UNDERSTANDING THE ROLE OF SPECIFIC MICRORNAS IN PSORIASIS PATIENTS, WITH AN IMMUNOLOGICAL PERSPECTIVE

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WAKNAGHAT

Dedicated to my

beloved parents for their

endless support.....

&

.....for all those who guided me in

the right path

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CERTIFICATE

This is to certify that project report entitled **"Understanding the role of specific microRNAs in Psoriasis Patients, with an immunological perspective",** submitted by **"Shivani Tomar"** in fulfillment for the award of degree of **Master of Technology** in Biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor:

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Date

SUMMARY

Psoriasis is a chronic incurable immune – mediated inflammatory disease. Disease etiology involves a complex interaction between keratinocytes and immune cells. Immunologically, new insights in the pathogenesis of psoriasis, points towards a major role of T-cells in the initiation and maintenance of the disease state. Despite our understanding of disease etiology, exact molecular details that regulates the complex interactions among these cells are not fully known. In this context , small non-codoing microRNAs (miRNAs) have emerged as major players that regulate many important processes such as development, survival, proliferation, differentiation, of immune cells and most important as regulators of the plasticity of CD4 T cells during inflammation and tolerance. In the present study, 8 untreated, moderate to severe psoriatic patient's blood and biopsy samples were collected. miRNA expression profiling of psoriasis patient versus control individuals, blood samples (plasma) was done and histopathological alterations in the psoriasis patient biopsy were studied in correlation with disease severity of 8 different psoriatic patients. A comparative, PBMC counts profile of psoriatic versus healthy controls was also done .

Signature of Student:-

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LIST OF ABBREVIATIONS

PBS	Phosphate bufferd saline
NBF	Neutral Buffered Formalin
FBS	Fetal bovine serum
DMSO	Dimethyl Sulfoxide
PBMC	Peripheral Blood Mononuclear cells
DEPC	Diethylpyrocarbonate
RNA	Ribose nucleic acid.
qRT PCR	Quantitative Real Time Polymerase Chain Reaction.
TAE	Tris Acetic acid EDTA.
BSA	Body surface area

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Chapter 1

INTRODUCTION

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INTRODUCTION

Psoriasis is a chronic immune – mediated inflammatory skin disease with itchy and painful skin manifestations. Despite the efforts of IFPA (International federation of psoriasis association) and national psoriasis organizations, psoriasis continues to be a serious disease worldwide affecting over 125 million people, or nearly three percent of the world's population (National Psoriasis Foundation).

Psoriasis continues to be a problem – first, because it is often misdiagnosed with other similar diseases with no standard differential diagnosis , – second, because there is a severe lack of recognition and awareness, a lack of understanding the nature of the disease both by the patient and the public (www.ifpa-pso.org) and – third, most importantly because there is no known cure as available treatment such as corticosteroids and immunosuppressants help in transient symptomatic relief with potential side effects and large number of non- responders. and not a single treatment works for everyone.

"Psoriasis has no known cure...no single treatment works for everyone…need global attention."

A vast body of literature 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 effect, the homeostatic crosstalk between different immune cells and resident skin cells normally seen in healthy skin is disturbed. Despite our understanding with respect to heterogeneous cell population viz. keratinocytes T-cell subsets, macrophages, dendritic cells, neutrophils involved in disease etiology, exact molecular details that regulates the complex interactions among these cells to create a chronic inflammatory environment are not full known.

Immunologically, new insights in the pathogenesis of psoriasis points towards a major role of cellular innate and adaptive immune responsive especially **T-cells** in the initiation and maintenance of the disease state (Cai, Fleming et al. 2012). Heterogeneous T-cell subset which Th1, Th17, Th22, Treg cells are associated with the disease, facilitated by considerable plasticity of one subset to transdifferentiate

into another subset under different environmental cues (Cai, Fleming et al. 2012; Fallen, Mitra et al. 2013). Thus unrevealing molecular switches that induce imbalance in immune cell subset and their functionality in Psoriatic patient compared to healthy controls can help in mechanism based understanding to the disease progression.

In this context **miRNAs** have emerged as major players. They are small ~ 22 bp non-coding RNAs that can regulate basic physiological processes such as development, survival, proliferation, differentiation, function of immune cells (Sethi, Kulkarni et al. 2013). In few independent studies comparison of miRNAs profile of psoriasis patient with healthy control (blood/biopsy) have revealed disregulation of specific miRNAs. But how changes in expression of different miRNAs actually contribute to disease process involving keratinocytes and infilterating immune cell is yet not clear. Thus, understanding the role of specific immunologically relevant miRNAs in psoriasis can further help in elucidating the complex pathogenesis of psoriasis.

1.1 Project Rationale

miRNAs as Early Prognostic and Diagnostic Biomarkers of disease progression in Psoriasis as changes in miRNA expression in body fluids occur earlier than conventional biomarkers like C-reactive protein, chemokines and cytokines.

miRNAs can also hold substantial potential for Targeted Therapeutic Intervention in psoriasis. This rationale can be supported by various ongoing clinical trials in evaluating the therapeutic potential of miRNA in various diseases and also in psoriasis e.g. the first microRNA-based therapeutic to reach clinical trials was for hepatitis C virus infection; also an anti-miR-21 therapy for psoriasis is under trial, showing promising results on the skin of mice bearing grafts of diseased tissue from human patients with psoriasis.

1.2 Objectives

1. To study the expression profile of immunologically relevant candidate miRNAs *miR-21 and miR-215* in psoriatic patient samples (plasma) compared to healthy control.

2. Histopathological correlation of lesional biopsy associated features with disease severity and miRNA profile.

Chapter 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

2.1 Epidemiology

Psoriasis is a chronic immune – mediated inflammatory skin disease with itchy and painful skin manifestations. It is an incurable inflammatory disease till date affecting over 125 million people, or nearly 3% of the world population, is common in Caucasians and affects equally men and women (National Psoriasis Foundation). Psoriasis *vulgaris* is the most common type of psoriasis, involving dry, red raised plaques with adherent silvery scales (Cai, Fleming et al. 2012). While relatively common in Japanese, it is less common in Chinese, Eskimos, West Africans and North American blacks, and very uncommon in North American and South American natives and aboriginal Australians (Langley, Krueger et al. 2005). In the USA, the prevalence 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 (Christophers 2001). In India the prevalence of psoriasis varies from 0.44 to 2.8%. The prevalence of psoriasis has been reported to be affected by latitude also (Vazquez, Carrera et al. 2006). The mean age of onset of psoriasis *vulgaris* is at 33 years, and 75% of the patients develop psoriasis before 46 years of age (Nevitt and Hutchinson 1996). Psoriasis also exhibits a bimodal distribution with a peak between 15 and 20 years of age and another peak between 55 and 60 years (Langley, Krueger et al. 2005; Myers, Gottlieb et al. 2006). On the basis of the bimodal distribution of the age at onset and inheritance, two types of psoriasis have been proposed: Type I psoriasis (approximately 65% of the psoriasis population) is associated with onset below the age of 40, a positive family history of psoriasis, a preceding streptococcal sore throat, and guttate lesions, Type II psoriasis (35% of psoriasis patients) appears to be associated with a population with onset after the age of 40 years and with no family history of psoriasis. Type II is not linked to a preceding infectious trigger (Gudjonsson, Karason et al. 2002). The onset 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 (Farber, Nall et al. 1974).

2.2 Environmental triggers

There are various triggers that are associated with psoriasis and often vary from person to person. Established triggers 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).

Several types of infections are known to be psoriasis triggers. *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 (Tomi, Kränke et al. 2005). The enterotoxins ,also called as Superantigens , acts as a polyclonal activation of CD4+ and CD8+ T cells (Petersson, Pettersson et al. 2003). Normal, healthy skin is colonised by *S. aureus* in 5 - 30% compared with approximately 60% of patients with psoriasis. (Leung, Harbeck et al. 1993; Brook 2002; Tomi, Kränke et al. 2005). Inverse psoriasis is sometimes associated with *candida albicans* (thrush) and other fungal infections of the skin (National Psoriasis Foundation 2011). Human immunodeficiency virus (HIV) infection as well as a rare form of the human papillomavirus (HPV), known as EVHPV can also be a psoriasis trigger (Farber and Nall 1974; Balato, Unutmaz et al. 2009).

Mechanical, ultraviolet, and chemical injuries of the skin can serve as triggers of psoriasis outbreaks (Peters, Weissman et al. 2000). The link between psoriasis outbreaks and skin injuries is known as the Koebner phenomenon. Approximately 50% of those diagnosed with psoriasis experience the Koebner phenomenon (American Academy of Dermatology 2011).

Psychological stress is a well-established trigger of psoriasis. Stress not only contributes to the onset of psoriasis, but it may increase the length of time of exacerbations. Because of the significant impact that psoriasis can have on quality of life, the disease process itself may also cause psychological stress that, in turn, perpetuates the cycle of psoriasis outbreaks (Langley, Krueger et al. 2005).

The use of certain medications like Lithium , Anti-malarial medications, Inderal (a beta blocker), quinidine (an antiarrhythmic agent) ,angiotensin-converting enzyme inhibitors, Indomethacin (a nonsteroidal anti-inflammatory (NSAID)) and overuse and sudden discontinuation of corticosteroids can lead to the onset of psoriasis outbreaks (University of Maryland Medical Center 2011).

2.3 Genetic Basis of Psoriasis

The molecular genetic basis of psoriasis involves multiple genes : nine chromosomal susceptibility *loci* have been revealed (PSORS1-9). The PSORS1 gene, in the major histocompatibility complex region on chromosome 6 (6p21), has been reported to be associated with most cases of psoriasis. The penetrance of PSORS1 *locus* is reported to be less than 15%, implying role of other genetic and/or environmental factors too. Moreover, an association of PSORS with functional polymorphisms in modifier genes that mediate inflammation (e.g., tumour necrosis factor (TNF-α) and vascular growth (e.g., vascular endothelial growth factor), has been found (Capon, Munro 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 (Watson, Cann et al. 1972). 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, Bruce et al. 2010) .

Moreover, a family history of psoriasis, an early-onset of the disease and the presence of HLA-Cw*0602 (the major determinant of phenotypic expression), have been associated to a more unstable and severe clinical course, as compared to those patients with late onset psoriasis and negative for HLA-Cw*0602 (Henseler and Christophers 1985; Gudjonsson, Karason et al. 2006).

Psoriatic patients present a substantial genetic heterogeneity leading to subtle differences in disease pathogenesis, explaining the different responses to treatment observed in the patients.

2.4 Disease etiology

Disease etiology involves a complex interaction among genetic, immunological, and environmental components and involves complex interplay between keratinocytes and immune cells. Psoriasis is characterized by hyperproliferation and abnormal differentiation of epidermal keratinocytes, lymphocyte infiltration consisting mostly of T lymphocytes and various endothelial vascular changes in the dermal layer (Guenther and Ortonne 2002). It does not involve a single cell type or a single inflammatory cytokine, rather 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 (Lowes, Bowcock et al. 2007).

In normal skin the ratio of proliferating to non-proliferating keratinocytes, is around 60% (Gelfant 1982) 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 with respect to normal 26 to 30 days (Fig. 2). The hyperproliferation does not allow for cell maturation or 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 (McCance and Huether 2014).

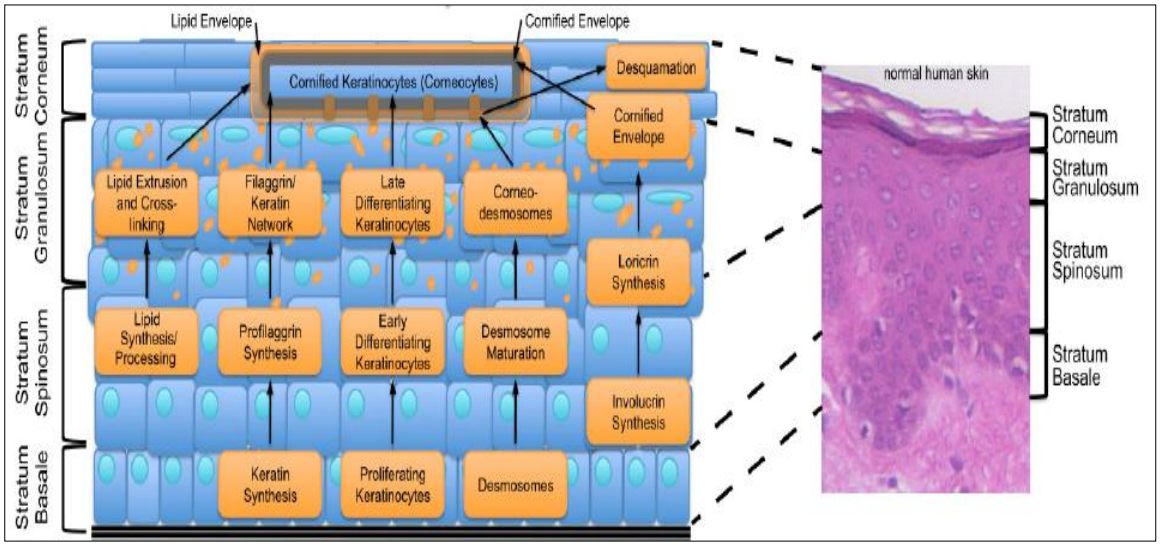


Fig 1: Schematic representation of the underlying differentiation process in keratinocytes
(Hänel, Cornelissen et al. 2013)

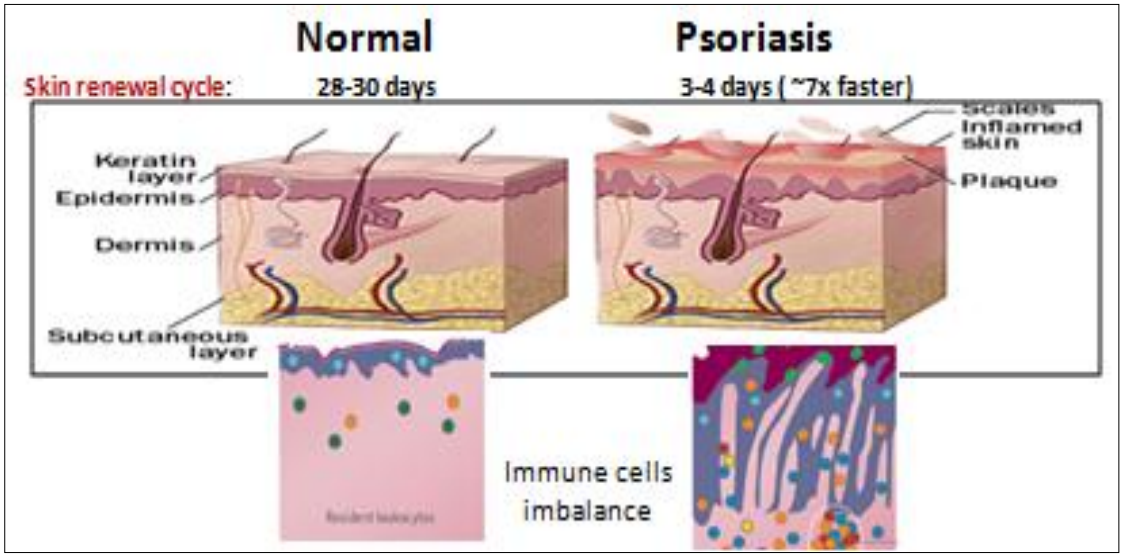


Fig. 2: Altered Skin renewal cycle and associated altered cellular events in Psoriasis (www. Mayoclinic.com)

2.5 Types of Psoriasis and associated clinical features

Psoriasis presents variable morphology, distribution, severity, and course. The various types of psoriasis include plaque (psoriasis vulgaris), guttate, inverse (flexural), pustular, erythrodermic, psoriasis arthritis, nail and scalp psoriasis. The different types of psoriasis may be localized or widespread and may range in severity from mild to severe. Typically, only one type of psoriasis will appear at a time.. However, it is possible for different types of psoriasis to be present at the same time. The course of psoriasis is characterized by wide inconsistencies in the length of time of exacerbations and remissions (National Psoriasis Foundation 2011); (Langley, Krueger et al. 2005; Nicolai, Aldenkamp et al. 2006).

Plaque psoriasis (psoriasis vulgaris) is the most prevalent form of the disease, affecting approximately 80% of individuals with psoriasis (National Psoriasis Foundation 2011). It commonly occurs on the elbows, knees, scalp, and lower back, as well as at sites of trauma. The lesions of plaque psoriasis are characterized by well-demarcated round or oval plaques. Initially, the lesions are erythematous macules or papules that enlarge, extend peripherally, and form larger inflammatory plaques ranging from one to several centimeters in diameter. The plaques appear scaly, thick, silvery, and erythematous, and are surrounded by normal skin. The scales are usually loosely cohesive and removal may cause small bleeding points, known as the Auspitz

sign. The amount of scaling in plaque psoriasis can vary widely among individuals and at various sites on the same individual.

Guttate psoriasis is the second most common form of the disease. It impacts about 10% of those with psoriasis. In children and young adults, guttate psoriasis often occurs as the initial outbreak of psoriasis. The lesions have a rapid onset and are small, tear-shaped papules that typically present on the trunk and extremities. The lesions are less thick than those of plaque psoriasis. The number of lesions can range from less than 10 to over 100. An initial outbreak of guttate psoriasis is often associated with a recent streptococcal respiratory or throat infection (National Psoriasis Foundation 2011)(Gordon and McCormick 2003; Nicolai, Aldenkamp et al. 2006).

Inverse (flexural) psoriasis involves lesions that develop in the axilla, groin, or folds of the skin. This type of psoriasis is more prevalent in obese and overweight individuals and those with deep skin folds. 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 (National Psoriasis Foundation 2011)(Langley, Krueger et al. 2005).

Pustular psoriasis is characterized by white blisters of noninfectious pus surrounded by reddened skin. There are three distinct forms of pustular psoriasis, including von Zumbusch, palmoplantar pustulosis, and acropustulosis (acrodermatitits continua of Hallopeau). Von Zumbusch psoriasis has a rapid onset that begins with reddened, painful patches of skin over large areas of the body, followed quickly by the development of the pustules. Within 24 to 48hours, the pustules will dry and peel and a new eruption of pustules will form. The cycle may continue for days or weeks. Von Zumbusch psoriasis is usually accompanied by fever, chills, dehydration, and muscle weakness and often requires hospitalization for treatment. Palmoplantar pustulosis is characterized by pustules that develop on the palms of the hands and soles of the feet. The pustules appear in a speckled pattern throughout reddened plaques, then turn brown, and crust over. Acropustulosis is a rare form of psoriasis in which the lesions are located on the ends of the fingers or toes. The lesions are extremely painful and cause deformity of the nails, and in some cases, bone changes (National Psoriasis Foundation 2011).

Erythrodermic psoriasis is the least common form of psoriasis and it affects 1 – 2% of individuals that have psoriasis. It is characterized by widespread red, scaling lesions located over the majority of the body surface. Severe itching and pain often accompany this form of psoriasis, with the scaling occurring in large sections of skin rather than the smaller scales associated with plaque psoriasis. Erythrodermic psoriasis can manifest as a result of the progression of chronic plaque psoriasis, the onset of von Zumbusch psoriasis, or it may evolve due to unstable psoriasis that is caused by infection, medications, or the abrupt withdrawal of corticosteroids. This form of psoriasis impairs the thermoregulatory function of the skin and it can lead to hypothermia, high output cardiac failure, and metabolic changes. Erythrodermic psoriasis can be life-threatening and requires immediate treatment (National Psoriasis Foundation 2011)(Langley, Krueger et al. 2005; Nicolai, Aldenkamp et al. 2006).

Psoriatic arthritis is the another major form . Approximately 10–30% of patients with psoriasis develop Psoriatic arthritis. In addition to inflamed, scaly skin, psoriatic arthritis causes pitted, discolored nails and the swollen, painful joints that are typical of arthritis. It can cause stiffness and progressive joint damage that in the most serious cases may lead to permanent deformity.

Nail psoriasis involves affected fingernails and toenails, causing pitting, abnormal nail growth and discoloration. Psoriatic nails may become loose and separate from the nail bed (onycholysis).

Scalp psoriasis appears as red, itchy areas with silvery-white scales. Flakes of dead skin can be seen in hair or on shoulders, especially after scratching scalp.

