UNDERSTANDING IMMUNE-ETIOLOGY OF PSORIASIS: AN AUTOIMMUNE DISEASE

Project report submitted

For the award of

M.Sc. Life Sciences (Biochemistry)

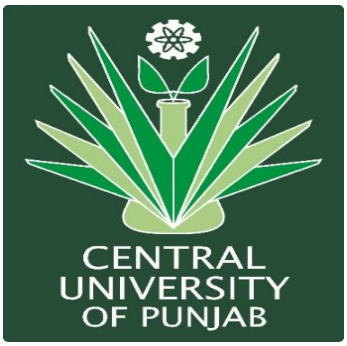
By

ANJNA KUMARI

Registration No: 16mslsbc04

Supervisor

Dr. Manju Jain



Department of Biochemistry and Microbial Sciences

School of Basic and Applied Sciences

Central University of Punjab, Bathinda, India

May, 2018

DECLARATION

I declare that the project entitled “UNDERSTANDING IMMUNE-ETIOLOGY OF PSORIASIS: AN AUTOIMMUNE DISEASE”, has been prepared by me under the guidance of Dr. Manju Jain, Assistant Professor, Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of project has formed the basis for the award of any degree or fellowship previously.

Anjna kumari
M.Sc. Life Sciences with specialization in Biochemistry,
Department of Biochemistry and Microbial Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda-151001
Date:

CERTIFICATE

I certify that ANJNA KUMARI has prepared her Project entitled "UNDERSTANDING IMMUNE-ETIOLOGY OF PSORIASIS: AN AUTOIMMUNE DISEASE", for the award of M.Sc. Life Sciences with specialization in Biochemistry degree of Central University of Punjab, under my guidance. She carried out this work at the Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab.

Dr. Manju Jain
M.Sc. Life Sciences with specialization in Biochemistry,
Department of Biochemistry and Microbial Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda-151001
Date:

ABSTRACT

Understanding Immune-etiology of Psoriasis: An autoimmune disease

Name of the student	: Anjna kumari
Registration Number	: 16mslsbc04
Degree for which submitted	: Master of Science
Supervisor	: Dr. Manju Jain
Department	: Biochemistry and Microbial Sciences
School	: Basic and Applied Sciences

Keywords: Autoimmune disease, Psoriasis, PBMCs, T cells, Cytokines, T Cell Receptor Excision Circles

Psoriasis is a chronic inflammatory autoimmune disease. Disease etiology is understood in terms of altered crosstalk between skin keratinocytes and immune cell infiltrates, specifically T cells leading to the development of characteristic psoriatic skin lesions. T cell alterations in skin lesions as well as in the peripheral blood of psoriatic patients have been shown to be associated with the disease condition. With a major research focus on keratinocyte abnormalities, more studies are required to understand the immune-etiology of disease. There are fewer reports with inconsistence findings on T cell changes in psoriatic patients. Towards this end, we performed a study using blood samples from psoriasis patients and healthy controls to access alterations in peripheral blood mononuclear cell and T cell count along with phenotyping of blood cells in terms of CD4+ Th and CD8+ Tc cells and expression pattern of T cell-associated cytokines in plasma samples. Furthermore, TREC analysis was done to understand possible origin of T cell alterations associated with psoriasis.

Dr. Manju Jain Anjna kumari

ACKNOWLEDGEMENT

I would like to express my special thanks of gratitude to my mentor **Dr. Manju Jain** who gave me the golden opportunity to do this wonderful project, which also helped me in doing a lot of research and I came to know about so many new things, I am really thankful to her. I am grateful to her for being patient with my mistakes and boosting my confidence.

Secondly I would also like to thank **Ms. Lovlesh Thakur** for her support and guidance, which helped me a lot in finalizing this project within the limited time frame.

I extend my heartfelt thanks to **Prof. Ramakrishna Wusirika** (HOD) and other faculty members of the Department for their mature and scholarly guidance in making this study fruitful.

My thanks and appreciations also go to my family and friends who have willingly helped me out with their abilities.

Anjna Kumari

Date:

LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1.	T-Cell Receptor Excision Circles	TREC
2.	Peripheral Blood Mononuclear Cell	PBMC
3.	Ethylenediamine Tetraacetic Acid	EDTA
4.	Neutral-Buffered Formalin	NBF
5.	Fetal Bovine Serum	FBS
6.	Phosphate-Buffered Saline	PBS
7.	Dimethyl Sulfoxide	DMSO
8.	Peridinin Chlorophyll Protein	PerCP
9.	Allophycocyanin	APC
10.	Fluorescein Isothiocyanate	FITC
11.	Propidium Iodide	PI
12.	Enzyme Linked Immunosorbent Assays	ELISA
13.	*4 Parameter Logistic* Regression	4PL
14.	Quantitative Real Time PCR	qRT-PCR
15.	Deoxyribonucleic Acid	DNA
16.	Room Temperature	RT

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENT

Chapter 1

INTRODUCTION

1.1 Background

Psoriasis is an inflammatory autoimmune skin disease. It is facilitated by cells and molecules of both the innate and adaptive immune system. It develops when immune system sends faulty signals that induce abnormal proliferation of skin cells, keratinocytes. Keratinocytes form in days rather than weeks. The excess skin cells are not shed by the body. The skin cells stack up on the surface of the skin, causing patches of psoriasis skin lesions to appear.

Psoriasis can appear in any location on the body, but it typically affects the knees or scalp, outside of the elbows. Some people report that psoriasis can be itchy, burns and stings. Many other health conditions are associated with it like diabetes, heart disease, depression (National Psoriasis Foundation). It affects approximately 0.5%–1% of children and 2%–3% of the world's population. Male are more susceptible to the disease compared to females (Dogra S. *et al.* 2016)**.** It is rarely seen in infants. Psoriasis likewise happens in all racial groups.

Current research implies that a complex interplay of genetic and environmental factors, along with immune regulatory abnormalities play a critical role in the pathogenesis of this disease. In psoriasis, the homeostatic crosstalk between different immune cells and resident skin cells seen in healthy skin is disturbed. Despite our understanding of heterogeneous cell population viz. keratinocytes, T-cells, macrophages, dendritic cells, neutrophils involved in disease etiology, exact molecular details that regulate the complex interactions among these cells to create a chronic inflammatory environment are not fully known.

Immunologically, new insights in the pathogenesis of psoriasis point towards a critical role of cell mediated immune response. Our current understanding suggests that T cells are the major players in disease etiology. Alterations in T cells, comprising a heterogeneous pool of subpopulations viz Th1, Th2, Th17, Th22, Treg subsets are reported to be associated with the disease.(Chiricozzi *et al,* 2018; Langewouters *et al*, 2008).Thus it is critical to decipher the nature of T cell alterations to understand the molecular and cellular mechanism of disease etiology. The mechanism behind T cell alterations reported in psoriasis is not much worked out. The origin of T cells alteration is also not known. One way to know the origin of the T cell turnover is to perform relative quantification of T-cell

receptor excision circles (TRECs) in diseased versus healthy individuals. Altered level of TRECs can happen either because of altered thymic output or altered proliferation of recent thymic emigrant cells in the periphery which may lead to change T cell numbers in the peripheral blood in the disease condition

1.2 Knowledge gap

- Limited and inconsistence report on quantitative and qualitative alterations in PBMCs, especially T cells.
- Limited knowledge on the origin of T cell alterations.

1.3 Hypothesis

A detailed unbiased study with a large sample size from Psoriatic versus healthy individuals is required for characterizing changes in PBMC count, total T cell number, T cell subsets numbers and functionality (Th & Tc cells) along with understanding the origin of the said changes.

1.4 Objective

- Evaluate alteration in PBMC count in psoriatic versus healthy individuals. (PBMC Isolation and Quantitation)
- Evaluate the T cell response in psoriatic patients compared to healthy controls. (Flow Cytometry and Cytokine expression analysis)
- Evaluate possible difference in TREC levels in psoriasis patients compared to healthy controls.

Chapter 2

REVIEW OF LITERATURE

2.1 Epidemiology

Psoriasis is a chronic, autoimmune skin disease. The disease is found worldwide, but the prevalence varies among different ethnic groups. It is an incurable inflammatory disease till date affecting over 125 million people, or nearly 3% of the world population, is typical in Caucasians and affects equally men and women (National Psoriasis Foundation). Psoriasis *Vulgaris* is a common type of psoriasis, involving dry, red raised plaques with adherent silvery scales. In India, the prevalence of psoriasis varies from 0.44 to 2.8% (Dogra S. *et al.* 2016).In the USA, the incidence of psoriasis was estimated to be around 4.6% while in Canada it was 4.7%. Data from Europe show little variation in countries with a range from 1.4% (Norway), 1.55% (Croatia) and 1.6% (UK). In East Africa, the figure was 0.7%, and in the Henan district of China, only 0.7% were found affected. While relatively common in Japanese, it is less frequent in Chinese, Eskimos, West Africans and North American blacks, and very uncommon in North American and South American natives and aboriginal Australians (Nevitt & Hutchinson 1996). Psoriasis also shows a bimodal distribution with a peak between fifteen and twenty years of age and another peak between fifty-five and sixty years. By the bimodal distribution of the age at onset and inheritance, two types of psoriasis have been proposed: Type 1 begins on or before age forty years; Type II starts after forty years. More than 75% of cases belong to type 1. The beginning of psoriasis can be at any time of life and, usually persists for life. Psoriasis is a relapsing disease, although natural remission occurs in about one-third of the psoriatic patients.

2.2 Disease Etiology

Psoriasis is a skin condition in which life cycle of skin cells speeds up such that, new skin cells build up rapidly on the surface of the skin. The extra skin cells form scales and red patches that are itchy and sometimes painful. There is no cure for psoriasis. One can manage the symptoms of psoriasis with Lifestyle measures, like moisturizing, quitting smoking and managing stress.

The cause of psoriasis is not apparent yet, but many environmental and genetic factors are known to cause psoriasis in a susceptible individual. Established causes of psoriasis include certain types of infections, skin injuries, stress, and the use of certain medications, Allergies, diet, weather, smoking, and alcohol (National

Psoriasis Foundation 2011; University of Maryland Medical Center 2011). Psychological stress is a well-established trigger of psoriasis. Exacerbating causes of plaque psoriasis can be divided into local and systemic factors (Ashwin B. Kuchekar *et al.* 2011).

Major causes of Psoriasis are:

Trauma: All types of trauma like Physical, chemical, electrical, surgical, infective, and inflammatory types of injury have been related to the development of plaque psoriasis. The development of psoriatic plaques at a site of injury is known as the Koebner reaction ((American Academy of Dermatology 2011).

Sunlight: Ultraviolet and chemical injuries of the skin can serve as triggers of psoriasis outbreaks (Peters *et al.* 2000). Most report a decrease in illness severity during the summer months or periods of increased sun exposure; however, a small minority find that intense sunlight aggravates their symptoms, and the condition becomes worse in the summer.

Infection: *Streptococcal* infections of the respiratory system, such as *streptococcal pharyngitis or sinusitis*, serve as a trigger for guttate psoriasis. Certain strains of *Staphylococcus aureus* can produce enterotoxin. The enterotoxins also called as Superantigens, cause polyclonal activation of CD4+ and CD8+ T cells. (Brook, 2002; Leung *et al*., 1993; Tomi *et al*., 2005) Inverse psoriasis is sometimes associated with *Candida albicans* (thrush) and other fungal infections of the skin.

HIV: It has been observed that in patients who are or become infected with HIV have an increase in psoriasis activity. The severity and extent of skin disease initially appear to parallel the disease stage. Psoriasis often becomes less active in advanced HIV infection.

Drugs: The use of certain medications like Lithium, Anti-malarial medications, Inderal, quinidine, angiotensin-converting enzyme inhibitors, Indomethacin and overuse and sudden discontinuation of corticosteroids can lead to the onset of psoriasis outbreaks.

Smoking & Alcohol: An increased risk of chronic plaque psoriasis exists in persons who smoke cigarettes. Alcohol is also a risk factor for psoriasis, particularly in young to middle-aged males.

As discussed above disease etiology involves a complex interaction among genetic, immunological, and environmental components. Critical players in Psoriasis are keratinocytes and immune cells. Psoriasis is characterized by hyperproliferation and abnormal differentiation of epidermal keratinocytes,

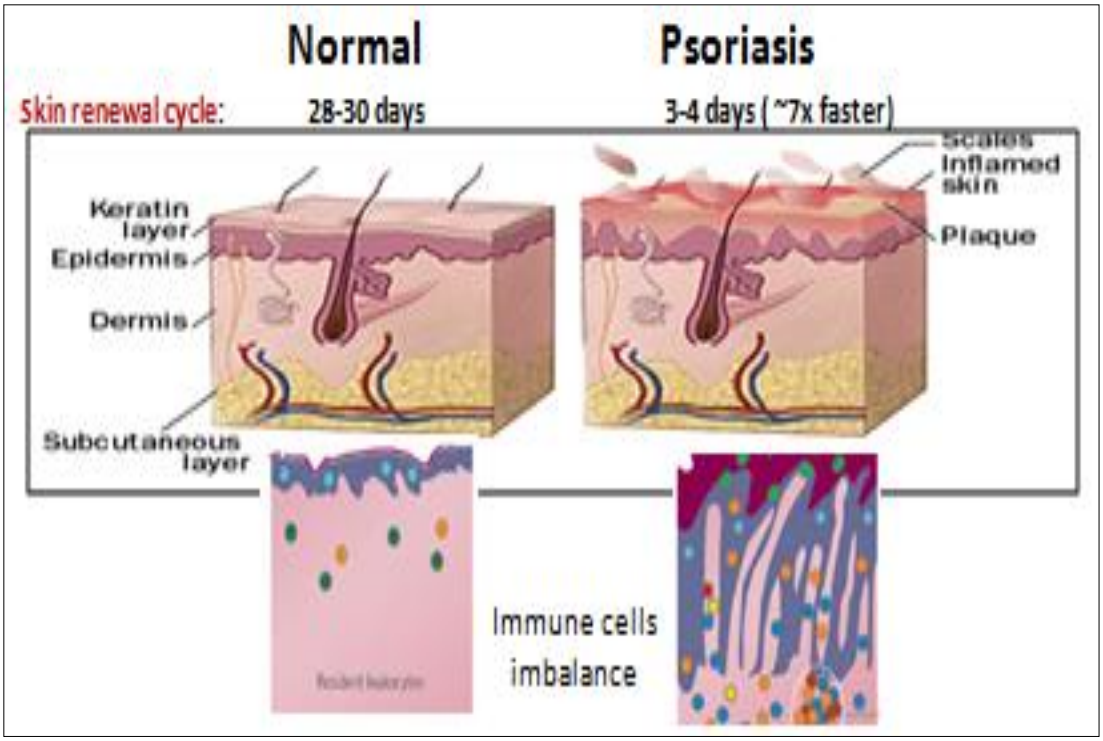


Figure 2.1: Altered Skin renewal cycle and associated altered cellular events in Psoriasis

Source (www. Mayoclinic.com)

lymphocyte infiltration consisting mostly of T lymphocytes and various endothelial vascular changes in the dermal layer (Guenther & Ortonne 2002). The current understanding of the pathogenesis of psoriasis suggests that interaction between acquired and innate immunity play an essential role in disease etiology. It does not involve a single cell type or a single inflammatory cytokine, preferably linked to complex interaction interactions among infiltrating leucocytes, resident skin cells (keratinocytes) and an array of pro-inflammatory cytokines, chemokines, and chemical mediators produced in the skin (Figure 2.1) (M. A. Lowes *et al.* 2007)

Genetics of Psoriasis: The mode of inheritance of psoriasis is complex. There are at least nine chromosomal loci with statistically significant linkage to psoriasis; *PSORS1* through *PSORS*9. In most cases of psoriasis, the PSORS1 gene, in the major histocompatibility complex region on chromosome 6 (6p21), has been reported to be associated with the disease. The penetrance of the PSORS1 *locus*

is said to be less than 15%, implying role of other genetic and environmental factors too. Moreover, an association of PSORS with functional polymorphisms in modifier genes that mediate inflammation (e.g., tumor necrosis factor (TNF-α) and vascular growth (e.g., vascular endothelial growth factor), has been found (Capon *et al.* 2002).

