expression in both T (CD8+ T_{CM}, $CD8^{+}$ T_{TD}) and NK cell subsets ($CD56^{bright}CD16^{-}$ NK cells, $CD56^{dim}CD16^{+}$ NK cells) was notable in PsA, interestingly this decrease was limited to $CD56^{dim}CD16^{+}$ NK cell and $CD56^{bright}CD16^{-}$ NK cell subsets in PsO,

4.3 Study III

Psoriasis patients on different biologics and healthy controls display different frequencies of DC populations and subsets

In our third study, we investigated the phenotypes of circulating DC in patients with severe plaque psoriasis, at inclusion and approximately 12 months after initiation of the treatment with different biologics, including anti-TNF etanercept, infliximab, anti-IL-17A secukinumab, and anti-IL12/IL-23 ustekinumab, to identify potential DC subsets as biomarkers for clinical response. We observed persistent low levels of peripheral blood pDC in patients treated with infliximab and secukinumab. In addition, secukinumab-treated patients showed sustained low levels of peripheral blood $CD5^+$ DC2. Etanercept treatment resulted in significantly increased levels of both cDC populations including subsets $CD5^+$ DC2, $CD5^-CD163^-CD14^-$ DC3 and $CD5^-CD163^+CD14^-$ DC3. Furthermore, we observed continuous low levels of pDC in patients with PsA and cDC2 increase in PsO after the introduction of biologics. The increase in cDC2 in patients with PsO upon treatment was mostly due to increased $CD5^-CD163^+$ $CD14^-$ DC3 subpopulation. Interestingly no differences in cDC populations and subsets were apparent in patients with PsO compared to HC.

5. Discussion

5.1 Methodological considerations

5.1.1 Common considerations for single cell analyses

Patients and controls: Study I included 58 patients, study II included 24 patients and 33 healthy controls and study III included 38 patients and 38 healthy controls. Success of each cytometry experiment is related to the use of relevant controls. In Study II and III, healthy donors were used as biological controls. Cryopreserved PBMC sample from one healthy donor (technical control) was included in each experimental run as an internal control to assess and adjust possible technical variations in experiments. We did not perform any power calculation in advance as this was an explorative study. Many variables were analyzed in each experiment with a risk of false positive results caused by multiple comparisons without making corrections[249]. However, all three studies were conducted to assess quantitative and qualitative differences in PBMCs and should therefore be taken into account in spite of low power.

Freezing and thawing of PBMC: Cryopreserved cells are widely used in research, as they are practical for simultaneous analysis of many samples. This avoid inter-assay variation, and allow for further testing if an obstacle occurs [250]. Via gradual freezing (1°C/minute) and use of cryoprotectant, dimethyl sulfoxide (DMSO), PBMC should preserve viability and avoid crystals forming and cell-membrane fracturing[251, 252]. Widely used freezing media usually contains serum support media, such as fetal bovine serum (FBS) which can cause unspecific stimulation of immune cells. To avoid this, our freezing medium was a mixture of non-animal support medium, ProFreezeTM-CDM, a serum-free media, X-VIVO-20^{TM} and DMSO. The temperature and osmotic change while thawing cells could affect the recovery and viability of the PBMCs, therefore the cells were thawed rapidly at 37° C[253]. As DMSO is toxic to lymphocytes, a washing step was performed during the thawing procedure[254].

Antibody selection and titration: Antibodies against surface antigens were chosen based on their ability to differentiate the cell population, subpopulation, and subsets in PBMCs, by their combinatorial expression on cells. Differences in cell frequencies

compared to healthy controls have been reported in the context of psoriasis. It has been reported that patients with psoriasis have higher total leukocytes, neutrophils, neutrophil to lymphocyte ratio (NLR), and lower lymphocytes [255]. $CD8^+$ T cells, monocytes, particularly intermediate monocytes, have been reported to be increased in active disease[256-259]. On the other hand, NK cells and pDC have been shown to be decreased in psoriasis, while contradictory results exist showing decreased or stable frequencies of circulatory NKT-like cells [65, 260-262]. In study I and II, we decided to look not only at the parental populations of T, B, NK, NKT-like cells and monocytes, but also at subpopulations. In study III, we primarily focused on the DC subpopulations and subsets. As we used PBMCs, we did not expect to see granulocytes and macrophages, and hence did not include those markers in our panels. All antibodies were titrated for their specific experimental conditions to find their optimal staining concentrations. For all surface molecules selected, the concentrations that gave clear separations between negative and positive populations and, at the same time, minimizing spillover.

