Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
SCIENZE BIOMEDICHE E BIOTECNOLOGICHE

CICLO XXXV

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Development of a new depigmenting cosmetic formulation

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Contents

List of abbreviations

ACTH	Adrenocorticotropic hormone
AHA	α-hydroxyacid
BM	Basal membrane
cAMP	Cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
c-kit	Tyrosine kinase receptor
COX	Cyclooxygenase
CPNP	Cosmetic Products Notification Portal
DAMP	Damage-associated molecular pattern
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
ECM	Extracellular matrix
EDN	Endothelin
ER	Oestrogen receptors
ERK	Extracellular regulated kinase

FGF	Fibroblast growth factor
GC	Guanylyl cyclase
GM-CSF	Granulocyte-macrophage colony stimulating factor
HGF	Hepatocyte growth factor
IL	Interleukin
KC	Keratinocyte
KGF	Keratinocyte growth factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MASI	Melasma area severity index
MC1R	Melanocortin type 1 receptor
MITF	Microphtalmia-associated transcription factor
MMP	Metalloproteinase
NF-κB	Nuclear factor κB
NGF	Nerve growth factor
NO	Nitric oxide
O/W	Oil-in-water
PAMP	Pathogen-associated molecular pattern
PG	Prostaglandin

PIF	Product Information File
PIH	Post-inflammatory hyperpigmentation
PKA	Protein kinase A
Plg	Plasminogen
Plm	Plasmin
POMC	Proopiomelanocortin
PRR	Pattern-recognition receptor
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase
SC	Stratum corneum
SCF	Stem cell factor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TXA	Tranexamic acid
TYR	Tyrosinase
TYRP-1	Tyrosinase-associated protein 1
TYRP-2 (DCT)	Tyrosinase-associated protein 2 (dopachrome tautomerase)
UV	Ultraviolet

UVA	Ultraviolet spectrum A
UVB	Ultraviolet spectrum B
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
VL	Visible light
W/O	Water-in-oil
α-Arb	α-arbutin
α-MSH	α-Melanocyte stimulating hormone

1. INTRODUCTION

1.1 Historical Overview of Skin Whitening in health and disease

Since ancient times people have used different methods for whitening the skin. White skin color has always, with major or minor impact, created false prejudices about the prevalence of white race rather than the other skin colors. It is known that in the ancient Mesopotamia, Egypt, Rome and Greece women used lightening cosmetics with the aim of having theatral semblance, they prepared make up formulations based on lead or chalk. Moreover, in China and Japan women used formulations based on chalk and white lead for resembling white jade or fresh lychee. In the colonization period white skin color was associated with beauty, intelligence and authority, while darker tonalities were associated with ugliness and inferiority. In 20s and 30s, whitening cosmetics went out of style because the consciousness of healthy lifestyle by passing free time on the open air was enlarged and studied by scientists. In the past, one of the historic elements used as lightening component was mercury. It has double lightening mechanism of action: inhibition of tyrosinase (TYR) and exfoliation of the superficial layers by the production of hydrochloric acid. However, harmful effect of mercury both on the skin and environment were noted after the massive poisoning in Minamata in Japan. From the 70s, mercury was prohibited in various part of the world, in US, in South Africa, Europe and Nigeria. Hydroquinone substituted mercury in this period, although actually it is prohibited by cosmetic regulation in skin topical products. In the 80s in South Africa a protest movement antiapartheid was born. The activists inspired from the movement Black consciousness were united under slogan “Black is beautiful” against the usage of whitening products. In the 90s this movement convinced the government to prohibit the commercialization and divulgation of lightening cosmetic products, and the industrial sector of lightening cosmetics was closed in that area. One of the alarming elements nowadays in South Africa is return on the market of whitening products based on mercury (Banodkar PD et al., 2022).

Whitening products are used nowadays by European and American countries also for medical purposes, for example for treating of melasma, which may be associated to a chronic inflammation of the skin with incurable or hardly curable consequences. Melasma can compare during the pregnancy period or as a side effect of contraceptive pills, based on oestrogens and progesterone, or administration of diphenylhydantoin, or again after

inflammation skin diseases like acne, psoriasis, lichen planus, atopic dermatitis or contact dermatitis.

The treatment of melasma is particularly difficult since it is a chronic disease and its pathogenesis is poorly known. Further, there are many restrictions on the usage of certain substances for their toxicity profile, thus they can be only used by specialised medical centers and sent at high costs, so these treatments can be only used by a little part of population. Other historic and safe active lightening substances, like azelaic acid, nicotinamide and many others have no shown satisfactory efficient profile. Indeed, there is a lack of studies on the efficacy and safety profile of new active components that can be potentially used by large part of population and treatment of melasma still represents a challenge that concentrates the efforts of the cosmetic industry towards the research and creation of new and more efficient products.

1.2 Skin organization

The skin is the most extended organ of the human body (from 1,6 to 1,9 m^2 and the thickness from 0,05 to 0,3 cm) and it plays such important roles as protection from the external environment, thermoregulation, secretion, adsorption, elimination, regulation of liquid balance, immunologic, sensorial, the function of storage and synthesis.

The skin covers the entire surface of body and it consists of three main layers: epidermis, dermis, subcutaneous layer and skin annexes (hair and nails) (Fig. 1). A specialised connection area between epidermis and dermis is called dermo-epidermic junction.

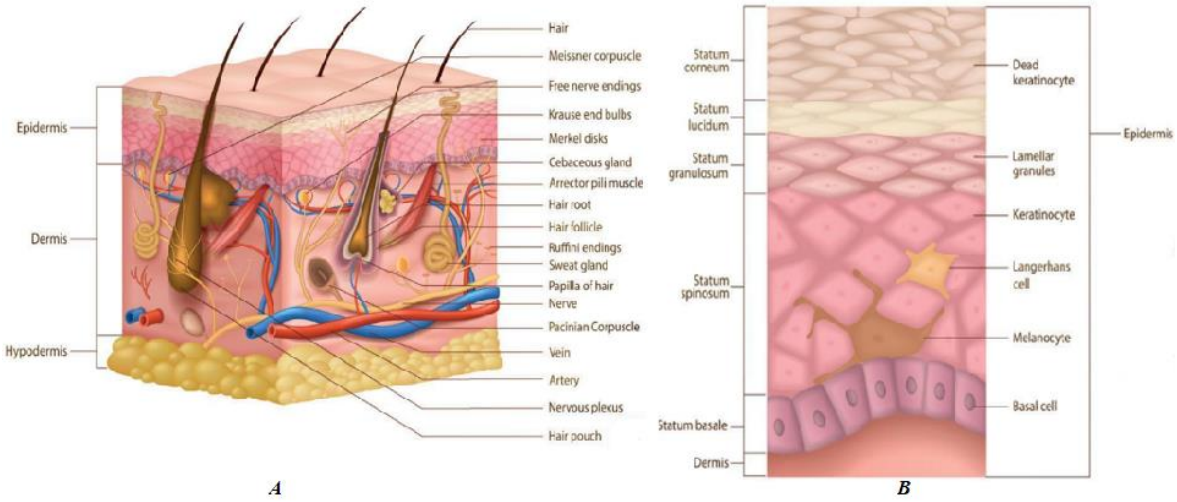

Figure 1. *A: the main three layers of the skin; B: cells of epidermis*

The superficial layer of the skin is epidermis which consists of several layer of specialised cells, the keratinocytes (KCs) which mainly play a protective role and contribute to the regulation of the activities of other skin cells, mainly melanocytes. The stratum corneum (SC), the outermost layer of epidermis, functions as a barrier to protect the skin from external stimuli. The protective role of epidermis is managed by the creation of physical barrier against external infections and is played mainly by lipid layer of epidermis, cornified layer of KCs and tight junctions among cells. In addition, a protective role of epidermis is performed by a complex system of melanin synthesis, which is essential to defend all the organism from UV damage.

The dermis is the vascularized connective tissue situated between hypodermis and epidermis and it consists mainly of collagen and elastin fibers, which represent the main extracellular matrix (ECM) components, synthesised by fibroblasts. Main functions of dermis are providing of resistance, strength, tone, sensitivity and nutrients to the epidermis. In addition, dermis plays an important role providing immune protection since it also includes immunoregulatory cells like dendritic cells, macrophages, mast cells, T-cells and innate lymphoid cells. Globally, skin provides epidermal barrier, immune response, resident memory and skin pigmentation. In addition, epidermis provides also the skin microbiota which plays an important role in modulation of immune response and generation of anti-inflammatory effect (Gallegos-Alcalá P et al., 2021).

The hypodermis is the deepest layer of the skin and it covers the deep fascia that lays on the skeletal muscle. It consists of connective tissue, fat and cells such as fibroblasts,

macrophages and adipocytes. Main functions of hypodermis are thermoregulation and energy reserve.

The skin plays an important role in protecting our bodies from the environment and is continuously exposed to external stresses such as ultraviolet (UV) radiation, infectious pathogens, and hazardous chemicals. These various external stimuli induce changes in the skin behaviour leading to biological reactions such as oxidative stress, inflammation, photoaging, alterations of melanogenesis and in some cases to skin cancer. Melanogenesis is the complex process leading to the production of melanin, in specialized cells called melanocytes. Melanin represent a group of natural pigments with essential defensive role as it protects skin from the adverse effects of ultraviolet radiation (UVR) and oxidative stress of environmental pollutants. Further melanin determines the color of the skin, hair and eyes, one of the topmost concerns of the human individuality, identity and represents the way of adaptation for the external environment (Hossain MR et al., 2021).

1.3 Melanocyte and melanin biosynthesis

Melanocytes play a central role in melanogenesis as producers of melanin and are situated in the basal layer of epidermis. They are specialised cells derived from unpigmented precursor cells called melanoblasts, originating from embryonic neural crest cells which migrate towards the skin and other tissues during embryogenesis. Epidermal melanocytes play a major role in protecting the skin from the drastic effects of solar UV, mainly photocarcinogenesis and photoaging. In mature melanocytes synthesis of melanin starts with formation of melanosomes, melanin-containing organelles, although skin pigmentation is a more complex mechanism that also involves transfer and accumulation of melanosomes in neighbouring KCs. Melanin pigment can be divided into two types—pheomelanin and eumelanin. Pheomelanin has a reddish to brownish color, and can cause reactive oxygen species (ROS) production under UVR stimulation (Koike S et al., 2020). Eumelanin has black color, which works as a physical barrier that reduces the penetration of solar UV rays. Additionally, eumelanin is a scavenger of ROS, highly involved in oxidative damage to cellular DNA, proteins, and lipids (Upadhyay PR et al., 2021).

Melanin is able to protect skin in dose-dependent manner. It is known that darker phototypes are less susceptible to carcinogenic processes than lighter types of skin. Darker phototypes have elliptical melanosomes and they are evenly distributed, that permits a

more efficient protection. In addition, in darker phototypes melanin is tightly stacked from KC nucleus, so it further contributes to create a strong sunscreen protection.

The variety of human skin color is determined by the amount and individual composition of pheo- and eumelanin mix, as well as size, number, mode of transfer, distribution and degradation of melanosomes inside KCs. On the other hand, the number of melanocytes generally remains relatively constant. Other functions of melanocytes include physical defence, metal adsorption, drug uptake, thermoregulation and adaptation to the environment.

A schematic representation of the synthesis of melanin and the main enzymes involved is shown in Fig. 2.

Synthesis of melanin occurs by means of sequential reactions (Fig. 2, Hushcha et al., 2021). It depends on three essential enzymes: TYR, tyrosinase-associated protein 1 (TYRP-1) and tyrosinase-associated protein 2 (TYRP-2) also called dopachrome tautomerase (DCT), which is involved in melanogenesis process in the later phase. The first enzyme, TYR, is a membrane-bound glycoprotein which determines a rate-limiting role in the melanogenesis. The reaction of oxidation, catalysed by TYR, transforms L-tyrosine into dopaquinone. Dopaquinone is a highly reactive molecule, and it can generate two distinguished downstreams of reactions leading to the synthesis of eumelanin (black-brownish) and pheomelanin (red-yellow).

1) In the chain of eumelanin synthesis, dopaquinone is transformed into leukodopachrome (cyclodopa) by intramolecular cyclization. Next, cyclodopa reacts with another molecule of dopaquinone by redox mechanism and forms dopachrome and DOPA. Further, dopachrome is transformed into eumelanin by two distinguished reactions. The first one involves TYRP-2 and leads to the formation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and therefore it is converted into eumelanin by TYRP-1 intervention. The second way consists in the conversion of dopachrome into 5,6-dihydroxyindole (DHI) and therefore TYR transforms it into eumelanin.

2) In pheomelanin downstream reactions dopaquinone is converted into 5-S-cysteinyldopa, or glutathionyldopa in the presence of cystein or glutathione. Next, 5-S-cysteinyldopa is transformed into quinoline and finally it is polymerized into pheomelanin.

The synthesized melanin is collected into melanosomes, followed by a complex maturation process and then it is ready to be transported into KCs along melanocyte's dendrites, driven by several stimuli.