About 30% of psoriatic patients present a family history of the disease in a first or second-degree relative. The risk to develop psoriasis appears to be about 20% if one parent has psoriasis and about 75% if both parents are affected. Psoriasis has been associated with certain HLA-types (HLA-Cw6, HLA-B13, HLA-B17, HLABw57, HLA-DR4), and those with HLA-Cw6 seem to have a 10-fold higher risk to develop the disease (Elder *et al.* 2010). (Gudjonsson *et al.* 2006; Henseler & Christophers, 1985).

2.3 Psoriasis: Clinical Manifestations

Psoriasis presents variable morphology, distribution, severity and course. The different types of psoriasis may range in severity from mild to severe and may be localized or widespread. Typically, at a time only one type of psoriasis will appear. However, it is possible for different types of psoriasis to be present at the same time. The course of psoriasis is characterized by vast inconsistencies in the length of time of exacerbations and remissions (R. Langley *et al.* 2005; Nicolai *et al*. 2006).

There are several types of psoriasis as shown in Figure 2.2. These include:

Plaque psoriasis (psoriasis vulgaris): It is the most prevalent form of the disease, affecting approximately 80% of individuals with psoriasis. It mostly appears on the scalp, elbows, knees, and lower back, as well as at sites of trauma. Well-demarcated round or oval plaques characterize the lesions of plaque psoriasis. The plaques appear scaly, thick, silvery, and erythematous and are surrounded by healthy skin. The scales are usually loosely cohesive, and removal may cause small bleeding points, known as the Auspitz sign.

- **Guttate psoriasis. It** is the 2nd most common form of the disease. It affects about 10% of people with psoriasis. This type mainly affects young adults and children. It's usually initiated by a bacterial infection such as strep throat. The lesions are small, tear-shaped papules on arms, legs, trunk, and scalp and have a rapid onset. The wounds are covered by an excellent scale and are

less thick than those of plaque psoriasis. (Gordon & McCormick 2003; Nicolai *et al*., 2006).

- **Inverse (flexural) psoriasis**: It involves lesions that develop in the axilla, groin, or folds of the skin. It is commonly seen in obese and overweight individuals. The lesions of inverse psoriasis are large, shiny, smooth, and have a deep red color. Inverse psoriasis lacks the scales associated with plaque psoriasis infection.
- **Pustular psoriasis**: It is not a common type of psoriasis. It can occur in widespread patches (generalized pustular psoriasis) or smaller areas on your hands, feet or fingertips. It is characterized by white blisters of non-infectious pus surrounded by reddened skin.
- **Erythrodermic psoriasis**: It is the least common form of psoriasis and affects 1 – 2% of individuals with psoriasis. It is characterized by red, scaling lesions which are located over the majority of the body surface. Pain and Severe itching accompany this form of psoriasis, with the scaling occurring in large sections of skin rather than the smaller scales associated with plaque psoriasis. (National Psoriasis Foundation 2011)(R. G. Langley *et al*. 2005; Nicolai *et al*. 2006).
- **Psoriatic arthritis**: It is another dominant form. Approximately 10–30% of patients with psoriasis develop Psoriatic arthritis. In addition to inflamed, scaly skin, psoriatic arthritis causes pitted discoloured nails and the swollen, painful joints that are typical of arthritis. It can cause progressive joint damage that in the most severe cases may lead to permanent deformity.
- **Nail & Scalp psoriasis** – In nail Psoriasis fingernails and toenails are mainly involved. It causes pitting, abnormal nail growth and discoloration. Psoriatic nails may get free and separate from the nail bed (onycholysis). Scalp psoriasis appears as red, itchy areas with silvery-white scales. Bits of dead skin can be imaged in the hair or on shoulders, especially after scratching the scalp.

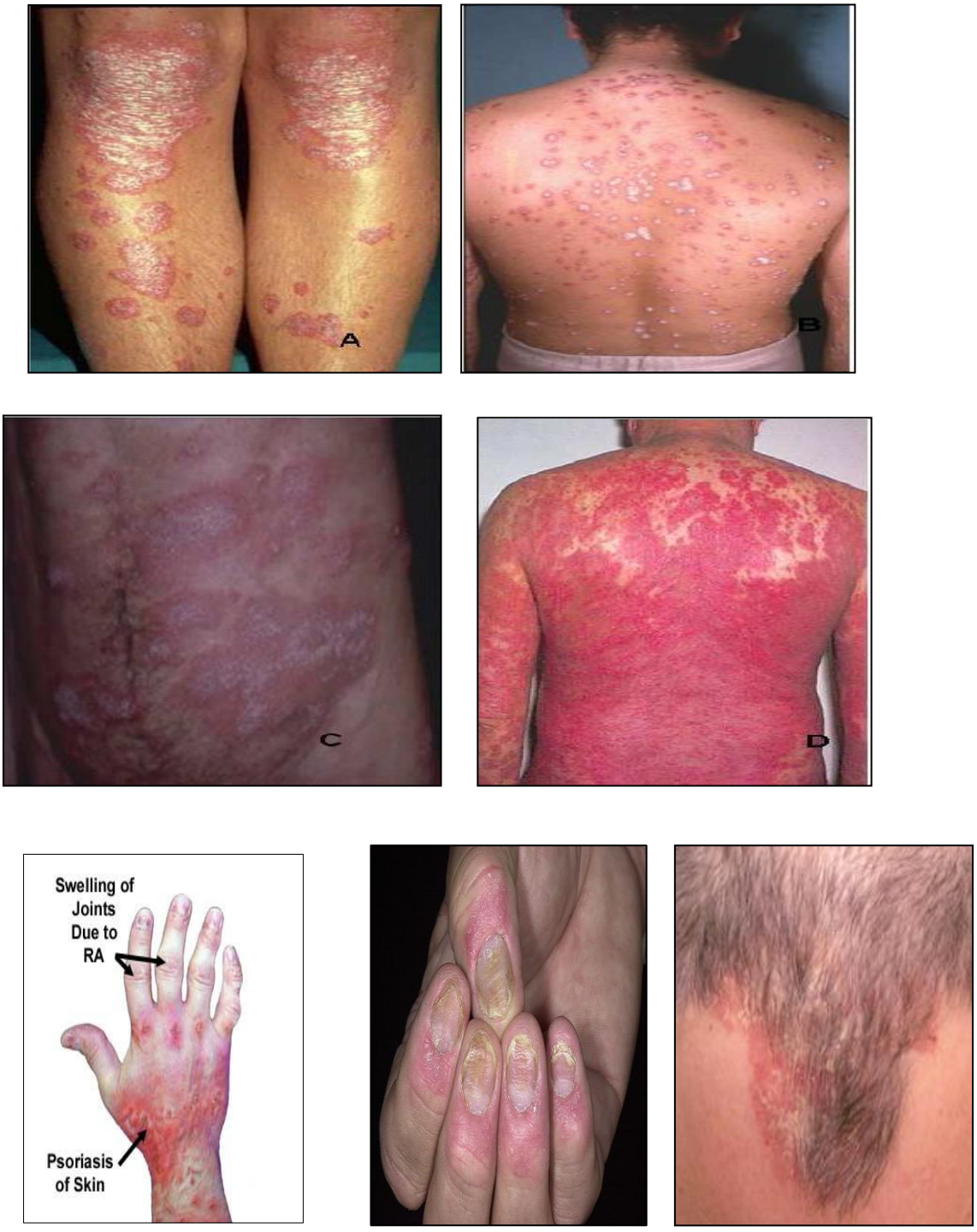


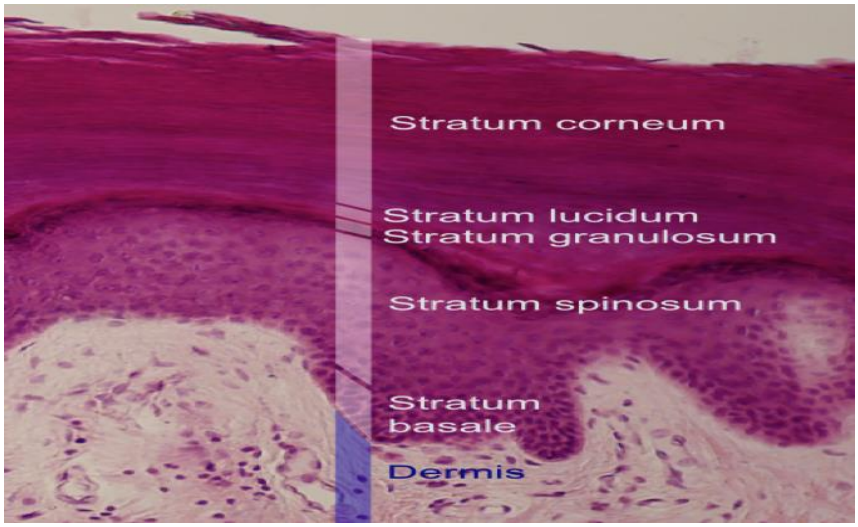
Figure 2.1: Psoriasis clinical manifestations:
A - chronic plaque psoriasis, B - guttate psoriasis, C - pustular psoriasis, D - erythrodermic psoriasis , E- Psoriasis Arthritis, F – Nail Psoriasis, G – Scalp Psoriasis

Source:(www.intechopen.com)

2.4 Histological feature

Healthy skin comprises of the epidermis, epidermal basement membrane, papillary and reticular dermis, with related adnexa, and subcutaneous fat. Epidermis shows an ordered maturation of keratinocytes from the basal (germinative) layer, toward the spinosum, the granular cell layer up to the keratinized layer. Psoriasis is a dynamic dermatosis identified by erythematous, raised, scaly skin lesions.

a.



b.

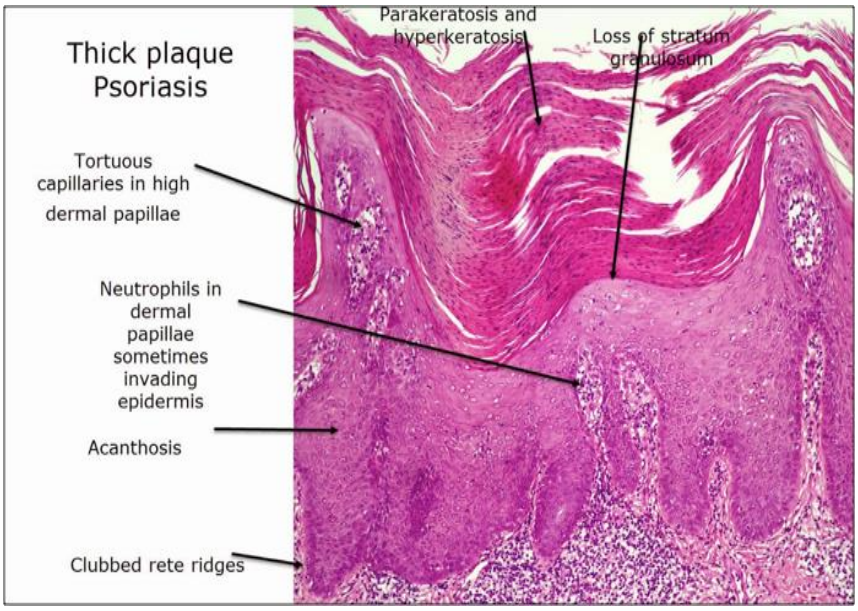


Figure 2.3: a. Skin layers exhibited in a H&E tissue section from normal skin. b) Histopathological alterations in psoriasis lesional skin seen in an H&E lesional section.

Numerous defined histologic changes are seen as shown in Figure 2.3:

- **Acanthosis**: due to rapid keratinocyte proliferation, epidermis became thick.
- **Hypergranulosis:** reduced or absent granular layer
- **Parakeratosis**: as a result of aberrant differentiation of keratinocytes, nuclei get retained in corneocytes
- Visible erythema is caused by marked dilation of blood vessels in the papillary dermis and
- A condensed inflammatory infiltrate composed of clusters of CD8+ T cells and neutrophils in the epidermis, and CD4+ T helper cells and antigen-presenting dendritic cells (DCs) in the dermis (E Nograles *et al*, 2010).

2.5 Assessment tools

A large variety of assessment tools are used to evaluate the severity of psoriasis are as follows:

- **Psoriasis Area Severity Index (PASI)**

It is a mostly used tool for the measurement of the severity. A single score range from 0 to 72 is used to measure the severity of lesions and surface area of the skin. The physical structure is split into four sections: head (10% of the body area), arms (20%), trunk (30%) and legs (40%). Each of these areas is graded individually, and the four scores are then aggregated. For each subdivision, the portion of the area of skin affected is judged and then translated into a grade from 0 to 6. The PASI is the most validated objective method to measure the severity of psoriasis and has high intra-rater reliability and an excellent inter observer correlation when used by trained assessors (Berth-Jones et al., 2006). The PASI system reflects disease improvement or deterioration, although the sensitivity to change for small areas of involvement is poor (Feldman & Krueger 2005; Finlay 2005). Achieving a seventy-five percentage improvement in the PASI is considered to be a successful treatment. PASI 50 (50% improvement) and PASI 90 (90% improvement) are sometimes also used.

- **Body Surface Area**

The Body Surface Area (BSA) is an method to estimate the extent of psoriasis involvement, calculating one palm represent 1% of the total body surface area

(Finlay 2005; N. Rossiter *et al.* 1996). The advantages of BSA are that it is quick and convenient to use, with a low test-retest variability for the same observer. However, there is moderately high interrater variability, and the method is likely to overestimate the extent of psoriatic lesions (N. D. Rossiter *et al.*1996).

The Dermatology Life Quality Index (DLQI): It is a ten-item questionnaire evaluating the quality of life in patients with dermatological diseases(Finlay & Khan 1994). It consists of six subscales: daily activities, symptoms and feelings, personal relationships, leisure, work and school, and treatment satisfaction. A higher score indicates poorer quality of life. The DLQI gives a total score of 30. An estimate of the minimal clinically significant difference of the DLQI total score is a 5 point improvement(Khilji *et al.* 2002). In DLQI score (Bowcock & Krueger, 2005)a set of intervals is proposed: 0 to 1=no effect 2 to 5=small effect, 6 to 10=moderate effect, 11 to 20=very considerable effect and 21 to 30= extreme effect. The reliability of the DLQI is well-established (Finlay & Khan 1994; Lewis & Finlay 2004; Shikiar *et al*. 2006).

