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miRNome analysis in psoriatic patients treated with anti IL-23: a cohort study

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Chapter One

INTRODUCTION

1.1 Psoriasis

1.1.1 Background

Psoriasis is a chronic immune-mediated inflammatory disease affecting between 2 and 3% of the population worldwide (1). It has a multifactorial etiology, caused by genetic risk factors that are associated with environmental triggers, such as intense mental and physical stress, surgery, infection, smoking or alcohol habits, and the use of some of drugs. The main genetic variations associated with the disease are single nucleotide polymorphisms (SNIPs) in gene sequences involved in the regulation of the immune response. To date, about 40 regions of the entire human genome associated with the risk of developing psoriasis have been identified (2). The most common form of the disease, called psoriasis vulgaris or plaque psoriasis, is characterized by the appearance of silvery erythematous plaques on the surface of the skin in different regions of the body, mainly knees, elbows, trunk, and scalp. In addition, like other immune-mediated inflammatory diseases, it is associated with a variety of comorbidities, such as psoriatic arthritis, metabolic disorders, and cardiovascular disease. These clinical manifestations are the result of persistent chronic inflammation with periods of exacerbation that results in altered proliferation and differentiation of keratinocytes and infiltration at the dermis and epidermis of immune effector cells (3), which induce increased cytokine production.

1.1.2 Pathogenesis

Psoriasis is considered a multisystem disorder, characterized by a generalized inflammatory state with predominant manifestation on the skin. This inflammation is triggered and maintained by a complex disruption of the immune system, particularly T cells. The pathogenesis of the disease is characterized by an initial phase, caused by the interaction between multiple genetic and environmental risk factors, and a maintenance phase, characterized by chronic clinical progression (4, 1). In the psoriatic plaque, keratinocytes produce large amounts of antimicrobial peptides (AMPs) capable of stimulating Toll-like receptors (TLRs) of plasmacytoid dendritic cells (pDc), specific cells of innate immunity

that mediate antigen presentation to cells of adaptive immunity. Thus, the maintenance phase of psoriatic inflammation is triggered, characterized by an adaptive immune response starring different groups of T lymphocytes (4). TNF-α stimulates proliferation and differentiation of T lymphocytes, Th1, Th17, and Th22 (1). IL-23 promotes the differentiation of Th17 and Th22 cells, while IL-12 promotes Th1 cells. Activated Th22 and Th17 cells secrete TNF-α, IL-17 and IL-22, which stimulate keratinocytes to proliferate and produce antimicrobial peptides, chemokines and inflammatory cytokines, such as IL-17 which is another therapeutic target. Consequently, a positive feedback loop is established, leading to the maintenance of the ongoing inflammatory process and the development of the psoriasis phenotype (1, 5). Although the pathogenesis of the disease is not yet fully elucidated, several studies suggest that dysfunction of regulatory T cells (Treg) and alteration of the balance between them and hyperactivated effector T cells (Th1, Th2, and Th17) may

also play a key role. Tregs are a subpopulation of T lymphocytes that can preserve immune balance and homeostasis, playing a key immunosuppressive role (1) (Figure 1).

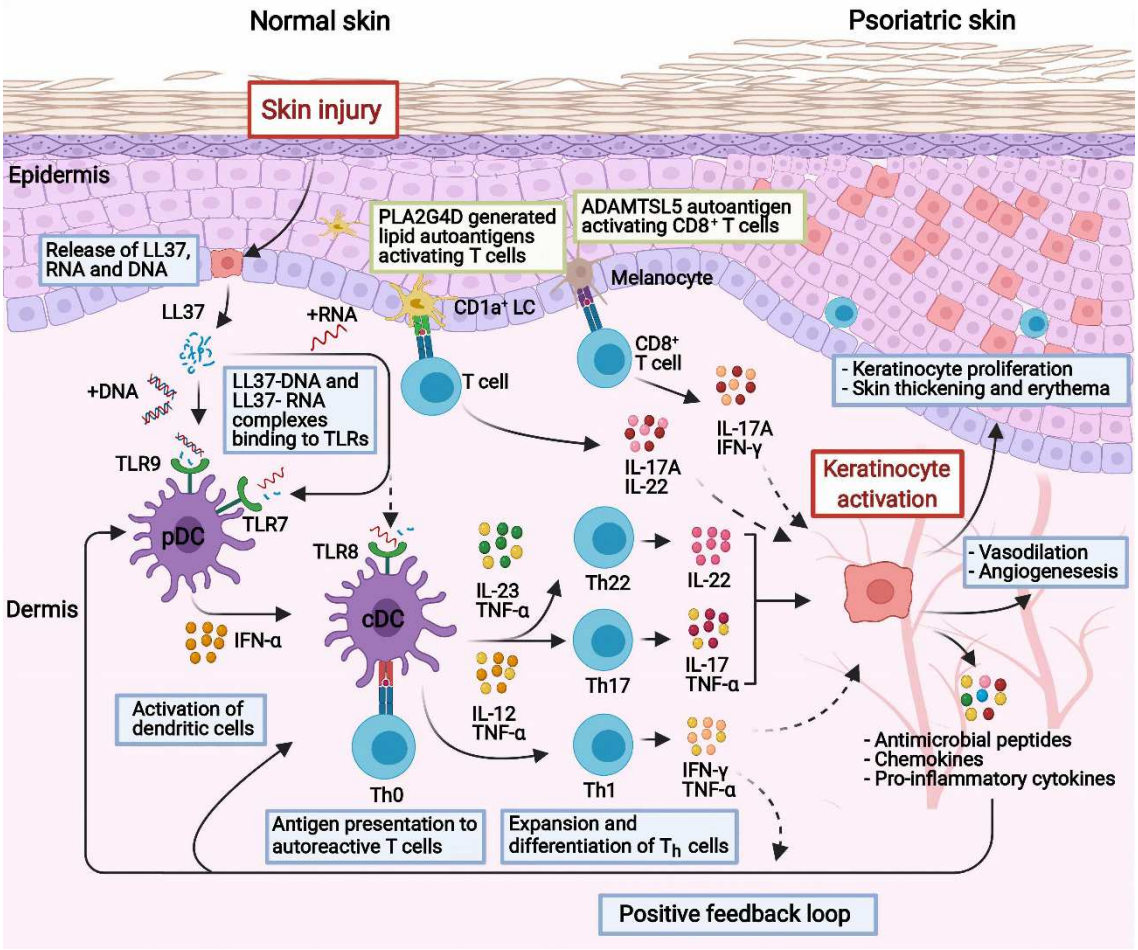

Figure 1. Outline of the pathogenesis of psoriasis. From: Ben Abdallah H, Johansen C, Iversen L. Key Signaling Pathways in Psoriasis: Recent Insights from Antipsoriatic Therapeutics. Psoriasis (Auckl). 2021 Jun 29;11:83-97. doi: 10.2147/PTT.S294173. PMID: 34235053; PMCID: PMC8254604.

1.1.3 Classification and clinical manifestations

The onset of psoriasis can occur at any age, with features varying from patient to patient. Its dermatologic manifestations reflect the pathogenetic mechanisms of inflammation, hyperproliferation, and angiogenesis that underlie the disease (6). The severity of the disease

is described according to several parameters, which allow defining mild, moderate, and severe psoriasis.

The main clinical indicators include:

- PASI (Psoriasis Area Severity Index). It takes into account the percentage of body surface area involved and the erythema, thickness and desuqmation of the plaques and is the most significant index for assessing disease severity. A PASI greater than ten defines moderate-severe psoriasis;
- BSA (Body Surface Area), expressed as a percentage value. It assesses the extent of the skin manifestation, and a BSA greater than ten defines moderate-severe psoriasis (7);
- PGA (Physician Global Assessment), does not quantify the body surface area affected or assess the locations of individual skin manifestations but is a global indicator of disease status. There are several forms of it, generally corresponding to a score of three or higher in moderate-severe psoriatic forms and zero or one in mild or successfully treated forms (8);
- DLQI (Dermatology Life Quality index), the most common indicator for assessing the impact of the disease on health-related quality of life and daily activities (9). It can be used for all skin diseases, allowing comparison between them. It consists of ten questions investigating symptoms and feelings, ability to perform daily activities in both work/school and leisure, the ability to maintain personal and social relationships and also any side effects of treatment in patients. The total score resulting from the questionnaire is between zero and thirty, where higher scores indicate greater impairment in quality of life (10).

There is no unified classification for the psoriatic clinical spectrum, so different approaches of evaluation are possible (11). It is possible to distinguish psoriasis into two groups according to the lesions that characterize it:

1) Non-pustular psoriasis

- Psoriasis vulgaris (early and late onset): the most frequently seen clinical form of psoriasis, psoriasis vulgaris, constitutes nearly 90% of cases. Clinically it is observed as erythematous plaques with sharp boundaries and covered with pearlescent squamae. Lesions demonstrate symmetric distribution, and they are most frequently localized on knees, elbows, scalp, and sacral region. Predilection for these lesions may be a result of traumatic incident results in "Koebner phenomenon" (12-13) . The plaques have a characteristic desquamation, referred to as "wax spot phenomenon", that represent a sign of parakeratotic hyperkeratosis. If psoriatic plaque is scraped further, a wet layer adhered to the lesion can be revealed. This is the last layer of the dermal papillae of the epidermis, and it is a pathognomonic sign of psoriasis, known as "last membrane phenomenon." Further scraping of the plaque reveals erythematous background and bleeding foci with appearance of small red pinpoints known as "Auspitz sign,'' signifying papillomatosis on tips of dermal papillae. Around healed psoriatic plaques, a hypopigmented macular ring can be observed, which is called "Woronoff ring" (12-14). The pathogenesis of this ring has not been fully clarified; however, it is thought to be related to decreasing levels of prostaglandin in healing lesions (15).

- Guttate psoriasis: this type of psoriasis is frequently seen in children and young adults. Lesions suddenly appear like small droplets and less frequently as squamous psoriatic papules, generally manifesting after streptococcal infections. This form of psoriasis is most frequently associated with HLA-Cw6 gene. Often, antistreptolysin titers are elevated. With regression of the infection, lesions generally disappear spontaneously. Lesions are usually seen on the trunk, proximal part of extremities, face, and scalp. They generally regress within 3–4 months. Sometimes, lesions enlarge and take the shape of psoriatic plaque (16).

- Erythrodermic psoriasis: psoriatic lesions affect nearly 80% of the body surface in this generalized form of psoriasis. Predominantly erythematous lesions are seen, and typical papules and plaques lose their characteristic features. Desquamation is not so distinct. In patients with erythrodermic psoriasis, hypothermia due to widespread vasodilatation can be seen. Desquamation may

also lead to protein loss and related systemic problems, such as oedema of the lower extremities, and cardiac, hepatic, and renal failure can occur. In addition, the protective barrier of the skin is impaired, leading to the potential development of systemic reactions. Most frequently, it develops as a complication of psoriasis vulgaris or can onset independently as erythrodermic psoriasis. Nail disorders may be very dramatic. Dermatopathic lymphadenopathy and severe pruritus may be observed. In the case of erythroderma, the presence of small areas of intact skin should be evaluated for psoriatic erythroderma or pityriasis rubra pilaris (PRP) erythroderma. There is no specific laboratory finding. There is a substantial risk of cardiovascular shock or septic shock; therefore, these findings should be followed closely. It is a severe, potentially fatal, and treatment-resistant clinical picture [12, 13, 17].

- Palmoplantar psoriasis: usually, this type of psoriasis symmetrically involves the palms of the hands and soles of the feet, and thenar regions are more frequently affected than hypothenar regions. Erythema is not always found, but it appears as a pinkish-yellow lesion when it exists. Squamae are the predominant lesions. Thick squamae may give the appearance of keratoderma (18).

- Inverse psoriasis: this form is localized in skinfolds. Lesions manifest as bright red, symmetric, infiltrative, fissured plaques with distinct contours (17). Fissured plaques with sharp contours are diagnostic for this form of psoriasis. It is more frequently seen in obese individuals, and there is a tendency to develop seborrheic lesions. This form is generally more resistant to classical treatments (17).

2) Pustular psoriasis

- Generalized pustular psoriasis (von Zumbusch type): this is a rarely seen form of psoriasis that progresses with pustules. It is most frequently seen in young individuals. It can develop independently or as a complication of psoriasis vulgaris, secondary to abrupt withdrawal of systemic steroid treatment, intervening triggering factors, hypocalcaemia, or irritant treatment. It onsets suddenly on an erythematous background in association with general symptoms,

such as high fever, lassitude, and polyarthralgia. An increase in sedimentation rate, leukocytosis, lymphopenia, and negative nitrogen balance can be seen. Pustules dry within a few days, followed by the eruption of new pustules. Peripustular erythema tends to disseminate, and thus, it can result in erythrodermia. It should be promptly treated. If the disseminated form is not treated, the acute phase may lead to a fatal course (17, 18, 19).

- Impetigo herpetiformis: this is a rare type of psoriasis, also known as generalized pustular psoriasis of pregnancy. It is characterized by erythematous lesions covered with pustules, which start and radiate from flexural regions and have a tendency to agglomerate. It may gain vegetative character at skin folds. During its course, involvement of mucous membranes and onycholysis secondary to subungual pustules can be seen. Lesions itch or cause a burning sensation and have a foul odour. In addition to deterioration of general health, symptoms of lassitude, fever, shivering, nausea, and vomiting may be present (12, 17). It is generally seen in association with hypocalcaemia. It may be seen in the last trimester of pregnancy or during the puerperal period. Frequently it recurs during subsequent pregnancies (12, 17).