Fixation of cells: To provide better time to analyze prepared cell, fixation of cells is an opportunity. The disadvantage of fixation in sample preparation is potential cell death, and therefore dynamic biological processes can not be investigated. The most common fixative is formaldehyde[263]. We utilized 1,6 % PFA by diluting stock 1:10 with FACS buffer in order to have the same conditions for all samples.

5.1.2 Flow cytometry: special considerations

Panel design: Which fluorophore will be used in the cytometry panel depends on wavelength of available lasers and filters on the flow cytometer, and availability of wanted fluorochrome-conjugated antibody. Usually, bright fluorochromes are used for rare antigens and dim for ubiquitous antigens. Next, to minimize compensation, it is necessary to reduce spillover into important and sensitive channels (low abundant antigens).

Control of cytometer setup and performance: We utilized cytometer setup and tracking beads to determine minimal baseline photomultiplier tube (PMT) voltages and to control cytometer performance including laser alignment, laser time delay and

sensitivity. The unstained cells and single-stained cells/beads were used during experiment setup aiming to optimize PMT voltages and spillover into other channels. In all flow cytometry (FC) experiments before running samples of interest, we prepared and run single stained compensation controls (beads or PBMCs) making a compensation matrix for the correction of fluorescent spillover. To set appropriate gates and have proper separation between negative and positive populations, particularly where it was not clear, we performed fluorescence minus one (FMO) controls, which included all antibodies in the panel but one.

Advantages and limitations of flow cytometry: FC as a single cell analysis method has several advantages and drawbacks. Advantages of FC include high throughput (measurement of up to several thousand events per second) and collected information on cell size and granularity through FSC and SSC measurements[264]. Analytical FC can be used to define cellular characteristics including the expression of specific intracellular markers or receptors, or to identify post-translational modifications of proteins. In addition, a step up from analytical FC is fluorescence-activated cell sorting (FACS), which provides the ability to extract cells for downstream analysis[265]. Limitations of FC include relatively low number of fluorochromes encompassed by panel, spectral overlap of fluorescent probes, that requires complicated compensations, autofluorescence and limited time for sample processing[266].

5.1.3 Luminex assay

Many factors can influence the measurement of cytokine levels. It includes the time required for sample preparation before cryopreservation, which should not exceed 4 hours[267]. Storage duration and temperature can influence cytokine levels as well[268, 269]. Type of sample (plasma, serum), and used anticoagulants (e.g., heparin, EDTA, citrate) can also affect measurement results [267, 270]. Plasma has some advantage over serum because the coagulation may influence cytokine release from cells[267]. Thus, we used heparin plasma for our analysis. Biological variations including intra- (like circadian rhythm, infections etc.) and inter individual variations in cytokine levels can be expected[271, 272]. To avoid technical variations, we analyzed the samples in random manner on the plate. Based on the earlier experience

with the assay, we performed adjustments to the manufacturer's recommendations. We added: (a) an extra dilution of standard in the serial dilution to be able to determine cytokine levels in the lowest end of the scale; (b) diluted samples 1:2 for 20-plex and IL-23, and 1:50 for CCL5. The median fluorescence intensity (MFI) of missing values and under the lowest standard values were not considered. Hence, we ended up with five analytes of 100% detection rate (CCL5, CCL2, CD25/sIL-2Rα, CXCL10 and IFN-γ R1) that were included in further analysis. Other cytokines were excluded from the analysis, as they were below detection limit in most samples. A dysregulated cytokine profile in psoriasis has been reported by many, with a systemic and local increase of proinflammatory cytokines[6, 97, 120]. Cytokine levels can be detected by several methods (ELISA, Luminex) with different sensitivity complicating direct comparison of cytokine levels from different studies [90, 96, 273].

5.2 Biological and clinical implications of the results

Psoriasis is a chronic inflammatory disease of the skin, joints or both, with autoimmune background, dysregulated immune response and systemic inflammation[4]. It is now recognized that other chronic inflammatory diseases such as Crohn's disease, diabetes mellitus and CVD represent co-morbidities of psoriasis[126, 274]. A vast majority of patients have mild disease, and their symptoms are usually controlled with local treatment, but an increasing number of patients reach for additional therapy such as ω3-PUFA in the hope of even better control[11, 140]. On the other hand, patients with severe disease may require treatment with biopharmaceuticals[275]. These targeted therapies are highly successful in psoriasis and the clinical efficacy of different biologics used in our project as measured by PASI and DLQI was beneficial in most patients. We employed high-throughput single-cell technology to compare peripheral blood immune cell composition and activation state of psoriasis patient with or without PsA and HC under different treatments. Biological explanation of the observations in immune cells, cytokines, and clinical parameters, will be presented in the following discussion.