2.6 Diagnosis: There are no individual blood tests or diagnostic procedures for psoriasis. Sometimes a skin biopsy or scraping may be needed to confirm the diagnosis. Some lab tests can be done like-

- the negative test result for rheumatoid factor (RF)
- The red blood cell sedimentation rate is usually reasonable (except in pustular and Erythrodermic psoriasis).
- Uric acid level (increased uric acid levels due to purine) may be elevated in psoriasis (especially in pustular psoriasis), confusing with gout in psoriatic arthritis.
- Fluid from pustules is sterile with neutrophilic infiltrate.
- Perform fungal studies. (This is particularly significant in cases of hand and foot psoriasis that seem to be worsening with the role of topical steroids.)

(http://emedicine.medscape.com/article/1943419-workup#aw2aab6b5b2aa)

2.7 Prognosis & treatment

Psoriasis is a lifelong condition. There is no cure for Psoriasis. Various treatments can reduce the symptoms. Effective agents used to treat severe psoriasis may have an increased risk of significant morbidity including skin cancers, lymphoma, and liver disease. If psoriasis is mild, it can be treated with topical medication. Psoriasis does get worse over time. Lifelong therapy requires for controlling the signs and symptoms typically. The recommended treatment for mild psoriasis is to start with topical therapy and move to phototherapy or systemic treatment in refractory cases. Phototherapy or systemic therapies are recommended for moderate to severe psoriasis.

- **Topical treatment**

Emollients

These are used to reduce irritation and soften scaling. The procedure has a positive effect on skin hydration and acts as a barrier function in psoriasis patients.

Corticosteroids

Corticosteroids have an anti-inflammatory and immunomodulating effect. Corticosteroids inhibit different proinflammatory cytokines such as TNF-α. Side-effects that may occur include cutaneous atrophy and the development of striae.

Calcipotriol

Calcipotriol is a vitamin D analog affecting epidermal proliferation and differentiation. It is mainly used for plaque psoriasis. Calcipotriol can cause irritant reactions (Mason *et al.* 2009).

Calcineurin inhibitor

Tacrolimus and pimecrolimus are immunomodulating agents and can be used for the treatment of intertriginous and facial psoriasis(Bigby M. 2005; Lebwohl *et al.* 2004). The main side effect is local burning. The long-term knowledge concerning a possible risk of developing skin cancer in areas exposed to the sun is limited.

Phototherapy

Ultraviolet B

For moderate to severe plaque psoriasis and guttate psoriasis UVB treatment is a standard treatment. The former use of broad-band UVB (BB-UVB) (290–320 nm) is now often replaced by narrow-band UVB (NB-UVB) **(**311±2 nm). The number of epidermal T lymphocytes and dendritic cells (DCs) decrease and there is a reduction in keratinocyte proliferation.

Psoralen + Ultraviolet A

PUVA treatment is psoralen (bath or oral) in combination with Ultraviolet A (320-400 nm). More Exposure to oral PUVA treatments increases the risk of developing squamous cell carcinoma (SCC).

Climate therapy

Sun exposure has an immunomodulating effect with a local and systemic reduction of T cells and cytokines, and Climatotherapy is the oldest form of phototherapy.

- **Systemic treatment**

Methotrexate

Methotrexate is a synthetic folic acid analog with anti-proliferative and anti-inflammatory properties. Polyglutamate is the primary metabolite in methotrexate. It competitively inhibits dihydrofolate reductase, preventing the reduction of folate cofactors. This results in inhibiting pyrimidine and purine synthesis and DNA methylation. Methotrexate empties the intracellular stores of activated folate. Cell replication is disrupted, and this leads to the inhibition of epidermal cell proliferation (Chan & Cronstein, 2010). Methotrexate is the first line treatment for moderate to severe psoriasis when systemic therapy is needed. Methotrexate can be administered orally, subcutaneously or intramuscularly.

Cyclosporin

Cyclosporin is a cyclic polypeptide consisting of eleven amino acids. It suppresses the activation of the calcium-dependent phosphatase calcineurin, inhibiting lymphokine secretion (e.g., IL-2, IFN- γ, GM-CSF, IL-3, IL-4, TNF-α and IL-17) which leads to diminished activation of T lymphocytes. Cyclosporin also inhibits antigen presenting cells. Cyclosporin is used for severe psoriasis. Cyclosporin is nephrotoxic and functional kidney damage can occur very fast after treatment has started.

Acitretin

Acitretin is a retinoid (synthetic vitamin A derivate) and has antiproliferative and immunomodulatory properties. In the epidermis, acitretin reduces the proliferative activity and favors the differentiation of epidermal keratinocytes. Acitretin inhibits the induction of Th17 cells and promotes the differentiation of T-regulatory cells. Acitretin is used for plaque psoriasis (especially in combination with UVB and PUVA) and also for pustulous psoriasis, hyperkeratotic hand- and foot psoriasis

and erythrodermic. Side effects are mainly hyperlipidemia and elevated liver enzymes (Van De Kerkhof 2006).

- **Biologics**

Biologics are drugs derived from living material, and that interfere with the immune system. Biologic therapies for psoriasis were introduced in Sweden in 2004. They are used for the treatment of moderate to severe psoriasis when traditional systemic therapies are contraindicated or cannot be used due to side effects or have not led to satisfactory treatment result(Smith *et al.* 2009). Higher risk of developing severe infections during treatment is a primary concern. Screening for tuberculosis and hepatitis is mandatory before treatment starts. Till now, there is no robust evidence of an increase in the risk of malignancy, but a possible future threat of lymphoma or other malignancies cannot be ruled out.

Etanercept

Etanercept is a human soluble TNF receptor fusion protein, binding free circulating TNF-α which competitively blocks TNF-α to bind to TNF-receptors. It is administered through subcutaneous injections.

Adalimumab

Adalimumab is a fully human anti-TNF- α monoclonal antibody, and it is administered through subcutaneous injections.

Infliximab

Infliximab is a chimeric human-mouse antibody that binds to both soluble TNFα and TNFα on the cell wall and is administered through intravenous infusions.

2.8 Comorbidity

Psoriasis is associated with several comorbidities, including psoriatic arthritis, metabolic syndrome and cardiovascular disease, gastrointestinal and liver disease, malignancy and depression. It has been suggested that the immune-mediated chronic inflammatory processes are a contributing and potentially independent risk factor for specific comorbidities associated with psoriasis.

- **Psoriatic arthritis**

The most well-known comorbidity in patients with psoriasis is psoriatic arthritis (PsA), with a prevalence of 10–30%. Development of pain, swelling, and tenderness of the joints surrounding ligaments and tendons are well-known characteristics of PsA. PsA can progress to an erosive, polyarticular disease with joint destruction and loss of functionality. Skin disease typically presents before

arthritis in more than 80% of the patients. By an average of ten years, psoriasis symptoms usually precede joint symptoms (Gladman *et al*. 2005; Gottlieb *et al.* 2006)

- **Metabolic syndrome**

Metabolic syndrome is frequently seen in patients with psoriasis. Obesity is a common comorbidity of psoriasis, and multiple studies have demonstrated that patients with psoriasis are more frequently overweight (BMI≥25) or obese (BMI≥30) compared with patients without psoriasis. It has been shown that a higher BMI coincides with a higher degree of psoriasis disease severity (Huerta *et al*. 2007; Naldi *et al.* 2005).

- **Cardiovascular disease**

Patients with severe psoriasis are more susceptible to developing cardiovascular disease. Systemic inflammation has been associated with the development of atherosclerosis which suggests that psoriatic patients may be at higher risk of developing cardiovascular disease (Mallbris *et al.* 2004).

- **Gastrointestinal disease and liver disease. In psoriasis**

patients, the prevalence of gastrointestinal illness and non-alcoholic fatty liver disease (NAFLD) is quite prevalent. NAFLD to be unrelated to psoriasis severity but revealed that psoriatic patients with NAFLD were much more likely to have psoriatic arthritis.

- **Malignancy**

Psoriasis is associated with an increased risk of malignancy, although the supporting data is inconsistent. The risk increase is most significant for patients with severe psoriasis treated with systemic therapies and minimal or no risk at all, for patients with milder disease. The increased risk is mainly for lymphoproliferative cancers and nonmelanoma skin cancers. The risk of psoriatic patients developing lymphoid malignancies may be attributable to the pathophysiology and also to the treatment of psoriasis. In addition to lymphoma and non-melanoma skin cancers, psoriatic patients are at higher risk of developing other malignancies, including those of the head and neck, solid organs (liver, pancreas, lung, breast, kidney), and genitals (Boffetta *et al.* 2001; Frentz & Olsen, 1999)).

- **Psychiatric disease**

Moderate to severe psoriasis is associated with marked physical and psychological morbidity, with up to 40% of patients reporting that their condition has adverse effects on their ability to daily function. High anxiety scores have been reported in over one-third of the psoriatic patients (Gupta & Gupta, 2003).

2.9 Pathogenesis of Psoriasis

Psoriasis is a common, inflammatory skin disorder which is characterized by red, scaly, well-defined, silvery-white, dry plaque formation in the skin. These plaques are derived from the excessive growth of skin epithelial cells. There are cellular changes occurs in the skin in psoriasis, such that there is rapid hyperproliferation and abnormal differentiation of keratinocyte along with infiltration of immune cells viz T lymphocytes, neutrophils, and other types of leukocytes in affected skin. Immuno-pathogenesis involves the complex crosstalk between skin resident keratinocytes and infiltrating immune cells.

In healthy skin the ratio of proliferating to non-proliferating keratinocytes is around 60% whereas in psoriasis it is almost 100%, also shedding of the epidermis is decreased to every 3 to 4 days due to the rapid cellular proliferation compared to average 26 to 30 days (Figure 2.4). The hyper-proliferation does not allow maturation and differentiation of skin cells, which results in a thickened epidermis and plaque formation. Due to the increased cellular metabolism caused by the rapid growth of skin cells, capillary dilation and increased vascularization of the skin occurs, leading to erythema.

Many factors can trigger psoriasis such as injury and trauma (termed the Koebner effect), infection, medications. Damage to the skin causes cell death and the production of the AMP LL37 by keratinocytes. DNA/LL37 complexes bind to intracellular TLR9 in plasmacytoid dendritic cells (pDCs), which causes activation and production of type I interferons IFN-α and -β. LL37/RNA complexes can activate plasmacytoid DCs through TLR7, and this complex can activate myeloid dendritic cells through TLR8(Lowes *et al.* 2014).

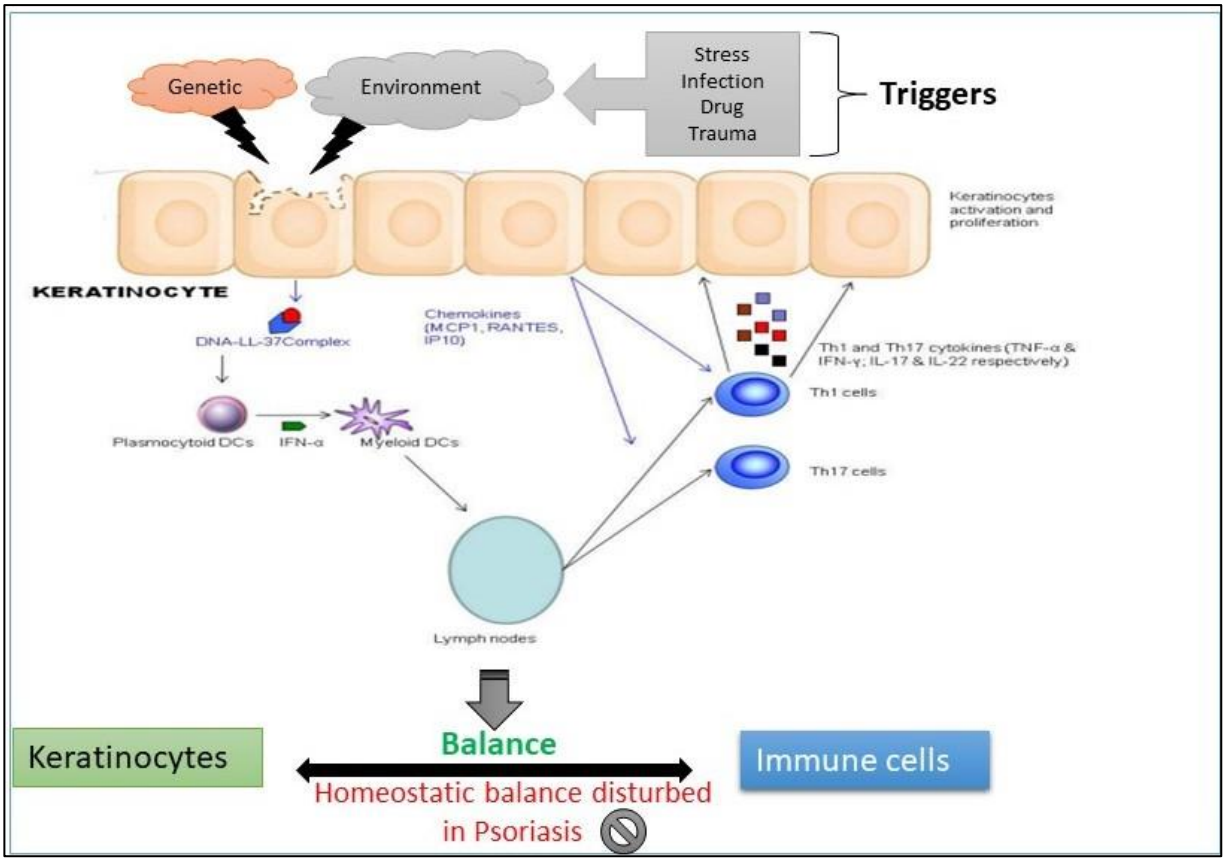


Figure 2.4: Disease Etiology: A result of dysregulated crosstalk between keratinocytes and immune cells. Source (Balato et al., 2012)

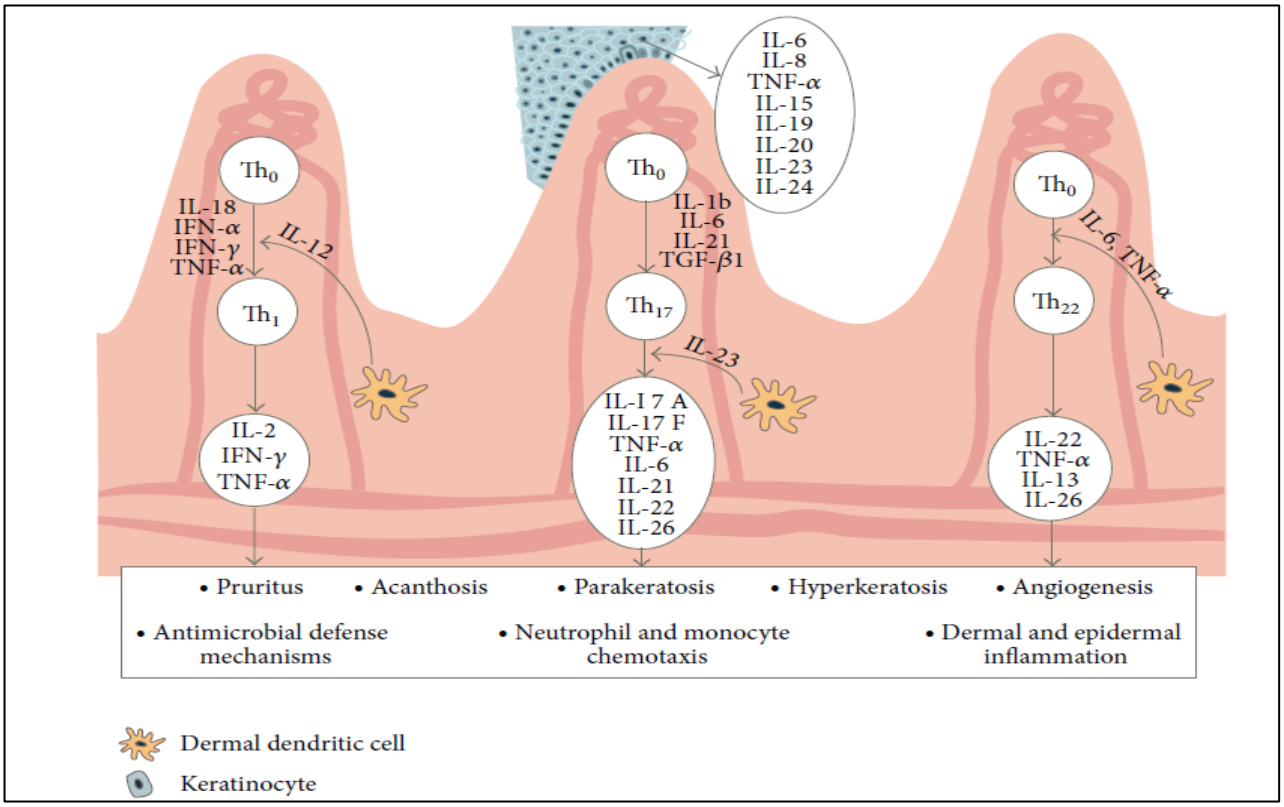


Figure 2.5: Role of different T cell subsets, Th1, Th17, and Th22 with subset specific cytokines in the pathogenesis of psoriasis.