- Palmoplantar pustular psoriasis (Barber type) it is a chronic, recurrent form more frequently seen in women and those with a family history of palmoplantar pustulosis. Clinically, it is observed as 2–4 mm-sized pustules localized on the palmoplantar region, especially erythematous thenar and hypothenar areas. While its etiology is not precisely known, underlying contact sensitivity is remarkable. Smoking, tonsillitis, humidity, and high temperature may activate the disease.

- Acrodermatitis continua of Hallopeau: it is a proximally progressive skin disorder characterized by sterile pustular eruptions involving fingers and toes, leading to loss of nails and distal phalanges in severe cases. Pustules become joined, resulting in small, polycyclic, purulent, fluid-filled vesicles. The presence of a variant of psoriasis is still debatable (20).

Significantly, the pathology does not affect only the skin district but has a systemic character, leading to inflammation of multiple organs or tissues. It is, therefore, associated with numerous comorbidities that may occur earlier, simultaneously or sequentially (Figure 2). Major systemic complications include depression (with severe anxiety and up to suicidal tendencies), cardiovascular disease, atherosclerosis, metabolic syndrome, diabetes, obesity, peptic ulcer disease, inflammatory bowel disease, and psoriatic arthritis (21). The general prevalence of PsA ranges between 0.02–0.1%, while its prevalence varies between 5.4–7% among psoriatic patients. In cases with severe skin involvement, particularly pustular psoriasis, the majority of PsA rises to 30–40%. Uncomplicated psoriasis usually onsets in the second or third decade of life, while the prevalence of PsA increases in the third decade. The average male-to-female ratio is 1:1 in PsA. In 75% of patients with PsA, psoriasis onsets before the appearance of arthritic symptoms, while in 15% of cases, skin lesions are seen concurrently with arthritis. In 10% of patients, arthritis manifests before emergence of skin lesions. Nail involvement is seen in 80% of patients with arthropathic psoriasis (10).

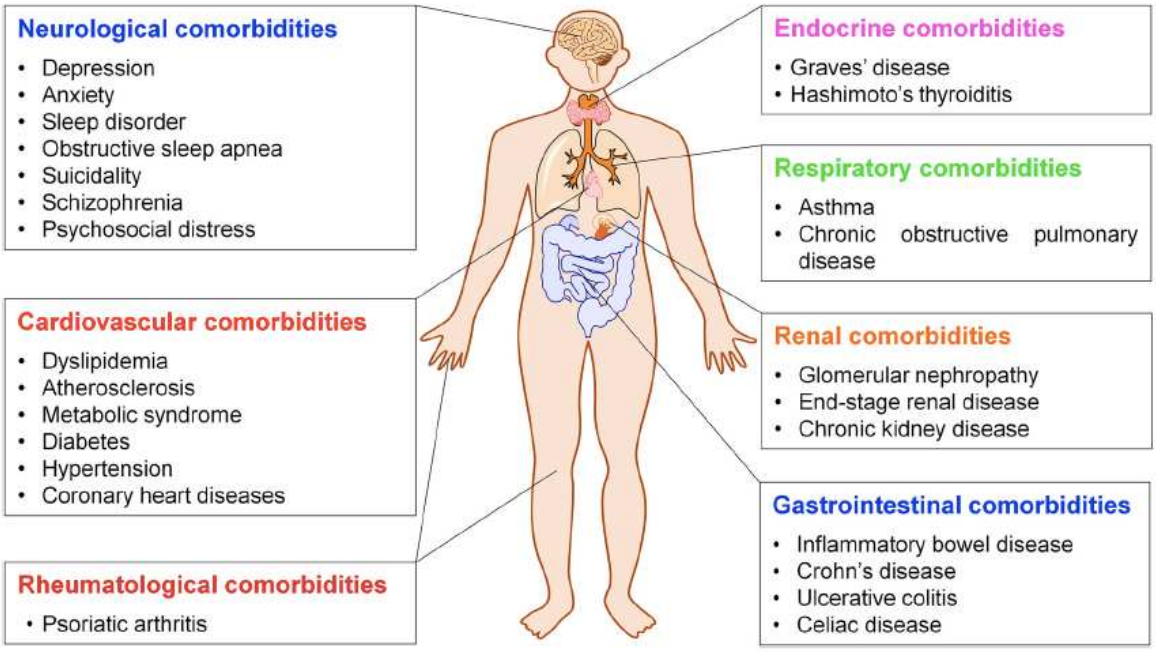

Figure 2. Comorbidities associated with psoriasis. From: Srivastava AK et al. Insights into interplay of immunopathophysiological events and molecular mechanistic cascades in psoriasis and its associated comorbidities. J Autoimmun. 2021 Mar;118:102614. doi: 10.1016/j.jaut.2021.102614. Epub 2021 Feb 9. PMID: 33578119.

1.1.4 Treatments

In recent decades, studies on the pathogenesis of psoriasis have focused on developing targeted and highly effective therapies. Although there is currently no definitive treatment for the condition, there are long-term therapies that allow complete, albeit temporary, remission (21). The clinician's choice of appropriate treatment is determined by the severity of the disease, following the guidelines of the International Psoriasis Foundation and the American Academy of Dermatology. Three main categories of therapies are available for treating plaque psoriasis: topical therapies, phototherapy, and systemic therapies (23). In mild to moderate forms of psoriasis, topical treatment of the lesions through glucocorticoids, vitamin D analogs, and phototherapy may prove sufficient. Conversely, moderate to severe forms of psoriasis often require systemic treatment (4).

Topical treatments

There are a large number of topical treatments for psoriasis. The most widely used are topical steroids and vitamin D analogs, used alone or in combination (22).

Glucocorticoids are a family of corticosteroids, steroid hormones synthesized by the adrenal cortex that act at the level of glucose metabolism. The mechanism of action of these hormones is involved in regulating the transcription of numerous genes, particularly those for proinflammatory cytokines. The effects of topical steroids, found in various formulations, dosages, and combinations, are anti-inflammatory, antiproliferative, immunosuppressive, and vasoconstrictive, allowing rapid symptom control and effectively decreasing patient itching (23-24).

Vitamin D decreases DNA synthesis and reduces hyperproliferation of keratinocytes. Due to their lower irritability, it is preferable to use synthetic vitamin D analogs, such as Calcipotriol, an active metabolite of vitamin D, and Calcipotriene, as topical treatment of the disease (23). It is believed that the mechanism of action of these molecules in psoriasis is mediated by their binding to vitamin D receptors, which leads to the modulation of keratinocyte proliferation and differentiation (24). Several studies have shown that vitamin D analogs are the most effective topical agents, except the more potent corticosteroids (25). The use of combination treatment of vitamin D analogues and topical corticosteroids is more effective than either agent alone for the treatment of psoriasis (22).

Phototherapy

Phototherapy is used in patients with extensive body surface area, and topical therapy with creams or ointments is impractical (23). Ultraviolet radiation can locally limit epidermal hyperproliferation, induce selective apoptosis of pathogenic T lymphocytes, and positively modulate levels of regulatory T cells (26). Due to its advantages in efficacy and safety, phototherapy is often considered the treatment of choice, suitable for almost all patients (27). Two types of phototherapies are mainly used for the treatment of psoriasis: narrow-band UVB (NB-UVB) and phototherapy using sensitization with psoralens (p-UVA) (22).

Systemic therapy

Traditional systemic therapy treatments involve the oral administration of drugs such as cyclosporine, acitretin, fumarates, apremilast, and methotrexate (28). These molecules

traditionally characterize systemic therapy, interacting differently with intracellular targets and showing a broad spectrum of action. However, they have proven less effective than the biologic drugs developed in recent years (21).

As our understanding of the pathogenesis of psoriasis improves, new engineered molecules capable of altering specific inflammatory pathways have been developed and approved. These biologic drugs include monoclonal antibodies and chimeric proteins and are administered subcutaneously or intravenously. The principal targets of these molecules include TNF-α, IL-12/IL-23, IL-17A, IL-17R, IL-17A/F, IL-23, and IL-36R (4, 21).

Risankizumab

Risankizumab is a humanized monoclonal antibody that targets the p19 subunit of interleukin 23 (IL-23). IL-23 is an inflammatory cytokine composed of two subunits, p40 and p19, which play a key role in the pathogenesis of psoriasis (4). It is produced in the early stages of the disease together with the cytokine IL-12 by myeloid dendritic cells (mDCs). IL-12 activates the Th1-dependent immune response, while IL-23 in the Th17-dependent one, along with IL-6 and TGFβ. Importantly, the two cytokines share the same p40 subunit and belong to the same family. This feature was exploited initially to develop biologic drugs targeting the shared p40 subunit, preventing the interaction of the two cytokines with the surface receptors of Th1 and Th17 lymphocytes and the subsequent activation of their respective inflammatory pathways (23). Subsequently, the development of IL-23-specific inhibitory biologic drugs targeting p19 achieved successful clinical results by sparing the Th1 signaling pathway (29). IL-23 interleukin inhibitors represent the most recently approved class of biological drugs for the treatment of moderate to severe psoriasis (30). Due to its molecular characteristics, including a high affinity for IL-23, risankizumab appears to have a rapid effect in reducing the symptoms and signs of psoriasis, with an improvement in patient's quality of life, even in individuals who do not respond to other biological drugs (31).

1.2 The inflammatory process in psoriasis

Inflammation is a typical mechanism of innate immunity established in response to pathogens and/or chemical, physical, or biological tissue damage. Inflammation encompasses a set of biological processes characterized by the activity of different cells to

defend the organism from potential pathogens and allow tissue damage to be repaired to restore normal body function. It is necessary, however, that the activation of inflammatory mechanisms be temporary and localized at the damaged site to avoid the development of a defensive response characterized by chronicity that may have systemic spread. In psoriasis, long considered an exclusively cutaneous disease, there is precisely the persistence of elevated levels of immune mediators, thus establishing chronic systemic inflammation. The endogenous danger signals released by keratinocytes at skin lesions fall into that numerous classes of molecules named Damage-Associated Molecular Patterns (DAMPs) and can trigger the inflammatory response. DAMPs share structural and functional similarities with exogenous danger signals, Pathogen-Associated Molecular Patterns (PAMPs), which, like DAMPs, are recognized by a series of receptors called pathogen recognition receptors (PRRs). The receptors involved in the early stages of the inflammatory process of psoriasis are located at the plasma membrane of dendritic cells or keratinocytes themselves. They are called Toll-like receptors (TLRs) (32). After stimulation, the cells can produce a series of proinflammatory cytokines, such as IFN-β, IL-1β, IL-36, TNF-α, IL-6, IL-8, IL-25, and CXCL-10, initiating a signaling cascade that culminates in the activation of nuclear transcription factor Kappa B (NF-kB) and the subsequent stimulation of cells that characterize adaptive immunity (33). The NF-kB signaling pathway plays a central role in the inflammatory process, as it controls the expression of proinflammatory cytokines and coordinates cell proliferation and differentiation. NF-kB consists of five protein monomers (p65/RelA, RelB, cRel, p50, and p52) that form homodimers or heterodimers capable of binding DNA (34). Usually, it is found in the cytoplasm sequestered by the inhibitor of the kB molecule (IkB), but in the presence of the proinflammatory stimuli described above, the signaling cascade leads to the activation of IkB kinase (IKK). The enzyme consists of two catalytic subunits, IKKα and IKKβ, and the essential NF-kB modulator regulatory subunit (NEMO) or IKKγ. Phosphorylation of the NEMO-containing IKK complex allows the enzyme to phosphorylate IkB kinase in two series at the N-terminal end of the molecule, resulting in its subsequent degradation. Consequently, NF-Kb is released from the cytoplasmic inhibitor and translocated into the nucleus to activate the transcription of target genes, mainly anti-apoptotic and proinflammatory (35). The messengers produced encode in particular for cytokines, such as IL-1, IL-2, IL-6, IL-8, and TNF-α, which in turn can

activate NF-kB by a positive feedback mechanism or for molecules that can activate T and B lymphocytes and allow the coordination of innate and adaptive immunity (36).

1.3 Epigenetic regulation of inflammation: microRNAs

MicroRNAs (miRNAs) are endogenous RNA molecules composed of 19-25 nucleotides, implicated in the post-transcriptional gene expression regulation of almost all human genes (37, 38, 39). They are noncoding molecules since they are transcribed from DNA but not translated into proteins and perform their function as RNA molecules by modulating physiological and pathological pathways, including cell growth and differentiation, metabolism, immunity, and numerous human diseases (39). They were first identified in 1993 in the nematode Caenorhabditis elegans, where a small RNA, encoded by the lin-4 locus, implicated in the regulation of the larval developmental process was identified (40). The subsequent identification of a homologous RNA in mammals made it possible to demonstrate high conservation of these molecules throughout evolution and further research into their function. Sequences coding for miRNAs have been shown to represent about 1 percent of the human genome, partly clustered and partly located within intronic sequences (41). To date, through genomic sequence analysis or computational studies, more than 2500 different miRNAs have been identified in humans (42, 43), which are listed and described in the available online miRBase database (http://www.mirbase.org). The rapid progress in recent years regarding the identification of new miRNAs has been supported by the development of next-generation sequencing techniques (Next Generation Sequencing), which have enabled rapid sequencing of all DNA and RNA present in a sample.

miRNAs are synthesized within cells but can be released externally by passive and active release mechanisms and thus be found in body fluids, including plasma and serum (circulating miRNAs). In circulation, miRNAs associate with proteins or are contained within vesicles so as to prevent the RNases present from catalyzing their digestion and degradation (44, 45). Deregulation of the synthesis and release of specific miRNAs in different diseases has allowed them to become potentially useful biomarkers for risk assessment, diagnosis, and prognosis of different diseases (37). Some characteristics of miRNAs could facilitate their future use in clinical practice: they can be quantified in peripheral blood draws (not particularly invasive draws); they have a 'high tissue-specificity

and show good sensitivity, modulating in response to therapy or disease progression. In addition, the existence of numerous detection techniques from different starting materials and the possibility of developing new tests quickly and inexpensively (46). Consequently, research has greatly investigated the role of miRNAs as biomarkers, highlighting their clinical potential. Currently, it has been shown that circulating miRNAs, through simple sampling, can contribute to diagnostic/prognostic assessments of various diseases, including autoimmune diseases, cardiovascular diseases, cancers, or to monitor the efficacy of treatment (37, 47).