5.2.1 The beneficial effect of HRO supplementation is reflected in the reduction of CD38 expression on $CD4^+$ and $CD8^+$ T cells, and $CD56^{bright}$ NK cells

Substantial research has been conducted to define CD38 as a marker of cell activation. It has a significant role in inflammation and autoimmunity, where CD38 plays roles in modulating cell differentiation and effector functions[276]. CD38 can establish lateral associations with various membrane proteins/complexes, including CD3/TCR, modulating activation thresholds from these complexes. Lipid rafts play an important role in the stability and function of such complexes[277]. Long-chain ω3-PUFAs could potentially interfere with signaling proteins in lipid rafts of the cell membrane, for which we were particularly interested in their effect on CD38 expression[212] In our study, patients treated with HRO showed decreased expression of CD38 in both $CD4^+$ and $CD8^+$ T cells, indicating a decreased activity of these cells.

In patients with psoriasis, reduction of $CD56^{bright}$ $CD16^-$ NK cells in circulation was followed by accumulated CXCR3 expressing $CD56^{bright}CD16^-$ NK cells in lesional skin, probably due to CXCL10 production by psoriatic keratinocytes[278]. In our study, stable plasma levels of CXCL10 chemokine in both patient groups during the 26 weeks could support steady frequencies of circulatory $CD56^{bright}CD16^-$ NK cells. Furthermore, during the HRO supplementation this subset showed lower expression of CD38 surface molecule indicating possible reduced capacity in transduction of activating signals.

5.2.2 The increased cytotoxicity of $CD8^+$ T cells after the oral intake of coconut oil, and decreased cytotoxicity of NK cells and $CD8^+$ T cells during IFX treatment

Few studies have investigated CD107a/lysosomal-associated membrane protein 1 (LAMP-1) in psoriasis, and the research was focused on tissue expression. Patients with the most pronounced PASI, BSA, and DLQI scores have been shown to have a higher incidence of CD107a-positive cells in psoriatic skin biopsies with a predominance of these cells in the epidermis compared to the dermis, but without the precise identification of cell types[279]. Interestingly, our patients treated with coconut oil had an increase in frequency of $CD107a^+$ T cells, particularly $CD107a^+$ $CD8^+$ T_{TD}

cells over time. These findings suggested their higher activity/cytotoxic potential, even though without clinical deterioration[280]. To date, no human studies (clinical or observational) have assessed potential preventive or therapeutic effects of orally administered coconut oil in patients with psoriasis. In contrast, numerous studies have been conducted to evaluate the effects of coconut oil on serum lipids and blood pressure in patients with CVD albeit with contradictory results[281]. In study II, the analysis of PBMC showed reduced cytotoxic activity in innate and adaptive immune cells including $CD56^{bright}CD16^{-}$ NK cells and $CD56^{dim}CD16^{+}$ NK cells in both patients groups as well as subpopulations of $CD8^{+}$ T cells (T_{CM}, T_{TD}) that were restricted to PsA as a confirmation of the favorable effect of IFX treatment[280].

5.2.3 The increase of IFN-γR1 and decrease in chemokine CCL2 could be related to the action of HRO in patients with mild psoriasis

A modest number of studies have been conducted to investigate the impact of ω3-PUFAs to the mediators of inflammation in psoriasis. In animal models, reduced production of proinflammatory cytokines, IL-17, IL-22, IL-23, have demonstrated the anti-inflammatory effect of n-3 polyunsaturated fatty acids and their bioactive metabolite[282-284]. Conflicting data exists regarding the effect of ω-3 PUFAs on binding of IFN-γ to IFN-γ receptor in animal models. It has been shown that a diet with high amount of ω-3 PUFAs induces reduction in IFN-γ binding due to the internalization and decrease in the number of expressed IFN-γR1 on the cell surface. The result is an immune cell hypo-responsiveness to IFN-γ with a consequent anti-inflammatory effect[285]. In contrast, another murine study showed that diminished IFN-γ signaling, but not reduction in the expression of the receptor, in murine macrophages is one mechanism by which ω-3 PUFAs affect in vivo responsiveness to this cytokine[286] To date, no investigation of the impact of HRO on cytokines in patients with psoriasis has been done. In our study, not all analyzed cytokines were detected, which was expected in patients with mild disease. In our study I, observed augmentation of circulatory IFN-γR1 in patients at week 65 could affect the clinical improvement of psoriasis, since we have determined negative moderate correlation between the lowest level of IFN-γR1 measured at week 12 and PASI score in patients

treated with HRO. One might speculate that the supplementation with HRO caused the disturbance in signal transduction through the IFN-γ receptor with the consequent beneficial effect on disease severity.