Source: (Anna *et al*.2013)

Activate plasmacytoid DCs through TLR7, and this complex can activate myeloid dendritic cells through TLR8 (Lowes *et al.* 2014). Activated plasmacytoid dendritic cells secrete interferon-α (IFN-α) which also promote myeloid DCs cell activation. Activated myeloid dendritic cells migrate into draining lymph node. There they can induce the differentiation of naive T cells into effector cells such as Th17 or type 17 cytotoxic T cells (Tc17) and Th1 or type 1 cytotoxic T cells (Tc1). Effector cells recirculate and a slowdown in skin capillaries in the presence of selectin-guided and integrin-guided receptor-ligand interactions. The Immune cells which are expressing the chemokine receptors CCR6, CCR4, and CXCR3 travel into skin tissue along chemokine gradients. In the presence of chemokines such as CCL19 produced by macrophages, Dendritic cells and T cells form perivascular clusters and lymphoid-like structures around blood vessels. Unconventional T cells, including natural killer T cells (NKT), also contribute to the disease process. Further vital methods include the release of IL-23 by dermal dendritic cells, the production of pro-inflammatory mediators such as IL-17A, IL-17F, IL-22 by Th17 and Tc17 cells and IFN-α and TNF-γ by Th1 and Tc1 cells. These cytokines act on keratinocytes leading to the activation, proliferation, and production of antimicrobial peptides (AMPs) (e.g., LL-37 cathelicidin and β-defensins) and chemokines (e.g., CXCL1, CXCL9 through CXCL11 and CCL20), and S100 proteins (e.g., S100A7-9) (Balato *et al.* 2012; Nestle *et al.* 2009). Soluble mediators feedback into the pro-inflammatory disease cycle and constitute the inflammatory infiltrate.

Concerning the involvement of multiple cell types and molecules involved in disease etiology as explained above, there are two schools of thoughts regarding a primary defect that leads to psoriasis:

Before 1990: Primary activation of keratinocytes leads to subsequent releases of cytokines and antigen-independent activation of T cells.

Post-1990 (current understanding): Persistent T-cell stimulation leads to abnormal keratinocyte proliferation (Cai *et al.* 2012). Our current understanding of the disease pathogenesis suggests that T cells are the dominant player in the initiation of the disease.

Thus, proceeding with the recent line of thought of T-cell being the principal initiators of the disease, the ongoing research focuses on how changes in T-cell subsets and their regulation is crucial in understanding the disease etiology.

Different T cell changes associated with Psoriasis:

I. Alteration in numbers and activation state T cells and T cell subtypes:

Many advances have been made in the last decades in understanding of disease pathogenesis leading to the identification of the T cell subsets that play a role in establishing inflammation in psoriatic skin lesions. Different subsets of T cells may have a different hierarchical role in the pathogenesis of the disease underlining the importance of distinguishing T cell subpopulations involved in the disease. In the literature there are few reports which show quantitative and qualitative changes in peripheral T cells. Moreover, these reports have varied results with respect to total T cells, Th cells and Tc cells numbers and their activation states. Some reports suggest a high number of PBMC count in peripheral blood of psoriasis patients in correlation with disease severity (W B Jeffes *et al*.1995) while alternative reports show no significant difference in PBMC count (Langewouters *et al*. 2008). According to A.L. Cameron et al. there is no change in total T cells but Aldona Pietrzak *et al.* show total T cells and Th cells are decreasing in psoriatic patients.

II. Altered T cell specific cytokines profile

As discussed before cytokines play a major role in development of psoriatic lesions (Figure 2.5). Higher concentration of IFN-γ is reported in patients with psoriasis (Kyung Roh *et al*. 2015). Alongside IL- 17 and IL-22 are also reported in higher concentration in serum (Michalak-Stoma *et al.* 2013; Y. Zhou *et al.* 2016). There is an inconsistency in different reports about IL-4 and IL-12 concentration in serum and plasma. (Bai et al., 2018; Jacob *et al.* 2003; Zalewska Janowska *et al.* 2004). There are few reports on plasma cytokine levels in psoriasis. Additonal studies are required for a clearer picture of cytokine signature associated with psoriasis.

III. T-cell Receptor Excision Circles (TRECs) assay as a way to understand origin of peripheral T cell changes

T cells play a critical role in the pathogenesis of psoriasis. The predominance of CD8+ T cells in the epidermis is a striking feature of psoriasis, while CD4+ T cells are predominant in the upper dermis.

As we know thymus is most active early in life, it plays a central role in a continued, controlled emission of T cells, referred to as recent thymic emigrants (RTEs). Due to lack of phenotypic markers for RTEs, T-cell receptor excision circles (TRECs) are used to evaluate thymic function in terms of T cell turnover.

At the point when the T-cell receptor is formed viaa signal joint, TREC is created amid the rearrangement of the T cell receptor α gene fragment in around 66% of αβ T cells. The δ gene is located within the T-cell receptor α gene locus. For αβ T cells, the gene rearrangement is initiated by the deletion of the δ gene from the α gene locus. Elimination of the δ gene usually occurs in both alleles; therefore, a maximum of 2 copies of TREC may exist in αβ T cells immediately after completion of a corresponding rearrangement event. Deletion of the δ locus from the α gene occurs during the double positive (CD4+CD8+) stage before the positive and negative selection events. The TREC DNA is localized in the cytoplasm of the T-cell and is not replicated during cell division. Therefore, the number of TRECs is halved for each cellular doubling. If a T-cell contains TREC, it is likely to be an RTE or a non-dividing cell (Figure 2.6) (Hazenberg *et al.* 2001).

There is only a single report which shows that patients with psoriasis have significantly low TREC contents compared with healthy controls. (Deleuran *et al.* 2008).

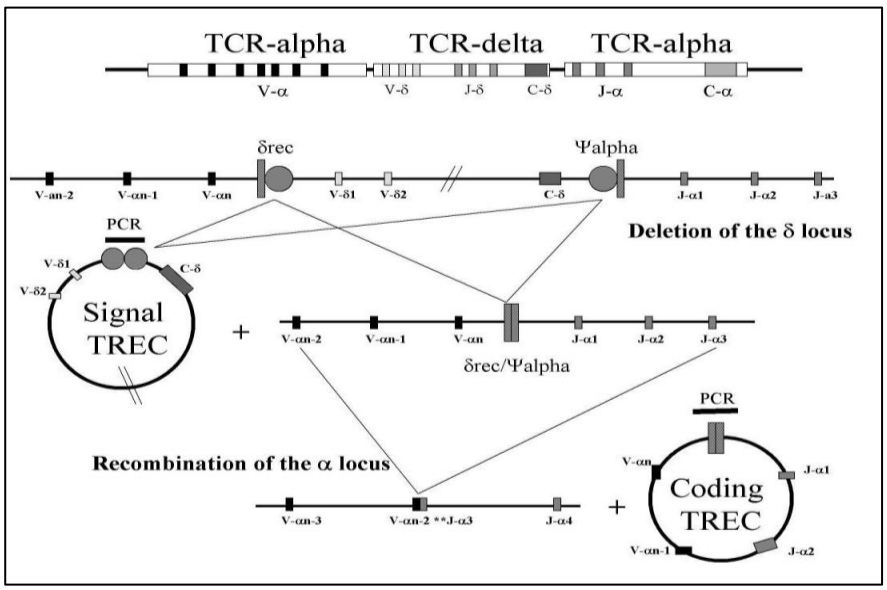
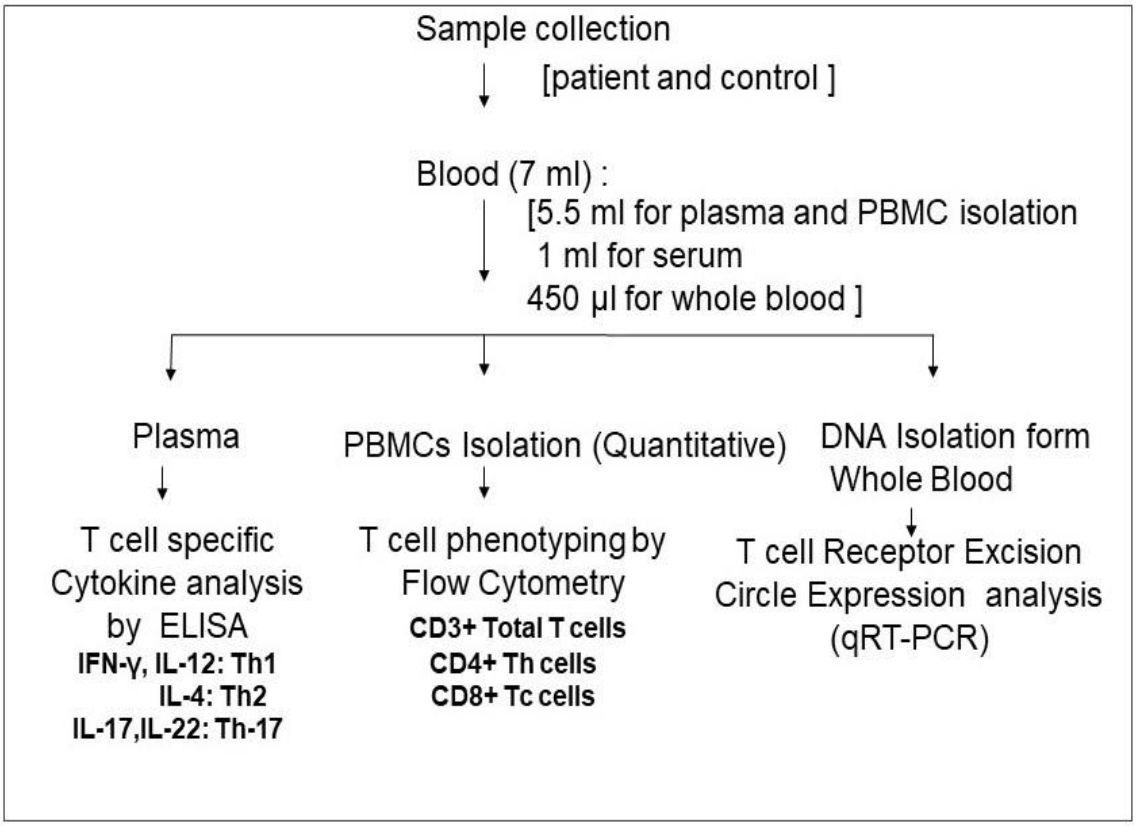


Figure 2.6: Molecular events in formation of T Cell Receptor Excision Circles (TRECs) during T cell development

Chapter 3

MATERIAL AND METHODS



EXPERIMENTAL WORK PLAN

3.1 Sample collection & processing

With due ethical approval and informed consent of study subjects, samples were cooled from healthy individuals and psoriatic patients.

Exclusion/ Inclusion criteria for Psoriasis Patient sample collection

- Patient age: 16 – 65 yrs
- Sex: M/F
- Psoriasis type: Psoriasis *Vulgaris* (moderate-severe)
- Body surface area: not less than 5% and preferably >10%
- Treatment priority of psoriasis patients (untreated patients)
  - Patients who have **not received topical therapy for at least two weeks**.
  - Patients who have **not received UV/PUVA for at least one month**
  - Patients who have **not received systemic therapy for at least one month.**

Materials required for blood sample collection & processing

Vacutainers with anticoagulant (EDTA),1X PBS (Appendix-A**)**, 10% NBF (Appendix-A**)**, Sterile gloves, surgical scalpel, 5 mm punches, 1ml and 10 ml sterile syringes, DMSO, Ficoll, 1X PBS (Appendix-A**)**, FBZ, Trypan blue dye.

- **Samples Collection Method:**

Control & Patient Blood: -Total 7 ml of blood collected through 10 ml sterile syringe

- 6 ml in EDTA vacutainers (with anticoagulant)

-1 ml in 1.5 ml vial (without anticoagulant)

- **Sample processing:**

Whole blood: 450ul of whole blood (from total 7 ml whole blood with anticoagulant) for each sample was stored in -80˚C till use.

Serum Isolation: 1ml blood was collected in 1.5 ml eppendorf (without anticoagulant) was processed for serum isolation by spinning at 3400 g for 10 mins at RT and later serum stored at -80ºC.

Plasma and PBMC Isolation by Ficoll density gradient centrifugation method: Peripheral Blood Mononuclear Cells (PBMCs) are blood cells with a round shaped nucleus, such as monocytes and lymphocytes, with the lymphocyte population comprised of T cells, B cells, and NK cells. PBMCs play an integral role in the defense system. Separation of PBMCs from whole blood is accomplished through density gradient centrifugation using Ficoll (density - 1.077 g/ml). After the centrifugation step, ficoll separates blood into layers according to the density of its components, topmost layer of pale yellowish layer of plasma, followed by a whitish buffy coat of PBMCs, then a layer of ficoll, followed by a reddish layer of granulocytes and erythrocyte**s**. PBMCs are widely used in both clinical laboratories and research. Separation of PBMCs from blood by centrifugation constitutes an essential step for all subsequent analyses, such as in immune monitoring. The protocol for PBMCs isolation was followed as shown in Figure 3.1.

