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THE NOVEL ANTI-IL-17A MONOCLONAL ANTIBODY SECUKINUMAB IN THE TREATMENT OF PSORIASIS: BIOLOGICAL EFFECTS ON ANGIOGENESIS AND EXTRACELLULAR VESICLES

Doctoral Thesis

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A mio figlio

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ABSTRACT

Psoriasis is a chronic inflammatory skin disease. The role of Interleukin-17A (IL-17A) is emerging in the pathogenesis of psoriasis and considered the main driver of inflammation and dysregulated angiogenesis promoting in psoriatic keratinocytes the release of soluble mediators including the vascular endothelial factor-A (VEGF-A).

The early growth response-1 (Egr-1) transcription factor, recently demonstrated upregulated in the skin of patients with psoriasis, has been suggested to influence angiogenesis by regulating VEGF-A expression. It is reported that IL-17A increases Egr-1 expression in human keratinocytes, however little is known about the biological function of Egr-1 in psoriasis.

Notably, the successful employment of the novel therapeutic monoclonal antibody anti-IL-17A Secukinumab (Cosentyx, Novartis) is ascribable to the indirect inhibition of angiogenesis.

Beside cell-to-cell contacts and release of cytokines, hormones and second messangers, cells communicate each other through the release of extracellular vesicles (EVs) containing DNA, RNA, microRNAs and proteins. It has been reported the alteration of EVs trafficking in several diseases, but there is scare evidence of the involvement of EVs trafficking in the pathogenesis of psoriasis.

Accordingly, we investigated the molecular mechanism by which Egr-1 could affect psoriasis angiogenesis in response to IL-17A signaling and if Secukinumab reduces angiogenesis in psoriatic keratinocytes by an Egr-1-dependent mechanism. We also aimed to characterize the release, the cargo content and the capacity to transfer bioactive molecules of EVs produced by keratinocytes following IL-17A treatment compared to untreated keratinocytes.

Results indicate that Secukinumab downregulates VEGFA mRNA and its soluble levels in human keratinocytes (HaCaT) treated with IL-17A. Conditioned medium from keratinocytes previously exposed to Secukinumab significantly decreases cell proliferation and migration of endothelial cells and inhibits capillary tube formation *in vitro*. The use of Egr-1 siRNA downregulates IL-17A-mediated VEGF-A expression. Overall, these data suggest that Secukinumab indirectly inhibits keratinocytes angiogenic property by interfering with IL-17A/Egr-1/VEGF-A axis.

As what concern the study of keratinocyte-derived EVs, results indicate that the treatment with IL-17A significantly modifies the EVs cargo and release. Vesicles from IL-17A-treated

cells display a specific pattern of mRNA which is abrogated by Secukinumab. Further, EVs are taken up by acceptor cells irrespective of their content but only those derived from IL-17A-treated cells enable recipient cells to express psoriasis-associated mRNA. These results imply a role of EVs in amplifying the pro-inflammatory cascade induced in keratinocytes by pro-psoriatic cytokines.

1. INTRODUCTION

1.1 PSORIASIS

Psoriasis is a chronic systemic inflammatory disease, with prevalent cutaneous involvement, characterized by a chronic relapsing course (Nestle FO *et al*., 2009). It is spread all over the world with a prevalence that varies considerably among the different populations and the various ethnic groups considered, in relation to the various geographical, environmental and genetic factors (Christophers E, 2001). Psoriasis seems to be most prevalent in Caucasian population and less frequent in Asian individuals and in black people (Gelfand JM *et al.*, 2005; Schafer T, 2006).

In the USA, the prevalence of psoriasis has been estimated between 2.2% and 2.6%; in contrast, in Japan, the prevalence is lower and practically absent in Australians and Indians from South America. African Americans have an approximately 52% reduction in the prevalence of psoriasis compared with Caucasians (1.3% vs. 2.5%) (Saraceno R *et al.*, 2008). In Europe the prevalence is higher in the Nordic countries than in the Mediterranean, probably due to climatic and environmental factors (Parisi R *et al.*, 2013). The estimated prevalence of psoriasis in Southern Europe varies from 1.3% in Germany to 1.55% in Croatia and 1.58% in the UK (Saraceno R *et al.*, 2008) (**Figure 1**).

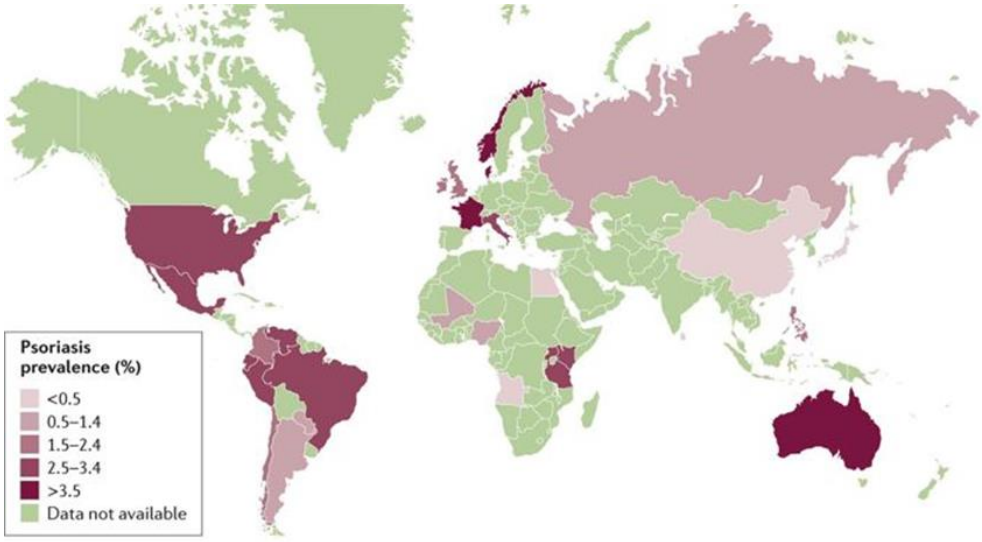


Figure 1. *Psoriasis. The prevalence of psoriasis worldwide (Greb JE et al., 2016).*

In Italy the prevalence of psoriasis is about 2.9% of the population, with a notable non-homogeneous distribution among the various regions; in fact, the calculated range varies from 0.8% to 4.5% with reference to the various locations considered (Saraceno R *et al.*, 2008). The regions that show a greater prevalence are Lazio, Abruzzo, Molise (4.5%) and Emilia Romagna (4%), while Sardinia (0.8%) is the region with the lowest prevalence, followed by Calabria, Puglia and Basilicata (1.6%) (Saraceno R *et al.*, 2008) (**Figure 2**).

Figure 2. *Psoriasis. Italian regions and percentage of people affected from psoriasis (Saraceno R et al., 2008).*

The disease affects males and females equally, although it has been suggested that the onset of psoriasis is generally earlier in women than in men in some European countries (Parisi *et al.*, 2013). Based on the age of onset, psoriasis is divided into two types (Griffiths CEM and Barker J, 2007):

- type I psoriasis, present in about 75% of cases, is characterized by an early onset (before the age of 40) with a peak incidence between 16 and 20 years of age, high familiarity and a strong tendency to evolve towards a serious and/or generalized clinical form (Nevitt GJ and Hutchinson PE, 1996);

- type II psoriasis, referred to as "late onset", with a peak incidence of around 50-60 years of age, a positive family history only in a limited number of cases and a less severe clinical course (Henseler T and Christophers E, 1985).

1.1.1 Clinical features and classification

The main clinical-pathological characteristic of psoriasis is the typical lesions consisting of chronic erythematous plaques, covered with ipercheratosic scales. Although lesions can be present throughout the body, they are most frequently located at the elbow, knee and scalp level (Nestle FO *et al.*, 2009).

The plaque contains histopathological hallmarks that include a thickening of the epidermis (acantosis) caused by hyperproliferation of keratinocytes, hyperkeratosis and paracheratosis, or retention of nuclei in the stratum corneum; there is also a lengthening of the epidermal ridges downwards and an increase in the dilation and tortuousness of the vessels of the dermal papillae. At the level of the dermis and, to a lesser extent, the epidermis there is a rich inflammatory infiltrator, mainly consisting of T cells, myeloid and plasmacytoid dendritic cells, NK lymphocytes, macrophages and neutrophil granulocytes (Mahil *et al.*, 2016).

The course is chronic, characterized by the alternation of acute phases and phases of remission difficult to predict. The disease can start silently for years until a stimulus of an undefined nature, sufficiently intense, determines the appearance of a psoriatic slick in the seat of injury.

Another typical, but not exclusive, feature of psoriasis is reactive isomorphism, which consists of the appearance of psoriasis patches in locations subject to mechanical stress (scarification, burns, surgical scars) (Pedace FJ *et al*., 1969).

From a clinical point of view, based on the form of the lesions and their localization, psoriasis can be distinguished in (**Figure 3**):

- **psoriasis vulgaris:** is the commonest form of psoriasis, accounting for 90% of all cases; it is characterized by red skin lesions (plaques) covered by silvery scales and well-delineated from surrounding normal skin. Lesions appear as erythematous, a few centimeters thick and typically located on elbows and knees, but can extend and affect other skin areas (Griffiths CE *et al.*, 2007);

- **guttate psoriasis**: this acute form primarily affects adolescents and children; it is marked by the rapid appearance of small (less than 1 cm), punctiform and scaling lesions (papules), scattered with regularity and symmetry over the entire surface of the skin, preferring the area of the trunk and face. It is generally triggered by a bacterial infection, usually of streptococcal origin (group A β-haemolytic streptococcus), such as tonsillitis or pharyngitis, or a viral

infection (Martin BA *et al.*, 1996; Griffiths CE and Barker J, 2007). Guttate psoriasis is self-limiting, resolving within 3–4 months of onset, although its long-term prognosis is unknown. One study indicated that only a third of individuals with guttate psoriasis develop classic plaque disease (Martin BA *et al.*, 1996);

- **pustular psoriasis**: there are three main forms;

  - generalized pustular psoriasis (von Zumbusch psoriasis) is an acute form in which small pustules develop in painful inflamed skin. The patient can also have fever, chills, severe itching and diarrhea (Gooderham MJ et al., 2019);

  - palmo-plantar pustulosis, consisting of yellow-brown pustules on palms and soles. About 25% of people with palmoplantar pustulosis also have chronic plaque psoriasis. Patients are predominantly women (9:1 female:male ratio) and either current or previous smokers (95%) (Kubeyinje EP and Belagavi CS, 1997);

  - Hallopeau's Acrodermatitis that occurs on the fingertips, on the peri-ungueal region, while the involvement of the nail matrix determines the complete loss of the nail. It is associated with an impairment of the general state with malaise, fever and asthenia (Baran R, 1979);

- **erythrodermic psoriasis:** the least common type of psoriasis; the whole body surface is affected by psoriasis, which can lead to hypothermia, hypoalbuminaemia, and high-output cardiac failure. Erythroderma can be caused by other diseases, including atopic dermatitis, drug eruptions, and cutaneous T-cell lymphoma. (Griffiths CE and Barker J, 2007);

- **inverse psoriasis**: is a form of psoriasis that selectively involves the folds, recesses, and flexor surfaces such as the ears, axillae, groin folds, inframammary folds, navel, intergluteal cleft, genitals, lips, and webspaces. Inverse psoriasis causes smooth patches of red, inflamed skin that worsen with friction and sweating. Fungal infections may trigger this type of psoriasis. (Griffiths CE and Barker J, 2007);

- **nails psoriasis**: a type of psoriasis that affect fingernails and toenails, causing pitting, abnormal nail growth and discoloration. Psoriatic nails might loosen and separate from the

nail bed (onycholysis) (Farber EM and Nall ML, 1974). This form is closely associated with artopathic psoriasis (Griffiths CE and Barker J, 2007);

- **psoriatic arthritis:** is a seronegative inflammatory arthritis that occurs in the presence of psoriasis. Five types of psoriatic arthritis have been proposed distal interphalangeal joint only; asymmetrical oligoarthritis; polyarthritis; spondylitis and arthritis mutilans. Classic psoriatic arthritis consists of oligoarthritis, distal interphalangeal joint involvement, dactylitis and calcaneal enthesitis. Recently presented data indicate that its prevalence has been greatly underestimated, and may be as high as 25% in people with psoriasis. In about 10% of people with psoriatic arthritis, the arthritis appears before skin manifestations of psoriasis (Moll JMH and Wright V, 1973; Gladman DD *et al.*, 2005; Helliwell PS and Taylor WJ, 2005).

Depending on the severity (extension on the body surface), psoriasis can be considered mild in the presence of a limited number of plaques, typically less than 3% of the skin surface is affected, moderate if 3 to 10% of the skin surface is occupied by plaques, and severe when more than 10% of the skin surface is affected (Meier M and Sheth PB, 2009).

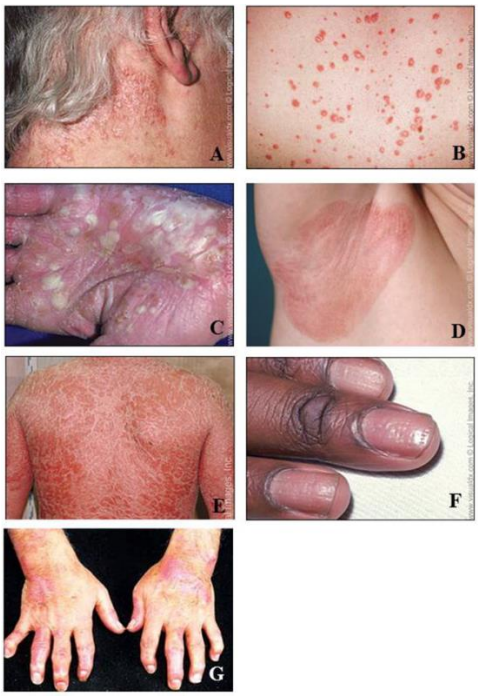


Figure 3. *Psoriasis. The different types of psoriasis. (A) plaque psoriasis; (B) guttate psoriasis; (C) pustular psoriasis; (D) reverse psoriasis; (E) erythrodermic psoriasis; (F) onychopsoriasis; (G) psoriatic arthritis (modified from Nestle FO et al., 2009; Boehncke WH and Schon MP, 2015).*

1.1.2 Comorbidities

Several diseases have a higher incidence in psoriasis patients than the unaffected population, including Crohn's disease (Cohen AD *et al.*, 2009), type 2 diabetes mellitus (Lee MS *et al.*, 2014), metabolic syndrome (Arias-Santiago S *et al.*, 2012), cardiovascular disease (Griffiths CE and Barker JN, 2007), depression and some cancers. A recent study showed that psoriasis patients are three times more likely to develop lymphomas than the unaffected population (Gelfand JM *et al.*, 2006). An increased risk of developing skin cancer (melanoma and non-melanoma) in psoriasis patients has not been proven, but a 14-fold increased risk of developing squamocellular carcinomas has been observed in patients of phototype I-II who received 250 UV phototherapy (PUVA) sessions compared to patients who received the least number of treatments (Griffiths CE and Barker JN, 2007). Treatments with metotrexate and

cyclosporin in high doses can also be associated with the development of tumors (De Oliveira Mde F *et al*., 2015).

The relationship between psoriasis, diabetes and cardiovascular disease is increasingly emerging Studies in psoriasis patients have found an increase in plasma levels of different inflammation markers, such as pro-inflammatory cytokines, or thrombosis regulators, suggesting that psoriatic pathology is associated with a state chronic flogosis (Fernández-Armenteros JM *et al*., 2019). This condition can lead to the onset of insulin resistance which, at the level of endothelial cells, leads to a reduction in the release of vasodilatory factors such as nitrogen monoxide. These events lead to the development of a state of endothelial dysfunction with increased expression of adhesion molecules, providing the basis for the development of ateroscletoric plaques and thus increasing the risk of cardiovascular pathology. Among the biomarkers identified in the plasma of patients and related to endothelial dysfunction are the soluble form of cell adhesion molecules VCAM-1 and E-selectine. To date the clinical relevance of these markers remains, however, unclear (Steyers CM and Miller FJ, 2014).

1.1.3 Pathogenesis and risk factors

The cause of psoriasis isn't yet fully understood; it is believed that clinical manifestations are determined by the combination of predisposing genetic factors and environmental triggers; among these, an important role is played by skin traumatism, infectious processes and some drugs (Kavli *et al.*, 1985; Elder JT *et al.*, 2009).

Its pathogenesis is therefore multifactorial and is conditioned by an abnormal immune response, probably triggered against an epidermal antigen (Lowes MA *et al.*, 2007). Since 1970 it has been thought that the massive presence in psoriasis patients of immune system cells, especially dendritic cells and T lymphocytes, suggested a possible pathogenic role for the disregulation of the immune system in psoriasis (Nestle FO *et al.*, 1994).

Although the literature data do not provide sufficient evidence that psoriasis is a real autoimmune disease, it shares with other immune-mediated diseases, such as Crohn's disease and diabetes mellitus, some characteristic manifestations chronic inflammation in the absence of a known antigen (Davidson A and Diamond B, 2001). For these reasons, psoriasis lesions

are believed to evolve from the interaction between cells and innate and adaptive immunity mediators and skin connective and epithelial tissues (Schon MP and Boechncke WH, 2005).

Several pathogenic hypotheses have been formulated to explain the origin and clinical expression of psoriasis. Among the various theories, the most accredited is that psoriasis depends on an altered response of psoriasis keratinocytes to disparate inflammatory stimuli, which originate from the intervention of innate immunity cells, such as dendritic cells, polymorphucleates, mastcells, NK cells, and from adaptive immunity cells, such as CD4+ and CD8+ T-cells (Liu et al., 2007). Under normal conditions, there are, at the dermal level, a small number of T cells and CD11+ cells, while at the epidermal level Langerhans cells are present evenly in the various layers. In the psoriatic lesion, there is an increase in CD11+ dendritic cells and CD8+ T lymphocytes, which migrate at the epidermis level, respectively, at the basal layer or throughout the area. Langerhans cells continue to be present in the epidermis, but migrate at the spinosum layer level, while neutrophils accumulate at the stratum corneum level forming small aggregates (Liu *et al*., 2007).

One of the pathogenic mechanisms for the development of psoriatic lesion involves, as the first event, the formation of a complex consisting of DNA fragments released by stressed or dying keratinocytes and the peptide LL-37 (**Figure 4**). This complex is able to activate plasmacytoid dendritic cells (pDCs) to produce IFN- α (Lande R *et al.*, 2007).

LL-37 belongs to the family of catelicides, which relate to the class of antimicrobial peptides. These molecules are able to promote the elimination of pathogenic microorganisms and to modify the inflammatory response. In psoriasis lesions, three types of peptides are overexpressed: catelicide, β defensine and S100 proteins (Morizane S *et al.*, 2012).

After the formation of the DNA-LL37 complex, the IFN-α produced by pDCs and the interleukin IL-1β, IL-6, the "tumor necrosis factor-α" (TNFα) produced by the keratinocytes activate the dermal dendritic cells (DDCs) that in turn produce IL23A, nitric oxide (NO) and TNFα (Zaba LC *et al.*, 2009).

DDCs migrate to the lymph nodes, where they present the antigen to naive T cells promoting differentiation inTh17, Th1 and Th22. These cells express on their surface the receptors for the cytokines CCR4 and CCR6, which bind CCL17 and CCL20 respectively, and the "cutaneous lymphcyte-associated antigen" (CLA), a surface glycoprotein capable of binding E-selectin (Yamanaka K *et al*., 2008).

Through the lymphatic and blood vessels, Th17 and Th22 lymphocytes arrive at the level of the psoriatic lesion in presence of chemotactic factors CCL20 and CCL17 released by

keratinocytes. IL23A, produced by activated dendritic cells, induces Th17 and Th22 to release IL-17A , IL-17F, IL-22 and IFN-a. IL-17A and IL-17F stimulate keratinocytes to produce chemokines capable of recruiting neutrophils, including CCL17, CCL20, CXCL8, CXCL10, and antimicrobial peptides (LL-37 and the S100 family). (Wilson NJ *et al.*, 2007).

CD8 T lymphocytes, which express on their Surface VLA-1 that can bind VCAM-1 expressed by endothelial cells, accumulate in the epidermis and produce IL-17 (Laggner U *et al*., 2011).

Th1 and Th22 reach the inflammatory site and induce the production, in synergy with the Th17, of IL-22, responsible for epidermal hyperplasia, altering the differentiation of keratinocytes, with a possible contribution of IL17A (Di Meglio P *et al*., 2011).

