“Establishment of in vitro models, assessment of genistein impact, and evaluation of lysosomal involvement in psoriasis”

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Psoriasis (Latin: *Psoriasis; Ps*) is a chronic autoimmune disease characterized by an abnormal process of keratinization, leading to the formation of characteristic skin lesions. The prevalence of psoriasis is estimated to be around 0.5-8.5% of the global population, with an increasing trend. Different clinical types of psoriasis have been described, each with distinct histological features: plaque psoriasis, which represents the most common type (accounting for approximately 90% of cases), guttate psoriasis, inverse psoriasis, pustular psoriasis, and erythrodermic psoriasis. During disease progression, even within the same patient, different clinical phenotypes can be observed. The symptoms depend on the type and severity of psoriasis and may include skin redness, itching, and burning, as well as scales that may crack and bleed, leading to disability due to joint and tendon involvement. The systemic inflammation associated with psoriasis is also linked to the development of comorbidities such as psoriatic arthritis, which occurs in approximately 20-30% of patients, metabolic syndrome, cardiovascular diseases, diabetes, cancer, and depression. Psoriasis affects both men and women equally, with an average age of onset of 33 years. The disease can have a bimodal onset with two genetically distinct subtypes: early onset, occurring before the age of 40 in 75% of cases, and late onset, occurring after the age of 40. The pathogenesis of psoriasis is complex and relies on interactions between genetic, immunological, and environmental factors. Over 60 loci have been discovered by genome-wide association studies (GWAS) to be associated with an increased risk of developing psoriasis. Potential genes correlated with psoriasis pathogenesis include factors involved in antigen presentation (*HLA-C* and *ERAP1*), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (*TNIP1*), type 1 interferon pathway (*RNF113* and *IFIH1*), interleukin (IL)-23/T helper (Th) 17 axis (*IL23R*, *IL12B*, and *TYK2*), and skin barrier function (*LCE3*). This suggests a complex interplay between innate and adaptive immune cells, primarily dendritic cells, T lymphocytes, and keratinocytes, with their leading roles shifting at different stages of the disease. The IL-23/Th17 axis is a key driver of immune activation, leading to chronic inflammation, excessive keratinocyte proliferation, and impairment of the skin barrier. Obesity, stress, physical trauma, infections, and certain medications, including nonsteroidal anti-inflammatory drugs, beta-blockers, and lithium salts, are the most common environmental triggers and/or exacerbating factors of psoriasis.

Over the past two decades, significant progress has been made in understanding the pathogenesis of psoriasis, which has subsequently translated into highly effective therapies for treating this dermatosis. A prime example of this progress is the development of targeted biologic drugs directed against IL-17, IL-23, and tumor necrosis factor α (TNF-α). However, side effects, loss of efficacy, and relapses of skin lesions upon treatment discontinuation have prompted researchers to explore new therapeutic strategies. Recent research highlights the significance of epidermal cells as prospective

therapeutic targets in the management of the illness by suggesting that keratinocytes, in particular, may play a significant etiological role in the development of psoriasis.

Lysosomes are cellular organelles that are essential for maintaining cellular homeostasis by degrading proteins, lipids, and other macromolecules through a process known as autolysis. Recent studies suggest that lysosomes, like many other cellular organelles, undergo global transcriptional regulation and can adapt to signals from the environment. These findings suggest that lysosomes not only serve a degradative function but also act as signaling hubs, regulating cellular homeostasis through communication with other organelles and cellular structures. Dysregulation of lysosomal function in the epidermis may contribute to the pathogenesis of psoriasis by influencing keratinocyte proliferation and differentiation processes and activating inflammatory responses.

The main aim of this study was to offer a thorough characterization of the lysosomal functions in the epidermal cells, as well as in both normal and psoriatic skin. Furthermore, the goal of this research was to find potential uses of the learned knowledge in the creation of novel therapeutic strategies that might serve as a foundation for the advancement of more effective techniques of treating skin illnesses.