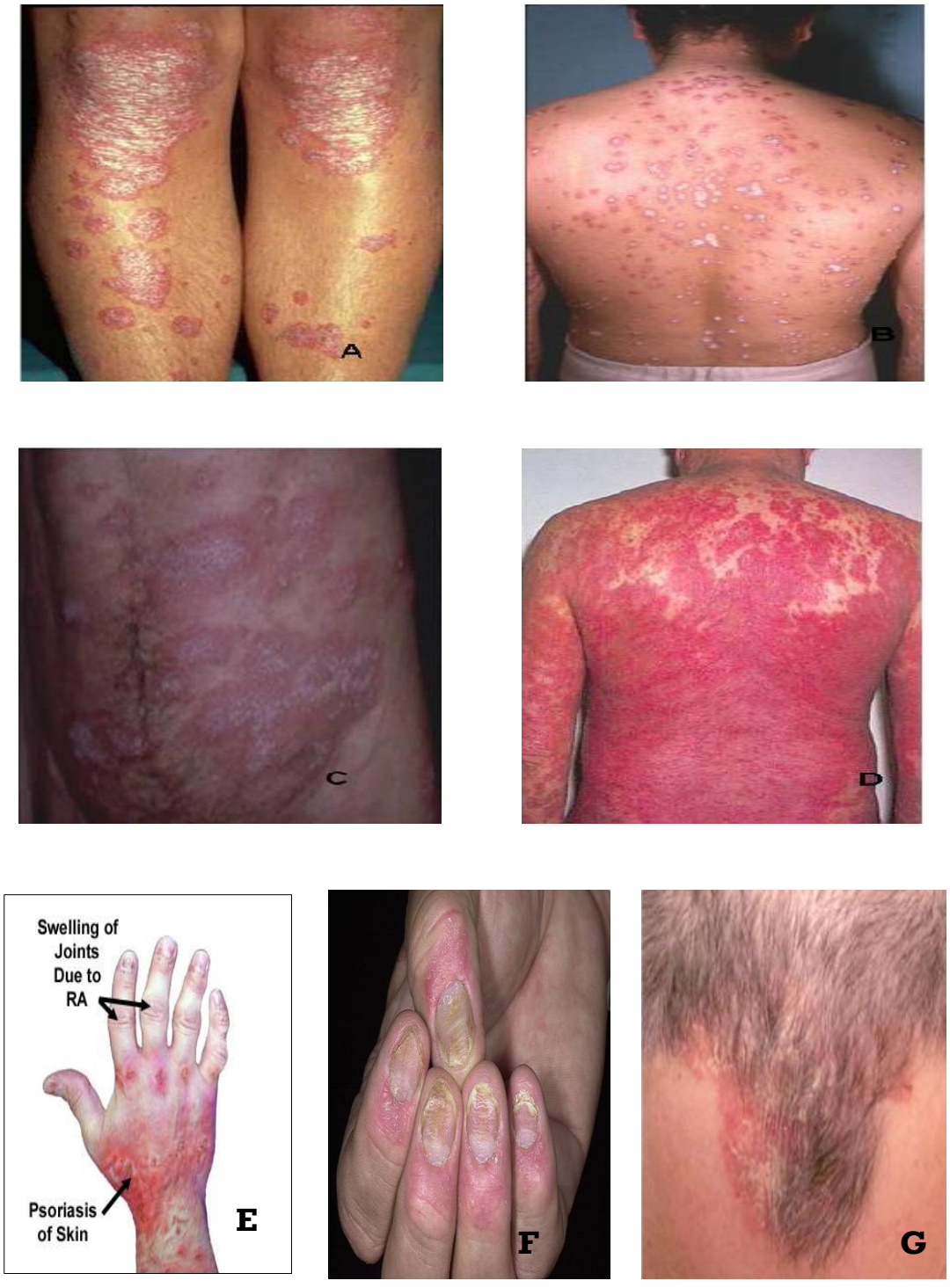


Fig. 3: Psoriasis clinical manifestations: A - chronic plaque psoriasis, B - guttate psoriasis, C - pustular psoriasis, D - erythrodermic psoriasis , E- Psoriasis Arthritis , F – Nail Psoriasis, G – Scalp Psoriasis (www.intechopen.com)

2.6 Histological features

Normal skin is constituted of 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.

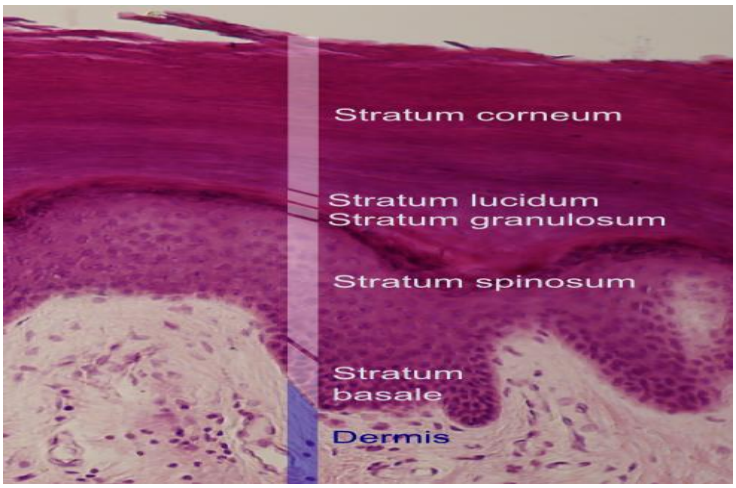


Fig . 4: Normal skin layers : H&E image

Psoriasis is a dynamic dermatosis with morphological changes during the course of an individual lesion (Rosa and Mignogna) , usually identified by erythematous, raised, scaly skin lesions. These clinical features are explained by impressive growth and dilation of superficial blood vessels (elongated/hyperplastic capillaries in the papillary dermal region) and equally impressive hyperplasia of the epidermis. Epidermal growth occurs in a pattern termed ‘‘psoriasiform’’ hyperplasia, which describes both elongated rete pegs, thickening (acanthosis), and differentiation changes (Krueger and Bowcock 2005). In psoriatic epidermis, keratinocytes proliferate and mature rapidly so that terminal differentiation, normally occurring in granular keratinocytes and then squamous corneocytes, is incomplete. Hence, squamous keratinocytes aberrantly retain intact nuclei (parakeratosis) and release few extracellular lipids that normally cement adhesions of corneocytes. The failure of psoriatic corneocytes to stack normally and to secrete extracellular lipids cause scaling and a break in the protective barrier whereas marked dilation of blood vessels in the dermis causes the visible redness of psoriatic skin lesions. The extensive infiltration of mononuclear immune cells in the dermis and epidermis (T cells and dendritic cells in the dermis and polymorphonuclear leucocytes such as neutrophils within small foci in the stratum

corneum) is another defining feature of psoriasis histopathology and a key point of its pathogenesis (Balato, Ayala et al. 2012).

The ***early or mild stage*** consists of elongation and dilatation of blood vessels of the papillary derma, with associated aedema and lymphocytic infiltrate (perivascular cuffing). Vessels are dilated and tortuous, with some neutrophils in their lumen. Lymphocytes and neutrophils emerge from the vessel reaching the epidermis ("squirting" papilla). Rare erythrocytes extravasated may be found. Epidermis during this phase, is quite normal . Shortly after, there is a thickening of epidermis with loss of granular cell layer and formations of mounds of parakeratosis, which is thought to result from a markedly shortened cellular turnover time. Keratinocytes proliferate and mature rapidly, so that terminal differentiation is incomplete. Thus, squamous keratinocytes aberrantly retain intact nuclei and release few extracellular lipids that normally cement adhesion of corneocytes. The resulting poorly adherent stratum corneum leads to the characteristic scales or flakes of psoriasis lesions(Krueger and Bowcock 2005). Scattered neutrophils are seen at the edge of mounds of parakeratosis: they represents the earliest manifestation of Munro microabscesses (Rosa and Mignogna)..

The ***advanced or moderate stage*** is characterized by regular acanthosis (thickening of stratum spinosum) and epidermal "psoriasiform hyperplasia" with regular elongation of the rete ridges and thinning of suprapapillary plates . Mitotic activity is often quite marked, a further indicator of the hyperproliferative nature of this condition. Parakeratosis become confluent, with loss of granular layer. There is transmigration of inflammatory cells through epidermis into parakeratotic scale resulting in intracorneal collections of neutrophils, the so called "Munro microabscesses" . Similar accumulation in the stratum spinosum are defined as "spongiform pustule of Kogoj" . When subcorneal abscesses are prominent the disease is designated as pustular psoriasis . Dermal inflammatory infiltrate is heavier than in early lesions, it is composed by T lymphocytes, containg a few Langherhan cells with occasional neutrophils (Rosa and Mignogna).. Under the epidermal basement membrane, macrophages CD11c positive are present (Weedon and Strutton 2002). In addition to neutrophils, T lymphocytes are also found interspersed between keratinocytes throughout the epidermis and in larger quantities in the dermis. With

immunohistochemical analysis it has been shown that epidermal lymphocytes are chiefly CD8+ T cells, while dermic lymphocytes are a mixture of CD4+ and CD8+ T cells, with a CD4+ predominance, similar to that seen in peripheral blood (Krueger and Bowcock 2005).

Later or severe stage lesions involves orthokeratosis, an intact granular layer and mild exocytosis of inflammatory cells. Munro microabscesses and Kogoj micropustoles are diagnostic clues of psoriasis, but they are not always present. All other features can be found in numerous eczematous dermatitis, such as allergic contact dermatitis and atopic dermatitis. However, in these lesions spongiosis and "oozing" (presence of coagulated serum in cornified layer) are marked. Moreover, in allergic contact dermatitis there is a characteristic eosinophilic infiltrate which is normally absent in psoriasis. Later lesions must be distinguished from Lichen simplex chronicus that in contrast to psoriasis shows an evident granular layer, marked acanthosis and dermic fibrosis. Seborreic dermatitis could be hardly differentiated from psoriasis by the evident spongiosis, follicular parakeratosis and irregular acanthosis. PAS and Gram stains are helpful to identify microorganisms, in cases of bacterial impetigo, candidiasis and pustolar dermatophytosis (Rosa and Mignogna).

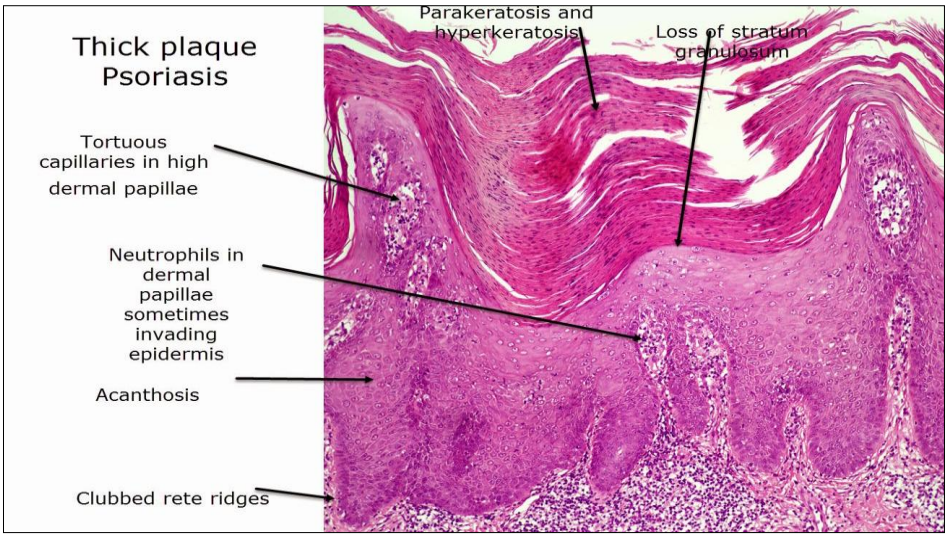


Fig. 5: Histopathological alterations in Psoriasis lesional skin: H&E image

2.7 Assessment tools

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

- **Psoriasis Area and Severity Index**

The Psoriasis Area and Severity Index (PASI) is a widely used tool for the measurement of the severity of psoriasis (Fredriksson and Pettersson 1978). The PASI combines the assessment of the severity of lesions and the area affected, into a single score within the range of 0 to 72. The body is divided into four sections: head (10% of the body area), arms (20%), trunk (30%) and legs (40%). Each of these areas is scored separately, and the four scores are then combined. For each section, the percentage of the area of skin involved is estimated and then transformed into a grade from 0 to 6. The PASI is the most validated objective method to measure the severity of psoriasis(Feldman 2004) and has a high intra-rater reliability and a good interobserver correlation when used by trained assessors (Berth-Jones, Grotzinger et al. 2006). The PASI system is sensitive to changes and reflects disease improvement or deterioration, although the sensitivity to change for small areas of involvement is poor (Feldman and Krueger 2005; Finlay 2005). PASI 75 is a widely used concept, meaning the percentage of patients achieving a 75% improvement in PASI from baseline to the primary endpoint, usually 12 to 16 weeks of treatment. Achieving a 75% improvement in the PASI is considered to be 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 instrument to estimate the extent of psoriasis involvement, calculating one palm of the hand represent 1% of the total body surface area (Rossiter, Chapman et al. 1996; Finlay 2005). 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 (Ramsay and Lawrence 1991) (Rossiter, Chapman et al. 1996).

- **Dermatology Life Quality Index**

The Dermatology Life Quality Index (DLQI) is a ten-item questionnaire evaluating the quality of life in patients with dermatological diseases (Finlay and Khan 1994). It consists of six subscales: symptoms and feelings, daily activities, leisure, work and school, personal relationships and treatment satisfaction. The DLQI can give a total score of 30 with a higher score indicating a poorer quality of life. An estimate of the minimal clinically important difference of the DLQI total score is a 5 point improvement(Khilji, Gonzalez et al. 2002). However, if patients score less than 5 points at baseline, the definition of a clinically meaningful response is expanded to include patients who achieved a DLQI total score of 0(Krueger and Bowcock 2005). A set of intervals of DLQI scores is proposed: 0-1=no effect at all on patient's quality of life, 2-5=small effect, 6-10=moderate effect, 11-20=very considerable effect and 21-30=extremely substantial effect. The reliability and validity of the DLQI is well-established (Finlay and Khan 1994; Lewis and Finlay 2004; Shikiar, Willian et al. 2006).

2.8 Diagnosis

- **Visual Examination**
  - There are no lab tests or blood tests available to confirm or deny the presence of psoriasis. Instead, a doctor must examine the skin and nails closely, sometimes analyzing dry skin under a microscope.
  - A primary care physician may be able to diagnose psoriasis. doctor may choose to refer to a dermatologist or other specialist for a more precise diagnosis.
  - In some situations, a conclusive diagnosis is impossible based on visual examinations, alone. Additional tests might be necessary to diagnose psoriasis or any other possible skin conditions.
- **Skin Biopsy**
  - The doctor might choose to perform a skin biopsy in order to rule out other possible skin conditions, sexually transmitted diseases, or infections.
  - Biopsy of the skin lesion may reveal basal cell hyperplasia, proliferation of subepidermal vasculature, absence of normal cell maturation, and

keratinization. A large number of activated T cells are present in the epidermis.

- With a skin biopsy, the doctor may order a series of lab tests to determine whether certain bacteria, viruses, or fungi are present.
- A doctor might also choose to perform an allergy test to determine whether your rash is the result of an allergic reaction to a food, plant, animal, or any other environmental conditions.

- **White Flake Of Skin**
  - When the doctor conducts a test with or without a microscope, he or she will pull a white flake of skin. If blood appears, the skin is likely affected with psoriasis.

- **Lab Tests**

  - Test result for rheumatoid factor (RF) is negative.
  - Erythrocyte sedimentation rate (ESR) is usually normal (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), causing confusion with gout in psoriatic arthritis.
  - (Uric acid formation may occur when the blood uric acid level rises above 7 mg/dL) (Normal Uric acid levels are 2.4-6.0 mg/dL (female) and 3.4-7.0 mg/dL (male))
  - Fluid from pustules is sterile with neutrophilic infiltrate.
  - Perform fungal studies. (This is especially important in cases of hand and foot psoriasis that seem to be worsening with the use of topical steroids.)

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

2.9 Treatment

Treatment goals

The treatment strategy is based on disease severity. Treatment goals (assessed after 10-16 weeks) are a reduction of PASI ≥75% and DLQI 0 or 1 (Pathirana, Ormerod et al. 2009). If a treatment regimen results in a reduction of PASI ≥75% or PASI ≥50% to <75% combined with a DLQI ≤5, treatment is successful and therapy should be

continued. When there is a reduction in PASI <50% or PASI ≥50% to <75% combined with a DLQI >5, treatment modifications should be considered, including increasing the drug dose, reducing intervals between drug doses, combining therapies or changing the drug (Mrowietz, Kragballe et al. 2011).

Treatment options

The recommended treatment for mild psoriasis is to start with topical therapy and move to phototherapy or systemic treatment in refractory cases. For moderate to severe psoriasis, phototherapy or systemic therapies are recommended.

❖ **Topical treatment**

- **Emollients**

Emollients are used to soften scaling and reduce irritation. The treatment has a positive effect on skin hydration and acts as a barrier function in psoriasis patients (Rim, Jo et al. 2005).

- **Corticosteroids**

Corticosteroids have an anti-inflammatory and immunomodulating effect. Corticosteroids inhibit different proinflammatory cytokines such as TNF-α (Norris 2005; Rhen and Cidlowski 2005). Corticosteroids with a low to mild potency are used for intertriginous psoriasis and face lesions. Potent and super potent corticosteroids are used on the body and the scalp. There has been concern regarding the long-term use of corticosteroids. Side-effects that may occur include cutaneous atrophy and the development of striae (Lebwohl, Ting et al. 2005; Mason, Mason et al. 2009).

- **Calcipotriol**

Calcipotriol is a vitamin D analogue affecting epidermal proliferation and differentiation (Bikle 1997). Calcipotriol is used for plaque psoriasis. Calcipotriol in a fixed combination with betamethasone dipropionate has a faster onset of action than monotherapy (Douglas 2002). Calcipotriol can cause irritant reactions (Mason et al 2009).

- **Calcineurin inhibitor**

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

❖ **Phototherapy**

- **Ultraviolet B**

UVB treatment is a standard treatment for moderate to severe plaque psoriasis and guttate psoriasis. 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 (Walters et al. 2003; Erkin 2007).The most common side effects of UVB therapy are erythema and burning. BB-UVB is not thought to lead to a risk of developing skin cancer (Stern and Laird 1994; Lee, Koo et al. 2005).

- **Psoralen + Ultraviolet A**

PUVA treatment is psoralen (oral or bath) in combination with Ultraviolet A (320-400 nm). Psoralen is a compound in a family of natural products known as furocoumarins. Psoralen intercalates into the DNA and, on exposure to ultraviolet UVA radiation, form covalent interstrand cross-links with thymine, inducing apoptosis. Exposure to more than 350 oral PUVA treatments greatly increases the risk of developing squamous cell carcinoma (SCC)(Stern and Study 2012).

- **Climate therapy**

Sun exposure has an immunomodulating effect with local and systemic reduction of T cells and cytokines(Søyland, Heier et al. 2011), Climatotherapy is the oldest form of phototherapy.

- **Grenz rays**

The exact mechanism of action of Grenz rays (Bucky) is unknown but it has effects on the Langerhans cells in the epidermis (Lindelöf, Liden et al. 1983). Grenz rays have wavelengths of around 20 nm, lying between x-rays and ultraviolet rays. Grenz rays are used mainly for scalp psoriasis, but also for psoriasis in the intertriginous

areas and for hand and foot psoriasis. Side effects are erythema and hyperpigmentation. One concern is skin malignancy, but the risk is considered to be low if the cumulative dose is less than 100 Gray (Lindelöf and Eklund 1986).

❖ **Systemic treatment**

- **Methotrexate**

Methotrexate is a synthetic folic acid analogue with anti-proliferative and anti-inflammatory properties(Shen, O'Brien et al. 2012). Polyglutamate, which is the primary metabolite in methotrexate, competitively inhibits dihyrofolate reductase, preventing the reduction of folate cofactors. This results in preventing 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 and Cronstein 2010). Methotrexate is the first line treatment for moderate to severe psoriasis when systemic treatment 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 (Thompson, Lindsten et al. 1989; Cooper, Baadsgaard et al. 1990; Tsuda, Yamanaka et al. 2012).Cyclosporin is used for severe psoriasis. Cyclosporin is nephrotoxic and functional kidney damage can occur quickly 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 favours the differentiation of epidermal keratinocytes. Acitretin inhibits the induction of Th17 cells and promotes the differentiation of T-regulatory cells (Xiao, Jin et al. 2008). Acitretin is used for plaque psoriasis (especially in combination with UVB and PUVA) and also for pustulous psoriasis, hyperkeratotic hand- and foot psoriasis and erythrodermia. 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, Anstey et al. 2009). There is a greater risk of developing serious infections during treatment, and screening for tuberculosis and hepatitis is mandatory before treatment starts. To date, there is no robust evidence of an increase in the risk of malignancy, but a possible future risk 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 (Leonardi, Powers et al. 2003).

- **Adalimumab**

Adalimumab is a fully human anti TNF- α monoclonal antibody and it is administered through subcutaneous injections(Gordon, Langley et al. 2006).

- **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(Gottlieb, Evans et al. 2004).

- **Ustekinumab**

Ustekinumab is a human monoclonal antibody that binds with high affinity and specificity to the p40 protein subunit that is used by both the interleukin (IL)-12 and the IL-23 cytokines. It is administered through subcutaneous injections (Krueger, Langley et al. 2007).