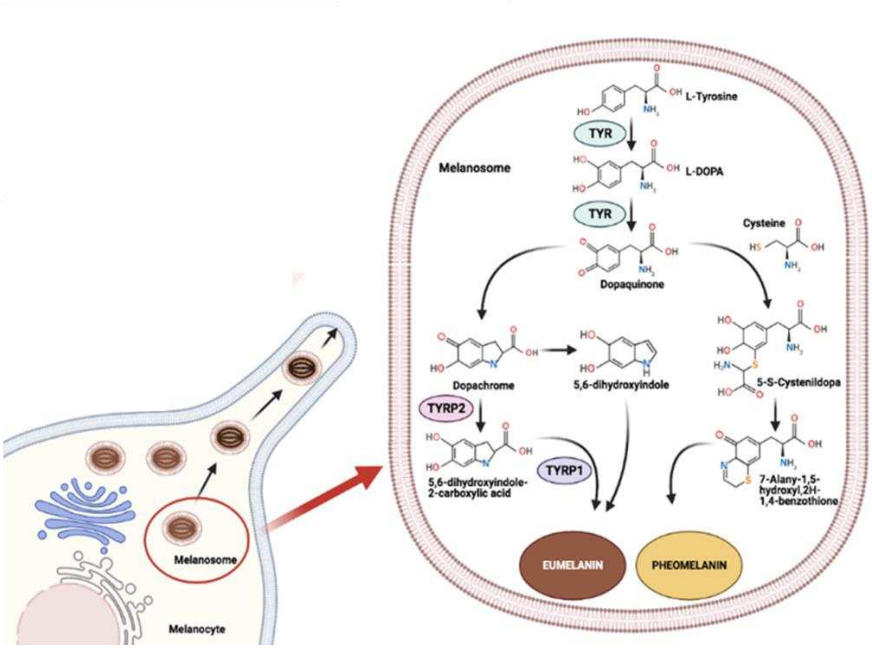

Figure 2. *Schematic representation of melanin biosynthesis (Hushcha Y et al., 2021)*

1.4 Signalling pathways regulating melanin biosynthesis

Control of melanogenesis is a very complex process occurring at different levels including regulation of melanocyte survival and differentiation, synthesis and transfer of melanin to the neighbouring KCs. Different signalling pathways and multiple molecules target melanocytes regulating melanin biosynthesis.

The critical transcription factor modulating melanogenesis, melanocyte survival, and proliferation is the melanocyte-specific microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix leucine zipper transcription factor. Activation of MITF results in the upregulation of the expression of the key genes TYR, TYRP-1 and TYRP-2 promoting melanin synthesis. In addition, MITF regulates several other genes involved in melanogenesis, including those required to control melanosome maturation, traffic, and

distribution to KCs. MITF expression is regulated by different transcriptional factors binding MITF promoter, such as cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB), paired box family of transcription factor 3 (PAX3), sex determining region Y-box 9 and 10 (Sox9, Sox10) and Wnt/β- catenin pathway effector lymphoid enhancer-binding factor 1 (LEF-1). The main signalling molecular pathways involved in the regulation of MITF expression and activity are shown in Fig. 3 (Hushcha Y et al., 2021).

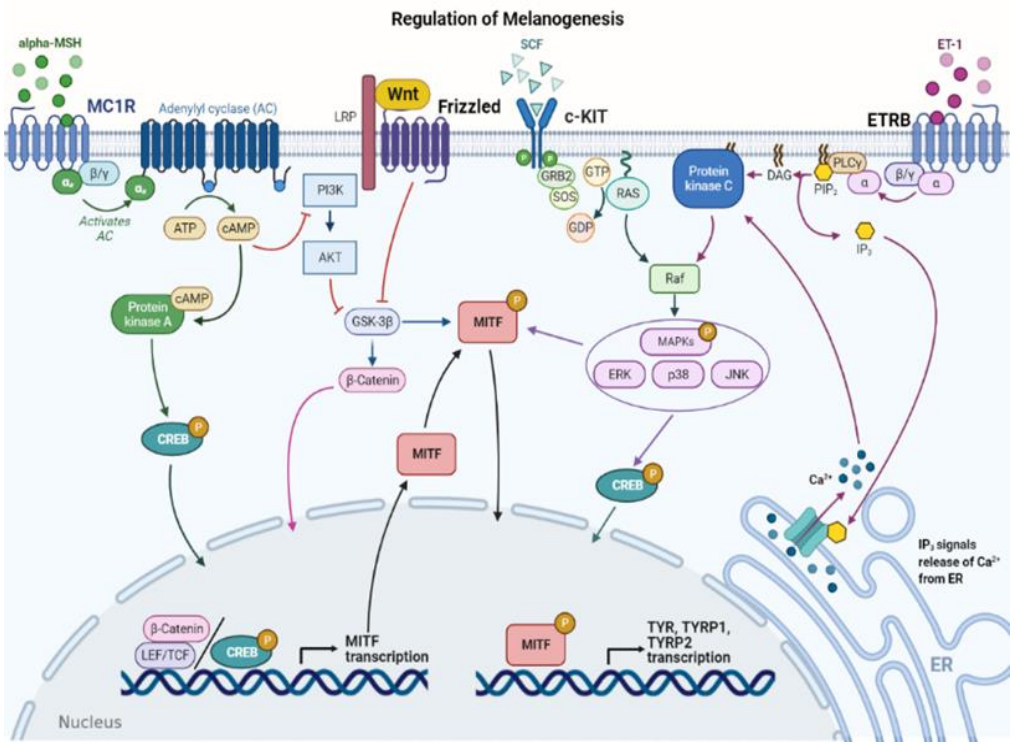

Figure 3. *Regulation of melanogenesis*

The most known and relevant pathway leading to MITF activation is the cAMP/ Protein kinase A (PKA)/CREB/MITF signalling pathway. The pathway is activated by the binding of α-Melanocyte stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH) which derive from pro-opiomelanocortin (POMC) and agonist stimulating protein (ASP) to the melanocortin 1 receptor (MC1R). MC1R activation, stimulates adenylyl cyclase and increases cAMP concentration. cAMP activates PKA, that phosphorylates cAMP response element (CREB) protein, leading to increased MITF transcription. Also, endothelin (EDN) signalling pathway plays a significant role especially in the paracrine interactions between KCs and melanocytes. The peptide EDN1, interacting with its G protein-coupled receptor (EDNRB), activates the inositol phosphate pathway, finally leading to the activation of mitogen-activated protein kinase (MAPK) cascade which increases CREB activity. In the

WNT pathway, activation of the Wnt receptor by the binding of a Wnt ligand, results in increased level of β-catenin which translocates to the nucleus where it binds to the lymphoid enhancing factor-1/T-cell factor (LEF-TCF) transcription factor, triggering MITF expression. MITF is also regulated post-transcriptionally, mainly by phosphorylation. MAPK, p38, ribosomal S6 kinase (RSK) and GSK3β are the main phosphorylation kinases involved. This phosphorylation process stimulates the recruitment of the transcriptional coactivator of CREB, triggering the transcription of TYR, TYRP-1 and TYRP-2 (Hushcha Y et al., 2021; Pillayar T et al., 2017; Lu Y et al., 2021).

A relevant factor leading to MITF phosphorylation is the stem cell factor (SCF), produced by KCs and fibroblasts which binds the KIT receptor, a tyrosine kinase receptor (c-kit) and activates MAPK signalling pathway (Niwano T et al., 2018). In addition, the MAPK signalling pathway can be activated by other growth factors including hepatocyte growth factor (HGF) and fibroblast growth factor type 2 (FGF-2), mainly produced by KCs which also operate through other c-kit. Also PKA signalling may be modulated by growth factors. In particular, transforming growth factor (TGF-β), produced by KCs and fibroblasts, inhibits PKA leading to the reduction of MITF transcription by CREB-dependent pathway thus playing an inhibiting role on melanogenesis (Wang Y et al., 2017).

Finally, several inflammatory factors may regulate melanin synthesis by means of both direct or indirect effects on melanocytes. The role of inflammatory molecules is described below.

1.5 The melanogenic unit and role of KCs in melanogenesis

In the skin, melanocytes are strictly associated to several KCs thus originating the so-called melanogenic unit in which each melanocyte is surrounded by 30 to 40 KCs through dendrites (N. T. Nguyen and D. E. Fisher, 2019). Melanocytes produce melanin pigment in melanosomes, which are transferred into the cytoplasm of the surrounding KCs, where they form supranuclear caps that protect genomic DNA from damage caused by impinging solar UV rays. The production of melanin in melanocytes and accumulation of melanin-containing melanosomes in KCs leads to skin pigmentation.

Therefore, skin pigmentation is very complex process involving several activities of both melanocytes and KC and many molecules, required for melanosome structure, melanin

synthesis, and melanosome maturation transport and transfer to KCs. Several transfer mechanisms have been suggested including exocytosis, cytophagocytosis, fusion of plasma membrane and membrane vesicles, although the exact events are still unclear. Melanosomes are transferred to the KCs in accordance with a coordinated transport activity. The most known mechanisms involved include the movement in opposite directions on microtubules (long-range anterograde and retrograde transport), regulated by a small protein melanoregulin, and on actin filaments (short-range anterograde transport), controlled by the protein complex Rab27A-Slac2-myosin Va. Several factors regulate this process. Among them, it was recently demonstrated that PAR2 is involved in the stimulation of phagocytic activity and melanocyte dendricity by releasing prostaglandin (PG), whereas MITF regulates the expression of melanosome transport protein (Rab27A). Since the number of melanocytes in human skin is the same, the final color is determined largely by the grade of melanin transport. Details of these processes have been recently reviewed. (Ohbayashi N et al., 2020; Firdaus A et al., 2019).

KCs play an essential role in the control of melanocyte functions also regulating melanocyte adhesion, proliferation, survival and morphology, as well as modulating their stress response (Hossain MR et al., 2021; Upadhyay PR et al., 2021). Further, KCs maintain skin homeostasis because of their capability of differentiation, regeneration and interaction with various environmental components, as well as with immunological skin cells, so that they are involved in regulating a state of balance within healthy skin and constitute the first line of defence against microbial pathogens, mechanical damage, as well as UV irradiation (Upadhyay PR et al., 2021; Lee JH et al., 2017). KCs regulatory activities are mainly mediated by several paracrine factors. Further, interactions among KCs, fibroblasts, and melanocytes play essential roles in stimulating pigmentation in hyperpigmentary disorders (Upadhyay PR, et al., 2021; Yang CY et al., 2022).

Many of the paracrine factors secreted by KCs can act on melanocytes to regulate melanogenesis. KCs produce and release SCF, EDN1, Nerve growth factor (NGF), HGF, granulocyte-macrophage colony stimulating factor (GM-CSF), α-MSH, ACTH, TGF-β (Hossain MR et al., 2021). As described above (in paragraph 1.4), many of these paracrine factors bind melanocyte surface receptors and may activate and modulate intracellular signalling pathways which can promote melanin synthesis. Also KCs can produce several proinflammatory molecules including NO, PGE2 and PGF2 and inflammatory cytokines such as interleukin (IL)-1α, IL-1β, tumor necrosis factor α (TNF-α) which directly or indirectly regulates melanocytes by stimulating the synthesis of melanogenic factors,

including SCF, keratinocyte growth factor (KGF), HGF in both KCs and fibroblasts. Production and release of these factors is regulated by several exogenous stimuli, including especially UV rays (Kang-Rotondo CH et al., 1993; Rome´ro-Graillet C et al., 1997; Chakraborty AK et al., 1996; Rousseau K et al., 2007; Wakamatsu Y et al., 1997) as well as by inflammatory molecules (Kondo S et al., 1995).

1.6 Regulation of skin pigmentation

In addition to the genetic influence, several nonspecific intrinsic factors (hormonal environment, inflammation) and extrinsic factors (UVR, environmental pollution, drugs) participate in the regulation of melanin levels and melanogenesis. As skin is the first line of defence in the human body to external stressors, most of these skin damage factors can induce either short-term or long-term skin inflammation, release of several molecular mediators with potential changes in skin pigmentation.

1.6.1 Inflammation

Recent findings indicate that skin inflammation involves not only typical inflammatory cells but a complex coordination among different cell types including epidermal KCs, melanocytes and dendritic Langerhans cells, as well as the cellular components of the dermis such as mast cells, macrophages, fibroblasts. During inflammation also increased blood supply and changes in the microvascular structure help plasma proteins enter from the blood stream and increase immunoregulatory cell migration. Inflammation can be triggered by pathogen-associated molecular patterns (PAMPs). Lipopolysaccharide (LPS) is the most common PAMP found in Gram-negative bacteria. Further, it is induced by damage-associated molecular patterns (DAMPs) derived from host cells exposed to environmental stimuli, including UVR, or from damaged tissue due to other events like ischemia, hypoxia. DAMPs and PAMPs bind to pattern-recognition receptors (PRRs) which consist of several families of receptors, including the most known Toll-like receptors (TLRs) and activate several signalling pathways, first of all nuclear factor κB (NF-κB) signalling (Hossain MR et al., 2021; Koike S, Yamasaki K., 2020). Several inflammatory molecules have been related to changes in melanocyte activities and skin pigmentation. They participate in the control of melanogenesis regulating proliferation and differentiation of human epidermal melanocytes, promoting or inhibiting melanogenesis-

related gene expression directly in melanocytes or by modulating the activities of other skin cells mainly KCs (Fu C et al., 2020, Hossain MR et al., 2021). The most known factors involved in melanogenesis regulation include PGE2, NO, as well as several cytokines. The roles of PGE2 and NO have been more clearly defined as they participate in maintaining inflammatory status but also may activate signalling pathway by the direct interaction with melanocytes leading to increased melanin biosynthesis.

In particular, PGE2, lipid signalling factors derived from the metabolism of arachidonic acid, bind PGE2 receptors EP1, EP3, and EP4, expressed in melanocytes, which couple to different G proteins, and increase cAMP levels thus contributing to CREB activation (Scott et al., 2004; Starner, 2010; Fu C et al., 2020).

Moreover, several studies show that nitric oxide (NO) is involved in the induction of melanogenesis by means of activation of the NO/cyclic guanosine monophosphate (cGMP)/PKG pathway. NO interacts directly with and activates heme-containing proteins, like guanylyl cyclase (GC), which induces the production of cGMP, which finally leads to MITF expression and melanogenesis (Pillayar T et al., 2017, Xue L et al., 2021).