Creating a two-dimensional (2D) *in vitro* model of keratinocytes was the first step in the study's effort to faithfully represent the phenotypic of psoriatic cells. Various *in vitro* 2D models were evaluated for this purpose, including monolayer cultures of the HaCaT cell line (Human Adult low Calcium Temperature keratinocytes) and primary keratinocytes (pKC), stimulated with a cytokine mixture (5MIX, consisting of IL-1α, IL-17A, IL-22, Oncostatin M (OSM), and TNF-α) in the presence of low or high calcium ion concentrations (i.e., $Ca^{2+} \leq 0.1$ mM or 2 mM, respectively). Other models included HaCaT or pKC monolayer cultures stimulated with imiquimod (IMQ), HaCaT monolayer cultures stimulated with serum (at concentrations of 1%, 5%, or 10%) obtained from patients with psoriasis or healthy individuals as controls, and co-cultures of HaCaT cells with human acute monocytic leukemia cell line (THP-1) treated with 12-O-tetradecanoylphorbol-13-acetate (PMA), lipopolysaccharide (LPS), and interferon-γ (IFN-γ). The induction of the psoriatic phenotype was assessed by analyzing the expression of selected marker genes, including 10 markers of keratinocyte proliferation and differentiation (i.e., *IVL, FLG, KRT1, KRT5, KRT6, KRT10, KRT14, KRT16, LOR*, and *MKI67*), 4 antimicrobial peptides (i.e., *DEFB4, PI3, S100A7*, and *S100A9*), and 4 chemokines (i.e., *CCL20, CXCL1, CXCL2*, and *CXCL8*), using real-time quantitative polymerase chain reaction (real-time qRT-PCR). To evaluate the suitability of the *in vitro* models for studying the molecular mechanisms of psoriasis, the expression of selected marker genes was compared with datasets from the Gene Expression Omnibus (GEO) database, including 6 profiles of *in vitro*-cultured psoriatic keratinocytes and 7 profiles of skin biopsies obtained from psoriasis patients. Based on the obtained data, it was observed that *in vitro* 2D pKC cultures supplemented with 5MIX (regardless of calcium ion concentration), compared to non-inflammatory activated cells cultured in the presence of 2 mM calcium ions (conditions corresponding to the environment of normal differentiated keratinocytes),

exhibited a synergy in the expression profile of the majority of tested genes with the gene profiles of psoriatic keratinocytes available in the GEO database (on average 84% of the analyzed transcripts, i.e., 78% and 89% for pKC cultured in the presence of 5MIX and low calcium ion level, respectively, and pKC cultured in the presence of 5MIX and high calcium ion level). Furthermore, it was demonstrated that the pattern of expression of selected marker genes in the GEO profiles of patients' data in relation to 7 sets of skin biopsy data (i.e., GDS2518, GDS3539, GDS4600, GDS4602, GDS4606, GDS5392, and GDS5420) was consistent with the literature data and the obtained results of gene expression characteristic of the psoriatic phenotype.

In later stages of the research, the status and function of lysosomes in HaCaT and pKC cells with a psoriatic phenotype were defined using the developed *in vitro* 2D model of keratinocytes. To analyze the quantity and localization of acidic organelles in the cell, dyes such as acridine orange and LysoTracker Red DND-99, as well as the lysosome marker protein LAMP1 (Lysosome-Associated Membrane Glycoprotein 1), were utilized. Immunofluorescence microscopy (IF) demonstrated that cells with a psoriatic phenotype exhibited an increased number of structures positive for LAMP1, accompanied by a decrease in the total pool of acidic organelles, as confirmed by statistical analysis of the mean fluorescence intensity compared to control conditions. EEA1 (Early Endosome Antigen 1), lysosome marker LAMP1, and autophagosome marker LC3B (Microtubule-Associated Proteins 1A/1B Light Chain 3B) colocalization serves as a critical indicator, allowing the analysis of interactions between organelles and the monitoring of intracellular processes such as endocytosis and autophagy. The quantity of the autophagy marker LC3B was lowest in cells with a psoriatic phenotype in the *in vitro* 2D model of both HaCaT and pKC cells. Additionally, cytokine-stimulated cells showed lower levels of LAMP1 and LC3B colocalization than control cells, according to the Pearson correlation coefficient, which may imply that the generation of autophagolysosomes has been blocked. Therefore, the autophagy process may be compromised despite elevated levels of LAMP1 in psoriatic keratinocytes. Next, in the *in vitro* 2D pKC model, nuclear translocation of the Transcription Factor EB (TFEB) was assessed using IF. TFEB stimulates the expression of genes encoding lysosomal proteins, leading to an increase in the number of lysosomes within the cell and enhanced intracellular degradative activity. The obtained results demonstrated that in cytokine-activated cells, regardless of the calcium ion level, a similarly high percentage of nuclear translocation of TFEB was observed compared to non-activated cells, as determined by calculating the ratio of nuclear fluorescence intensity to cytoplasmic fluorescence intensity.