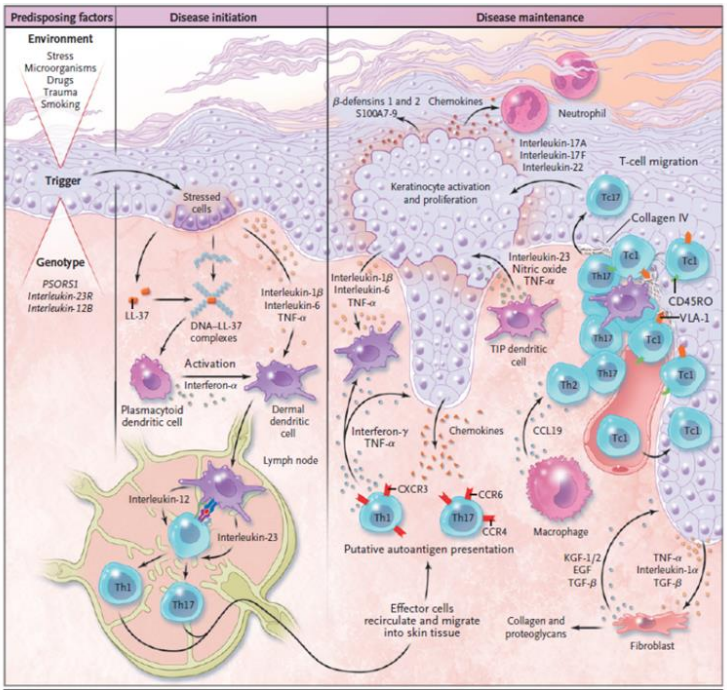


Figure 4. *Psoriasis. Pathogenetic model of psoriasis* (*Di Meglio P et al., 2011).*

The greatest risk factor for psoriasis is the positive family history for the disease (Farber EM *et al*., 1974). It is estimated that about 30% of psoriasis patients have at least one family member with the same condition (Andressen C and Henseler T, 1982). This highlights a strong genetic component in the etiology of psoriasis.

In recent years linkage analysis studies have led to the mapping of 15 gene loci, distributed on different chromosomes, that are associated with psoriasis susceptibility, numbered 1 to 15 (**Table 1**). PSORS (psoriasis susceptibility) loci correspond to regions in which localize genes

involved in inflammatory processes or genes involved in the differentiation phases of epidermis.

Locus	Cytogenetic location	Genomic coordinates (GRCh38)	Candidate genes[a]
PSORS1	6p21.33	31,268,748–31,272,135	HLA-C
PSORS2	17q25.3	80,169,991–80,209,330	CARD14
PSORS3	4q	50,000,000–190,214,555	NFKB1, CFI, KIAA1109, IL2, IL21, IL21-AS1, BBS12
PSORS4	1q21	143,200,000–155,100,000	HFE2, FLG, LCE3C, LCE3B, LCE3A, LCE3E, LCE2C, LCE1C, LCE1A, SMCP, IVL, SPRR2C, SPRR2G, LELP1, PRR9, LOR, PGLYRP3, PGLYRP4, S100A9
PSORS5	3q21	122,200,000–129,500,000)	SLC12A8
PSORS6	19p13	19:0-19,900,000	BSG, SMARCA4, OR7A10
PSORS7	1p	0-123400000	TNFRSF9, TNFRSF1B, KAZN, IGSF21, PAX7, CAPZB, IFNLR1, RUNX3, AZIN2, CSMD2, OMA1, IL23R, GNG12-AS1, LRRC7, AK5, SPATA1, DDAH1, GBP6, KIAA1107, CEPT1, DENND2D, PTPN22
PSORS8	16q	36,800,000–90,338,345	CYLD, NOD2, FTO, CDH8, SMPD3, CDH3, IL34, MLKL, CMIP, CDH13, SLC38A8, MBTPS1, WFDC1, KIAA0513
PSORS9	4q31-q34	138,500,000–182,300,000	RNF150, DCHS2, MSMO1, SPATA4
PSORS10	18p11.23	7,200,000–8,500,000	*No gene reported to be mapped conclusively*
PSORS11	5q31.1-q33.1	131,200,000–153,300,000	RAD50, IL13, IL4, STK32A, TNIP1
PSORS12	20q13	43,100,000–64,444,167	SPATA2, RNF114, CYP24A1
PSORS13	6q21	111,555,377–111,606,873	TRAF3IP2
PSORS14	2q14.1	113,058,637–113,064,743	IL36RN
PSORS15	2q36.1	223,755,330–223,837,602	AP1S3

Table 1. *Psoriasis. PSORS loci with cytogenetic location, genomic coordinates and candidate genes (Singh et al., 2019).*

The first of these loci, the PSORS1 locus, is the major genetic determinant of psoriasis which is confined to ~300 kb region in the major histocompatibility complex (MHC) (Russell TJ *et al*., 1972; Nair *et al*., 2006; Liu Y *et al.*, 2007). The SNPs detected through genomic association studies revealed about fifteen genes (DDR1, DPCR1, MUC21, MUC22, HCG22, C6orf15, PSORS1C1, CDSN, PSORS1C2, CCHCR1, TCF19, POU5F1, PSORS1C3, HCG27, HLA-C) in the 300-kb critical region of PSORS1 are strongly associated with psoriasis (Capon et *al.*, 2002). The association with the locus HLA is responsible for 30-50% of cases of the disease (Trembath *et al*., 1997, Nair RP *et al*., 2006). Fourteen PSORS regions (PSORS2-15) outside the MHC are reported to be associated with psoriasis. PSORS2 is associated with psoriatic patients having family history (Nair *et al.*, 1997; Enlund *et al*., 1999;). PSORS3 locus represents psoriasis associated SNPs in IL2 and IL21, that takes part in T lymphocytes proliferation, Th17 differentiation, and keratinocyte proliferation (Liu *et al*., 2008). PSORS4 genes mainly participate in keratinization process and expressed in upper strata of the epidermis. Mutations in PSORS4 genes are reported to have high probability of triggering psoriasis vulgaris (PsV) (Capon F *et al*, 2001; Hüffmeier *et al.*, 2009).

SNPs in SLC12A8 that encode a potassium/chloride transporter, is reported to be associated with psoriasis in PSORS5 locus (Hewett *et al.*, 2002). SNPs in PSORS6 locus enhances risk of early-onset psoriasis vulgaris (Hüffmeier *et al*., 2009). IL23R with 19 psoriasis associated

SNPs, at PSORS7, has been established as one of the major susceptible gene that can trigger psoriasis (Di Meglio and Nestle, 2010). PSORS8 is reported as an overlapping susceptibility locus for both psoriasis and Crohn disease (Nair *et al*., 1997). The locus PSORS9, PSORS10,PSORS11 and PSORS12, are not reported to show significant association towards a distinct clinical subtype of psoriasis. As that of PSORS4, mutations in PSORS13 locus were observed to be associated with psoriasis vulgaris (Ellinghaus *et al*., 2010). The locus PSORS14 and PSORS15 have high association with pustular psoriasis cases (Setta-Kaffetzi *et al*., 2014).

There are various type of environmental factors that act on the polygenic substrate; those most involved are traumatic, infectious, pharmacological, endocrine-metabolic, alimentary and psychological (Barker J, 1991; Enamandram M and Kimball AB, 2013). Any physical trauma, such as burns and scars, can cause psoriatic lesions to appear in affected sites in predisposed persons. The phenomenon of reactive isomorphism, which implies the appearance of a psoriatic lesion on apparently healthy skin following traumatic insults, is an example of the role played by traumatic factors on the development of psoriasis. Generally it occurs within two weeks of the traumatic event at the affected site but the latency period can be even shorter (3 days) or much longer (even a year) (Weiss G *et al.*, 2002).

Even the sunrays, which usually improve the clinical picture of most patients, in some subjects (especially those with phototype I and II) can cause an aggravation of psoriasis acting as a traumatic factor. In fact, photosensitive psoriasis is defined as the form of dermatosis in which there the appearance of new lesions following sun exposure (Rutter KJ *et al.*, 2009). The incidence of photosensitive psoriasis varies from 14% to 24% depending on the different studies, while the phototype represents the most important factor of susceptibility (Nalluri R *et al*., 2010).

Infections, not only affecting the skin, but also internal organs are important elements that can trigger a latent psoriasis. The role of streptococcal infections in the pathogenesis of guttate psoriasis is known, which occurs within 15 days of disease onset (McFadden *et al*., 2009). More rarely, exanthematous diseases (measles and chickenpox) and HIV can be considered triggers (Naldi L *et al*., 2005; Patel RV and Weinberg JM, 2008).

Even some drugs can be a risk factor, that can interfere with psoriasis in various ways: aggravating a pre-existing psoriasis, causing the appearance of lesions on previously uninvolved skin areas, causing the appearance of psoriasis ex novo, favoring a resistance to treatment (Milavec-Puretić V *et al*., 2011). Among the most frequently implicated drugs are

antimalarials, lithium, β-blockers and nonsteroidal anti-inflammatory drugs (NSAIDs) (Basavaraj KH *et al.*, 2010).

As far as endocrine factors are concerned, the existence of peaks of incidence at puberty and in menopause has always suggested the interference of phenomena of hormonal nature. Psoriasis may aggravate during estrogen intake and in the pre-menstrual period (Hall G and Phillips TJ, 2005).

There is numerous clinical evidence regarding the influence of metabolic disorders on the appearance or aggravation of psoriasis. In particular, dyslipidemias and obesity have considerable effects on skin disease (Naldi L *et al.*, 2005).

Anxiety and stress are very often triggers of psoriasis that alter immune mechanisms (Raychaudhuri SP and Gross J, 2000). It is widely recognized that stress plays an important role as a trigger for psoriasis both in the first manifestation and as an aggravation of an already existing form (Heller MM *et al.*, 2011). Stressful events can worsen psoriasis severity and even extend the period of exacerbations. The percentage of psoriatic subjects who think that stress alters the condition of their skin (stress-responders) is particularly high: from 37% to 78% (Basavaraj KH *et al.*, 2011). Numerous studies have confirmed a higher alcohol consumption in the psoriatic population. Alcohol abuse not only acts as a triggering factor, but also as an aggravating factor in the disease, causing a greater extension of the lesions with a more evident inflammatory component (Jankovic *et al.*, 2009).

Finally, it is estimated that in about 25% of psoriatic patients the disease is triggered by cigarette smoking, which has been particularly associated with palmar-plantar pustular psoriasis (Naldi L *et al.*, 2005; Jin Y *et al.*, 2009). There is a statistically significant dose-response association between the number of cigarettes smoked and the risk of disease, with a more consistent correlation for females (Setty AR *et al.*, 2007).

1.1.4 Diagnosis

The diagnosis of psoriasis is mainly carried out on clinical observation which takes into consideration the appearance of the skin lesions, with particular attention to the color of the lesions, the size, the morphology, the distribution of the different body regions involved.

The PASI index (Psoriasis Area and Severity Index) is used to monitor the pathology through the evaluation of some parameters, and combines the assessment of the severity of lesions and

the area affected into a single score in the range from 0 (absence of psoriasis) to 72 (severe psoriasis).

The body is divided into four sections: head (H) (10% of skin surface); arms (A) (20%); trunk (T) (30%); legs (L) (40%). For each area, the percent of area of involved skin is estimated and then transformed into a grade from 0 to 6:

0. 0% of involved area
1. < 10% of involved area
2. 10–29% of involved area
3. 30–49% of involved area
4. 50–69% of involved area
5. 70–89% of involved area
6. 90–100% of involved area.

Within each area, the severity is estimated by three clinical parameters: erythema (redness), induration (thickness) and desquamation (scaling). Severity parameters are measured on a scale of 0 to 4, where 0 indicates a complete lack of skin involvement and 4 represents the highest possible involvement. The sum of all three severity parameters is then calculated for each section of skin, multiplied by the area score for that area and multiplied by weight of respective section (0.1 for head, 0.2 for arms, 0.3 for body and 0.4 for legs) (Oji V *et al*., 2015).

Although this is a validated and widely used method, recommended for attributing a shared degree of psoriasis severity to both scientific and clinical applications, it has a very low sensitivity. This limit is particularly evident in the case of mild or moderate intensity diseases, with a low percentage of the body surface area (BSA) involved, and does not take into consideration the symptoms that affect the hands, nails, feet, face, or genitals . The European Medicine Agency (EMA) recommends the use of PASI in combination with the PGA index (Physician Global Assessment), which uses a 4 to 10 point scale, evaluating erythema, plaque extension and injuries involved (Robinson A *et al*., 2012).

Psoriasis can be considered mild, in the case in which less than 3% of the skin surface is affected, moderate, if a surface is involved from 3 to 10%, and severe, when it extends over 10% (Meier M and Sheth PB, 2009).

1.1.5 Treatments

In the treatment of mild and moderate psoriasis, topical drugs containing various active ingredients are often used, including corticosteroids, analogues of vitamin D and retinoids ie. metabolites derived from vitamin A.

Glucocorticoids belong to the corticosteroid family, a group of hormones produced by the cortex of the adrenal glands. These molecules penetrate the cell by passive diffusion and bind to cytosolic glucocorticoid receptors (GC) forming a glucocorticoid-receptor complex that translocates to the nucleus and interacts with specific nuclear sequences, glucocorticoid responsive elements (GRE), regulating the expression gene. The anti-inflammatory action of glucocorticoids is caused by the activation of several genes such as IκB (NF-κB inhibitor), DUSP 1, also known as mitogen activated protein kinase (MAPK) phosphatase 1, IL-10, glucocorticoid-induced leucine zipper (GILZ) and annexin A1 (Spies CM *et al.*, 2011). All these genes are involved in the inhibition of proinflammatory factors or in the expression of anti-inflammatory molecules (Guichard A *et al.*, 2015). Following treatment with corticosteroids there is the appearance of local secondary effects due to the application and systemic effects due to drug absorption. The local effect is represented by cutaneous atrophy caused by the inhibition of proliferation, mitosis and protein synthesis in dermal fibroblasts causing cell cycle arrest and induction of apoptosis (Hein R *et al.*, 1994; Amsterdam A *et al.*, 2002). Among the systemic effects there is a decrease in the concentration of the corticotropin-releasing hormone (CRh) and of the adrenocorticotropic hormone (ACTH). Following absorption, corticosteroid levels increase in the blood leading to the appearance of clinical signs of Cushing's syndrome (Castela E *et al.*, 2012).

Calcipotriol is an analogue of vitamin D that is used in psoriatic therapy alone or in combination with betamethasone (steroid drug). The pharmacological target is the vitamin D receptor (VDR) expressed by the keratinocytes present in the basal layer of the epidermis and, following the receptor binding, inhibits proliferation and normalizes keratinocyte differentiation (Naga Sravan Kumar Varma V *et al.*, 2014). Several clinical studies have demonstrated an excellent efficacy and safety profile of this compound in the treatment of this pathology (Trémezaygues L and Reichrath J, 2011).

Tazarotene is a synthetic retinoid, recently introduced for the treatment of mild psoriasis; is a prodrug that is rapidly hydrolyzed in the active form that binds receptors for retinoic acid (RARs) by normalizing keratinocyte differentiation and proliferation. Although able to induce

a remission of the psoriatic lesion, its use is limited by the irritant action, which exerts both on healthy skin and on the plaque itself.

Among the various therapeutic options, phototherapy for psoriasis may be indicated in particular cases of the disease.

Narrowband ultraviolet B (UVB) rays are often used in psoriasis patients. The treatment involves exposure to artificial UVB rays, naturally present in sunlight. Applications are administered several times during the week and the therapy cycle can last a long time, up to twenty or thirty applications.

Another source of ultraviolet light used for the treatment of psoriasis is PUVA, which involves the association of ultraviolet radiation A (UVA) with the use of psoralens taken orally which sensitize the skin, making it more prone to treatment. The mechanism of action of the PUVA therapy is not well known, but probably determines the activation of the psolarenes, which inhibit cell reproduction. Treatment with PUVA and psolarenes is associated with an increased risk of skin cancer, especially malignant melanoma and squamous cell carcinoma, which is why PUVA therapy cannot be used in the long term.

Systemic treatment is reserved for subjects with severe or particularly extensive forms, or for those patients in whom topical or phototherapeutic treatments have proved ineffective. Systemic therapy for the treatment of psoriasis uses retinoids, methotrexate, cyclosporine A, mofetil mycophenolate and tacrolimus.

Several retinoids have been used in the treatment of psoriasis. Currently, acitretin is used, which has favorable pharmacokinetic properties, the most important of which is the shorter half-life. Acitretin has a marked effectiveness in pustular and erythrodermic psoriasis. When this drug is used on its own, the daily dosage varies from 25 to 50 mg; treatment with dosages of 10 to 20 mg is started and increased gradually (if necessary). Although complete remission is only achieved in 50% of cases, the majority of patients see the number of plaques, their extension or flaking decrease dramatically. Higher doses of Acitretin (50-75 mg/day) are more effective in less time, but are associated with more important side effects. Like all retinoids, Acitretin is also teratogenic, so it is necessary to adopt safe contraceptive measures in women of childbearing age. These must be maintained for three years after treatment has been suspended. The most easily observed side effects are dryness of the lips, nose, eyes and the thinning and fragility of the nail plates. Hyperostosis and tendon calcifications have been reported.

Methotrexate inhibits the enzyme dehydrofolate reductase, which is necessary for the synthesis of nucleotides and amino acids. In this way, the drug reduces DNA synthesis and inhibits mitoses, especially of rapidly proliferating cells. It is able to alter lymphocyte and neutrophil. Methotrexate should not be administered in patients with active infection, alcoholism, cirrhosis, viral hepatitis, immunological defects, renal failure and in women who do not take contraception measures.

Side effects can range from simple nausea to cancer. The recommended starting dose is 7.5 mg once a week. The use of methotrexate for the treatment of psoriasis is today less frequent than in the past.

Ciclosporin is an immunosuppressant, which inhibits the production of interleukin 2 and therefore the immune response mediated by T cells. The initial dosage is 2.5-4 mg/kg/day, divided into two doses. Given the side effects of the drug, it is necessary to monitor both blood pressure, renal function and bone marrow function. In fact, the major side effects are nephrotoxicity and hypertension, as well as hirsutism and gingival hyperplasia. Unfortunately, in addition to the side effects during treatment with Cyclosporine, the suspension usually leads to a reignition of the disease with worsening and difficulty in responding to other treatments.

Mofetil mycophenolate is able to prevent transplant rejection being an immunosuppressive agent; it has been used in the treatment of psoriasis. The recommended dose is 1 gr for 2 times a day or 500mg for 4 times a day. This drug is generally well tolerated and has a good safety profile. The main side effects are gastrointestinal disorders, anemia, leukopenia and onset of infections.

Tacrolimus has a mechanism of action very similar to that of cyclosporine and is used at a dosage of 0.1-0.15 mg/ kg/day. The most common side effects are paresthesia and diarrhea.

The new biological agents, that act on the various steps of the autoimmune-inflammatory process, offer an important alternative in the treatment of moderate to severe plaque psoriasis in adult patients who have not responded, or who have intolerance or contraindications to other systemic therapies, including cyclosporine, methotrexate and PUVA therapy (**Table 2**). Pro-inflammatory cytokines such as TNF-α play a central role in the pathogenesis of psoriasis. High levels of TNF-α have been found in psoriatic plaques. In plaque psoriasis, the infiltration of inflammatory cells, including T cells, leads to an increase in TNF levels in psoriatic lesions compared to levels in unaffected skin. (Gearing AJ *et al.*, 1990). This explains why anti-TNF-α, such as Etanercept, Infliximab and Adalimumab, led to an

improvement in the treatment of the disease. Etanercept is a fusion protein of p75 tumor necrosis factor (TNF) receptor (p75TNF-R) with the Fc fraction of human IgG1 immunoglobulin. The protein act as a soluble receptor for TNF-α; it is a competitive inhibitor of TNF-α binding to its surface cellular receptors, p55 and p75, and therefore inhibits the biological activity of TNF. Etanercept is indicated for plaque psoriasis, psoriasis arthritis, rheumatoid arthritis and ankylosing spondylitis (Rønholt K and Iversen L, 2017). Infliximab is chimeric human-murine monoclonal antibody that binds with high affinity to both the soluble and trans-membrane forms of TNFα, inhibiting its activity. It is currently approved for the treatment of rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis and psoriasis (Laws PM and Young HS, 2012). Adalimumab is a recombinant human monoclonal antibody produced in Chinese Hamster Ovary cells. It selectively binds to TNF and neutralizes its biological function by blocking its interaction with cell membrane TNF receptors. The drug has been approved for use in psoriasis and psoriatic arthritis (Laws PM and Young HS, 2012).