Layering on ficoll

5.5ml blood

Dilution with PBS

Layering on ficoll

Centrifuge at 2000 rpm for 15 min @ RT

↓

Plasma & PBMCs Seperation

Plasma Preservation at -80°C

↓

Washing (2X)

Wash PBMCs with 1X PBS(5% FBS)

Centrifuge at 2000 rpm for 15 min @ RT

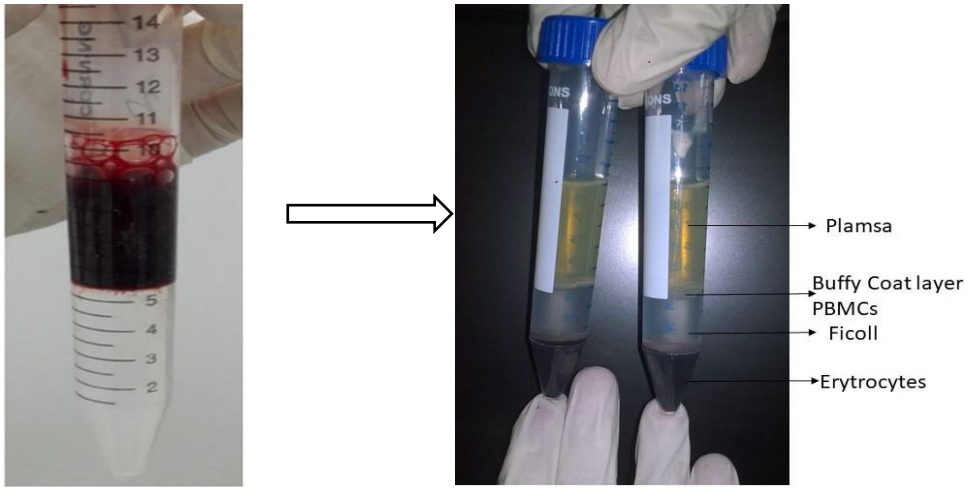
Discard supernatant & wash Pellet with 1X PBS

Centrifuge at 2000 rpm for 10min @ RT

↓

Cryopreservation

PBMCs in 900µl FBS +100µl DMSO & store at -80°C



Blood Overlaying on Ficoll

Layers after centrifugation

Figure 3.1: Flow chart of PBMC Isolation protocol

Quantification of PBMCs yield was done using haemocytometer

Viable cell count/ml (10^6 cells /ml) **=** Average viable cell count x Dilution factor x 10^4/ml

3.2 Peripheral T Cell Phenotyping using CD3+, CD4+and CD8+ markers

Flow cytometry is a commonly used technique for analyzing the expression of cell surface and intracellular markers. It can characterize and define different cell types in a heterogeneous cell population. It allows simultaneous multi-parameter analysis of single cells. It is primarily used to measure fluorescence intensity generated by fluorescent-labeled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules.

Expression of CD3 can define major T cells in a heterogenous population comprising PBMCs. From there, subtypes can then be split into CD4+ T helper cells and CD8+ cytotoxic T lymphocytes.

Materials required: Cells to be stained, 1XPBS, anti-human CD3+ antibody, anti-human CD4+ antibody, anti-human CD8+ antibody, centrifuge machine, vortex, ice bucket, pipette, tips, Eppendorf tubes, Flow cytometer**.**

Method: For immune staining the protocol was followed as shown in Figure 3.2. The gating strategy as shown Figure 3.3 was used for the study clearly defines T cell subsets. According to run sheet (Table1) samples were stained. 50000 events were taken. For analysis BD accuri TM C6 software was used.

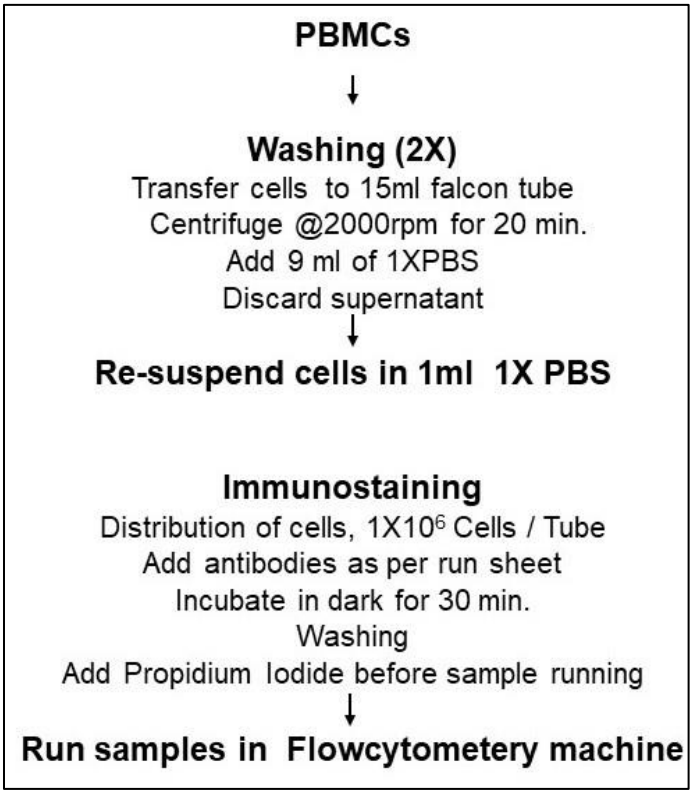
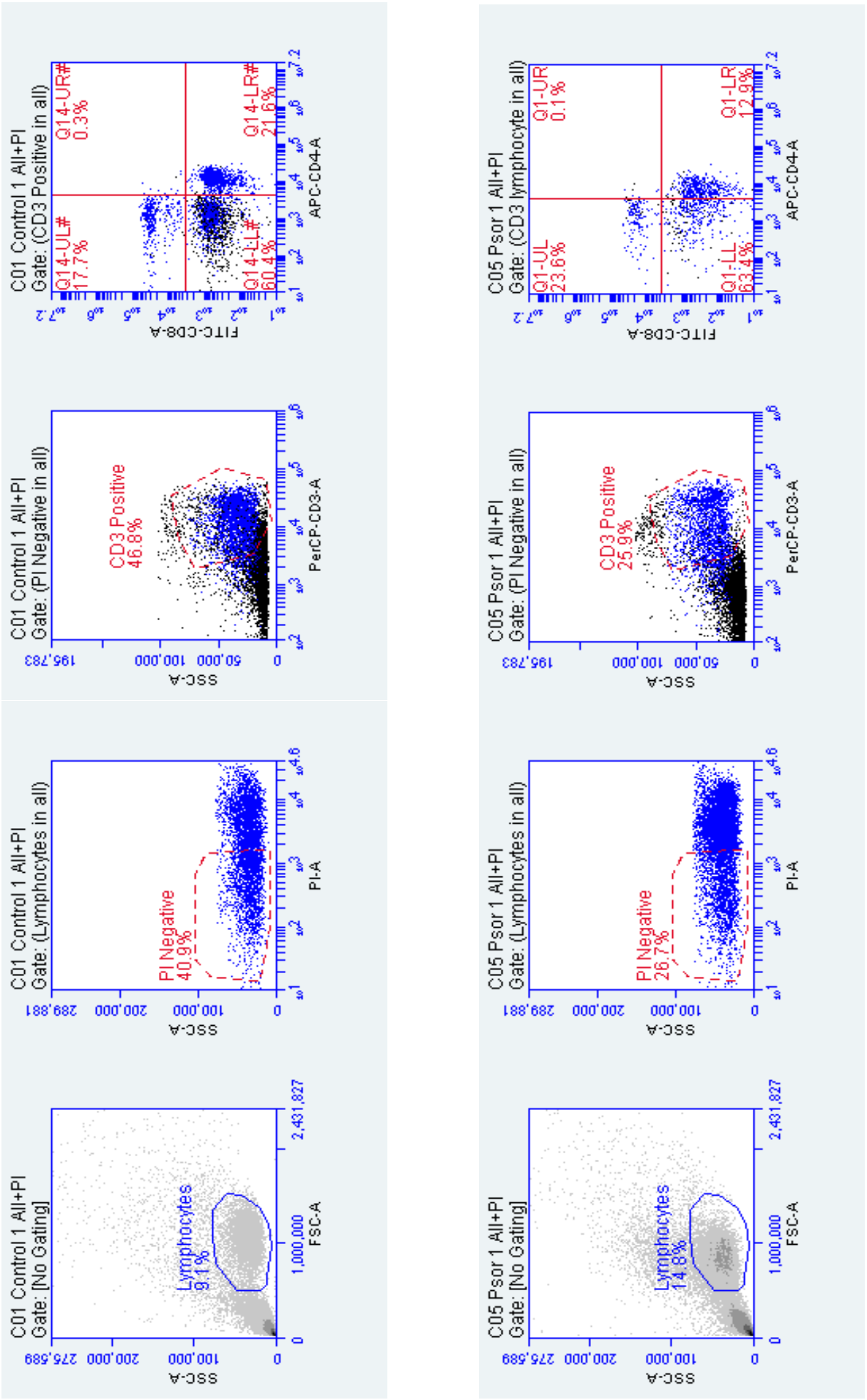


Figure 3.2: Flow chart of PBMC Staining for Flow Cytometry

Table 1: Run sheet for samples staining

Tube	Sample Name	Antibody Fluorochrome	Tube	Sample Name	Antibody Fluorochrome
1	Unstained	-	1	Psor 1	All
2	Control PBMCs	CD3-PerCP	2	Psor 2	All
3	Control PBMCs	CD4-APC	3	Psor 3	All
4	Control PBMCs	CD8-FITC	4	Psor 4	All
5	Control PBMCs	PI	5	Psor 5	All
6	Control PBMCs	All	6	Psor 6	All
7	Control 1	All	7	Psor 7	All
8	Control 4	All			
9	Control 5	All			
10	Control 6	All			



Control

Psoriasis

Figure 3.3: Gating strategy used for T cell phenotypic analysis by Flow Cytometry

3.3 T cell-specific cytokine expression analysis by ELISA

Cytokine analysis in plasma samples was done by Sandwich ELISA as shown in Figure 10. Sandwich ELISA is a technique that quantifies antigens between two layers of antibodies (i.e., capture and detection antibody). The benefit of Sandwich ELISA is that the sample does not have to be purified before analysis, and it is more sensitive (up to 2 to 5 times more sensitive than direct or indirect ELISA).

Material required: 96-well plate, Multi-channel pipette, Plate-reader [capable of measuring absorbance at 450nm], Capture Antibody- 1:250 in coating buffer, ELISA/ELISPOT diluent (1X)- 1:5 in deionized water, Detection Antibody- 1:250 in coating buffer, Avidin-HRP- 1:250 in Diluent, Coating Buffer, Wash buffer- 1X PBS, 0.05% Tween, Stop solution N H2SO4.

Method: Protocol for the assay was followed according to Figure 10. Further the concentration of different cytokines in samples is analyzed with the help of standard curve using 4PL regression equation in GraphPad Prism 6.

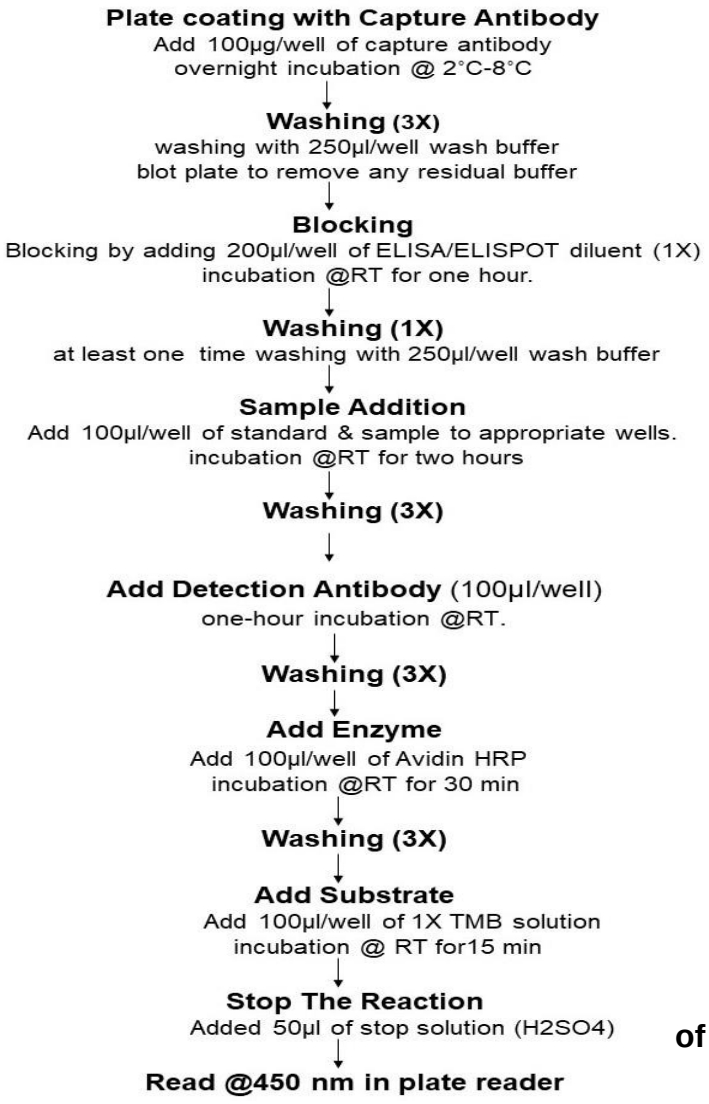


Figure 3.4: Flow chart of ELISA Protocol

3.4 TREC analysis

3.4.1 DNA isolation from whole Blood was done as shown in figure 3.5

Materials required

Lysis buffer, proteinase K, Chloroform, phenol, Isoamyl alcohol, 75% Ethanol, 3M sodium acetate, Nuclease-free water, centrifuge, vortex, pipette, tips, Eppendorf tubes, etc.

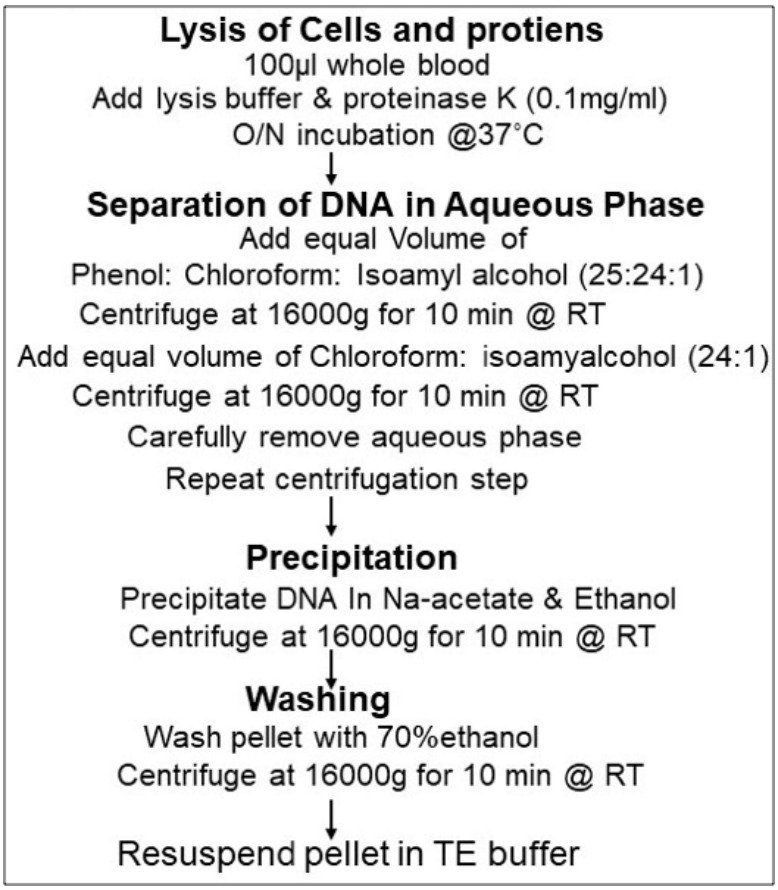


Figure 3.5: Flow chart showing protocol for DNA Isolation from Whole blood

Nanodrop estimation of DNA purity and concentration:

Nucleic acids are quantified based on UV absorption using a spectrophotometer. The absorbance is measured at 260 and 280 nm. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.