Transcription Factor EB (TFEB) and Mammalian Target Of Rapamycin Kinase (mTOR) play key roles in controlling cellular metabolism, including autophagy, endocytosis, and lysosomal degradation. The mTOR protein is essential for regulating the immunological response as well as keratinocyte proliferation, differentiation, and homeostasis. Furthermore, research points to increased mTOR complex 1 kinase (mTORC1) activity in psoriasis, indicating its potential role in the disease's pathogenesis. Additionally, mTORC1 suppresses TFEB activity, which prevents autophagy and

lysosomal breakdown. Conversely, calcineurin, acting as an antagonistic factor, can counteract these effects by dephosphorylating and dissociating TFEB from the 14-3-3 protein complex, enabling TFEB activation and translocation into the cell nucleus. Calcineurin is a serine-threonine phosphatase whose activity is calcium-dependent. Increased calcium concentration leads to the binding of calmodulin to calcineurin, activating it and regulating various cellular processes. On the other hand, calcineurin is inactive when calcium levels are low. Psoriatic epidermis exhibits a reduced calcium ion concentration gradient, which affects the disruption of keratinization process and proper keratinocyte maturation. Analysis of the 93 lysosome-associated genes expression in the *in vitro* 2D pKC model revealed that pro-inflammatory cytokines had the strongest impact on the number of regulated genes. The study's findings showed that, in comparison to non-activated cells cultured in the presence of 2 mM Ca^{2+}, the addition of 5MIX decreased the expression of *PPP3CA* and *PPP3CB* genes encoding calcineurin subunits in 50 and 100% of cultures of keratinocytes derived from 4 independent donors, respectively, while simultaneously increasing the expression of *MTOR* in 75% of these cultures. To understand the role of lysosome biogenesis-related pathways in the pathogenesis of psoriasis, RNA interference (RNAi) technique was applied to silence the expression of genes encoding *TFEB*, its inhibitor mTORC1 complex (*MTOR*), and its activator calcineurin (*PPP3CA*) in HaCaT and pKCs cultures. Subsequently, the characterization of these cells was performed in terms of psoriatic phenotype by analyzing the expression of 23 selected marker genes. The obtained results indicate that silencing the *MTOR* gene leads to the normalization of selected markers' expression characteristic of the psoriatic phenotype. Conversely, silencing the mTOR antagonist, *PPP3CA*, enhances the psoriatic phenotype in HaCaT and pKC cells, indicating its significant role in potential modulation of signaling pathways occurring in the epidermal cells.

The next stage of the study involved expanding to an *in vitro* 3D model of skin tissue, including a layer of fibroblasts (non-psoriatic normal, NN) or psoriatic plaque (PP), as well as a layer of keratinocytes (non-psoriatic normal, NN). The *LAMP1* and *TFEB* genes were found to be overexpressed in PP tissues, confirming the results obtained from immunofluorescence analysis of *in vitro* 2D keratinocyte cultures. Following the application of specific lysosomal system modulators, such as chloroquine (CQ), rapamycin (RAP), and wortmannin (WORT), in both 3D NN tissues and 3D PP tissues, additional research involved the measurement of gene expression. The use of CQ resulted in an elevation of psoriasis marker genes (*DEFB4*, *KI67*, and *PI3*) in the 3D PP tissue. Additionally, in the case of 3D PP tissue, the expression of LAMP1 was reduced while TFEB was increased, regardless of the type of modulator used, compared to non-modulated PP tissue.

In subsequent steps, the status and role of lysosomes were characterized in skin biopsies obtained from patients with psoriasis (lesional skin: psoriatic plaque, PP, and non-lesional skin: psoriatic normal, PN) as well as healthy individuals as controls (non-psoriatic normal, NN). A positive correlation was found between the results of the psoriasis indices - PASI (Psoriasis Area and Severity

Index), BSA (Body Surface Area) and DLQI (Dermatology Life Quality Index) and the amount of LAMP1 protein.

Transcriptomic analysis was conducted to examine the expression of 93 genes related to lysosomes in PP, PN, and NN tissues. Statistical analysis showed that 23 out of the 93 deregulated genes exhibited significant changes in a continuous pattern of PP > PN > NN. Among them, genes with significantly altered activity encoded: lysosomal enzymes (*ASP5*, *CTSS*, *GBA*, *GLA*, *GLB1*, *GM2A*, *HPSE*, *IFI30*, *NEU1*, and *PPT2*), lysosomal biogenesis factors (*BLOC1S2* and TFEC), acidic organelle markers (*EEA1*, *LAMP3*, *MAP1LC3B*, *RAB5A*, *RAB7A*, and *RAB34*), and cation channels (*MCOLN2* and *MCOLN3*). Additionally, it was found that the pattern of gene regulation was similar in patient tissues from PP and PN compared to NN, indicating that these changes take place before the emergence of the typical illness phenotype.