Elevated levels of serum/plasma sIL-2Rα (CD25) has been observed in individuals with various immune-mediated diseases including psoriasis[287]. Moreover, its serum concentration as well as the frequency of $CD25^+$ cells in lesional skin correlate with disease severity expressed by PASI. We observed a significant increase of sIL-2Rα following treatment with HRO despite the clear improvement in PASI which needs further analysis.

It has been shown that, increased serum level of CXCL1, CCL5 and CCL2 in psoriasis patients decreases after treatment with coal tar and UV (Goeckerman regime) resulting in improvement of skin lesions[288]. In patients with psoriasis, the main source of CCL2 is keratinocytes. The binding of CCL2 to the chemokine receptor CCR2, mainly expressed on the surface of monocytes, induces migration from the circulation into lesional skin and differentiation into macrophages. These cells are able to act as APC and secrete TNF responsible for maintaining skin inflammation[289]. In psoriatic skin, CCL2 is effectively induced by IFN-γ or complement component (C3) with consecutively transepithelial migration of monocytes, DCs and $CD4^+$ T cells[290-292]. It has also been observed that ω–3 PUFA consumption inversely correlates with the levels of peripheral C3 and CRP concentration[293]. Accordingly, we can assume that consumption of HRO interferes with the decrease of C3, affecting the production of CCL2 with the subsequent decrease in migration of monocytes to the site of inflammation. Elevated levels of circulatory CCL2 in patients with psoriasis have been observed in several studies[294, 295]. Various therapeutic modalities such as anti-TNF, anti-CD11, combination of coal tar and UV light, and narrowband UVB phototherapy induce the decrease of CCL2 in the circulation[288, 294, 296]. According to the results of study I, HRO supplementation has a similar effect.

5.2.4 Lower frequency of NK cells in patients with psoriatic arthritis

In concordance with previous findings that psoriasis patients have deviant immune cell frequencies which normalize with TNF treatment, no differences in cell frequencies of main populations except for NK cells were observed in our study II[258]. The observed decrease of circulatory NK cells relative to total live PBMC in PsA compared to HC and PsO could indicate increased disease activity due to the ongoing NK cell recruitment from the circulation to synovium, as suggested in previous studies[297]. On the contrary, our patients were on stable IFX treatment with low skin activity. We can only speculate that the decrease in peripheral NK, i.e. $CD56^{dim}CD16^{+}$ NK cells in PsA could be a result of chronic systemic inflammation with impaired cell survival[260]. In addition, the state of NK cell activity through the expression of the investigated surface receptors did not differ from HC, except for CD107a, which was significantly reduced in both NK cell subpopulations of patient groups, thus indicating their reduced cytotoxicity.

5.2.5 Continuous impairment of Tregs in psoriasis

Numerous studies have observed a contributing role of B cells in the pathophysiology of psoriasis[25]. Conflicting results have shown variations in quantity and quality of peripheral B cells and their subsets depending on disease activity and applied treatment[298-300]. In our patients in study II, the disturbed ratio of B cell subsets with the shift from naïve $CD27^{-}$ $CD38^{+/-}$ to memory $CD27^{+}CD38^{+/-}$ B cells and significant reduction of transitional $CD27^{-}CD38^{high}$ B cells was in concordance with a previous study[301]. In humans, immature transitional B cells, through IL-10 secretion display regulatory functions[302]. Transitional B cells suppress autoreactive $CD4^{+}$ T cell proliferation, $CD8^{+}$ T cell activation, production of proinflammatory cytokines, differentiation of $CD4^{+}$ T cells into Th1 and Th17 and contribute to the transformation of effector $CD4^{+}$ T cells into Tregs[303-305]. Recent studies have shown that transitional B cells are decreased and functionally impaired in both psoriasis and PsA as well as in the circulation and skin[82, 301, 306, 307]. In our PsO, a decrease in transitional B cells indicated their reduced suppressive capacity that contributes to the dysfunctional state of Tregs, even though they were on stable IFX treatment. Unlike

PsO, patients with PsA showed complete restoration of B cell frequencies as noted in previous research[297]. Although memory $CD27^{+}CD38^{+/-}$ B cells were increased in PsO, their activity in both patient groups was reduced through the decrease of CD38 which is fundamental in BCR activation [308]. Down-regulation of CD38 was probably IL-4 induced as it has been noticed in IFX treated patients with rheumatoid arthritis [309-311]. The confirmation for this can be the increase of IL-4 in the blood of patients with psoriasis after 12 weeks of treatment with etanercept[312].