1.3.1 Biogenesis of miRNAs

The process of miRNA biogenesis begins in the nucleus, where genes coding for miRNAs are transcribed by RNA polymerase II, generating RNA fragments a hundred nucleotides long, the primary miRNAs or pri-miRNAs. These immature RNAs exhibit a characteristic hairpin structure (48) and are targeted by an RNase III enzyme, Drosha, which cleaves them to form shorter molecules, the intermediate precursor miRNAs or pre-miRNAs. The pre-miRNAs are translocated from the nucleus into the cytoplasm by the GTP-dependent shuttle system mediated by Exportin 5. There they are cleaved by another enzyme of the RNase III family, the enzyme Dicer, into a mature miRNA duplex of 22 nucleotides. Subsequently, the enzyme interacts with the Ago protein to form the RNA-induced silencing complex (RISC) and remains associated with the mature miRNA. Consequently, the two strands of the miRNA duplex are separated, generating the guide strand and the passenger strand or miRNA*: the former remains integrated into the complex while the latter is degraded (49, 50). In most cases, it appears that guide strand selection by the Ago protein is based on the stability of the 5' end of the duplex strands (51). In this form, the miRNA guide strand is able to recognize and bind the noncoding 3'UTR end of the target mRNA by complementarity and result in its translational repression or degradation (Figure 3).

It is easy to infer that disruption of this regulatory process can cause abnormal expression of various cytokines, resulting in inflammation and loss of immune tolerance to autoantigens, a common feature in chronic inflammatory diseases (37, 52).

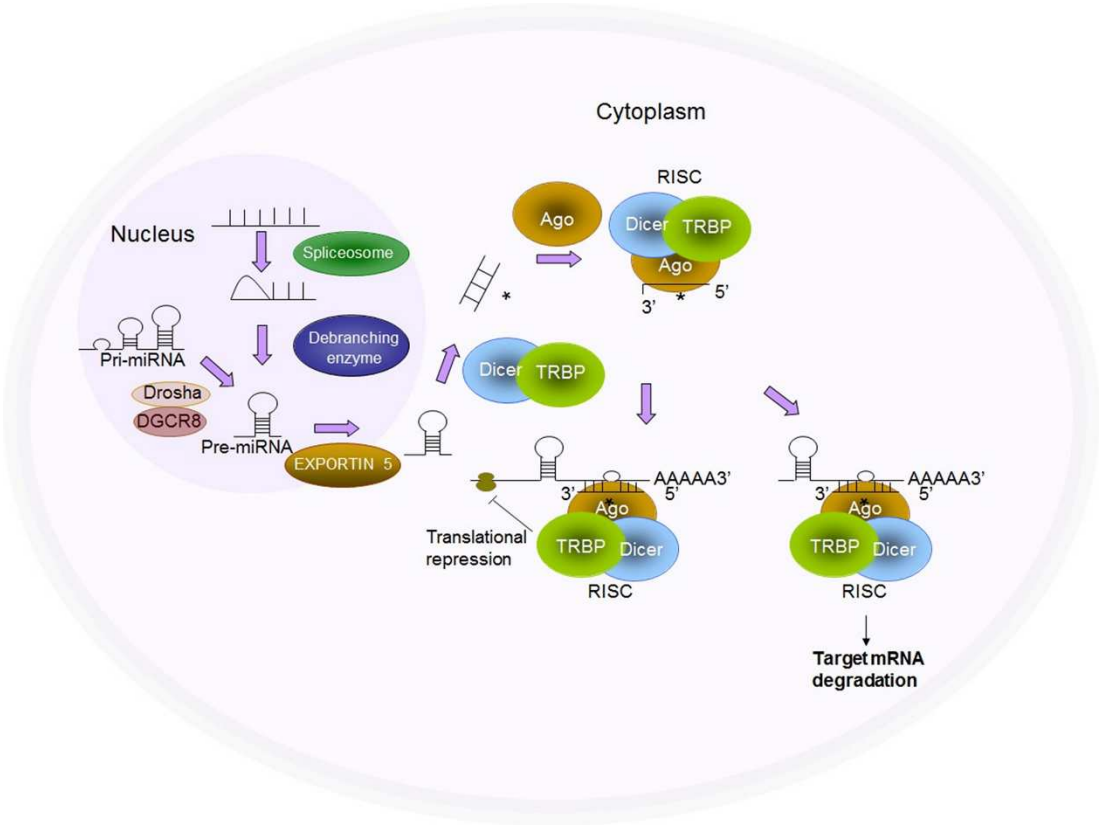

Figure 3. miRNA biogenesis. From: Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009 Mar;11(3):228-34. doi: 10.1038/ncb0309-228. PMID: 19255566.

1.3.2 Mechanism of action of miRNAs

The main action of RISC-bound mature miRNAs is to inhibit gene expression of a given target, causing its reduced protein synthesis. This activity is generally manifested through the pairing of a short sequence at the 5' end of the miRNA, called the seed sequence, with the 3' untranslated region (UTR) of the target mRNA, blocking its translation (48). This interaction is essential for proper modulation of target expression, although it may occur with incomplete complementarity, as is often the case in animals, and not perfect complementarity, as in plants (53). Extensive studies on this recognition have made it possible, through computational analysis, to predict potential target messengers for specific

miRNAs (54). Thus, it has been shown that a single miRNA can bind to several mRNAs and that several miRNAs can cooperatively regulate the same target. In this way, miRNAs are able to simultaneously regulate multiple pathways and biological processes by interacting with messenger RNA molecules that code for proteins of the same or different pathways (37).

Taking into account the overall mechanism of action, miRNAs might be able to control the proliferation, apoptosis or differentiation of immune cells and keratinocytes, play a central role in the regulation of immune response in the context of psoriasis.

1.3.3 miRNAs and psoriasis

Altered expression of miRNAs in psoriasis patients was first described in 2007 (52, 55). Subsequently, numerous studies have shown that they play a key role in several chronic inflammatory skin diseases and that the expression profile of specific miRNAs is shown to be different in psoriatic skin and plasma samples compared with the physiological condition (37). Recent studies have shown that approximately 251 miRNAs are differentially expressed in psoriasis lesions compared with healthy skin, among which 139 are over-expressed and 122 are under-expressed. Moreover, the target mRNAs of these miRNAs, appear to be implicated in a wide range of pathways all associated with psoriasis (56). It is well known that hyperproliferation and aberrant differentiation of keratinocytes are the key pathological features of psoriatic epidermis (57). Recent studies have shown that most of the differentially expressed miRNAs in the disease are associated precisely with the impairment of the balance between proliferation and differentiation of these cells. Furthermore, although the pathogenesis of psoriasis remains unclear, imbalance of CD4+ T-cell subsets has been shown to be a critical pathogenic factor, involving Th1 and Th17 cell expansion and Treg cell dysfunction (58-60). To date, numerous miRNAs regulating T-cell fates and behaviors have been reported but it is still not entirely clear which of them is specifically associated with CD4+ T lymphocytes (52).

Among the miRNAs most frequently associated with psoriasis, with different tendencies to be upregulated or downregulated, circulating or expressed in tissue samples from psoriatic patients, the following should be noted: miR-21, which maintains the cutaneous inflammatory state in psoriasis patients; miR-31, which stimulates the production of

inflammatory cytokines and chemokines via TNF-α; miR-146, which modulates the NF-kB pathway and has a marked correlation with IL-17 expression; miR-155, which leads to TNF-α production; miR-203, which induces epithelial differentiation and suppresses skin immune responses; miR-99, which inhibits keratinocyte differentiation; miR-125, which inhibits cell proliferation; miR-197, which reduces keratinocyte proliferation and migration; and miR-520, which suppresses keratinocyte proliferation (49, 61).

In addition, studies in recent years have demonstrated a probable modulation of specific psoriasis-related miRNAs in patients receiving systemic therapies. For example, phototherapy appears to be able to reduce the expression of miR-21 in the epidermis and increase the expression of miR-125b (62). Again, there is a decrease in miR-143 and miR-223 in peripheral blood mononuclear cells of patients who have undergone methotrexate treatment, compared with the initial condition (63). Pivarcsi et al. verified an increase of 38 miRNAs in the serum of patients undergoing therapy with the biologic drug etanercept, a result not achieved by methotrexate therapy, however. In conclusion, a correlation can be seen between therapies and changes in miRNA levels in psoriatic patients. However, further studies are needed to clarify the exact role of miRNAs in psoriasis, considering the significant interindividual variability in therapeutic response (54).

1.4 Regulatory T cells (Treg)

The pathogenesis of psoriasis is mainly driven by type 1 and type 17 T lymphocytes, which produce cytokines and are modulated by regulatory T cells (Treg) in healthy individuals. Tregs play a key role in immune homeostasis and help prevent autoimmune diseases by suppressing immune responses. In psoriasis, Tregs are impaired in their suppressive function, leading to an altered balance between T-helper 17 lymphocytes and Tregs themselves (2). Tregs are characterized by the expression of the transcription factor Foxp3, and most of them also turn out to be CD4+ CD25+ (65). Their suppressive function develops through several pathways: cell-to-cell interaction, production of suppressive cytokines such as IL-10, TGF-β1 and IL-35, and direct cytotoxic action (66). Based on their origin, natural Tregs can be divided into thymus-derived Tregs (tTregs) and peripherally induced Tregs (pTregs) (67). Tissue-resident Tregs are heterogeneous populations that exhibit tissue-specific adaptation closely related to the environmental and physiological signals they

receive, developing specific features of their own phenotype, transcriptome, and metabolism that distinguish them from circulating Tregs and those located in secondary lymphoid organs. (68, 69).

Tregs localized in skin tissue modulate important skin processes, including wound healing, hair follicle regeneration, and adaptive immune tolerance to skin commensals (70). The role of Tregs in psoriatic pathology has not yet been fully elucidated. The number of Tregs present in skin samples and in the blood of patients seems to be related to the severity of the disease. Several authors have reported a reduced percentage of Tregs in the peripheral blood of patients with psoriasis, but the severity of the condition was variable (71); other patients examined, however, showed no difference in the percentage of circulating Tregs (72). Regarding Treg infiltration in psoriatic lesions, most studies show a higher frequency of Treg than in healthy skin (73). Zhang et al., for example, reported that increased Treg in psoriatic skin correlated with PASI scores in patients with moderate to severe disease (74). Furthermore, while Bovenschen et al. observed a higher frequency of CD4+ CD25+ Foxp3+ Tregs in the dermis than in the epidermis in patients with plaque psoriasis, Fujimura et al. reported the opposite (73, 75). These conflicting results may be a consequence of the different disease states, the different definition of Treg, the sites of biopsy from psoriatic plaques, and the subtypes of psoriasis studied. However, it remains clear that Tregs actively contribute to the pathogenesis of psoriasis.

Recent studies indicate that most patients with psoriasis are characterized by the altered function of Tregs, isolated from both skin lesions and peripheral blood (76). The suppressive function of Tregs could be impaired by the pro-inflammatory cytokine environment of the lesions, such as high levels of IL-6 (77). Additionally, inhibition of Foxp3, operated by miR-210 overexpressed in CD4+ T cells, appears to cause decreased levels of the suppressive cytokines IL-10 and TGF-β and increased levels of the pro-inflammatory cytokines IFN-γ and IL-17A, demonstrating how overexpression of miR-210 also contributes to Treg dysfunction (78).

Th17 cells are a subset of CD4+ T cells that secrete a characteristic profile of cytokines, including IL-17A, IL-17F, IL-21, and IL-22. They play an important role in immunity to extracellular pathogens and in the pathogenesis of inflammatory and autoimmune diseases,

including psoriasis (79, 80). In this disease, highly activated cells infiltrate psoriatic lesions once polarized by elevated levels of the cytokine IL-23 (81, 82). Treg and Th17 cells share expression of TGF-β for differentiation from their common precursor, the naïve T cell, resulting in constant evolutionary competition (83). In psoriasis, an alteration in the existing balance in the skin of the ratio of Th17 to Treg has been noted. Zhang et al. showed elevated levels of both Th17 and Treg cells in the peripheral blood and injured skin of patients. Furthermore, the change in the ratio of Th17 to Treg cells in skin lesions was exactly the opposite of what was observed in peripheral blood, suggesting a probable recruitment of Treg to the sites of injury to inhibit the hyperimmune response in inflammation (84).