The relationship among pigmentation and inflammatory cytokines is less defined and more complex as they may impact melanogenesis process in multiple ways which require to be clarified. In fact, although the levels of many cytokines are increased under UVR which induce skin pigmentation, however many inflammatory cytokines have been also associated to vitiligo which in contrast is characterized by skin depigmentation (Hossain MR et al., 2021). To date it is known that some cytokines, IL-18, IL-33, GM-CSF appear to act as promelanogenic factors, activating MAPK and CREB with consequent increase in MITF and the melanogenic enzymes TYR, TYRP1 and TYRP2 expression, whilst others like IFN-γ inhibits melanogenesis by regulating pigmentation genes, blocking melanosome maturation and inhibiting IL-18-induced melanogenesis. IL-1, TNF-α, IL-6, released by inflammatory cells as well as KC, are largely known for their contribute to maintain inflammation. These cytokines may induce releasing of pro-melanogenic factors especially by KCs but also regulate directly melanocyte functions. It has been reported that in vivo in mouse skin TNF-α increased expression of END1 (Ahn GY et al., 1998). On the other hand, TNF-α in combination with IL-17, increase melanocyte number but inhibit melanogenesis reducing c-kit receptor, MC1R, MITF, and TYRP2 through PKA and MAPK signalling pathways (Hossain MR et al., 2021). IL-1 exists in two forms, IL-1α and IL-1β). Also IL-1α has been reported to inhibit melanin biosynthesis in melanocytes by suppressing TYR activity, and in addition it stimulates the generation of KGF by

fibroblasts, which induce TYR expression and favours melanogenesis in primary melanocytes. Combined IL-1α and KGF activity has been observed in the first phase of the development of solar lentigines. In addition, IL-1α can regulate components of the plasminogen/plasmin (Plg/Plm) synthesis increasing the expression of both uPA and tPA in KCs (Lian X et al., 2008). More debated is the role of IL-1β on melanocytes, as both inhibition and stimulation of melanin synthesis have been described. Further it has been observed that IL-1β can promote UV-induced pigmentation by increasing α-MSH and EDN1 secretion (Hossain MR et al., 2021; FU C et al., 2020; Yang CY et al., 2022).

1.6.2 UV radiation

Indeed, UV is the main environmental factor involved in regulation of skin colour and the induction of hyperpigmentation disorders such as freckles, melasma and solar lentigines. Among three UV lights, UVC (wavelength 180–280 nm) is completely absorbed by the ozone layer, while both UVA (wavelength 320–400 nm) and UVB (wavelength 280–320 nm) can reach and potentially induce damage in the skin. However, UVB radiation penetrates only into epidermis, whilst UVA radiation into the dermis (Yardman-Frank JM et al., 2021). Especially UVB originates various biological effects inducing erythema and vasodilation, oedema, sunburn, hyperplasia, inflammation, regulation of melanogenesis and hyperpigmentation. The UVB irradiation induced skin immune response leads to neutrophil accumulation in the skin, mast cells activation and the release of several inflammatory mediators. Early and late events are induced by UVR. The early inflammatory events include erythema and redness due to vasodilation of cutaneous blood vessels, followed by oedema and apoptosis of KCs (also called sunburn KCs). Chronic UV exposure induces profound skin changes which also involves dermis, called as “photoaging”, characterised by skin dryness, wrinkling, elastosis, altered pigmentation and the activation of fibroblasts which cooperate to the production of inflammatory molecules. In the acute response, UVR can induce direct DNA damage, the activation of several receptor-mediated signalling pathways, and the increased production of reactive oxygen species (ROS; $O2^-$, OH, and $H2O2$) also involved in DNA mutations. In KCs, DNA damage, triggered by UVB, increases p53 overexpression, that directly leads to the transcription of pro-opiomelanocortin (POMC) gene, increasing α-MSH level and MITF transcription. This p53 driven α-MSH/MC1R/m-MITF is the most known UVB induced pathway regulating skin pigmentation. Further, p53 activation results in the downstream release of other promelanogenic factors like SCF and ET-1 (Yardman-Frank JM et al.,

2021). The high levels of ROS may provoke KC death, elicit the activation of many signalling pathways mainly in KCs and fibroblasts leading to the release of inflammatory mediators (Ansary TM et al., 2021). Signalling pathways involved include nuclear factor-κB (NF-κB), the signal transducer and activator of transcription 3 (STAT3), p38/MAPK, or c-Jun N-terminal kinase (JNK) signalling, which affect apoptosis, cytotoxicity, or inflammation (Bito T. Nishigori C et al., 2012). Therefore, in response to UV, KCs produce various growth factors, cytokines, and other inflammatory molecules including NO (Romero-Graillet C et al., 1997) and PGE2, as result of increased cyclooxygenase-2 (COX-2) expression (Scott G et al., 2004, 2005) which may impact melanocyte activity and therefore, melanin production. Several inflammatory cytokines including IL-1, IL-6, IL-8, IL-10, and TNF-α, colony-stimulating factors, high-mobility group box 1 (HMGB1) have been reported to increase in KCs cultured in vitro exposed to UV radiation (Ansary TM et al., 2021; Yang CY et al., 2022). Further, in co-culture experiments it has been proven that paracrine cytokine interactions between KCs and melanocytes, is able to modulate melanocytes activities inducing the increased expression and activity of the key melanin synthetic enzymes finally resulting in increased melanin amount (Niwano T et al., 2015; Yang CY et al., 2022). As a consequence of the release of inflammatory and promelanogenic factors, MITF expression, melanin production and melanosome maturation increase in melanocytes. In addition, the induced increase in α-MSH is associated with the increased sensitivity of melanocytes to the hormone, as UV radiation leads also to an upregulation of MC1-R which amplifies the MSH response (Upadhyay PR et al., 2021).

Finally, it has been reported that also melanocytes can produce inflammatory mediators like PGE2 in response to UV (Maeda K et al., 1998). Prolonged UV radiation causes also dermal inflammation and activates fibroblasts, which participate to the release of SCF, PGE2 as well as matrix degrading enzymes, mainly metalloproteinases (MMP) 2 and 9 and hyaluronidase which degrade ECM components, such as type IV and VI collagen and hyaluronic acid (HA) inducing damage in the basal membrane (BM) (Esposito et al., 2022) and in the dermis (Amano S et al., 2016). Further, it has been observed that UV radiation induces activation of the system Plg/Plm which, by means of Plm, a wide-range serin protease, provides MMP activation from proenzymes (Ogura Y et al., 2008). The Plg/Plm system is also involved in other events which may impact on melanogenesis and is described in detail below. However, the impact of this activation on melanogenesis is poorly known.

1.7 Plg/Plm system

1.7.1 Molecular Components and general activities

Plg is an essential plasma protein, mainly synthesized in the liver, existing at a concentration of approximately 2 μM in the plasma as a proenzyme which is proteolytically activated to form Plm, a broad-spectrum serine protease, mainly known for its essential role in fibrinolysis. Plg is a multidomain protein, comprised of an amino (N)-terminal peptide domain, five kringle domains rich in Lysine binding sites (LBSs), and a serine protease domain (Bharadwaj AG et al., 2021). The Plg/Plm system consists of several molecular components involved in the enzymatic cascade which converts Plg in Plm, which is considered the key player of the system. This system has been largely investigated for its key role in the removal of fibrin deposits and blood clots by Plm. Now, it is known that apart from fibrinolysis, this system is involved in the control of several other processes, including matrix turnover, phagocytosis, inflammation, cell migration, wound healing, and consequently in several pathological conditions, such as cardiovascular diseases, angiogenesis and more recently also in cancer invasion (Keragala CB et al., 2021; Bharadwaj A G et al., 2021). Fig. 4 shows the main components of the system and the multiple activities of Plm.

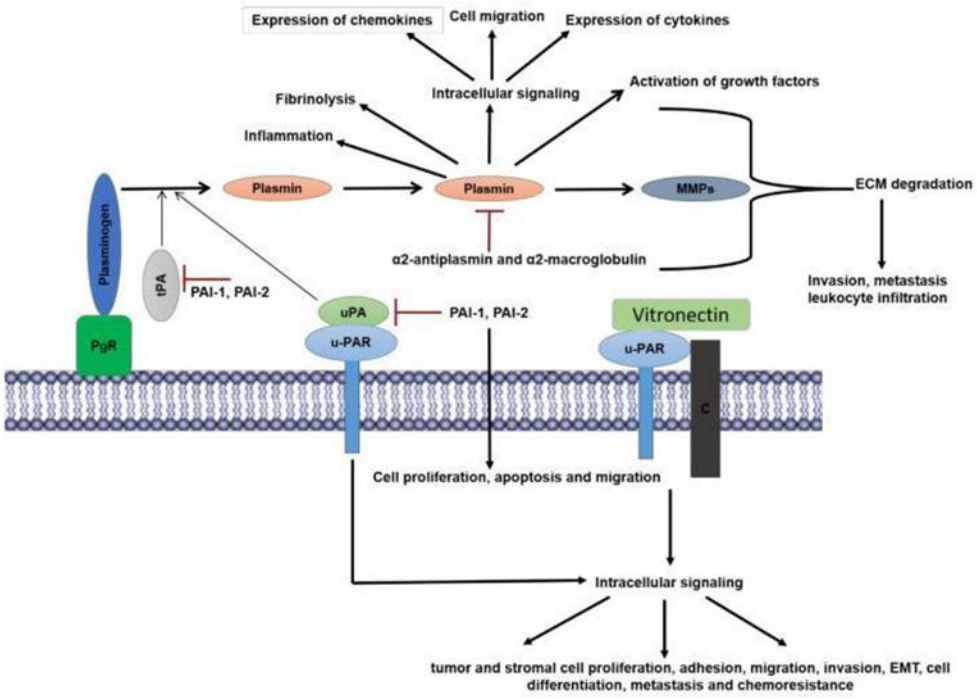

Figure 4. *Schematic representation of the Plg/Plm system (Bharadwaj AG et al., 2021)*

The main components of the Plg activation system, include two physiological Plg activators (PAs), the tissue Plg (tPA) and the urokinase activator (uPA), which facilitate Plg–Plm conversion, and the Plg activator inhibitors PAI-1 and PAI-2, which regulate PA activity tightly controlling Plg conversion into Plm. Plm is also directly inhibited by α2-antiplasmin and α2-macroglobulin. tPA is mainly found in the ECM of most tissues and is the primary activator in the circulation. uPA is the predominant form of Plg activator on cells surface. uPA-induced Plg activation is associated to the binding of the single-chain uPA to the glycosylphosphatidylinositol (GPI)- anchored uPA receptor (uPAR), where the proenzyme is activated by Plm, as well as by other proteases including cathepsin B and L, trypsin, kallikrein, thermolysin, and mast cell tryptase. Notably, both uPA and tPA are expressed by activated or even normal cells and may interact with other cell surface proteins with consequent relevant effects on cell behaviour. uPAR may also interact with vitronectin, integrins, EGFRs, TLRs and activate signalling pathways like FAK, Src, Ras, Rac, MAPK, PI3K, JAK1, etc., thus participating in the regulation of many events such as cell proliferation, migration, adhesion, inflammation, angiogenesis and the epithelial–mesenchymal transition (EMT), especially in tumor cells (Zhai BT et al., 2022; Kiyan Y et al., 2020; Alfano D et al., 2022). tPA interacts with a distinct receptor complex that includes the N-methyl-D-aspartate (NMDA) receptor and Low-Density Lipoprotein Receptor-related Protein-1 (LRP1) to trigger cell-signalling (Gonias SL et al., 2021). The complex coordination of the multiple components of the Plg system is involved in the events related to activation of Plg. Plm results from the conversion of the inactive proenzyme Plg into the active enzyme, by means of the cleavage of the Arg561–Val562 peptide bond by tPA and uPA, (Bharadwaj, AG et al., 2021). In fibrinolysis lysine residues on fibrin surface provide docking sites for the critical LBSs located within kringle domains of the Plg molecule. Binding to fibrin induces unfolding of the Plg from its inactive conformation, exposing the sites to tPA and uPA cleavage which generates Plm. Similarly, Plg can be activated to Plm on cell surface where it binds Plg receptors by means of the LBSs. At present, over 12 distinct cell Plg receptors have been identified, including α-enolase; actin; annexin A2 in cooperation with S100A10, cytokeratin 8, histone H2B, integrins, and the most recent identified Plg-RKT (Madureira PA et al., 2012; Bharadwaj AG et al., 2021). These receptors are broadly distributed in several cell types including macrophages, endothelial cells, fibroblasts, platelets, adrenal medullary cells and carcinoma cells. Although to date the exact role of Plg receptors in different cell types

remain to be clarified, some events related to Plg receptor–dependent cell modulation begin to be elucidated (Keragala CB et al., 2021). As expected, Plg-/- mice animal show defects in fibrinolysis, but they also display growth retardation, delayed wound healing, and reduced overall survival showing the Plg involvement in several other processes beyond fibrinolysis (Keragala CB et al., 2021). The Plg system linked events have been mainly investigated in relation to the generation of Plm which displays several activities. Plm can act by means of its proteolytic activities and/or activating signalling at the cell surface. As a protease, Plm directly degrades proteins in BM and ECM, such as laminin and collagen type IV, but also activates MMPs further favouring disruption of ECM and cell–cell and cell–ECM adhesions (Bharadwaj AG et al., 2021; Szabo I et al., 2004). Consequently, this may result in the increase of cell mobility or spreading thus playing a role in wound healing, tumour cell invasion or embryonic development. Plm may also favour release of growth factors such as FGF-2, HGF, and vascular endothelial growth factor (VEGF) from ECM or induce their activation like TGF-β (Bharadwaj AG et al., 2021; Khalil N et al., 1996). In addition, Plm may activate several signalling pathways, such as Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), MAPK in several cell types including macrophages, fibroblasts, endothelial cells, epithelial cells, and platelets, thus linking the Plg system to the release of cytokines, chemokines and inflammation (Madureira PA et al., 2012; Keragala CB et al., 2021; Burysek L et al., 2002). The link of the Plg system with inflammation, is very complex. Indeed, it is related to the effects on ECM but also to the regulation of immune cell behaviour (Baker SK et al., 2020). It has been mainly investigated in monocytes and macrophages, which express several surface Plg receptors including Plg-RKT, only recently identified, which accounts for the majority of the Plg binding capacity of macrophages (Vago JP et al., 2020). It is clear that both *in vitro* and *in vivo*, Plg system is associated to macrophage migration and recruitment during the inflammatory response and that cell surface binding is crucial for many of Plg's functions (Baker SK et al., 2020; Silva LM et al., 2019; Vago JP et al., 2019). In addition, first in vitro studies showed that stimulation of monocytes by Plm could enhance the expression of several inflammatory cytokines like TNFα, IL-6, IL-1α/β, CD40, chemokines as well as and lipid mediators and these effects have been associated to the Plm induced activation of several signalling pathways including AP-1 and NF-κB, MAPKs (Extracellular regulated kinase (ERK) 1/2; p38) and janus kinase (JAK)/STAT following binding of Plg and its receptors (Syrovets T et al., 2012; Carmo AA et al., 2014). These events have been related to binding of Plg to Plg receptors, mainly Plg-RKT, as well as to the activation Protease-activated Receptor (PAR family) such as PAR-1 and PAR-2

induced by Plm (Carmo AA et al., 2014; Zalfa C et al., 2019). However, exact molecular mechanisms of these activations still remain poorly clarified.

