Biomarkers for Cardiovascular Disease in Psoriasis Patients and Effects of Treatment

Ástríður Pétursdóttir
Supervisor: Charlotta Enerbäck
Co-supervisors: Anna-Karin Ekman and Gunnþórunn Sigurðardóttir

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Ástríður Pétursdóttir
Main Supervisor: Charlotta Enerbäck

Co-supervisors: Anna-Karin Ekman and Gunnþórunn Sigurðardóttir

1 Faculty of Medicine, University of Iceland, Reykjavík, Iceland
2 Ingrid Asp Psoriasis Resarch Center, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth factor β</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>PsA</td>
<td>Psoriatic Arthritis</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>IL-1 Ra</td>
<td>Interleukin-1 Receptor Antagonist</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular Adhesion Molecule</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>tPAI</td>
<td>Total Plasminogen Activation Inhibitor</td>
</tr>
<tr>
<td>sE-selectin</td>
<td>Soluble E-selectin</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix Metalloprotease-9</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like Receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PASI</td>
<td>Psoriasis Area Severity Index</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albamine</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzedine</td>
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Ástríður Pétursdóttir
Biomarkers for Cardiovascular Disease in Psoriasis Patients and Effects of Treatment

ABSTRACT

Introduction: Psoriasis is a papulosquamous skin disease, today regarded as a common T cell mediated inflammatory disorder. The disease is characterized by inflammatory skin lesions on various sites of the body. Since cardiovascular diseases have been connected to the inflammatory diseases, specifically atherosclerosis, there is a possibility that psoriasis and cardiovascular diseases are linked through a partly common inflammatory process. This study aims to investigate this relationship and the effect of psoriasis treatments, ultraviolet B (UVB) phototherapy and systemic therapy by a TNFα inhibitor, on the cardiovascular risk factors.

Materials and Methods: Two methods were used for the execution of this study. The Multiplex assay technology was used to measure cardiovascular biomarkers in serum and immunohistochemical staining was used to determine the expression of the Nod-like receptor NLRP-1, which may play a role in the inflammatory process of both psoriasis and atherosclerosis.

Results: When comparing psoriasis patients and healthy controls, five biomarkers were notably increased in the serum of patients. When matching for BMI for the same comparison, only tPAI-1 was significantly up-regulated. Comparison of patients before and after 12 weeks of UVB therapy revealed no significant change in the cardiovascular biomarkers. Comparing patients before and after treatment with a TNFα inhibitor showed a significant decrease in all biomarkers. Immunohistochemical staining of NLRP-1 demonstrated equal staining in patients and controls.

Discussion: The results suggest an increased level of CVD biomarkers in psoriasis patients compared to healthy controls. They also point towards a negligible effect of UVB treatment on the expression levels of the biomarkers. In contrast, a therapeutic effect of TNF-α inhibitor on biomarker concentration is demonstrated. In conclusion, our data suggest that patients with high risk of cardiovascular disease may benefit from the use of systemic treatment.
1 Introduction

1.1 Psoriasis

Psoriasis is a papulosquamous skin disease, today known as a common immunological disorder (1). It was first recognized in 1808 by Robert Willan, when he described the disease and took the first step in distinguishing it from leprosy (1). In 1836, Thomas Bateman put all doubt to an end and established psoriasis as a disease of its own, different from leprosy (3).

1.1.1 Epidemiology and aetiology

Psoriasis is a common inflammatory disease, its’ incidence is estimated to be 60 individuals per 100 000 per year (4, 5). It is also noteworthy that the annual incidence almost doubled in 30 years, from the 1970s to the 2000s, for unknown reasons (6).

The prevalence of 2-3% in the world is mostly affected by ethnicity and latitude, with the disease being most common in Caucasian people and in the northernmost part of the world. Sunlight has proven to have a bettering effect on the psoriatic lesions, which might explain the prevalence distribution (7). There seems to be no difference in prevalence between the genders (7), though the disease onset is slightly earlier in women. The incidence of the disease also varies between ethnic groups, 0.3% in China and 1.5-3.0% in Northern Europe (1).

The development of the disease can occur at any age, the age of onset varies between studies. The average age of onset has been reported to be from 12 years to 36 years of age and most studies agree that majority of patients have onset before 40-45 years (5). These studies, collectively with those that have taken older patients into consideration, strongly suggest a bimodal distribution of the age of onset with two peaks, between 16-22 years and 57-60 years of age (8).

1.1.2 Classification

Different forms of the disease are recognized today, chronic plaque psoriasis vulgaris and acute psoriasis, guttate and pustular variants.

In 90% of cases, psoriasis refers to the common chronic clinical variant, psoriasis vulgaris, where scaled papulosquamous plaques are well demarcated from normal skin (Figure 1). The skin lesions are red or pink in colour, raised, of any size, covered by silvery scales and are most active at the edge. Lesions are most commonly localized at elbows, knees, scalp, lumbosacral region, umbilicus and 50% of patients experience the psoriatic nail disease (1, 3). The guttate form most commonly occurs in children post-infection and usually resolves in 3-4 months, although it associates with an increased risk of developing classic plaque disease (1,
Generalized pustular psoriasis (von Zumbuch psoriasis) can be described as systemic infection-like symptoms, including fever, along with small pustules on the inflamed skin. The development of the pustular form may be triggered by an infection or sudden withdrawal of cortical steroids (1).