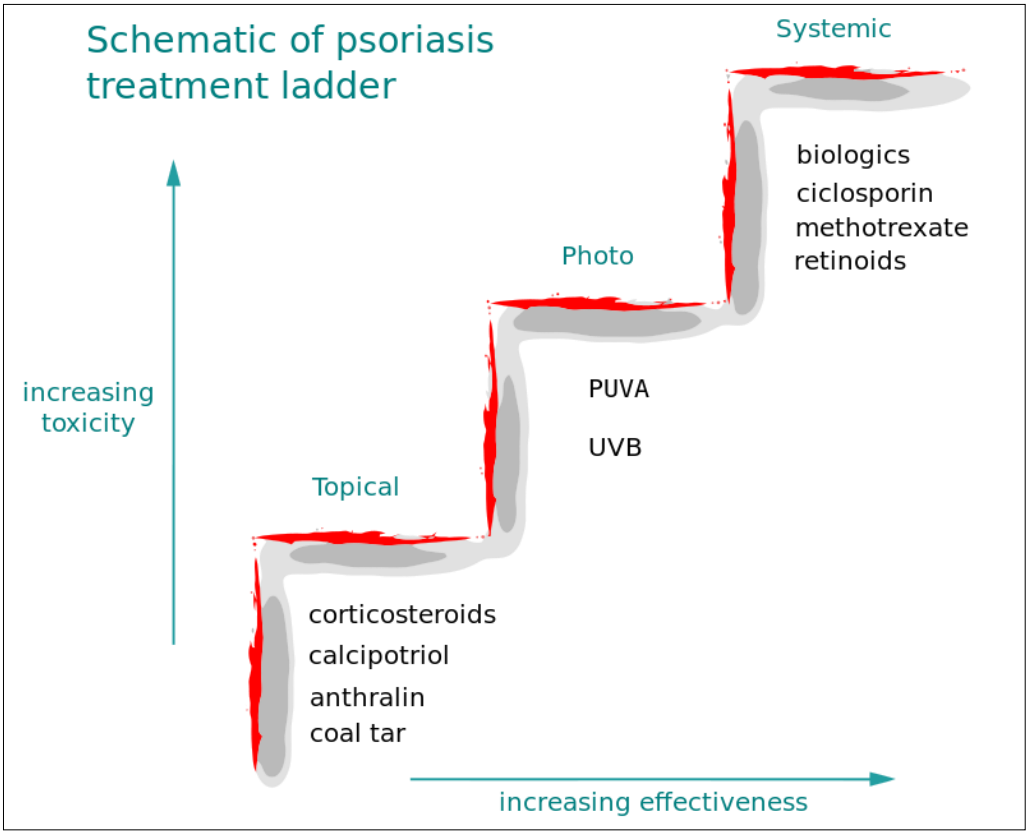


Fig. 6: Psoriasis treatment Ladder

(http://commons.wikimedia.org/wiki/File:Psoriasis_treatment_ladder.svg)

2.10 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 certain 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% (Zachariae, Zachariae et al. 2002; Madland, Apalset et al. 2005). PsA is characterised by the development of pain, swelling, and tenderness of the joints surrounding ligaments and tendons. PsA can progress to an erosive, polyarticular disease with joint destruction and loss of functionality (i.e. arthritis mutilans). Skin disease typically presents before arthritis in more than 80% of

the patients, and psoriasis symptoms usually precede joint symptoms by an average of 10 years (Gladman, Antoni et al. 2005; Gottlieb, Mease et al. 2006).

- **Metabolic syndrome**

Metabolic syndrome is frequently seen in patients with psoriasis (Sommer, Jenisch et al. 2007). Metabolic syndrome can be defined as central obesity and the presence of two or more of the components: 1. Raised triglycerides or history of treatment for this lipid abnormality, 2. Reduced HDL cholesterol or history of treatment for this lipid abnormality, 3. Raised blood pressure or treatment of previously diagnosed hypertension, 4. Raised fasting glucose level or previously diagnosed type 2 diabetes mellitus. 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 demonstrated that a higher BMI coincides with a greater degree of psoriasis disease severity (Naldi, Chatenoud et al. 2005; Huerta, Rivero et al. 2007).

- **Cardiovascular disease**

Patients with severe psoriasis are at greater risk of developing cardiovascular disease (Mallbris, Akre et al. 2004). Systemic inflammation has been associated with the development of atherosclerosis (Hansson 2005) which suggests that psoriatic patients may be at greater risk of developing cardiovascular disease. Studies report that plasma acute-phase protein levels (C-reactive protein, fibrinogen, and plasminogen activator inhibitor-1) were significantly elevated in patients with psoriasis compared with healthy controls (Kural, Örem et al. 2003; Chodorowska, Wojnowska et al. 2004).

- **Gastrointestinal disease and liver disease**

The prevalence of gastrointestinal disease and non-alcoholic fatty liver disease (NAFLD) is greater in psoriasis patients. The prevalence in psoriasis patients compared to the general population for NAFLD is 48-59% / 20-30%, Crohn's disease (CD) 0.5% / 0.004-0.04%, ulcerative colitis (UC) 0.5% / 0.05-0.07% and for celiac disease 0.2–4.3% / 1%. The magnitude of the association with psoriasis seems to be greater for CD compared with UC (Gisondi, Del Giglio et al. 2010). Psoriasis and CD are inflammatory disorders primarily mediated by Th1 lymphocytes producing cytokines such as TNF-α and IFN-γ. In recent years, an important role for Th17 cells has also been found, in CD as well as in psoriasis (Tesmer, Lundy et al. 2008).

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 greatest 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 (Margolis, Bilker et al. 2001). The risk of psoriatic patients developing lymphoid malignancies, may be attributable to the pathophysiology and also to the treatment of psoriasis(Margolis, Bilker et al. 2001; Gelfand, Shin et al. 2006). In addition to lymphoma and non-melanoma skin cancers, psoriatic patients are at greater risk of developing other malignancies, including those of the head and neck, solid organs (liver, pancreas, lung, breast, kidney), and genitals (Frentz and Olsen 1999; Boffetta, Gridley et al. 2001).

- **Psychiatric disease**

Moderate to severe psoriasis is associated with marked physical and psychological morbidity, with up to 40% of patients reporting that their disease has negative effects on their ability to daily function. (Stern, Nijsten et al. 2004). High anxiety scores have been reported in over one-third of the psoriatic patients(Gupta and Gupta 2003). There also appears to be an association between psoriasis and lifestyle choices that can have a negative impact on the general health of patients, which can contribute directly to both medical and psychological comorbidities.

- **Oral disease**

Dental cavities and periodontal (gum) disease are the most common oral diseases. Dental caries is a major oral health problem in most industrialised countries, affecting 60-90% of schoolchildren and the vast majority of adults. Severe periodontitis, which may result in tooth loss, is found in 5-20% of middle-aged adults (Oral health – WHO Fact sheet).

Intraoral psoriatic lesions are relatively uncommon and can affect the buccal mucosa, palate and gingiva. Lesions can manifest as yellow to white borders, frank ulcerations and desquamative gingivitis, and be asymptomatic or with tenderness and a burning

sensation. There also appears to be an increase in the frequency of geographic tongue and fissured tongue in patients with psoriasis.(HIETANEN, SALO et al. 1984; Pogrel and Cram 1988).

2.11 Understanding Psoriasis from an immunological perspective

Immunopathogenesis involves complex crosstalk between skin resident **keratinocytes** and infiltrating **immune cells**. The cycle starts with an initial triggers such as physical trauma or bacterial products start a cascade of events that include the formation of DNA-LL-37 complexes, activation of plasmocytoid dendritic cells and secretion of interferon-α (IFN-α). IFN-α secreted by plasmocytoid dendritic cells promotes the activation of myeloid dendritic cells ((Nestle, Conrad et al. 2005). Activated myeloid dendritic cells migrate into draining lymph nodes and 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) (Nestle, Kaplan et al. 2009; Balato, Ayala et al. 2012). Effector cells recirculate and slow down in skin capillaries in the presence of selectin-guided and integrin-guided receptor-ligand interactions. Immune cells expressing the chemokine receptors CCR6, CCR4, and CXCR3 emigrate into skin tissue along chemokine gradients. Dendritic cells and T cells form perivascular clusters and lymphoid-like structures around blood vessels in the presence of chemokines such as CCL19 produced by macrophages. Unconventional T cells, including natural killer T cells (NKT), also contribute to the disease process. Further key processes 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 mediators 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) (Nestle, Kaplan et al. 2009; Balato, Ayala et al. 2012). soluble mediators feed back into the pro-inflammatory disease cycle and constitute the inflammatory infiltrate (Fig.2).

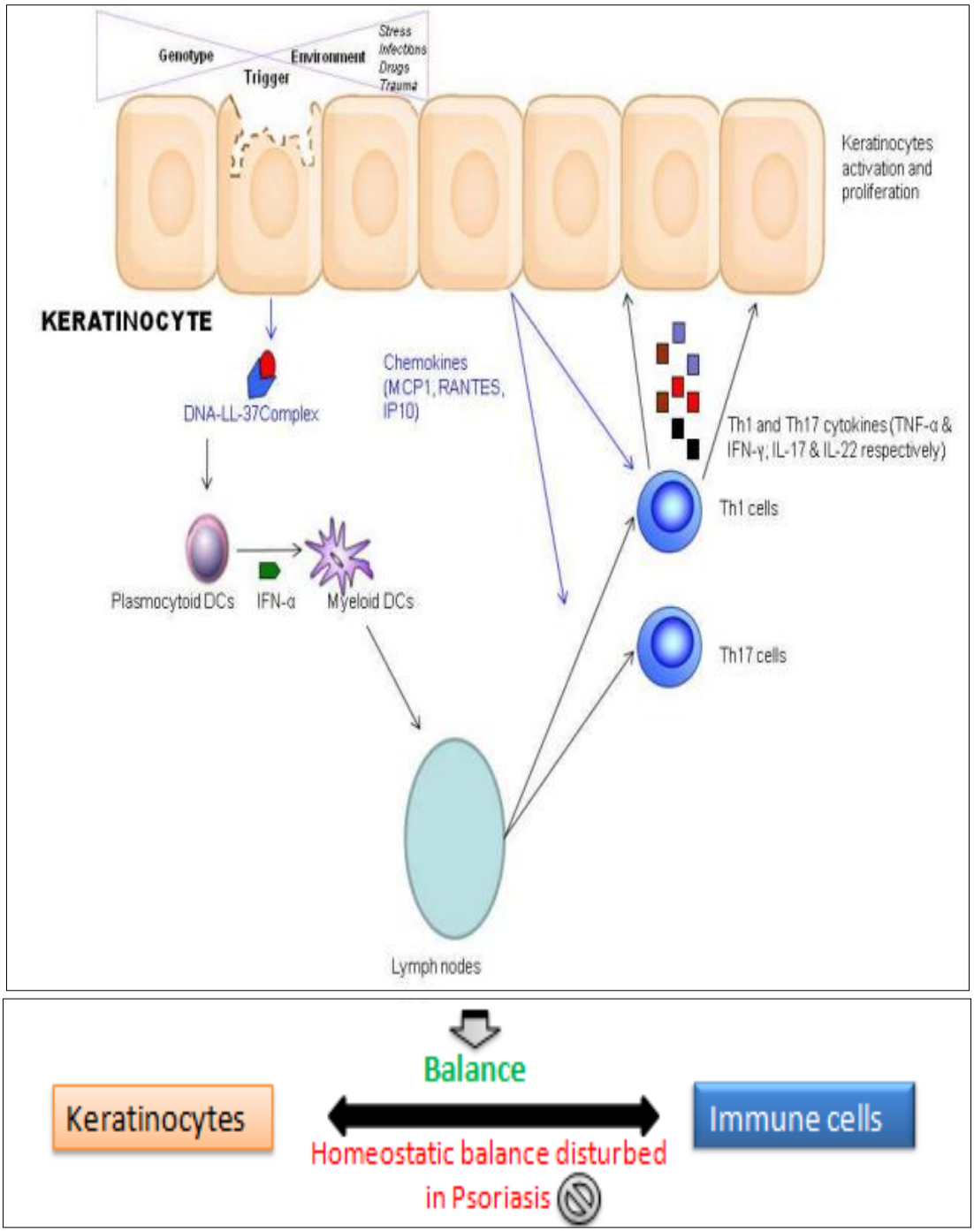


Fig. 7: Complex interplay between keratinocytes and immune cells (Balato, Ayala et al. 2012)

With respect to involvement of multiple cell types and molecules involved in disease etiology as explained above there are two school of thoughts regarding primary defect that leads to psoriasis :

Prior to 1990 : Primary activation of keratinocytes leads to subsequent release of cytokines and antigen-independent activation of T cells.
Post 1990 (current understanding): Persistent T-cell stimulation leads to abnormal keratinocyte proliferation (Cai, Fleming et al. 2012).

Immunologically, new insights in the pathogenesis of psoriasis, points towards a major role of cellular innate and adaptive immune response **especially T-cells** in the initiation and maintenance of the disease state.

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

Role of T-cell-mediated inflammation in Psoriasis

T lymphocytes are central in pathogenesis of psoriasis. Heterogenous T-cell subset viz Th1, Th17, Th22, Tc17, Tc22, Treg cells are associated with the disease, exhibiting considerable plasticity in transdifferentiating from one subset to another subset under different environmental cues. The interaction of T cells and DCs creates a 'type 1' inflammatory environment by secreting large amounts of Th1 type cytokines, leading to the development of psoriasis as **Th1** cell-mediated disease. A new population of IL-17-producing CD4+ Th cells, named **Th17**, and their related cytokines such as IL-17A, IL-17F, IL-22, IL-21 and IL-26 play essential roles in psoriasis too. Recently, another distinct population of Th cells, which preferentially express CCR10, CCR6 and CCR4 and produce only IL-22, but not IL-17 or IFN-γ, has been characterized and called IL-22-producing **Th22** cells. This unique subset of human skin-homing memory T cells may be involved in epidermal immunity and remodelling and dedicated to the skin homeostasis and pathology (Cai, Fleming et al. 2012).

In addition to the well-established role for conventional T cells in psoriasis pathogenesis recent studies have indicated a potential contribution of innate gamma-delta **(γδ) T cells**. Dermal γδ T cells constitutively express IL-23 receptor and rapidly produce large amounts of IL-17 upon stimulation with IL-23. Thereby, γδ T cells may amplify Th17 responses and induce autoimmunity(Flatz and Conrad 2013).

Inconsistent T cell profile in psoriasis : Literature review reveals an inconsistency in T cell profile in psoriasis in two respects: First, with respect to both variable quantitative and qualitative profile of T cells showing **aberrant number and types** in periphery and lesion, focus of immunosuppressive therapies in psoriasis. Second, in terms of its **aberrant activation state,** which is the main target of various immunomodulatory therapies in psoriasis.

The above inconsistency correlated with the **functionally compromised Treg cells** in psoriasis , which are functionally deficit in regulation T cell function ,differentiation and proliferation

T cell in lesional skin of psoriasis : Magnitude of T cell infiltration in skin lesions is quite impressive in Psoriasis biopsies . T cell subsets are not uniformly distributed in psoriasis lesions and are found interspersed between keratinocytes throughout the epidermis and in somewhat larger quantities in the dermis. Epidermal T cells are chiefly CD8+ T cells and dermal T lymphocytes are a mixture of CD4+ and CD8+ cells, with a CD4+ predominance. Most T cells in skin lesions are memory cells that express cutaneous lymphocyte antigen (CLA), the skin addressin (Krueger and Bowcock 2005).

T cell in peripheral blood in psoriasis : Peripheral profile of T cells in psoriasis shows variable reports in literature with quantitative and qualitative alterations in different subpopulations of T cells.

A study showed percentage of the CD4+Tcells and CD8+ T cells decrease in peripheral blood of the psoriatic patients in comparison with the controls (Lecewize, Pietrzak et al. 2001). In another report, CD4+ T cells and CD8+Tcells percentage are shown to decrease significantly in patients in comparison with the controls either due to decreased production or transmission of these cells from the blood to the skin and higher rate of apoptosis (Bueeni, Ramezanpour et al. 2013).. This abnormal proliferation of auto reactive lymphocytes that perpetuate the inflammatory response can be attributed to decrease p53 expression in peripheral blood lymphocytes in the patients or inactivation of p53 due to mutation leading to its abnormal expression that can disturb cell cycle regulation. A deficiency in T-regulatory cells in the peripheral blood is also postulated as one of the rational for altered T cell profile (Bueeni, Ramezanpour et al. 2013).

Another report show a contrasting feature of T cell profile in periphery .Acoording to the report, large numbers of CD4+ Th1 and CD8+ cytotoxic T cells type 1 (Tc1) in psoriatic plaques and peripheral blood of psoriatic patients and more of Th22 cells along with Th17 cells are seen in the circulation of psoriatic patients which may collaboratively interact with each other and contribute to the disease pathogenesis (Cai, Fleming et al. 2012).

Therefore, psoriasis cannot be simply defined as a particular Th subset based disease. Instead, different pathogenic Th subsets are implicated in the disease development, which interact DCs and neutrophils to create a chronic inflammatory environment for the maintenance of psoriatic plaque.

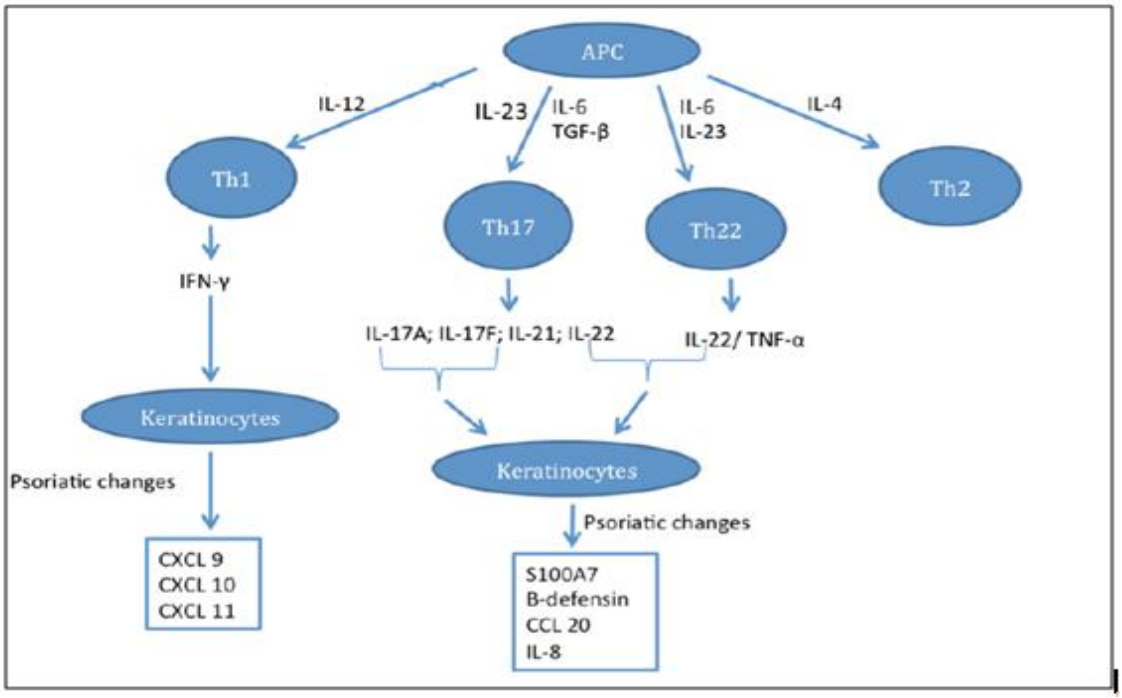


Fig. 8: Involvement of different subsets of T cells as major players in pathogensis of Psoriasis (Fallen, Mitra et al. 2013)

2.11 miRNAs and T cells: molecular mechanistics in Psoriasis

Different subsets of CD4 T cells originate from a common precursor CD4 T naive cell. Recent studies showed that these cells are not terminally differentiated but have potential to differentiate into other CD4 T subset under the regulation of specific cytokines and other extracellular signals present in the tissue microenvironment. Unrevealing molecular switches that induce imbalance in immune cell subset and their functionality in psoriatic patient

compared to healthy controls can help in mechanism based understanding of the disease progression (Sethi, Kulkarni et al. 2013).

In this context , **microRNAs** (miRNAs) have emerged as major players that regulate many important processes such as development, survival, proliferation, differentiation, function of immune cells and most important orchestrate plasticity of CD4 T cells during inflammation and tolerance.