5.2.6 Relation to comorbidities

Several studies have confirmed association of psoriasis with a higher prevalence of cardiovascular risk factors, such as obesity, high blood pressure, diabetes mellitus, dyslipidaemia, and metabolic syndrome [1, 313, 314]. Pathophysiology of psoriasis and atherosclerosis has a common denominator, such as monocytes[315]. Circulating monocytes are important cellular players in the development and progression of both diseases. In peripheral blood of patients with psoriasis, elevated levels of intermediate $CD14^{+}CD16^{+}$ monocytes and reduced levels of classical $CD14^{+}CD16^{-}$ monocytes have been observed[258, 259]. These two subsets of monocytes act as pro-atherogenic cells while non-classical $CD14^{-}CD16^{+}$ monocytes exert an atheroprotective effect as they maintain vascular homeostasis[316]. In study I, HRO treatment did not affect frequency of monocyte subsets. However, we observed a decrease in $CD14^{-}CD16^{+}$ non-classical and $CD14^{+}CD16^{-}$ classical monocytes in the control patients treated with coconut oil indicating a higher risk of developing atherosclerosis in these obese patients[314]. This needs further investigations and a follow-up over time. In study II, PsO had higher frequencies of intermediate $CD14^{+}CD16^{+}$ monocytes. It has been shown that these cells could be involved in creation of monocyte-platelet aggregates (MPA) with consequent increased adhesion to vascular endothelium and trans-endothelial migration[317]. As a marker of a platelet activation, MPA is an indicator of coronary artery disease that may contribute to atherosclerosis progression[318]. Moreover, patients with psoriasis have a higher risk of death due to CVD caused by an increase in MPA, and intermediate monocytes that correlate with severity of the disease[259, 319]. Conflicting data exists regarding the effect of infliximab treatment to the risk of CVD in patients with severe psoriasis[320, 321]. Compared to

conventional systemic therapies, or phototherapy, treatment with TNF inhibitors reduces the risk of CVD in patients with severe psoriasis [320, 321]. However, there is a lack of evidence that can equalize reduced CVD risk by TNF inhibitors in psoriasis to CVD risk in a general healthy population. In study 2, elevated level of activated intermediate $CD14^{+}CD16^{+}$ monocytes through increased expression of CD69 in PsA and CD38 in PsO supports the hypothesis of ongoing systemic inflammation in both patient groups, even after resolution of skin lesions. As systemic inflammation promotes CVD, this result raises the question if these patients have a persisting increased risk of CVD due to the activity of different surface molecules and poor balance between pro-atherogenic and anti-atherogenic monocytes[126, 322].

5.2.7 The effect of IFX treatment through increased expression of CD38 on CD4+ T cells and NKT-like cells

Within immune cells, CD38 is expressed constitutively on plasmablasts and plasma cells, and after stimulation on macrophages, NK cells, monocytes, NKT like cells, T cells, DCs, and innate lymphoid cells[276]. In study II, the increased expression of CD38 on $CD4^{+}$ T_{EM} in PsO group could point to their greater ability to traffic through peripheral organs and blood[323]. In addition, the role of elevated $CD38^{+}CD4^{+}$ T_{TD} cells in patient with PsO is not known, although we can speculate that these cells have an active profile with reduced proliferation capacity but improved potential to produce cytokines, as it has been observed in studies conducted both, in mice and humans[324, 325]. This raises the question of whether antigens/autoantigens in psoriasis trigger terminal effector differentiation and T_{TD}/T_{EMRA} cell formation, which may be related to antigen loading or persistence.