Recently, a new class of biological drugs blocking both interleukins (IL)-12 and 23 was identified. This cytokines induce CD4+ naïve lymphocytes to differentiate into T helper1 (Th1) and T helper17 (Th17) lymphocytes respectively. Ustekinumab is a fully human IgG1κ monoclonal antibody that binds the p40 protein subunit of human cytokines IL-12 and IL-23 with high affinity and specificity, inhibiting its activity and preventing binding of these cytokines with the respective receptor protein IL-12Rβ1 expressed on the surface of immune cells. Ustekinumab currently can be used in psoriasis and in psoriatic arthritis treatment (Thibodaux RJ et al., 2018). Currently there are no strong criteria to guide the choice between anti-TNF drugs available and between Etanercept, Infliximab, Adalimumab and Ustekinumab. However, the Etanercept vs Ustekinumab comparison suggests that Ustekinumab shows greater efficacy to Etanercept over a 12-week period (Sterry *et al*., 2004).

Biologic Drug	Target	Administration	Treatment Algorithm	Stage of Development	Approved for Psoriasis Arthritis	Withdrawn
Alefacept	LFA [1]-3	Intra-muscular	15 mg once weekly for 12 weeks	Approved 2003		2011
Efalizumab	CD [2]11a	Subcutaneous	0.7 mg/kg initial dose, then 1 mg/kg (max 200 mg) once weekly	Approved 2003		2009
Etanercept	TNF [3]-α	Subcutaneous	50 mg twice weekly for 12 weeks, then 50 mg once weekly	Approved 2004	+	
Infliximab	TNF-α	Intra-venous	5 mg/kg on week 0, 2 and 6, then every 8 weeks	Approved 2006	+	
Adalimumab	TNF-α	Subcutaneous	80 mg initial dose, then 40 mg every 2 weeks, starting one week after initial dose	Approved 2008	+	
Ustekinumab	IL-12/IL-23 p40	Subcutaneous	45 mg (≤100 kg) or 90 mg (>100 kg) on week 0 and 4, then every 12 weeks	Approved 2009	+	

Tab.2 *Psoriasis. Biologics approved for psoriasis by the United States Food and Drug Administration (Rønholt K and Iversen L, 2017).*

1.2. SECUKINUMAB: THE NEW ANTI-IL-17A BIOLOGIC AGENT

IL-17A is a dimeric glycoprotein and belongs to a family of cytokines family that includes other five members, i.e. IL-17B, IL-17C,IL-17D, IL-17E and IL-17F, which are involved in inflammatory disorders and autoimmune diseases such as psoriasis and cancer (Kolls & Linden 2004). IL-17A was first described in 1993 in human peripheral blood, as an important pro-inflammatory cytokine with a critical role against extracellular microorganisms and in the pathogenesis of different autoimmune diseases (Rouvier E *et al.*, 1993). Within the IL-17 family, IL-17A and IL-17F are central players in the adaptive immune response, particularly against bacteria and fungi while the function of IL-17B, IL-17C and IL-17D is less understood (Kolls JK and Lindén A, 2004; Iwakura Y et al., 2011). The induction and production of IL-17A during CD4+ or CD8+ T-cell differentiation is up-regulated by TGF-β, IL-6, IL-21 and IL-23 (Heidenreich *et al.* 2009). Specialized Th17 cell subsets of the adaptive immune response are characterized as main sources of IL-17A *in vivo* (Kirkham BW et al., 2014). IL-17A exists as either a homodimer of two IL-17A chains or a heterodimer with IL-17F. IL-17A and IL-17F signal through the same receptor subunit (IL-17RA and IL-17RC). However, IL-17A is approximately 10-30 times more potent than IL-17F in activating gene expression, cause to different ligand-receptor affinities (Gu C et al., 2013). IL-17A plays a critical role in the pathogenesis of a range of inflammatory diseases, including psoriasis, rheumatoid arthritis, psoriatic arthritis, autoimmune uveitis (Kirkham BW et al., 2014); it is known that this cytokine is elevated in lesions of psoriasis and in the serum of patients (Raychaudhuri SP, 2013; Chiricozzi et al., 2014). IL-17A has many functions that are relevant to psoriasis, including the direct activation of keratinocytes leading to increased production of other inflammatory mediators and the enhancement of angiogenesis (Marinoni et al., 2014).

Secukinumab is a human monoclonal IgG1k antibody that has been developed to target and block the actions of IL-17A. Secukinumab was developed by Novartis and the first publication was a Phase I trial published in 2010 (Frieder J *et al*., 2018). In January 2015, the FDA (Food and Drug Administration) approved Secukinumab to treat adults with moderate-to-severe plaque psoriasis (Sanford M and McKeage K, 2015; Rønholt K and Iversen L, 2017). It was the first IL-17A inhibiting drug ever approved. In January 2016, the FDA approved it to treat adults ankylosing spondylitis and psoriatic arthritis (Roman et al., 2015). The recommended dose is 300 mg by subcutaneous injection (into the front of thighs, lower

abdomen or outer upper arms but not into areas of skins that is affected by psoriasis) at week 0, 1, 2, 3 and 4 followed by 300 mg every four weeks. The mean half-life range from 22 to 31 days. At the therapeutic concentrations used in psoriasis, Secukinumab fully neutralizes the activity of IL-17A, does not neutralize IL-17F, leaves other function of TH17 cells intact and does not directly influence the TH1 pathway. This specific mechanism of action is unique to IL-17 inhibitors and leads to the normalization of skin histolgy, including achievement of clear to almost clear skin for the majority of patients (Garnock-Jones KP, 2015).

In clinical studies, Secukinumab has demonstrated the achievement and maintenance of skin that is free or almost free of lesions (from PASI 90 to PASI 100) in 80% of patients. During the studies, 70% of patients treated with Secukinumab obtained the resolution complete (PASI 100) or almost complete (PASI 90) of cutaneous manifestations after the first year of treatment. During Phase III clinical trials, 70% or more of patients receiving Secukinumab 300 mg achieved complete (PASI 100) or near complete (PASI 90) resolution of cutaneous manifestations during the first 16 weeks of treatment (Hueber W *et al*., 2010; Godse K, 2017).

1.3 THE ROLE OF ANGIOGENESIS IN PSORIASIS

The formation of new capillaries from pre-existing blood vessels is described as angiogenesis. This process is tightly regulated by a balance between pro- and anti-angiogenic mediators. Physiological angiogenesis is induced only transiently during processes such as wound healing or pregnancy. Dysregulated angiogenesis occurs under pathological conditions such as tumor growth and chronic inflammation, as observed during psoriasis. In this condition, angiogenesis is needed for disease development and progress (Folkman 1995; Kneilling et al. 2007; Heidenreich *et al.* 2008). The dermis of patients with psoriasis presents peculiar histological features, including elongated, tortuous, blood capillaries, increased in diameter and number (Braverman & Sibley 1982) (**Figure 5**). Vascular alterations occur prematurely and precede the characteristic epidermal hyperplasia of the disease (Schon & Boehncke 2005; Griffiths & Barker 2007).

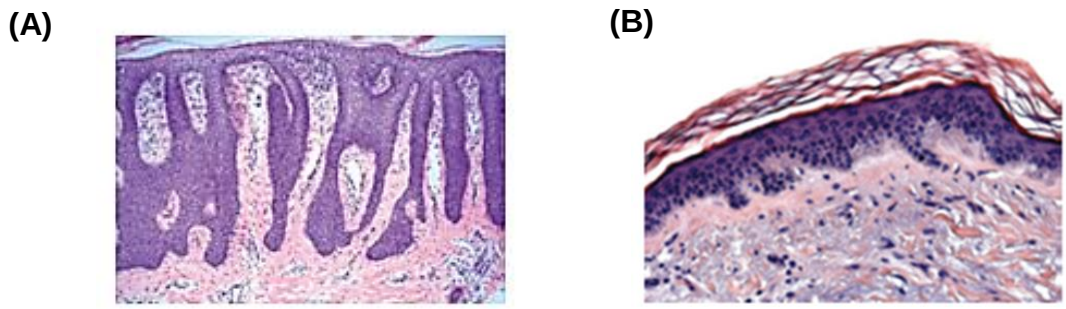


Figure 5. *The role of angiogenesis in psoriasis. Psoriasis histology. (A) H&E staining of psoriasis skin. (B) Healthy skin (Heidenreich et al., 2009).*

As angiogenesis is one of the key features of psoriasis, various studies have been focused on the identification of pro-angiogenic mediators in psoriatic skin. It is of interest that the remodeling of the vasculature in lesional psoriatic skin depends considerably on factors derived from the epidermis, whereas blood vascular remodeling is essential for nutrient supply of the hyperproliferative epidermis. Evidence for keratinocyte-derived pro-angiogenic signals came from a study comparing the angiogenic activity of conditioned media from keratinocytes isolated from either lesional or non-lesional skin of psoriasis patients. Conditioned media from lesional or non-lesional keratinocytes stimulated endothelial cell migration *in vitro* and showed strong angiogenic activity in the rat cornea micropocket assay

in vivo. In contrast, conditioned media from keratinocytes of healthy donors showed no pro-angiogenic response (Nickoloff *et al*. 1994).

Several pro-angiogenic factors are up-regulated in psoriasis development, among them vascular endothelial growth factor (VEGF), hypoxia-inducible factors (HIFs), angiopoietins (ANGs), tumor necrosis factor (TNF)-α, interleukin (IL)-8, interleukin (IL)-17, transforming growth factor (TGF)-α e TGF-β (Elder *et al.* 1989; Christophers 1996; Starnes *et al.* 2001; Creamer *et al* 2002; Numasaki *et al.* 2003; Ghoreschi *et al.* 2007; Heidenreich *et al.* 2008). Combined action of these factors stimulate endothelial cells from dermis to form new blood vessels.

VEGFs are a class of secreted polypeptides structurally similar to those of the "platelet derived growth factor" family (PDGF). The family members expressed by mammalian cells are VEGF-A, VEGF-B, VEGF-C, VEGF-D and the placental growth factor (PGF). VEGFs and its tyrosine kinase receptors VEGFR-1 and VFGR-2 (KDR) are essentially involved in vascular embryogenesis and adult neovascularization. VEGF-A, PGF and VEGF-B act mainly at the level of blood vessels, while VEGF-C and VEGF-D are essential for lymphangiogenesis. The VEGF receptors VEGFR-1 or -2 are primary expressed by vascular endothelial cells (De Vries *et al*. 1992; Shibuya 1995; Ferrara *et al*. 2003; Shibuya & Cleasson-Welsh 2006).

VEGF-A is a major regulator of physiological and pathological angiogenesis. This factor was strongly up-regulated in psoriatic skin lesions. Indeed, although this angiogenic factor can be produced by mast cells, monocytes/macrophages and infiltrating neutrophils, in psoriatic lesions the primary source of VEGF-A is keratinocytes. Physiological production of VEGFA contributes to normal proliferation, differentiation and function of the epidermis, such as epidermal barrier homeostasis (Man *et al.* 2006; Elias *et al.* 2008); in psoriasis VEGFA overexpression might contribute to keratinocytes hyperproliferation in an autocrine manner and to the epidermal changes observed in this disease (Canavese *et al.* 2010). Experiments based on in situ hybridization techniques and immunohistochemistry have demonstrated an increase in VEGF-A expression, both at the transcriptional and translational level, in psoriatic keratinocytes, and an increase in the expression of its receptors tyrosine kinase at endothelial cell level in dermal papillae. In psoriatic skin, VEGFR-1 and VEGF-2 receptors are detectable and functional in keratinocytes. In addition to an increased expression in the skin, psoriatic patients show a high secretion of VEGF-A in the bloodstream and these elevated plasma (serum) levels are correlated with an early onset (esordio) of the disease, with disease severity

and with the subsequent development of psoriatic arthritis (Creamer *et al*. 1996; Bhushan *et al*. 1999; Nielsen *et al*. 2002). VEGFA pathologically increased secretion by keratinocytes are triggered by a wide variety of factors, including pro-inflammatory cytokines secreted by leukocytes and the tumor growth factor TGF-α found in high concentrations in patients with psoriasis (Detmar *et al*. 1994). In humans, some polymorphisms of the gene encoding VEGF-A, rs2010963 and rs833061, have been associated with early development of psoriasis. The VEGFA gene is in the vicinity of the PSOR1 gene on chromosome 6p21; however, there is no linkage disequilibrium between these two genes, suggesting that they are inherited independently (Young *et al*. 2004, 2006).

HIFs represent heterodimeric transcription factors composed of a constitutively expressed β subunit (aryl hydrocarbon receptor nuclear translocators: ARNT, ARNT2, ARNTL) and a regulatory α subunit (HIF-1α, HIF-2α, HIF-3α) (Harris 2002; Maxwell & Ratcliffe 2002; Wenger 2002). At physiological oxygen tension, HIF-α subunits are continuously synthetized and degraded by the proteasome. For degradation, prolyl residues of HIF-α subunits are hydroxylated by prolyl hydroxylase, which are active only in the presence of normal oxygen concentrations. Under hypoxic conditions, prolyl hydroxylases are inactive; in consequence, HIF-α subunits are no longer degraded and the increasing HIF concentrations lead to nuclear translocation. Among the HIF target genes are mains regulators of angiogenesis such as VEGF, VEGFR-1, VEGF-2 and IL-8 (Elvert *et al.* 2003; Takeda *et al.* 2004; Kim *et al.* 2006). In psoriasis lesions, HIF-1α and -2α expression are both increased (Rosenberger *et al.* 2007). In epidermal keratinocytes, HIF-1α colocalizes with VEGF expression, whereas HIF-2α is expressed in the epidermis and in dermal capillaries. Epidermal hypoxia and increased HIF expression may result from the strong epidermal proliferation (Tovar-Castillo *et al.* 2007).

Angiopoietins are a family of secreted oligomeric proteins composed of 4 proteins, ANG1, ANG2, ANG3 and ANG4. ANG1 is widespread in the adult vascular system where it is mainly produced by pericytes that surround the endothelium of the vessels and, to a lesser extent, by monocytes and neutrophils. ANG2 is generally not expressed in healthy tissues, but is released following the presence of stimuli such as thrombin and histamine (Fiedler *et al.* 2004). ANG1 and ANG2 bind to the receptor tyrosine kinase Tie2. This receptor is ubiquitous at the level of the vascular endothelium, where it is constitutively phosphorylated in quiescent endothelial cells suggesting an active role in vascular homeostasis (Dumont *et al.* 1994; Davis *et al.* 1996) . Tie2 is also expressed by other cell types such as monocytes, neurons and tumor cells. ANG1 is an agonist of Tie2 and, following interaction with the receptor, induces a

protective response, inhibiting cell apoptosis, promoting both the migration of endothelial cells and smooth muscle cells (Davis *et al.* 1996). Moreover, ANG1 has a clear pro-angiogenic effect and an anti-inflammatory effect on endothelial cells, reducing the expression of E-selectin, of ICAM-1 and VCAM-1 induced by VEGF-A, thus inhibiting leukocyte diapedesis (Kim *et al* 2000). ANG2, on the other hand, is able to antagonize the effect of ANG1 at the receptor level (Yuan *et al.* 2009). Following inflammatory stimuli, ANG2 is released very quickly from pericytes and inhibits the ANG1-Tie2 bond in a competitive manner, destabilizing the extracellular cell-matrix contact and activating the expression of adhesion molecules for leukocytes (Moss 2013). In psoriasis, ANG1 is expressed at the level of fibroblasts and dendritic cells, while ANG2 is expressed at the level of endothelial cells. It has been demonstrated in transgenic mouse models that the over-expression of the ANG1 / ANG2 / Tie2 system is able to increase epidermal hyperplasia, hyperkeratosis, parakeratosis and induce an increase in vascularization at the dermal level. In particular, ANG2, synthesized by endothelial cells in response to inflammatory stimuli such as TNF-α, increases the expression of ICAM-1 and VCAM-1 at endothelial cell level, facilitating leukocyte adhesion and extravasation. In this way, ANG2 could contribute to the development of psoriasis (Heidenreich *et al.* 2009).

Several cytokines exhibit a profound impact on angiogenesis by influencing endothelial cells proliferation, migration or survival, or by modulating the expression of pro- or anti-angiogenic factors. Among the cytokines with pro-angiogenic activity, TNF, IL-8 and IL-17 are overexpressed during psoriasis.

TNF induces various pro-angiogenic factors, such as VEGF, IL-8, bFGF, in endothelial cells and exerts both pro-angiogenic and anti-angiogenic effects (Yoshida *et al.* 1997). Elevated levels of TNF mRNA and protein are detectable in psoriasis skin (Johansen *et al.* 2006). Therapies blocking the activity of TNF lead to clinical improvement of psoriasis and decreased expression of pro-angiogenic factors. The data confirm that TNF contributes to angiogenesis associated with psoriasis. It remains open whether it directly causes angiogenesis or indirectly through the induction of pro-inflammatory or angiogenic factors.

IL-8 or CXCL8 belongs to the CXC family of chemokines (Baggiolini *et al.* 1997). IL-8 is a strong chemoattractant for neutrophils, basophils and T lymphocytes, and is involved in autoimmune, inflammatory and infectious diseases (Brat *et al.* 2005). Enhanced IL-8 and IL-8 receptor mRNA and protein is detected within the epidermis of psoriatic lesions. Immunohistochemistry localizes IL-8 protein to suprabasal keratinocytes and neutrophils

(Duan *et al.* 2001; Gillitzer & Goebeler 2001). IL-8 is a strong chemoattractant for neutrophils, basophils and T lymphocytes, and is involved in autoimmune, inflammatory and infection diseases. IL-8 can be induced by IL-1, TNF, IL-6, IFN-γ, reactive oxygen species and other mediators of cellular stress (Strieter *et al.* 1992). IL-8 is also a potent pro-angiogenic factor (Koch *et al.* 1992); it has been described to stimulate endothelial cells proliferation, migration, survival and expression of MMPs (Koch *et al.* 1992; Szekanecz *et al.* 1994; Shono *et al.* 1996; Li *et al.* 2003). Moreover, IL-8 can stimulate keratinocyte proliferation (Tuschil *et al.* 1992). Various cell types are capable of producing IL-8, including immune cells such as mast cells, neutrophils or T cells, keratinocytes and EC (Nickoloff *et al.* 1994; Biedermann *et al.* 2000; Gillitzer & Goebeler 2001; Karl *et al.* 2005).

IL-17A is a pro-angiogenic factor; it can induces new vessel formation in the rat cornea micropocket assay and IL-17A overexpressing tumor cells can induce more rapid tumor growth with significantly enhanced tumor vascularization *in vivo* (Numasaki *et al.* 2003). Although it does not have a direct effect on the proliferation of endothelial vascular cells, IL-17 stimulates their migration and the production of capillary-like structure *in vitro* (Heidenreich *et al.* 2009). Moreover, IL-17A triggers the production of chemokines, growth factor and adhesion molecules by epithelial cells, fibroblasts, and EC including IL-6, IL-8, IL-1 and ICAM-1, an adhesion molecule secreted by fibroblasts and keratinocytes. Thus IL-17 enhances neutrophils accumulation. This interleukin is also capable of stimulating VEGF production by keratinocytes and fibroblasts. Consequently, by inhibiting the activity of IL-17A, Secukinumab inhibits angiogenesis indirectly reducing the production of VEGF by keratinocytes associated to vascular neo-formation.

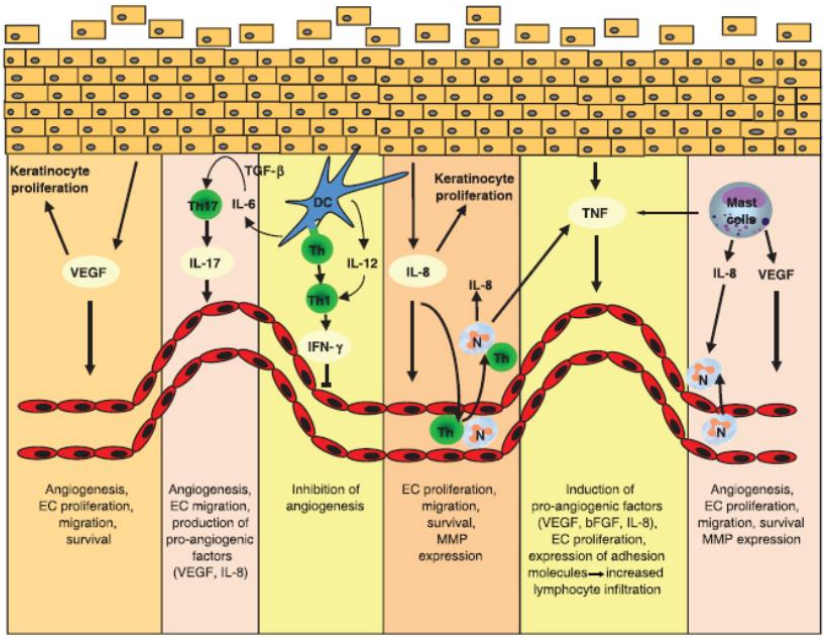


Figure 6*. The role of angiogenesis in psoriasis. Schematic representation of angiogenic signaling pathways (Heidenreich et al., 2009).*

1.3.1 Angiogenesis as a potential target of novel therapies

Although angiogenesis is a central process in the evolution of psoriasis and is closely linked with the clinical manifestation, treatments aimed directly to the inhibition of this process have received little attention so far. Systemic therapies currently used in the management of psoriasis patients modulate the immune response but also have indirectly an additional anti-angiogenic effect. As an example, there is evidence that methotrexate or cyclosporine therapies, that interfere with immune activation, can indirectly inhibit/target important mediators of angiogenesis (pro-angiogenic mediators) targeting the VEGF pathway (Hirata *et al.* 1989; Hernandez *et al* 2001; Yamasaki *et al.* 2003). Anti-TNF-α treatments such as Infliximab, Etanercept and Adalimumab, that target a cytokine that exerts multiple effects on angiogenesis, proved to exhibit potent anti-angiogenic activities in the therapy of psoriasis (Vassalli 1992; Pandya *et al.* 2006). The epidermal expression of VEGF, Ang-1, An-2, MMP-9 decreases significantly during psoriasis therapy with the TNF-α antagonist Infliximab (Markham *et al.* 2006). The same antibody was shown to reduce the expression of VEGF and

its receptors during psoriasis arthritis (Cordiali-Fei *et al.* 2006). Similar effects occur with other TNF antagonists, Etanercept and Adalimumab, that reduces VEGF-A and thereby reduces blood supply at the site of the tissue damage and psoriatic inflammation (Wcislo-Dziadecka *et al.* 2015). In addition some traditional therapies are associated with a reduction in plasma VEGF-A levels and, among these, topical use of mineral tar in combination with UVB therapy.