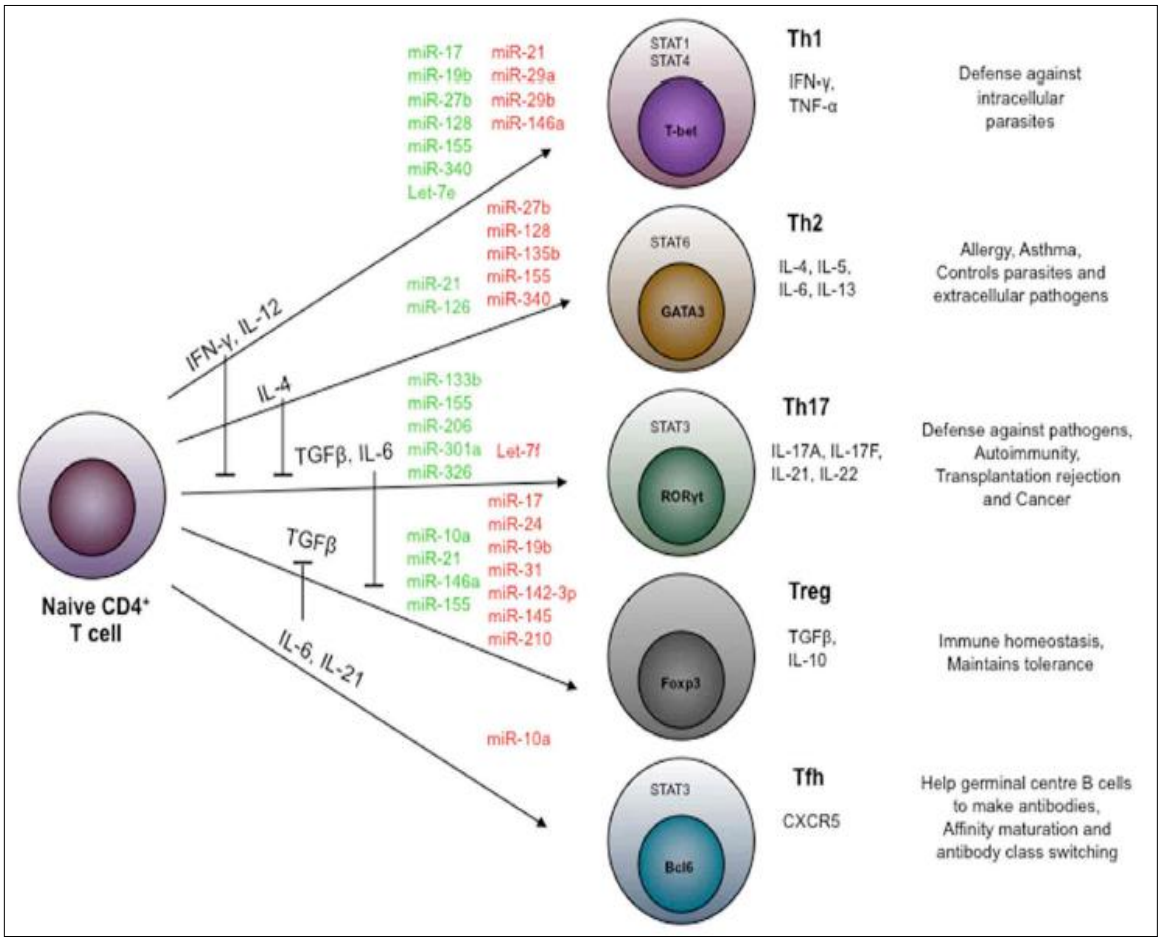


Fig. 9: miRNAs control the differentiation of CD4+ T helper cell subsets (Sethi, Kulkarni et al. 2013)

2.12 miRNAs

miRNAs are small ~ 22 bp non-coding RNAs that can regulate gene expression in a cell specific manner and are themselves amiable to changes with respect to expression level and functionality. In few independent studies comparison of miRNAs profile of Psoriasis patient with healthy control (blood/biopsy) have revealed dysregulation of specific miRNAs. How changes in expression of different miRNAs actually contribute to disease process involving

keratinocytes and infilterating immune cell is not yet clear. Also the miRNAs expression profile may differ from one population to other based on location, environment and genetics.

In lieu with the prior understanding of primary defect- keratinocytes being the major initiators of the disease cycle and psoriasis being considered as skin inflammatory disease, much of the work till date is focused on miRNA profile of the psoriasis skin lesion with scarce reports on its systemic nature of circulatory mirna alterations.

A vast body of literature has been centred around profiling and characterization of the keratinocyte specific miRNAs from patient sample biopsies showing keratinocyte mediated effector functions viz. miR-203, miR- 125b, miR-31, miR-135, miR-205 , miR-34a/c etc

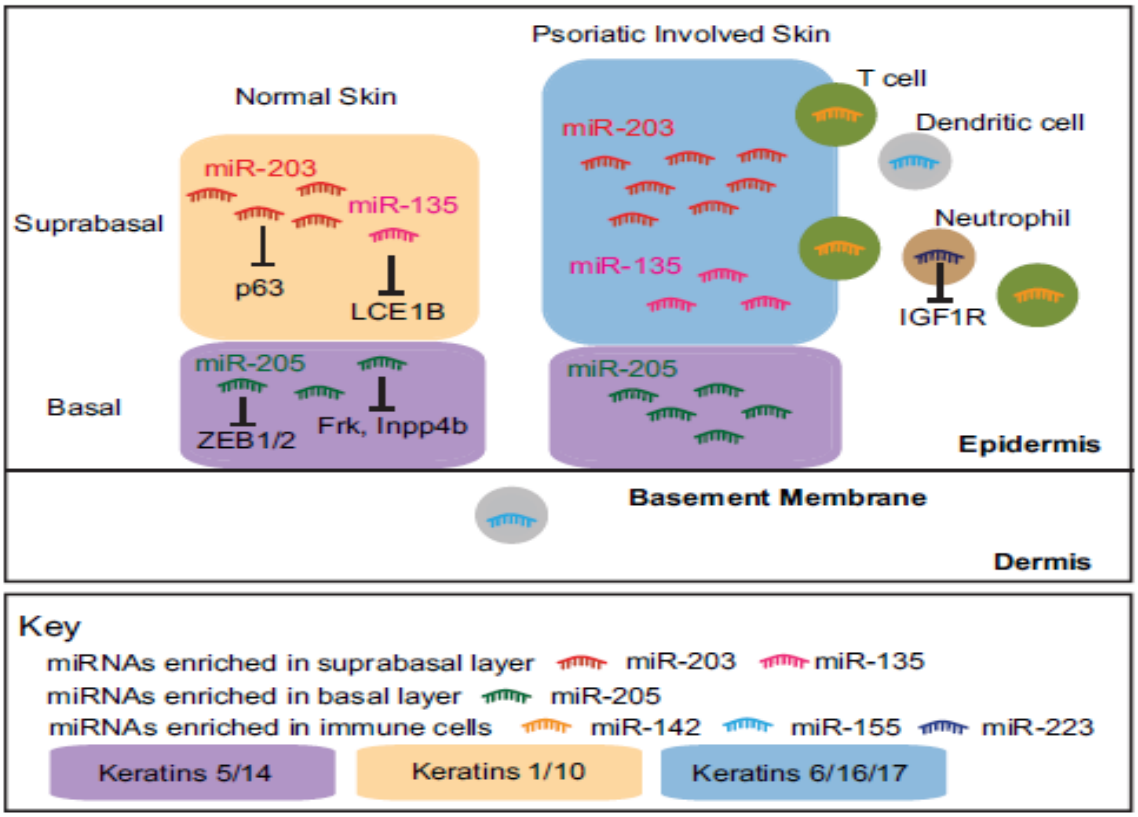


Fig. 10: Localization of miRNAs in normal and psoriatic-involved skin
(Xia and Zhang 2014)

Circulating miRNAs are attractive candidates for disease monitoring to serve as valuable prognostic indicators of disease progression or resolution. Markers of inflammation and repair like troponin, C-reactive protein, chemokines and cytokines , by the time these proteins are detectable in the circulation, much of the tissue damage has already occurred, which makes it crucial that better biomarkers be uncovered for the early detection of

diseases. Changes in miRNA expression in body fluids occur earlier than conventional biomarkers (Moldovan, Batte et al. 2014).

Activated lymphocytes release a large amount of nano-sized vesicles (exosomes), containing miRNAs, in circulation upon altered cellular profile under an inflammatory condition (de Candia et al. 2014) . As psoriasis is also associated with altered T –cell profile, it is expected that the disease is associated with altered (upregulated /downregulated) circulatory miRNA profile in patient plasma or serum samples compared to normal healthy control.

Inspite of significant research in understanding the association of miRNAs and psoriasis there remains a substantial gap in understanding the systemic nature of the disease in regard to the circulatory miRNA profile in psoriasis, more specifically the circulatory miRNAs leading to a T-cell based dysfunctionality in disease progression.

According to a recent report by Sen, Chao et al.**, there is no substantial research on plasma miRNA expression profiling in psoriasis (Sen, Chao et al. 2012).** Few studies have reported specific plasma/serum miRNAs in psoriasis that too not specific to T-cells associated changes (Table 1).

miRNA reported	Body fluid and expression	Ref.
miR-424	Serum (downregulated)	(Ichihara, Jinnin et al. 2011)
miR-1266	Serum (upregulated)	(Ichihara, Jinnin et al. 2012)
miR-369-3p	serum (upregulated)	(Guo, Zhang et al. 2013)
mir-33	plasma (upregulated)	(García-Rodríguez, Arias-Santiago et al. 2014)
miR-128a,let-7d, miR-142-3p, miR-181a	Serum (upregulated)	(Pivarcsi, Meisgen et al. 2013)

Table 1: Reported miRNA in circulation (Serum/Plasma) in Psoriasis

Among various circulatory miRNAs , **miR-21 and miR-215** are an important class of circulatory miRNAs that have been reported to be deregulated in various diseases like cancer, T-cell lymphoma and myriad of other inflammatory diseases.

Literature suggests a correlation of these miRNAs with respect to T-cell specific role in psoriasis with a possible altered expression profile in skin lesions but not in circulation although reported in other diseases .

2.11.1 miR-21 : A crucial regulator of T-cell biology

miR-21 playes a crucial role in a plethora of biological functions and diseases including development, cancer and inflammation, especially correlated with the pathogenesis of autoimmune diseases like psoriasis. miR-21 was identified to regulate a variety of immune cells like B-cells, T –cells , DCs etc. miR-21 is an important regulator of different aspects of T-cell biology depending on the differentiation status of the T-cell.

miR-21, NF-kB and Tipe2 - a unique regulatory axis controlling T-lymphocyte apoptosis.

MiR-21 acts as an anti-apoptotic agent in a variety of cell types including human primary T cells and modulates cytokine production. Proliferative expansion of lymphoid cells is required for effective immune responses against invading microorganisms, but the expanded effector cells must be eliminated to prevent overaccumulation of cells after the infection is controlled. The cell number, repertoire diversity, and self-tolerance of mature T lymphocytes are tightly controlled by a process called programmed cell death or apoptosis. Apoptosis of mature T lymphocytes is regulated by extensive networks of signal-transduction pathways which ensures controlled activation and expansion of cells during immune responses and apoptotic deletion of lymphoid cells that are no longer needed at the end of immune responses. T-cell apoptosis occurs in at least two major forms: antigen-driven and lymphokine withdrawal-induced (Ruan, Wang et al. 2014). In this regard , it has been reported that the three factors**,** NF-kB, miR-21, and Tipe2, form a unique regulatory axis that controls T-lymphocyte apoptosis. This axis is involved in NF-kB-mediated inhibition of T-cell death and may serve as a unique target for the regulation of immune responses (Ruan, Wang et al. 2014).

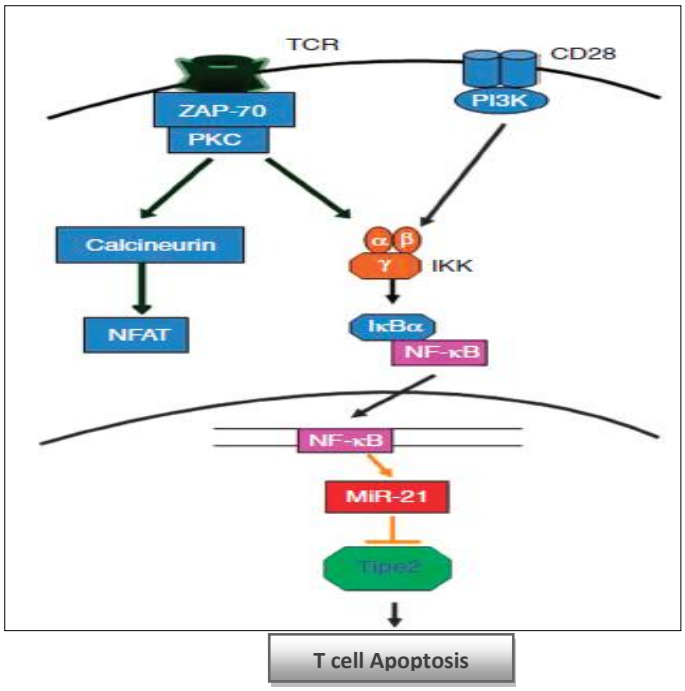


Fig. 11: NF-kB inhibits activation-induced T-cell apoptosis through the miR-21–Tipe2 axis (Ruan, Wang et al. 2014)

In addition to apoptosis-related genes, bioinformatic analysis of putative miR-21 targets relevant for T-cell biology revealed several immune-related genes, including CC chemokine receptor 7 (CCR7), which is substantially expressed on naive T-cells . By binding with its ligands (CCL19 and CCL21) presented on the surface of high endothelial venules, CCR7 enables entry of T-cells into lymph nodes, and as such ensures (re)circulation of naive T-cells through the lymphatic system (Förster, Davalos-Misslitz et al. 2008).

Dual Role of miR-21 in two key aspects of T-cell biology of CD4+ T-Cells: Activation-Induced miR-21 Supports Survival of Memory T-Cells and Regulates CCR7 Expression in Naive T-Cells in relation to potential homing properties .As per their reports miR-21 regulates the potential migration capacity and transition towards the memory T-cell phenotype of activated naive T-cells, and supports survival of activated memory T-cells (Smigielska-Czepiel, van den Berg et al. 2013).

miRNAs can influence TH1 cell differentiation indirectly by targeting genes in immune cells other than CD4+ T cells. For example, miR-21 regulates TH1 cell differentiation by modulating IL-12 production by dendritic cells (DCs) (Baumjohann and Ansel 2013). IL-12 is an important cytokine that induces T-bet and IFNγ expression and supports the growth and survival of TH1 cells (Lazarevic and Glimcher 2011). miR-21 is highly expressed in DCs,

and directly targets the mRNA that encodes IL-12p3565 (Lu, Munitz et al. 2009). miR-21 deficiency increases IL-12 expression in DCs, and enhances TH1 cell development and delayed type hypersensitivity responses *in vivo* (Lu, Hartner et al. 2011). Also **miR-21 negatively regulates programmed cell death 4 (PDCD4)** and suggest the effects of miR-21 on T cells by virtue of diminishing PDCD4 expression (Xu, Pan et al. 2013).

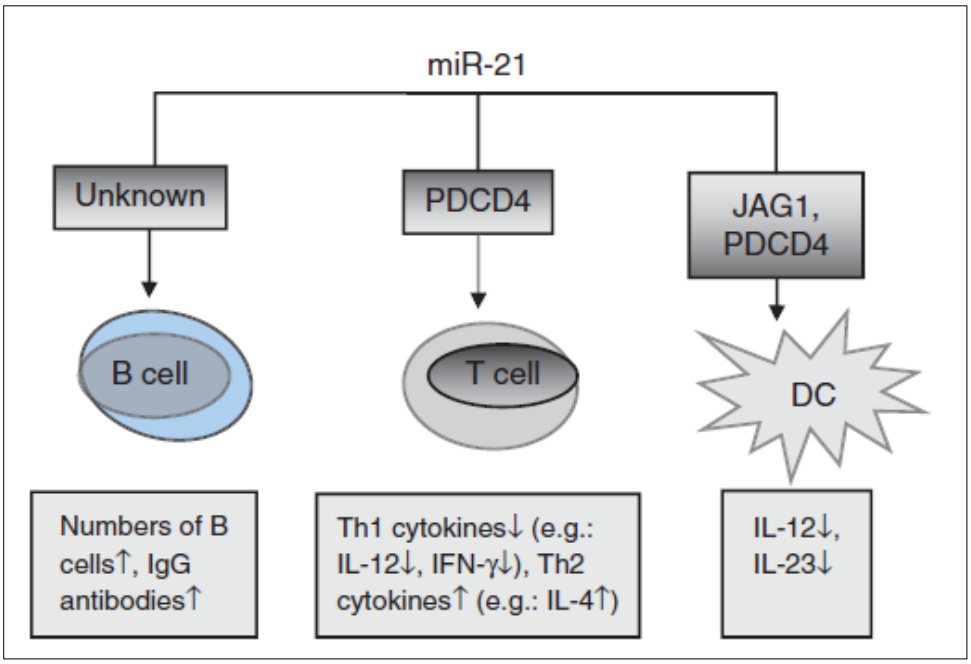


Fig. 12: The role of miR-21 in immune cell function (Xu, Pan et al. 2013)

miR-21 as a negative modulator of signal transduction downstream of TCR in T-lymphocytes: Memory T-lymphocytes express higher levels of miR-21 compared to naïve T-lymphocytes and that miR-21 expression is induced upon TCR engagement of naïve T-cells. miR-21 plays opposing roles in naïve and memory T-cells show that miR-21 expression affects ERK phosphorylation, AP-1 activity and T-cell activation and interleukins (IFN-γ) production upon TCR engagement, highlighting a role of miR-21 as a negative regulator of T-lymphocyte activation(Carissimi, Carucci et al. 2014).

miR-21 profile in Psoriasis

miR-21 profile has been studied by various groups in psoriasis in lesional biopsy . miR-21 is among the most abundant miRNAs with **upregulation** (4-fold) (Joyce, Zhou et al. 2011) and 2.51 fold (Sonkoly, Wei et al. 2007) in psoriatic lesions. In another study, the expression of miR-21 in epidermal cells and dermal T cells between psoriatic and healthy skin showing an elevated expression in both cell types . Also expression of miR-21 in epidermal and dermal

cells of psoriatic skin biopsies, exhibits a 2.8-fold increase compared with healthy epidermis, and a 2.6-fold increase in psoriasis dermis in comparison with healthy dermis up-regulated miR-21 in psoriasis skin lesions arises from the infiltration of activated T cells (Meisgen, Xu et al. 2012) . Inhibition of miR-21 increases the rate of apoptosis in activated T cells, suggesting that miR-21 suppresses apoptosis in activated T cells. Therefore, overexpression of miR-21 in psoriatic lesions may reflect the infiltration of activated T cells in psoriatic skin, contributing to T cell-derived psoriatic skin inflammation.

Inspite of the skin based studies of miR-21 and its relation with T-cell , its circulatory nature has not been yet ruled out in blood samples (plasma/serum) in psoriasis although reported in other diseases like breast cancer, multiple sclerosis (upregulated), obesity, Crohn's disease (upregulated), ulcerative colitis (downregulated), rheumatoid arthritis (upregulated).

Thus understanding the circulatory miR-21 profile in psoriasis and its relation with T cell biology in lesional skin needs to be ruled out , with a view to shed light on the systemic nature of the disease.

2.11.2 miR-215 : A crucial regulator of cellular proliferation , cell death and immune cell migration.

miR-215 , a functional homologoue of miR-192 (sharing virtually identical transcriptional profiles) has been studied in various diseases like multiple myeloma, renal cell carcinoma, gastric cancer, breast cancer, psoriasis (in skin lesion).

miR-215 as regulators of the MDM2/p53 auto regulatory loop

p53 is tumor-suppressor gene that controls cellular proliferation, migration and apoptosis. Activation of the p53 protein suppresses the over proliferation of cells and regulate cellular homeostasis under disease conditions by cell cycle arrest and apoptosis. MDM2, a p53-specific E3 ubiquitin ligase, is the principal cellular antagonist of p53, acting to limit the p53 growth-suppressive function in unstressed cells.Under normal conditions there is a homeostatic balance between in the interaction between p53 and MDM2 which is tightly regulated at multiple levels. Disruption of the p53-MDM2 complex by multiple routes under diseased conditions may lead to either p53 activation leading to more of cellular apoptotic response or MDM2 activation leading to more of cellular proliferation. Thus, the two

molecules are linked to each other through an autoregulatory negative feedback loop aimed at maintaining low cellular p53 levels in the absence of stress.

miR-215 is an important regulator of MDM2/p53 autoregulatory loop ,through binding to the p53 REs in their promoters . p53 is also a functional transcription factor in activated T cells and induces the expression of MDM2. A study gave evidence that 215 is down-regulated in mulitiple myeloma, can be transcriptionally activated by p53 and then modulate MDM2 expression (Pichiorri, Suh et al. 2010). Another study revealed that miR-215 can act as effectors as well as regulators of p53 and seems to suppress cancerogenesis and cell cycle arrest. Accordingly, miR-192 and miR-215 suppressed proliferation and cell adhesion in a partially but not completely p53-dependent manner (Braun, Zhang et al. 2008).

Futher a study report that, SAP (SLAM-associated protein) is a target of p53 in activated primary T cells and contributes to T cell homeostasis through the induction of the pro-apoptotic SAP. T cells expressing high levels of SAP undergo apoptosis. Modifying p53 levels which specifically dissociates the MDM2-p53 interaction, was sufficient to upregulate SAP expression, indicating that SAP is a target of p53 in T cells (Madapura, Salamon et al. 2012).Expression of miR-215 is also severely reduced in many colon cancers targeting a number of regulators of DNA synthesis and the G1 and G2 cell cycle checkpoints in cells (Georges et al. 2008). It was further showed that miR-215 and other two miRNAs have tumor suppressor effects on Renal cell carcinoma by targeting MDM2 thereby reducing the cellular migration and invasion abilities(Khella, Bakhet et al. 2013).