1.7.2 The Plg/Plm system in the skin

In agreement with the role of Plm on cell migration, several findings emphasize the role of Plg in the skin, especially in favouring re-epithelialization and wound healing by increasing KC proliferation and migration. In 1996, the observation that Plg deficient mice exhibited significant delays in wound healing (Romer J et al., 1996) has led to further studies showing that Plg participates in the different phases of wound healing process including inflammation in several models of skin wound healing (Keragala CB et al., 2021; Ny L et al., 2020). Because of the essential role of KCs in wound healing, studies on Plg/Plm in the skin have been mainly focused on these cells. Plg has been detected in the basal cell layer of the epidermis and Plg binding sites have been identified in normal epidermis (Burge SM et al., 1992). Further, KCs express several components of the Plg system like Plg activators uPA and tPA, the inhibitors PAI-1 and PAI-2 and show uPA receptors which efficiently activates generate Plm (McNeill H et al., 1990). However, whereas in normal human and murine epidermis uPA, uPAR, and tPA are only detectable at low basal levels, they are upregulated in KCs of the epithelial outgrowth skin wounds (Bechtel MJ et al., 1996.; Szabo I et al., 2004). Moreover, it has been shown that Plm directly promotes KCs migration in vitro (Reinartz J et al., 1996), and recently the Plg-RKT has been identified as the Plg receptor involved. Indeed, the role of the Plg Plm system in wound healing is more complex, as Plg-RKT also regulates myeloid cell activities, and in vivo participate in different phases of the wound healing including the inflammatory, proliferative, and remodelling phases (Ny L et al., 2020). In addition, uPAR overexpression has been identified during the morphogenesis of hair follicles suggests also its involvement in KC migration into the dermis (Gao Q et al., 2010). Interestingly, some findings show that the Plg system may play other roles in the regulation of skin homeostasis and functions. In fact, it has been recently shown that KCs activated by Plg increase the synthesis and production of VEGF, suggesting a role for Plg/Plm in the modulation of the KC paracrine activities and angiogenesis (Cheng TL et al., 2018). Several factors are able to modulate components of the Plg system and generate Plm in KCs, including retinoic acid that increase tPA secretion in HaCaT (Braungart E et al., 2001), the cytokines IL-1 and TNF-alpha which induce uPA and tPA in HaCaT and murine

KCs (Rox JM et al., 1996; Reinartz J et al., 1996, Lian X et al., 2008), growth factors like TGF-β (Wilkins-Port CE et al., 2009), as well as UV rays (Marschall C et al., 1999). For what concerns UV effects, increased Plg activator activity and uPAR cell surface expression have been identified in irradiated cultured KCs (Takashima A et al., 1992; Miralles F et al., 1998; Marschall C et al., 1999). Further, in vivo, increased Plm activity has been identified in the SC in photodamaged skin and tightly correlated with transepidermal water loss, showing that Plm can be considered as a barrier impairing enzyme even in non-diseased skin (Voegeli R et al., 2008). The observation that UV rays increase Plm activity appears relevant, especially in the context of this thesis, as UVR are the main skin stressor which highly stimulate melanogenesis. However, to our knowledge no significant data are present in literature which directly associate Plg or Plm to melanogenesis (Prudovsky I et al., 2022). Link of alteration in the Plg Plm system and skin homeostasis is also supported by the identification of changes in the expression of components of the system in some skin pathologies such as psoriasis in which an increase in uPAR uPA, uPAR, and PAI-1 expression has been shown in psoriatic skin (Rubina KA et al., 2017, Voegeli R et al., 2019) and atopic dermatitis in which overexpression of ENO1, a Plg receptor, and increased Plm activity have been identified in KCs and associated to alterations of tight junction and decreases integrity of the cellular barrier (Tohgasaki T et al., 2018).

1.8 Melasma

Melasma is a multifactorial common acquired condition of symmetric hyperpigmentation induced by sun exposure, typically occurring on the face, with higher prevalence in females and darker skin types. It is more typical for asian or hispanic woman with skin types III-IV in according to Fitzpatrick classification (Ward WH et al., 2017). Melasma is generally diagnosed clinically by the identification of symmetric reticulated hypermelanosis in three predominant facial patterns: centrofacial, malar and mandibular. The major clinical pattern in 50-80% of cases is the centrofacial pattern, which affects forehead, nose and upper lip, excluding the philtrum, cheeks and chin. The malar pattern is restricted to the malar cheeks on the face, while mandibular melasma is present on the jawline and chin. A newer pattern termed extra-facial melasma can occur on non-facial body parts, including the neck, sternum, forearms, and upper extremities.

1.8.1 Diagnosis

The diagnosis is generally made with the dermatoscope which identifies pronounced hyperpigmentation in the pseudo-rete ridges of the skin. Using a Wood's lamp, the hyperpigmentation can be accentuated when the pigment is epidermal. Reflectance confocal microscopy (RCM) which uses the diode laser as a source of monochromatic and coherent light is also used. It consists of a non-invasive imaging technique that enables in vivo visualisation of the epidermis at a depth of 200–300 μm down to the papillary dermis. It allows to obtain images which give information at the cellular level, identifying hyperpigmented basal KCs or activated melanocytes, similarly to conventional histology.

1.8.2 Epidemiology

Various epidemiologic studies have estimated the prevalence of melasma in the general population at 1% and in higher-risk populations at 9-50%. These wide ranges are secondary to variations in prevalence in women rather than in men (with average ratio 9:1) among darker skin types, pregnancy, different ethnic heritages, and different levels of UV exposure within various geographic locations. Although the approach of calculation the prevalence and incidence of melasma can give underestimated numbers, there are several clinical studies that have produced statistics of melasma. A prospective study in Latin patients in New York private dermatology practice indicates 8,2%, in Saudi Arabia a 2,9% of patients revealed melasma, and in Arab-American population in Michigan a prevalence of 13,4-15,5% has been indicated. A health center in Ethiopia demonstrated 1,5% prevalence of melasma.

1.8.3 Pathogenesis

The pathogenesis of melasma is very complex and still poorly characterized, although it is becoming clear that it does not involve only melanocytes, but also the other skin cells including KCs, inflammatory cells, and dermic fibroblasts. Melasma has been associated with several factors including external factors (such as solar exposure), hormonal factors, as well as skin inflammation, and genetic predisposition (Espósito ACC et al., 2022). Indeed, sun exposure is the main environmental factor in melasma pathogenesis. It is known that any radiation may influence skin participating in inducing melasma, although to date effects associated to specific wavelengths have not been elucidated and most

knowledge is relative to the effects induced by UV, described above (Yardman-Frank JM et al., 2021). The other main factors involved are here summarized. Genetic predisposition plays an important role for development of melasma. This is mainly based on the observation that 55-64% of patients with melasma have a positive family history. Some transcriptomic studies have been performed to examine associated genes, and findings have identified changes in the expression of several genes involved in melanin biosynthesis, the transfer of melanosomes, inflammatory, hormonal and vascular responses (Chung BY et al., 2014). In addition, comparison among fibroblasts derived from normal and affected skin has shown differences in multiple genes. These data suggest the involvement of a genetic background in melasma, although to date no gene has been definitively associated with melasma. Interestingly, a polymorphism in MC1R has been associated with hyperpigmentation prevalence in Javanese women (Espósito ACC et al., 2022). Hormonal influences are also relevant in the development of melasma, since the observation of its appearance in pregnancy, administration of oral contraceptives and other hormonal therapies. Oestrogen and progesterone are known to play a role in melasma pathogenesis. To date, measurements of hormone levels in subjects affected by melasma, have not elucidate this connection, and provided contradictory results. However, melasma skin shows increased expression of oestrogen receptors (ER2) in the epidermis and dermal fibroblasts and it is known that oestrogen can induce melanogenesis in melanocytes by means of ER2. Further, it was demonstrated that in the facial melasma ER2 are more present than ER1, in contrast with abdominal and breast melasma. In addition, in vitro studies have shown that estradiol can stimulate KC proliferation as well as KGF release. Also progesterone receptors are elevated in melasma affected skin, although the role of progesterone on melanogenesis is not completely established.

1.8.4 Histopathological changes

From the histological point of view changes in melasma may involve both the epidermis and dermis with tissue alterations compared to normal skin and the involvement of several skin cell types. Historically melasma has been classified in three histologic types: epidermal, dermal and mixed, based on the involved tissues. Several tissue and cellular alterations have been identified in melasma affected skin compared to normal skin.

Most melasma lesions (95.8%) show alteration of the BM associated to melanocyte migration to the dermal layer. BM degradation is linked to the activation of MMPs, mainly

MMP2 and MMP9 and degradation of several matrix components, including type IV and VI collagen. Melasma lesions show also significant changes in vascularity, characterized by the increase of vessel density, vessel size and vessel area in melasma spots rather than in perilesional healthy skin. Melasma skin presents an increased epidermal density of eumelanin in all layers, including SC. At a cellular level, Increased activity of melanocytes is a typical feature of melasma, which can also move to the dermal layer. This process seems to be the consequence of the BM degradation, as well as the loss of cadherin expression and adhesion to KCs. Melanocytes present in melasma lesion show increased volume and more prominent dendrites if compared with healthy adjacent skin. In addition, melanosomes transported to KCs are larger, more mature and numerous if compared to healthy skin. Nevertheless, there is no evidence of an increased number of melanocytes. Moreover, the recent research has shown that pendulum melanocytes, which protrude from the basal layer to the dermis, are more frequent in melasma skin, although the role of these cells is still to be clarified. Growing evidence has revealed that other cellular components are involved in melanocyte dysregulation, especially KCs which show overproliferation and abnormal differentiation. Altered skin barrier is present in melasma, as the SC is thinner than non-affected skin. SC results more compact than in the adjacent healthy skin, the granular layer is atrophic, it usually presents ridge flattening and epidermal thinning. Basal KCs display large nuclei with irregular shape, chromatin heterogeneity and loss of polarization (Esposito ACC et al., 2022). In some cases, melasma skin shows a deep dermis damage, described by increased solar elastosis, which indicates the involvement of fibroblasts in the development of hyperpigmentation. Solar elastosis is associated with abnormal accumulation of elastic tissue in the dermis and thicker and more curled and fragmented elastic fibers and correlated with collagen fragmentation, increase in blood vessel and superficial dermis cells, including mast cells (Kwon SH et al., 2016). Moreover, melasma skin shows the increase of the number of fibroblasts, mainly senescent fibroblasts, present a proinflammatory and melanogenic secretory profile (e.g., SCF, HGF, and NGFb) (Espósito ACC et al., 2022).

1.8.5 Molecular changes

Some studies have investigated changes in specific regulators of melanogenesis by serum and immunohistological studies showing dysregulation of several paracrine factors and their receptors involved in signalling pathways leading to increased melanin biosynthesis. The main relevant data show increased levels of α-MSH as well as its receptor MC1R in

the epidermis of melasma lesions when compared to perilesional normal-appearing skin (Espósito ACC et al., 2018). Similarly, the higher expression of SCF and c-kit receptor has been more often observed within the lesional sites than in the perilesional areas. In particular, increased SCF expression has been identified and reported especially in the dermis layer, mainly in fibroblasts and perivascular areas. Also increased Wnt1, inducible NO synthase and VEGF expression have been demonstrated in affected skin. Globally these data are in agreement with their roles identified mainly in *in vitro* studies, thus confirming their role in melasma. Some studies have also recognized systemic changes associated with disease severity, mainly related to oxidative stress. Specifically increased serum levels of NO and malondialdehyde (MDA), derived from tissue damage following lipid peroxidation of cell membranes have been identified h among patients with melasma, while a strong negative correlation has been found between plasma glutathione (GSH), a major endogenous antioxidant and melatonin (Espósito ACC et al., 2022).

1.9 Hyperpigmentation and melasma treatment

Actually there are different ways of treating melasma. Mainly they are divided into physical, chemical and preventive treatments.