1.1.3 Histopathology

There are three main histological criteria for psoriasis: thickened epidermis (acanthosis), increased vascularity in the dermis, and dermal inflammatory infiltrate (3) (Figure 2). Hyperproliferative epidermal changes include premature keratinocytes with an increased mitotic rate, incomplete cornification resulting in parakeratosis, loss of the granular cell layer and elongation of rete ridges. Clinically symptomless areas and hair follicles are unaffected and histologically normal (1). Angiogenesis is evidently caused by angiogenic factors produced by the hyperproliferative keratinocytes, notably Vascular endothelial growth factor, VEGF, which is significantly elevated in psoriatic plaques (10). It has also been suggested that after VEGF synthesis by keratinocytes, it is released into the systemic circulation (11). The inflammatory infiltrate consists mainly of dendritic cells, macrophages, T cells and neutrophils (3), and precedes epidermal changes (5).
Until approximately 30 years ago, psoriasis was believed to be primarily localized epidermal keratinocyte proliferation (1). Since then, many studies have demonstrated that the presence of immune cells in psoriatic lesions is a factor in the disease (1). Compelling evidence has accumulated supporting their pathogenic role. For example, it has been suggested that both innate and adaptive immune systems are necessary to initiate and maintain psoriatic plaques (1). One of the strong links between the local and systemic affects is the cell-mediated adaptive immune response (1). The dermal inflammatory infiltrate, which is one of the histological criteria for psoriasis, consists mostly of CD4-positive and CD8-positive T-cells and may precede epidermal hyperplasia (12).

Along with T cells, there are also detected endothelial cells, dendritic cells, monocytic cells, neutrophils, keratinocytes, as well as elevated levels of chemokines and cytokines. Each cell type supposedly playing a distinct role at different stages of the disease (13). Dendritic cells in psoriatic lesions have the ability to activate T cells, while dendritic cells in the healthy skin do not have the same ability (13). It has not yet been established why psoriatic dendritic cells have this ability (13).

Studies have also shown an increase in the T helper-1 (Th1) pathway cytokines in psoriatic plaques, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2) and interleukin-12 (IL-12). For that reason, psoriasis is sometimes referred to as a Th1 disease (13, 14). Noticeably, correlation between high levels of Th1 cytokines in serum of psoriasis patients and the severity of the disease has also been reported. However, it is not clear what triggers the increased cytokine production or the elevated serum levels (13, 15).
Of all the Th1 cytokines, researchers have especially been interested in IFN-γ. IFN-γ empowers the migration of immune cells to the skin, activates monocytes/macrophages, dendritic cells and endothelial cells, and decreases keratinocyte apoptosis (13, 16). In other words, IFN-γ may have the ability to exaggerate the immune response and assist the keratinocyte proliferation, hence, can potentially be very important in the early stages of psoriasis.

Another important member of the Th1 pathway is TNF-α. TNF-α is vital to local T cell proliferation also contributing to proliferation, activation and differentiation of other cell types and cytokines (17). In psoriasis, it is produced primarily by dermal macrophages, T cells and keratinocytes, so soon it escalades into a vicious circle (18). Together, IFN-γ and TNF-α enable the infiltration of the dermis by T cells and other immune cells and accelerate the pathogenesis of psoriasis.

Together with Th1 cells, Th17 type T cells have been shown to be important in development of the disease, through their role in the autoimmunity and chronic inflammation (19). Cytokines of the Th17 pathway include IL-1, IL-6, transforming growth factor-β (TGF-β), IL-23, and IL-12. All together, these cytokines have the effect of activating memory Th17 cells, driving Th17 cell proliferation and inducing Th1 and Th17 cells, which stimulate macrophages and dendritic cells to release inflammatory mediators which also activate keratinocyte proliferation (13, 19). Thus it is now evident that psoriasis can be defined as an inflammatory disease with an increase in Th1 and Th17 cytokines (13). Three cytokines, IL-23, IL-17 and IL-22, are considered specifically important. IL-23 and IL-17 are both produced by Th17 cells and serve as mediators of cytokine regulation in psoriasis. IL-23 can activate other Th17-cells, induce hyperproliferation of keratinocytes and dermal infiltration, thereby enhancing the type I immune response in the skin (13, 20). IL-17 can enhance the pro-inflammatory cytokine production, mainly by endothelial cells, keratinocytes and macrophages. The cytokines activated by IL-17, i.e. IL-8, have been shown to be overexpressed in psoriatic lesions (21). Interestingly, IL-17 in blood samples from psoriasis

<table>
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<tr>
<th>Cytokines</th>
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<tr>
<td>Tumor Necrosis Factors (TNFs)</td>
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<tr>
<td>Interleukins (ILs)</td>
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<td>Transforming Growth Factors (TGFs)</td>
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<tr>
<td>Chemokines</td>
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<tr>
<td>Interferons (IFNs)</td>
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<td>Colony Stimulating factors (CSFs)</td>
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Table 1
patients was found to be elevated and correlate with disease severity (22, 23). The third important cytokine, IL-22, mediates the communication between the immune system and the epithelial cells, as well as assisting Th-17 with its enhancement of the pro-inflammatory cytokine production. The activation of IL-22 is directly controlled by IL-23 (13).

An important effect of inflammation in psoriasis is skin angiogenesis. Capillaries grow, expand, dilate and reach between the dermis and epidermis. This pathological angiogenesis does not differ from other, i.e. in tumor growth or atherosclerosis and it is activated by the local expression of angiogenic factors (13). TNF-α, TGF-α, IL-8, thymidine phosphorylase, endothelial cell-stimulating angiogenesis factor, angiopoietin and VEGF are reportedly all angiogenic peptides and are all overexpressed in psoriatic lesions (13, 24). VEGF is the most potent of them, known to be epidermis-derived and vessel-specific. It plays an essential role in the inflammatory process by stimulating epidermal hyperplasia, vascular growth and leukocyte infiltration. Furthermore, it has also been found to be elevated in plasma of psoriasis patients, along with TNF-α (24, 25), contributing to the hypothesis that systemic inflammation is an important part of the disease and suggesting a new therapeutic pathway (13).

At present, most of the therapeutic targets in psoriasis, belong to the immune system i.e. IL-12, IL-23 and TNFα (13).