Increasing experimental data show that directly blocking angiogenic pathways that drive cutaneous inflammation could represent a promising therapeutic strategy for psoriasis treatment. The antiangiogenic agent Neovastat may represent a new potential treatment modality in patients with moderate to severe psoriasis. Neovastat it is a VEGF antagonist and has been shown to inhibit endothelial cells proliferation *in vitro* and angiogenesis *in vivo* (Dupont *et al*. 2002). In a phase I/II clinical trial which involved 49 plaque psoriasis patients, was noted a statistically significant decrease in PASI score (Sauder *et al.* 2002). Moreover, Neovastat have an excellent tolerability. Further studies need to be performed before the drug is approved by FDA for the treatment of psoriasis. Despite the promising results obtained in pre-clinical murine models (Schonthaler *et al*. 2009), further studies are required before for a possible use of Bevacizumab, a humanized monoclonal antibody directed against VEGF-A and used in adjuvant therapy of colon cancer, also in psoriatic pathology (Weidemann *et al.* 2013). To date, only one case of a patient has been reported, subjected to Bevacizumab treatment for metastatic colon cancer, who has had a simultaneous and complete remission of his psoriasis (Akman A *et al*. 2009).

1.4 THE EARLY GROWTH RESPONSE (EGR)-1 IN THE PATHOGENESIS OF PSORIASIS

The early growth response (Egr)-1 is a zinc finger transcriptional factor (Sukhatme VP, 1990) which plays an important role in the regulation of cell growth, differentiation, cell survival and immune response (McMahon SB and Monroe JG, 1996; Qu Z *et al*., 1998; Sakamoto KM *et al*., 2004). Egr-1 is rapidly induced by a broad spectrum of extracellular signals including growth factor, cytokines and many physiologic stimuli (Thiel G *et al*., 2002). Egr-1 induces the expression of growth factors, growth factor receptors, extracellular matrix proteins, proteins involved in the regulation of cell growth or differentiation, and proteins involved in apoptosis, growth arrest, and stress responses (de Belle I *et al*., 1999; Virolle T *et al*., 2001; Krones-Herzig A *et al*., 2005; Zwang Y *et al*., 2011). Comparable to some other transactivators, Egr-1 associates with corepressor proteins that can modulate transcription of Egr-dependent genes. Two corepressors of Egr-1, Nab1 and Nab2, have been identified using yeast two-hybrid screening (Svaren J *et al*., 1996; Swirnoff AH *et al*., 1998). These factors bind to Egr-1 by direct protein-protein interactions, thus inhibiting the transactivating potential of Egr-1. Whereas Nab1 is constitutively expressed in most tissues and appears to be a general transcriptional regulator (Swirnoff AH *et al*., 1998), Nab2 may function as an important inducible regulator of gene expression (Svaren J *et al*., 1996).

Egr-1 was originally identified as a tumor suppressor gene in a variety of human tumor cell lines including osteosarcomas, fibrosarcomas (Huang *et al.*, 1995), hepatocellular carcinoma, and esophageal carcinoma (Hao *et al.*, 2002). Lung and breast carcinoma can be entirely lacking in Egr-1 expression (Huang *et al*., 1997; Levin *et al.*, 1994), and deletion of the Egr-1 containing locus 5q31 has been observed in acute myelogenous leukemia (Fairman *et al.*, 1995) and breast cancer (Ronski *et al.*, 2005). In contrast, prostate cancer and metastatic gastric cancer can exhibit high levels of Egr-1 (Kobayashi *et al.*, 2002; Thigpen *et al.*, 1996). The mechanisms underlying these tissue-specific effects of Egr-1 are under active investigation.

Egr-1 also regulates inflammatory genes in many diseases such as atherosclerosis and pancreatitis (Harja E *et al*., 2004; Gong LB *et al*., 2005).

Furthermore, Egr-1 plays a crucial role in angiogenesis (Khachigian LM and Collins T, 1997; Thiel G and Cibelli G, 2002; Fahmy RG *et al*., 2003; Lucerna M *et al*., 2006). Egr-1 is critically involved in the regulation of the expression of proangiogenic genes, such as

VEGFA, FGF2 and IL-6 in endothelial cells or TNF-α in macrophages (Fahmy RG *et al.*, 2003; Yan SF *et al*., 2000; Hao F *et al*., 2008; Park PH *et al*., 2007). Moreover, Egr-1 knockdown, or overexpression of Nab2, attenuates the proangiogenic effects of fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) on proliferation and differentiation of ECs (Fahmy RG *et al*., 2003; Lucerna M *et al*., 2003). Several studies have reported that Egr-1 expression in cancer cells, endothelial cells and macrophages is related to tumor progression (Lucerna M *et al*., 2006; Abdulkadir SA *et al.*, 2001; Guha M *et al*., 2001). Collectively, these findings suggest that Egr-1 plays important roles in tumor-associated angiogenesis and tumor progression.

Interestingly, in human epidermis, it was observed that Egr-1 was decreased in both basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) but was strongly increased in the skin lesions of patients with psoriasis. (Wee S *et al*., 2001; Fang M *et al*., 2017), raising the possibility that Egr-1 may contribute to differences between benign and malignant proliferation in epidermis. Emerging evidence demonstrate that IL-17A increases Egr-1 expression in human keratinocytes (Jeong *et al*., 2004). However, the role and the regulatory mechanism of Egr-1 in psoriasis are still unknown.

1.5 EXTRACELLULAR VESICLES (EVs)

Besides cell-to-cell contacts and the release of cytokines, chemokines, growth factors, hormones and other soluble messengers, it has been recently demonstrated the ability of cells to communicate each other via the delivery of extracellular vesicles (EVs). The secretion of EVs is a universal cellular process occurring from simple organism (Archea or Gram-negative and Gram-positive bacteria) to complex multicellular organism, suggesting that this EV-mediated communication is evolutionarily conserved (Kim *et al.* 2013).

EVs mediate normal and pathologic intercellular communication (Thèry *et al.* 2009, György *et al.* 2011). Their function depends on the cargo that they carry and the cell type from which they originate.

Many cell types are known to secrete EVs, and they include epithelial cells (van Niel et *al.* 2001; El Andaloussi *et al.* 2013), fibroblasts (Ji *et al.* 2008), erythrocytes (Geminard *et al.* 2002), platelets (Heijnen et al. 1999), mast cells (Raposo *et al.* 1997), tumor cells (Atay & Godwin 2014), stem cells (Zhang *et al.* 2014), and immune cells such as dendritic cells (DCs) (Sobo-Vujanovic *et al.* 2014), monocytes (Akao *et al.* 2011), macrophages (McDonald *et al.* 2014), NK cells (Lugini *et al.* 2012), B lymphocytes (McLellan *et al.* 2009), and T lymphocytes (Zhang *et al*. 2011).

The presence in accessible fluids (blood plasma, urine, saliva, or broncho-alveaolar lavage fluid) of exosomes containing pathology-specific biomarkers, is already exploited in clinical studies and may be useful for early detection of a disease status. Furthermore, considering the role of EVs in the intercellular communication and the potential to carry out this process at a considerable distance from the production area, the study of EVs has aroused great interest for the possible application as a transporter of iatrogenic substances to be conveyed to a specific target.

One of the major problems is the difficulty in purifying MVs and exosomes, given the low number and lack of standardized techniques and protocols (Lakhal and Wood 2011; Zhang *et al.*2019).

Another critical point to solve is the optimal loading of EVs with bioactive components without affecting their architecture. In fact, it has been shown that, based on the nature of the bioactive loading, the vesicles could become immunogenic, and therefore degradable.

1.5.1 Biogenesis and composition

Depending on their biogenesis and size, EVs are classified as apoptotic bodies, ectosomes or microvesicles (MVs) and exosomes (Raposo and Stoorvogel, 2013).

MVs are 50 to 1000 nm in diameter, are generated by outward budding and fission of membrane vesicles from the cell surface and may travel considerable distances in the interstitial or other body fluids until taken up by their target cells (Théry *et al.* 2009, György *et al.* 2011, Lee et *al.* 2011).

As for the other EVs, even MVs, for their formation and secretion, require specialized proteins in the transport and folding of membranes, such as escortins (van Niel *et al.* 2018).

Exosomes, referred to small membrane vesicles, are 20 to 100 nm in diameter; their secretion requires the fusion of multivesicular bodies with the plasma membrane. Exosomes biogenesis starts from the formation of an early multivescicular endosome (MVE) in which the accumulation of intraluminal vesicles (ILV) begins. The organization of this complex of vesicles, called multivesicular body (MVB), is regulated by transport proteins such as escortins (ESCRT), although an independent ESCRT pathway is shown (van Niel *et al.* 2018).

The final phase of the exosome formation process is their secretion which occurs following the fusion of the MVB with the plasma membrane. Their molecular composition reflects their origin in endosomes as intraluminal vesicles; exosomes are rich in endosomal proteins (i.e. Rab GTPase, SNARE, Annessin), some of which (Alix and Tsg101 among others) are used as exosomal markers (van Niel *et al.* 2006). Exosomes from different cellular origins sequester a common set of molecules but also display cell-type specific components (2).

EVs are essentially information carriers that can be macromolecules contained inside them or exposed on their own membrane. All the different types of EVs contain DNA and RNA (mRNA, microRNA, circRNA, lncRNA) and proteins able to modulate the functions of recipient cells. The demonstration of the presence of mRNA and miRNA in the EVs and the translation in the target cell (Ratajczak *et al.* 2006; Valadi *et al.* 2007; Mittelbrunn *et al.* 2011; Montecalvo *et al.* 2012) represented an important discovery.

The EV content profile also appears to be compatible with the cell of origin, especially with regard to MVs, with a selected and non-random transported material (Valadi *et al.* 2007).

To date, a continuously updated database is available, ExoCarta (http: www.exocarta.org) which catalogs the cargo of the EVs according to scientific evidence.

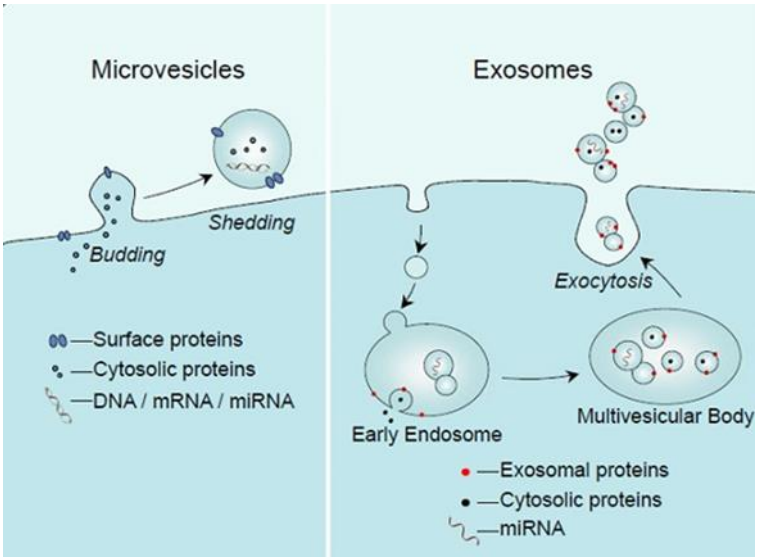


Figure 7. *Extracellular vesicles (EVs). Schematic representation of the mechanisms of formation of microvesicles and exosomes (Lawson et al. 2016).*

1.5.2. The involvement of exosomes in psoriasis

The function of EVs is closely linked to the cell of origin and to the possibility of interaction with the target cell, so that information can be transmitted in the form of mRNA, DNA, proteins or miRNAs.

Emerging evidence demonstrates a role of EVs in a variety of fundamental physio- and pathological processes ranging from T-cell activation to Alzheimer's disease, also correlating their levels to poor prognosis in hematological malignancies (Raposo *et al.* 1996, Kim *et al.* 2003, Valadi *et al.* 2007).

The role of vesicles for the functioning of the nervous system is known, in the intersynaptic release of neurotransmitters, although recently the role in myelin formation has been observed (Bakhti *et al.* 2011). Pathological processes involving EVs are also known in the transmission of prions (Fevrier *et al.* 2004) and of beta-amyloid peptides in Alzheimer's disease (Rajendran *et al.* 2006).

EVs are also involved in the progression of autoimmune diseases and tumor growth. EVs secreted by immune cells (including T cells, B cells, dendritic cells and macrophages), can

both stimulate and inhibit the immune system; these effects on immunity include T-cell activation, T-cell polarization into regulatory T cells, immune suppression, anti-inflammation and others (Zhang *et al*. 2014). EVs secreted by tumor cells support tumor progression and metastasis by promoting tumor initiation, angiogenesis, epithelial-to-mesenchymal transition (EMT), matrix remodelling and immune modulation (Liu *et al.* 2018). In particular, increasing evidence has shown that exosomes are also involved in the pathogenesis of chronic inflammatory skin diseases and skin tumors. Studies have shown the involvement of changes in exosome cargo in the regulation of chronic inflammatory skin diseases and skin tumors, such as systemic lupus erythematosus (SLE) and melanoma.

The serum levels of exosomes in SLE patients were found to be significantly upregulated compared with healthy controls. The level of exosomes was correlated with disease activity in the SLE patients (Lee *et al.* 2016). In addition, exosomes purified from SLE patients induce higher cytokine production by peripheral blood mononuclear cells (PBMC) from healthy controls, including the production of interferon (IFN)-α, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (Lee *et al.* 2016). Recently it has been demonstrated that, in SLE patients, urinary miRNAs are contained primarily in exosomes and are significantly increased compared with controls, especially for miR-146a (Perez-Hernandez *et al.* 2018).

Melanoma-derived exosomes promote the invasion and migration of primary melanocytes through an EMT-resembling process (Anderson *et al.* 2010). A research has revealed that the dysregulation of miRNAs may be associated with melanoma progression (Mione & Bosserhoff 2015). The expression levels of miR-532-5p and miR-106b were significantly higher in exosomes collected from the serum of the melanoma patients compared with healthy controls. This study also suggested that miR-532-5p and miR-106b in exosomes can be used to distinguish melanoma patients from healthy individuals, patients with metastasis from non-metastatic patients (Tengda *et al.* 2018). Another study found miR-17, miR-19a, miR-21, miR-126 and miR-149 in exosomes derived from the plasma of metastatic melanoma patients (Pfeffer *et al.* 2015). However, the expression of miR-125b was significantly lower in exosomes from melanoma patients than those from healthy controls (Alegre *et al.* 2014). Overall, whether the differential expression of miRNAs in exosomes from different melanoma patient cohorts can be used as biomarkers for melanoma metastasis requires further examination.

Taken together, these data indicate that exosomes cargo may play a crucial role in deseases development and progression, and may be useful potential predictive biomarkers for diagnosis and therapeutic response.

To date, only few data correlate EV release to psoriasis. Seminal observations reported an increase of both endothelial- and platelet-derived circulating EVs in psoriatic patients (Tamagwa-Mineoka et al. 2010; Pelletier et al. 2011). It has been recently reported the ability of T cell–derived EVs to induce mast cell production of IL-24 (Shefler et al. 2014) as well as the ability of mast cell–derived exosomes to present neolipid antigens to psoriatic T cells (Cheung et al. 2016).

No data are, instead, available on the ability of IL-17 to modulate the release of EVs by keratinocytes and/or their content in term of bioactive products.

2. AIMS OF THESIS

Experimental evidence underlines the essential role of IL-17A in the pathogenesis of different inflammatory pathologies including psoriasis. In psoriasis, IL-17A is considered the main driver of inflammation and dysregulated angiogenesis, as it promotes in psoriatic keratinocytes the release of soluble mediators such as cytokines, chemokines and several pro-angiogenic factors including VEGF-A that contribute to the pathogenesis of the disease. More importantly, it has been recently reported that IL-17A increases Egr-1 expression in human keratinocytes although currently data concerning the role of Egr-1 in psoriasis are really scare. The anti-IL-17A monoclonal antibody Secukinumab is the most recent biologic medication approved for the treatment of moderate-to-severe plaque psoriasis. Secukinumab exhibited a strong efficacy clinically and its successful employment is ascribable to the indirect inhibition of angiogenesis.

Furthermore, it has been recently reported the alteration of extracellular vesicles (EVs) trafficking in several diseases, but there is no data available on the ability of IL-17A to modulate the release of EVs by keratinocytes and/or their content in terms of bioactive products.

The main purpose of this work is to investigate the molecular pathway affected by Secukinumab, and to clarify the involvement of EVs in psoriasis pathogenesis. In greater detail, we hypothesized that Egr-1 could influence angiogenesis in response to IL-17A signaling and that Secukinumab might be involved in the regulation of the IL-17-Egr-1-VEGFA axis negatively influencing angiogenesis. Regarding the study of EVs, we aimed to characterize the release, the cargo content and the capacity to transfer bioactive molecules of EVs produced by keratinocytes following IL-17A treatment compared to untreated keratinocytes.

3. MATERIALS AND METHODS

3.1 CELLS AND REAGENTS

The spontaneously transformed aneuploid immortal keratinocyte HaCaT cell line, derived from adult human skin, was purchased from Cell Line Service GmBH and was cultured in Dulbecco's modified Essential Medium (DMEM), 4.5 g/L glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 UI/mL penicillin and 100 µg/mL streptomycin (Mediatech/Corning). Human umbilical vein endothelial cells HUVEC were purchased from Lonza, Pharma & Biotechnology and were cultured in Endothelial Cell Growth Medium-2 (EGM-2) BulletKit (Lonza, Pharma & Biotechnology). Cells were routinely maintained in culture in a humidified atmosphere of 5.5% CO2 at 37°C. Recombinant human IL-17A (rIL- 17A) was purchased from Peprotech and used at 100 ng/mL unless otherwise specified. 5(6)-CFDA-SE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester, CFSE) was purchased from Invitrogen/Thermo Fischer Scientific. PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane Labeling and Actinomycin D were both purchased from Sigma-Aldrich/Merck. CellTiter 96 AQueous MTS was purchased from Promega. Secukinumab (Cosentyx) was a kind gift of Novartis Pharmaceuticals. All the experiments were performed using Exosome-depleted FBS Media Supplement (System Biosciences).

3.2 PREPARATION OF KERATINOCYTES CONDITIONED MEDIA

To prepare conditioned media, HaCaT cells were seeded at 1.5×10^6 cells in 100-mm-diameter dishes (Falcon/Corning) and let them attach overnight. Then, cells were stimulated for 24 hours with rIL-17A or Secukinumab. At the end of stimulation, supernatants were discarded, cells were washed three times with PBS and media were replaced/changed. After 24 hours supernatants were collected and stored at -80°C until further use/before use.

3.3 EVs ISOLATION AND STAINING

Cells were seeded at 1.5×10^6 cells in 100-mm-diameter dishes (Falcon/Corning). After 24 hours, cells were washed in complete medium and stimulated for 48 hours with rIL-17A. At the end of stimulation, supernatants were collected and EVs isolated by serial centrifugations. Supernatants were spun 500 × g for 10 minutes and 2000 × g for 10 minutes to remove detached cells and cellular debris, and then ultracentrifugated 100 000 × g for 60 minutes. Pellet containing EVs was either lysed to isolate total RNA or resuspended in complete medium or phosphate-buffered saline (PBS) depending on the subsequent use. PHK67 fluorophore staining was performed on pelleted EVs resuspended in 0.5 µL of PHK67 in 250 µL of Diluent C buffer, for 5 minutes at RT. The staining was stopped by adding 250 µL of 1% bovine serum albumin, 1× PBS for 1 minutes at RT. Exceeding dye was removed by ultracentrifugation.