Many current or future treatments for psoriasis seem to target the number and function of Treg. Phototherapy and biologic drugs, such as anti-IL-17 or anti-IL-2, can, at least partially, safeguard the suppressive function of Treg. The balance between Th17 and Treg cells, on the other hand, seems to be restored through several mechanisms, for example: induction of Treg, by anti-TNF-α, anti-IL-23 or phototherapy; underregulation of Th17 cells, by phototherapy or anti-IL-17; and inhibition of Treg plasticity, by anti-IL-23 (85, 86) (Figure

4)

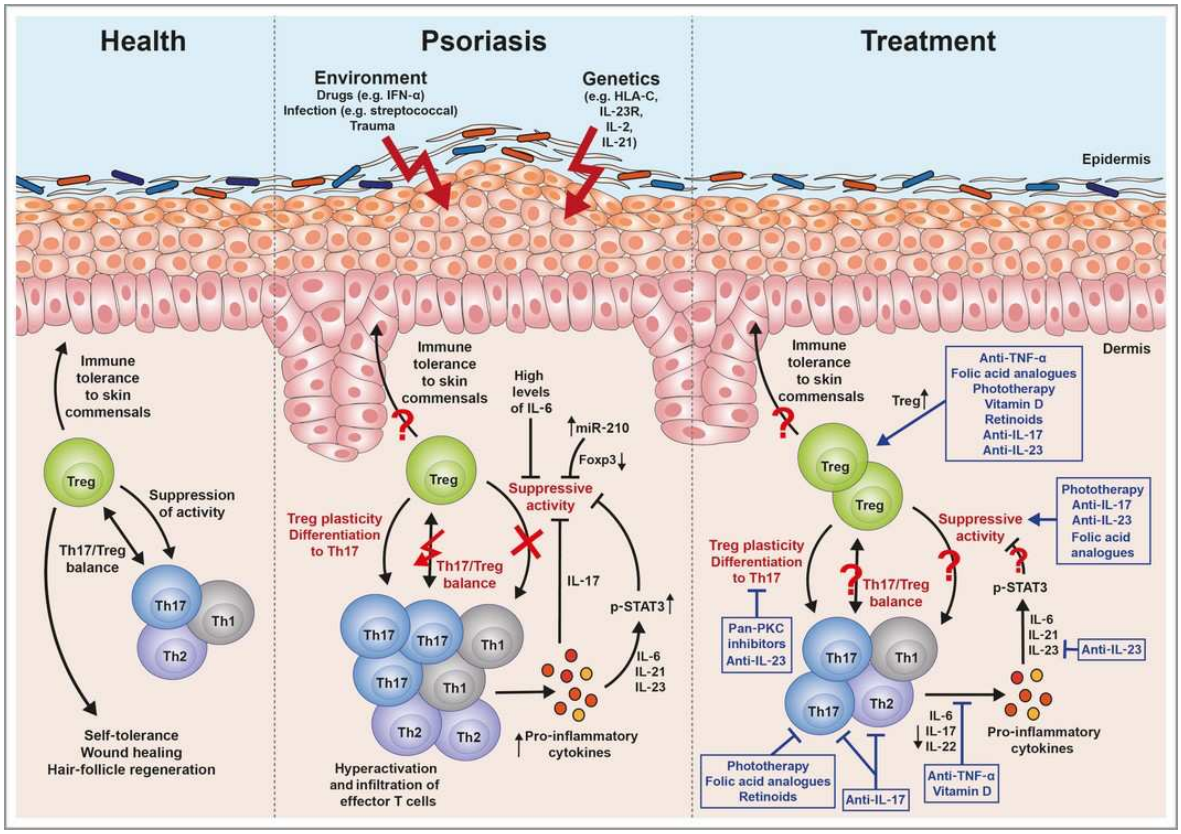

Figure 4. Treg cells and psoriasis. *Br J Dermatol*, Volume 184, Issue 1, 1 January 2021, Pages 14–24

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AIM AND SCOPE

Psoriasis is an immune-mediated inflammatory disease supported by sustained inflammation and characterized by altered proliferation and differentiation of keratinocytes and infiltration of immune cells into the skin tissue. The role of miRNAs in disease pathogenesis has been widely discussed in recent years, suggesting that miRNAs that modulate the inflammatory response might be involved in the heterogeneity of psoriatic features and clinical responses to treatments. Consequently, analysis of miRNA levels could be a novel approach to explore both the unresolved aspects of disease pathogenesis and the biological mechanisms underlying the effectiveness or ineffectiveness of the most innovative therapies in psoriasis patients.

In addition, biological therapy with risankizumab, an anti-IL-23 monoclonal antibody, is known to be effective for the treatment of psoriasis. To date, however, there are no studies evaluating the modulation of circulating miRNAs in relation to individual response to anti-IL-23 therapy. Thus, the aim of this thesis is to identify, using NGS technologies and RT-PCR for subsequent validation, the circulating miRNAs significantly modulated before and following risankizumab treatment in patients with psoriasis.

Furthermore, since the pathogenesis of psoriasis seems to be characterized by alteration of regulatory T-cell function and frequency, further aim of this thesis is to evaluate how the Treg population varies before and after biological therapy with anti-IL-23 drug.

Finally, considering the predominant involvement of inflammatory process in psoriasis diseases, changes in some typical inflammatory cytokines pre and post therapy were also evaluated.

Chapter Three

MATERIALS AND METHODS

3.1 Characteristics of the study population (discovery phase)

During the period between January 2021 and July 2022, 12 subjects with moderate-severe plaque psoriasis were selected from the Clinic of Dermatology of the Università Politecnica delle Marche (UNIVPM)- Azienda Ospedali Riuniti Hospital in Ancona. Baseline characteristics (T0) of the study patients are summarized in Table 1.

Patients were screened for HBV, HCV, HIV, TB, as recommended by guidelines (7). Objective examination and assessment of disease severity was then performed using the parameters: PASI (Psoriasis Area Severity Index), BSA (Body Surface Area), PGA (Physician Global Assessment), DLQI (Dermatology Life Quality index).

The selection criteria adopted were:

- Patients older than 18 years, with moderate to severe plaque psoriasis according to national and international guidelines (7);

- Patients who did not receive any local or systemic corticosteroid treatment or immunosuppressive therapy one month prior to enrolment;

- Patients eligible for biological therapy with anti-IL-23 agents.

Exclusion criteria were:

- Patients with severe cardiovascular, cerebrovascular, hepatic, renal and hematopoietic diseases;

- Pregnant or lactating patients;

- Patients noncompliant for other clinical evaluations.

Patients underwent biological therapy with Risankizumab. Treatment administration was by two 75-mg injections of Risankizumab subcutaneously at week 0, after 4 weeks and every 12 weeks. Thereafter, patients underwent monthly follow-up for clinical evaluation and assessment of any adverse events and reassessed at one year (T1) after the first administration.

Blood samples were collected from all 12 patients before and after the start of risankizumab therapy by blood sampling in tubes containing anticoagulants (5 ml serum in citrate and 5 ml in EDTA for plasma and whole blood).

The UNIVPM ethics committee approved the study protocol, and all enrolled participants provided written informed consent.

Table 1. Baseline demographic and clinical characteristics of patients. Values in table are presented as the mean ± standard deviation (SD), with or without the range in parentheses, or as the count (number of patients) with the percentage in parentheses BMI Body mass index, PASI Psoriasis Area Severity Index, BSA body surface area, PGA Physician Global Assessment, DLQI Dermatology Quality of Life Impairment.

Variables	**N=12**
Age (years) - m (DS)	44.9 ±12.5
Gender (M) - n (%)	9 (75)
Weight (kg) - m (DS)	83.3±16.6
BMI (kg/m2) - m (DS)	27.3 (5.1)
Smokers – n (%)	6 (50)
Comorbidities – n (%)	
Dyslipidemia	2 (16.7)
Hypertension	1 (8.3)
Diabetes mellitus type II	1 (8.3)
Atopy	1 (8.3)
Anxiety and depressive disorders	1 (8.3)
Thyroiditis	2 (16.7)
Age of onset of psoriasis (years) - m (SD)	29.3 ± 15.1 (10-64)
Duration of psoriasis (years) m (SD)	15.6 ± 14 (1-46)
Familiarity n (%)	3 (25)
Patients with special sites involvement (%)	10 (83.3)
Two or more sites	3 (25)
Facial lesions	2 (16.7)
Palmoplantar lesions	3 (25)
Scalp lesions	7 (58.3)
Genital lesions	2 (25)
Previous biological tratment n (%)	
No	
Yes	10 (83.3)

1	2 (16.7)
2	1 (8.3)
3 o più	0
	1 (8.3)
PASI m (DS)	19.9 ± 14.8 (3- 33.2)
BSA m (%) (DS)	27.8 ± 0.2 (3- 75)
PGA m (DS)	3.3 ± 0.5 (3-4)
DLQI m (DS)	7.1 ± 3.6 (2- 15)

3.2 Total RNA extraction from whole blood

For evaluation of mRNA expression of specific circulating cytokines, it was necessary to perform extraction and isolation of total RNA from whole blood samples of the selected patients.

Total RNA was isolated from 50 µl of whole blood using the Total RNA Purification Kit from Norgen Biotek Corporation (#37500, Thorold, ON, Canada). The kit involves the use of ion exchange chromatography columns composed of a separation matrix resin for RNA binding. The binding allows isolation of RNA from other components in the blood. The procedure is performed at room temperature, with gloves, filter tips and RNase-free plastic to avoid possible contamination by RNases that can degrade or damage RNA. Eppendorfs used during the procedure but not contained in the kit must previously undergo autoclave sterilization. The main extraction and purification steps involve: preparation of the lysate, to be carried out on ice; binding of the RNA to the chromatographic column; washing of the column through several steps; and elution of the RNA (Figure 5).

In detail, the kit extraction protocol includes the following steps:

1. Dilute whole blood (50 µl) with 50 µl of PBS;

2. Transfer the unclotted whole blood into RNase-free eppendorf;

3. Add 350 µl of Buffer RL and vortex for 10 seconds to obtain a clear solution;

4. Add 200 µl of 95-100% ethanol and vortex for 10 seconds;

5. Assemble the chromatographic column to a Collection Tube, provided in the kit;

6. Transfer a maximum lysate volume of 600 µl to the column and centrifuge at 6500 RPM for one minute. Discard the eluate afterwards;

7. Check that the entire volume in the column passes through the filter and if not, centrifuge further at 14000 RPM for one minute;

8. Repeat the previous two steps until the sample is exhausted, so that all the blood passes through the column filter;

9. Add 400 µl of Wash Solution and centrifuge for one minute at 11000 RPM;

10. Repeat washes with Wash Solution two more times, discarding the Collection Tube eluate each time;

11. Centrifuge for 2 minutes at 13000 RPM to dry the resin;

12. Discard the Collection tube and transfer the column to an Elution tube, provided by the kit;

13. Add 20 µl of Elution Solution to the column and centrifuge for 2 min at 2000 RPM and then for 1 min at 13000 RPM;

14. Transfer the eluted RNA back to the corresponding column and repeat the centrifugation steps in order to increase the recovery of RNA from the resin. Keep the purified RNA samples on ice until use or storage at -80°C.

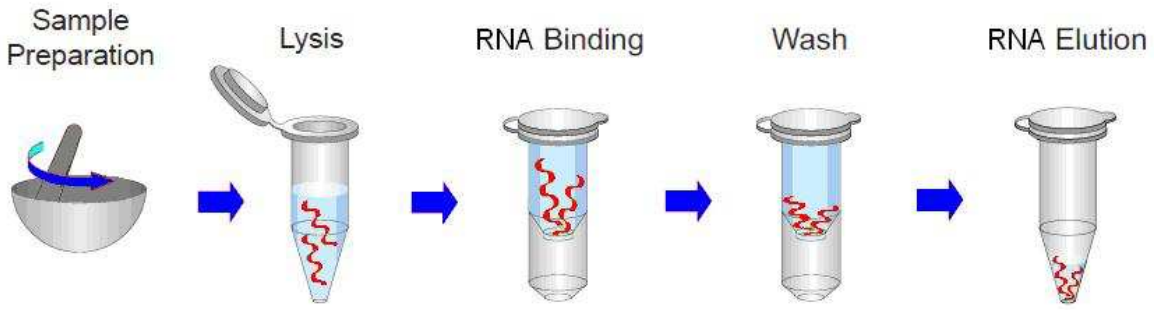


Figure 5. Total RNA extraction and purification steps using ion exchange chromatography columns.

Then, the quantization of the extracted RNA is carried out using the Nanodrop ONE (NanoDrop Technologies, Wilmington,DE, USA), in ng/µl. This instrument uses the ability

of nucleic acids to absorb UV light in order to obtain an accurate measurement of the amount of RNA present in 1 µl of sample in a few seconds.

3.3 Retrotranscription of mRNAs

Retrotranscription (Reverse Transcription RT) results in single-stranded DNA (cDNA) molecules complementary to starting RNA molecules. Several reagents provided by PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio Europe) are needed and stored in a freezer at -20°C. They are: Deoxyribonucleosides triphosphate (dNTPs), buffer containing Mg+ ions, Reverse Transcriptase (RT) enzyme, and random Primers (Forward and Reverse) capable of binding different points of the sequences. Water (H2O) is also required for the reaction, which is first autoclaved and filtered to avoid contamination. The enzyme Reverse Transcriptase is an RNA-dependent DNA-polymerase capable of synthesizing a DNA sequence complementary to a guide RNA strand from a primer for polymerization, namely random primers.

The specific protocol for the retrotranscription reaction involves two steps:

- The elimination of genomic DNA present in the sample using DNases, enzymes that can specifically degrade DNA;
- The actual retrotranscription reaction operated by reverse transcriptase.

For the first step, a single reaction mix is prepared on ice with a small excess added.

Next, 0.2-mL eppendorfs are set up for each sample, containing: a volume of RNA corresponding to the amount to be back-transcribed; the amount of H2O missing to reach a total volume of 7 µl; and a volume of 3.0 µl of vortexed and centrifuged mix. The solution of 10 µl total, for each sample, is then placed at 42°C for 2 min.

For the second step, a single reaction mix is prepared on ice, with a small excess added.

Thereafter, the mix is vortexed and centrifuged, and then dispensed into each sample set up in the previous step, with a volume of 10 µl. Finally, the samples undergo three passes at different temperatures by means of the PTC-200 Peltier Thermal Cycler, namely:

1. 16 °C for 30 min;

2. 42 °C for 30 minutes;

3. 85 °C for 5 minutes.

After the cycles are finished, the instrument keeps the samples at 4°C indefinitely to prevent damage, before they are transferred to ice to proceed with the next Real-Time PCR.