$CD3^{+}CD56^{+}$ NKT-like cells take part in the pathogenesis of psoriasis[298]. Their number in skin inflammatory infiltrate decrease with the treatment[326]. In untreated patients with psoriasis, levels of circulating NKT-like cells are stable[261]. However, recent findings in patients with RA have shown that $CD38^{+}$ NKT-like cells co-cultured with T cells stimulate the differentiation of $CD4^{+}$ T cells into Tregs and reduce the Th17 level in synovial fluid[327]. In study II, highest expression of CD38 on NKT-like cells in PsO but not in PsA, could indicate a beneficial effect of IFX treatment, but it

remains unknown whether these cells reach lesional skin and restore impaired immune tolerance, and whether they are sufficient for the normal functioning of Tregs, which are already disturbed in psoriasis.

5.2.8 Altered DC frequencies in patients with psoriasis compared to healthy controls

Biologics have been used in the treatment of psoriasis more than two decades, but we still lack predictive markers to foresee which patients will need biological treatment and what the efficacy of different drugs will be on individual psoriasis patients [328]. For this reason, studies focusing on the phenotypic characteristics of immune cells involved in the pathophysiology of psoriasis are increasing in order to better stratify patients in the context of adequate targeted therapy. DC play a significant role in pathophysiology of psoriasis, and they have been extensively studied in this context in the skin [66, 329]. However, the discovery of DC heterogeneity and new subsets has renewed research interest analyzing the function of these cells in initiating and maintaining inflammation in psoriasis [330-332]. Early during disease development, pDC infiltrate the skin and become activated to produce IFN-α continuing the pathophysiological cascade[65]**.** Thus, lower levels of pDC in the peripheral blood of patients with psoriasis may suggest their active involvement in inflamed tissues [65]. Conversely, normalization of peripheral blood pDC may be interpreted as a suspension of their active recruitment to the skin due to the favorable therapeutic effect observed in our PsO group (study III). However, we did not expect the maintenance of low levels of pDC in PsA, and in patients treated with IFX and SEC over the course of effective treatment. An explanation for this could be phenotype switching of blood-to-skin/joint recruited pDC from an immunogenic to a tolerogenic phenotype that promote self-antigen-specific $CD4^+$ T-cell tolerance and induce Treg differentiation, as observed in RA and type 1 diabetes [333, 334]. This shift in function of pDC might be partly related to presence of PsA and the application of certain biologics but not to the success of the treatment, considering that it was observed in patients with both higher and lower PASI improvement. We could not further stratify the patients into PsA and PsO within the groups treated with a specific biologic due to too small number of patients in these groups.

5.2.9 Treatment responsiveness in patients with psoriasis could be associated with changes in frequencies of cDCs and cDC2 subsets

Recently, extended analysis of DC populations in human skin revealed terminally differentiated $CD5^+$ DC as a superior activators of inflammatory T cell responses [335]. Their distribution in circulation has not been investigated much until now. In our results, the observed decrease in circulatory $CD5^+$ DC2 in PsA patients was probably due to their recruitment to the site of inflammation. This is where their capacity for local enhanced $CD4^+$ T cell proliferation comes to the fore [335, 336]. However, their frequency returned to HC levels during 12 months of biological treatment. Still, the normalization of $CD5^+$ DC2 frequency in the secukinumab-treated patient group was absent which can be related to the presence of PsA and lower treatment responsiveness. These patients had the lowest PASI and DLQI improvement, as well as the longest disease duration (29.6 years), the highest BMI (32.5), and the highest number of those who were previously treated with other biologics, therefore, all the characteristics that make the clinical control of the disease difficult [6, 337]. Conversely, the increased levels of both cDCs and cDC2 subsets including, $CD5^+$ DC2, $CD5^-CD163^-CD14^-$ DC3 and $CD163^+CD14^-$ DC3 during follow-up in etanercept-treated patients, could be related to beneficial effect of the treatment mirrored in reduction of PASI for 74.5% and DLQI for 91.3%. It has been shown that etanercept specifically induces apoptosis of $CD11c^+$ cDC in lesional skin of psoriasis patients with PASI 75 improvement for 6 months follow-up [338]. Local decrease in number and proinflammatory activity of $CD11c^+$ cDC produces substantial therapeutic effects [338]. Moreover, clinical improvement could also be related to patient group characteristics including the absence of PsA, low average age (27.2 years), shorter disease duration (12.2 years), lower BMI (28.3) and absence of prior biological treatment.