Physical treatments consist in the usage of microneedling, laser and light technologies, which must be carried out under strict dermatologist's supervision. Microneedling is generally performed with a roller. Although the exact mechanism of microneedling is unknown, it is reported that this procedure favours KC turnover, stimulating melanin transcutaneous elimination and induces skin whitening. However, it can cause slight skin injury, including diffuse erythema and bleeding spots, and therefore this technique is not largely diffused in dermatological practice (Bailey AJM et al., 2022). A diffused physical method consists in the use of laser and light technologies. The most used laser is Q-switched Nd-YAG technique, which produces pulses with high peak of energy delivered in a short time (nano- and picoseconds). The treatment leads to destruction of melanosomes and melanin by a photoacoustic effect without excessive damaging of MCs and KCs, thus producing less inflammation and post-inflammatory hyperpigmentation (PIH). Another type of laser treatments is the use of ablative fractional laser (CO2 and Er: YAG), which induces damage of KCs containing more melanin. However, this type of laser produces a

high thermal effect, which may result in hyperpigmentation as a side effect. (Cassiano DP et al., 2022)

Chemical treatments are divided into the usage of peeling and melano modulators. Chemical peelings consist in the use of α-hydroxyacids (AHAs), which act by destruction of the upper epidermal layer by a disruption of the cohesion of corneocytes in order to accelerate the turnover of KCs and remove superficial corneal cells and melanin. The acidic nature of AHAs also reduces pH, thus inactivating enzymes like kinases and transferases, and thus contributes to the desmosome resolution and desquamation. The final effects are epidermal remodelling and dermal reorganisation as a consequence of an induced inflammatory process which stimulates the production of collagen and elastin by fibroblasts. As described above, several inflammatory cytokines can induce melanogenesis and thus the treatment may lead to the worsening of melasma. In addition, one of the further complications of chemical peelings are PIH, infections, allergic reactions, persistent erythema and textural changes. Because of these strong side effects the use of chemical peelings require caution and professional supervision. The most used chemical peels are trichloroacetic acid (TCA) (in concentration of 15% and 20%) and (AHAs), including glycolic acid (30%, 50% or 70%), salicylic acid (20% or 30%) and lactic acid (82% or 92%) (Sheau-Chung Tang et al., 2017).

A class of chemical molecules used for the melasma treatment are called melano modulators. Their mechanism of action is related to the direct interaction with melanocytes and the system of melanogenesis. There are two main groups of melano inhibitors: the first acts by direct binding to the active site of TYR enzyme, leading to its inhibition. The most known molecules are hydroquinone, arbutin, kojic acid, resorcinol, ellagic acid, aloesin and glabridin. The second group acts via inhibition of the melanosome transfer from MCs into KCs, including niacinamide and soy derivatives. Despite their skin whitening capacity, some of these molecules demonstrated to induce several side effects. Severe ochronosis and teratogenic features were reported for hydroquinone, thus leading to the prohibition of its usage in cosmetic products for whitening purposes. Similarly, resorcinol tends to damage melanocytes, leading to the hypopigmented spots occurrence and allergic reactions, while contact dermatitis and sensibilisation are typical side effects of kojic acid. Therefore, European Commision put restrictions on the concentration of usage of these substances. Among the other largely used molecule is Arbutin (Arb). In according to the literature, Arb participates in the direct inhibition of TYR activity without suppressing TYR gene expression, thus leading to the reduction of melanin content. There are two

isoforms of Arb, α- and β-Arb, and the first one resulted more active in the TYR inhibition then the second one, so less concentration of α-Arb is needed to reach the whitening effect. In addition, Arb has demonstrated an efficacious hydroxyl radical scavenging activity, involved in the melanin synthesis during the oxidation of L-tyrosine and L-DOPA. However, an exact mechanism of its action needs to be completed, as well as data related to the clinical assessment. According to the indications of the European Commission regarding their safety assessment, the recommended concentration of α-Arb use is 2% (w/w) for face products and 0,5% (w/w) for body products (Boo YC et al., 2021). For the other molecules like ellagic acid, glabridin and aloesin more information about their safety profile and efficacy in vitro and in vivo clinical assessment is needed for the determination of their functionality. For what concerns the inhibitors of melanosome transfer into KCs on one hand it was reported that high concentration is needed to reach the melano inhibitory efficacy. On the other hand, a high concentration of their usage is associated with the occurrence of side effects as irritation and burning.

Preventive and protective treatment. Sun exposure is the major triggering factor of melasma and it can significantly limit the quality of clinical treatment. Further, UV and visible light (VL), are present during all the seasons and are able to penetrate clouds and the glass of windows. Therefore, in dermatological practice the use of sunscreens is strongly recommended to prevent melasma exacerbation as well as during and after the melasma treatment. To obtain a valid protection level, high quality products containing both UVA and UVB spectrum sunscreens with a very high sun protection factor (SPF 50+), as well as the other components suitable for the usage on sensitive skin, should be constantly used (Lyons AB et al., 2021).

Till date none of its existing treatment modalities has provided quick and sustained results. Considering multiple severe side effects and limited whitening efficacy of the described treatments, the research efforts are focused on identifying new functional molecules or treatments. Among recent studies a molecule largely investigated is Trans-4-(Aminomethyl)cyclohexanecarboxylic acid, also known as tranexamic acid (TXA), is a lysine analogue. Its activities and functions are described in detail below.

1.10 Tranexamic acid

As indicated above, it is known that UV light, the main inducer of melanogenesis, acts as a Plg/Plm system activator in the skin (Marschall C et al., 1999). This finding associated to recent data showing that inflammation contributes to altered skin pigmentation (Rox JM et al., 1996), and the link of the Plg/Plm system with inflammation has led to the investigation of TXA, for treating melasma (Zhang L et al., 2018; Sahu PJ et al., 2020; Feng X et al., 2021; Austin E et al., 2019).

TXA is a lysine analogue that blocks lysine binding sites (LBSs) on Plg, thereby inhibiting its activation to Plm by tPA and uPA. As Plm drives fibrin hydrolysis, TXA has been largely used for over 50 years as an antifibrinolytic agent in a broad range of formulations and dosages to control excessive bleeding during and after surgery or in haemophilia and menorrhagia (Ismail AA et al., 2021, Prudovsky I et al., 2021). Mechanistically, TXA works as a synthetic lysine-analogue, which reversibly forms a complex with Plg. It binds to the LBSs of Plg with 1 high-affinity (1.1 μM) and 3 medium-affinity (~0.75 mM) binding sites. Accordingly, TXA at submicromolar concentrations significantly attenuates Plm formation. In addition, TXA can also directly inhibit Plm activity (Wu G et al., 2019). In agreement, it has been shown that the interactions of Plg with several cell types and the skin are blocked by TXA, lysine and lysine analogs in macrophages, fibroblasts, monocytes and basal layers of keratinocytes (Miles LA et al., 2013, Burge SM et al., 1992). Further in an acellular in vitro system it has been shown that TXA can also bind to the other components of the Plg/Plm system, like uPA attenuating uPA activity (Wu G et al., 2019).

In addition to the inhibition of fibrinolysis TXA, reducing Plm formation and activity, can potentially control many different other biological events which involve Plm activities, including inflammation. Indeed, in vivo recent publications report the anti-inflammatory effects of TXA in animal models of trauma. TXA reduced the level of proinflammatory cytokines IL-1β, IL-6 and TNF-α and the anti-inflammatory cytokine IL-10 in serum or bronchoalveolar fluid, as well as other inflammation parameters such as invasion of neutrophils, macrophages, and platelets. A similar anti-inflammatory activity of TXA has been also reported in human surgery patients, although with more conflicting data. Decrease in inflammatory molecules including NO and PGE2 has also been reported in vitro in mouse bone marrow-derived macrophages (Baranowsky A et al., 2021) and UV treated human fibroblasts (Endo K et al., 2021).

Besides treatment of trauma, surgery, and angioedema, TXA has been recently used for treatment of melasma. The inhibition of skin pigmentation by TXA was firstly reported by Kazuhisa Maeda in 1998, showing a reduction of TYR activity and skin pigmentation in Weiser-Maples guinea pigs exposed to UV radiation (Maeda K et al., 1998). From that observation, TXA attracted interest for its potential efficacy in the treatment of melasma and has been used recently for successful melasma treatments in several clinical studies. (Colferai MMT et al., 2018; Karn D et al., 2012; Nagaraju D et al., 2018, Prudovsky I et al., 2022, Kim HJ et al., 2017). However, to our knowledge, few studies investigated the *in vitro* effects of TXA on melanin content and melanogenesis with conflicting and fragmentary results (Desai S et al., 2019; Maeda K and Tomita). Recently, Ying Liu et al. have confirmed the inhibition of melanin content in human A375 melanoma cells as well as *in vivo* in UV treated guinea pigs, by means of TXA-loaded liposomes packed in HA gels (Liu Y et al., 2021). Concerning mechanisms of action, it has been suggested that TXA can interfere with PGE2 signalling in endothelial cells (Chang WC et al., 1993), melanocyte (Desai S et al., 2019) and UVA irradiated senescent human fibroblasts (Endo K et al., 2021). In UVB exposed normal human melanocytes, TXA decreased TYR activity and melanin production via inhibiting activation of VEGFRs and subsequent expression of melanogenic enzymes (Zhu JW et al., 2020). Also, a link among TXA, melanogenesis and autophagy has been shown (Cho H et al., 2017). Further, the inhibition of KC inflammatory molecules has been proposed, as TXA suppressed melanogenesis in normal and malignant murine melanocytes stimulated with a medium conditioned by UV irradiated KCs (Kim M et al., 2015).

Because of the pleiotropic properties of Plm, the antiplasmin activity of TXA has been generally indicated as the primary mechanism for its inhibition of pigmentation (Desai S et al., 2019; Wu G et al., 2019), however, this link has not been proved and the underlying mechanism for the whitening effects and modulation of melanogenesis still remains unclear.

1.11 Design of a cosmetic product

1.11.1 Phases and rules

The process of development of a cosmetic product in industry is divided into several phases and it requires collaboration among different roles, such as research and development, control of quality, marketing and commercial offices, procurement, and

regulatory affairs. Generally, at least 9 months are required to develop and launch a cosmetic product. Actually, all the cosmetic products in their phases of existence are subjected to European Regulation n. 1223/2009 (Official Journal of the European Union, https://eur-lex.europa.eu).

The development of a new product includes four main phases.

Firstly, an ideation of the product should be performed in order to determine its exact final function. In this phase a bibliographic and market research is the main instrument which helps to determine and describe the skin problem, existing commercial solutions and to detect limits of proposed treatments. These limits can include the lack of efficacy, the usage of ingredients with doubtful safety profiles, the lack of information about the possibility of the application in particular populations, such as sensitive skin, the lack of clinical or laboratory confirmation of declared claims. This information is the basis for principles of innovation and gives the possibility to determine essential requirements for a new product, its claims and consequent selection of ingredients, cosmetical form and scientific and commercial communication.

Next, the laboratory design of the product and the production of the pilot sample establish the second phase of development. In this phase the product is formulated in the laboratory and its primary stability is evaluated. During the formulation it is important to establish the most efficient method of manufacturing, possible incompatibilities between ingredients, the compatibility with packaging, the behaviour of the product during and after the manufacturing process. Thereafter, it is important to test the stability of the formulation and determine the final specifications of the product. Finally, since a cosmetic product requires a high acceptability by the consumer, the sensory analysis is performed to determine the pleasantness and therefore a continuity of the treatment.

Further, the testing of declared efficacy is mandatory as established by the European Regulation 655/2013. Based on the declared information about the functionality of the product, which will be reported in the communication for consumers, it is necessary to execute laboratory tests and clinical trials in order to prove the claim. For some claims it is indispensable the collaboration with dermatologists, as for example in case of the possibility of application on sensitive skin or a declaration about specific function (e.g. “whitening” or “anti hair loss”). In case of absolute innovation the industry can evaluate to patent the discovery. In this phase a mandatory technical document, Product Information

File (PIF) must be prepared and the product should be registered on the Cosmetic Products Notification Portal (CPNP).

Finally, when all the necessary information is produced, it is possible to proceed to the manufacturing phase and to develop commercial and graphic communication. After the launch of the product on the market, the industry should conduct post-marketing analysis and cosmetovigilance in order to guarantee a continuous collection of the information on the efficacy and safety of the product.

1.11.2 Selection of functional components

In case of a functionalized cosmetic product which claims some specific activity, it is necessary to select the most performant and safe active ingredient or a combination of them. A bibliographic research and collaboration with dermatologists are an essential instrument for this process. A quantity and a quality of available scientific information about the activity of the component *in vitro* and *in vivo*, the presence of clinical assessments and its regulatory status, as well as the information about its safety profile should be clarified before proceeding to the formulation of the product. On the other hand, the finished product should correspond to the innovation requirement, therefore the lack of some information in the literature can be assessed by the industry, which will constitute the advantage for the industry. The innovation can consist of synthesis of an innovative active ingredient, the use of the known ingredient in combination with the other components, the use of known ingredient in a particular delivery system or in the type of packaging that improves its performance. As from the indication of European Commission, it is always needed to support the claimed information with scientific demonstration, so the development of laboratory and clinical methods is of particular interest for the cosmetic industry.

1.11.3 Delivery system selection

In dermatology topical delivery is an important issue since it permits to improve the efficacy of the product, transporting active ingredients into epidermic layers or leaving

them on the surface, thus increasing its effectiveness as well as reducing potential systemic and local side effects (Nastiti CMRR et al., 2017).

Therefore, it is fundamental to know all the most important endpoints of a dermatological problem, if it involves epidermic, dermic or both layers, the chemical features of the actives and vehicle ingredients in order to select the most suitable system in the function of the skin depth and the compatibility of ingredients between them. Several topical delivery systems are commonly used by the cosmetic industry, including monophasic and multiphasic systems. Monophasic include only one phase represented by water or oil, while bi- and triphasic systems include both water and oil in different ratios. The most known multiphasic systems include macro- and microemulsions.

Macroemulsions

Macroemulsions are one the most diffused cosmetic forms not only in cosmetology, but also in pharmaceutical and food industries. They can take a form of fluids (milk), cremes (emulsions), pastes, and able to perform as hydrating, detergent, humectant or nourishing products depending on added components.

Generally, emulsions are considered oil-in-water (O/W) if the external phase is water and internal phase is oil; and vice-versa, water-in-oil (W/O) if the external phase is oil and internal phase is water. The orientation of the phases depends on several factors as the ratio between volumes of phases, the emulsifier, the density of phases and the preparation method. All types of emulsions require energy to be formed and the use of procedures and special equipment.

In cosmetology O/W emulsions are used for their capacity to incorporate hydrophile active components, to obtain a light texture and a final refreshing effect, and in cases that do not require a deep penetration. On the contrary, this structure does not permit a prolonged emollient function.