1.1.5 Genetic and environmental factors of the disease

Previous research has demonstrated a strong genetic factor in the disease. Population studies show that the probability of developing the disease is greater in the first and second degree relatives than in the general population (26). A major psoriasis susceptibility locus has been identified within the Major histocompatibility complex (MHC) region on chromosome 6p21 (27), named PSORS1 (5), along with the less important PSORS2-PSORS9. PSORS1 is responsible for 35-50% of cases of familial psoriasis (5). However, not all first degree relatives develop the disease, indicating an environmental factors in the pathogenesis (26).

As studies on the disease have mainly been focusing on its genetic factors, no conclusive evidence exists regarding the role of environment (5). As a result, only a few factors have been studied and identified as promising candidates for psoriasis pathogenesis. Among those are trauma, infection, drugs, sunlight, female hormones, hypocalcaemia, stress, anxiety, alcohol, smoking and Human immunodeficiency virus (HIV) (5), all of which can have provocative effects on pathogenesis of the disease. Sunlight has been proven to have
beneficiary effect on the disease, but strong sunlight can provoke it in a minority of patients (5).

1.2 Comorbidities

The term “comorbidities” is defined as: “The occurrence of one or multiple disorder(s) in association with a given disease [...]” (28). Psoriasis comorbidity research has taken a front seat in the last decade, with researchers finding a number of disorders which have common pathogenic pathways with psoriasis. The first comorbidity was described in 1818 by Alibert as inflammatory joint disease, today called psoriatic arthritis (PsA) (28) and is defined as: “a seronegative inflammatory arthritis that occurs in the presence of psoriasis” (1, 29). Some studies state that PsA is seen in approximately 5% of guttate psoriasis and 15% of plaque psoriasis patients (28) and other studies state that it can be found in up to 25% of all psoriasis patients (1, 30). Symptoms of PsA often overlap with other inflammatory joint diseases and have at least five different clinical forms. Research has also shown that the HLA-B27 marker, carried by 8% of the European population, is predictive of whether patients develop PsA (28).

The second comorbidity is Crohn’s disease (CD), an inflammatory bowel disease. Epidemiologic research has shown that prevalence of psoriatic lesions in CD patients is 7 times higher than in the general population (28, 31). A common genetic variant has also been suggested as pathogenic genes, as both diseases have been found at the same locus, 16q21 (32).

Cardiovascular diseases (CVD) have been recognized as comorbidities and they will be discussed later in the following section.

Other comorbidities include diabetes mellitus (particularly type 2), metabolic syndrome and obesity (1, 33, 34). Adding to the physiological changes, psoriasis can have an astounding impact on the patients’ psychological state. Self-awareness and disgust can lead to despair and isolation, impairing patients’ quality of life. High levels of anxiety and depression are common. Patients are predominantly afraid of the chronicity of the disease, the absence of a cure and even those in remission fear a relapse (1).
1.3 Psoriasis and cardiovascular disease

The cytokines work first and foremost as gene-regulatory proteins and affect the inflammatory process via protein synthesis (35). Some of them have a positive effect on immune cell differentiation and proliferation, as discussed earlier (35). In recent years, studies on the link between psoriasis and cardiovascular disease (CVD) have yielded clearer results. Systemic increase of cytokines in psoriasis patients has been connected to CVD risk factors, such as vascular dysfunction, atherosclerosis and hypertension (35, 36). This correlation has been suggested for TNF-α, as treatment with TNF-α inhibitors blocks the formation of atherosclerosis, hence decreasing the risk for CVD (37). Atherosclerosis, a hallmark of CVD, and psoriasis have many pathological mechanisms in common (Figure 3): Chronic inflammation, Th1/Th17 response, endothelial cell dysfunction, angiogenesis, metabolic processes, oxidative stress and common genes(34).

The psoriasis patients’ higher risk of developing CVD might be explained in part by pre-existing risk factors for atherosclerosis. CVD and its risk factors are very common in the general population and therefore psoriasis patients could possibly be affected by them, purely by chance. The obvious link between the two diseases has not yet been found but proposals have been made (34). The “psoriatic march” is a concept introduced in 2011. The concept’s authors propose that systemic inflammation can lead to insulin resistance and evidently to endothelial cell dysfunction. Eventually, this can lead to atherosclerosis and a stroke or myocardial infarction (38).

1.4 Treatment

In this study, the effect of two treatment options for psoriasis patients were analyzed. Treatment with ultraviolet radiation has been one of few solutions for psoriasis patients since the 1970s, when scientists observed the beneficiary effect of sunlight on the skin of psoriasis patients.
patients. Ultraviolet treatment is available in three wavelengths, both narrowband and broadband. In this study, ultraviolet B (UVB) treatment refers to narrowband radiation, a wavelength of 311-313 nm. Despite the common use of UVB treatment and its effectiveness on psoriatic lesions of the skin, the underlying mechanisms remain unknown (39).

Another option for psoriasis patients is systemic treatment. The three most common treatments include methotrexate, retinoids and biologics. Etanercept (Enbrel®), a biologic product, has been chosen for investigation in this study. The medicine, which is administered subcutaneously and has shown to be effective in rheumatoid arthritis, juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis and plaque psoriasis. Etanercept works by blocking the activation of TNF, including TNF-α, and therefore reduce the inflammation. It has been demonstrated to reduce psoriatic lesions although it is still unknown whether Etanercept eliminates the systemic inflammation. The most common side effects of Etanercept are injection site reactions and infection, occurring in up to 1 of every 10 patients (40).

1.5 Specific aims

As previously discussed, psoriasis is associated with elevated levels of chemokines, cytokines and growth factors in its plaques. A recent study from this group reported elevated serum levels of epidermal growth factor (EGF), IL-1 receptor antagonist (IL-1Ra), TNFα, macrophage inflammatory protein (MIP) - 1α and IL-6 (41) of psoriasis patients compared to matched controls (41). Markedly increased serum concentration of EGF and IL-1 Ra was observed in psoriasis patients, which did not correlate with the severity of the disease and did not decrease after phototherapy (UVB) treatment. The study suggested another source of elevated cytokines than psoriatic lesions, which might lead to finding a possible mechanism linking psoriasis and its extra-cutaneous comorbidities.