3.4 RNA ISOLATION AND REAL-TIME RT-PCR ON CELLS AND EVs

To extract RNA, cultured cells were grown in a 60-mm-diameter dish to 60–80% confluency and lysed. Supernatants were collected, and EVs were isolated as previously described, whereas cells were washed twice in PBS. Cellular mRNA was isolated using Total RNA Purification Kit (Norgen Biotek Corp.) according to the manufacturer's instructions. mRNA concentration was quantified using a DU 800 Spectrophotometer (Beckman Coulter, Indianapolis, IN). One microgram of mRNA was converted to cDNA using the Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instructions. Gene expression was quantified by real-time PCR using the 7900HT Fast Real-time PCR System and the SensiMix SYBR Hi-ROX Kit (Bioline) according to the manufacturer's instructions and analyzed. Each experiment was performed in triplicate and is expressed as mean ± SD. Experiments were independently repeated three times. Data were normalized using as endogenous control genes HPRT-1, or 18S rRNA for EV analysis, and expressed using the $2^{-\Delta\Delta CT}$ method. The following gene-specific primers were used:

hu β2-Defensin	FW 5′-GACTCAGCTCCTGGTGAAGC-3′ RV 5′- GCAGGTAACAGGATCGCCTA-3′
hu IL-6	FW 5′-GGTACATCCTCGACGGCATCT-3′ RV 5′- GTGCCTCTTTGCTGCTTTCAC-3′
hu CCL20 (MIP3α)	FW 5′-TGGCCAATGAAGGCTGTGA-3′ RV 5′- GATTTGCGCACACAGACAACTT-3′
hu CXCL1 (GROα)	FW 5′-CCAAACCGAAGTCATAGCCA -3′ RV 5′- CTTCCTCCTCCCTTCTGGTC-3′
hu CXCL3 (GROγ)	FW 5′-ATCCCCCATGGTTCAGAAA -3′ RV 5′- ACCCTGCAGGAAGTGTCAAT-3′
hu CXCL5	FW 5′-TTGTCTTGATCCAGAAGCCC-3′ RV 5′- CTTCTCTGCTGAAGACTGGGA-3′
hu CXCL6	FW 5′-TGAAGAACGGGAAGCAAGTT-3′ RV 5′- AAAACTGCTCCGCTGAAGAC-3′
hu CXCL8 (IL-8)	FW 5′-CTTGGCAGCCTTCCTGATTT-3′

	RV 5′- TTCTTTAGCACTCCTTGGCAAAA-3′
hu TNF-α	FW 5′- ATCTTCTCGAACCCCGAGTGA-3′ RV 5′- CGGTTCAGCCACTGGAGCT -3′
hu TGF-β	FW 5′-GCAGCACGTGGAGCTGTA-3′ RV 5′-CAGCCGGTTGCTGAGGTA-3′
hu VEGFA	FW 5′-CTACCTCCACCATGCCAAGT-3′ RV 5′-CCACTTCGTGATTCTGC-3′
hu KDR	FW 5′-TCAACAAAGTCGGGAGAGGA-3′ RV 5′-GAGTCTTCTACAAGGGTCTCA-3′
hu MMP9	FW 5′-TTGGTCCACCTGGTTCAACT-3′ RV 5′-ACGACGTCTTCCAGTACCGA-3′;
hu MMP2	FW 5′-GGAAAGCCAGGATCCATTTT-3′ RV 5′-ATGCCGCCTTTAACTGGAG-3′
hu PDGFA	FW 5′-TTTGGACACCAGCCTGAGAG-3′ RV 5′-AGACAGCGGGGACAGCTT-3′
hu bFGF	FW 5′-CCTGGGGAGAAAGCTAT-3′ RV 5′-GCTTCACGGGTAACAG-3′
hu EGF	FW 5′-GGATAGCCAACAAACACACT-3′ RV 5′-GGCACGTGCAGTAATAGGAT-3′
hu EGR-1	FW 5′-AGCCCTACGAGCACCTGAC-3′ RV 5′-GGTTTGGCTGGGGTAACTG-3′
hu HPRT-1	FW 5′-AATTATGGACAGGACTGAACGTCTTGCT-3′ RV 5′-TCCAGCAGGTCAGCAAAGAATTTATAGC -3′
hu RNA 18s	FW 5′-CTACCTCCACCATGCCAAGT-3′ RV 5′-CCACTTCGTGATTCTGC-3′

3.5 WESTERN BLOT

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM Dithiothreitol (DTT) and 0.5 mM sodium orthovanadate (Sigma–Aldrich). Protein concentration was determined by the Bradford assay and 40 μg of proteins per sample were resolved on an 8% SDS-PAGE gel and blotted onto a PVDF membrane (Amersham HyBond-P GE Healthcare). After blocking at room temperature for 1 hour with 5% dry-milk in Tris Buffer Saline (TBS) containing 0.1% Tween-20, membranes were incubated overnight at 4°C with rabbit polyclonal anti-Egr-1 antibody (sc-110, Santa Cruz Biotechnology, Dallas, dilution 1:500) and mouse monoclonal anti-β-actin (sc-47778, Santa Cruz Biotechnology, Dallas, dilution 1:2000) antibody was used for normalization. Membranes were, then, incubated with anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies (dilution 1:10,000, GE Healthcare Bio-Sciences). Immunocomplexes were detected by ECL Western Blotting detection system (GE Healthcare Bio-Sciences). Densitometric analysis was performed with Image Studio software (LI-COR Biosciences).

3.6 MTS CELL PROLIFERATION ASSAY

HUVEC cells were seeded into 96-well plates (5×10^3 cells/well) and maintained overnight. Then, cells were treated with HaCaT conditioned media for 72 hours. Every 24 hours MTS reagent (Promega, Madison, WI) was added to each well 2 hours before absorbance evaluation with a microplate reader (Tecan, Männedorf, Switzerland). Three independent experiments were performed in quintuplicate. Data are presented as mean±SD.

3.7 CELL MIGRATION ASSAY

Semi-confluent HUVEC cells were harvested, disaggregated, resuspended in serum free medium and seeded at 5 x 10^4 /well in 24-well cell culture inserts with 8 µm pore size membrane (Boyden chambers, BD Biosciences). The lower chamber contained HaCaT cells conditioned media. Cells were incubated at 37°C for 18 hours. After this time, cells were fixed with 100% methanol and non-migrated cells were removed from the inner side of the insert with a cotton swab. Migrated cells, which had the ability to push themselves through the 8 µm pores, were gently rinsed with PBS, stained with 0.25% crystal violet (Sigma–Aldrich) for 15 minutes, rinsed again and allowed to dry. Five random fields per insert were photographed with a light microscope (Leica DM 4000B) at 10x magnification and ImageJ software was used to count cells. In all experiments, data were collected from three chambers.

3.8 TUBE FORMATION ASSAY

A 96-well plate was coated with 60 µl of a matrigel matrix (Corning Matrigel Growth Factor Reduced), which was allowed to polymerize and solidify at 37°C for 30 minutes. The HUVECs were seeded onto the gel layer (2 x 10^5 HUVEC cells per well) in the presence or absence of HaCaT conditioned cell media and incubated at 37°C for 6 hours. Cell rearrangement and tube formation were visualized by inverted microscope (Nikon Eclipse TS100). Five random fields per well were photographed at 10x magnification and ImageJ software was used to quantify endothelial tube parameters. Each treatment was performed in duplicate and the experiment was independently repeated three times.

3.9 SiRNA TREATMENT

For silencing, EGR1 pre-designed siRNA (Invitrogen, Grand Island, NY USA, 4390822 or scrambled sequence RNA oligonucleotide, Negative Control siRNA 4390846) were transiently transfected at 25 nM or 50 nM into HaCaT cells using High-Perfect Transfecting Agent (Quiagen, Valencia, CA) following the Quiagen protocol.

3.10 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

VEGF-A levels were measured in HaCaT conditioned media using a human VEGF-A ELISA Kit (Invitrogen, ThermoFisher Scientific) according to the manufacturer's protocol. The absorbance was then evaluated with a microplate reader (Tecan, Männedorf, Switzerland). Each experiment was repeated three times.

3.11 IMMUNOHISTOCHEMISTRY

For this study, five patients affected by chronic plaque-type moderate to severe psoriasis (minimum PASI greater than 12) for at least 6 months were recruited. We included subjects inadequately controlled by prior treatments (topicals, phototherapy, and/or systemic therapies), with a minimum age of 18 years, with a body mass index (BMI) from 18.5 – 24.9 (normal or healthy weight). Exclusion criteria were: other psoriasis treatment during the study; pregnancy or lactation status; significant medical problems; alcohol and tobacco products use. Patients included in the study received the recommended dose of 300 mg of Secukinumab by subcutaneous injection for 4 weeks, followed by monthly maintenance dosing. From a psoriatic plaque, selected at the discretion of the clinical dermatologist, a biopsy of 4 mm of diameter was taken for each patient using punch under local anesthesia. The samples obtained were fixed in 10% buffered formalin (4% formaldehyde) and subsequently included in paraffin. Paraffin-embedded tissues were deparaffinized, rehydrated in descending graded alcohols, incubated for 15 minutes in methanol containing 3% H_2O_2 to block endogenous peroxidase activity, and then subjected to microwave antigen retrieval for 20 minutes in sodium citrate buffer (10 mM trisodium citrate dihydrate, 0.05% Tween 20, pH 6.0). After pre-incubation in Super Block (ScyTek Laboratories) for 10 minutes, sections were incubated overnight with rabbit monoclonal anti-EGR-1 antibody (15F7, Cell Signaling Technology, diluition 1:200) and rabbit polyclonal anti-VEGFA antibody (Ab46154, Abcam, diluition 1:200) at 4 °C in humid chamber, washed with PBS, incubated for 10 minutes at room temperature with UltraTek Anti-Polyvalent (ScyTek Laboratories), washed with PBS and then incubated with the UltraTek HRP (ScyTek Laboratories) according to the manufacturer's instructions. The sections were then stained with 3-3-diaminobenzidine (ScyTek Laboratories) as chromogen to visualize the reaction product, and were finally counterstained with hematoxylin. Images were acquired with Nikon Eclipse Ni motorized microscope system at 20x magnification.

3.12 EVs CYTOMETRIC ANALYSIS

Cells were seeded at 5×10^5 cells in a 60-mm-diameter dishes. Cells were washed twice in PBS and stained for 10 minutes at 37°C in serum-free medium plus 5 µmol/L CFSE according to the manufacturer's instruction. Cells were, then, washed twice in complete medium and stimulated with rIL-17A. After 48 hours, supernatants containing EVs were collected, by sequential centrifugation at 500 × g for 10 minutes and 2000 × g for 10 minutes. Samples were acquired on a FACS ARIA II Cell Sorter equipped with FACS DiVa software v6.1.1 (Becton Dickinson) set with a minimal threshold (ie 200) on FITC channel and no thresholds on forward and side scatter. Acquisition field was calibrated using Megamix-Plus SSC beads (Biocytex). Samples were acquired for 30 seconds at minimal flow rate. To avoid carry-over, fluidic was extensively washed with 0.1 µm filtered, distilled water before each sample acquisition. Results were analysed using Flowing software v2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland).

3.13 STATISCAL ANALYSIS

All statistical analyses were performed using GraphPad Prism software (La Jolla, CA, United States). Statistical comparisons were performed by Student's t-test and one-way analysis of variance (ANOVA). *: $p < .05$; **: $p < .01$; ***: $p < .001$; ****: $p < .0001$. All results are presented as mean ± SD.

4. RESULTS

4.1 VALIDATION OF *IN VITRO* PSORIATIC MODEL

In order to reproduce the *in vitro* psoriatic model, we treated the spontaneously transformed keratinocyte cell line derived from adult human skin (i.e. HaCaT) with rIL-17A reported to be a driver cytokine in the pathogenesis of psoriasis (Raychaudhuri SP, 2013; Chiricozzi *et al*., 2014; Kirkham BW *et al*., 2014 Marinoni *et al*., 2014), and according to a specific time course (1, 6, 24, 48 and 72 hours). Given that the treatment of human keratinocytes with IL-17A induces an inflammatory specific molecular signature characterized by the up-regulation of 28 genes including CXCL1, CXCL3, CXCL5, CXCL6, CXCL8, CCL20 and β-Defensin 2 (Nograles *et al.*, 2008), therefore we analyzed the expression of the chemokines and cytokines aforementioned by RT real-time PCR in our *in vitro* model. More importantly, we also evaluated the effect of the new clinical approved neutralizing anti-IL-17A antibody (Secukinumab), on the expression of inflammatory chemokine pattern. All experiments were performed by employing a concentration of Secukinumab currently used in the clinical practice to treat psoriasis (Sanford M and McKeage K, 2015; Rønholt K and Iversen L, 2017; Roman *et al*., 2015; Garnock-Jones KP, 2015). Result show that in our experimental system, CXCL1, CXCL3, CXCL5, CXCL6, CXCL8, CCL20 and β-Defensin 2 were significantly upregulated by IL-17A, at different degrees and with different kinetics. We also detected the up-regulation of IL-6 mRNA as already observed in IL-17-treated murine keratinocyte (Wu L et al., 2015). Both the combined pre-treatment of rIL-17A and Secukinumab and Secukinumab alone confirm the downregulation of the inflammatory pattern compared to rIl-17A stimulus (**Figure 8**).

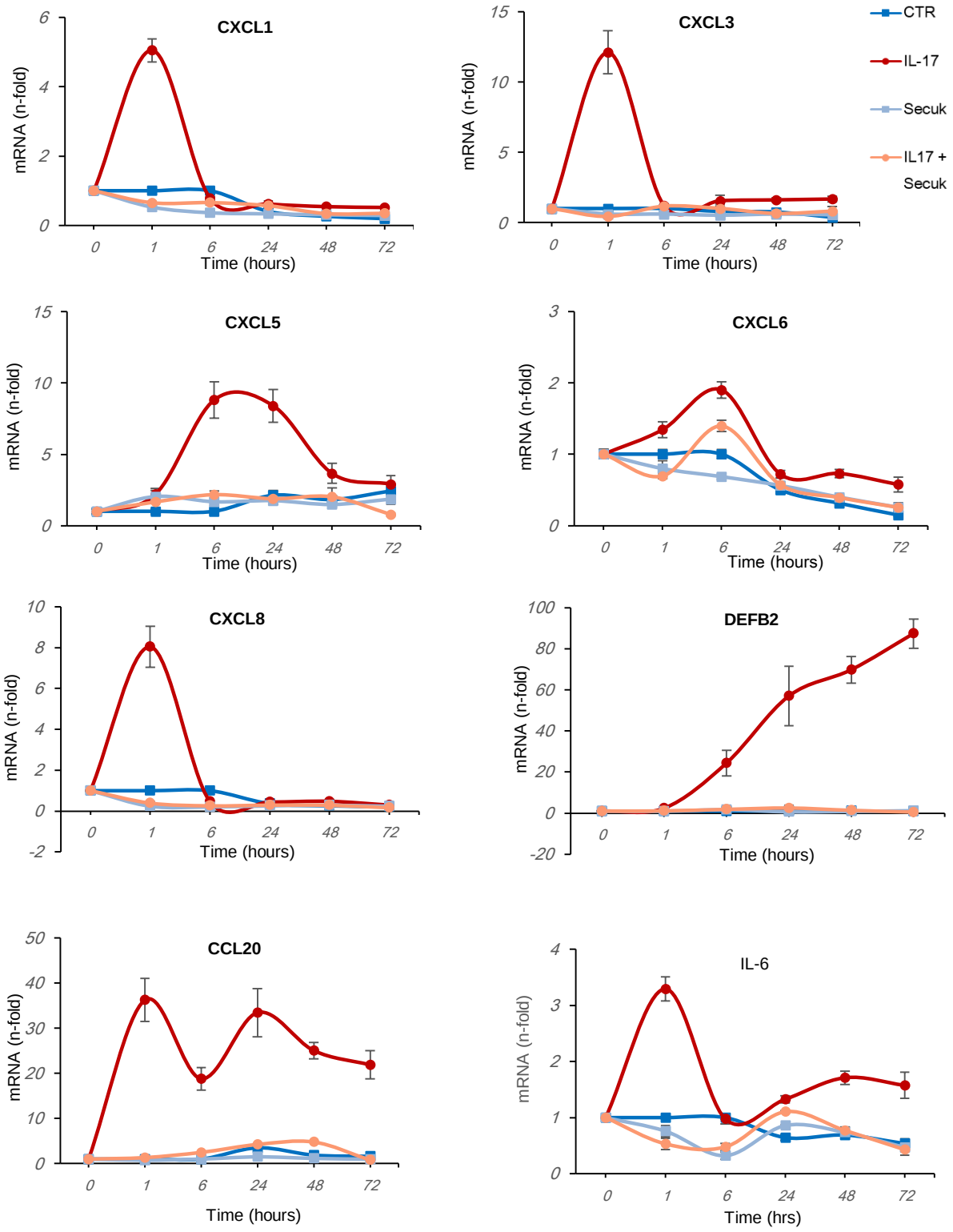


Figure 8. *Validation of in vitro psoriatic model. CXCL1, CXCL3, CXCL5, CXCL6, CXCL8, β-Defensin 2, CCL20 and IL-6 gene expression levels using RT real-time PCR in HaCaT cells. Cells were stimulated for 1, 6, 24, 48 and 72 h with rIL-17A (100 ng/ml) pre-incubated for 1 h at 37° C with or without Secukinumab, with Secukinumab alone or left untreated. Blue*

line: untreated cells (Ctr); red line: rIL-17A; light blue line: Secukinumab (Secuk); light red line: Secukinumab plus rIL-17A (rIL-17A+Secuk).

4.2 THE EFFECTS OF SECUKINUMAB ON IL-17A-DEPENDENT ANGIOGENESIS

Dysregulated angiogenesis is one of the key features of psoriasis. Many different growth factors and pro-inflammatory cytokines involved in the modulation of the angiogenic process are up-regulated in psoriasis development, such as fibroblast growth factor (FGF), angiopoietins, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), platelet-activating factor, ephrins, soluble adhesion molecules and angiogenin. Among these, the vascular endothelial growth factor (VEGFA) plays a major role in regulating endothelial cell functions, including proliferation, migration and activation (Lamalice L *et al.*, 2007). IL-17A is known to regulate angiogenesis inducing the expression of VEGFA in keratinocytes. The VEGFA is upregulated in the psoriatic epidermis (Marina ME *et al.*, 2015; Gerkowicz A *et al.*, 2018). Thus, we evaluated by RT real-time PCR the expression levels of a panel of angiogenic factors, such as EGF, FGF, PDGF, TGF-β, TNF-α, including VEGFA, after rIL-17A treatment. In addition, the effect of the treatment with Secukinumab on the transcriptional levels of angiogenesis-related genes was also evaluated. To this purpose, HaCaT cells were treated for 24 hours with rIL-17A in presence or absence of Secukinumab; at the end of each treatment, cell culture derived supernatants were also collected, as described in Materials and methods, and tested by ELISA for the secreted angiogenic protein. We detected the up-regulation of EGF, FGF, PDGF, TGF-β and TNF-α mRNA levels after rIL-17A treatment, as expected. The pre-treatment with Secukinumab significantly reduced the induction of these mRNAs compared to rIL-17A-treated cells as well as Secukinumab alone. The treatment with rIL-17A increased the expression of VEGFA at both mRNA and secreted protein levels. Conversely, the pre-treatment with Secukinumab decreased the expression of VEGFA to control levels (**Figure 9 A-B**).