3.4 Real Time-PCR

Real Time-PCR (qPCR) is a PCR that allows simultaneous amplification and quantitative evaluation of the cDNA present in the sample, by using a fluorescent dye capable of intercalating into double-stranded DNA sequences. In this case, the reaction is conducted using TB Green Premix Ex Taq (Takara). The detected fluorescence intensity is converted through software into a numerical value, which is directly proportional to the amount of RNA present in the starting sample.

Similar to retrotranscription, it is necessary to set up specific mix for the primers, considering the number of duplicate samples plus an excess.

Next, 8.5 µl of mix, vortexed and centrifuged, is dispensed into 0.1-mL tubes; a volume equal to 1.5 µl of cDNA sample is then added to obtain a final volume for the reaction of 10 µl. The prepared samples are then analyzed with the Rotor-Gene Q instrument (Qiagen, Hilden, Germany), which performs the denaturation, annealing and elongation steps according to the amplification protocol (Figure 6):

1. 95°C for 10 min;

2. 95°C for 15 seconds;

3. 60°C for 1 minute.

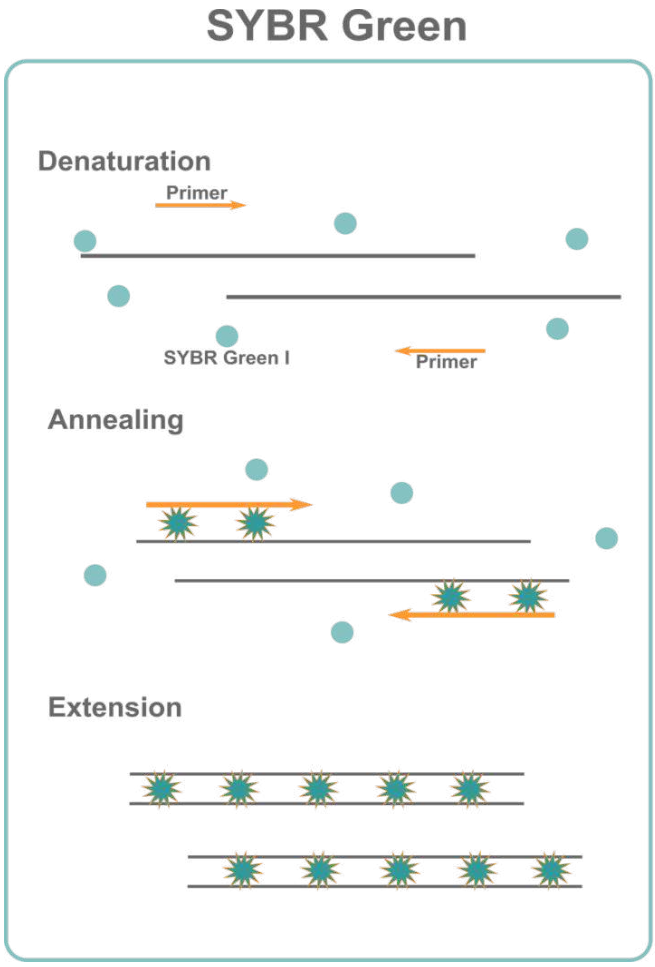


Figure 6. Mechanism of action of SYBR green.

The last two steps are repeated for 40 cycles. With each cycle there is an exponential increase in the fluorescence of the SYBR green proportional to the amount of product in the sample. The entire run lasts about 80 to 90 minutes, after which the data recorded during amplification can be evaluated.

3.4.1 RT-PCR data analysis.

The reading of the signal by the instrument occurs at the end of each cycle and allows the fluorescence trend to be represented in real time in individual samples with amplification curves. To perform data analysis, it is important to set the threshold line *(Threshold line)*, which allows the value of the threshold cycle *(Ct or threshold cycle)*, the cycle corresponding to the intersection of the curve being analysed and the threshold line, to be derived. This value indicates the cycle at which fluorescence is detected that exceeds the background

signal and is inversely proportional to the initial RNA concentration. It is possible, therefore, to trace the amount of RNA present in the sample by comparing the Ct values of the mRNAs of interest with the Ct values of normalizing mRNAs, i.e., encoded by constitutively expressed reference genes (housekeeping genes). In this case, β-actin was used as an endogenous control. The analysis steps were:

- for each sample the Ct of actin was subtracted from that of the mRNA of interest, yielding the ΔCt;
- for each sample the relative expression was calculated, which is 2-ΔCt, or the relative expression level of the normalized mRNA.

3.5 miRNome analysis by Next Generation Sequencing (NGS) technology

Analysis of miRNOMA by NGS technology was conducted in collaboration with the Experimental Pathology group of the Faculty of Medicine, University of Bologna.

Blood samples were collected from all 12 patients before and after the start of Risankizumab therapy by blood sampling, including 5 ml in EDTA for plasma and whole blood. The samples were centrifuged, within two hours after collection, at 1800 RPM for 10 minutes to separate the plasma, which was aliquoted and frozen at -80°C. RNA for analysis was obtained from 100 µl of plasma. TruSeq Small RNA Library PrepKit v2 (Illumina; RS-200-0012/24/36/48) was used for library preparation according to the manufacturer's instructions. Briefly, 35 ng of purified RNA was ligated to 3' and 5' adapters, converted to cDNA, and amplified using Illumina primers containing unique sequences for each sample. Each library was quantified using Agilent Bioanalyzer and High Sensitivity DNA Kit (cat. no. 5067-4626, Agilent Technologies, USA), and an equal amount of libraries were pooled together. Size selection allowed fragments of 130-160 bp to be retained. After precipitation with ethanol, the library pool was quantified with Agilent High Sensitivity DNA Kit, diluted to 1.8 pM and sequenced using NextSeq 500/550 High Output Kit v2 (75 cycles) (Illumina; FC-404-2005) on the Illumina NextSeq500 platform.

Raw base identification data generated by the Illumina NextSeq 500 system were demultiplexed using Illumina BaseSpace Sequence Hub (https://basespace.illumina.com/home/index) and converted to FASTQ format. After quality

control with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), adapter sequences were trimmed using Cutadapt (http://cutadapt.readthedocs.io/en/stable/index .html), which also removed sequences with less than 10 nucleotides. The reads were mapped using the STAR algorithm (https://www.ncbi.nlm.nih.gov/pubmed/23104886). The reference genome consisted of human miRNA sequences from the miRbase 21 database. Unprocessed values from the mapped reads were obtained using the htseq-count script, from the HTSeq tools (http://www-huber.embl.de/HTSeq/doc/overview.html).

Raw counts were further normalized using the DESeq2 bioconductor package (//bioconductor.org/packages/release/bioc/html/DESeq2.html). NGS raw data are available through the European Nucleotide Archive (http://www.ebi.ac.uk/).

MiRNAs with normalized expression above the 40th percentile in at least one sample in the group were selected as expressed.

3.6 Validation phase of miRNome analysis

For the validation phase a group of 23 patients was considered. The validation group was composed of 11 patients with plaque psoriasis selected from the Dermatology Clinic of Ospedali Riuniti delle Marche database based on the availability of serum samples and clinical data collected at the time of admission. The 11 patients were selected considering the same inclusion and exclusion criteria as in the discovery group and enrolled from 1st January 2023 to 31st March 2023, The validation group contained also the previous 12 patiens consideredfor the discovery phase. A summary of anthropometric and clinical data of the total 23 patients can be found in Table 2.

Table 2. Demographic and clinical characteristics of the validation group. Values in table are presented as the mean ± standard deviation (SD), with or without the range in parentheses, or as the count (number of patients) with the percentage in parentheses. BMI Body mass index, PASI Psoriasis Area Severity Index, BSA body surface area, PGA Physician Global Assessment, DLQI Dermatology Quality of Life Impairment.

Variables	**N=23**
Age (years) - m (DS)	52.9 ±15.1
Gender (M) - n (%)	18 (78.2)

Weight (kg) - m (DS)	72.3±15.6
BMI (kg/m2) - m (DS)	26.8 (4.1)
Smokers – n (%)	8 (34)
Comorbidities – n (%)	
Dyslipidemia	8 (34.8)
Hypertension	2 (8.7)
Diabetes mellitus type II	2 (8.7)
Atopy	1 (4.3)
Anxiety and depressive disorders	1 (4.3)
Thyroiditis	2 (8.7)
Age of onset of psoriasis (years) - m (SD)	34.2 ± 14.1 (10-68)
Duration of psoriasis (years) m (SD)	17.2 ± 13 (1-52)
Familiarity n (%)	6 (26)
Patients with special sites involvement (%)	10 (83.3)
Two or more sites	
Facial lesions	5 (21.7)
Palmoplantar lesions	3 (13.0)
Scalp lesions	2 (8.7)
Genital lesions	7 (30.4)
	2 (8.7)
Previous biological tratment n (%)	
No	
Yes	18 (78.3)
1	5 (21.7)
2	2 (8.7)
3 o più	1 (4.3)
	2 (8.7)
PASI m (DS)	15.8 ± 12.3 (2- 60)
BSA m (%) (DS)	25.8 ± 17.1 (3- 75)
PGA m (DS)	3 ± 0.7 (1-4)
DLQI m (DS)	9.2 ± 3.2 (2- 18)

3.7 Quantitative RT-PCR of mature miRNAs

MiRNAs expression was quantified by quantitative real-time PCR (RT-qPCR) using TaqMan miRNA assay (Catalog #4427012 - ThermoFisher Scientific), according to the manufacturer's protocol. Briefly, miRNA was reverse transcribed with the TaqMan MicroRNA reverse transcription kit (4366596 – ThermoFisher Scientific), using a miR specific stem-loop primer.

10 µl of RT mix contained 2 µl of each miR-specific stem-loop primer, 3.34 µl of input RNA, 1 µl of 10 mM dNTPs, 0.67 µl of reverse transcriptase, 1 µl of 10× buffer, 1.26 µl of RNase inhibitor diluted 1:10, and 0.73 µl of H2O. The mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min. The 10 µl RT-PCR reaction mix included 0.5 µl 20x TaqMan MicroRNA Assay, which contained the PCR primers and probes (5'-FAM), 5 µl 2x TaqMan Universal Master mix no UNG (4440040 –ThermoFisher Scientific), and 2.66 µl RT product. The reaction presented an initial step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Data were analysed with Rotor Gene Q (Qiagen, Hilden, Germany) with the automatic comparative threshold (Ct) setting for adapting baseline. qRT-PCR data were standardized to RNU44. The 2−ΔCT method was used to determine miRNA expression.

3.8 Luminex multiplex assays

Multianalyte profiling was performed on the Luminex-100 system (Bioplex, BIORAD). Calibration microspheres for classification and reporter readings as well as sheath fluid were also purchased from BIORAD. Acquired fluorescence data were analysed by the Bioplex manager software v.4.0. The Premixed Human Cytokine 8-Plex Panel (Biotechne) was used to analyse TNF-α, IL-8, and IL-6, IL-12, IL-18, IL-1□, IL-17 and INF-□ in the serum of the 23 patients of the validation phase. All analyses were performed according to the manufacturers' protocols.

3.9 Treg analysis with permeabilization

Lymphocyte subpopulation analysis of regulatory T cells (Treg) was performed by cytofluorimetry or flow cytometry (CFM). CFM is a technique to detect, identify and count specific cells by their fluorescent and physical characteristics (internal complexity, size, granularity). The analysis consists of two steps: a first step of marking with dyes specific to the sample under examination and a second step of analysis by cytofluorometer.

The treatment of the sample to be discriminated into the different cellular subtypes involves the use of monoclonal antibodies, conjugated to fluorochromes, directed toward particular cellular districts or surface antigens. The protocol to be followed for such treatment involves the following steps:

- Centrifuge, within two hours of collection, at 1800 RPM for 10 minutes, the blood samples to separate the plasma;
- Prepare the antibody mixes in the tubes in the dark after initialing them. Add the anti-FOXP3 antibody only after the lysis and permeabilization step, as it is an intracellular dye;
- Isolate the plasma and take 200 µl of blood, after shaking the tube, to be added to the antibody mix;
- Incubate for 15 min at room temperature in the dark to perform surface labeling;
- Prepare 4 ml per sample of 1:10 Lysing solution in distilled H2O and add to the sample at the end of incubation;
- Vortex the solution and incubate for 10 min at room temperature in the dark;
- Centrifuge at 1800 RPM for 10 min in the dark and then discard the supernatant;
- Prepare 1 ml per sample of Permeabilization buffer solution 1:10 in distilled H2O and add to the sample at the end of centrifugation;
- Vortex the solution and incubate for 10 min at room temperature in the dark;
- Add 4 ml of PBS and centrifuge at 1800 RPM for 10 min in the dark;
- Discard the supernatant and add anti-FOXP3. Incubate for 30 min at room temperature in the dark;
- Add 4 ml of PBS and centrifuge at 1800 RRM for 10 min in the dark;
- Discard the supernatant and dilute the obtained sample with 200-400 µl PBS for cytofluorometer acquisition.

The obtained sample containing the labeled cells is subsequently analyzed at the cytofluorometer. This instrument consists of several parts: a fluidic system, an excitation system, an electronic component system to acquire the signal, and software to analyze the data (88). Cells in the sample pass through a laser beam aligned one behind the other. For each cell passing through the laser beam, the cytofluorometer records how the cell scatters

the incident laser light and emits fluorescence, generating a characteristic scatter The data obtained are shown as graphs, from which the percentage amount of specific subpopulations

3.10 Statistical analysis

The statistical software used was SPSS/Win (version 25.0, SPSS, Chicago). Continuous variables with normal distribution were reported as mean ± standard deviation (SD). Dichotomous variables were reported as number, percent (%). Comparisons between continuous variables were performed using the t-test, Paired t-test or Mann-Whitney test, as appropriate; comparisons between dichotomous variables were performed using the Chi square test, McNemar test or Z-test, as appropriate. Spearman's rho correlation test was used to estimate correlations between miRNA expression levels and clinical parameters. A p-value<0.05 was considered statistically significant.