Similarly, W/O emulsions are able to incorporate more of lipophile active ingredients, and they are able to restore a hydro-lipidic layer of the skin, to create a non-penetrating film in order to distribute active components on the surface (as in case of solar creams) or to penetrate in the deep. A main disadvantage of these emulsions is their fatty and heavy texture and they are hardly spreadable.

Microemulsions

Microemulsion is a transparent, isotropic, thermodynamically stable system, formed spontaneously by mixing two immiscible liquids due to the presence of surfactant and cosurfactant that reduces interfacial tension. Similarly to emulsions, microemulsions are able to form O/W or W/O versions.

From the technological point of view, low energy is requested, therefore, it is very easy to produce microemulsion on an industrial scale using a simple equipment such as a shovel or helix mixer. A thermodynamic stability of microemulsions permit to improve a shelf-life of a finished product. In some cases, microemulsions are used to protect unstable active components (e.g., vehiculation of Arb into microemulsion improves its photostability) (Gallarate M et al., 2004). Furthermore, another advantage in cosmetology is their reduced colloidal dimension of particles (from 1nm to 1μm), therefore, externally they appear completely transparent since light rays are not refracted through particles, differently from macroemulsions, thus improving cutaneous penetration. In addition, microemulsions permit a gradual release of the drug and reduce side effects of pharmaceutical or cosmetic active components. Microemulsions are generally composed of organic phase (oil), water phase, a surfactant and co-surfactant. The numeric balance between these components can be described with the help of ternary diagrams, that are generally used to determine a microemulsion behaviour in the dilution path (Fig. 5)

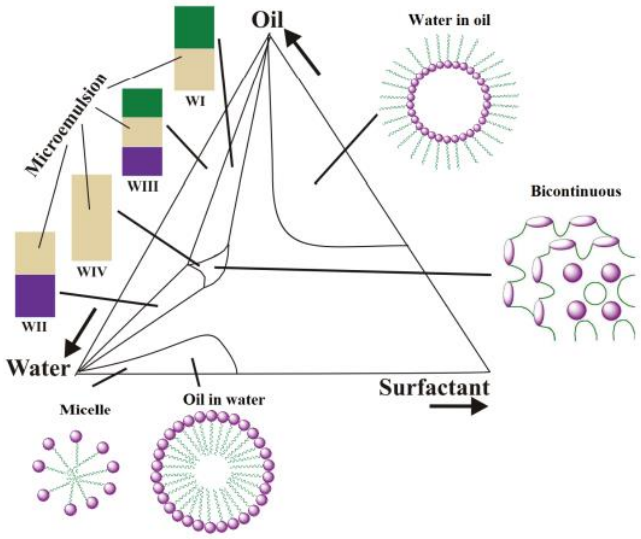

Figure 5. *Ternary diagram of microemulsion formation and types of microemulsion*

1.11.4 Stability evaluation

The stability test provides important information about the integrity and the capacity of the product to maintain its characteristics for a definite period. It is essential for its safety evaluation for a definition of a standard reference for the compliance of the product acceptability, as well as for the determination of transport and storage conditions. The stability testing is performed at the Quality Control Office of the industry and consists in the evaluation of several physico-chemical and microbiological parameters including mass variation, humidity, pH, density, viscosity, phases integrity, etc. Further, the efficacy of the preservative system of the finished product from pathogens is evaluated by current microbiological analysis, collected in the *Challenge test* (Annex I of Cosmetic Regulation 1223/2009)**.** The product is generally also tested under different temperature and humidity conditions both in the neutral packaging (glass) and in the hypothesised final packaging in order to verify potential influence of packaging on the product and vice versa of the product on the packaging.

1.11.5 Packaging

The choice of the type of packaging depends on the kind of product, its chemical composition, physical parameters, the site of the application and on the marketing strategy.

The chemical composition of the cosmetic product can determine the damage of the packaging, as in the case of strong acidic or basic components (e.g. strong peeling or depilatory emulsions), sunscreens that can migrate to the plastic of low-density polyethylene (LDPE) or of high density polyethylene (HDPE), resulting in the instability of the formulation. Furthermore, phenomena like water evaporation are to be considered as they may change concentration of active components, potentially giving product instability (Briasco B et al., 2017).

Physical parameters of the product should be also considered because the type of packaging dispensing can influence the simplicity and practicality of its usage. Such parameter as viscosity can determine limits for the type of the product dispensing, leading in some cases to the impossibility of the usage of the product. More viscous is the product, more difficult is its dispensing, therefore, the forms of jars or tubes with a large hole should be preferred, while sprays, airless dispensers, pumps, brushes, etc. are preferred in case of liquid forms.

The site of the application is also the determining aspect for the choice of packaging. The level of precision of the applied dosage, the vastness of a zone of application, the thickness of the skin and its anatomic and metabolic particularity can determine the type of dispensing system. Furthermore, the safety aspect must be always considered in the choice of packaging, as in case of the nearness of eyes.

The evaluation of the packaging is regulated by the Directive 94/62/CE in Italy. It foresees such principles as the usage of less packaging as possible for the single product, minimization of dangerous substances, the recycling and reuse possibility of the material and the guarantee of hygiene and safety.

1.11.6 Safety evaluation and clinical trial

Following the safety evaluation of a cosmetic product, according to European Regulation n. 1223/2009, and the stability study, it is possible to execute a clinical evaluation *in vivo* on healthy volunteers. Notably, from 2009 it is not possible to execute this type of evaluation on animals, which has significantly slowed the development of new cosmetic ingredients since an essential safety information can be obtained only from animal testing. However, the European Commission is developing new alternative testing methods in order to support the development and innovation of the cosmetic industry.

In the industry, the safety evaluation consists in the collection of all available information about ingredients and finished products in an official document, called PIF, which permits to establish safety of the finished product and is performed by the *Responsible Person*, a qualified and specialised worker. As no specific guidelines for trials involving cosmetic products exist, clinical evaluation should respond to the ethical principles, based on Good Clinical Practices of pharmaceutical substances and Helsinki declaration (1964 and following revisions). It is to note that clinical trials performed in the cosmetic industry do not always require submission to an ethical committee. In fact, safety and stability are assured passing the safety evaluation and stability testing. Afterwards, the new cosmetic product can be notified in the CPNP, for control by health authorities including the Ministry of Health. Notification of the products allows the beginning of the clinical evaluation and commercialization. This is different from pharmaceutical drugs which always need the approval of an ethical committee. The protocol of the study is elaborated in collaboration by dermatologists and the researchers of the Industrial Research and development unit, defining the inclusion and exclusion parameters, treatment conditions,

evaluation parameters and timing. The dermatologist enlists participants to the study, performs diagnostic evaluation and follows subjects during treatment. Qualitative and quantitative instrumental data are then collected and analysed to establish efficacy of the product, as well as potential undesirable effects. In some cases, especially for products which require long treatment periods, a satisfaction questionnaire is also prepared and proposed to the enrolled subjects. Globally results of the clinical study provides important information for claim declarations and advertising scientific and professional communication (Commission regulation n.655/2013, Official Journal of the European Union).

2. AIM OF THE STUDY

The first aim of this study was the development and application of an innovative cosmetic formulation for the topical treatment of skin hyperpigmentation disorders, mainly melasma. Design of the new formulation was performed according to requirements of actual legislative cosmetic regulations and European guidelines. In addition to safety and efficiency, from a strict industrial point of view the new product should also meet other characteristics, including good availability of the ingredients, low costs of production, decrease of energy consumption and easiness of usability for consumers. Innovation was based on: 1. the choice of the specific whitening ingredients combined in a single formulation; 2. the selection of the delivery system. As active ingredients, molecules with different suggested roles in the melanogenic process were selected. Specifically, α-arbutin (α-Arb), a well-known inhibitor of TYR, the main enzyme involved in melanin synthesis, and tranexamic acid (TXA), a molecule known as an inhibitor of the Plg/Plm system potentially involved in skin inflammatory activities related to melanogenesis. Further, TXA has been recently investigated in some clinical studies with beneficial effects on melasma. The new formulation could potentially show advantages over other cosmetic products containing a single ingredient or irritating treatments, like peeling and laser therapy, increasing amelioration and/or reducing side effects. As a vehicle system, we selected a microemulsion, an uncommon topical formulation in cosmetics, more used for pharmaceutical drugs, which has gained recent interest to improve transdermal delivery.

The project development included the following different phases:

- the first phase was the analysis of the *in vitro* effects of the selected functional components used alone or combined on skin cells, including melanocytes, KCs and macrophages. Specifically, we evaluated potential effects on cell viability and melanin production in B16/F10 murine melanoma cells, a model largely used *in vitro* to investigate the activity of melanogenesis inhibitors. Further, the effects of TXA and α-Arb, alone or combined, were investigated on the release and expression of inflammatory mediators involved in altered melanogenesis, in KC and macrophage cell lines.

- the second phase included the design and establishment of the preparation method of the new cosmetic formulation. Further, evaluation of its characteristics and properties, as well as a study of stability and safety assessment were performed according to standard

procedures to gain essential information necessary to the industrial development of the product.

- finally, a clinical study, was planned in collaboration with dermatologists and performed to evaluate safety and efficacy of the cosmetic formulation in subjects affected by melasma.

3. MATERIALS AND METHODS

3.1 Cell culture

The murine melanoma cell line B16-F10 (CliniSciences, Italy), the immortal human KC cell line HaCaT (ACCEGEN, Fairfield, NJ, USA) and RAW 264.7 cells derived from murine macrophages (ATCC, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, United States) supplemented with 10% foetal bovine serum (FBS), 1% penicillin G and 1% streptomycin sulphate (all from Sigma-Aldrich, St. Louis, MO, USA), and maintained at 37 °C in a cell incubator with 5% CO2. Cells were harvested by trypsinization when they were about 70% confluent, counted and seeded at the appropriate numbers into wells of cell culture plates for further experiments.

3.2 Cell Viability Measurement by MTT Assay

To evaluate the effects of TXA and α-Arb, used alone or combined, on cell viability, cells were exposed to increasing doses of TXA (2,5 – 40 mg/ml) (Fagron Italia S.R.L) and α-Arb (500 μM) (Kangcare Bioindustry Co., LTD, Hong Kong, China). The range of doses of TXA were derived from previous studies (Eikebrokk TA et al., 2019). α-Arb, alone or in combination with TXA, was used at 500 μM, the concentration preferentially used in previous studies showing the inhibitory effect on melanin synthesis in B16 cells (Shim E et al., 2017). Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Sigma Aldrich, UK), according to the Scientific Committee of Consumer Safety (SCCS) notes of Guidance for the Testing of Cosmetic Ingredients (11^{th} revision) (De Mattei M. et al., 2009). This method is based on the reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan by mitochondrial enzymes in viable cells. The quantity of formazan formed is proportional to the number of viable cells and can be measured spectrophotometrically. In brief, cells were plated into a 96-well plate at a density of 1×10^4 cells/well. After 24 h plating, cells were treated with TXA or α-Arb, alone or in combination, for 24 h and 72 h. At the end of treatments, cells were incubated with MTT solution (5 mg/mL in phosphate buffered saline, PBS) for 4 h. The medium was then discarded, and cells treated with isopropanol/HCl 0.04 N for the formazan solubilization. The solution absorbance was

measured at 540 nm using an optical density reader (Tecan Sunrise Basic Microplate Reader, Tecan Trading AG, Switzerland). Cell viability was determined relative to the control group.

3.3 Measurement of Melanin Content

The intracellular melanin content was measured according to a previously reported method (Chan YY et al., 2011). Briefly, B16-F10 cells were seeded in 24-well plates (2×10^4 cells/well) and incubated for 24 h. Then, cells were treated with TXA and α-Arb, used alone or in combination, in the absence and in the presence of 10 ng/ml α-MSH (Merck Life Science, Germany) for 48 h according to previous studies (Chung S et al., 2019). At the end of treatments, cells were washed with phosphate buffered saline (PBS), collected by trypsinization and pellets were suspended in 300 µL of 1 N NaOH (Merck, Germany) in 10% dimethyl sulfoxide (Sigma-Aldrich). After heating at 80 °C for 30 minutes, relative melanin content was determined by measuring absorbance at 490 nm using a microplate reader (Tecan Sunrise Basic Microplate Reader, Tecan Trading AG, Switzerland). The melanin content was determined from a standard curve prepared from an authentic standard of synthetic melanin (0-20 µg/mL) (Sigma, USA). Images of control and treated cells were obtained by a standard light microscope (Nikon Eclipse TE 2000-E microscope, Nikon Instruments Spa, Sesto Fiorentino, FI, Italy) equipped with a digital camera (DXM 1200F; Nikon Instruments Spa, Italy).

3.4 Scratch cell migration assay in HaCaT cells

As TXA is an inhibitor of the Plg/Plm system, we evaluated the effects of TXA on HaCaT cell migration in selected controlled experimental conditions. Specifically, cells were cultured in the absence of serum and activation of the Plg/Plm system was induced by treating cells with Plg, or Plg + tPA (Alifax S.r.l., Polverara (PD) Italy), according to recent studies in other cell types (Pontecorvi P et al., 2019; Zalfa C et al., 2019). For scratch assay, HaCaT cells were seeded at a density of 5×10^4 cells/cm^2 into six-well plates and used at confluence. After serum starvation for 12 h, cells were scratched using a 10 µL pipette tip to generate a cell-free zone. Medium and dislodged cells were aspirated, and the remaining cells were incubated in serum-free DMEM medium. Cells were treated for 18 h

as follows: Plg (500 nM) in the absence and in the presence of TXA (10 mg/ml); Plg (500 nM) and tPA (12 nM) in the absence and in the presence of TXA (10 mg/ml). TXA concentration was based on cell viability data. Control cells were maintained in serum-free medium. Cell migration was monitored at different times and the wound area was photographed through a microscope with a photomicrographic equipment (Nikon Eclipse TE300, Nikon Instruments S.p.A, Sesto Fiorentino, FI, Italy) at time 0 and 18 h after the scratch wound. The image acquisition software (NiSElements Advanced Research, Nikon Italia, Italy) was used to measure width of the cell free zone of the artificially created wound (10 measures for each image). The wound area of cells was calculated based on the formula as below according to previous studies (Mittraphab Y et al., 2022):

Wounding closure (%) = $((At_0 – At_{18})/At_0) \times 100$,

where At_0 is wound area measured at 0 h, At_{18} is wound area measured at 18 h after treatments.