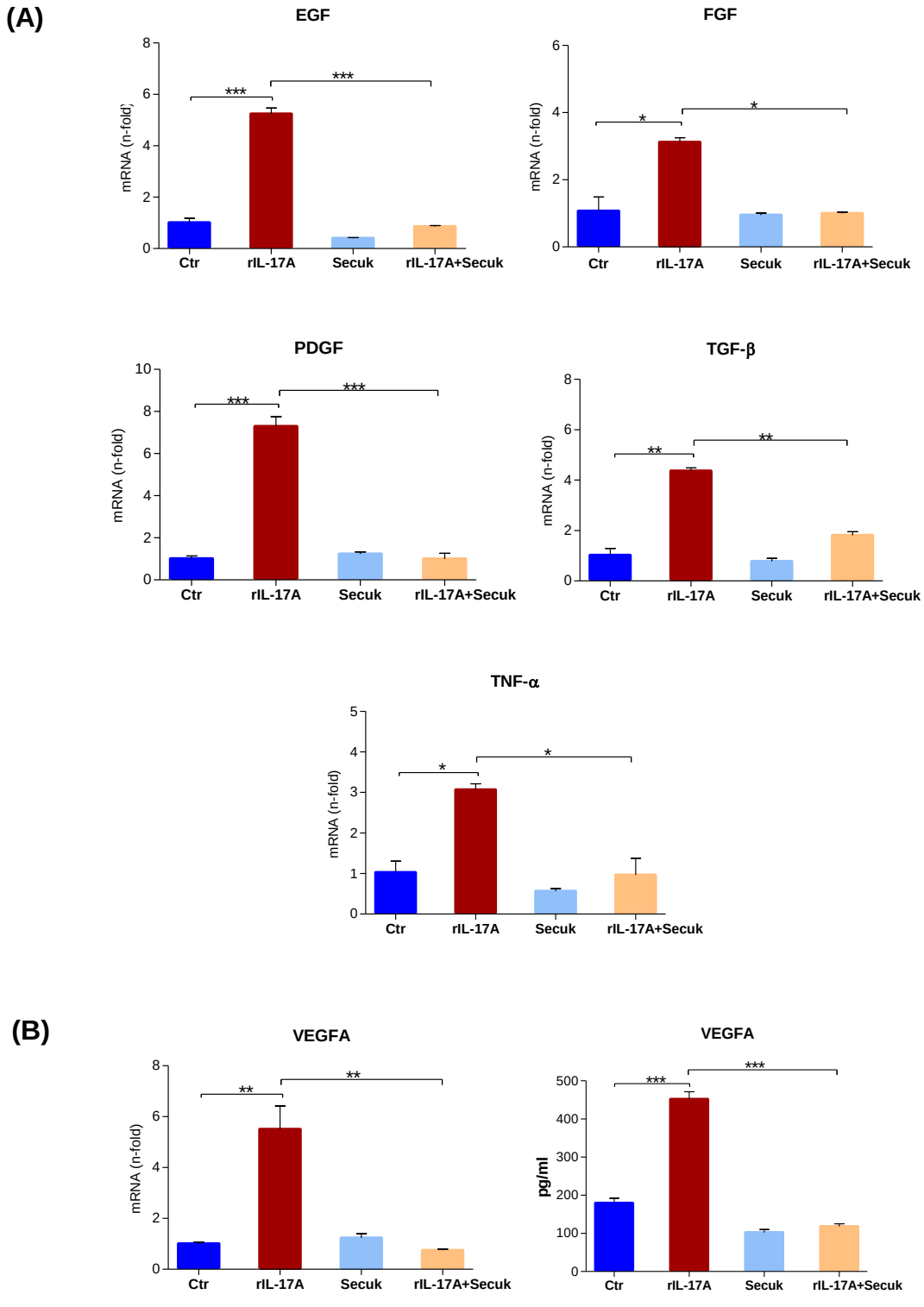


Figure 9 A-B. *The effects of Secukinumab on IL-17A-dependent angiogenesis. (**A**) EGF, FGF, PDGF, TGF-β and TNF-α gene expression levels in HaCaT cells. (**B**) VEGFA gene expression levels in HaCaT cells analyzed by RT real-time PCR and VEGFA protein levels in the supernatants of HaCaT cells measured by ELISA. Cells were stimulated for 24 h with rIL-*

*17A (100 ng/ml) pre-incubated for 1 h at 37° C with or without Secukinumab, with Secukinumab alone or left untreated. Blue column: untreated cells (Ctr); red column: rIL-17A; light blue column: Secukinumab (Secuk); light red column: Secukinumab plus rIL-17A (rIL-17A+Secuk). *: p < .05; **: p < .01; ***: p < .001.*

Dysregulated angiogenesis appears to be a first-order event in psoriasis pathogenesis (Heidenreich R et al., 2009). Coherently to this, we tested the biological effects of the same supernatant derived from HaCaT cells already tested by RT real-time PCR on human umbilical vein endothelial cells (ie HUVECs). Thus, we evaluated proliferation and migration of endothelial cells which are reported as two main endothelial properties during angiogenesis. In order to evaluate the capacity of HaCaT derived conditioned media to influence proliferation of endothelial cells, HUVECs were treated 24, 48 and 72 hours with these supernatants and cell proliferation was determined by MTS assay. After 48 hours, the conditioned medium from rIL-17A-treated HaCaT was able to induce HUVECs proliferation. Conversely, the conditioned medium from Secukinumab and rIL-17A-treated HaCaT cells significantly decreased HUVECs proliferation (**Figure 10**).

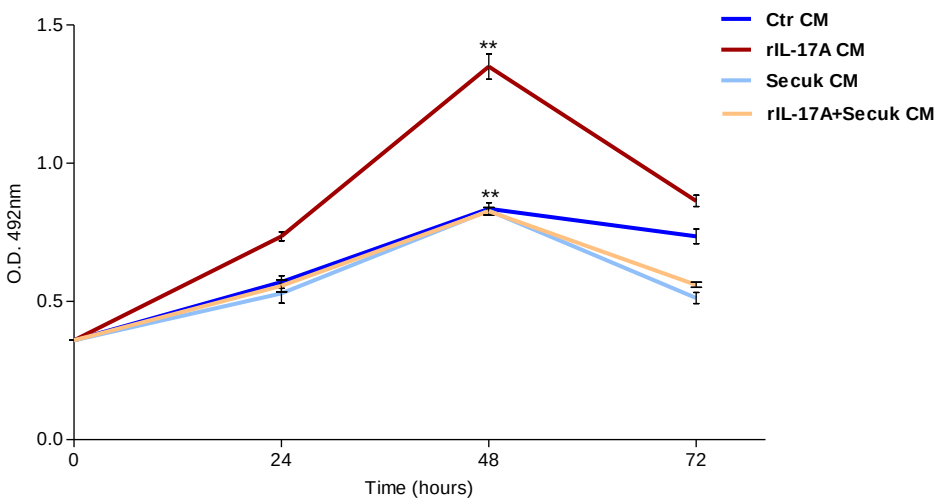
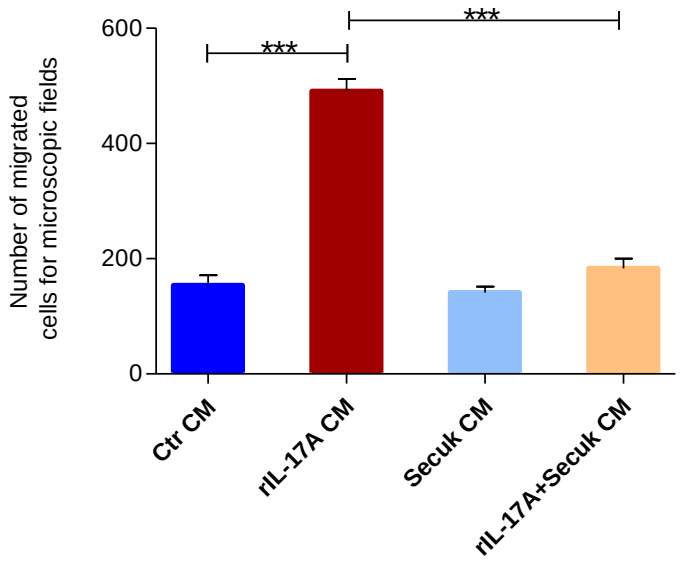


Figure 10. *The effects of Secukinumab on IL-17A-dependent angiogenesis. MTS cell proliferation assay. HUVECs were seeded into 96-well plates with conditioned media (CM) from HaCaT cells treated with rIL-17A pre-incubated for 1 h at 37° C with or without Secukinumab, with Secukinumab alone or left untreated. Blue line: CM from untreated HaCaT cells (Ctr CM); red line: CM from rIL-17A-treated HaCaT cells (rIL-17A CM); light blue line: CM from Secukinumab-treated HaCaT cells (Secuk CM), light red line: CM from Secukinumab plus rIL-17A-treated HaCaT cells (IL-17A+Secuk CM).*

In order to evaluate the capacity of HaCaT derived conditioned media to influence migration of endothelial cells, the supernatants were used as chemoattractant in cell migration assay. Results demonstrated that conditioned medium from rIL-17A-treated HaCaT cells stimulated migration capability of HUVECs. The conditioned medium from Secukinumab and rIL-17A-treated HaCaT cells significantly decreased the number of migrated cell (**Figure 11 A-B**).

(A)



(B)

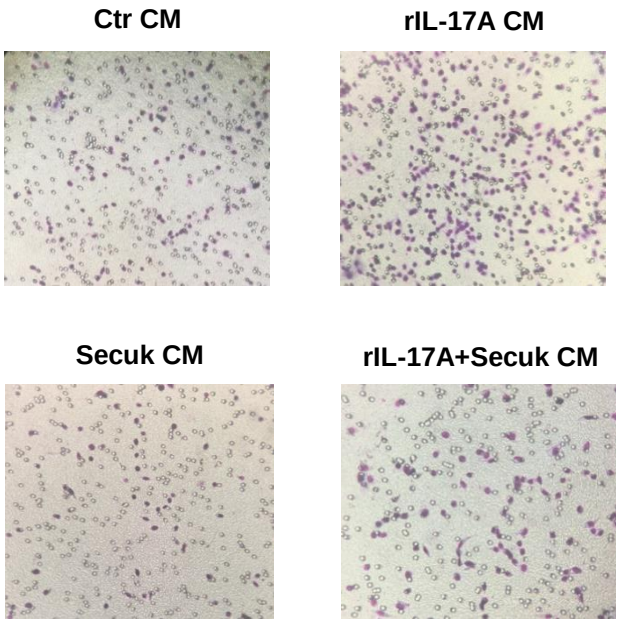
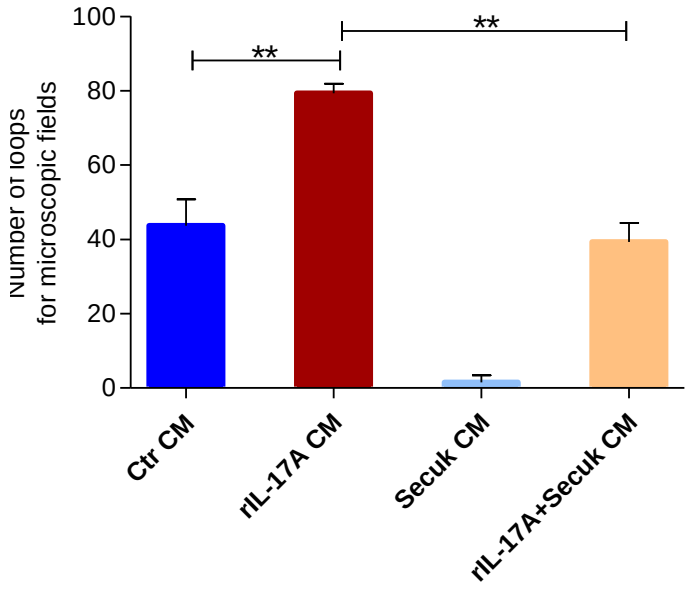


***Figure 11 A-B**. The effects of Secukinumab on IL-17A-dependent angiogenesis. (**A**) The number of migrated cells for microscopic fields. Blue column: CM from untreated HaCaT cells (Ctr CM); red column: CM from rIL-17A-treated HaCaT cells (rIL-17A CM); light blue column: CM from Secukinumab-treated HaCaT cells (Secuk CM), light red column: CM from Secukinumab plus rIL-17A-treated HaCaT cells (IL-17A+Secuk CM). (**B**) Representative*

*images of the transwell migration assay on HUVECs after staining with crystal violet (10X). HUVECs were seeded in 24-well cell culture inserts with 8 µm pore size membrane with CM from untreated HaCaT cells (Ctr CM), CM from rIL-17A-treated HaCaT cells (rIL-17A CM), CM from Secukinumab-treated HaCaT cells (Secuk CM) and CM from Secukinumab plus rIL-17A-treated HaCaT cells (IL-17A+Secuk CM). ***: p < .001.*

To further corroborate the observation that Secukinumab has an antiangiogenic effect on HUVECs, a functional tube formation assay was performed. Quantitative analysis of endothelial cell networks revealed that the number of loops was significantly increased in presence of the conditioned medium from rIL-17A-treated HaCaT cells compared to conditioned medium from untreated cells. The conditioned medium from Secukinumab and rIL-17A-treated HaCaT cells significantly decreased the number of loops compared to IL-17A treatment (**Figure 12 A-B**).

(A)



(B)

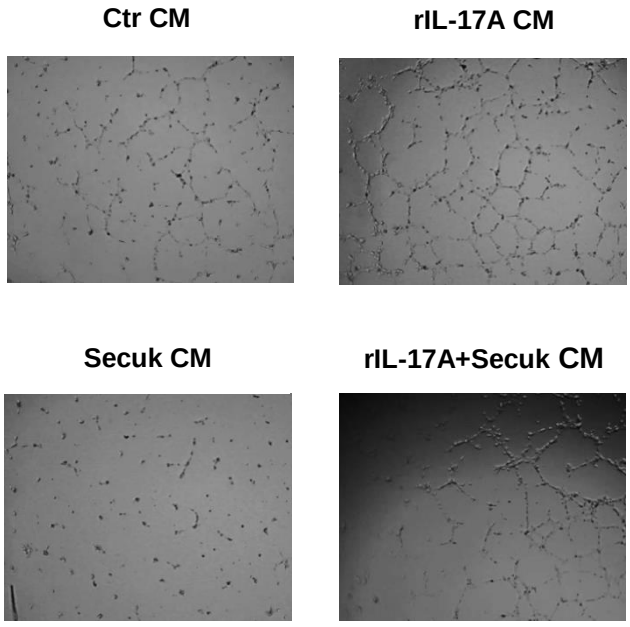


***Figure 12 A-B**. The effects of Secukinumab on IL-17A-dependent angiogenesis. (**A**) The number of loops cells for microscopic fields. Blue column: CM from untreated HaCaT cells (Ctr CM); red column: CM from rIL-17A-treated HaCaT cells (rIL-17A CM); light blue column: CM from Secukinumab-treated HaCaT cells (Secuk CM), light red column: CM from*

*Secukinumab plus rIL-17A-treated HaCaT cells (IL-17A+Secuk CM). (**B**) Representative images of the tube formation assay on HUVECs (10X). HUVECs were seeded in 96-well Matrigel-coated plate with CM from untreated HaCaT cells (Ctr CM), CM from rIL-17A-treated HaCaT cells (rIL-17A CM), CM from Secukinumab-treated HaCaT cells (Secuk CM) and CM from Secukinumab plus rIL-17A-treated HaCaT cells (IL-17A+Secuk CM). Capillary-like structures (ie loops) formation was photographed after 6 h. **: $p < .01$.*

We also detected the up-regulation of several pro-inflammatory and angiogenesis-associated mRNAs in HUVECs treated for 24 hours with HaCaT conditioned media. The conditioned medium from rIL-17A treated HaCaT cells strongly enhanced mRNA levels of kinase insert domain receptor (KDR, also known as VEGFR-2), PDGF, FGF, matrix metalloproteinase-2 (MMP-2) and MMP-9 in HUVECs. The pre-treatment with Secukinumab significantly reduces the induction of these mRNAs whose levels are comparable to control cells (**Figure 13**).

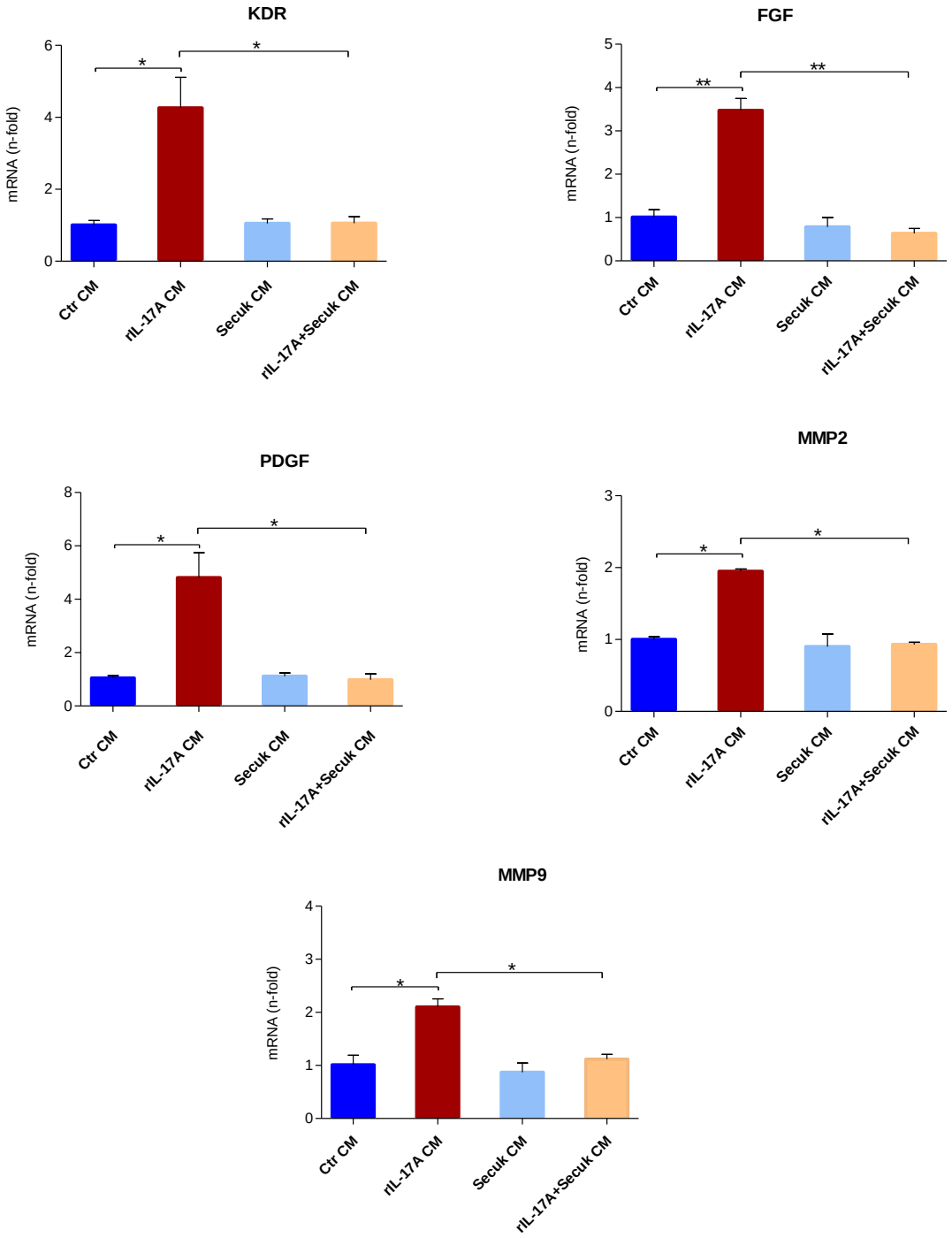


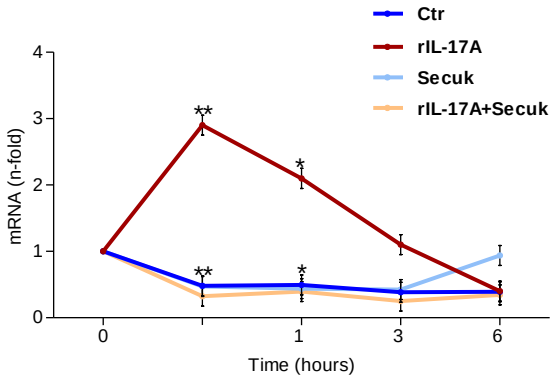
Figure 13*. The effects of Secukinumab on IL-17A-dependent angiogenesis. KDR, FGF, PDGF, MMP-2 and MMP-9 gene expression levels in HUVECs. HUVECs were stimulated with CM from untreated HaCaT cells (Ctr CM), CM from rIL-17A-treated HaCaT cells (rIL-17A CM), CM from Secukinumab-treated HaCaT cells (Secuk CM) and CM from*

*Secukinumab plus rIL-17A-treated HaCaT cells (rIL-17A+Secuk CM). Blue column: CM from untreated HaCaT cells (Ctr CM); red column: CM from rIL-17A-treated HaCaT cells (rIL-17A CM); light blue column: CM from Secukinumab-treated HaCaT cells (Secuk CM), light red column: CM from Secukinumab plus rIL-17A-treated HaCaT cells (rIL-17A+Secuk CM). *: $p < .05$; **: $p < .01$; ***: $p < .001$.*

4.3 THE ROLE OF EGR-1 AS TARGET OF IL-17A/SECUKINUMAB AXIS

The transcriptional factor Egr-1 is strongly increased in the skin lesions of patients with psoriasis (Wee S *et al*., 2001; Jeong *et al*., 2004; Fang M *et al*., 2017). It has been suggested that Egr-1 influences angiogenesis by regulating the transcriptional activity of VEGFA (Lee KH and Kim JR, 2009; Shimoyamada *et al*., 2010). We have hypothesized an Egr-1-dependent regulation of the VEGFA by Secukinumab. Thus, to verify our hypothesis we first evaluated the levels of Egr-1 in HaCaT cells after rIL-17A and/or Secukinumab treatment. To this purpose, HaCaT cells were stimulated with rIL-17A in presence or absence of Secukinumab, by analyzing early time points (½, 1, 3 and 6 hours) given that Egr-1 is a rapidly and transiently inducible gene upon a variety of different stimuli including growth factors, cytokines and environmental stresses (Sukhatme VP *et al*., 1988; Bernstein SH *et al*., 1991; Baron V *et al*., 2006). The expression levels of Egr-1 were evaluated by RT real-time PCR and Western blot analysis. According to Jeong SH *et al.* we observed that Egr-1 gene expression levels showed a maximal upregulation at 30 minutes following a decreasing trend up to 3 hours of stimulation with rIL-17A. In addition, we detected a rapid and transiently increasing of Egr-1 protein levels after 30 minutes and 1 hour of treatment with rIL-17A. The pre-treatment with Secukinumab decreased the induction of Egr-1 mRNA and protein compared to rIL-17A stimulation (**Figure 14 A-B**).