3.11 Bioinformatic analysis

The prediction of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was achieved with the DNA intelligent analysis (DIANA)-mirPath online software suite (89) in order to individualize the pathways potentially altered by the deregulated miRNAs.

Chapter 4

RESULTS

4.1 Patient characteristics.

Twelve subjects (n=12) who visited the Dermatology Clinic of Università Politecnica delle Marche (UNIVPM) - Ospedali Riuniti delle Marche between January 2021 and July 2022 were recruited.

All selected patients met the study's inclusion criteria as being 18 or older, with moderate-severe plaque psoriasis, not receiving treatment one month before enrolment, and eligible for anti-IL-23 therapy with Risankizumab. Of the 12 individuals, 9 men and 3 women, none had other variants of psoriasis (erythrodermic, suberitrodermic, pustular psoriasis, etc.) and psoriatic arthritis. At the time of dermatologic examination at baseline (T0), the following was found: the mean duration of disease was 15.6 years (range 1-46, standard deviation [SD] 14); the mean age at diagnosis of the disease was 29. 3 years; 25% of patients reported a positive family history of psoriasis; 10 out of 12 patients showed special site involvement (83.3%), including 3 with involvement of two or more special sites; 2 out of 12 patients (16.7%) had lesions on the face, 3/12 (25%) palmoplantar, 7/12 (58.3%) on the scalp, and 2/12 (25%) on the genital area.

Regarding previous therapies, all patients had unsuccessfully performed treatments with disease-modifying antirheumatic drugs (DMARDs): 4/12 patients (33.3%) with acitretin, 7/12 with cyclosporine (58.3%), 3/12 (25%) with methotrexate. and 1/12 (8.3%) with apremilast. Of the 12 patients in the study, 10 (83.3%) were naïve to biologic therapy, while for the remaining 2 (16.7%), therapy with at least one biologic drug was unsuccessful. Of the latter, 1 (8.3%) failed therapy with adalimumab and apremilast, and 1 (8.3) with adalimumab, certolizumab, and etanercept. Baseline demographic and clinical characteristics of patients were shown in Table 1.

4.1.1. Changes in PASI, BSA, PGA, DLQI

In patients observed in T0 (pre- Risankizumab), the mean PASI score was 19.9 (3-33.2, SD 14.8). 7 out of 12 patients (58.3%) had a PASI score between 10 and 20, corresponding to moderate psoriasis, while 4/12 (33%) had a PASI score above 20, corresponding to severe

psoriasis. Mean BSA was 27.8% (3-75, SD 0.2), mean PGA was 3.3 (3-4, SD 0.5) and mean DLQI was 7.1 (2-15, SD 3.6).

After one year of treatment with Risankizumab (T1), all achieved PASI 50, or a PASI improvement of 50% following treatment, and PASI 75 (75% improvement); 11/12 (91.7%) achieved PASI 90 (90% improvement) and 9/12 (75%) achieved PASI 100, reaching complete remission of symptoms (78). Mean PASI score at T1 was 0.4 (0-2, SD 0.8; $p < 0.0007$), mean BSA was 0.4% (0-2, SD 0.01; $p < 0.0018$), mean PGA was 0.25 (0-1, SD 0.5; $p<0.0001$), and mean DLQI was 0.25 (0-1, SD 0.5; $p < 0.00008$). The results are shown in Table 3, Figure 7 and Figure 8. No patients reported adverse events during follow-up.

Table 3. PASI, PGA, BSA, DLQI in patients pre-Risankizumab (T0) and after one year risankizumab treatment (T1).

	T0	T1	p
PASI m (DS)	19.9 ± 14.8 (3-33.2)	0.4 ± 0.8 (0–2)	0.0007*
BSA m (%) (DS)	27.8 ± 0.2 (3–75)	0.4 ± 0.01 (0-2)	0.0018*
PGA m (DS)	3.3 ± 0.5 (3–4)	0.25 ± 0.5 (0-1)	0.0001*
DLQI m (DS)	7.1 ± 3.6 (2– 15)	0.25 ± 0.5 (0-1)	0.00008*

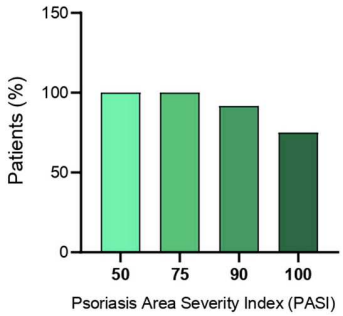


Figure 7. Percentage of patients with psoriasis achieving PASI 50, PASI 75, PASI 90, PASI 100 at 1 year after treatment initiation with risankizumab. PASI 50, PASI 75, PASI 90, PASI 100 represent the 50%, 75%, 90%, 100% (complete resolution) improvement after 1 year risankizumab treatment (T1) compared to baseline (T0).

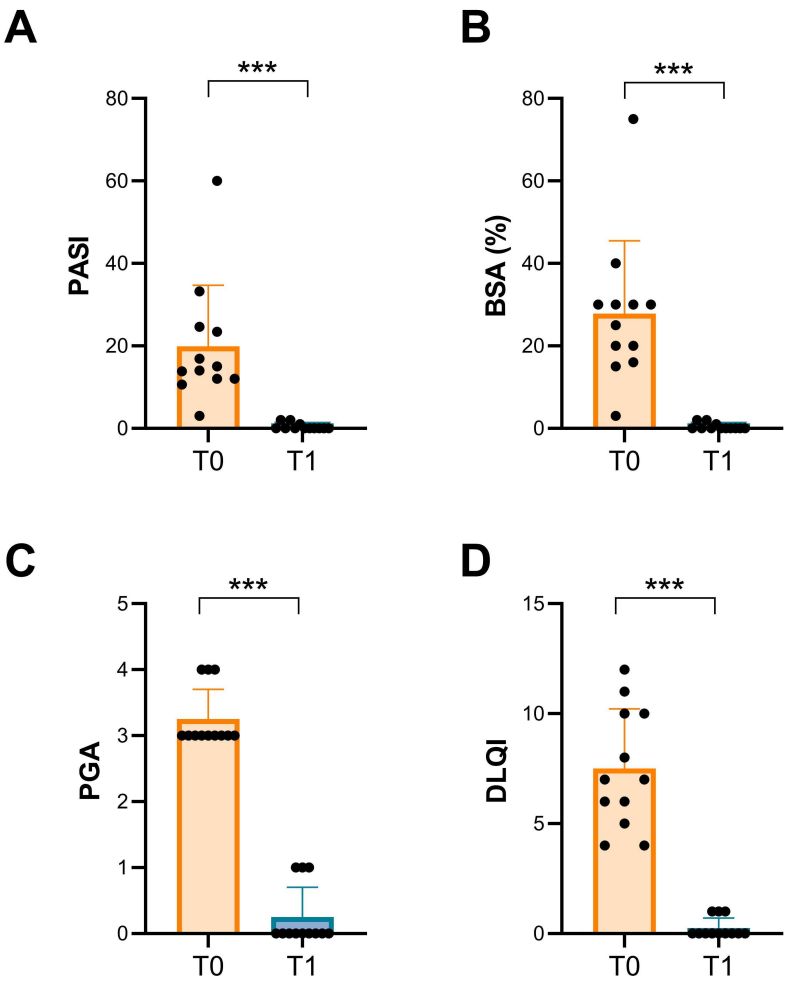


Figure 8. Changes in PASI (A), BSA (B), PGA (C), DLQI (D) after 1 year of treatment (T1) compared to baseline (T0).

4.2 Analysis of pro-inflammatory cytokines expression in PBMCs and analysis of Treg lymphocyte population in psoriatic patients

Psoriasis is not an exclusively cutaneous disease but there is persistence of elevated levels of immune mediators, thus establishing chronic systemic inflammation. Furthermore, this disease is characterized by the impairment of Treg regulatory function Therefore, we evaluated by cytofluorimetric analysis, the percentage of the Treg population (CD4+ CD25+ FOXP3+), selected from the CD4+CD25+ lymphocyte population.

The Treg population, which literature data indicate is reduced in psoriatic patients, increases significantly (p=0.025) after one year of Risankizumab treatment (Figure 9 A).

In addition, we measured gene expression levels of three major pro-inflammatory cytokines, interleukin 1β (IL-1 β), interleukin 23 (IL-23), interleukin 8 (IL-8) in PBMCs of T0 and T1 patients. Following treatment with anti-IL-23 therapy, we observed that IL-1β, IL-23 and IL-8 expression levels were significantly reduced compared to T0. (Figure 9 B-D).

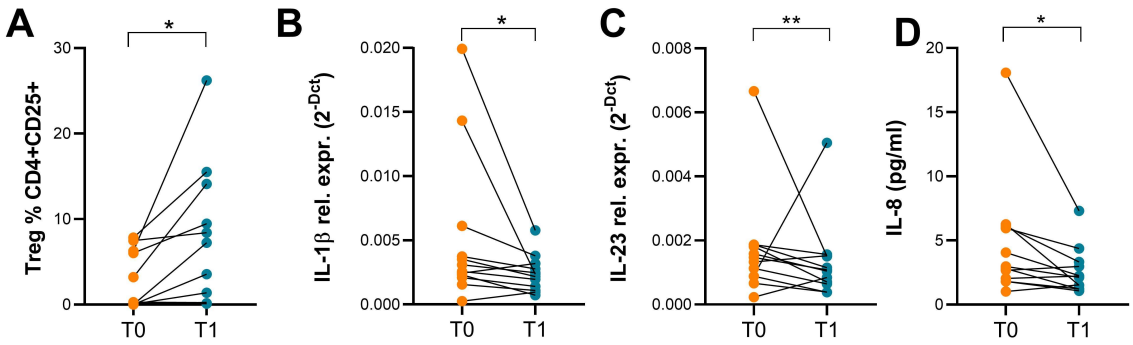
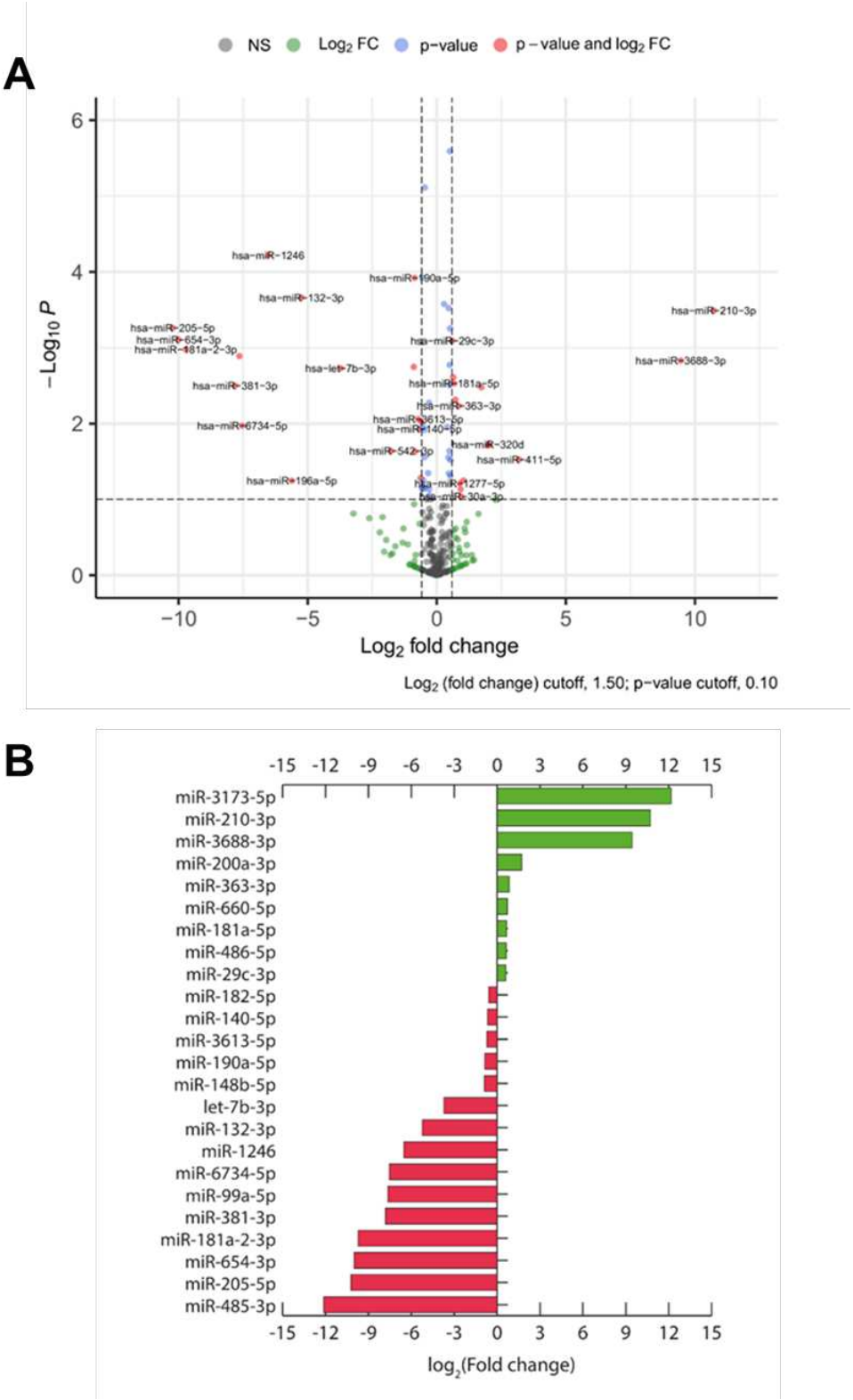


Figure 9. Percentage of Treg CD4+CD25+population in T0 and T1 patients(A), IL-1β (B), IL-23 (C) and IL-8 (D) expression levels in PBMCs of T0 and T1 patients.