In another publication on the subject, from the same group, plasma chemokine levels were compared between psoriasis patients and healthy controls, an increased expression of Th1-, Th2- and Th17- associated chemokines was detected/confirmed in psoriasis patients compared to controls. The comparison of psoriasis patients, before and after UVB phototherapy treatment revealed that while UVB therapy reduced skin symptoms, it was more effective against the local inflammation than the systemic one. These results suggest that the UVB-induced remission is not entirely due to a decrease in systemic antigen presenting cell (APC) and T-cell activation, but is rather focused on reducing local immune response. The link between psoriasis and cardiovascular disease, both being considered as inflammatory
diseases to date, has been thoroughly discussed above. Psoriasis has been shown to be independently associated with cardiovascular disease such as myocardial infarction and stroke and an increase in mortality in those with severe disease. The increase in mortality is more marked/pronounced in young patients (42, 43). The cause is assumed to be effects of systemic inflammation on vessels and other organs (38). It is therefore of interest to investigate cardiovascular biomarkers, many of them being mediators of inflammation, in psoriasis patients and the effects of treatment.

The aim of this study was to analyze links between psoriasis and CVD:

To address this aim, known biomarkers of CVD were measured and following comparisons performed:

a) Psoriasis patients and healthy controls
b) Psoriasis patients and Body Mass Index (BMI) matched controls
c) Psoriasis patients before and after narrowband UVB treatment
d) Psoriasis patients before and after systemic treatment with Etanercept.

Controlling for BMI is expected to eliminate a bias and hence lead to clearer results, because high BMI is one of the most common risk factors for CVD in the world (44). The CVD biomarkers measured are intracellular adhesion molecules (ICAM-1), vascular cell adhesion molecules (VCAM-1), total plasminogen activation inhibitor (tPAI-1), soluble E-selectin (sE-selectin), matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO). VCAM-1 and ICAM-1 have been observed to be overexpressed in atherosclerotic plaques and it has been implied that they are also upregulated in psoriasis (2). tPAI-1 and MMP-9 play a role in plasminogen activation and therefore in thrombosis. They could be important factors in development of atherosclerosis (45). E-selectin is a mediator of leukocytes in the immunologic response and thus takes part in the inflammation process. MPO is under control of reactive oxygen species and is in close connection with the deteriorating vessel wall, implying a role in the pathogenesis of atherosclerosis (46).

1.6 Inflammasomes

Another way to study the relationship between psoriasis and CVD is through the analysis of inflammasomes, complex macromolecules, responsible for activation of IL-1 and IL-18 (47). Inflammasomes are a part of the Nod-like receptor (NLR) family, which are classified as
pattern recognition receptors (PRRs) (48). The NLR family is divided into a three groups, NLRPs, NLRCs and NAIPs, based on different effector domains. Inflammasomes might play a role in activating inflammatory chemokines or cytokines and thereby have effect on the pathogenesis of CVD (48). The most investigated CVD in relation to inflammasome activation is atherosclerosis (48), suggesting a connection between inflammasome activation and disease progression (49). Because of the inflammatory effect of inflammasomes there is a possible link between psoriasis and inflammasomes. This is supported by two genetic studies from the group. Therefore I hypothesized that NLRP-1 might be expressed in higher levels in psoriatic lesions than in the skin of healthy controls. Immunohistochemistry was used to analyze the skin biopsies from psoriasis patients and healthy controls for expression of NLRP-1.
2 Materials and methods

2.1 Summary

Two methods were applied in this study. For measuring the cardiovascular biomarkers in serum and to compare their concentration between patients and controls Multiplex assay technology was used. That was done in four distinct experiments, psoriasis patients compared to age- and gender matched controls, patients compared to age-, gender- and BMI – matched controls, psoriasis before and after the UVB treatment and finally, patients before and after systemic treatment with the TNF α inhibitor.

For analyzing the expression of NLRP-1 in skin biopsies, immunohistochemistry was used. Skin tissue samples were compared between psoriasis patients and healthy controls.

2.2 Milliplex™ map, Luminex® xMAP® Technology

The Multiplex assay technology is a procedure able to measure multiple analytes in each sample at once, compared to single analyte measuring procedures. In this particular study, the CVD1 Panel, 96-well plate assay was used, analyzing ICAM-1, VCAM-1, tPAI-1, sE-selectin, MMP9 and MPO all at once. A 1:50 dilution was required for the blood samples for a sample size of 25µL.

The process includes to internally color-code microspheres with two fluorescent dyes, creating up to 100 distinctly coloured bead sets, each coated with a specific antibody. The beads then capture analytes from the test sample, before a biotinylated detection antibody is introduced. The reaction is then completed on the surface of each hemosphere by incubating the reaction mixture with the reporter molecule, Streptavidin-PE conjugate. Thereafter, the microspheres then pass through the first laser, that excites the dyes marking the microsphere set, then the second laser that excites the PE conjugate. Finally, high-speed digital processors identify the microspheres and quantify the results.

2.2.1 Patients and controls

The study was approved by the local ethics committee and every participant gave his or her written informed consent.