Pro-inflammatory $CD5^-CD163^+CD14^+$ DC3 have recently come into focus [331]. These cells, identified in the blood of patients with systemic lupus erythematosus (SLE), were correlated with disease activity. Dutertre and colleagues hypothesized that $CD5^-CD163^+CD14^+$ DC3 with the ability to stimulate Th17 differentiation could also play a pathogenic role in psoriasis inflammation [331]. However, conversely to the

SLE patients, in our PsO/PsA patients we did not find significant differences in the proportions of peripheral blood $CD5^{-}CD163^{+}CD14^{+}$ DC3 compared to HC, and neither any statistical correlation between their frequency, and clinical parameters of disease severity, PASI and DLQI [331]. The peripheral blood compartment might therefore not be appropriate to assess the role of proinflammatory DC3 in the development and maintenance of psoriasis. In lesional skin of patients with psoriasis an increase of $CD14^{+}$ DC3s were identified [339].

5.3 Limitations of the studies

In the present work, the sample size was a trade-off between the ability to detect differences between groups and the methods used, which involved expensive reagents, technologies, and time-consuming and laborious work. Consequently, cohorts were not optimal. We did not include healthy controls in study I, as the main objective was to assess intra-individual changes in cytokine levels and immune cells activation states depending on the severity of the disease and the applied HRO treatment. There was also a lack of PBMC samples at the end of follow up, week 65. Healthy controls were included in study II with one timepoint, but not biologics-naive samples from patients. All patients, except one, were treated with infliximab and methotrexate simultaneously. It has been shown that methotrexate helps in the restoration of the immune balance by decreasing Th1 and Th17 cells and increasing Th2 and Treg cells in circulation, thus resulting in a significant reduction in disease severity[340]. In study III, we included pre-treatment samples, but only one time point for healthy controls. We had a small number of patients in the individual PsO and PsA groups treated with different biologics

A larg sample size is necessary to reveal differences in cytokines, activation status, PBMC populations and subpopulations between different psoriasis patients (i.e, early/late onset of the disease, presence/absence PsA, naïve/non-naïve to treatment, responders and non-responders to treatment) receiving different treatments.

Our studies were exploratory in nature; therefore, we did not adjust results for multiple comparisons, which is needed when analyzing high-dimensional datasets. Sometimes,

in small sample size, these adjustments, although statistically correct, could negatively affect p-value, and thereby hide biologically relevant results.

In studies II and III, disease severity in patients with psoriasis and psoriatic arthritis was evaluated by use of PASI and DLQI, and we did not assess peripheral joint activity, pain, or several other domains including spinal disease, dactylitis, enthesitis, and nail disease.

All samples from patients and HC were cryopreserved, which could have affected cytokines and cell activation, but since we performed the same procedures, the error was reduced to systematic. Duration of storage in liquid nitrogen for patient samples was longer than for HC, but it did not obviously affect the activity state of immune cells.

Limitation of study I was also low sensitivity of the cytokine assay. Five analytes were detected in all plasma samples (CCL5, CCL2, CD25/sIL-2Rα, CXCL10 and IFN-γ R1), while other cytokines were excluded from the analysis, as they were below detection limit in most samples. Detection of cytokines in blood could have been better with the use of more sensitive kits. Plasma measurements are probably not sufficient and sensitive enough to investigate cytokines, but additional measurements in local tissue, such as skin or synovium and synovial fluid, can complete the picture and possibly provide more information.

A limitation of all three studies is the lack of skin biopsy specimens from psoriatic lesions. Future studies exploring simultaneously immune cells in circulation and skin biopsy specimens in subgroups of psoriasis patients and healthy controls might shed further light on the utility of different treatment options.

We observed variation within immune cell subsets, and this could be explained by the variety of underlying profiles within each individual having different characteristics (i.e., age, sex and BMI). Therefore, the interpretation of the results must be done with caution. More studies with a larger number of patients with different characteristics and healthy controls in different time points are needed to confirm our results.

6. Conclusions

The work presented in this thesis identifies single-cell analysis as a useful approach towards understanding the complex immunological interplay between cells and cytokines network in psoriasis. Unique immune profiles allow stratification of patients, which could lead us to more personalized treatment of the disease.

6.1 Study I

Psoriasis patients could have a beneficial effect of HRO treatment due to the reduction of CD38 expression on $CD4^+$ and $CD8^+$ T cells, and $CD56^{bright}$ NK cells. This effect is complemented by the increase of IFN-γR1 and decrease in chemokine CCL2. Patients treated with coconut oil have an increased cytotoxic capacity of $CD8^+$ T cells and a higher risk of developing atherosclerosis due to decrease in $CD14^-CD16^+$ non-classical monocytes.