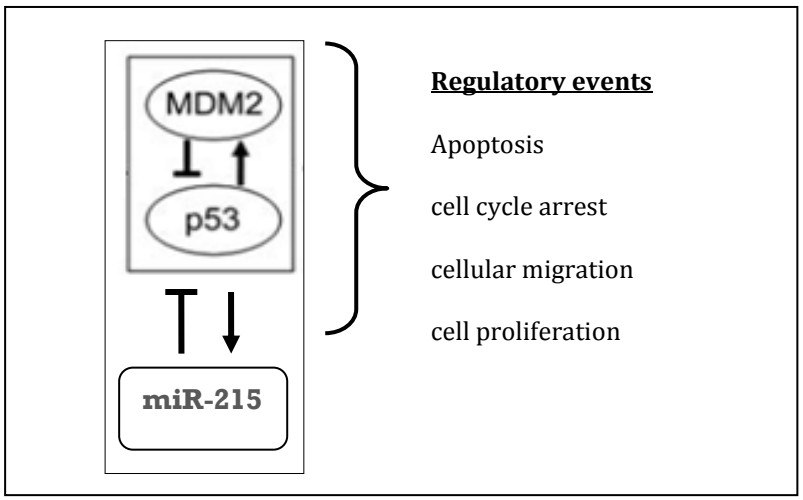


Fig. 13: miR-215 as regulators of the MDM2/p53 auto regulatory loop (Feng, Zhang et al. 2011)

miR-215 directly target IL-17 Receptors (IL-17Rs) mRNAs

Recently, a study has provided evidence that the IL-17/miR-215/192/IL-17Rs regulatory feedback loop is manifested in MM and might represent a promising and efficient prognostic marker and therapeutic target for MM. miR-215 along with its homologue miR-192, and directly targeted the 3'-untranslated regions of IL-17 Receptors (IL-17Rs) , including IL-17RA and RE mRNA. Interleukin-17 (IL-17), produced by a lineage of CD4+ T helper cells (TH17 cells), has been shown to be involved in several chronic pathologies, such as inflammation, autoimmune disease and tumors having a pro-inflammatory role in inducing cell proliferation and inhibiting cellular apoptosis and facilitated cell migration (Sun, Pan et al. 2014).

miR-215 profile in Psoriasis

miR-215 has been reported to be **downregulated** in psoriasis skin lesion by 0.56 fold change with respect to normal healthy individual(Sonkoly, Wei et al. 2007).The peripheral blood profile of the miRNA in psoriasis has not been reported till date.

With reference to its correlation with transcription factor p53 in many inflammatory diseases ,there may be a possible correlation in psoriasis also. p53 has been reported to be overexpressed in skin lesion and decreased in peripheral blood lymphocytes in psoriasis .Also p53 is crucial in maintaning T cell homeostasis which are major effector cells in psoriasis. (Bueeni, Ramezanpour et al. 2013).Thus, decreased miR-215 expression and its subsequent interaction with p53/MDM2 regulatory axis along with other cell cycle check points targets, may be a part of complex regulation of increased T cell proliferation and diminished T-cell apoptosis in psoriasis etiology.

As per another study in multiple myeloma that miR-215 along with its homologue miR-192, directly target the 3'-untranslated regions of IL-17 Receptors (IL-17Rs) , including IL-17RA and RE mRNA (Sun, Pan et al. 2014), this functionality of miR-215 may also be important in psoriasis also as IL-17A –IL-17 Rs axis is crucial in psoriasis pathogenesis. Numbers of IL-17A-positive cells are increased in lesional skin, with majority of cells being T cells (CD8+ T cells) and neutrophils. Interleukin-17 receptors are detected on a wide variety of immune and connective tissue cells including monocytes/macrophages, dendritic cells, neutrophils, keratinocytes, mast cells, epithelial cells and vascular endothelial cells. These interactions can have possible multiple inflammatory responses like recruitment of

neutophiles, memory T cell, keratinocytes and T cell proliferation and associated inhibition of cellular apoptosis in psoriasis.

The moleculer mechanistics, as to how miR-215 is related to T cell alterations in context of psoriasis is not clear and needs futher studies to arrive at a wholistic picture including lesional as well as circulatory profile and effector mechanism. .

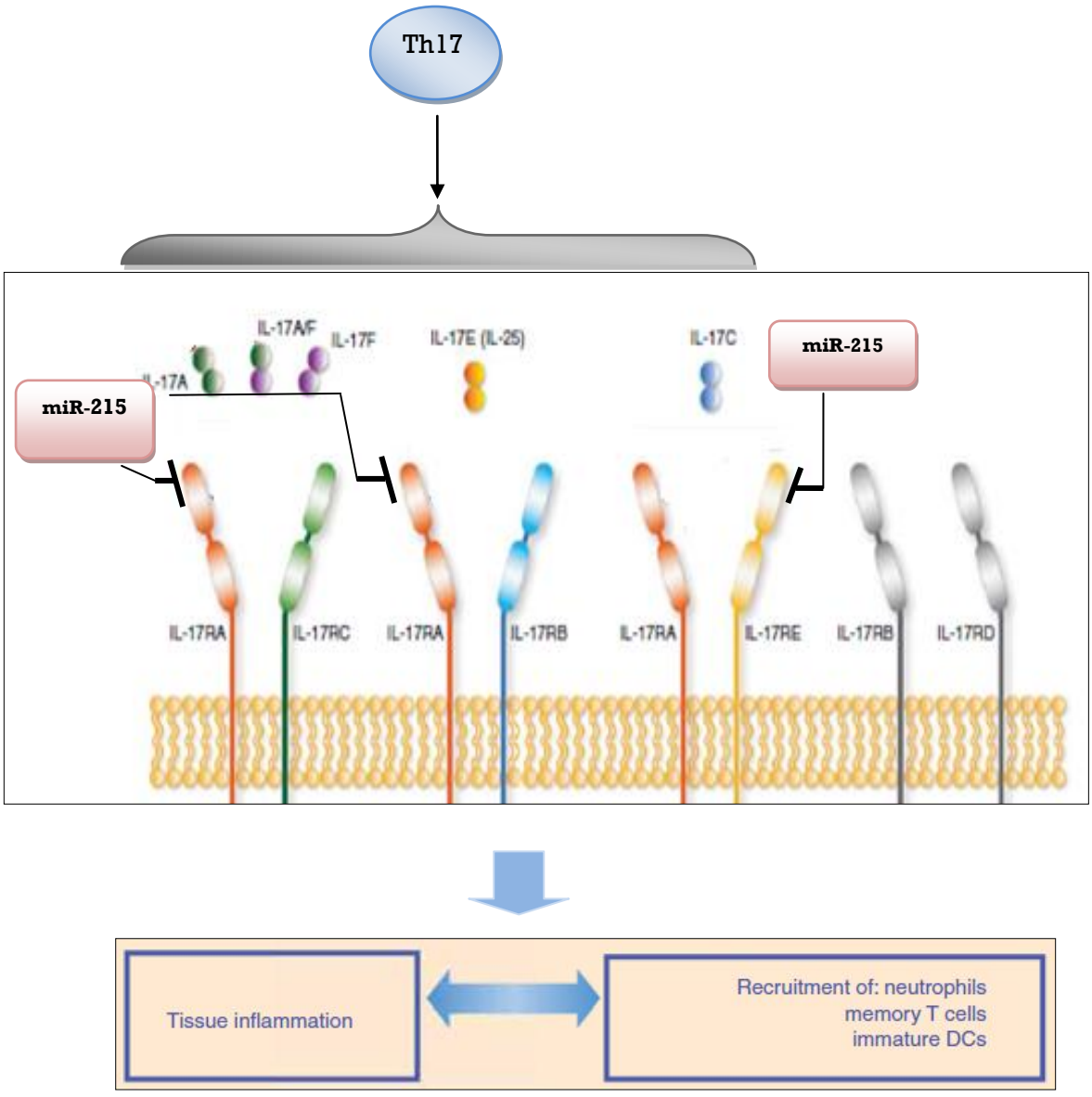


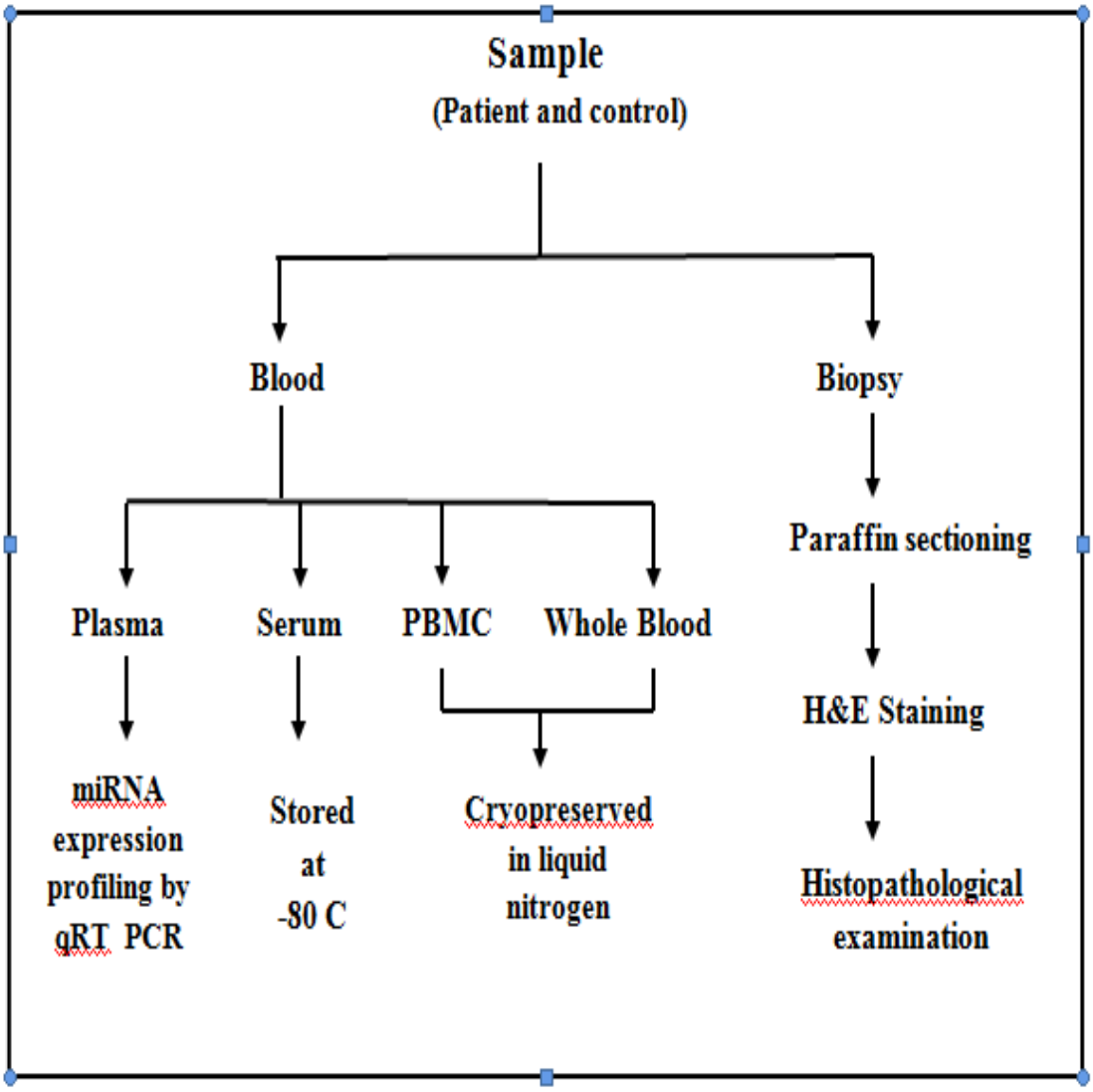
Fig. 14 : Interaction among Interleukin-17 (IL-17) release by Th17 cells and IL-17 receptor family members and resultant biological roles (Kirkham, Kavanaugh et al. 2014)

Chapter 3

MATERIAL AND METHODS

CHAPTER 3

MATERIAL AND METHODS



Experimental work Plan

3.1 Sample collection

Psoriasis patient samples **(Blood and Biopsy)** were collected with specific criterias from Dermatology Department, **Indira Gandhi Medical College (IGMC),** Shimla , Himachal Pradesh with due patient consent.

Specifications 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)
    1. Patients who have **not received topical therapy for at least 2 weeks** .
    2. Patients who have **not received UV/PUVA for at least 1 month**
    3. Patients who have **not received systemic therapy for at least 1 month.**

Materials required

Vacutainers with anticoagulant (sodium citrate),1X PBS (Appendix-A**)**, 10% NBF (Appendix-A**)**, Sterile gloves , surgical scalpel, 5 mm punches, 1ml and 10 ml sterile syringes.

Samples Collection Method :

- **Patient Blood**: Total 7 ml of blood collected through 10 ml sterile syringe
    - 6 ml in sodium citrate vacutainers (with anticoagulant)
    - 1 ml in 1.5 ml vial (without anticoagulant)

  Control Blood : collected from a healthy individual ,as above in sodium citrate vacutainers (with anticoagulant)

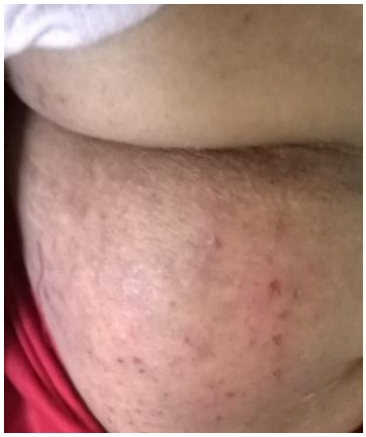
- **Patient Biopsy**: The skin is swabbed with an antiseptic and then injected with a local anaesthetic 5 mm punch biopsy of patient lesional skin collected by 5 mm punches and cut with surgical scalpel. **(**cut in two half: 2 mm in PBS and 3 mm in 10% NBF, collected in 2 ml vial)

  Control Biopsy: collected as above from uninvolved skin (non-lesional) of the patient, 5 cm away from the lesion. **(**cut in two half: 2 mm put in PBS and 3 mm put in 10% NBF, collected in 2 ml vial)

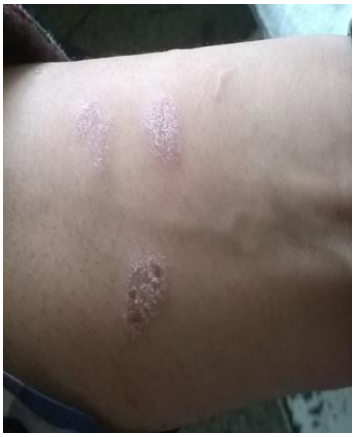
Patient ID No.	Age(years)	Sex	BSA (%)	Severity
PSOR 1 (P1)	43	Female	15%	Severe
PSOR 2 (P2)	70	Female	>10%	Severe
PSOR 3 (P3)	25	Male	11%	Severe
PSOR 4 (P4)	18	Male	5-10%	Moderate
PSOR 5 (P5)	30	Male	5%	Moderate
PSOR 6 (P6)	23	Male	>15%	Severe
PSOR 7 (P7)	30	Male	20%	Severe
PSOR 8 (P8)	30	Male	10%	Severe

Table 2 : Details of Psoriasis patients (Psoriasis *vulgaris*) selected for samples collection

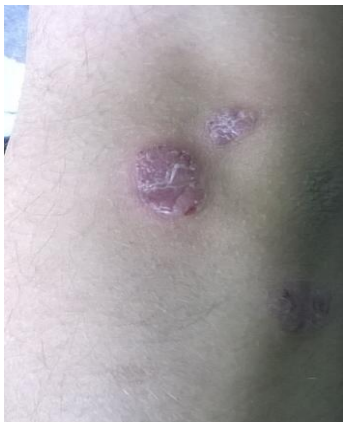
PSOR 1



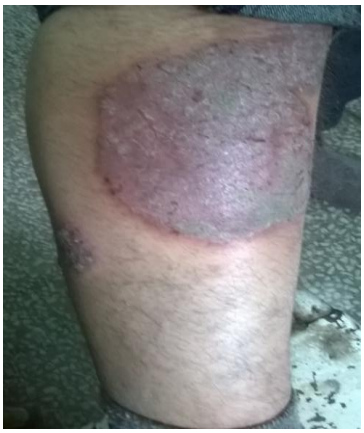
PSOR 2



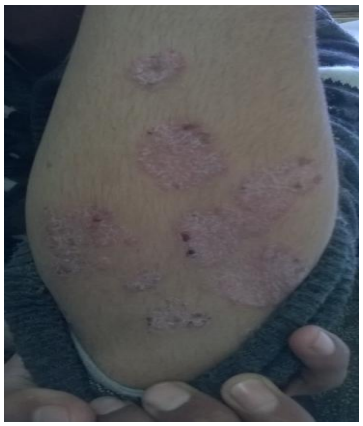
PSOR 3



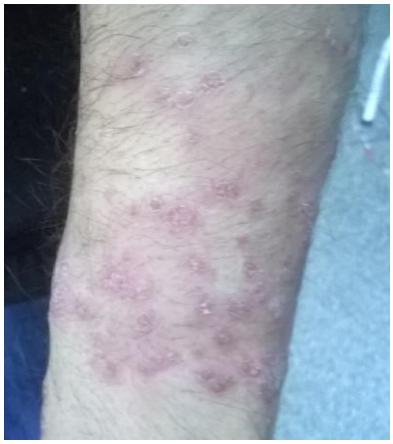
PSOR 4



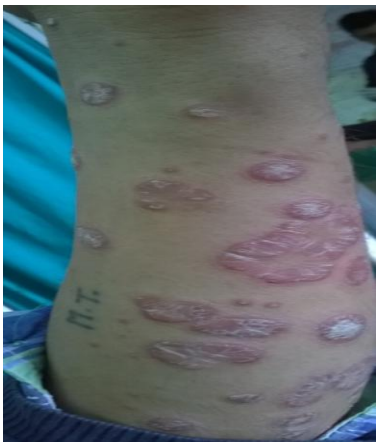
PSOR 5



PSOR 6



PSOR 7



PSOR 8

Fig. 15: Photographs of patients affected by Psoriasis having variable lesional morphology and disease severity of affected body parts

3.2 Blood sample processing

Materials required

DMSO, Ficoll , 1X PBS (Appendix-A**)**, FBS , Trypan blue dye.

Methodology

Patient and control blood samples were processed in Biosafety Laminar hood as follows:

3.2.1. Whole blood cryopreservation: 1 ml of whole blood (from total 6 ml whole blood with anticoagulant) for each sample is cryopreserved by adding 100µl DMSO (10% of volume of whole blood) in a well labelled cryovial and stored in liquid nitrogen.

3.2.2. Serum isolation: 1ml blood collected in 1.5 ml vial (without anticoagulant) is proceded for serum isolation by spining at 3400 g for 10 mins at RT and later serum stored at -80ºC.

3.2.3. 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. These cells are a critical component of the immune system, playing an integral role in the body's defenses. 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 lesser or greater than itself as : top most layer of pale yellowish layer of **plasma,** followed by whitish buffy coat of **PBMCs ,**then a layer of **ficoll**, followed by reddish layer of **granulocytes** and **erythrocytes**. PBMCs are widely used in both research and clinical laboratories and the separation of PBMCs from blood by centrifugation constitutes an extremely important step for all subsequent analyses, such as in immune monitoring.

Protocol: Remaining 5 ml whole blood (with anticoagulant) is proceeded for plasma and PBMC isolation as follows:

- Diluted whole blood with an equal volume of 1X PBS
- Overlaing of diluted whole blood above ficoll (1:3 ratio)
- Centrifuged (swinging bucket rotor centrifuge) at 2000 rpm for 15 mins at RT

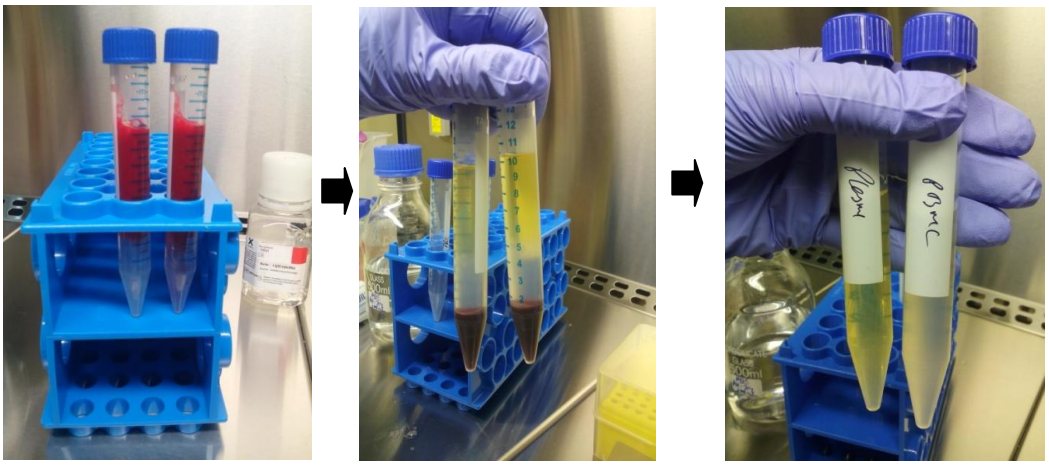
- Whole blood separated into **plasma** (top yellowish layer , stored at -80°C**)** and **PBMC** lower whitish buffy coat)
- Collected the PBMCs (whitish layer) from the diluted plasma/Ficoll interface and transfer it into another 15 ml falcon tube
- 1st washing with 1X PBS + FBS (5% of volume of PBMC) and centrifugation at 2000 rpm for 10 mins at RT and discarded the supernatant
- 2nd wash with 1X PBS + FBS (5% of volume of PBMC) and centrifuged at 2000 rpm for 10 mins at RT and discarded the supernatant
- Suspended the PBMC pellet in 1ml 1X PBS and took 10 µl of diluted PBMC in for PBMC count and rest is centrifuged at 2000 rpm for 10 mins
- Discarded the supernatant , resuspend the pellet in 900 µl FBS and transfer it into well labelled cryovial and cryopreserved by adding 100 µl of DMSO .
- Stored in -80ºC and transfered the cryovial in liq.N_2 next day.