2.2.1.1 Psoriasis patients and age- and gender-matched healthy controls

Biomarkers measured were compared between psoriasis patients and controls. Patients with a diagnosis of plaque psoriasis were invited to participate in the study. Patients were acceptable if they had received local treatment or UVB treatment, but for no more than 3 runs.
Patients who had received systemic treatment or UVB treatment for more than 3 runs the previous four weeks, were not accepted. 28 patients and 28 controls were recruited, 20 men and 8 women in each group. The participants were not asked for BMI. The average Psoriasis Area Severity index (PASI) was 7.8, with a range from 2.0 to 25.3. Of each group, 15 patients were recruited in Linköping University Hospital and 13 in Gothenburg Sahlgrenska University Hospital Department of Dermatology. Background information is available for the patients recruited in Linköping. They are all Caucasian, 2 have mild psoriasis arthritis which does not acquire any regular intake of medication, 7 suffer from hypertension where 5 of them also have dyslipoproteinemia. Of those with dyslipoproteinemia one has ischemic heart disease and another has experienced stroke. 3 have diabetes type II, 2 of those also have hypertension. Patients with underlying cardiovascular disease had appropriate treatment. Their average age of diagnosis is 31.6 years, ranging from 11 to 58 years of age. Of the 15 controls recruited in Linköping, they are all of Nordic origin and 2 suffer from hypertension where both have dyslipoproteinemia and the other also diabetes mellitus type II. Those 2 controls had appropriate treatment for the underlying condition. The average age of both groups is 56.5 years.

2.2.1.2 Psoriasis patients and age- gender and BMI – matched healthy controls

This part of the study was executed the same way, but now the patients were also BMI – matched. The conditions for recruitment were the same for this part of the study. 29 patients were recruited in Linköping, along with 29 controls, 15 men and 14 women in each group. The average age of both groups is 50.3, ranging from 24 to 82 years. The mean BMI for patients respective controls is 26.01, ranging from 22.0 to 34.7, and 25.93, ranging from 22.8 to 34.6.

Of the 29 patients, all are of Nordic origin except for one who was from Hungary, all are Caucasian. 12 suffered from cardiovascular disease (hypertension, ischemic heart disease, post myocardial infarction), 4 from diabetes mellitus type II and 1 from diabetes mellitus type I. Their mean age of onset is 32.97 years, ranging from 12 to 77 years Of 29 controls, all of Nordic origin, 3 suffered from cardiovascular disease (hypertension) and no one from diabetes mellitus.

2.2.1.3 Psoriasis patients before and after UVB treatment

In this part of the study, no controls participated, but the same group of patients was investigated before and after 12 weeks of UVB treatment. Again, the same conditions for
recruitment applied. 21 patients participated, 15 recruited in Linköping, the same patients that were age and gender matched to controls, and 6 in Gothenburg. Their PASI scores are 8.00 of average, ranging from 2.20 to 17.20. Improvement in PASI was significant for all patients (p<0.001). Seventeen of the patients improved at least 75% in PASI. The average age of the patients was 54.0, ranging from 16 to 78 years.

2.2.1.4  **Psoriasis patients before and after systemic treatment**

For this part, the same approach applied as for the UVB study. No healthy controls were enrolled as the patients were investigated before and after 12 weeks of systemic treatment. The same conditions for recruitment applied. All of the serum samples in this part of the study, come from psoriasis patients recruited in Stockholm Karolinska University Hospital, Department of Dermatology. No background information is available for this group of patients, only gender, age and BMI. 15 psoriasis patients participated, 8 women and 7 men, their age range was 19-78 years of age with an average of 46.07 years. BMI ranged from 21.0 to 36.0, the mean BMI being 22.5 for the patient group and 22.95 for the control group.

2.2.2  **Blood Samples**

Blood was collected in CPT™ tubes (Becton Dickinson, Stockholm, Sweden) coated with sodium heparin anticoagulant or in serum tubes with clot activator (Terumo Europe, Västra Frölunda, Sweden) for the isolation of plasma and serum, respectively. The CPT tubes were centrifuged at 2,600 rpm for 25 minutes, separating the leukocytes from the plasma. The serum tubes were allowed to sit for 30 minutes before separating the serum from the clotted blood by centrifugation at 3,000 rpm for 10 minutes.

2.2.3  **Measurements**

The levels of the biomarkers ICAM-1, VCAM-1, tPAI-1, sE-selectin, MMP-9 and MPO were measured in plasma. The measurements were performed using a Milliplex™ MAP kits (Millipore Corporation, Billerica, USA) according to the manufacturer’s instructions(Appendix A). The samples were analysed on a Luminex 200 instrument (Biosource, Nivelles, Belgium) and the data were analysed using StarStation 3.0 software.

2.2.4  **Statistical analysis**

Data analysis was performed in Graph Pad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Data were compared using Wilcoxon matched-pair signed rank test or Mann-Whitney test, unless otherwise stated. Correlations were determined by Spearman’s test. A p-value of less than 0.05 was considered significant.
2.3 Immunohistochemistry (IHC)

The method was first described in 1941 as an “immunofluorescence technique for detecting cellular antigens in tissue sections” by Coons et al (50) and combines immunology, histology and chemistry. IHC has been a breakthrough method in detecting certain antigens in certain tissues. The method is based on the technique of binding antibodies to antigens in a tissue section and a colored histochemical reaction is used to visualize the binding, in this case, in a microscope(50). The research group responsible for this research, has made variations to the current IHC method and evolved their own protocol (Appendix B).

2.3.1 Patients and controls

Skin biopsies from patients with plaque psoriasis and controls without inflammatory skin disease were retrieved at Linköpings University Hospital. Background information is not available for these patients.

2.3.2 Biopsy retrieval

Biopsies were executed by a 4mm punchbiopsy in local anaesthesia with a 10 mg/mL lidocaine and 5µg/mL adrenaline. The samples were subsequently fixated in formalin, before they were further handled in the laboratory.