4.3 Analysis of miRNoma from plasma of psoriatic patients treated with Risankizumab

miRNome was analysed in the plasma of 12 patients before (T0) and after treatment with Risankizumab for 12 months (T1). Figure 10 A shows the volcano plot of up- and down-regulated miRNAs after Risankizumab treatment. Only miRNas with a T1/T0 fold change (expressed as Log2) of at least 1.5 and a p-value ≤0.10 were selected. In Figure 10 B, the bar plot shows the 9 upregulated miRNAs (in green) and the 15 downregulated miRNAs (in red). The heatmap (Figure 10 B) reveals the expression of each miRNAs in individual patients at T0 and T1.


A
NS
Log2 FC
p-value
p – value and log2 FC
−Log10 P
Log2 fold change
hsa-miR-1246
hsa-miR-190a-5p
hsa-miR-132-3p
hsa-miR-210-3p
hsa-miR-205-5p
hsa-miR-654-3p
hsa-miR-181a-2-3p
hsa-miR-29c-3p
hsa-miR-3688-3p
hsa-let-7b-3p
hsa-miR-381-3p
hsa-miR-181a-5p
hsa-miR-363-3p
hsa-miR-6734-5p
hsa-miR-3613-5p
hsa-miR-140-5p
hsa-miR-320d
hsa-miR-542-3p
hsa-miR-411-5p
hsa-miR-196a-5p
hsa-miR-1277-5p
hsa-miR-30a-3p
Log2 (fold change) cutoff, 1.50; p−value cutoff, 0.10
B
miR-3173-5p
miR-210-3p
miR-3688-3p
miR-200a-3p
miR-363-3p
miR-660-5p
miR-181a-5p
miR-486-5p
miR-29c-3p
miR-182-5p
miR-140-5p
miR-3613-5p
miR-190a-5p
miR-148b-5p
let-7b-3p
miR-132-3p
miR-1246
miR-6734-5p
miR-99a-5p
miR-381-3p
miR-181a-2-3p
miR-654-3p
miR-205-5p
miR-485-3p
log2(Fold change)

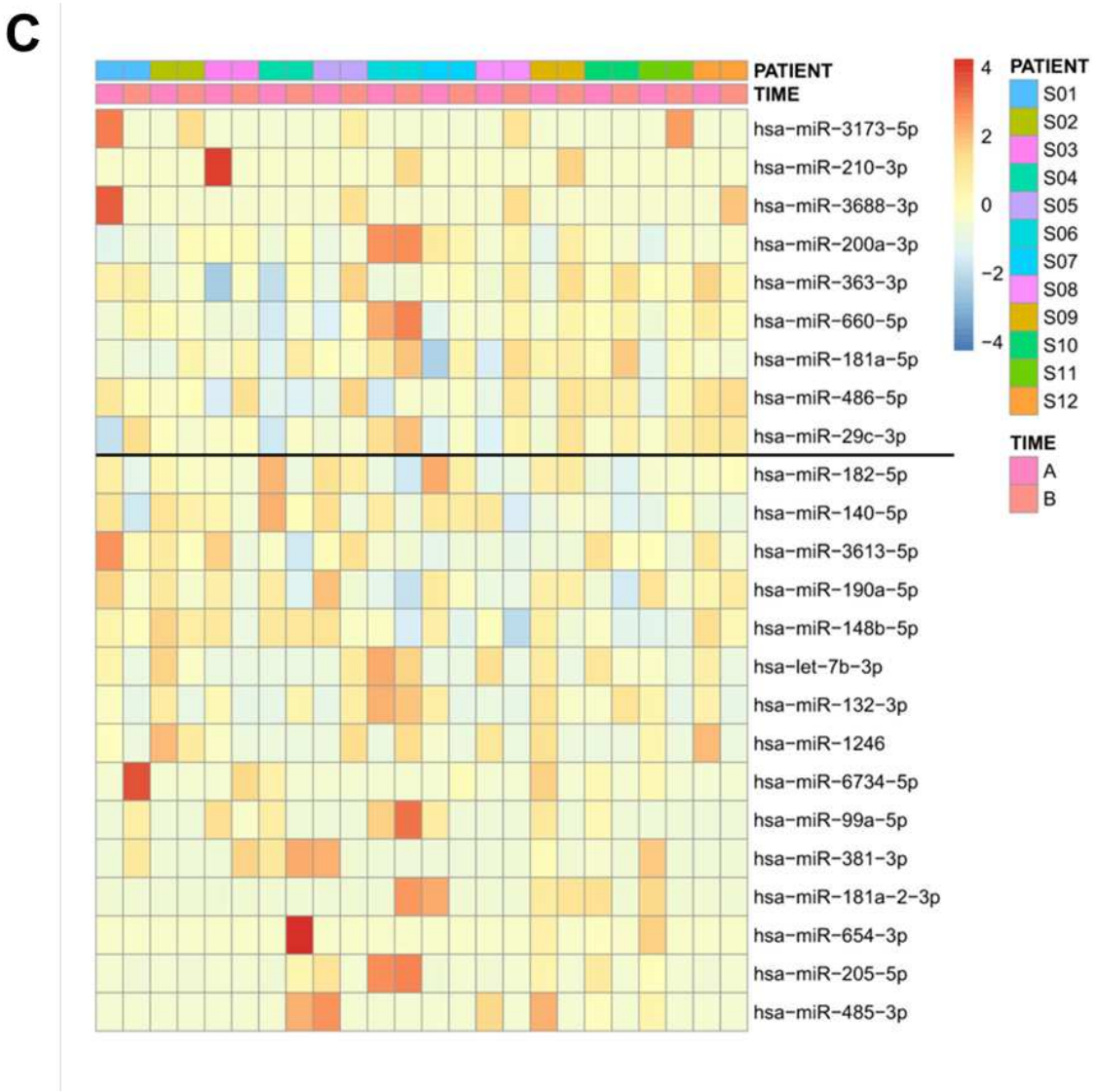


Figure 10. (A) Volcano plot of up- and downregulated miRNAs after treatment with Risankizumab. (B) Bar graph of the 9 upregulated miRNAs (in green) and the 15 downregulated miRNAs (in red). (C) Heatmap of the expression of each miRNAs in individual patients at T0 and T1.

4.3.1 Identification and analysis of predicted targets for the cluster of upregulated and downregulated miRNAs

Once the 9 significantly upregulated miRNAs and the 15 significantly downregulated miRNAs were identified, they were analyzed using the DIANA-miRpath v.3 program (90).

This tool allows identification of the target messenger RNAs of the selected miRNAs and the pathways in which they are involved using the DIANA-microT-CDS target prediction algorithm.

Table 4 shows the 14 pathways significantly regulated by the 9 up-regulated miRs, listed in order of significance. Interestingly, the first pathway identified is related to the extracellular matrix and interaction with membrane receptors (i.e., integrins). These interactions control various cellular activities such as adhesion, migration, and proliferation.

Among other pathways, the focal adhesions and PI3K-Akt signaling pathways are also noteworthy.

Table 4. KEGG pathways of the up-regulated miRNAs.

KEGG pathway	p-value	genes	miRNAs
ECM-receptor interaction	1.52E-55	26	7
Amoebiasis	6.49E-07	28	7
Focal adhesion	4.84E-05	54	8
Protein digestion and absorption	0.000177	29	5
Thyroid hormone signaling pathway	0.000516	25	8
PI3K-Akt signaling pathway	0.000622	72	8
Steroid hormone biosynthesis	0.001352	10	3
Amphetamine addiction	0.001352	15	7
Glioma	0.001352	17	7
Small cell lung cancer	0.002607	25	6
Renal cell carcinoma	0.002607	20	7
Proteoglycans in cancer	0.004966	38	8

mTOR signaling pathway	0.00579	19	6
Cocaine addiction	0.009535	11	6

Similarly, analysis was performed for down-regulated miRs. Table 5 shows the 136 pathways significantly regulated by the 15 down-regulated miRs, listed in order of significance.

As we can observe, the first three pathways potentially regulated are those of adherens junctions, mucin biosynthesis and TGF-β signaling.

Table 5. KEGG pathways of down-regulated miRNAs.

KEGG pathway	p-value	genes	miRNAs
Adherens junction	2.65E-12	45	13
Mucin type O-Glycan biosynthesis	2.85E-11	14	11
TGF-beta signaling pathway	1.20E-08	42	15
Proteoglycans in cancer	1.56E-08	79	15
Signaling pathways regulating pluripotency of stem cells	3.32E-08	64	15
Biosynthesis of unsaturated fatty acids	2.06E-07	8	5
Pathways in cancer	8.54E-07	151	15
GABAergic synapse	2.87E-06	33	14
Renal cell carcinoma	1.07E-05	35	15
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	1.08E-05	30	14

Hippo signaling pathway	2.34E-05	62	15
Bacterial invasion of epithelial cells	7.19E-05	37	14
Phosphatidylinositol signaling system	7.21E-05	35	13
Thyroid hormone signaling pathway	7.21E-05	55	14
Axon guidance	7.65E-05	53	13
Rap1 signaling pathway	8.87E-05	83	15
Prostate cancer	0.000119	43	15
Glioma	0.000129	30	13
Wnt signaling pathway	0.000662	59	15
Vasopressin-regulated water reabsorption	0.001185	22	11
Colorectal cancer	0.001597	31	11
Estrogen signaling pathway	0.001685	39	13
ErbB signaling pathway	0.003464	42	13
Glutamatergic synapse	0.004274	42	13
cGMP-PKG signaling pathway	0.005323	66	14
Cell adhesion molecules (CAMs)	0.005323	75	15
Endocytosis	0.005323	75	15
AMPK signaling pathway	0.005323	51	15
Choline metabolism in cancer	0.005323	44	15
Endometrial cancer	0.005511	24	11
Adrenergic signaling in cardiomyocytes	0.005511	52	14
Tight junction	0.005511	53	14

Focal adhesion	0.00555	78	15
Nicotine addiction	0.007569	18	9
Ras signaling pathway	0.007582	81	15
Pancreatic cancer	0.008279	29	13

4.4 Correlations between de-regulated miRNAs and clinical characteristics of patients before Risankizumab treatment

The 24 miRNAs found significantly deregulated by miRNome analysis were correlated with indices of disease severity in the 12 psoriatic patients before Risankizumab treatment.

Among down-regulated miRs, we observed that miR-190a-5p correlated significantly and positively with PASI (r=0.690; p=0.013) (Figure 11 A) and BSA (r=0.595; p=0.041) (Figure 11 B), while miR-148b-5p correlated significantly and positively with BSA (r=0.834; p=0.001) (Figure 11 D). Also, among the up-regulated miRNAs, miR-200a-3p showed a

significant negative correlation with PASI (r=0.620; p=0.032) (Figure 11 C).

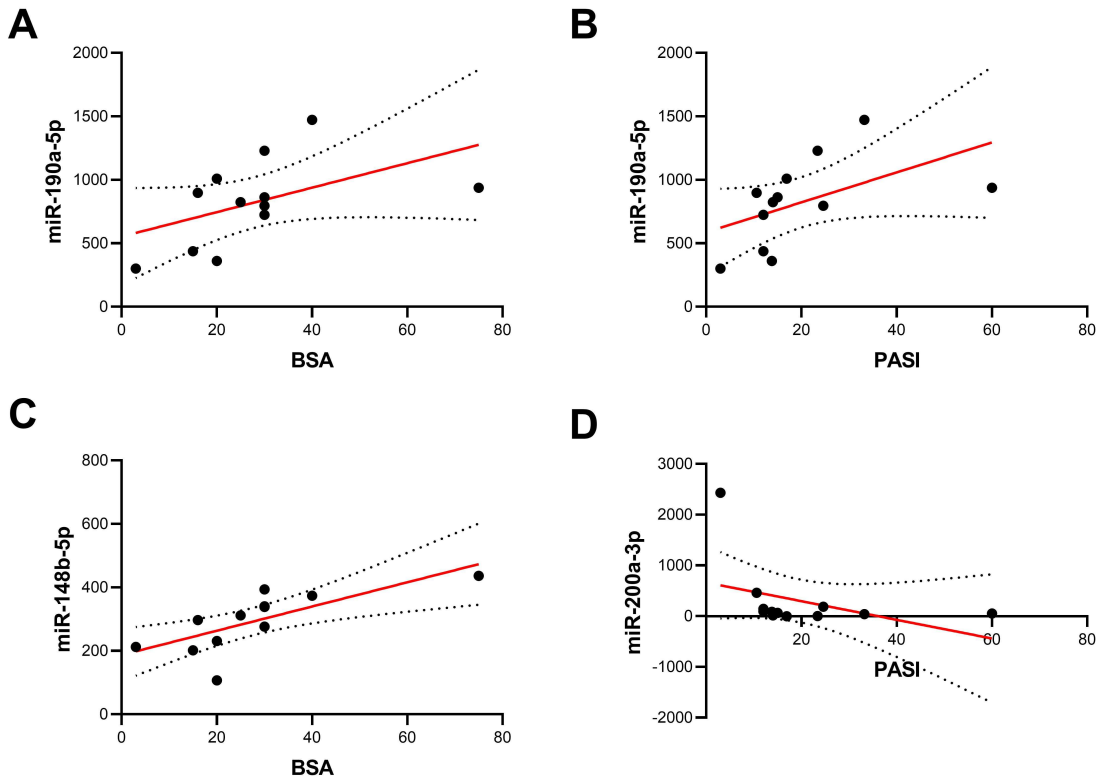


Figure 11. (A, B) miR-190a-5p positive correlation with BSA (r=0.595; p=0.041) and PASI (r=0.690; p=0.013); (C) miR-148b-5p positive correlation with BSA (r=0.834; p=0.001) and miR-200a-3p negative correlation with PASI (r=0.620; p=0.032) (D).