The concentration of DNA should be determined by measuring the absorbance at 260 nm (*A*260). An absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml.

Purity check of DNA

DNA has its absorption maxima at 260 nm, and the ratio of the absorbance at 260 and 280 nm is used to estimate purity of DNA and potential contaminants that absorb in the UV spectrum, such as protein. Pure DNA has an A260/A280 of 1.8. DNA was checked for purity and concentration.

3.4.2 PCR based target amplification & gel electrophoresis

DNA isolated from whole blood was used to amplify TREC specific region using specific primers by PCR. Different annealing temperatures were used to standardize the reaction conditions. After DNA amplification, gel electrophoresis was done to check the expected product.

Material required: PCR master mix, Reverse and forward primer, nuclease-free water, 70% ethanol, agarose, tips, pipette, ice bucket, PCR machine, TBE buffer, Gel electrophoresis setup**.**

Method: Total 10µl of reaction volume was made.5µl of master mix, 0.3 µl of each forward and reverse primer, 50ng of sample DNA was used then the volume was makeup to 10µl using nuclease-free water. Temperatures for Denaturation, Annealing, and extension were 95˚C, 62˚C and 72˚C respectively. The reaction was run till 30 cycles.

For gel electrophoresis, 2% agarose was mixed in the 1XTBE buffer. 0.6µl of EtBr dye was added then comb was inserted into a gel. After solidifying, the gel was put into TBE buffer filled the tank. Samples and a ladder were loaded in wells, and after attaching electrodes, the gel was run at 100volt.

3.4.3 Quantitative real-time PCR (qRT PCR):
Materials required

SYBR green master mix, forward primer and reverse primer for TREC and Housekeeping gene (Albumin), nuclease-free water, 70% ethanol, vortex, ice bucket, tips, pipette, PCR tubes, centrifuge, qRT- PCR machine.

Method: 5 µl of SYBR Green PCR Master Mix, 0.3µl of each Forward primer& Reverse primer and 30ng of Template DNA is used, and volume is made up to 10 µl with RNase-free water. Temperatures for initial activation, denaturation was 95ºC for 10 min and 10 sec respectively then for annealing and extension 60˚C, and the 72˚C temperature is used for 60 sec. and 2 min. respectively. The reaction was run till 40 cycles.

Chapter 4

RESULTS AND DISCUSSION

4.1 PBMC count in psoriasis patients vs. healthy controls:

Table 2: PBMC count in psoriasis patients vs healthy controls. S: Severe, M: Mild

Patient ID	**Age /Sex**	**PBMC Count** (Cells/ml)	**Control ID**	**Age /Sex**	**PBMC count** (Cells/ml)
PSOR1 (S)	43 / F	4.2 x 10^6 Cells /ml	C1	21 / M	4.2x10^6 Cells/ml
PSOR 2(S)	70 / F	2.6x 10^6 Cells /ml	C2	19 / M	2.16x10^6 Cells/ml
PSOR 3(S)	25 / M	2.4x 10^6 Cells /ml	C3	21 / M	5.12x10^6 Cells/ml
PSOR4(M)	18 / M	3.97x 10^6 Cells /ml	C4	18 / F	3.14x10^6 Cells/ml
PSOR5(M)	30 / M	4.45x 10^6 Cells /ml	C5	21 / M	1.46x10^6 Cells/ml
PSOR6(S)	23 / M	7.3x 10^6 Cells /ml	C6	20 / F	1.82x10^6 Cells/ml
PSOR7(S)	30 / M	4.25 x 10^6 cells/ml	C7	19 / M	5.68 x10^6 Cells/ml
PSOR8(S)	30 / M	5.2x 10^6 cells/ml	C8	19 / M	4.90 x10^6 Cells/ml
			C9	20 / F	2.36x10^6 Cells/ml
			C10	19/ F	3.30x10^6 Cells/ml

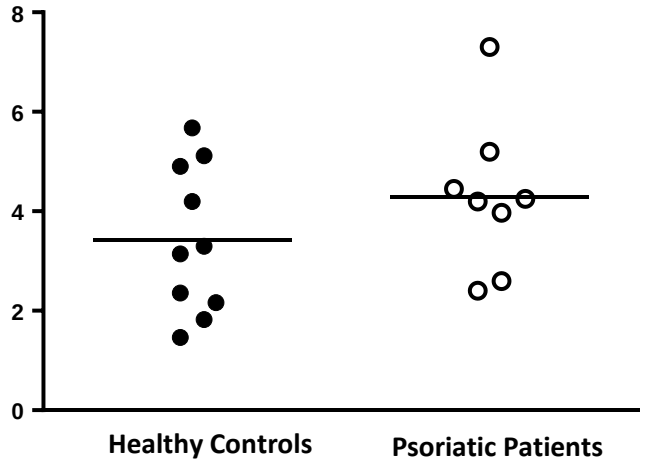


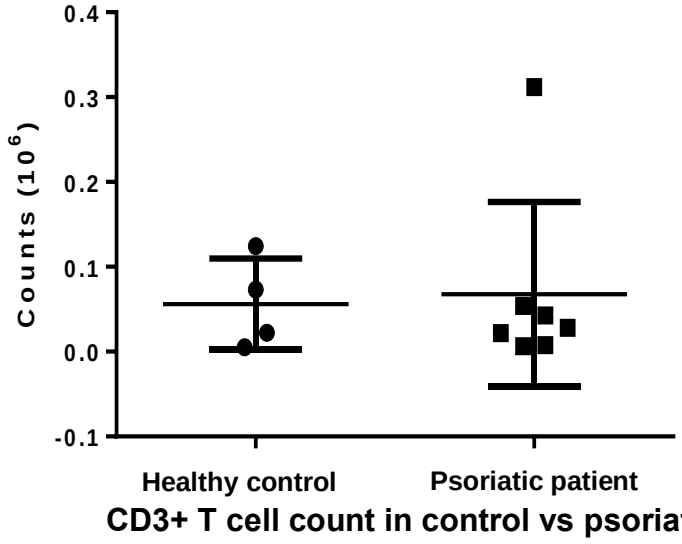
Figure 4.1: Scatter Plot (with mean) of PBMC count in Control Vs Psoriatic patients. Y axis unit (10^6)

There is no significant difference observed in PBMC count of control versus psoriatic patients with p-value =0.2464 as analyzed by t-test (Mann Whitney Test) ($p < 0.05$) using Graphpad Prism Software (Table 2 and Figure 12). There is no gender biasedness observed in the counts and no significant difference in mild vs. severe psoriatic patients in PBMC count. Few earlier studies on quantitation of PBMCs in psoriatic patients versus healthy controls report inconsistent finding. Some reports suggest a high number of PBMC count in peripheral blood of psoriasis patients in correlation with disease severity (W B Jeffes *et al*.1995) while alternative reports show no significant difference in PBMC count (Langewouters *et al*. 2008). Our result, with a small sample size shows no change in PBMC count.

4.2 Peripheral T Cell Phenotyping using CD3+, CD4+and CD8+ markers

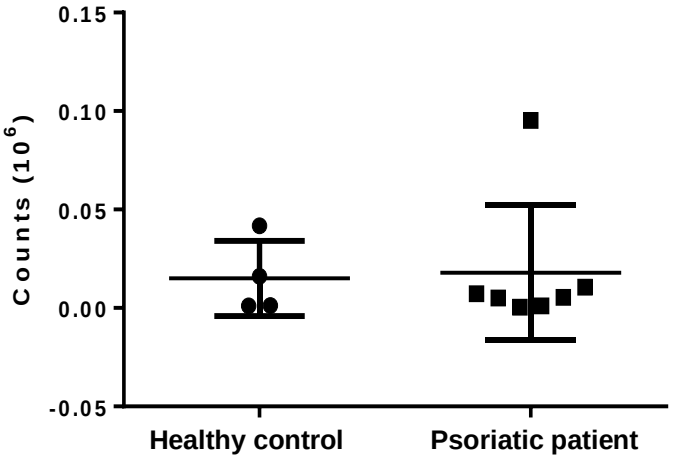
Our study shows no significant change in percentage and absolute numbers total T cells (CD3+) in control versus psoriatic PBMC samples. Also no change is seen in Th cells (CD4+) percentages and absolute numbers. A trend towards increased percentage and absolute count of CD8+ T cells is observed such that the ratio of Th cells (CD4+) and Tc cells (CD8+) is seen to decrease compared to healthy controls which generally have CD4+ and CD8 in a ration near to 2:1. The data is shown in figure 4.2 and 4.3. Thus absolute number and percentage of Tc cells are increased in diseased condition. There are limited and inconsistent reports about total T cells (CD3+), Th cells (CD4+) and Tc cells (CD8+) in psoriatic patients as compared to healthy controls (Cameron *et al*. 2002; Koreck *et al.* 2002; O'Daly *et al*. 2010) Due to small sample size and varied severity level of patients the assay needs to be repeated with more sample.

A.



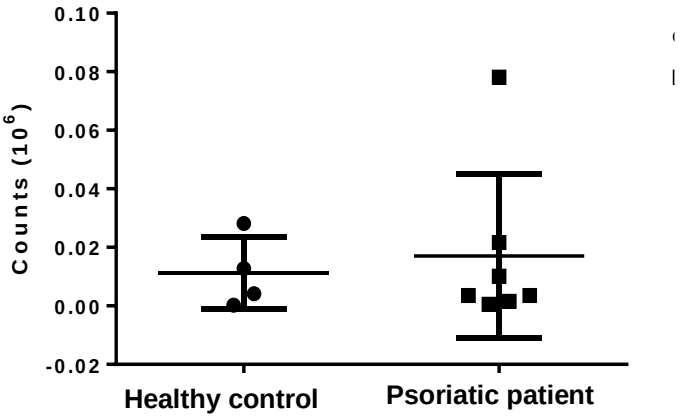
CD3+ T cell count in control vs psoriatic

B.



CD4+ T cell count in control vs Psoriatic patients

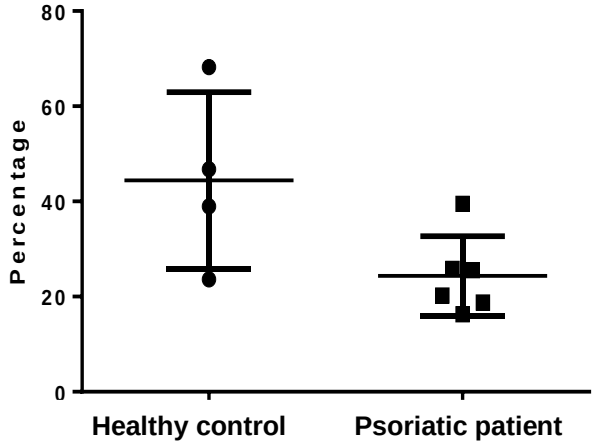
C.



CD8+ T cell count in control vs psoriatic patients

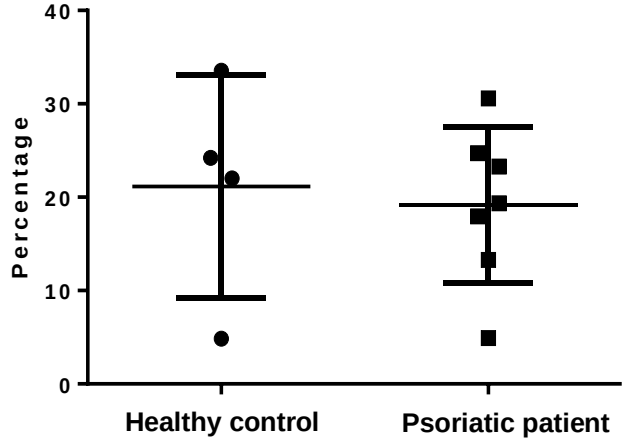
Figure 4.2: Scatter Plot (with mean) of absolute count of A. CD3+ (total T cells), B. CD4 + (Th cells), C. CD8+ Tc cells) cells in Control Vs psoriatic patients

A.



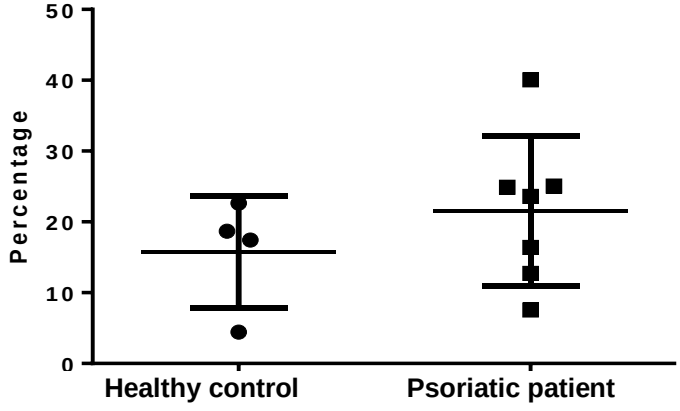
CD3+ T cell % in control vs psoriatic

B.



CD4+ T cell % in control vs psoriatic patients

C.



CD8+ T cell % in control vs psoriatic patients

Figure 4.3: Scatter Plot (with mean) of percentage of A. CD3+ (total T cells) B. CD4 + (Th cells), C. CD8+ (Tc cells) cells in control Vs psoriatic patients

4.3 T cell-specific cytokine analysis by ELISA

A. IFN-γ

Our study shows a significant increase in concentration of IFN-gamma in patients compared to healthy controls (p value= 0.0041) as shown in figure 4.4. Our results corroborate earlier findings (Arican, Aral *et al.* 2005; Chodorowska 1998; Kyung Roh *et al.* 2015). Massive quantities of IFNγ is known to be produced only under pathologic conditions such as trauma, infection, cancer, and autoimmunity. IFN-γ is understood to be more critical as an immunoregulator than as an antiviral agent. IFN-γ regulates cellular activities responsible for nearly all phases of immune and inflammatory responses: the activation, growth, and differentiation of T cells, B cells, macrophages, NK cells and other cell types such as endothelial cells and fibroblasts(Pietrzak *et al.* 2008).

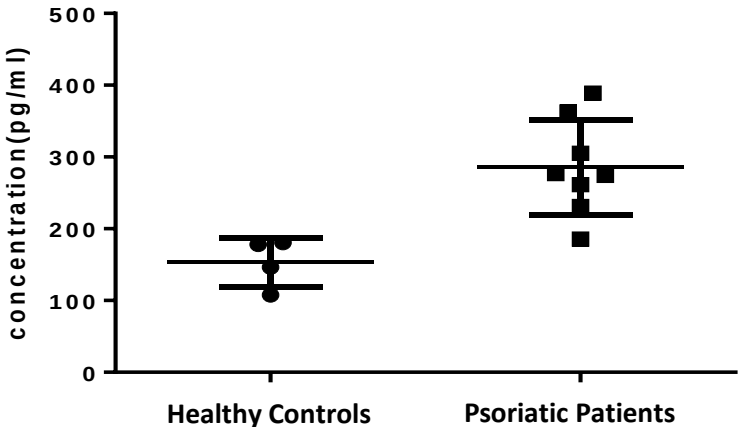


Figure 4.4: Scatter Plot (with mean) of IFN γ concentration of Control versus Psoriatic Patients

B. IL-12

The result of our study shows a low expression of IL-12 in psoriatic patients as compared to control (p value= 0.0135) as shown in figure 4.5. There is no report on plasma IL-12 concentrations in psoriasis. Interestingly data on serum IL-12 levels in psoriasis are conflicting. Some authors have reported lower concentration or no difference in IL-12 expression in serum of psoriatic individuals compared controls (Jacob *et al.* 2003). A higher level of IL-12 in serum of psoriatic patients has also been reported (Kyung Roh *et al.* 2015). So it is an important cytokine to understand the disease etiology. acts on the proliferation, function, cytotoxic activity of T cells and NK cells and stimulates the production of IFN-g and TNF (Pietrzak *et al.* 2008) .