2.3.3 Protocol

After retrieving the biopsies, the samples were fixated, paraffin embedded and sectioned. Dehydration, deparaffination and target retrieval from the sections was achieved by a preparation procedure by PT Link®, a pre-treatment module for tissue specimens, at low pH. Endogenous peroxidases were blocked by using a H$_2$O$_2$ solution. Unspecific protein binding was blocked in a moisture chamber using Bovine serum albamine (BSA) from cows and finally the primary antibody (mouse monoclonal anti-NLRP-1) was diluted (1:30) and applied to the sections, overnight. The secondary antibody was subsequently applied and the sections incubated in a moisture chamber. 3,3’ diaminobenzidine (DAB) was applied to visualize the staining pattern with the help of horseradish peroxidase catalysis. The slides were finally stained with hematoxylin and mounted with aqua/polymount.
3 Results

3.1 Comparison between age- and gender matched controls and untreated psoriasis patients

When using the Multiplex assay technology to measure the six cardiovascular biomarkers as mentioned before, the unit ng/mL is used.

When measuring the cardiovascular biomarkers in psoriasis patients, there is a statistically significant increase in sICAM-1, sE-selectin, MPO, MMP-9 and tPAI-1 in psoriasis patients as compared to the healthy controls. While there is a significant increase in all the biomarkers measured, except for sVCAM-1, there is a tendency for increase of sVCAM-1 in psoriasis patients.

3.2 Comparison between age-, gender and BMI matched controls and psoriasis patients

The results clearly state a statistically significant elevation in tPAI-1 in psoriasis patients compared to controls. But the difference between the group in concentrations of the other five biomarkers seems to be dependent of BMI leaving tPAI-1 as a BMI-independent cardiovascular biomarker significantly higher in patients than in controls.
Age- and gender matched

- **sICAM-1**: p=0.0005
- **MPO**: p=0.0003
- **tPAI-1**: p=0.0005
- **sE-selectin**: p=0.0010
- **MMP9**: p<0.0001
- **sVCAM-1**: p=0.07
3.3 **Comparison between psoriasis patients before and after 12 weeks of UVB phototherapy**

Twelve weeks of treatment effectively reduced the PASI scores but did not affect the systemic levels of the measured cardiovascular biomarkers.

The results reveal that none of the biomarkers measured decreased after UV-treatment. In all measurements, there are tendencies both to increase and decrease in concentration. The tendency to increase seems to be more common than to decrease, though overall the concentrations remain the same. There is no statistically significant change in any of the biomarkers’ concentrations, as p-value was >0.05 in all measurements.

3.4 **Lower concentration of the cardiovascular biomarkers in psoriasis patients after systemic treatment compared to before treatment**

After 12 weeks of systemic treatment with Etanercept, there is a significant decrease in concentrations of all measured biomarkers compared to pre-treatment measurements.

3.5 **Comparing NLRP-1 levels in skin biopsies between psoriasis patients and healthy controls**

A microscopic analysis of the immunohistochemically stained tissue was performed and a comparison made between psoriasis patients and healthy controls. In preliminary data, very faint staining of NLRP1 in the skin tissue from psoriasis patients was noticed. The tissue samples from patients and controls had the same expression pattern.
Age-, gender-, and BMI matched
Before and after UVB treatment
Before and after systematic treatment
4 Discussion

4.1 The Milliplex™ method

4.1.1. Summary

The aim of this research was fourfold:

a-b) To investigate the change in CVD biomarkers in psoriasis patients compared to healthy controls and then to eliminate BMI as a confounding factor

b) To study the effectiveness of UVB treatment on the concentration of the CVD biomarkers in psoriasis patients

c) To study the effectiveness of a TNFα inhibitor on the concentration of the CVD biomarkers in psoriasis patients

Measuring six cardiovascular biomarkers in serum/plasma of psoriasis patients and controls, the results implicate:

a-b) Five cardiovascular biomarkers have significantly higher concentrations in patients than controls, and one has a tendency, but not significantly, of being higher in patients than controls. When controlling for BMI, only tPAI-1 is significantly elevated.

b) The overall expression levels of the biomarkers remain the same in psoriasis patients before and after 12 weeks of UVB treatment, no significant difference was expressed.

c) All six biomarkers show a significant decrease in concentration after the patients have been treated with a TNFα inhibitor.

4.1.2. The risk of developing CVD

For some time now, researchers have been pointing towards this risk of developing CVD for psoriasis patients. Research after research is providing evidence, each leading us one step closer to understanding this relationship(34). This study does not disagree with others, and makes a contribution to reaching the common goal.

The first experiment was only age- and gender matched, resulting in 5 biomarkers being significantly elevated in psoriasis patients compared to healthy controls: tPAI-1, sE-selectin, MPO, MMP9 and sICAM-1. When controlling for BMI, in the second study, only one biomarker, tPAI-1 is significantly elevated. This difference can be explained by the BMI-
matching. sE-selectin, MPO, MMP9 and sICAM-1 are elevated in the first study but are not significantly elevated in the second study. That suggests that those four biomarkers are increased along with increased BMI, but do not necessarily associate with psoriasis. The biomarkers are equally elevated between both groups in the results, suggesting a correlation between high BMI and psoriasis. Not knowing whether psoriasis precedes high BMI or vice versa, there is still a possibility of psoriasis causing high BMI, which then again causes higher risk of developing CVD. High BMI could also trigger psoriasis development.

tPAI-1 is significantly elevated, pointing towards another verification of the relationship between psoriasis and elevated risk for CVD. tPAI-1 plays a role in thrombosis and is an important factor in development of atherosclerosis. These results suggest a significant risk for psoriasis patients, of developing atherosclerosis, which can be the beginning of many CVDs and, if goes untreated, could have serious repercussions. These results also suggest a correlation between psoriasis and high BMI, which then again correlates with elevated risk of CVD.