In addition, we analysed the pathways potentially deregulated by these three miRs (miR-190a-5p, miR-200a-3p and miR-148b-5p) by DIANA-miRpath-v.3. The heatmap in Figure 12 shows the pathways in which target genes of at least one of the three miRNAs analysed are present.

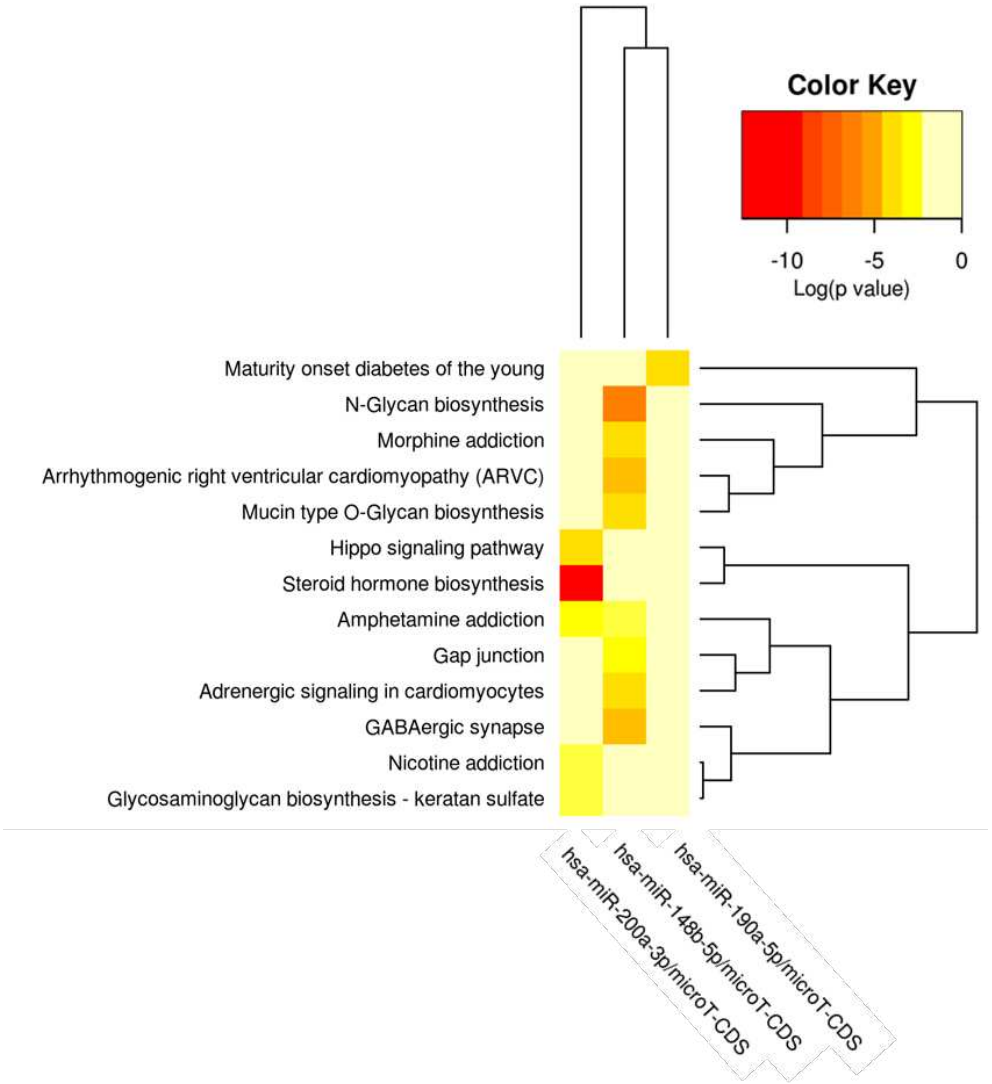


Figure 12. Heatmap of pathways potentially deregulated by miR-190a-5p, miR-200a-3p and miR-148b-5p.

4.5 Validation analysis by RT-qPCR and investigation of circulating cytokines by multiplex assay.

In the validation phase we performed RT-qPCR of the three miRNAs which we found significantly correlated with the clinical characteristics of the psoriatic patients, such as miR 200a-3p, miR-190a-5p and miR-148b-5p. After validation, we confirmed a negative correlation between miR-200a expression level and PASI in the 23 psoriatic patients of the validation group (Figure 13A).

Finally, we measured a multiplex panel of 8 pro-inflammatory cytokines (TNF-α, IL-8, and IL-6, IL-12, IL-18, IL-1α, IL-17 and INF-γ) in the serum of the 23 psoriatic patients by Luminex-100 system. Then, we correlated the circulating cytokines concentration levels with the indices of disease severity in the 23 psoriatic patients before risankizumab treatment. We found that only the INF-γ showed a significant and positive correlation with PASI (Figure 13 B).

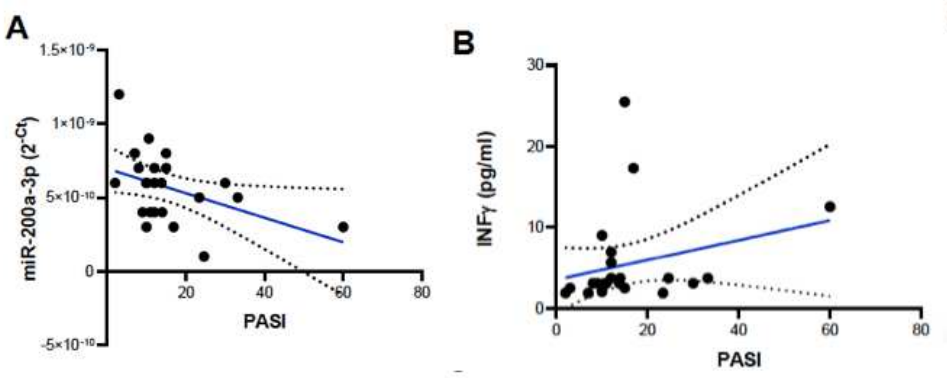


Figure 13. (A) MiR-200a-3p negative correlation with PASI. (B) Positive correlation between circulating INF-γ and PASI.

4.6 Different expression of miRNAs in subjects over and under 60 years old.

Finally, the difference in the expression of the 3 main deregulated miRNAs that correlated with clinical parameters of disease severity before treatment was studied in the same patients divided into two groups according to age: patients under 60 years old (mean age: 43.6 (±10.4)), defined as the "*young group*," and patients over 60 years old (mean age: 68.5 (±7.6)) defined as the "*elderly group*." We observed that the expression of miR-148b was significant different betweenthe two groups being more highly expressed in the "*young group*" (Table 6).

Table 6. miR-148b, miR-190a and miR-200a differential expression in patients < 60 years old and patients > 60 years old.

	Patients<60years (n=15)	**Patients>60years** (n=8)	***p value***
Age (years)	43.6 (±10.4)	68.5 (±7.6)	**0.0001**
miR-148b	0.00031320 (±0.000142073)	0.00020550 (±0.000101545)	**0.05**
miR-190a	0.00003100 (±0.000026739)	0.0002913 (±0.000015009)	0.832
miR-200a	0.00002473 (±0.000010840)	0.00001813 (±0.000008026)	0.381

Chapter Five

DISCUSSION

Psoriasis is a complex, multifactorial disease caused by immune-mediated inflammation and characterized by erythematous-desquamative skin lesions that may occur in some limited regions or extend over the entire body. It is a condition that mainly affects the skin but can involve other organs and apparatuses, such as the joints, which is why it is considered a systemic disease (1). Regarding treatments in recent years, there has been a real "revolution", moving from the use of broad-spectrum immunosuppressants (cyclosporine, methotrexate, or other drugs in this category) to biologic drugs. The first biologic drugs developed were the anti-TNF alpha. Due to discoveries about the mechanisms involved in psoriatic plaque formation and maintenance, anti-interleukin 17 (IL-17) and anti-interleukin 23 (IL-23) drugs were developed in later years. As for IL-23, it is now considered the major player in the pathogenetic mechanisms of the onset of cutaneous and/or joint systemic inflammation by stimulating the release of the cytokines IL-17, IL-22, and TNF-alpha. IL-23 also appears responsible for disease relapse in patients, probably because it is involved in reactivating specific memory T cells located at the skin level (TRM) (91, 92). Their reactivation correlates with the occurrence of psoriatic disease after a period of inactivity. Thus, the use of anti-IL-23 drugs should be associated not only with excellent efficacy against the clinical manifestations of the disease but also with helping reduce relapse (92).

Therefore, to better understand the mechanisms underlying the clinical efficacy of anti-IL-23 treatment of patients with psoriasis, we evaluated possible changes in circulating microRNA and pro-inflammatory cytokines levels as well as in the percentage of regulatory T cells. The aim is identifying potential biomarkers associated with clinical response to biological therapy which is characterized by high costs.

All treated patients included in the study showed marked improvement in clinical symptoms, with improvement in all clinical assessment indices (PASI, BSA, PGA, DLQI). Regarding the most significant changes in circulating microRNAs, we identified three de-regulated miRNAs, miR-200a, miR-190a-5p and miR-148b-5p, after risankizumab therapy whose levels correlate with disease severity indices detected at baseline.

MiR-200a appears to be closely related to Th17 cells in psoriasis. In fact, Wang et al. found a statistically positive correlation between miR-200a expression and Th17 percentage, Th17/Treg ratio, IL-17 and IL-23 levels, and RORγt mRNA expression, as well as PASI score. In contrast, there was a negative correlation between miR-200a and Treg percentage, TGF-β level and FoxP3 mRNA expression (93). A study in multiple sclerosis validated the key role of miR-200a, as well as miR-141, in inducing Th17 cell differentiation by inhibiting Treg cell differentiation (94).

However, in our study, miR-200a levels were found to be increased after treatment, and a negative correlation was demonstrated between circulating values of this miR at baseline and PASI value. Patients with the most clinically evident pathology had the lowest circulating levels of miR-200a.

Despite appearing to contradict the existing literature, it is worth mentioning that previous studies only looked at miR levels within cells, while this study considered the plasma level. Therefore, we could hypothesize a therapy-induced translocation of miR-200a from the cytoplasm to the circulation, and this could explain the increase in plasma after therapy(95).

Regarding the two other circulating miRNAs, whose levels were found to be reduced after treatment, we observed that miR-190a-5p correlated positively with PASI ($r=0.690$; $p=0.013$) and BSA while miR-148b-5p correlated with BSA ($r=0.834$; $p=0.001$). To date, there are no studies that have identified the role of these two miRNAs in the pathogenesis of psoriasis; however, they appear to play an important role in other diseases. The miR-190a-5p would appear to be involved in the pathogenesis of cardiac arrhythmias (96) and would also appear to be involved in ferroptosis, promoting the onset of myocardial infarction (97).

MiR-148b-5p was mentioned in several studies analysing its implications at the level of neuronal tissue: it would appear that a blockade of miR-148b can induce a process of neuroprotection through Wnt/β-catenin signaling (98). We know that psoriasis is a disease burdened by numerous comorbidities (21) so it cannot be ruled out that the levels of these miRNAs may give us information about neurodegenerative comorbities. However, further studies are needed to confirm this.

Validation analysis confirmed ~~only~~ a negative correlation between miR-200a expression level, which is increased after anti-IL23 therapy, and PASI index.

Regarding the T regulators analyzed in our study, the increase in circulating Tregs after treatment is confirmed in all treated patients. IL-23 induces the conversion of Tregs to Th17, promoting inflammatory phenomena (99). Anti-IL-23 restores circulating Treg values that are functional in modulating inflammation.

Moreover we analysed a panel of pro-inflammatory cytokines and we observed a reduction in IL-23, IL-1β and IL-8 expression levels in PBMCs of psoriatic patients after risankizumab therapy, and this is just a confirmation of the drug's effect on reducing the systemic inflammation characteristic of the disease. In particular IL-8 plays an essential role in psoriasis as expressed in the stratum granulosum, attracts polymorphonuclear cells, and stimulates angiogenesis and keratinocyte mitogenesis. The decrease in this cytokine is a confirmation of the biological efficacy of the drug. Finally, the positive correlation of serum INF- γ with PASI index represents a confirmation of the biological effect of these cytokines on disease severity.

CONCLUSIONS

The study of miRNome with subsequent identification of miRs involved in pathogenetic disease processes and their changes after therapy can be extremely useful in increasing the level of knowledge of psoriasis and its treatment. In particular, with regard to miR-200a, we hypothesized a possible transport of the miR from the lymphocyte to the circulation as a result of therapy, assumably mediated by extracellular vesicles. Thus, miR-200a might be considered as a good biomarker of disease activity or response to therapy.

In contrast, miRNAs that we observed to be significantly reduced in circulation after treatment with anti-IL-23 were found to be associated with progression of cardiac or neurological disease. This result suggests that treatment with anti-IL-23 could also have an influence on the comorbidities of psoriatic disease.

Finally, the effect of risankizumab on the reduction of systemic inflammation is demonstrated by the increase in Treg and reduction of the inflammatory cytokines IL-1β and IL-8 expression level after risankizumab therapy.

LIMITATIONS

The main limitation of the study is the limited size of the study population. Larger, multicenter, and possibly prospective studies are needed to identify miRNA cut-off values and validate our preliminary observations' clinical relevance.

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