In this research, tissues taken from PP locations also showed a rise in *PPP3CA* expression and a concurrent decrease in *PPP3CB* expression. This could be explained by the presence of immune cells in the skin biopsies. In immune cells, calcineurin is a key enzyme that activates transcription factors of the NFAT (Nuclear Factor of Activated T Cells) family, regulating the expression of genes involved in the inflammatory response. Due to their capacity to prevent T cell activation by blocking the NFAT pathway, calcineurin inhibitors, such as tacrolimus (FK506) or cyclosporine, are utilized in the treatment of numerous autoimmune illnesses, including psoriasis. Studies have shown that the use of calcineurin inhibitors can reduce the number of T cells in the skin and decrease the activity of pro-inflammatory cytokines such as IL-17 and IFN-γ. It is worth noting that therapy with calcineurin inhibitors in psoriasis treatment is not always effective and may lead to unwanted side effects. This may be because calcineurin is also required for the activation of the transcription factor TFEB in most cell types, which, as was previously indicated, is in charge of the transcription of genes involved in autophagy, a process required for the development of a functional skin barrier. In this regard, it is worth mentioning that in the studies using *in vitro* 2D cell cultures, treatment of psoriasis-like HaCaT and pKC cells with tacrolimus was shown to decrease the expression levels of transcription factors from the MiT family (mainly *TFE3*, *TFEB*, and *TFEC*), suggesting the inhibition of lysosomal biogenesis. Additionally, strong inhibition of genes associated with the autophagy process (*ATG7*, *MAP1LC3A*, and *LAMP1*) was observed in HaCaT cells after the use of tacrolimus.

In psoriatic epidermis, in addition to impaired autophagy, quantitative changes occur in ceramides (CER) and sphingolipids (SL), whose metabolism is partially regulated in lysosomes. Maintenance of the skin barrier relies on SL, which regulates cellular processes such as proliferation, differentiation, and apoptosis of keratinocytes. To characterize the pathways of sphingolipid and ceramide metabolism in keratinocytes, an analysis of lysosomal enzyme levels, such as acid ceramidase (ASAH1), acid sphingomyelinase (ASM/SMPD1), beta-glucocerebrosidase (GBA), and sphingosine 1 kinase (SPHK1), was conducted using the enzyme-linked immunosorbent assay (ELISA) in a previously established 2D model of HaCaT and pKC. The reduction in the level of the

lysosomal enzyme acid sphingomyelinase (ASM) in psoriasis-like keratinocytes, both in HaCaT and pKC cells, suggests that the conversion of sphingomyelin to ceramide may be inefficient under these conditions. Therefore, the deficiency of ceramides in the psoriasis-affected epidermis may result from impaired ceramide synthesis in the lysosomal salvage pathway. A similar tendency was observed for ASAH1 in HaCaT cells, which may disrupt the process of converting ceramide to sphingomyelin. GBA hydrolyzes glucosylceramides into ceramides. In pKC cells, the level of GBA increased after activation with pro-inflammatory cytokines, regardless of the calcium ion concentration in the environment. According to the literature data, changes in transepidermal water loss in psoriatic epidermis may serve as a stimulus for the GBA enzyme, enabling ceramide synthesis. SPHK1 is a key enzyme in the conversion of sphingosine to sphingosine-1-phosphate (S1P), which is a bioactive lipid involved in regulatory functions in various biological processes, including inflammatory reactions. Increased levels of SPHK1 in psoriasis-like HaCaT and pKC cells lead to increased conversion of sphingosine to S1P, ultimately resulting in increased pro-inflammatory processes in the skin. In HaCaT cells, the level of the SPHK1 enzyme was regulated similarly to GBA, although these differences were less statistically significant. On the other hand, the level of SPHK1 in pKC cells treated with 5MIX increased compared to cells not treated with cytokines (regardless of the applied calcium ion concentration).

To provide a comprehensive characterization of molecular pathways in the psoriatic *in vitro* 2D model of HaCaT and pKC keratinocytes, a multiplex analysis was conducted using Luminex xMAP® technology. The study included the assessment of protein levels involved in the Akt/mTOR pathway and the degree of their phosphorylation. The mechanism of Akt/mTOR pathway activation in psoriasis is not fully understood, but multiple studies suggest that various stimuli such as growth factors, cytokines, and oxidative stress may influence its activation in keratinocytes, resulting in accelerated proliferation and resistance to apoptosis. The degree of protein phosphorylation was compared between psoriasis-like keratinocytes (with the addition of 5MIX at low Ca^{2+} concentration) and normal, differentiated keratinocytes (without 5MIX at high Ca^{2+} concentration) in HaCaT and pKC cell lines.