PBMC cell counting by Hemacytometer

- **Cell count Calculation**

Dilution factor = [sample vol. + vol. of trypan blue] / Vol. of sample

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



Blood overlay on Ficoll **Plasma and PBMC separation** **layers after centrifugation**

Fig. 16 Sequential steps in Plasma and PBMC isolation by Ficoll density gradient centrigugation

3.3 RNA isolation from Blood Plasma

3.3.1 Total RNA isolation using TRI Reagent BD

Total RNA present in blood plasma samples was isolated using TRI Reagent BD solution, which is a mixture of guanidine thiocyanate and phenol in a mono-phase solution. When a sample of blood derivatives is lysed with it, and chloroform is added, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA and an organic phase containing proteins.

Materials required

TRI Reagent BD (sigma-aldrich Cat. No.T3809) ,Chloroform, Isopropanol, 75% Ethanol , 5 N Acetic acid(Appendix-A**)**, Nuclease free water, DEPC-treated water(Appendix-A**)**, spike – in control (ce-miR-39)

Protocol

- Added 200 µl of plasma to 750 µl of TRI Reagent BD , supplemented with 20 µl of 5 N acetic acid per 0.2 ml of whole blood or plasma and allowed to stand for 5 minutes at RT.
- Added 3.5 µl of Spike-In Control.
- Added 200 µl of chloroform per 0.75 ml of TRI Reagent BD, shaked vigorously for 15 seconds, allowed to stand for 2-5 minutes at RT and Centrifuged the mixture at 12,000 x g for15 mins at 4 °C to separate the phases
- Phase separation occurs as : A colorless upper aqueous phase (**containing RNA**) ,an interphase (containing DNA) and lower red organic phase (containing protein).
- Transfered the aqueous phase to a fresh vial and added 0.5 ml of isopropanol per 0.75 ml of TRI Reagent BD ,Incubated at -20ºC for 30-60 mins after inverting vials and Centrifuged at 12,000 x g for 8 minutes at 4-25 °C.
- Removed the supernatant and washed the RNA pellet by adding 1 ml (minimum) of 75% ethanol per 0.75 ml of TRI Reagent BD
  (Samples can be stored at this point in ethanol at 4 °C for 1 week and up to 1 year at –20 °C)
- Vortexed the sample and centrifuged at 7,500 x g for 5 minutes at 4-25 °C
- Briefly dried the RNA pellet for 5-10 minutes by air-drying or under a vacuum and Resuspend the pellet in an appropriate volume of nuclease free water and stored at -20ºC

3.3.2 Total RNA including miRNA isolation using *miRNeasy Serum/PlasmaKit (QIAGEN)*

The **miRNeasy *Serum/Plasma Kit*** method combines phenol/guanidine-based lysis of samples and silica membrane–based purification and isolation of cell-free total RNA, which primarily includes small RNAs such as miRNAs, from small volumes(up to 200 µl) of plasma, using QIAzol Reagent which is a monophasic solution of phenol and guanidine thiocyanate using the miRNeasy Serum/Plasma Kit. Blood samples are homogenized in QIAzol Lysis Reagent. After addition of chloroform, the homogenate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The sample is then applied to the RNeasy MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of RNase-free water. Serum and plasma contain primarily small RNAs, therefore enrichment of miRNAs and other small RNAs in a separate fraction is usually not required.

Materials required

miRNeasy Serum/PlasmaKit (QIAGEN) with the following components -RNeasy® Mini Spin Columns (each packaged with a 2 ml Collection Tube) ,Collection Tubes (2 ml), QIAzol ® Lysis Reagent, Buffer RWT, Buffer RPE , RNase-Free Water ,Chloroform (without added isoamyl alcohol) ,Ethanol (70% , 80% 96–100%), Sterile, RNase-free pipette tips, spike in control(ce-miR-39)

Protocol

- Added 1000 µl QIAzol Lysis Reagent (5 times the volume of sample) to 200 µl of plasma samples and mixed by vortexing or pipetting up and down and placed the tube containing the lysate on the benchtop at RT (15–25°C) for 5 min
- Added 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 108 copies/µl working solution) and mix thoroughly.

- Added 200 µl of chloroform of an equal volume to the starting sample to the tube containing the lysate and placed the tube containing the lysate on the benchtop at room temperature (15–25°C) for 2–3 min.
- Centrifuged for 15 min at 12,000 x *g* at 4°C and the sample separated into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase
- Transfered the upper aqueous phase to a new 2 ml collection tube and added 1.5 volumes of 100% ethanol .*Continue without delay with next step
- Pipetted up to 700 µl of the sample, into an RNeasy MinElute spin column in a 2 ml collection tube (supplied with the kit) and centrifuged at 8000 x *g* (10,000 rpm) for 15 s at RT.
- Discarded the flow-through and reuse the collection tube in next step
- Repeated the previous step using the remainder of the sample
- Discarded the flow-through and reuse the collection tube in next step
- Added 700 µl Buffer RWT to the RNeasy MinElute spin column and centrifuged for 15 s at 8000 x *g* (10,000 rpm) to wash the column.
- Discarded the flow-through and reuse the collection tube in next step
- Pipetted 500 µl Buffer RPE onto the RNeasy MinElute spin column and centrifuged for 15 s at 8000 x *g* (10,000 rpm) to wash the column.
- Discarded the flow-through and reuse the collection tube in next step
- Pipetted 500 µl of 80% ethanol onto the RNeasy MinElute spin column and centrifuged for 2 min at 8000 x *g* (10,000 rpm) to wash the spin column membrane.
- Discarded the collection tube with the flow-through
- Placed the RNeasy MinElute spin column into a new 2 ml collection tube (supplied),opened the lid of the spin column, and centrifuged at full speed for 5 min to dry the membrane.
- Discard the collection tube with the flow-through
- Placed the RNeasy MinElute spin column in a new 1.5 ml collectiontube (supplied) , added 14 µl RNase-free water directly to the center of the spin column membrane and centrifuge for 1 min at full speed to elute the RNA

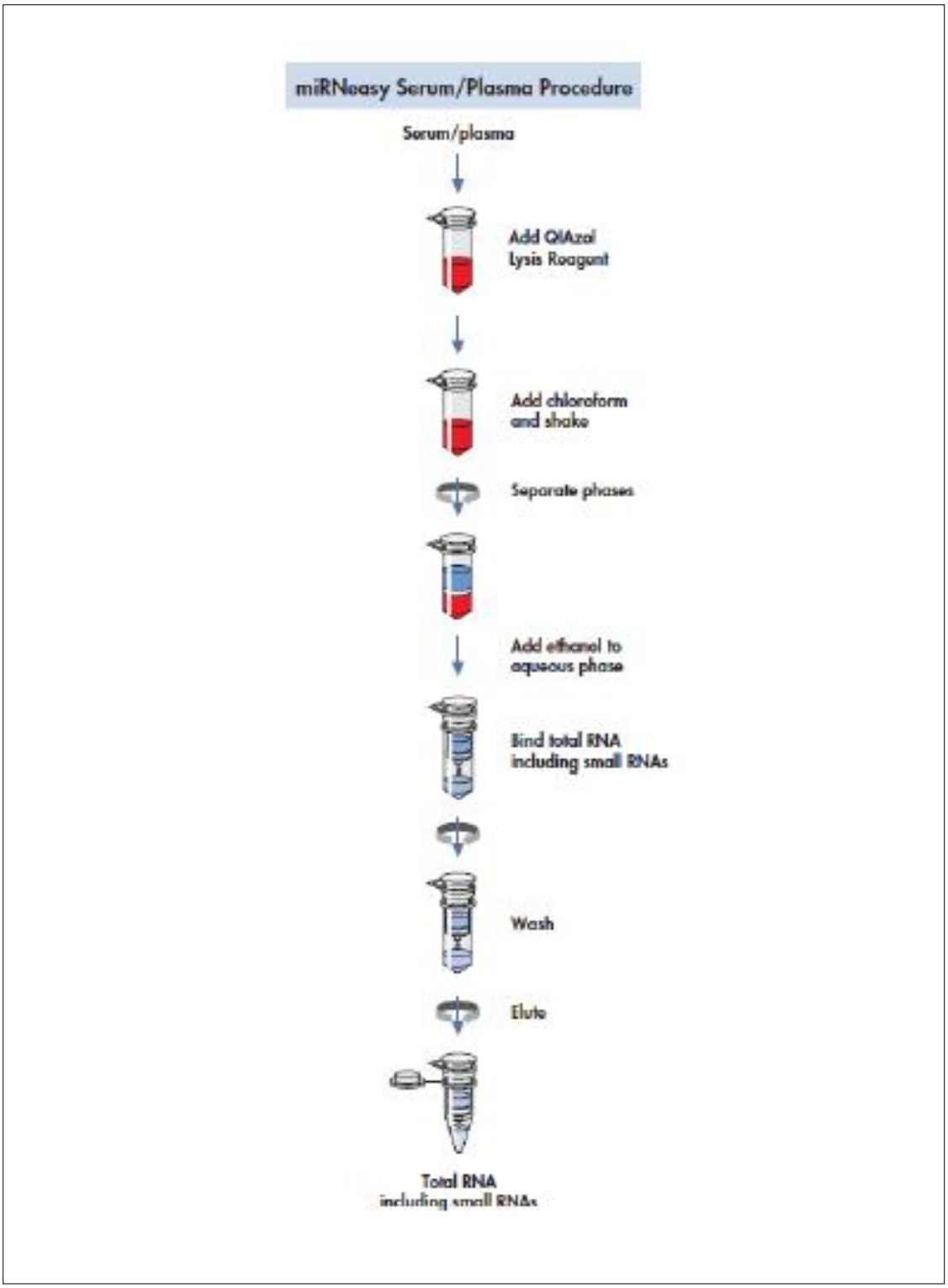


Fig. 17: Sequential diagramatic representation of steps involved in Total RNA including miRNA isolation by miRNeasy *Serum/Plasma Kit*

Nanodrop estimation of RNA purity and concentration

Quantification of RNA

Nucleic acids are traditionally quantified using UV absorption using a spectrophotometer. In its simplest form 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 RNA should be determined by measuring the absorbance at 260 nm (*A*260) . (Small amounts of RNA can be accurately quantified using an Agilent® 2100 Bioanalyzer).

An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml .

Purity check of RNA

RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. Pure RNA has an A260/A280 of 2.1.

Also , it is important that not only the OD **A260/A280** ratio should be very close to 2.0, but that in addition, also the OD **A260/A230** ratio should be very close to 2.0. Specially, when isolating low amounts of RNA the OD A260/A230 ratio drops significantly to sometimes under the 1.0. This clearly indicates, contamination with chaotropic salts or rests of phenol or protein in the RNA solution .

Method: RNA quality check for purity and concentration was done by Multiskan™ GO Microplate Spectrophotometer (Thermo)

3.4 miRNA Expression Analysis

The deregulated miRNA expression under diseased condition can be assessed by miRNA expression analysis of specific miRNA in patient samples versus healthy control by qRT PCR. Mature miR-21 expression analysis for psoriasis patient samples versus control was done using miScript II RT Kit (Qiagen).

3.4.1 cDNA Synthesis

Mature miRNAs are naturally occurring, 22-nucleotide, noncoding RNAs that mediate posttranscriptional gene regulation. Unlike mRNAs, miRNAs are not polyadenylated in nature. When reverse transcription reactions is performed using miScript HiSpec Buffer, mature miRNAs and certain small nucleolar RNAs and small nuclear RNAs are selectively converted into cDNA. Mature miRNAs are polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. Polyadenylation and reverse transcription are performed in parallel in the same tube. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of mature miRNA in the real-time PCR step.

Materials required

miScript II RT Kit (Qiagen) with the following components for cDNA synthesis -10x miScript Nucleics Mix, miScript Reverse Transcriptase Mix, RNase free water, 5x miScript HiSpec Buffer

Protocol

cDNA synthesis was done using miScript II RT Kit (Qiagen) in in Thermocycler as per the following reaction conditions:-

- **Reverse transcription reaction components** :

Component	Volume/reaction
5x miScript HiSpec Buffer	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water + Variable Template RNA	13 µl
miScript Reverse Transcriptase Mix	1 µl
Total volume	**20 µl**

- **Thermel cycler incubation conditions:**
  - 60 min at 37ºC
  - 5 min at 95ºC

* cDNA dilution prior to PCR

For mature miRNA quantification depending on abundance of miRNAs of interest; ensure 50 pg–3 ng cDNA per PCR

Reaction dilution: As per the requirement, added at least 200 µl RNase-free water, (or more if necessary) to the 20 µl reverse transcription reaction

3.4.3 Real-Time PCR

cDNA prepared using the miScript II RT Kit in a real-time cycler (Bio-Rad CFX96).This protocol enables real-time PCR quantification of mature miRNA or noncoding RNA using target-specific miScript Primer Assays (forward primers) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix.

Materials required

miScript II RT Kit (Qiagen) with the following components for qRT- miScript Primer Assays (forward primers) , miScript Universal Primer (reverse primer) ,QuantiTect SYBR Green PCR Master Mix, RNase free water, cDNA Template

Protocol

Qrt PCR was done using miScript II RT Kit (Qiagen) in a real-time cycler (Bio-Rad CFX96) as per the following reaction conditions:-

- **Reaction setup for real-time PCR**

Component	Volume/reaction
2x QuantiTect SYBR Green PCR Master Mix	6.25 µl
10x miScript Universal Primer	1.25 µl
10x miScript Primer Assay	2.5µl
RNase-free water	1.25µl
Template cDNA	1.25µl
Total volume	**12.5**µl

Step	Time	Temperature	Additional comments
PCR Initial activation step	15 min	95ºC	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	15 s	94ºC	
Annealing	30 s	55ºC	
Extensions	30 s	70ºC	Perform fluorescence data collection.
Cycle number	40 cycles		

Cycling conditions for real-time PCR

- **Dissociation curves /Melting curve analysis**

A dissociation curve analysis performed to aid in verifying the specificity and identity of PCR product(s). Dissociation curve analysis is an analysis step built into the software of real-time cyclers.

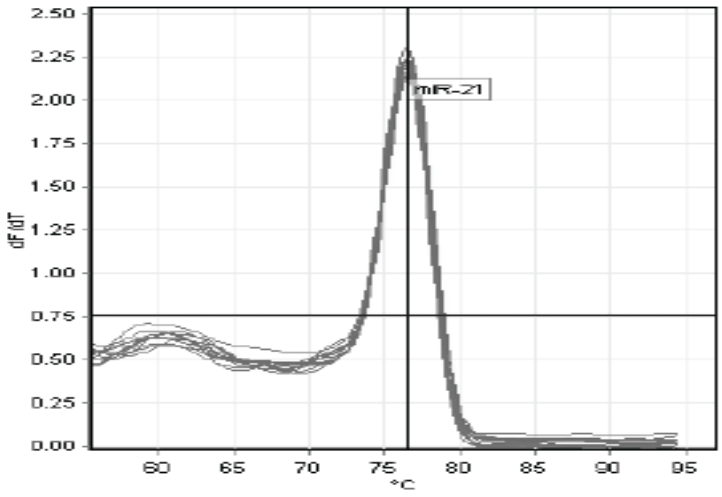


Fig. 18 . Dissociation curves. A Typical dissociation curve analysis of mature miRNA miR-21 PCR products showing single peaks from the specific amplification products.

3.5 Histopathological Examination (skin lesional biopsy samples):Paraffin sectioning and H&E staining

Histopathological examination (cutaneous pathology) is the microscopic examination of skin biopsies , transported in fixative (usually 10% neutral buffered formalin) in order to estimate the disease severity or to determine a specific diagnosis or a list of differential diagnoses. Diagnosis can be determined by the routine stain for tissue sections (Haematoxylin and Eosin).

H&E stain, or haematoxylin and eosin stain, is a popular staining method in histology. It is the most widely used stain in medical diagnosis. The stain works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features Haematoxylin is a basic stain that stains basophilic structures such as chromatin andribosome's a deep purple or blue. Eosin is an acidic stain that stains acidophilic structures red. The advantage of this stain is that it provides a clear stain of the cell nuclei; it provides a good stain for the cell membrane. These stains are not as specific for the tissue it stains. Nonetheless, this stain aided pathologists in diagnoses.

The staining method involves application of haematoxylin, which is a complex formed from aluminium ions and oxidised haematoxylin. This colors nucleus of cells (and a few other objects, such as keratohyalin granules) blue. The nuclear staining is followed by counterstaining with eosin, which colors eosinophilic other structures in various shades of red and pink.

Optimization may be necessary to achieve staining (haematoxylin may be diluted in H2O and eosin may be diluted in ethanol as needed).

Materials Required: Patient’s lesional and non-lesional skin biopsies stored in 10% NBF; Cassettes, 50 %, 70%, 80%, 90% and absolute alcohol, xylene, paraffin, tissue cutter, glass slides, eosin and haematoxylin stains, distilled water, 1% acid alcohol, DPX, coverslip.

Instruments required : Microtome, water bath, freezer, incubator, Hot plate

Protocol

3.5.1 Pre-processing of the tissue biopsy

- Biopsy samples transported in **fixative** (usually 10% NBF).*(It remains in this preservative for a minimum of 24 hours prior to processing. Formalin is a good fixative media by forming cross-links between lysine residues in the proteins of the tissues without alterations to their structure.)
- **Grossing** (Checking sample size,shape etc. and packing tissue in blotting sheets in cassettes with proper sample ID no.)
- The biopsies are processed in the tissue processing machine for 24 hours; this machine automatically processed the tissues in graded alcohols and xylene.
- **Dehydration** in ascending grades of alcohol 50%,70%, 80%, 90% or absolute alcohol .
- **Clearing with xylene** (4 steps -10 mins each)
- **Impregnation in molten paraffin** to remove xylene as it is not water soluble
- Tissue is ready for embedding

3.5.2 Embedding or block preparation

- Skin biopsies are **embedded in metal moulds filled with paraffin wax** using ice to hold the tissue in place.(with particular attention to the position of the biopsies to ensure orientation of the tissues from the epidermis down to the subcutaneous tissues)
- The cassette is positioned and the blocks are left in the ice to set.
- The mould is removed whilst cold and the excess wax is trimmed manually using a dissecting blade.
- The wax blocks are cooled in ice and sectioned at a thickness of 4 μm using a microtome.
- The sections are floated on a 40 ºC water bath and collected on adhesive slides to minimize section loss during heat-mediated retrieval.
- They are then incubated at 37 ºC overnight for 24 hours on slide racks, and kept in special slides container at room temperature.
- Slide ready for H&E staining

3.5.3 Haematoxylin & Eosin staining of Paraffin sections

- Put the slide on a hot plate to melt the wax with continuous observation
- Emmersed into three sets of xylene for 10 minutes each.
- Passed through descending grade of alcohols : 95%, 80%, 70%, 60% -2 mins each (to remove xylene as it will alter staining and does not dissolve in water).
- Rinsed with tap water (to remove the wax and dehydrate the sections).
- Slides are placed into **haematoxylin** for 20 minutes and rinsed by tap water for 4-5 minutes.
- Excess haematoxylin is removed by adding 1% acid alcohol (1% HCl in 70% (v/v) alcohol) for 5 seconds followed by a tap water wash.
- The pink haematoxylin stain is converted to blue by adding Scott's tap water, for approximately 10 seconds until the sections turned blue and then rinsed in tap water
- Stained in **eosin** (1% (w/v)) for 15 seconds with a subsequent wash in running tap water for 1-5 minutes.
- The sections are then dehydrated by ascending grades of alcohol.
- Two washes of xylene for 10 minutes each.
- Mounted in DPX mountant and covered with glass cover slips.