6.2 Study II

Although the clinical stability of patients with psoriasis and PsA treated with infliximab was evident, our exploratory study indicated a preserved pathophysiological process including continuous systemic inflammation through the increase of active intermediate $CD14^+CD16^+$ monocytes. Moreover, in patients with psoriasis, the decrease in transitional B cells indicated their reduced suppressive capacity that contributes to the sustained dysfunctionality of Tregs. However, the beneficial effect of IFX treatment was reflected in reduced cytotoxicity of NK cells in both patient groups and $CD8^+$ T cells in patients with PsA.

6.3 Study III

Biological treatment in patients with psoriasis and PsA caused differences in DC frequencies. The persisting low levels of pDC in peripheral blood in patients with PsA might relate to the presence of arthritis. In addition, the sustained low levels of $CD5^+$

DC2 frequency in the secukinumab-treated patient group could be associated with the presence of PsA and lower treatment responsiveness. In contrast, the increased levels of cDC1, cDC2, and cDC2 subsets during follow-up in etanercept-treated patients could be related to beneficial effect of the treatment.

7. Future perspectives

Numerous studies conducted on patients with psoriasis have led to a better understanding of the disease and better treatment, but we still do not have answers to the questions regarding the initiation of psoriasis, pathophysiology, and prediction of treatment response. Combining immunological and translational research through the technologies that measure features of the single-cell, genome, transcriptome, and proteome, we can generate data that will facilitate stratification of patients according to their cellular profiles[312, 341]. However, we still have a methodological problem in implementing the results of this research. To overcome these difficulties and identify good biomarkers we need well designed prospective studies[312].

Our findings of decreased expression of CD38 in both $CD4^+$ and $CD8^+$ T cells of psoriasis patients with mild disease treated with HRO, indicated a decreased activity of these cells. Therefore, those treated with HRO warrant further investigation of CD3/TCR activation thresholds due to the potential interaction of ω3-PUFAs with signaling proteins in cell membrane lipid rafts, with a particular interest in the effect on CD38 expression[212]. Additionally, possible reduced capacity in transduction of activating signals due to lower expression of CD38 surface molecule on circulatory $CD56^{bright}CD16^-$ NK cells, during the HRO treatment, may be the subject of further study. Namely, it would be interesting to simultaneously analyze expression of CD38 on $CD56^{bright}CD16^-$ NK cells in lesional skin and circulation and explore their potential immunoregulatory function observed in different autoimmune diseases, such as multiple sclerosis and osteoarthritis[342, 343].

In patients on stable IFX treatment, we observed a difference in frequency of peripheral NK, cells between PsO and PsA patients. Further functional research, based on study II results, could investigate the decrease and potentially impaired survival of NK cells. In study II, we found an increased frequency of $CD38^+CD4^+$ T_{TD} cells. These cells likely have an active profile and an enhanced potential for cytokine production that may be related to antigen loading or persistence[324, 325]. Future studies could analyze simultaneous expression of CD38 on $CD4^+$ T_{TD} cells in lesional skin and

circulation and explore their capacity to produce cytokines. The potential influence of transitional B cells and NKT-like cells on Treg in our patients requires further investigation since we obtained results in PsO patients that could have a contradictory effect on Treg. Furthermore, analyses of Tregs including absolute counts could be useful, particularly in clinical practice, as it is less sensitive to the influence of the counts of other cell subsets.

The aberrant frequencies in pDC, cDC1, cDC2, and cDC2 subsets in untreated/treated patients with different disease onset, clinical manifestations and associated comorbidities, disease severity and treatment modalities, should be analyzed further. Also, identifying patients who are naïve or non-naïve to biologics, non-responders, or responders to several biologics, with subsequent functional analysis of immune cells in peripheral blood and skin are in the planning.

Although skin is an easily accessible organ for investigation of cellular features in detail, blood biomarkers may be an informative and less invasive predictor of response[312]. Our project was based on peripheral blood, as PBMC can be used as a presenter of treatment response providing insight into the pathophysiology of psoriasis. Since psoriasis is a skin disease with systemic inflammation, our plan is to collect simultaneously blood samples and skin biopsies from patients with psoriasis for analyzes by imaging mass cytometry. As a next generation immunohistochemistry, imaging mass cytometry give us an opportunity to explore tissue biology with up to 35 antibodies simultaneously, while maintaining the tissue architecture[344]. Imaging mass cytometry combined with functional analysis could give us a guidepost or answer to the questions raised by this project.

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