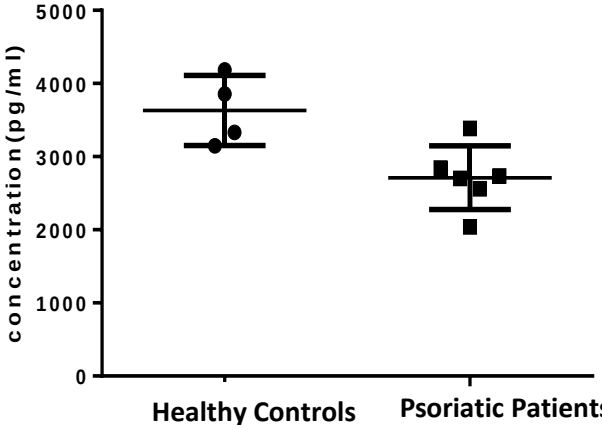


Figure: 4.5: Scatter Plot (with mean) of IL-12 concentration of Control versus Psoriatic Patients

C. IL-4

With a trend towards lower concentration, there is no significant difference observed in IL-4 concentration in control versus psoriatic patients (p-value =0.3614) as shown in figure 4.6. In literature, there are varied reports. Fan Bai *et al.* show no significant difference in IL-4 concentration. Although variable reports with a decrese in serum and an increase in serum and plasma are also reported (Zalewska Janowska *et al.* 2004). IL-4 applies pleiotropic effects on the immune system and may directly suppress Th1-mediated inflammation by skewing the T helper cell phenotype towards Th2 cells.

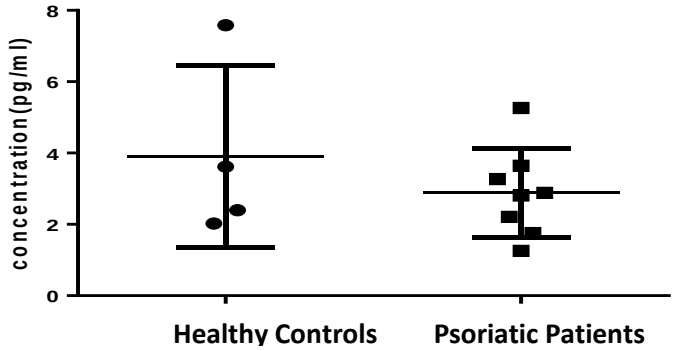


Figure 4.6: Scatter Plot (with mean) of IL-4 concentration of Control Vs Psoriatic Patients

D. IL-17

In this study, the psoriatic patients show a trend towards higher expression of IL-17 as compared to healthy controls, with no statistically significant difference in

values (p-value = 0.2820) as shown in figure 4.7. In literature, IL-17 is reported to be higher in patients with psoriasis than in healthy controls. (Y. Zhou *et al.* 2016). The increased expression of IL-17 at sites of inflammation in psoriasis strongly suggests its role in promoting autoimmune pathology (Baliwag *et.al* 2015).

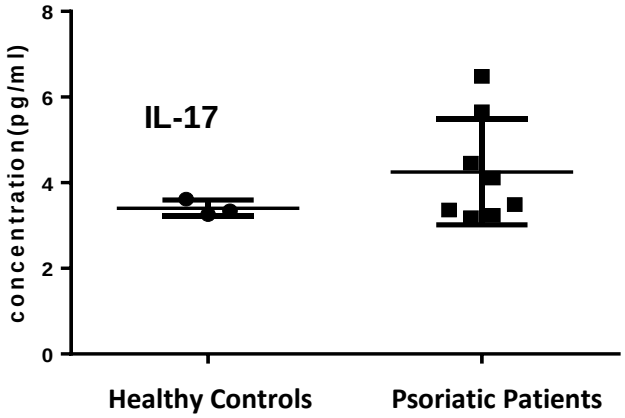


Figure 4.7: Scatter Plot (with mean) of IL-17 concentration of Control Vs Psoriatic Patients

E. I**L-22**

We found a higher concentration of IL-22 in psoriatic plasma sample compared to control (p value= 0.0045) as shown in figure 4.8. Literature also supports similar findings (Michalak-Stoma *et al.* 2013). IL-22 is a crucial cytokine and has a vital role in psoriasis pathogenesis. IL-22 induces epidermal hyperplasia but not keratinocyte proliferation; it inhibits epidermal differentiation either alone or in synergy with IL-1 and IL-17, drives the induction of pro-inflammatory gene expression.

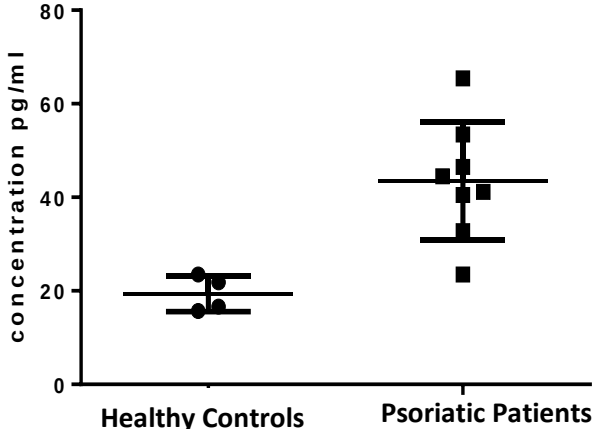


Figure 4.8: Scatter Plot (with mean) of IL-22 concentration of control versus Psoriatic Patients

4.4 TREC analysis

4.4.1 Quantitation of DNA isolated from of Psoriatic patients and Control samples

Quantification of DNA isolated from whole blood is done using nanodrop. Absorbance at 260/280 is approximately 1.8 in most samples which simply good quality of DNA.

Table 3: Nanodrop readings of Psoriatic patients and controls DNA samples

control	**Concn (ng/µl)**	**260/280**	**260/230**	Psoriasis	**Concn (ng/µl)**	**260/280**	**260/230**
C1	27.2	1.87	0.05	Psor 2	22.0	1.85	0.28
C2	10.0	1.88	0.16	Psor 3	13.6	1.97	0.15
C3	32.2	1.80	0.13	Psor 4	16.9	1.99	0.29
C4	8.2	2.22	0.34	Psor 5	33.9	1.84	0.81
C5	76.2	1.76	0.57	Psor 6	20.5	1.98	0.15
C6	26.4	1.97	0.14	Psor 7	15.7	1.64	0.24
C7	39.9	1.84	0.20	Psor 8	10.4	1.68	0.06
C8	11.9	2.31	0.21				
C9	27.0	1.96	0.10				
C10	34.7	1.81	0.30				

4.4.2. PCR based target amplification to check primer specificity

To check primer specificity, PCR based target amplification was done using PCR followed with gel electrophoresis. Different temperature conditions were used to standardize PCR for amplification of target DNA. Expected amplification of TREC DNA was obtained at 62˚C in both control and patient samples as shown in figure 4.9.

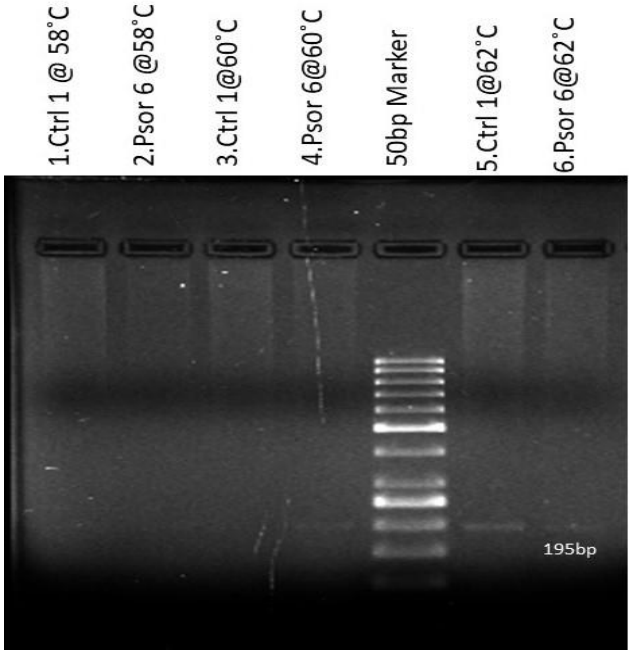
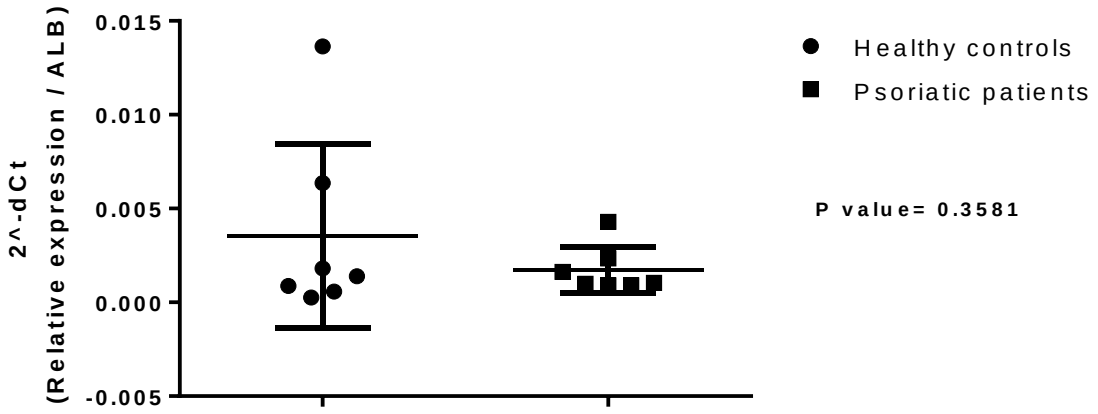


Figure 4.9: Agarose Gel showing, expected target PCR amplification product, ~ 195 bp at different temperature conditions.

4.4.3 qRT PCR expression analysis of TREC in psoriasis patient samples versus healthy control:

A decrease in TREC level normalized against housekeeping gene (ALB) is found in psoriatic individuals as compared to control although we didn't go for statistical analysis due to small sample size. Low expression of TREC can happen either because of lower thymic output or higher proliferation of RTEs in the periphery which may lead to high T cell numbers in the peripheral blood in a disease condition. A single study on TREC analysis in psoriatic patients report a significantly low TREC contents in psoriatic patients compared with healthy controls (Hazenberg et al., 2001). We did TREC analysis using whole blood samples while previous study has been done on purified T cells. We need to run the assay on more samples for the final conclusion.

A.



B.

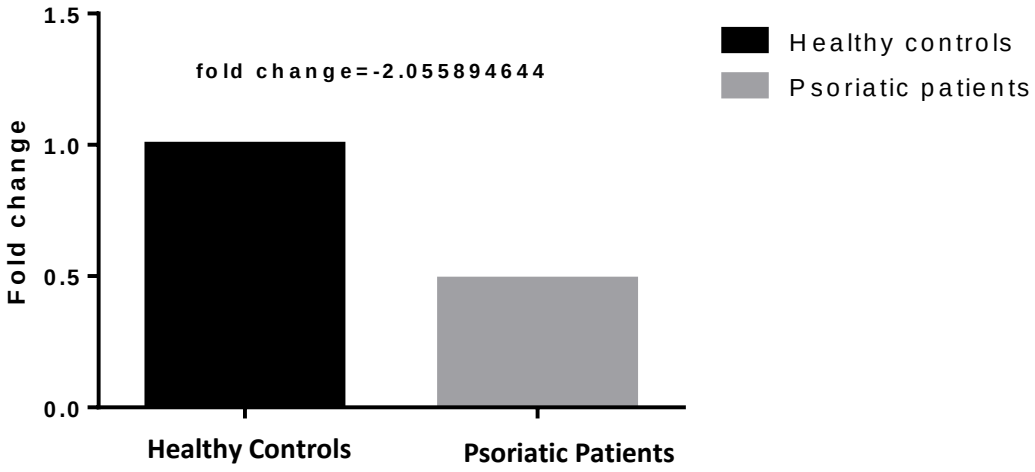


Figure 4.10: TREC analysis in psoriatic individuals compared to control. A. Scatter Plot (with mean) of 2^-dCt of Control Vs Psoriatic B. Fold change in Control versus Psoriatic Patients

Chapter 5

CONCLUSION AND FUTURE PROSPECTS

CONCLUSION

Psoriasis is an incurable disease till date affecting a significant fraction of world's population. Knowledge on T cell associated changes in psoriasis will help to understand the pathogenesis of the disease better. Till now psoriasis is seen as a Th cells mediated disease but our results and a recent study (Marco Diani *et al* 2016) point towards potential role Tc cells in psoriasis. We got a higher percentage and number of CD8+(Tc cells) in psoriatic patients with an altered CD4/CD8 ratio such that the ratio decreased in diseased individuals. This is in line with a higher secretion of Th1 cytokines (IFN-γ) that can lead to a polarized Th1 immune response, possibly leading to increased CD8+ cells in psoriatic patients. Our TREC assay results also suggest more proliferation of naïve T cells in periphery in diseased condition. Our small sample size and unavailability of age and sex matched controls, reflect limitations of our study. We need to repeat the experiments on a large cohort of patients samples along with appropriate control to reach final conclusion. Nonetheless, our preliminary data suggest that CD8+ cells increase in number as a result of a biased Th1 response induced in psoriasis possibly due to increased peripheral proliferation of CD8+ naïve cells leading to a decrease in TREC levels in diseased individual compared to control.

FUTURE PROSPECTIVES

Project has been undertaken in light of the various research gaps in understanding of psoriasis etiology, pathophysiology, associated comorbidities, lack of early diagnostic biomarker and targeted therapies, and mainly on lack of understanding the profile of T cells. Future prospects of the project include an extension of the current project in the following areas: -

- Use of enriched T cells for further work.
- IHC (Immunohistochemistry) of paraffin embedded biopsy sections for cell specific lesional studies
- Comparing T cell profile in different psoriasis types
- Validation of candidate immune-miRNAs and their putative T cell-based targets in a larger cohort of people, to develop possible disease biomarkers
- Accessing the profiles of chosen candidate miRNAs before or after systemic therapies.

REFERENCES

Arican, O., Aral, M., Sasmaz, S., & Ciragil, P. (2005). Serum Levels of TNF-α, IFN-γ, IL-6, IL-8. Mediators of Inflammation **2005**: 273-279.

Ashwin B. Kuchekar, R. R. P., Shantanu B. Kuchekar, Shashikant N. Dhole and Payal M. Mule. (2011). Psoriasis: A comprehensive review*. International Journal of Pharmacy & Life Sciences* **2**: 857-865.