Fig. 19: Tissue trek automated machine for pre-processing of biopsy

Fig.20: Metal moulds for embedding

Fig. 21: Microtome

Fig. 22:Tissue floating waterbath

Fig.23: Paraffin embedded slides

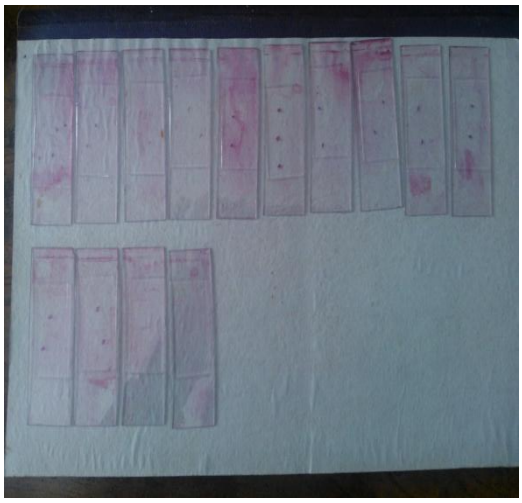


Fig. 24: H&E stained slides

Chapter 4

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results of PBMC count of Psoriasis Patient samples vs Healthy control

- **Psoriasis Patient samples count**

Patient Id	Age (Years) (☐ < 40yrs , ☐ > 40yrs)	Sex	PBMC Count (Cells/ml)
PSOR 1	43	Female	4.2 x 10^6 Cells /ml
PSOR 2	70	Female	2.6x 10^6 Cells /ml
PSOR 3	25	Male	2.4x 10^6 Cells /ml
PSOR 4	18	Male	3.97x 10^6 Cells /ml
PSOR 5	30	Male	4.45x 10^6 Cells /ml
PSOR 6	23	Male	7.3x 10^6 Cells /ml
PSOR 7	30	Male	4.25 x 10^6 cells/ml
PSOR 8	30	Male	5.2x 10^6 cells/ml

Table 3 : Psoriasis Patient samples count (Av. count : 4.30 x 10^6 Cells /ml)

- **Control samples count :**

Control ID	Age (years)	Sex	PBMC count (Cells/ml)
C1	21	Male	4.2x10^6 Cells/ml
C2	19	Male	2.16x10^6 Cells/ml
C3	21	Male	5.12x10^6 Cells/ml
C4	18	Female	3.14x10^6 Cells/ml
C5	21	Male	1.46x10^6 Cells/ml
C6	20	Female	1.82x10^6 Cells/ml
C7	19	Male	5.68 x10^6 Cells/ml
C8	19	Male	4.90 x10^6 Cells/ml
C9	20	Female	2.36x10^6 Cells/ml
C10	19	Female	3.30 x10^6 Cells/ml

Table 4 : Control samples count (Av. count : 3.41 x 10^6 Cells /ml)

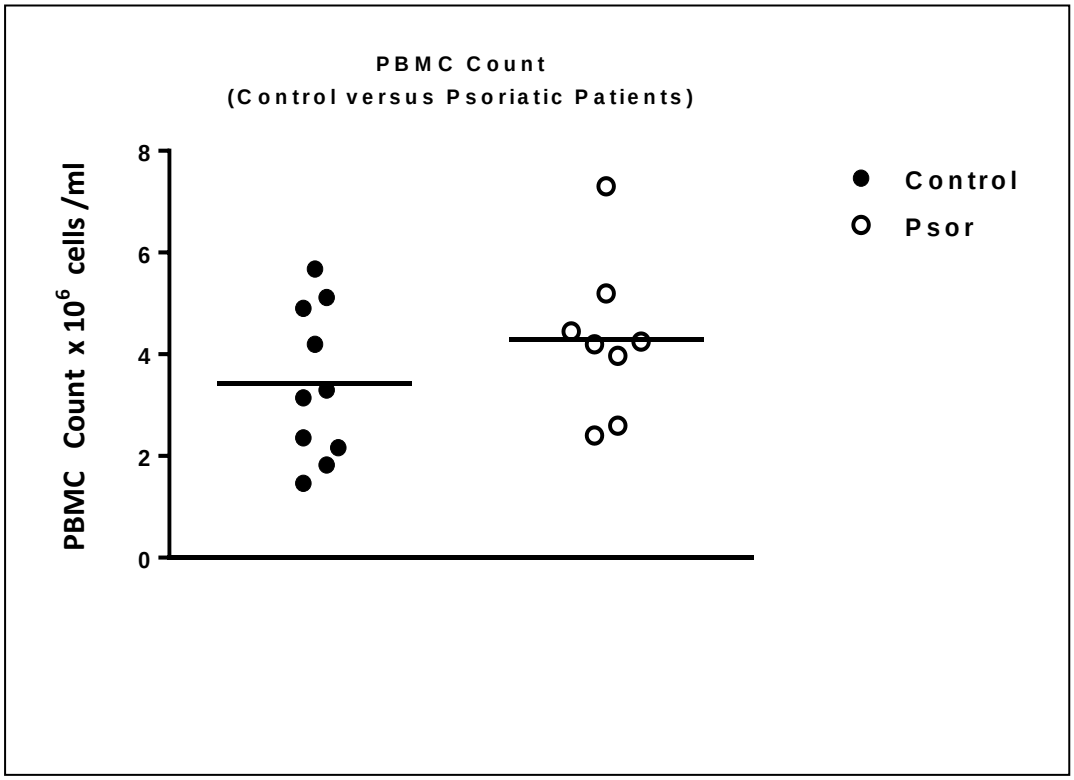


Fig. 25 : Scatter Plot (with mean) of PBMC count of Control versus Psoriatic Patients:

PBMC count of Control versus Psoriatic Patients (not statistically significant, p value = 0.2464 using Mann Whitney Test, $p < 0.05$ is significant, GraphPad Prism 5)

4.1.1 Discussion

There is no statistical significant difference in the PBMC count of control versus psoriatic patients. The reason for our result can be, small sample size and variability of PBMCs alterations depending on severity profile of patient. Here in the study moderate to severe patients have been sampled thus having variable severity profile which has direct relation with cell count alterations in psoriasis. All the more, there are variable reports suggests elevated number of PBMC count in peripheral blood of psoriasis patients correlate with disease severity but there are many other reports that show no significant difference in PBMC count of psoriasis versus control supported by the fact that individual cell type count may differ.

4.2 **Results of qRT PCR expression analysis** of miR-21 in psoriasis patient samples versus healthy control

Quantitation Data

A

Well	Fluor	Content	Target	Sample	Threshold Cycle (C(t))
A01	SYBR	Unkn	Ce_miR-39	P1	19.99
A12	SYBR	NTC	Ce_miR-39		28.81
B01	SYBR	Unkn	Hs_miR-21	P1	27.18
C01	SYBR	Unkn	Ce_miR-39	P3	17.00
D01	SYBR	Unkn	Hs_miR-21	P3	21.68
E01	SYBR	Unkn	Ce_miR-39	Cntrl R	26.04
F01	SYBR	Unkn	Hs_miR-21	Cntrl R	16.36
G01	SYBR	Unkn	Ce_miR-39	Cntrl An	27.26
H01	SYBR	Unkn	Hs_miR-21	Cntrl An	16.16
H12	SYBR	NTC	Hs_miR-21		N/A

B

Quantitation Data

Well	Fluor	Content	Target	Sample	Threshold Cycle (C(t))
A01	SYBR	Unkn	Ce_miR-39	P1	23.20
H01	SYBR	Unkn	Ce_miR-39	P3	19.27
H12	SYBR	Unkn	Ce_miR-39	Cntrl An	27.06

Table 5 : Threshold cycle C(t) values : A. For expression of miR-21 and Ce-miR-39 for psoriatic samples and control . B. for standardization of Ce-miR-39 expression in psoriatic samples .

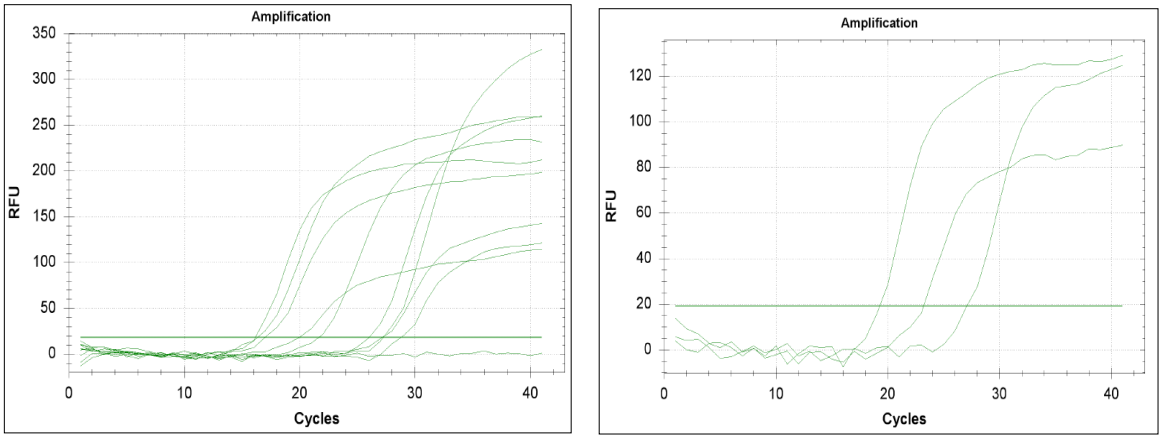
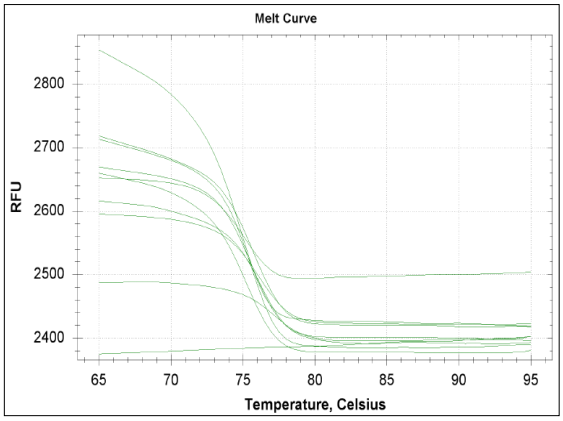


Fig . 26 : Amplification Plots for data A and B in table 5



Melt Curve Data

Well	Fluor	Content	Sample	Melt Temp
A12	SYBR	NTC		75.00
B01	SYBR	Unkn	P1	76.00
C01	SYBR	Unkn	P3	76.00
D01	SYBR	Unkn	P3	75.00
E01	SYBR	Unkn	Cntrl R	75.50
F01	SYBR	Unkn	Cntrl R	75.50
G01	SYBR	Unkn	Cntrl An	75.00
H01	SYBR	Unkn	Cntrl An	75.50

Fig. 27 : Melt Curve and Melt curve data showing melting temperature values for qRT expression analysis for samples in table 5 .A

Samples	Concentration (ng/µl)	A260/A280	A260/A230
PSOR 1	11.2	1.57	0.21
PSOR 2	31.8	2.25	0.406
PSOR 3	7.2	- 1.7	0.164
PSOR 4	22.7	1.81	0.416
PSOR 5	1.84	- 0.213	0.00141
PSOR 6	2.0	- 0 368	0.109
PSOR 7	29.8	1.57	0.34
PSOR 8	8.4	-1.75	0.0809

Table 6 : Nanodrop readings for isolated psoriasis RNA samples

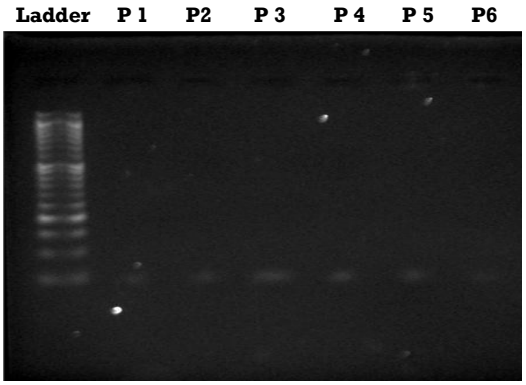


Fig. 28 :2.5% agarose gel in 1 X TBE for samples P1-P 6

4.2.1 Discussion

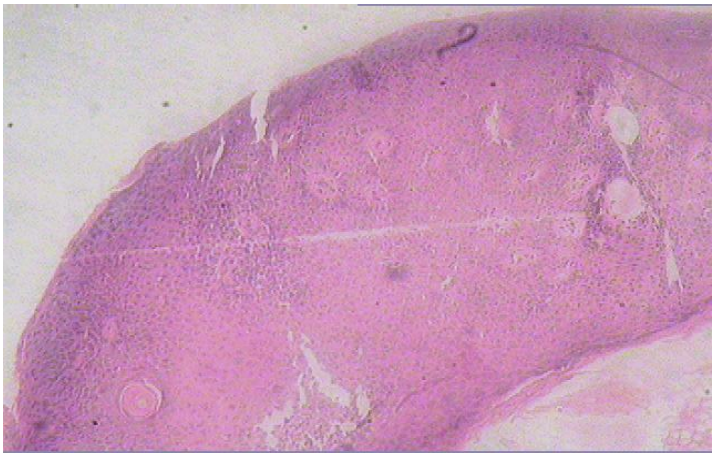
Expression analysis of miR-21 by qRT PCR show high expression (low Ct values) of internal control, i.e. Ce-miR-39, that generally falls in a range of range of Ct-21-27.This was due to instrument error (Nanodrop) showing inaccurate variable readings. Due to the erroneous readings of RNA concentration in psoriasis samples, approximated RNA sample was taken for cDNA synthesis (100 ng of RNA concentration in each sample). Furthermore, to carry on the work, agarose gel semi-quantitative analysis of cDNA psoriasis samples was done to get an idea of comparative concentration of cDNA in different samples . Dilution of cDNA was done as per the band intensity for different samples obtained on agarose gel to proceed for qRT PCR. PSOR 1(P1) and PSOR 3(P3) samples were analysed for miR-21 expression with respect to aged matched controls by qRT PCR but results again showed high expression in internal controls. Thus optimization of Ce-miR-39 expression baseline was needed to normalize the expression of internal controls in all samples. Thus another qRT PCR was set for the above standardization after further dilution of the above two psoriasis samples and the resultant expression of internal control were still not in the range and further work could not be carried out.

Thus, because of semi-quantitative estimations, the results of miR-21 expression in psoriasis samples cannot be relied on to have an estimate of comparative fold change between psoriasis versus control samples. Experiments need to be repeated further, to report the miR-21 and miR-215 expression in psoriasis vs control samples in light of the current literature to arrive at concrete expression pattern of the said miRNAs

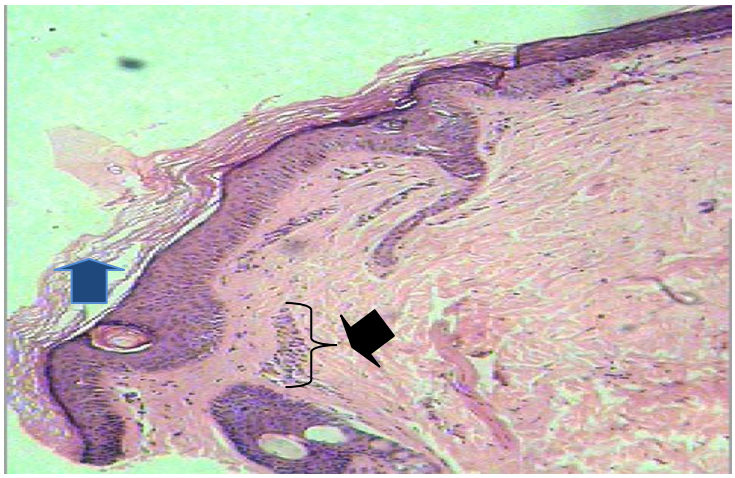
4.3 Results of Histopathological examination of Psoriasis lesional skin vs non - lesional skin

Fig.29: 10X H&E images of lesional versus non lesional skin biopsy

- **PSOR 1 (10X, H&E)**



A. Lesional skin

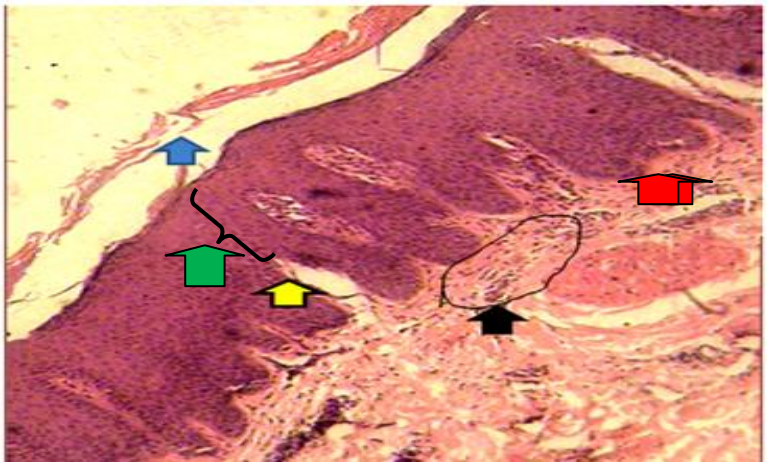


B. Non- lesional skin

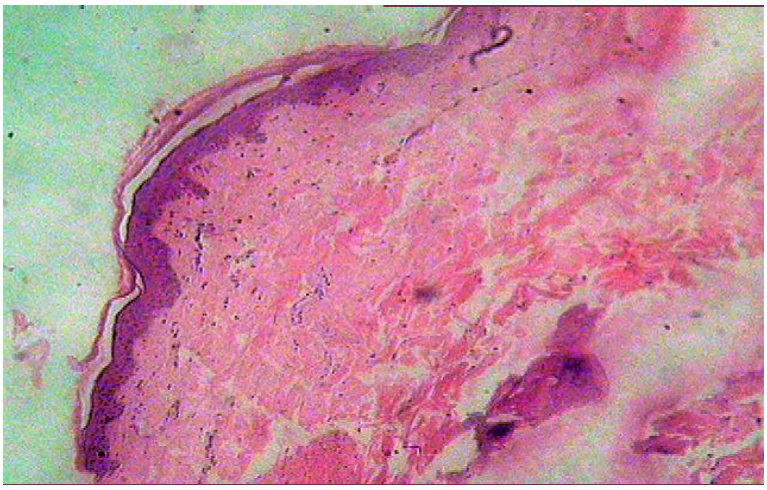
Observations

- Bad slide lesional skin due to improper sectioning of biopsy.
- Non-lesional skin section also shows scaling over epidermis with slight inflammatory cells

- **PSOR 2 (10X, H&E)**



A. Lesional skin

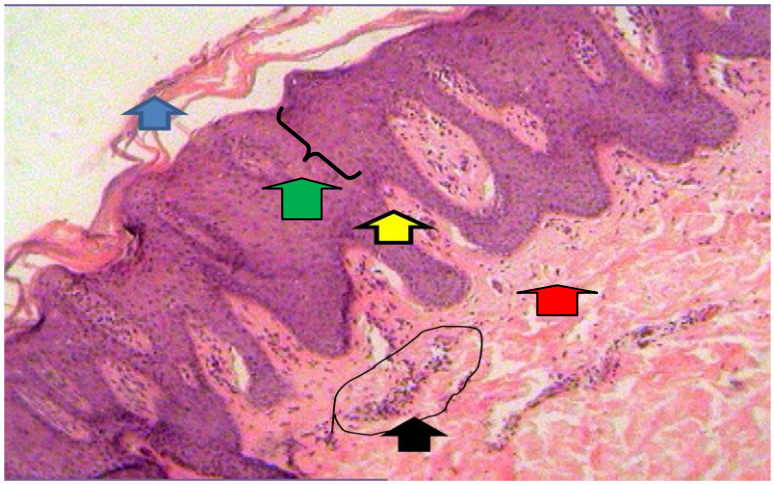


B. Non- lesional skin

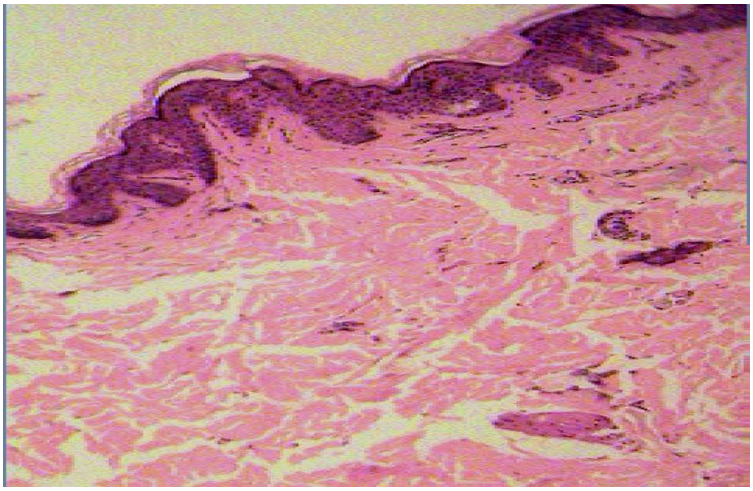
Observations

- Mild Hyperkeratosis and Parakeratosis in the stratum corneum in the scales over epidermis in lesional skin as compared to non lesional skin. ( )
- Regular acanthosis or slightly club shaped elongated rete ridges with thickened epidermis in the lesional skin as compared to non lesional skin.( )
- Papillomatosis (elongation of dermal papillae)( ) & suprapapillary thining in the lesional skin as compared to non lesional skin ( )
- Increased concentration of Inflammatory infiltrate in the lesional skin as compared to non lesional skin. ( )

- **PSOR 3 (10X, H&E)**



A. Lesional skin

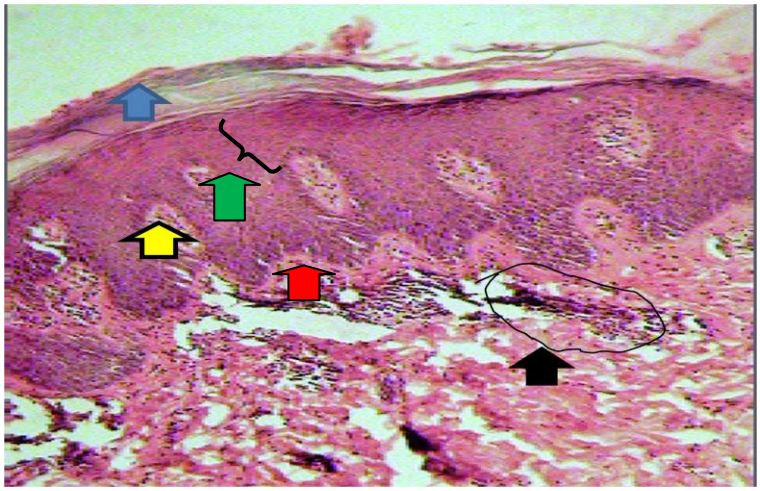


B. Non- lesional skin

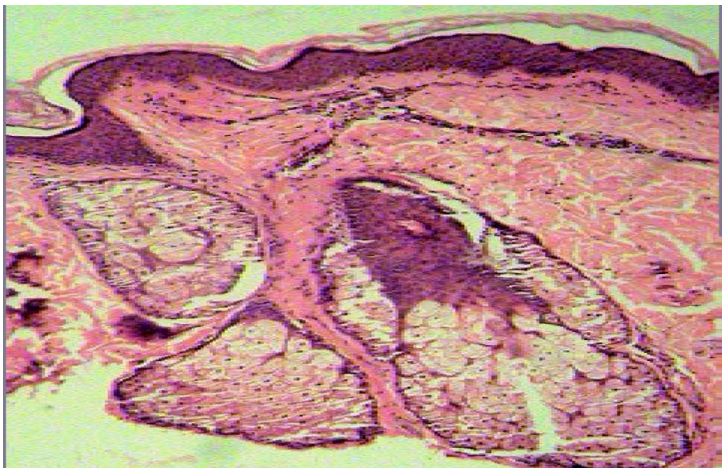
Observations

- Moderate Hyperkeratosis and Parakeratosis in the stratum corneum in the scales over epidermis in lesional skin as compared to non lesional skin. ( )
- Regular acanthosis or slightly club shaped elongated rete ridges with thickened epidermis in the lesional skin as compared to non lesional skin.( )
- Papillomatosis (elongation of dermal papillae)( ) & suprapapillary thining in the lesional skin as compared to non lesional skin ( )
- Increased concentration of Inflammatory infiltrate in the lesional skin as compared to non lesional skin. ( )