(A)



(B)

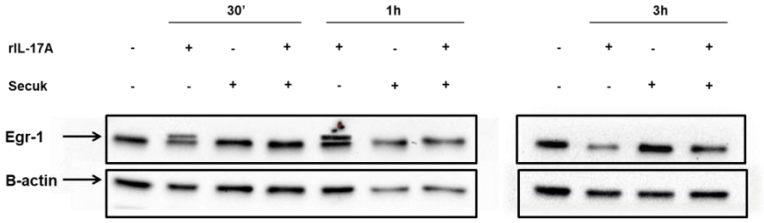


Figure 14 A-B*. The role of EGR-1 as target of IL-17A/Secukinumab axis. (**A**) Egr-1 gene expression levels in HaCaT cells. Cells were stimulated for ½, 1, 3 and 6 hours with rIL-17A (100 ng/ml) pre-incubated for 1 h at 37° C with or without Secukinumab, with Secukinumab alone or left untreated. Blue line: untreated cells (Ctrl); red line: rIL-17A; light blue line: Secukinumab (Sec); light red line: Secukinumab plus rIL-17A. (**B**) Representative images of Western blot analysis for Egr-1 after½, 1 and 3 hours of treatment with rIL-17A, Secukinumab alone and Secukinumab plus rIL-17A. *: p < .05; **: p < .01; ***: p < .001.*

Considering that *in vivo* psoriatic keratinocytes are chronically exposed to IL-17A produced by activated Th17 lymphocytes (Miossec P and Kolls JK, 2012), we also reproduced a chronic *in vitro* exposure by stimulating HaCaT cells for 7 days with rL-17A (daily stimulation) in presence or absence of Secukinumab to test EGR-1 and VEGF mRNA expression. The expression levels of Egr-1 and VEGF were evaluated by RT real-time PCR. The effect of up-regulation of Egr-1 and VEGFA mRNA levels was maintained in presence of chronic stimulus by rIL-17A. The pre-treatment with Secukinumab decreased the expression of Egr-1 and VEGFA to control levels (**Figure 15**).

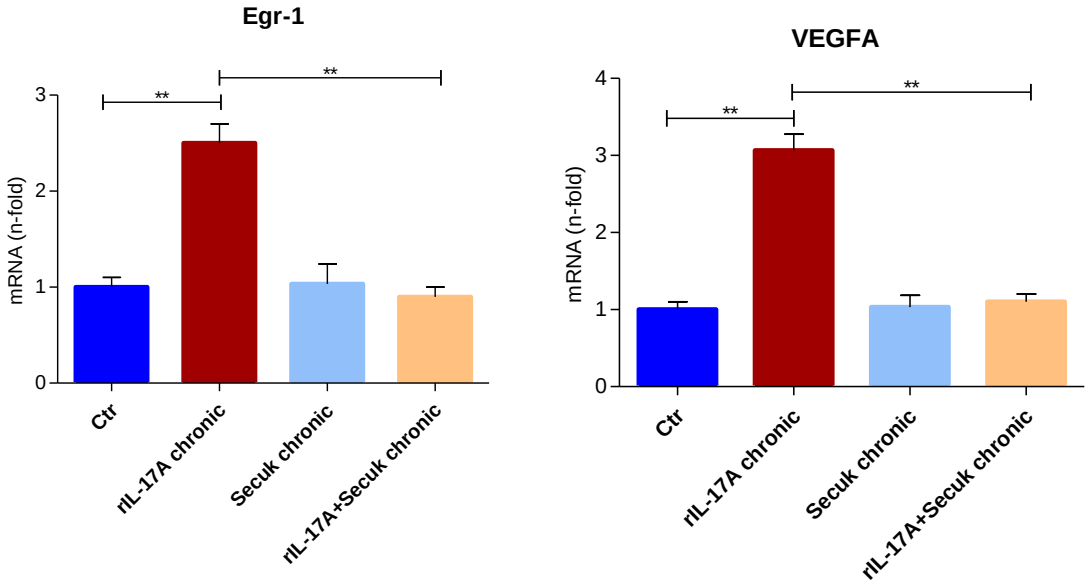


Figure 15. *The role of EGR-1 as target of IL-17A/Secukinumab axis. Egr-1 and VEGFA gene expression levels in HaCaT cells. Cells were daily stimulated for 1 week with rIL-17A pre-incubated for 1 h at 37° C with or without Secukinumab, with Secukinumab alone or left untreated. Blue column: untreated cells (Ctr); red column: rIL-17A; light blue column: Secukinumab (Secuk); light red column: Secukinumab plus rIL-17A (rIL-17A+Secuk). **: p < .01.*

To further confirm our hypothesis, we evaluated the effects on the expression of VEGFA in HaCaT cells by reducing the levels of Egr-1. HaCaT cells were treated with a specific Egr-1 small interfering RNA (siRNA) oligos and then stimulated with rIL-17A. The expression levels of Egr-1 and VEGFA were measured by RT real-time PCR. By lowering the Egr-1 mRNA levels with siRNA specific to Egr-1 sequence, we observed a significant reduction of VEGFA mRNA levels after rIL-17A stimulation compared to cells treated with non-specific (scrambled) siRNA. Similar changes were observed with the decreased levels of secreted VEGFA under the same experimental conditions (**Figure 16 A-B**).

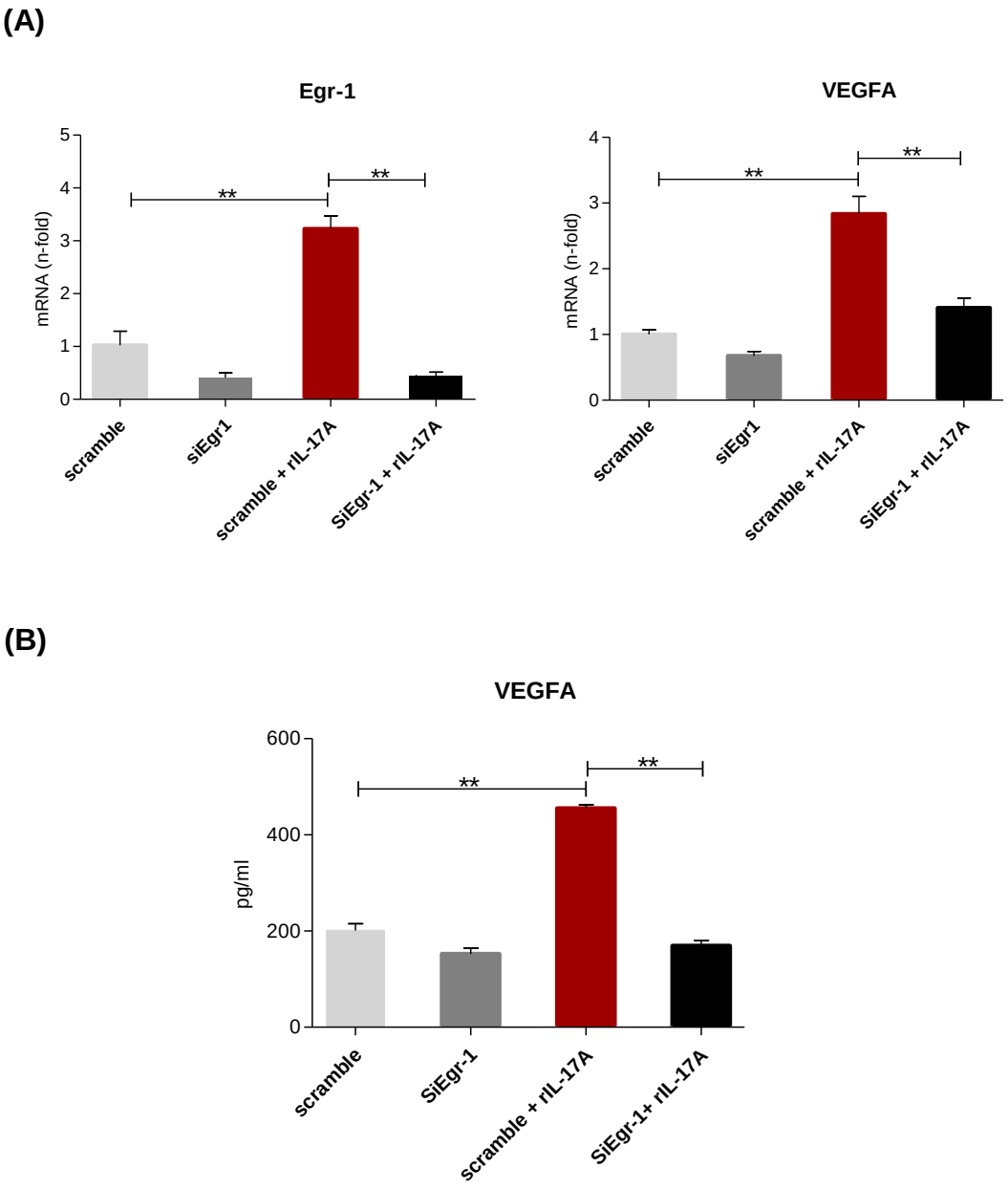


Figure 16 A-B*. The role of EGR-1 as target of IL-17A/Secukinumab axis. (**A**) Egr-1 and VEGFA gene expression levels in HaCaT cells. Cells were transfected with scramble and Egr-1 siRNA oligos and then were treated with rIL-17A or left untreated. (**B**) Levels of VEGFA in the supernatants of HaCaT cells after Egr-1 silencing measured by ELISA. Cells were transfected with scramble and Egr-1 siRNA oligos and then were treated with rIL-17A or left untreated. Light grey column: cells transfected with scramble; grey column: cells transfected with Egr-1 siRNA oligos; red column: cells transfected with scramble and treated with rIL-17A; black column: cells transfected with Egr-1 siRNA oligos and treated with rIL-17A. **: p < .01.*

Finally, we validated these results *in vivo* by analyzing the expression levels of both Egr-1 and VEGFA in skin sections of patients with plaque psoriasis, before and after the treatment with Secukinumab. The immunohistochemistry analysis of skin lesions showed the presence of Egr-1 positive cells both in the basal layer, the immediate suprabasal layer and the spinous layer of psoriatic epidermis. Moreover, a strong nuclear immunopositivity was noted among the extended spinous layer than among the basal layer and the immediate suprabasal layer. A similar result was found in the IHC analysis of VEGFA. Following treatment with Secukinumab, the clinical response in patients was associated with a reduction of histo-morphological signs of acanthosis and epidermal hyperplasia. The immunohistochemistry analysis showed that the expression of Egr-1 was markedly decreased following treatment with Secukinumab; the most interesting phenomenon was seen in the basal layer of the psoriatic involved epidermis, where only a few nests of dividing keratinocytes maintain a nuclear immunopositivity. The analysis of VEGFA expression showed that treatment with Secukinumab reduced the expression of this growth factor, indicating a decrease of VEGFA staining in the spinous layer. Only moderate VEGFA staining was detected in the in the basal layer and the immediate suprabasal layer (**Figure 17**).

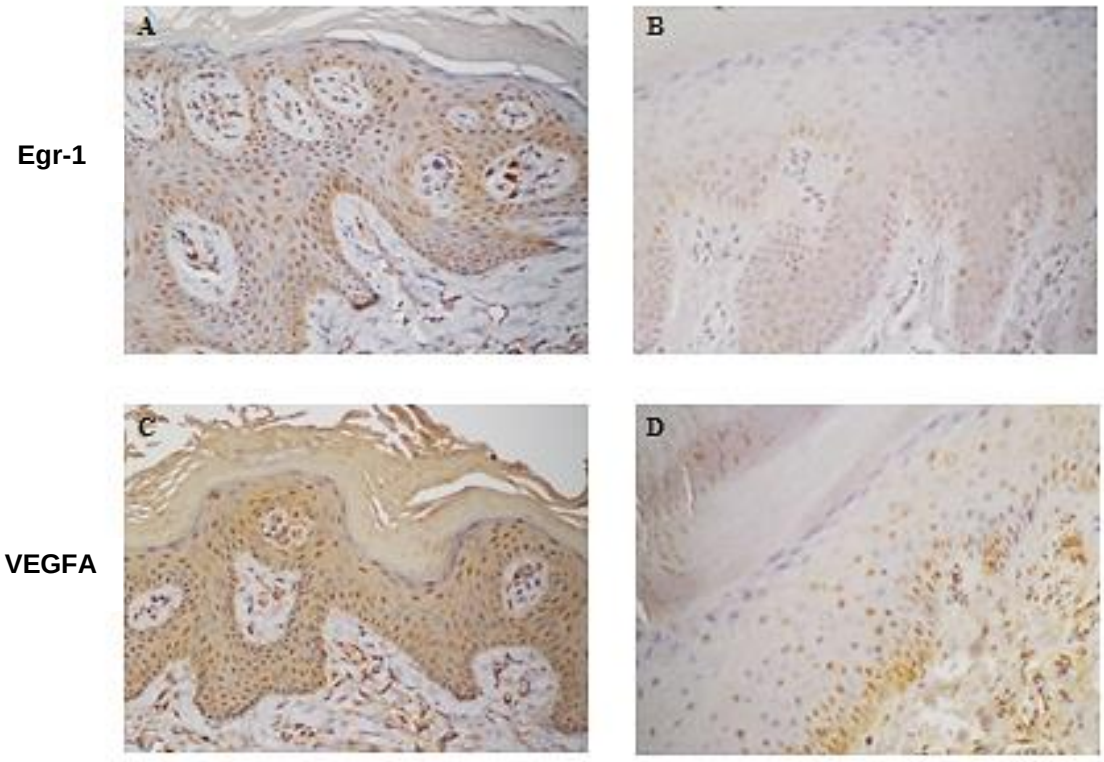


Figure 17 A-B-C-D. *The role of EGR-1 as target of IL-17A/Secukinumab axis. Representative images of IHC analysis of EGR-1 psoriatic epidermis, before (**A**) and after Secukinumab therapy (**B**). IHC analysis of VEGF psoriatic epidermis, before (**C**) and after Secukinumab therapy (**D**) (20X).*

4.4 IL-17A REDUCES THE RELEASE OF EVs

In the second part of this work, we aimed to test the involvement of EVs in psoriasis pathogenesis. First we tested the hypothesis that the treatment of keratinocytes with rIL-17A could affect both quantitatively and qualitatively the synthesis and release of EVs. For this purpose, HaCaT cells were stained with CFSE, treated for 72 hours with rIL-17A and released EVs quantified by flow cytometry. The treatment with rIL-17A decreased the release of EVs in a statistically significant manner. Furthermore, the scatter analysis revealed a change in the size of EVs released by rIL-17A-treated cells compared to untreated cultures. A reproducible increase in the percentage of EVs < 450 nm isolated from rIL-17A treated cells compared to untreated cells was observed. Accordingly, the release of both EVs > 800 nm and those included in the range of 450-800 nm was reduced in IL-17A-treated cells compared to control (**Figure 18**). As the CFSE dye does not leak out from undamaged particles, we also conclude that EVs were intact. Cytotoxic or cytostatic effects of IL-17A treatment did not account for the reduced EV release since MTS assays revealed no significant differences between IL-17A-treated and untreated controls (data not shown).

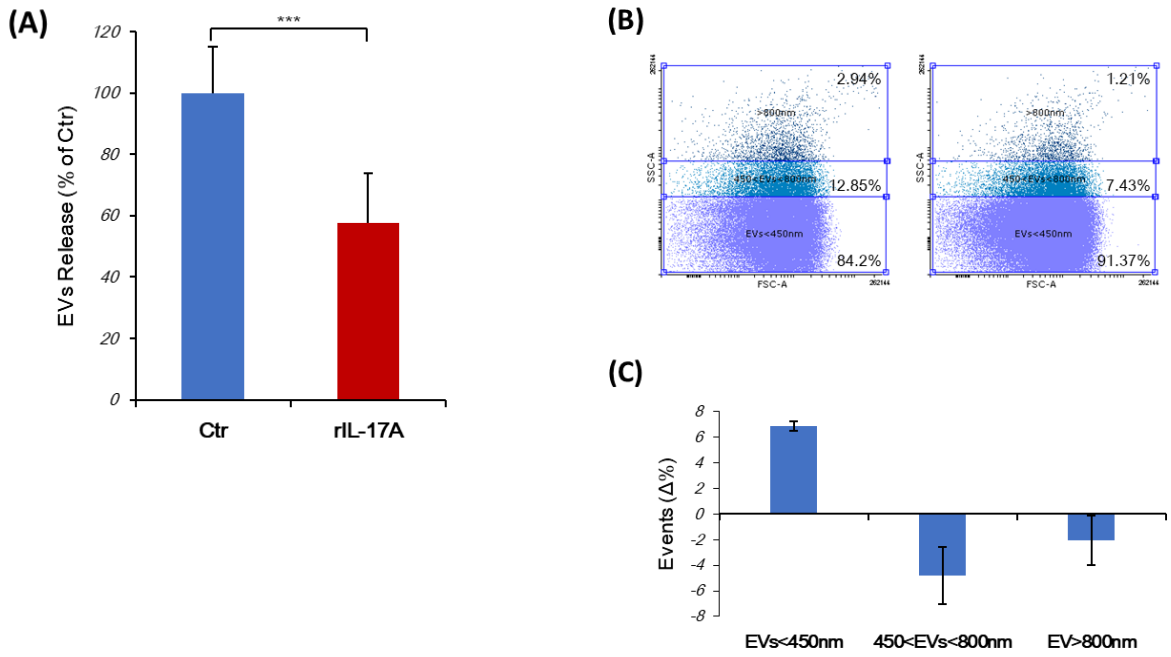


Figure 18 A-B-C*. IL-17A reduces the release of EVs. HaCaT cells were stained with CFSE and stimulated for 72 h with rIL-17A. Supernatants were collected and EVs enumerated on a FACs ARIA II cytometer. (**A**) CFSE-positive EVs were normalized to cell counts, and results are expressed as percentage compared to untreated cells. Blue bar: EVs from untreated cells (Ctr); red bar: EVs from IL-17A-treated cells. (**B**) A representative dot plot of CFSE stained EVs derived from untreated (left panel) and rIL-17A-treated cells (right panel). (**C**) The variation in the EVs subpopulation in response to rIL-17A treatment was expressed as the difference between the percentage of each EVs subpopulation measured in rIL-17A-treated cells and the percentage in untreated controls (Δ%). **: p < .01 (Mangino G et al., 2019).*

4.5 EVs ARE INTERNALIZED BY ACCEPTOR CELLS

Once we recorded the ability of HaCaT cells to release EVs, we tested the hypothesis that these vesicles could be transferred to acceptor cells. Then, we isolated EVs from untreated (Ctr-EVs) or IL-17A-treated (IL-17A-EVs) HaCaT cells, staining them with PHK67, a green fluorescent dye able to label membranes. In both cases, acceptor unstained HaCaT cells were incubated for different time points with stained EVs. Incubation was performed at both 37 and 4°C to discriminate between EV endocytosis and surface adsorption, respectively. EVs were rapidly internalized by acceptor cells as 30-40% of acceptor cells were positive to PHK67 when incubated at 37°C for 5 minutes but not at 4°C, thereby excluding any adsorption to acceptor cell surface (**Figure 19**).

We also performed immunofluorescence analysis on EVs-treated cells using structured illumination microscopy. Both Ctr-EVs and IL-17A-EVs were internalized at 37°C with the same efficiency, and no major differences in the intracellular localization were detected, whereas no fluorescence was detected at 4°C (data not shown).