In the case of HaCaT cells, a statistically significant increase in the phosphorylation level was observed for glycogen synthase kinase 3 beta (GSK3β) protein in psoriasis-like cultures. For pKC cells, a statistically significant decrease in the activity of Akt and IGF1R proteins (as indicated by reduced phosphorylation) was observed in psoriasis-like cultures compared to the control, suggesting possible disruptions in signaling pathways associated with cell proliferation. The reduced activity of these proteins may affect processes regulating cell growth, differentiation, and survival. Statistically significant decreases in the phosphorylation levels of GSK3α (increased activity), p70S6K (decreased activity), PTEN (increased activity), and TSC2 (increased activity) were observed only for the psoriasis-like pKC cultures, which may influence cell homeostasis and their interactions with the environment. Increased activity of GSK3α can impact various biological aspects such as metabolic

regulation, cell proliferation, differentiation, and apoptosis, while reduced activity of p70S6K can inhibit growth processes. Increased activity of PTEN can affect cell cycle regulation and apoptosis, and increased activity of TSC2 can influence cell proliferation regulation, growth control, and differentiation.

The next step involved conducting this multiplex analysis on an *in vitro* 3D skin tissue model, where the phosphorylation levels of Akt/mTOR pathway proteins were evaluated in PP tissue compared to the NN control in the first step. A statistically significant increase in the activity (phosphorylation increase) of insulin-like growth factor 1 receptor (IGF1R) protein was observed in PP compared to NN. IGF1, mainly synthesized in fibroblasts, is a key factor stimulating keratinocyte proliferation processes. On the other hand, a decrease in activity (hence phosphorylation) of insulin receptor substrate 1 (IRS1) in PP *vs.* NN was observed. Subsequently, the activity of the Akt/mTOR pathway was examined after the application of autophagy modulators (CQ, RAP, or WORT) in both NN and PP tissues. For NN tissue treated with CQ, RAP, or WORT compared to untreated NN tissue, the activity of the analyzed proteins remained mostly unchanged, except for an increase in phosphorylation levels of IGF1R, IRS1, and PTEN proteins after CQ treatment. Effects of modulator application to PP tissue compared to untreated PP control were observed only for IRS1 protein activity. Its activity (phosphorylation level) was elevated in PP tissue after the addition of CQ, RAP, or WORT compared to the untreated modulator PP.

The performed research found that both *in vitro* 2D keratinocyte models, *in vitro* 3D psoriatic tissue models, and skin biopsies taken from patients *in situ* exhibited an increase in biogenesis and an elevation in the number of lysosomes. It is worth noting that despite the increased lysosome count, their function may shift from autophagy processes to immune-related processes, such as antigen presentation, cytokine production, and the generation of pro-inflammatory molecules like S1P. This shift in lysosomal activity may have a significant impact on the pathogenesis of psoriasis, indicating the crucial role of lysosomes in the development of the disease.

An additional research step involved evaluating the therapeutic potential of genistein in the context of psoriasis as a modulator of TFEB, regulating lysosomal biogenesis and proper metabolism. In order to determine the molecular mechanism of genistein action, *in vitro* HaCaT and pKC keratinocytes stimulated with cytokines IL-17, TNF-α, or their combination IL-17A/TNF-α for 24 hours were subjected to cytometric analysis of the MAPK (*Mitogen-Activated Protein Kinases*) pathway by assessing the degree of (*Extracellular Signal-Regulated Kinases*) phosphorylation. Statistical analysis revealed significant differences in ERK1/2 phosphorylation in IL-17A-stimulated HaCaT cells. Furthermore, a significantly lower level of ERK1/2 phosphorylation was observed in HaCaT cultures preincubated with genistein for one hour and subsequently activated with IL-17A. Subsequently, the activity of PI3K (*Phosphoinositide 3-Kinases*) was analyzed in *in vitro* cultures of HaCaT and pKC keratinocytes preincubated with genistein for one hour and then stimulated with IL-17A, TNF-α, or IL-17A/TNF-α. A significant increase in PI3K activity was demonstrated following

IL-17A and IL-17A/TNF-α activation in pKC, while a decrease in PI3K phosphorylation was noted in cells preincubated with genistein, indicating potential anti-inflammatory properties of the investigated isoflavone. Additionally, after one-hour preincubation with genistein, a decrease in the expression of genes *CAMP, CCL20, DEFB4, S100A7,* and *S100A9*, which are involved in the inflammatory response in psoriasis, was observed in HaCaT and pKC cells regardless of the type of stimulation (IL-17A, TNF-α, or IL-17A/TNF-α), compared to the control group.