3.5 Quantification of inflammatory mediators

In selected experiments, levels of inflammatory mediators including NO, PGE2, and the proinflammatory cytokine TNF-α were measured in culture media. To evaluate potential changes in inflammatory molecules, due to activation of Plg into Plm and to the micro-emulsion functional ingredients, HaCaT and RAW 264.7 cells were treated with Plg (500 nM); Plg (500 nM) and tPA (12 nM); TXA (10mg/ml); α-Arb (500 µM) for 24 h, in culture medium without serum. The doses of TXA and α-Arb were based on cell viability data. LPS (1µg/ml) was used as a proinflammatory control stimulus. In RAW 264.7 cells, treatments with TXA and α-Arb were also evaluated in the presence of LPS at 24 h.

3.5.1 Measurement of NO

The release of nitrite, a stable breakdown product of NO was measured as an indicator of NO synthesis, according to the Greiss method, as previously reported (Varani K et al., 2008). In brief, medium was mixed with the same volume of Griess reagent (Sigma-Aldrich Chemical Co.). NO levels were then determined using a microplate reader at 540 nm (Tecan Sunrise Basic Microplate Reader, Tecan Trading AG, Switzerland). Nitrite concentration was calculated from a standard curve of sodium nitrite. All samples were assayed in triplicate.

3.5.2 Measurement of PGE2 and TNF-α

PGE2 and TNF-α levels in culture media were quantified using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN, USA; Abcam, Cambridge, UK), according to the manufacturer's specifications (Varani K et al., 2008). Absorbance was measured using a microplate reader (Tecan Sunrise Basic Microplate Reader, Tecan Trading AG, Switzerland). Concentration was calculated according to a standard curve calibration. All samples were assayed in triplicate.

3.6 Preparation of the microemulsion

Selection of active components. In our study we have assessed about 150 active anti melanogenic substances by the inserting of the following key words: "melanoinhibitor, melasma", "whitening treatment, melasma" "bleaching treatment, melasma", "melanoinhibitor, hyperpigmentation", "treatment, hyperpigmentation", available in the data bases PubMed, Scopus and Cohrain library. We have divided all these substances into several groups: botanical extracts and active substances (46), newly synthesised molecules (12), pharmaceutical active compounds (31), known cosmetic compounds (61). The group of newly synthesised compounds was excluded for the absence of any information about their safety profile and the commercial unavailability. Also the major part of pharmaceutical active compounds were excluded for the prohibition of usage in cosmetic products, established by the Annex II of Cosmetic Regulation 1223/2009 and its updates. The groups of botanical extracts and known cosmetic anti melanogenic ingredients were analysed for the availability of the information about their *in vitro* and *in vivo* efficacy and safety. The increasing interest for TXA lead us to its selection, as well as for α-Arb, which is a well-known botanical active ingredient, although the information about its clinical assessment was insufficient. The concentration of 4% (w/w) of TXA was chosen based on the available literature data about its whitening efficacy and the evaluation of its safety profile evaluated in according with the Commission Directive 93/67/EEC, Council Regulation 793/93, Technical Guidance Document on Risk Assessment 2003 and Commission Regulation n. 1488/94. The concentration of 2% of α-Arb (w/w) is suggested by the SCCS guidelines (SCCS opinion on α-Arb of May, 27, 2015), based on the sufficiency of its efficacy and safety.

Actives: TXA (Fagron Italia s.r.l.) is a white crystalline powder, well-soluble in water at room temperature (18 g/L, https://go.drugbank.com/). α-Arb (Kangcare Bioindustry Co., LTD, Hong Kong, China) is a white crystalline powder, soluble in water at room temperature (151 g/L - SCCS opinion on α-Arb). Fig. 6 shows chemical structures of TXA and α-Arb.

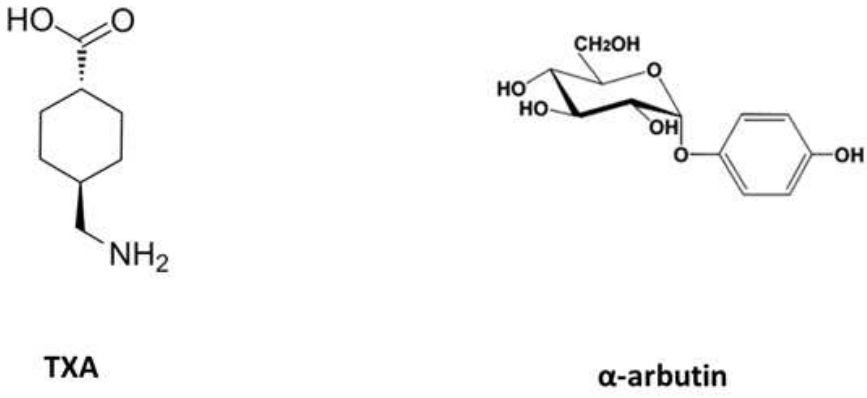

Figure 6. *Chemical structure of TXA and α-Arb*

Microemulsion components. As the selected actives of the new preparation, TXA and α-Arb are hydrophilic molecules, they require topical formulations able to pass through the lipid barriers of the skin SC. Therefore, a microemulsion was selected as a topical delivery system. The generation of the microemulsion requires mixing of surfactant and cosurfactant, oil and water phases. Here, the components of the oil phase, the concentration of the active ingredients and any other additive have not been disclosed because part of the intellectual property of the company and at present the cosmetic product is not commercially available.

Method. For the weighing of ingredients analytical scales with a precision 0,01g was used (Electronic scales Ohaus Adventurer Pro AV812, NJ, USA). The components of the oil phase were added to the graduated becker one by one and then stirred for 2 minutes in order to obtain a homogeneous and transparent system. Separately, TXA and α-Arb were weighed and dissolved in water. The preservative was added to the mix and finally pH was adjusted with buffer solution till the value of 3,5. To conclude the preparation the oil phase (A) was added to the water phase (B) drop by drop and stirred for 5 minutes till a homogeneous transparent and yellowish system was obtained.

3.7 Dimensional analysis of microemulsion

The microemulsion has been analysed for the particle dimension to prove its formation. This analysis has been performed in the accredited laboratory ECSIN (Padua, Italy) with the help of a scanning electronic microscopy with energy dispersion X-ray spectroscopy (SEM-EDX) Nova 600i (FEI). The images have been produced with the help of dedicated software ImageJ. For the calculation of descriptive parameters of dimensional distribution about 500 particles were analysed. The images magnifications were: 15000x, 80000x, 125000x and 240000x. The evaluation of 500 particles for their length and strength has been performed for the dimensional characterization, measuring minimum diameter, the first quartile, median, absolute median deviation, media, standard deviation, the third quartile, maximum diameter, moda, D10, D50 and D90.

3.8 Microemulsion stability evaluation

Physico-chemical parameters of the prepared microemulsion were measured and analysed with analytical instruments:

- Mass variation: Electronic scales Ohaus Adventurer Pro
- pH: pH-meter Delta Ohm HD2105.1
- Density: Pycnometer Hubbard 25mL
- Humidity: Humidifier OHaus Mb35 Halogen
- Phase separation: Centrifuge Hettich Zentrifugen EBA20

All parameters were measured after 30, 60, 90 and 180 days under different conditions: at room temperature with the exposure to sunlight, at the dark at -20°C, +8°C, +40°C, and in changing condition (stress test) including cycles of testing conditions applied at 30, 60, 90 and 180 days. Each cycle included 24 h at +40°C followed by 24 h at +8°C for 5 consecutive days. The product was tested under these conditions both in the neutral packaging (glass) and in the final packaging (polyethylene, PE). After preparation, parameters of the study were measured at T0. 200mL of microemulsion was put into 5 glass containers, each for every test condition, and they were left at the selected temperature and evaluated at each time point. To test mass loss, 10mL was put into separate appropriate containers, each for every condition of testing, closed until the end of the test. The same procedure was repeated with the final packaging. The protocol of the study has been designed in accordance with cosmetics (Unipro Guideline n.32/2003; COLIPA/CTFA Guideline 2004; PCPC Guideline 2011; ISO/TR 18811:2018; the SCCS's

notes of guidance for the testing of cosmetic substances and their safety evaluation (SCCS/1564/15 Revised version of 25 April 2016) and pharmaceuticals guidelines (Q1B; Q1A (R2), IPEC 2011) of The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (European Medicine Agency). In according with guidelines UNIPRO regarding stability testing (Unipro Guideline n.32/2003), we have compared parameters, obtained in different temperature conditions, with the standard. The standard is a sample of the product with a defined specification, which is able to maintain all the parameters stable, and conserved in the most possible inert conditions, +8°C in our case.

Acceptance criteria were defined as follows: variation of more than ±5% was considered non acceptable for weight loss and humidity, whereas a variation of more than ±15% was considered non acceptable for pH and density parameters (ICH guidelines Topic Q 6 A of EMA).

Moreover, as established by the Regulation 1223/2009, it is mandatory to guarantee a microbiologic safety of cosmetic products. We have performed a Challenge Test, a standard method for the evaluation of the adequacy of preservative system, which is generally executed on products with high water content. Challenge test has been performed in external laboratory Bio Basic Europe S.r.l., and it consists in the exaggerated microbiological pollution with pathogenic Gram+, Gram- bacteria, yeasts and moulds (Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Candida albicans and Aspergillus brasiliensis) and finally the evaluation of the capacity of preservative system to break down their proliferation.

3.9 Clinical evaluation of the lightening effect of the microemulsion

Treatment. Whitening microemulsion in PE tube of 8mL with a brush. Patients were instructed to use the product twice a day, in the morning and in the evening, directly on the hyperpigmented spot, for 2 months. They were also instructed to avoid any other whitening treatment during the period of clinical trial.

Volunteers. 21 healthy volunteers with hyperpigmented spots (melasma, post acne spots, PIH) were enrolled by a dermatologist of an Italian dermatological center Dermovicenza in Vicenza (VI, Italy). One patient did not finish the study. Due to the cosmetic nature of the

product rather than pharmacological action of drugs, Ethics Committee approval was not required. Nevertheless, the present clinical trial was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice (https://www.ema.europa.eu/en/ich-e6-r2-good-clinical-practice-scientific-guideline) and their revisions. The tolerability of the protocol treatment has been evaluated with the Patch Test, previously executed on sensitive skin and described below. Inclusion criteria comprised patients of minimum 18 years old, further they were selected in according to the classification of Fitzpatrick phototypes I-IV; the treatment was proposed to subjects who presented from slight to severe hyperpigmentation based on dermatological visit. Before starting the study, volunteers must have finished previous treatments for at least 1 month. In case of hypersensitivity to active ingredients or to any other component of the microemulsion volunteers were instructed to inform the dermatologist and to suspend the treatment. Pregnant and breastfeeding patients, patients with the other dermatoses, immunosuppression and pathologies connected with thrombosis or administration of anticoagulants were also excluded from the present clinical trial.

3.9.1 Total MASI score

MASI score has been calculated to test the efficacy of the dermocosmetic protocol. It has been evaluated by two independent dermatologists, using a professional dermatoscope at T0 and after two months of treatment, T2. It was calculated in according with the following formula:

(Darkness (D) + homogeneity (H)) × area (A) of involvement (total scoring from 0 to 48 points),

where A is rated on a scale 0-6 in according with the Table 1A, while D and H were rated on a scale of 0–4 represented in the Table 1B. A factor was assigned to each area (forehead 0.3; right malar 0.3, left malar 0.3, chin 0.1) (Berardesca E et al., 2019). 20 patients have participated in this evaluation.

Table 1. *The score of MASI evaluation for area of involvement (A), darkness of the spot and homogeneity (B)*

Area of involvement (A)	
0	no involvement
1	<10% involvement
2	10-29% involvement
3	30-49% involvement
4	50-69% involvement
5	70-89%involvement
6	90-100% involvement

A

Darkness (D) and homogeneity (H)	
0	absent
1	slight
2	mild
3	marked
4	maximum

B

3.9.2 VISIA® Complexion Analysis System

To test the efficacy of dermocosmetic protocol the skin of 10 subjects included in the study has been analysed at T0 and at T2 with the help of VISIA (7th Generation VISIA® Complexion Analysis System), shown in Fig 7.

Figure 7. *VISIA® Complexion Analysis System*

VISIA® uses patented technology IntelliFlash® and RBX® of Canfield. IntelliFlash® measures and registers the skin condition, in the surface and in the deepest layers with the help of cross-polarised light. As a result, it provides the analysis of skin damages caused

by sunlight. RBX® of Canfield is able to divide chromatic components of the skin of red and brown color, analysing in this way pathological situations like teleangiectasia, hyperpigmentations, inflammation, etc. During the analysis VISIA® Complexion Analysis System catches photos with standard, cross-polarised, parallel-polarised and UV light. Images were taken in three views (right lateral 37°, left lateral 37°, front 0°) to determine patient's phototype and to quantify the score of dark brown spots in the face indicating the presence of melasma. Evaluation of the subject's complexion was performed through a comparison between the individual's scores and those of people of the same sex, generation and skin type provided by VISIA® Complexion Analysis System database. Score provides a comprehensive measurement of the impact that the feature has on the patient's melasma status. Score determines the total size and area as well as intensity of evaluated parameters.