4.1.3. Should UVB therapy not be the first option?

UVB phototherapy has shown to have beneficiary effect on the psoriatic skin lesions most patients suffer from, as anti-inflammatory therapy. However, the results from this study suggest that the UVB therapy does not eliminate the elevated risk of developing CVD, but the effect of the treatment does not seem to penetrate the body past the skin. The question remains if the treatment has been giving false hope, providing an acceptable recovery for the symptoms the eye can see, while the underlying, cardiovascular comorbidities could still cause problems. In the meantime, patients could possibly have the wrong idea about their health, thinking they are healthier than they are in reality. In addition, UVB treatment requires a patient to attend therapy at least 2-3 times each week for a period of time for it to work properly. That could be difficult for a number of patients. The question remains, if the benefit of bettering psoriatic lesions is worth a higher risk of developing CVD than the general population. Is UV phototherapy an obsolete method for psoriasis treatment?

The results from this study point to no change in risk for CVD after UVB treatment. If that is correct, it can make the assessment of whether to take the risk much easier in each case. The CVD risk could be taken into consideration, making UVB treatment only an option to cure the lesions, not the disease itself, an option which does not limit the risk of CVD development. The results also suggest that UVB treatment is not an ideal therapy for patients
who already are at risk of developing CVD, i.e. have high blood pressure, high BMI or a family history of CVD.

4.1.4. Is systemic treatment the solution?

Etanercept, the TNF-α inhibitor, has proved to be effective in battles against various inflammatory diseases, including plaque psoriasis (40). Like with UVB treatment, the only victory that patients experience physically is curing of the psoriatic lesions. It does make sense, after seeing how psoriasis, CVD and inflammation intertwine, that the TNF-α inhibitor is more effective in eliminating the CVD risk than the UVB therapy. That is exactly what the results suggest, the systemic treatment having a reducing effect on the cardiovascular biomarkers, contrary to the UVB treatment. So, if the systemic treatment can reduce the psoriatic lesions and lower the risk for CVD development, is there a reason why not all psoriasis patients should seek this treatment?

Like all medication, of course Etanercept has side effects and risks to take into consideration. Being a TNF-α inhibitor, Etanercept has a suppressing effect on the immune system and the inflammatory response. Immediately it is not susceptible for the immuno-compromised and has a serious side effect of infection affecting more than 1 in 10 patients (40). Etanercept is also a therapy of much higher costs than UVB treatment but, on the other hand, the patient is more likely to follow medical treatment instructions when accepting systemic treatment.

If psoriasis is categorized as an autoimmune disease, it can be identifiable with other autoimmune diseases. Scientists still do not agree in which disease category to put psoriasis. It is becoming clearer that psoriasis is more than just a cutaneous disease, it is a systemic inflammatory disease, more serious than thought at first. If psoriasis really is a systemic disease, why only treat the symptoms but not the systemic inflammation? The pathogenic role of the immune system supports the theory of psoriasis being an autoimmune disease. The only way of response to various autoimmune diseases is to suppress the immune system of the patient. Immunosuppressant therapy is a serious treatment and should only be used for severe cases with caution. The anti-inflammatory systemic treatment could also be an option for psoriasis patients who already have one of the many risk factors for CVD excluding psoriasis, i.e. hypertension, obesity and family history. Thereby, the patient can avoid adding the CVD risk associated with psoriasis to the pre-existing risk.

This study cannot be used as ground for assuming that one treatment is better than the other. First, future research needs to be performed regarding, for example, how much greater
the risk of CVD is for psoriasis patients and if the amount of risk correlates with the severity of the psoriasis lesions.

4.1.5. Possible bias

The general risk factors for CVD are very familiar to the world. The non-modifiable risk factors are higher age, family history of CVD, male gender and African or Asian ethnicity. The modifiable risk factors are high cholesterol, hypertension, lack of exercise, unhealthy diet, tobacco use, alcohol consumption and diabetes mellitus. In this research, the participants were age-, gender-, and BMI matched. That leaves all the other cardiovascular risk factors to be possible biases. There is high correlation between high cholesterol, hypertension, obesity, lack of exercise, unhealthy diet and BMI. There is a possibility of decrease in the effect of these risk factors on the results, due to the BMI matching. The participants were all asked about these cardiovascular risk factors and their answers exist in the lab’s database. However, with only 29 participants in each run, 14-15 in each group, it was quite difficult to control for all the cardiovascular risk factors.

When studying the UVB and systemic therapies, the same patients were measured before and after treatment. This eliminates any possible bias and provides a clean result of receiving these treatments. The only variable between the compared groups is the treatment under surveillance.

This could be the reason why not all of the patients’ cardiovascular biomarkers are elevated. When the overall concentration of a biomarker is increased, it does not necessarily mean that each and every patient has increased concentration of this particular biomarker, as shown in the results. As seen in the result of the BMI-matched study, a lot of this can be explained by BMI. When matching BMI, the results become a lot more cohesive, suggesting the largest bias has been eliminated.

4.2. Immunohistochemical staining of NLRP-1

The results report an indifferent staining of NLRP-1 in skin tissue of psoriasis patients compared with healthy controls. This most likely results from lack of expression of the NLRP-1 inflammasome of the psoriatic lesions, at least there is no difference in expression between patients and controls. These results can not be reviewed as evidence supporting the absence of higher inflammasome expression in patients compared to controls. There are other types of inflammasomes in the NLR family that could very much be the link between psoriasis, systemic inflammation and CVD. Most likely, that link is not NLRP-1. Moreover,
there might be different expression levels in blood cells, that was not investigated in the present study.

4.3. The other way around

If psoriasis is a systemic autoimmune disease, one might wonder if the systemic inflammation is actually the core of the disease and the skin lesions only symptoms. In the beginning, scientists could not tell psoriasis from leprosy, most likely because since then and until the later half of the 20th century, everyone assumed it was only a cutaneous disease. Since the 1970s, scientists have been interested in the immune system’s involvement and now, the relationship with CVD. Until now, the disease has been treated and researched as it has origin in the skin. There is no reason why it should not be an option, the cutaneous lesions being preceded by the systemic inflammation. That theory could state an accumulation of immune system malfunction within the body, up until it breaks out and presents in psoriatic lesions of the skin. That could be the subject for another study in the future.