- **PSOR 4 (10X, H&E)**



A. Lesional skin

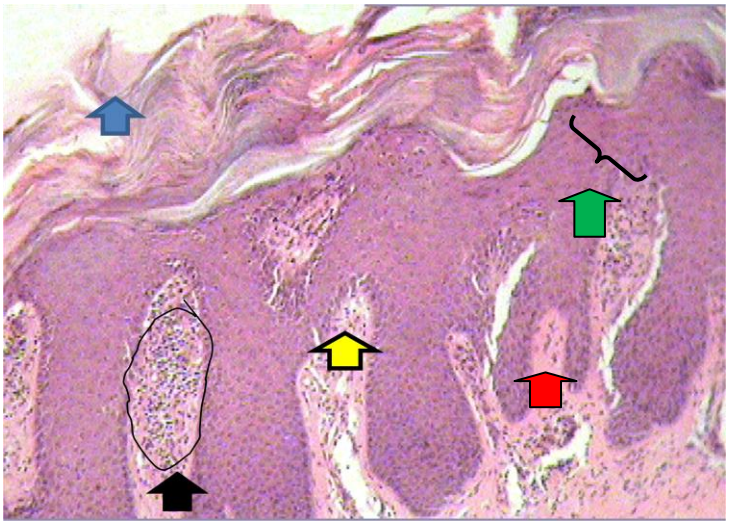


B. Non- lesional skin

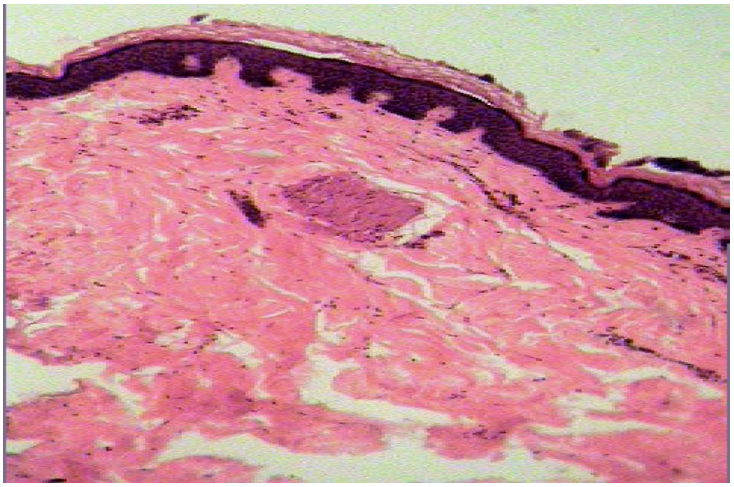
Observations

- Moderate Hyperkeratosis and Parakeratosis in the stratum corneum in the scales over epidermis in lesional skin as compared to non lesional skin. ( )
- Regular acanthosis or slightly club shaped elongated rete ridges with thickened epidermis in the lesional skin as compared to non lesional skin.( )
- Papillomatosis (elongation of dermal papillae)( ) & suprapapillary thining in the lesional skin as compared to non lesional skin ( )
- Increased concentration of Inflammatory infiltrate in the lesional skin as compared to non lesional skin. ( )

- **PSOR 5 (10X, H&E)**



A. Lesional skin

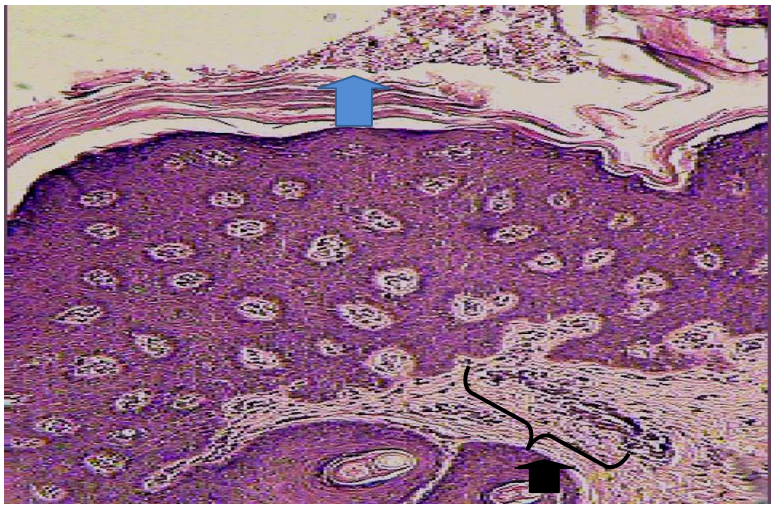


B. Non- lesional skin

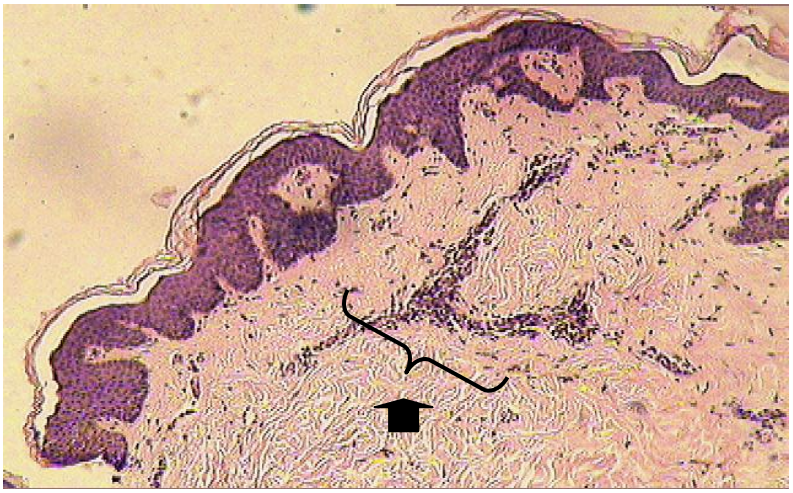
Observations

- Severe Hyperkeratosis and Parakeratosis in the stratum corneum in the scales over epidermis in lesional skin as compared to non lesional skin. ( )
- Regular acanthosis or slightly club shaped elongated rete ridges with thickened epidermis in the lesional skin as compared to non lesional skin.( )
- Papillomatosis (elongation of dermal papillae)( ) & suprapapillary thining in the lesional skin as compared to non lesional skin ( )
- Increased concentration of Inflammatory infiltrate in the lesional skin as compared to non lesional skin. ( )

- **PSOR 6 (10X, H&E)**



A. Lesional skin

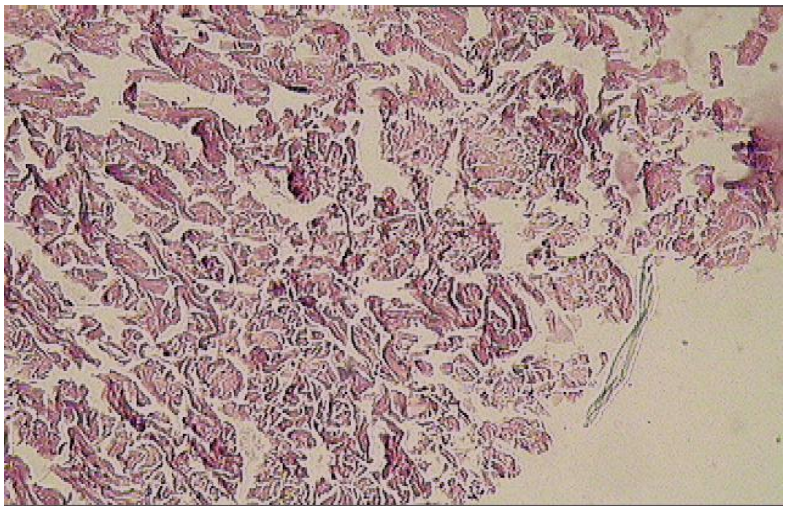


B. Non- lesional skin

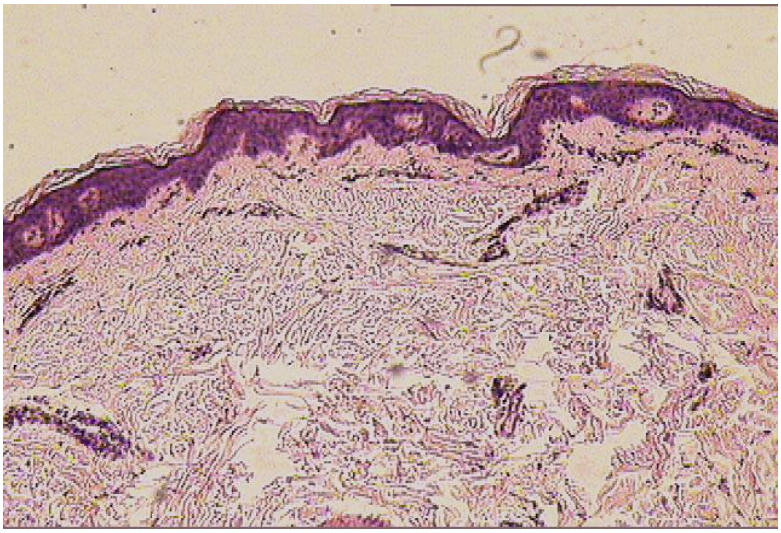
Observations:

- Improper lesional skin section due to wrong orientation
- Severe hyperkeratosis and parakeratosis in the stratum corneum in the scales over epidermis in lesional skin as compared to non lesional skin. ( )
- Comparable Inflammatory infiltrate in the lesional skin as well as in non- lesional skin. ( )

- **PSOR 7 (10X, H&E)**



A. Lesional skin

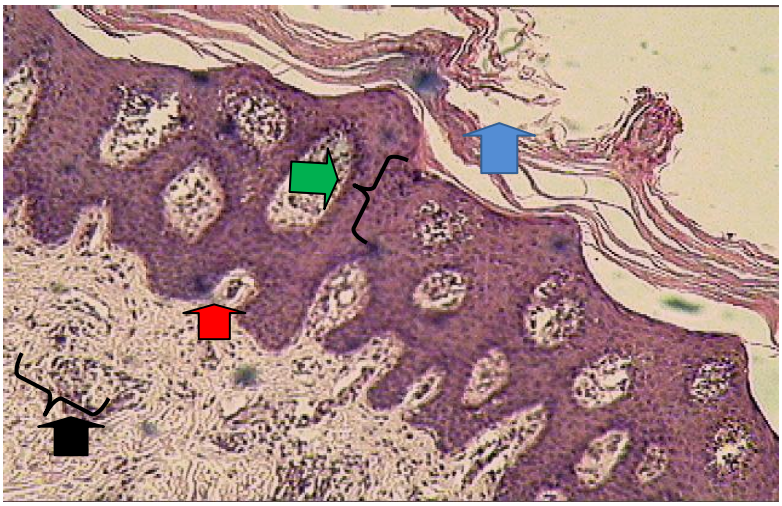


B. Non- lesional skin

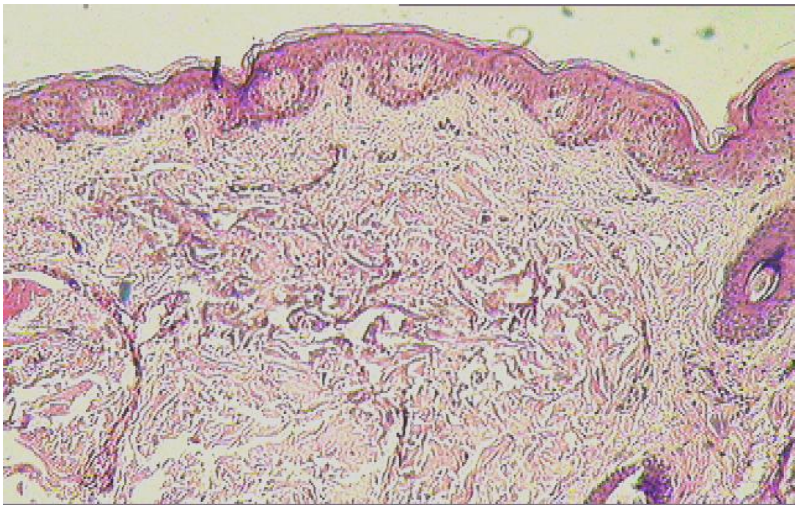
Observations:

- Improper lesional skin section due to sectioning in wrong orientation
- Non-lesional skin do not show any acanthosis and para- or hyperkeratosis with slight inflammatory infiltrate

- **PSOR 8 (10X, H&E)**



A. Lesional skin



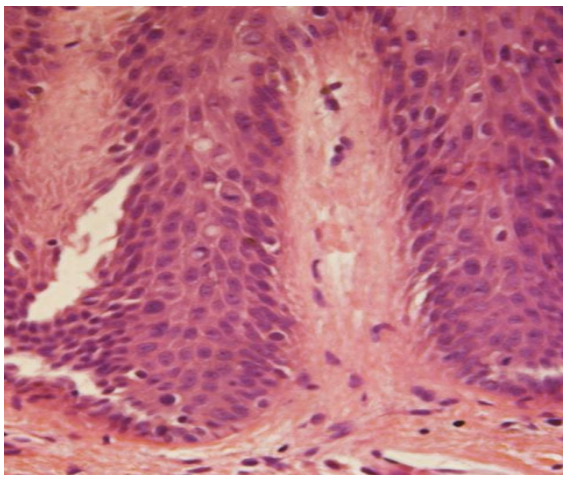
B. Non- lesional skin

Observations:

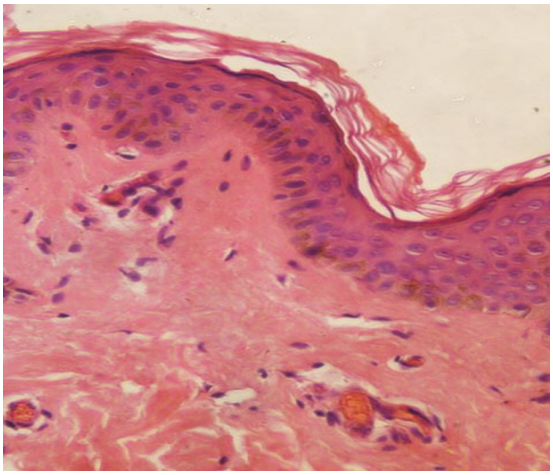
- Severe Hyperkeratosis and Parakeratosis in the stratum corneum in the scales over epidermis in lesional skin as compared to non lesional skin. ( )
- Regular acanthosis or slightly club shaped elongated rete ridges with thickened epidermis in the lesional skin as compared to non lesional skin.( )
- Suprapapillary thining in the lesional skin as compared to non lesional skin ( )
- Increased concentration of Inflammatory infiltrate in the lesional skin as compared to non lesional skin. ( )

Fig. 30: 40 X H&E images of lesional versus non-lesional skin of psoriasis patients

- **PSOR 3 (40 X H&E)**



A. Lesional skin

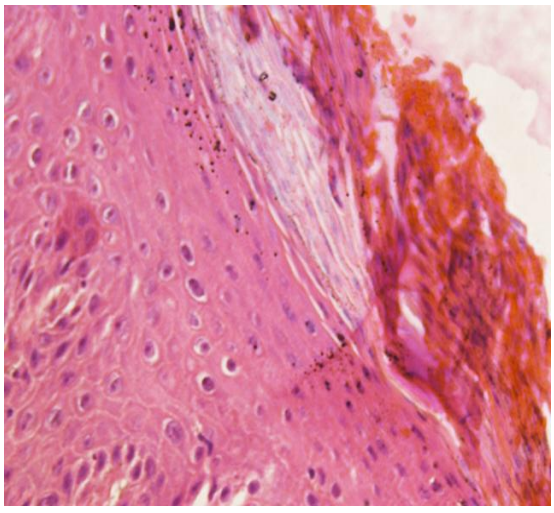


B. Non lesional skin

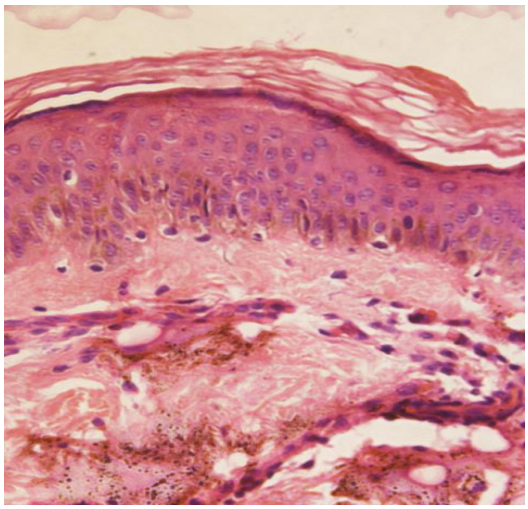
Observations:

- Lesional skin showingregular acanthosis , papillomatosis and inflammatory infiltrate in dermis as compared to non- lesional skin

- **PSOR 4 (40 X H&E)**



A. Lesional skin



B. Non lesional skin

Observations:

- Lesional skin showing profound scaling and severe parakeratosis above epidermis as compared to non- lesional skin

Sample No.	Acanthosis	Hyperkeratosis & Parakeratosis	Suprapapillary thining	Papillomatosis	Inflammatory infiltrate
PSOR 1	Bad slide (Improper Sectioning)				
PSOR 2	++	mild	+	+	+
PSOR 3	+++	Moderate	++	++	++
PSOR 4	+++	Moderate	++	++	+
PSOR 5	+++	severe	+++	+++	+++
PSOR 6	-	severe	-	-	+++
PSOR 7	Bad slide (Improper Sectioning)				
PSOR 8	+++	severe	+++	+	+++

Table 7: Histopathological features as observed in psoriatic patient lesional biopsy sections (10X , H&E)

4.3.1 Discussion

As per the observations, psoriasis lesional biopsy H&E images shows clear distinct charactersistic, histopathological features like acanthosis, para-and hyperkeratosisas, scaling, elongated dermal papillae ,inflammatory infliterates as compared to non lesional psoriasis biopsy, which is in accordance with what is reported in the literature .Out of 8 patients, **3 show severe** (PSOR 5, PSOR 6, PSOR 8) and **2 show moderate**(PSOR 4) and **1 show mild** (PSOR 2) histopathological disease profile. The observed features can be correlated with variable severity levels and extent of body surface area covered.

Although non-lesional uninvolved biopsy H&E image also shows marked inflammatory infilterate more than as seen in normal skin. This observation, corroborates with reports stating that potent inflammatory alterations also affect the uninvolved skin as an effect of disease on entire body.

Chapter 5

CONCLUSION AND FUTURE WORK

CONCLUSION

The present work explores

- the role of specific immunologically relevant circulatory miRNA candidates in psoriasis (that have not been profiled in circulation till date)
- associated histopathological alterations in correlation with disease severity

Specific miRNA expression analysis experiments could be done based on semi-quantitative estimations and thus needs to be repeated further due to unexpected results. Although histopathological analysis of lesional skin biopsy shows significant distinctive features as compared to non- lesional skin , showing significantly correlation with disease severity as reported in the literature.

Psoriasis is an incurable disease till date affecting a significant fraction of world's population, with no cure till date. The potential of miRNA to serve as an early biomarker and a more promising targeted therapy is envisioned. Thus further research is needed to understand the complete picture of role of miRNAs in immne-etiology of psoriasis.

Future Work

Project has been undertaken in light of the various research gaps in understanding of Psoriasis etiology, pathophysiology, associated cor-mobidities, lack of early efficient diagnostic biomarker and targeted therapies, and mainly on lack of understanding the role of miRNA as early prognostic and diagnostic biomarker in immune cells specific manner through profilling studies ,with a view of contributing a little to the research society as to have an impact on the lives of people living with and suffering from psoriasis.

Future prospects of the project include an extension of the current project in the following areas :-

- Validation of the profilled miRNAs and its putative T cell based targets in a larger cohort of people ,to predict it as a possible disease biomarker.
- FACS (Fluorescence Activated Cell Sorting) of the cryopreserved sample PBMCs to access the relative numbers of different cell types in patient samples versus healthy control .
- IHC (Immunohistochemistry) of sampled Paraffin biopsy sections for specific miRNA or its lesional targets and correlating it with peripheral profile .

- Analysis of the plasma/serum specific protein or cytokine levels in patient versus healthy control.
- Choosen candidate miRNA target gene polymorphism study in psoriasis Patient samples (blood or biopsy)
- Accessing the profiles of choosen candidate miRNAs before or after systemic therapies.
- Comparing candidate miRNA profile of miRNA in different psoriasis types.

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

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Brief Bio-data of Student

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