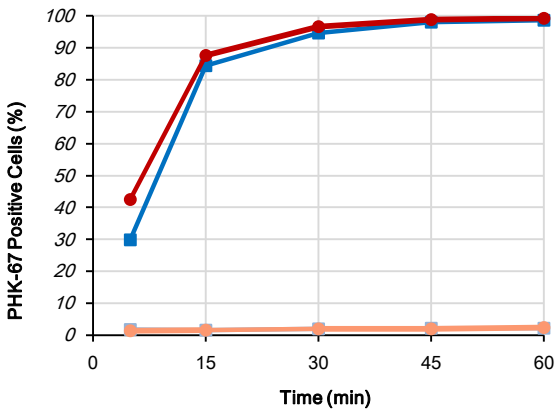


Figure 19. *EVs are internalized by acceptor cells. Cells were stimulated with rIL-17A; at the end of stimulation, supernatants were collected and EVs were isolated and stained with PHK67 as described in materials and methods. HaCaT cells were incubated with PHK67-labeled EVs for 5', 15', 30', 45' and 60' at both 37 or 4°C. Incubation was stopped by adding 900 µl of cold PBS and cells were analyzed by flow cytometry. Results were expressed as percentage of PHK67 positive cells. Blue line: HaCaT cells treated with EVs from untreated cells, 37°C; red line: HaCaT cells treated with EVs from rIL-17A-treated cells, 37°C; light*

blue line: HaCaT cells treated with EVs from untreated cells, 4°C; light red line: HaCaT cells treated with EVs from rIL-17A-treated cells, 4°C (Mangino G et al., 2019).

4.6 EVs FROM IL-17A-TREATED CELLS CONTAIN SPECIFIC mRNAs

As we have shown previously (**Figure 8**), HaCaT cells treated with rIL-17A contain specific cytokines/chemokines mRNAs. Therefore, we tested the presence of these mRNAs in EVs isolated from rIL-17A-treated cells. HaCaT cells were treated for 72 hours with rIL-17A and EVs were isolated from collected supernatants. Then, we analyzed the mRNA content in EVs isolated from both untreated keratinocytes and treated with rIL-17A in the presence or absence of Secukinumab. EVs isolated from rIL-17A-treated cells embedded mRNA for all chemokines and β-Defensin 2 but not IL-6 and CXCL8. This feature was completely abrogated by Secukinumab pre-treatment (**Figure 20**).

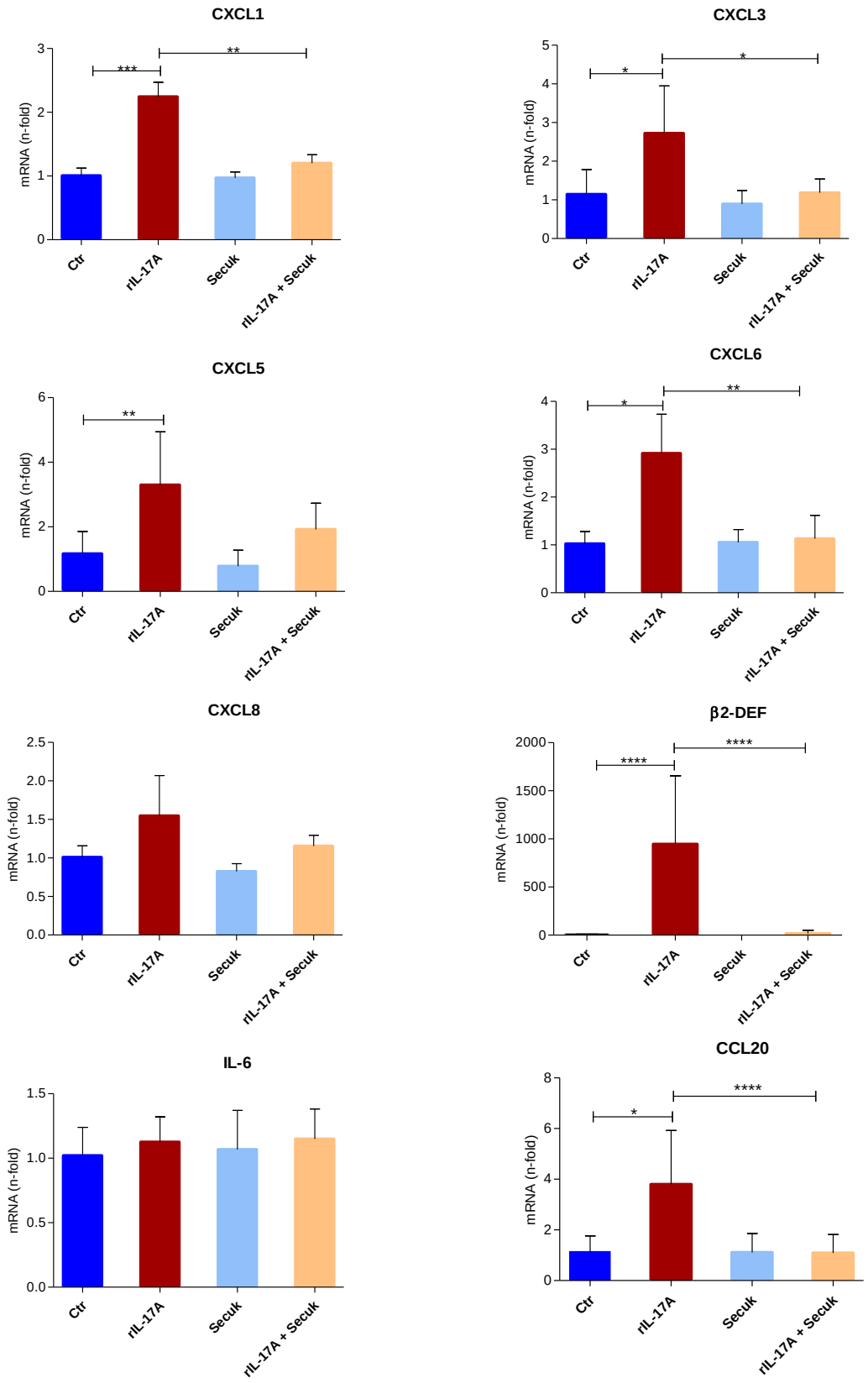


Figure 20. *EVs from IL-17A-treated cells contain specific mRNAs. B-Defensin 2, IL-6, CCL20, CXCL1, CXCL3, CXCL3 and CXCL8 gene expression levels in HaCaT cells EVs.*

*Cells were stimulated for 72 h with rIL-17A pre-incubated for 1 h at 37°C with or without Secukinumab, with Secukinumab alone or left untreated. Supernatants were collected, EVs were isolated, total RNA was purified, and real-time RT-PCR was performed. Blue bar: EVs from untreated cells (Ctr); red bar: EVs from rIL-17A-treated cells (rIL-17A); light blue bar: EVs from Secukinumab-treated cells (Secuk); light red bar: EVs from Secukinumab plus IL-17A-treated cells (rIL-17A+Secuk). *: $p < .05$; **: $p < .01$; ***: $p < .001$; ****: $p < .0001$ (Mangino G et al., 2019).*

4.7 β-DEFENSIN 2 mRNA IS OVER-REPRESENTED IN EVs ACCEPTOR CELLS

In vivo psoriatic keratinocytes were continuously exposed to IL-17A signaling. We simulated chronic exposure by stimulating HaCaT cells for 5 days with IL-17A either in acute mode (ie one stimulation at the beginning of the experiment) or in chronic stimulation (ie daily stimulation with the cytokine), to test whether there are any differences between these conditions. Among the previously tested cytokines/chemokines, we measured higher levels of β-Defensin 2 mRNA in samples exposed to IL-17A in chronic rather than in acute manner in both cells and EVs.

In view of previously results, we wondered if cargo mRNAs could be transfer to acceptor cells. Donor cells were daily stimulated with IL-17A for 5 days; therefore, HaCaT cells were treated with both Ctr-EVs and IL-17A-EVs and to monitor the cargo transfer, we measured the β-Defensin 2 mRNA as readout (**Figure 21**).

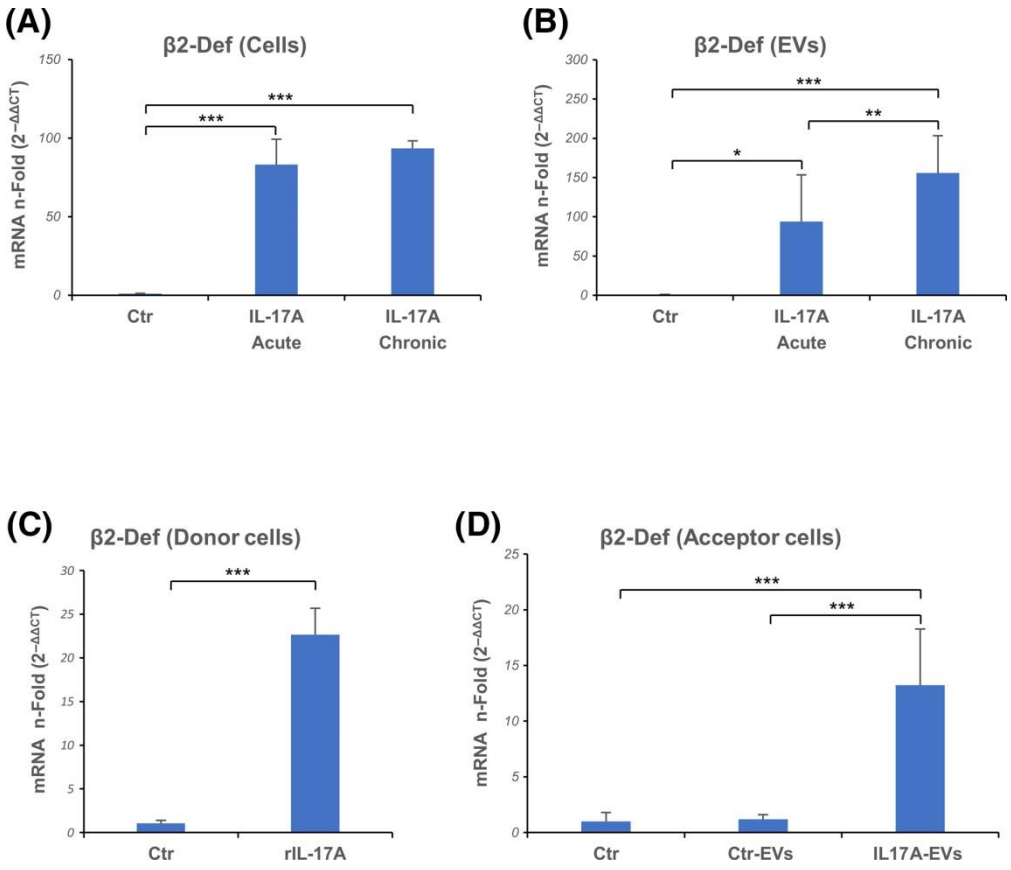


Figure 21 A-B-C-D. *β-Defensin 2 mRNA is over-represented in EVs acceptor cells. β-Defensin 2 gene expression levels in HaCaT cells and EVs. Cells were stimulated for 5 d, daily (IL-17A Chronic) or at the beginning of stimulation (IL-17A Acute) with rIL-17A or left untreated (Ctr). Supernatants were collected, and EVs were isolated. B-Defensin 2 real-time RT-PCR was performed on both cells (**A**) and EVs (**B**). Donor cells were stimulated for 5 d, daily (IL-17A Chronic) with rIL-17A or left untreated (Ctr). Supernatants were collected, and EVs were isolated. EVs from rIL-17A-treated cells (IL-17A-EVs) and from unstimulated cells (Ctr-EVs) were then added to acceptor cells. Acceptor cells were incubated for 24 h at 37°C with or without EVs. β-Defensin 2 real-time RT-PCR was performed on both donor (**C**) and acceptor cells (**D**). *: p < .05; **: p < .01; ***: p < .001 (Mangino G et al., 2019).*

Overall, these results suggest that chronic exposure to IL-17A induces a higher β-Defensin 2 mRNA expression in EVs. These vesicles are taken up by acceptor cells and β-Defensin 2 mRNA was specifically upregulated in cells treated with IL-17A-EVs.

4.8 IL-17A-EVs SPECIFICALLY INDUCE ENDOGENOUS B-DEFENSIN 2 mRNA IN ACCEPTOR KERATINOCYTES

Based on the previous result, two possible scenarios are conceivable: (a) IL-17A-EVs transfer their cargo to acceptor cells enabling them to express 'exogenous' mRNAs or (b) IL-17A-EVs allow acceptor cells to express endogenous β-Defensin 2 mRNA. To discriminate between these two hypotheses, acceptor cells were pre-treated with Actinomycin D, thereby interfering with cellular mRNA synthesis, then incubated with EVs collected from both donor untreated and IL-17A-treated cells, containing β-Defensin 2 mRNA (**Figure 22**). Cells pre-treated with Actinomycin D failed to upregulate β-Defensin 2 mRNA following IL-17A-EV challenge.

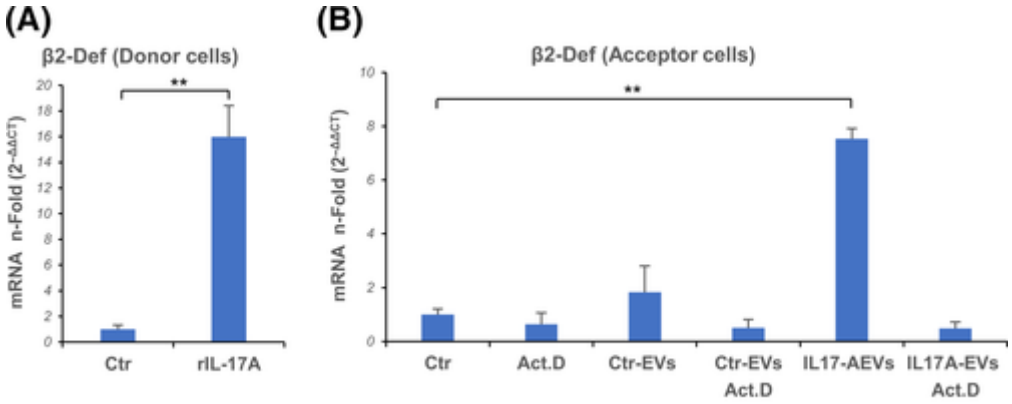


***Figure 22 A-B**. IL-17A-EVs specifically induce endogenous β-Defensin 2 mRNA in acceptor keratinocytes. β-Defensin 2 gene expression levels in HaCaT cells. Donor cells were daily stimulated for 5 d with rIL-17A or left untreated. Supernatants were collected; EVs from rIL-17A-treated cells (IL-17A-EVs) and from unstimulated cells (Ctr-EVs) were isolated. Acceptor cells were pre-treated with Actinomycin D or left untreated. Cells were, then, stimulated with or without EVs for 24 h. β-Defensin 2 real-time RT-PCR was performed on both donor (**A**) and acceptor cells (**B**). Ctr: untreated cells; Act D: Actinomycin D. **: p < .01 (Mangino G et al., 2019).*

Taken together, these results indicate that EVs derived from IL-17A-treated keratinocytes, but not from untreated cells, are able to induce the expression of specific mRNAs (*ie* β-Defensin 2) in acceptor cells in the absence of the primitive stimulus (*ie* IL-17A).

5. DISCUSSION AND CONCLUSIONS

The presented study represents the first analysis of a potential molecular mechanism underlying the therapeutic effect of Secukinumab in psoriasis. First, the results obtained from this study confirm that treatment with Secukinumab has anti-inflammatory effects. This can be detected by reducing the inflammatory cytokine and chemokine pattern in our *in vitro* psoriatic model. In addition, Secukinumab also exhibits anti-angiogenic effects by downregulating the gene expression of several pro-angiogenic factors, such as EGF, FGF, PDGF, TGF-β, and TNF-α, known to be over-expressed during psoriasis inflammation and therefore to promote suitable deregulated inflammatory microenvironment (Elder *et al.* 1989; Christophers 1996; Starnes *et al.* 2001; Creamer *et al* 2002; Numasaki *et al.* 2003; Ghoreschi *et al.* 2007; Heidenreich *et al.* 2008). More importantly Secukinumab downregulates the gene expression of VEGFA, the most powerful pro-angiogenic factor able to also exert a paracrine action on endothelial cells promoting their proliferation and migration (Lamalice L *et al*., 2007). The results obtained by ELISA assay confirm that the secreted levels of VEGFA decrease following Secukinumab treatment. We also observed the effects of Secukinumab in modulating the angiogenic properties of keratinocytes-derived supernatants, probably due to VEGFA alterations. The supernatants from keratinocytes previously exposed to Secukinumab significantly decreases cell proliferation and migration of endothelial cells. Interestingly, Secukinumab strongly inhibits capillary tube formation by endothelial cells, as demonstrated by Matrigel assay. We then investigated the molecular pathway by which Secukinumab affects angiogenesis. As already described in literature (Jeong SH *et al*., 2014), we confirm in our experimental model that Egr-1 is a target of IL-17A, that is emerging as a potent pro-angiogenic cytokine (Numasaki *et al.*, 2003). In addition, we provide evidence that the involvement of Egr-1 in angiogenesis is correlated to the enhancement of VEGFA levels by IL-17A. As shown by results obtained *in vitro,* IL-17A-mediated VEGFA expression was reduced after silencing of Egr-1. Immunohistochemical analysis of the expression of Egr-1 and VEGFA in patients with plaque psoriasis confirm that Egr-1 is downregulated in response to therapeutic treatment with Secukinumab and this phenomenon is associated with a decrease of VEGFA expression. Consequently, the effect of IL-17A blockade by Secukinumab, prevents the activation of Egr-1-VEGFA molecular axis and therefore negatively influence angiogenesis.

As what concern the role of keratinocyte-derived EVs in psoriasis, the second part of this work, our *in vitro* model suggests that IL-17A reduces the delivery of the vesicles highlighting the role of keratinocytes-derived EVs in psoriasis. We also observed an effect of IL-17A in the size of the EV population released by keratinocytes. EVs produced from IL-17A-treated cells are smaller in size if compared to those released from untreated cells, suggesting the presence in the former sample of a heterogeneous population of EVs mostly composed of exosomes and/or small MVs (in the range of 100 nm). The exposure of HaCaT cells to IL-17A also affects the EVs cargo in terms of mRNA. Results obtained in EVs isolated from IL-17A-treated HaCaT cells demonstrate the presence of mRNAs for chemokines representing an IL-17A specific signature overlapping the intracellular expression pattern in keratinocytes (Nograles KE *et al.*, 2008). According to Nograles *et al.*, the most induced mRNA in both cells and EVs is β-Defensin 2 also in our experimental system. β-Defensin 2 is a psoriasis associated marker together with CCL20, another mRNA we founded upregulated in IL-17A EVs (Kolbinger F *et al*., 2017; Liu Y *et al*., 2010). Another streaking feature is the ability of Secukinumab to inhibit the expression of IL-17A related mRNAs in EVs and in keratinocytes.

In addition to the prognostic/diagnostic use, EVs represent an almost unexplored way of cell to cell and cell to extracellular matrix communication. From this point of view, one of the critical step is the interaction between EVs and target/acceptor cells. Our results indicate that not only EVs derived by keratinocytes interact with acceptor cells, but also that these EVs are internalized. Fluorescent-labelled EVs stain at 37°C, but not at 4°C, acceptor HaCaT cells, independently to rIL-17A treatment of donor cells (data not shown). This suggests that the interaction between EVs and acceptor cells is due to the presence of a molecule constitutively expressed on EV surface. Nevertheless, only EVs derived from cells treated with IL-17A induce the expression of β-Defensin 2 mRNA in acceptor cells. Results obtained pre-treating acceptor cells with Actinomycin D indicate that the β-Defensin 2 mRNA expression is due to de novo synthesis of endogenous mRNA rather than the transfer from donor to acceptor cells. Taken together, our results indicate that IL-17A affects the production of EVs and their size in keratinocytes also reshaping the cargo content through the overexpression of psoriasis-associated mRNAs like β-Defensin 2. These EVs interact and are internalized by acceptor cells which, in turn, express β-Defensin 2 in the absence of primitive stimulus (ie IL-17A). This mechanism could play a role in the amplification of the chronic inflammatory state in the surrounding of the psoriatic lesions. It may be hypothesized that, if EVs reach the blood

stream, they could contribute to the dissemination of the disease in other anatomical sites as heart, eye or joints in the case of psoriatic arthritis.

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"È una follia odiare tutte le rose perché una spina ti ha punto, abbandonare tutti i sogni perché uno di loro non si è realizzato, rinunciare a tutti i tentativi perché uno è fallito.

È una follia condannare tutte le amicizie perché una ti ha tradito, non credere in nessun amore solo perché uno di loro è stato infedele, buttare via tutte le possibilità di essere felici solo perché qualcosa non è andato per il verso giusto.

Ci sarà sempre un'altra opportunità, un'altra amicizia, un altro amore, una nuova forza. Per ogni fine c'è un nuovo inizio.”