Bai, F., Zheng, W., Dong, Y., Wang, J., Garstka, M. A., Li, R., Ma, H. (2018). Serum levels of adipokines and cytokines in psoriasis patients: a systematic review and meta-analysis. *Oncotarget* **9**: 1266-1278.

Balato, A., Ayala, F., Schiattarella, M., Megna, M., Balato, N., & Lembo, S. (2012). Pathogenesis of psoriasis: the role of pro-inflammatory cytokines produced by Keratinocytes: *INTECH Open Access Publisher.*

Baliwag, J., Barnes, D. H., & Johnston, A. (2015). Cytokines in psoriasis. *Cytokine 73:* 342-350.

Berth-Jones, J., Grotzinger, K., Rainville, C., Pham, B., Huang, J., Daly, S., Hotchkiss, K. (2006). A study examining inter-and intrarater reliability of three scales for measuring severity of psoriasis: Psoriasis Area and Severity Index, Physician's Global Assessment and Lattice System Physician's Global Assessment. *British Journal of Dermatology* **155:** 707-713.

Bigby, M. (2005). Pimecrolimus and tacrolimus for the treatment of intertriginous and facial psoriasis: are they effective? *Archives of dermatology* **141:** 1152-1153.

Boffetta, P., Gridley, G., & Lindelöf, B. (2001). Cancer risk in a population-based cohort of patients hospitalized for psoriasis in Sweden. *Journal of Investigative Dermatology* **117:** 1531-1537.

Bowcock, A. M., & Krueger, J. G. (2005). Getting under the skin: the immunogenetics of psoriasis*. Nat Rev Immunol* **5:** 699-711.

Brook, I. (2002). Secondary bacterial infections complicating skin lesions. *Journal of medical microbiology* **51:** 808-812.

Cai, Y., Fleming, C., & Yan, J. (2012). New insights of T cells in the pathogenesis of psoriasis. *Cellular and Molecular Immunology* **9:** 302-309.

Cameron, A., Kirby, B., Fei, W., & Griffiths, C. (2002). Natural killer and natural Killer-T cells in psoriasis. *Archives of Dermatological Research* **294:** 363-369.

Capon, F., Munro, M., Barker, J., & Trembath, R. (2002). Searching for the major histocompatibility complex psoriasis susceptibility gene. *Journal of investigative dermatology,* **118:** 745-751.

Chan, E. S., & Cronstein, B. N. (2010). Methotrexate—how does it really work? *Nature Reviews Rheumatology* **6:** 175-178.

Chiricozzi, A., Romanelli, P., Volpe, E., Borsellino, G., & Romanelli, M. (2018). Scanning the Immunopathogenesis of Psoriasis. *Int J Mol Sci* **19**.

Chodorowska, G. (1998). Plasma concentrations of IFN-gamma and TNF-alpha in psoriatic patients before and after local treatment with dithranol ointment. *J Eur Acad Dermatol Venereol* **10:**147-151.

Dogra, S., & Mahajan, R. (2016). Psoriasis: Epidemiology, clinical features, co-morbidities, and clinical scoring. *Indian Dermatology Online Journal* **7:** 471-480.

E Nograles, K., Davidovici, B., & G Krueger, J. (2010). New Insights in the Immunologic Basis of Psoriasis. *NIH Public Access* **29:** 3-9.

Elder, J. T., Bruce, A. T., Gudjonsson, J. E., Johnston, A., Stuart, P. E., Tejasvi, T., . . . Nair, R. P. (2010). Molecular dissection of psoriasis: integrating genetics and biology. *Journal of Investigative Dermatology* **130:** 1213-1226.

Feldman, S., & Krueger, G. (2005). Psoriasis assessment tools in clinical trials. *Annals of the rheumatic diseases* **64:** ii65-ii68.

Finlay, A. (2005). Current severe psoriasis and the rule of tens. *British Journal of Dermatology* **152:** 861-867.

Finlay, A., & Khan, G. (1994). Dermatology Life Quality Index (DLQI)—a simple practical measure for routine clinical use. *Clinical and experimental dermatology* **19:** 210-216.

Frentz, G., & Olsen, J. (1999). Malignant tumours and psoriasis: a follow-up study. *British Journal of Dermatology* **140:** 237-242.

Gladman, D., Antoni, C., Mease, P., Clegg, D., & Nash, P. (2005). Psoriatic arthritis: epidemiology, clinical features, course, and outcome. *Annals of the Rheumatic Diseases* **64(suppl 2)**: ii14-ii17.

Gordon, K. B., & McCormick, T. S. (2003). Evolution of biologic therapies for the treatment of psoriasis. *Skinmed* **2:** 286-294.

Gottlieb, A. B., Mease, P. J., Mark Jackson, J., Eisen, D., Amy Xia, H., Asare, C., & Stevens, S. R. (2006). Clinical characteristics of psoriatic arthritis and psoriasis in dermatologists' offices. *Journal of dermatological treatment* **17:** 279-287.

Gudjonsson, J. E., Karason, A., Runarsdottir, E. H., Antonsdottir, A. A., Hauksson, V. B., Jónsson, H. H., Valdimarsson, H. (2006). Distinct clinical differences between HLA-Cw* 0602 positive and negative psoriasis patients–an analysis of 1019 HLA-C-and HLA-B-typed patients. *Journal of investigative dermatology* **126:** 740-745.

Guenther, L. C., & Ortonne, J. P. (2002). Pathophysiology of psoriasis: science behind therapy. *J Cutan Med Surg* **6(3 Suppl):** 2-7.

Gupta, M. A., & Gupta, A. K. (2003). Psychiatric and psychological co-morbidity in patients with dermatologic disorders. *American journal of clinical dermatology* **4:** 833-842.

Hazenberg, M. D., Verschuren, M. C., Hamann, D., Miedema, F., & van Dongen, J. J. (2001). T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med (Berl)* **79:** 631-640.

Henseler, T., & Christophers, E. (1985). Psoriasis of early and late onset: characterization of two types of psoriasis vulgaris. *Journal of the American Academy of Dermatology* **13:** 450-456.

Huerta, C., Rivero, E., & Rodríguez, L. A. G. (2007). Incidence and risk factors for psoriasis in the general population. *Archives of dermatology* **143:**1559-1565.

Jacob, S., Nassiri, M., A Kerdel, F., & Vincek, V. (2003). Simultaneous measurement of multiple Th1 and Th2 serum cytokines in psoriasis and correlation with disease severity. *Tyaylor and francis Health Sciences* **12:** 309-312

Just, H. L., Deleuran, M., Vestergaard, C., Deleuran, B., & Thestrup-Pedersen, K. (2008). T-cell receptor excision circles (TREC) in CD4+ and CD8+ T-cell subpopulations in atopic dermatitis and psoriasis show major differences in the emission of recent thymic emigrants. *Acta Derm Venereol* **88:** 566-572.

Khilji, F., Gonzalez, M., & Finlay, A. (2002). Clinical meaning of change in Dermatology Life Quality Index scores. *British Journal of Dermatology-supplement* **147**.

Koreck, A., Surányi, A., Szöny, B. J., Farkas, Á., Bata-Csörgö, Z., Kemény, L., & Dobozy, A. (2002). CD3(+) CD56(+) NK T cells are significantly decreased in the peripheral blood of patients with psoriasis. *Clinical and Experimental Immunology,* **127:** 176-182.

Krueger, J., & Bowcock, A. (2005). Psoriasis pathophysiology: current concepts of pathogenesis. *Annals of the rheumatic diseases* **64(suppl 2):** ii30-ii36.

Kyung Roh, N., Hee Han, S., Jeong Youn, H., Ri Kim, Y., Lee, Y., Beom Choe, Y., & Joong Ahn, K. (2015). Tissue and Serum Inflammatory Cytokine Levels in Korean Psoriasis Patients: A Comparison between Plaque and Guttate Psoriasis. *Ann Darmatol* **27:** 738-743

Langewouters, A. M. G., van Erp, P. E. J., de Jong, E. M. G. J., & van de Kerkhof, P. C. M. (2008). Lymphocyte subsets in peripheral blood of patients with moderate-to-severe versus mild plaque psoriasis. *Archives of Dermatological Research* **300:**107-113

Langley, R., Krueger, G., & Griffiths, C. (2005). Psoriasis: epidemiology, clinical features, and quality of life. *Annals of the rheumatic diseases* **64(suppl 2):** ii18-ii23.

Lebwohl, M., Freeman, A. K., Chapman, M. S., Feldman, S. R., Hartle, J. E., & Henning, A. (2004). Tacrolimus ointment is effective for facial and intertriginous psoriasis. *Journal of the American Academy of Dermatology* **51:**723-730.

Leung, D., Harbeck, R., Bina, P., Reiser, R., Yang, E., Norris, D., Sampson, H. (1993). Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis. Evidence for a new group of allergens. *Journal of Clinical Investigation* **92:**1374.

Lewis, V., & Finlay, A. Y. (2004). 10 years' experience of the Dermatology Life Quality Index (DLQI). *Journal of Investigative Dermatology Symposium Proceedings.*

Lowes, M. A., Bowcock, A. M., & Krueger, J. G. (2007). Pathogenesis and therapy of psoriasis. *Nature* **445:** 866-873.

Lowes, M. A. S. F., M. Krueger. (2014). Immunology of psoriasis. *Annu Rev Immunol* **32:** 227-255.

Macro Dilani, Gianfranco Altomare and Eva Reali (2016) T helper cell subsets in clinical menifestations of Psoriasis. *Journal of immunology research* **2016.**

Mallbris, L., Akre, O., Granath, F., Yin, L., Lindelöf, B., Ekbom, A., & Ståhle-Bäckdahl, M. (2004). Increased risk for cardiovascular mortality in psoriasis inpatients but not in outpatients. *European journal of epidemiology* **19:** 225-230.

Mason, A. R., Mason, J., Cork, M., Dooley, G., & Edwards, G. (2009). Topical treatments for chronic plaque psoriasis. *Cochrane Database Syst Rev*.

Michalak-Stoma, A., Bartosińska, J., Kowal, M., Juszkiewicz-Borowiec, M., Gerkowicz, A., & Chodorowska, G. (2013). Serum Levels of Selected Th17 and Th22 Cytokines in Psoriatic Patients. *Disease markers* **35:** 625-631.

Naldi, L., Chatenoud, L., Linder, D., Fortina, A. B., Peserico, A., Virgili, A. R., Solaroli, C. (2005). Cigarette smoking, body mass index, and stressful life events as risk factors for psoriasis: results from an Italian case–control study. *Journal of Investigative Dermatology* **125:** 61-67.

Nestle, F. O., Kaplan, D. H., & Barker, J. (2009). Psoriasis. *N Engl J Med* **361:** 496-509.

Nevitt, G. J., & Hutchinson, P. E. (1996). Psoriasis in the community: prevalence, severity and patients' beliefs and attitudes towards the disease. *Br J Dermatol* **135:** 533-537.

Nicolai, J., Aldenkamp, A. P., Arends, J., Weber, J. W., & Vles, J. S. (2006). Cognitive and behavioral effects of nocturnal epileptiform discharges in children with benign childhood epilepsy with centrotemporal spikes. *Epilepsy Behav* **8:** 56-70.

O'Daly, J. A., Rodriguez, B., Ovalles, T., & Pelaez, C. (2010). Lymphocyte subsets in peripheral blood of patients with psoriasis before and after treatment with leishmania antigens. *Archives of Dermatological Research* **302:** 95-104.

Peters, B. P., Weissman, F. G., & Gill, M. A. (2000). Pathophysiology and treatment of psoriasis. *American Journal of Health System Pharmacy* **57:** 645-662. Pietrzak, A. T., Zalewska, A., Chodorowska, G., Krasowska, D., Michalak-Stoma, A., Nockowski, P., Rolinski, J. M. (2008). Cytokines and anticytokines in psoriasis. *Clin Chim Acta* **394:** 7-21.

Rossiter, N., Chapman, P., & Haywood, I. A. (1996). How big is a hand? *Burns* **22:** 230-231.

Shikiar, R., Willian, M. K., Okun, M. M., Thompson, C. S., & Revicki, D. A. (2006). The validity and responsiveness of three quality of life measures in the assessment of psoriasis patients: results of a phase II study. *Health and quality of life outcomes* **4:** 71.

Smith, C. H., Anstey, A., Barker, J., Burden, A., Chalmers, R., Chandler, D., McHugh, N. J. (2009). British Association of Dermatologists' guidelines for biologic interventions for psoriasis 2009. *British Journal of Dermatology* **161:** 987-1019.

Tomi, N. S., Kränke, B., & Aberer, E. (2005). Staphylococcal toxins in patients with psoriasis, atopic dermatitis, and erythroderma, and in healthy control subjects. *Journal of the American Academy of Dermatology* **53:** 67-72.

Van De Kerkhof, P. (2006). Update on retinoid therapy of psoriasis in: an update on the use of retinoids in dermatology. *Dermatologic therapy* **19:** 252-263.

W B Jeffes, E., C. Lee, G., Said, S., Sabahi, M., L. Mccullough, J., Herrod, R., Edwards, S. (1995). Elevated Numbers of Proliferating Mononuclear Cells in the Peripheral Blood of Psoriatic Patients Correlate with Disease Severity. *The society for investigative dermatology* **105**: 733-738.

Y. Zhou, X., Bao, J., Huang, B., & Jin, Y. (2016). Association between plasma interleukin-17 levels and risk of psoriasis: a meta-analysis. *Clinical and Experimental Dermatology* **42:** 161-166.

Zalewska Janowska, A. W., Janina Dziankowska-Bartkowiak, Bozena Sysa-Jedrzejowska, Anna. (2004). Interleukin 4 plasma levels in psoriasis vulgaris patients. *Med Sci Monit* **10:** CR156-162.

APPENDICE-A

- **1 X PBS**

Components of 1X PBS (100 ml)

NaCl	0.8 g
KCl	0.02 g
Na_2HPO_4	0.144 g
KH_2PO_4	0.024 g
Distilled Water	80 ml

*Adjust pH to 7.4 , raise volume to 100 ml and autoclave.

- **10% NBF**

Components of 10% NBF (500 ml)

37% Formaldehyde	50 ml
Na_2HPO_4	3.25 g
NaH_2PO_4	2 g
Distilled Water	450 ml

- **5 N Acetic acid**

1 ml of Glacial acetic acid (>99%) with 2.48 ml of water.

- **DEPC-treated water (0.1 %)**

1 ml in 999 ml distilled water , keep overnight and autoclave next day.

- **1% Acid alcohol**

1% HCl in 70% ethanol (v/v)

- **1X TAE(1000 ml)**

100 ml 10 X TAE in 900 ml distilled water

*(10 X TAE : 48.4 G Tris base,11.4 ml Glacial acetic acid,0.5 M EDTA in 750 ml deionised water and make up final volume to 1L)

APPENDIX - B

List of instruments:

Hemacytometer (Neubauer)

Multiskan™ GO Microplate Spectrophotometer (Thermo)

Thermal cycler (Applied Biosystem)

CFX 96 Real Time Cycler (Biorad)

Bio-safety laminar hood

Centrifuge 5804 R (Eppendrof)

Spinwin (Tarsons)

Micropipette (Eppendorf and Axygen)

RNAse free microtips and vials (Tarsons)

Autoclave (NSW, India)

Vortex (Spinx)

Glassware (Borosil)

Gel Doc (Biorad)

Tissue Tek system

Microtome

Tissue floating water bath

Freezer

Incubator

Hot plate