4.4. Execution

The only obvious fault in this study, apart from the possible bias, is how few the participants were. If there had been more participants, it would have been possible to control for other risk factors of CVD. On the other hand, the research has a significant affirmation in controlling for BMI and other CVD risk factors, like age and gender.

Otherwise, the research was well executed with attention to detail.

4.5. Conclusion

In conclusion, there is a relationship between psoriasis, systemic inflammation and CVD that is a serious extension of the psoriasis disease that should have an impact on how the condition is treated today. Very possibly, psoriasis patients have an increased risk of developing cardiovascular disease, including atherosclerosis. These results suggest a negligible effect of UVB treatment on the systemic inflammation and on the risk of CVD development, pointing towards the potential obsolescence of the phototherapy. Data is also recited, implying a therapeutic effect of TNF-α inhibitor on the systemic inflammation and the CVD risk. This research could be a step towards proving the relationship between psoriasis and CVD, potentially alternating the treatment options for psoriasis patients and hopefully a step towards making their disease tolerable.
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References


22. Arican O, Aral M, Sasmaz S, Ciragil P. Serum Levels of TNF-alpha, IFN-gamma, IL-6, IL-8, IL-12, IL-17, and IL-18 in Patients With Active Psoriasis and Correlation With Disease Severity. Mediators of Inflammation. 2005;2005(5).


Appendix A

The Luminex/Multiplex protocol

Day One

Supplies: The kit, water for day 2 (270 mL day 2), if using cell supernatant, cell culture medium is needed for the background (25 µL per standard/background/QC = <750 µL)

1. Bring reagents to room temperature.
2. Thaw samples, vortex (centrefuge if necessary) (dilute in assay buffer, if necessary).
3. Dilute QCs in 250 µL water. Invert several times, vortex, allow to sit for 5-10 minutes.
4. Prepare standards according to the guidelines (for CVD by reconstituting Human cytokine Standard with 250 µL water). Invert several times and vortex for 10 seconds. Allow to sit for 5-10 minutes and then prepare a 1:5 dilution series by adding 50 µL to 200 µL assay buffer.
5. Use a holder for the plate. Prewet the plate by pipetting 200 µL of wash buffer to each well. Seal.
6. Mix on a plate shaker for 10 minutes.
7. Sonicate each Ab-flask of the antibody beads for 30 seconds and vortex for 1 minute before use (sonicate in flow room, tubes closed). Add 60 µL from the flask to the mixing bottle and bring it up to 3 mL in Bead diluent. Vortex well.
8. Remove assay buffer from plate with vacuum, blot bottom.
9. Add 25 µL of each standard or QC to their wells. Use assay buffer for background.
10. Add 25 µL assay buffer to the sample wells.
11. Add 25 µL assay buffer to background, standards and QCs.
12. Add 25 µL samples to the sample wells.
13. Vortex the mixing bottle and add 25 µL of the bead-mixture to each well.
14. Seal the plate.
15. Put on a shaker over night at 4°C.
Day Two

1. Bring reagents to room temperature.
2. Make wash buffer; 30 mL buffer to 270 mL water.
3. Remove fluid by vacuum.
4. Wash twice with 200 µL/well of wash buffer, vacuum, blot.
5. Add 25 µL of detection antibody in each well.
6. Seal and cover, incubate with agitation on a shaker for 1 hour.
7. Add 25 µL streptavidin-phycoerythrin to each well.
8. Seal and cover, incubate on shaker for 30 minutes.
9. Remove fluid by vacuum.
10. Wash twice with 200 µL/well wash buffer, vacuum.
11. Add 100 µL sheath fluid to all wells, re-suspend on shaker for 5 minutes.
12. Analyze.
Appendix B

The Immunohistochemistry protocol

Day One:

1. Warm up the PT-Link machine to 65°C.
2. Prepare wash buffers
   a. PBS-Tween : 1 tablet PBS, 1 mL Tween-20 and 1 L MilliQ water
   b. PBS + 0.5% BSA: 1 tablet PBS, 5 g BSA, 1 mL MilliQ water
   c. PBS + 5% BSA: 10 mL PBS, 0.5 g BSA.
3. Put the slides in a low pH bath when the machine is warm, press run. The protocol takes approximately 1 hour.
4. Place 30 µL of 3% H₂O₂ (100 µL H₂O₂, 900 µL PBS) on each slide and keep them dark for 10 minutes.
5. Dry the glass with Kleenex® around the tissue and mark around the section with a paraffin pen.
6. Prepare a moisture chamber.
7. Drop a few drops of 5% BSA on the sections and incubate for 20 minutes in a moisture chamber.
8. Dilute the primary antibody to yield 1/30 NLRP-1 to PBS + 0.5% BSA.
9. Carefully drop off the protein block, dry if necessary. Make sure the slides are properly labeled with date, antibody and dilution.
10. Put 60 µL of the NLRP-1 dilution on each section.
11. Put the slides in a moisture chamber and in a refrigerator overnight.

Day Two:

1. Wash slides with PBS + 0.5% BSA for 2 min x 2 times, in a slide holder.
2. Dry the glass with Kleenex® around the tissue.
3. Put 2 drops of the secondary antibody on the tissue and incubate for 30 minutes in a moisture chamber at room temperature.
4. Pour off the secondary antibody.
5. Wash with PBS + 0.5% BSA for 2 minutes x 2 times in a slide holder.

6. Apply the DAB solution on the sections and incubate for 7 minutes at room temperature.

7. Wash the slides in PBS + 0.5% BSA quickly, followed by water for 5 minutes.

8. Color with hematoxylin for 3 minutes.

9. Wash the slides in distilled water for 10 minutes

10. Dry the slides with Kleenex®, mount with aqua/polymount.

11. Allow to dry for more than 1 hour