Endpoints of the efficacy of treatment are the following (situation at T2 respectively T0):

- variation of UV spots, which correspond to the superficial epidermic damage;
- variation of brown spot count, which corresponds to the deepest dermal damage; the detection is released by RBX® Technology using cross-polarized imaging;
- variation of the red spot count, which corresponds to the inflammatory status of patients. Red Areas are photographed with cross-polarized lighting and outlined in light blue.

Results were also presented with photos, compared at T0 and T2, generated by the technology VISIA® Complexion analysis System.

3.10 Patch Test

The human occlusive patch test is a standardised application of products applied in single dose on the intact skin which exaggerate exposure compared to actual use. The objective was to assess the compatibility of the product with the human skin, defined as absence of skin irritation after the first application. The test was performed by an external laboratory EcamRicert (Monte di Malo (VI), Italy) in collaboration with Tuscany USL and University of Florence. The evaluation was assessed in accordance with Colipa guidelines: Cosmetic product test guidelines for the assessment of human skin compatibility, "Notes of guidance for testing of cosmetic ingredients for their safety evaluation", SCCNFP/0321/00; SCCS "Memorandum on use of Human Data in risk assessment of skin sensitization",

SCCS/1567/15, 15 December 2015; Linee Guida SIDAPA su Dermatite da Contatto, annali italiani di dermatologia allergologica 63, (2), 2009.

The evaluation was carried out by a dermatologist-allergist. The test was performed on 20 healthy volunteers with sensitive skin, with exclusion criteria as children, pregnancy and lactation period, the presence of dermatitis, allergic and inflammatory reactions and previous execution of analogue test in the last two months. The visual assessment of skin irritation is made 30 minutes and 24 hours after removal of the patch (24 and 48 hours after product application), according to scoring scale showed in the Table 2. The sum of erythema and oedema score is defined “irritation index”, which is expressed in according to the Table 3

Table 2. *Graduation of erythema and oedema parameters*

Evaluation parameters	**Grading of skin reactions**	
Erythema	no evidence of erythema	0
	minimal or doubtful erythema	0.5
	slight redness, spotty and diffuse	1
	moderate, uniform redness	2
	strong uniform redness	3
	fiery redness	4
Oedema	no sign of oedema	0
	slight, hardly visible oedema	1
	slight, clearly visible oedema	2
	moderate oedema	3
	strong oedema (extended beyond the application area)	4

Table 3. *Classification of average irritation index*

Average irritation index (AII)	Classification
<0.5	non irritating
0.5≤AII≤1.0	slightly irritating
1.0≤AII≤3.0	moderately irritating
≥3.0	highly irritating

3.11 Statistic analysis

In laboratory analysis every experimental condition was tested in triplicate. All experiments were repeated three times independently. Statistical analysis was performed by two-way ANOVA followed by Tukey's posthoc test used for multiple comparison. A p value < 0.05 was considered statistically significant. GraphPad Prism 8 (GraphPad Software, San Diego, CA) was employed. The Student's t test was used in comparisons between two groups. All data were expressed as means ± standard error. Changes in clinical data between pre-treatment and post-treatment were analysed and compared using Wilcoxon test for non-parametric data. For all analyses, $P<0.05$ was considered to indicate a statistically significant difference.

4. RESULTS

4.1 In vitro analysis

4.1.1 Cell viability

One essential aspect for the ingredients used as functional components of a cosmetic formulation, is the absence of cell toxicity (SCCS guidelines 11 rev.). Therefore, at first, we verified potential cytotoxicity of different doses of TXA (2.5-40 mg/ml), used alone or in combination with α-Arb (500 μM) on the skin cell lines used in the study. At 24 hours no effect on cell viability was observed at any dose of TXA in all cell lines investigated. Data obtained in B16 cells at 72 h treatment are reported in Fig 8. As shown, TXA had no effect on cell viability up to 10 mg/ml, whilst it decreased cell viability to 77.2% ± 8.3 and 25.4% ± 7.7, when used at 20 and 40 mg/ml respectively. The combination of TXA with α-Arb did not further significantly modify cell viability at all TXA doses. Similar behaviours were observed in the other cell lines investigated (data not shown). Based on these results, TXA was used at 10 mg/ml in subsequent experiments both when used alone or combined with α-Arb.

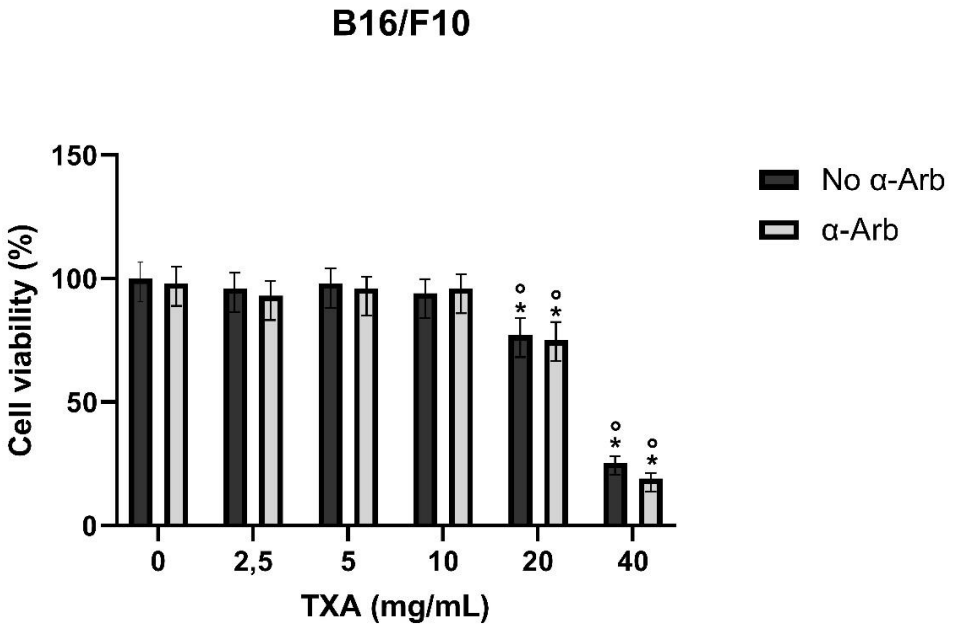

Figure 8. Effects of TXA and α-Arb on cell viability of B16/F10 cells at 72 h treatment. *Cell viability with respect to cells cultured in the absence of TXA (0) was evaluated by MTT assay. TXA was tested at different concentrations (2.5-40 mg/ml) alone or combined with α-arb (500 μM). All values are the mean ± S.D. * Significance vs. TXA 0 mg/ml, $p < 0.05$, ° significance vs. the lower TXA concentration, $p < 0.05$.*

4.1.2 Melanin content

To determine the potential ability of TXA and α-Arb in attenuating the production of cellular melanin, melanin content was measured in untreated and α-MSH-treated B16/F10 cells at 48 hours (Fig. 9). As expected, MSH increased melanin content in comparison to untreated control cells. In the absence of MSH, α-Arb and TXA used alone inhibited melanin content respectively of 30±5,1% and 19±3,7%, whilst in the presence of MSH, respectively of 36±7,2 % and 26±4,9 %. When α-Arb and TXA were used in combination, melanin content was significantly reduced in comparison to cells treated with TXA or α-Arb used alone.

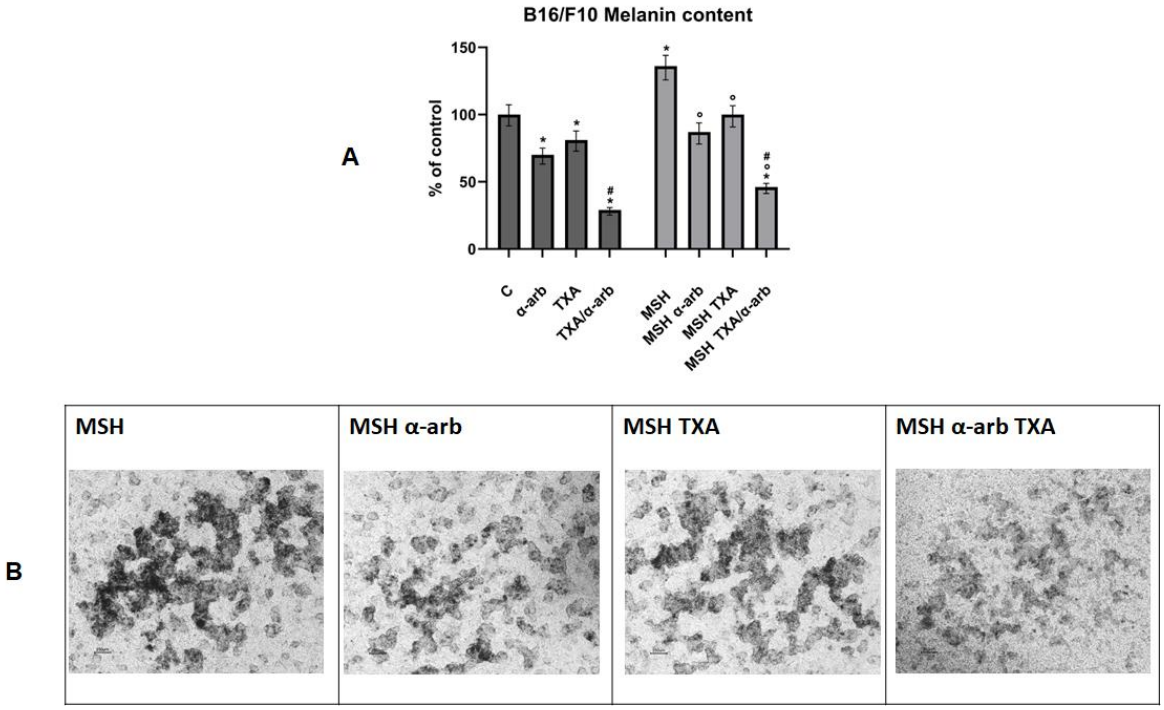

Figure 9. A: Melanin content in B16/F10 cells at 48 h treatment*. Cells were treated with TXA (10 mg/ml) and α-Arb (500 µM), used alone or combined, both in the absence and in the presence of α-MSH (10 ng/ml). All values are the mean ± S.D.*

** significance vs. control (C), p < 0.05. ° significance vs. α-MSH, p < 0.05, # significance vs. TXA or α-Arb, p < 0.05.* ***B:*** *Representative images of B16/F10 cells treated with α-Arb and TXA in the presence of α-MSH. (magnification 4x).*

4.1.3 PGE2 production

As TXA activity has been previously associated to the negative modulation of PGE2 activity (Maeda K and Tomita Y, 1998; Endo K et al., 2021), we analysed PGE2 levels in B16/F10 cells cultured in the same experimental conditions showing the melanin content

modulation induced by α-Arb and TXA. As reported in Fig. 10, PGE2 production was not modified respectively to control cells in all the tested experimental conditions. The ability of B16/F10 cells to produce PGE2 was verified in LPS-treated cells showing a significant increase in PGE2 level compared to control cells.

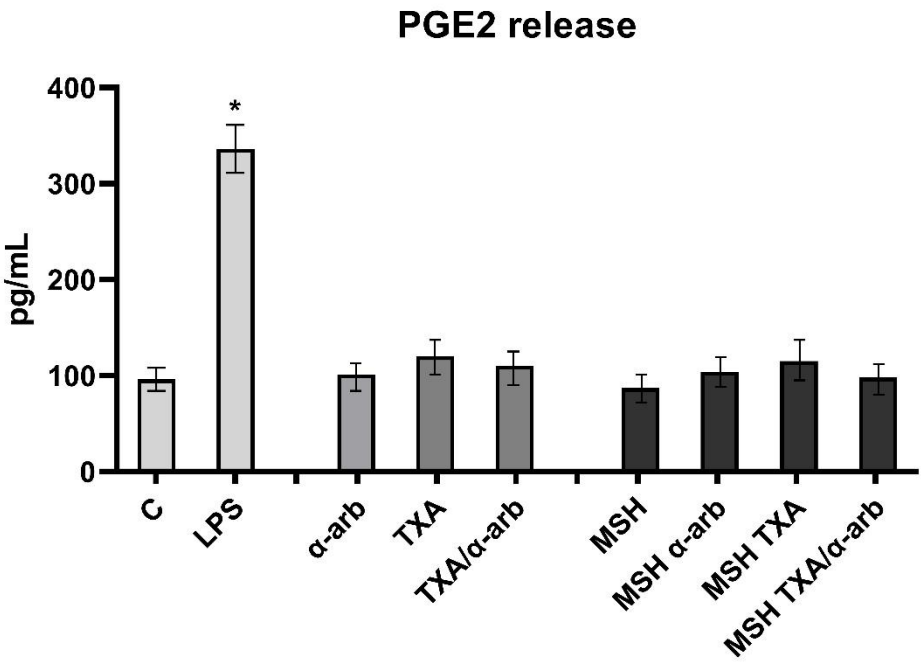

Figure 10. *PGE2 levels in B16/F10 cell conditioned media at 48 hours treatment. Cells were treated with TXA (10 mg/ml) and α-Arb (500 μM), used alone or combined, in the absence and in the presence of α-MSH (10 ng/ml). LPS (1 ug/ml) was used as a control inflammatory stimulus. All values are the mean ± S.D. * significance vs. control, $p < 0.05$.*

4.1.4 Effects of Plg (±tPA), TXA and α-Arb in HaCaT and RAW 264.7 cells

4.1.4.1 Scratch assay

The Plg/Plm system, has been previously involved in KCs migration (Cheng TL et al., 2018). Therefore, at first in a typical scratch assay experiment, we verified the effect of Plg in the absence and in the presence of tPA on HaCaT cell migration and the potential activity of TXA as a cell migration regulator. As shown in Fig. 11, both Plg and Plg + tPA treatments promoted wound recovery in a similar manner. In both experimental conditions, TXA significantly inhibited wound recovery, showing that in the selected experimental conditions, it was able to modulate KC behaviour and inhibit cell migration.

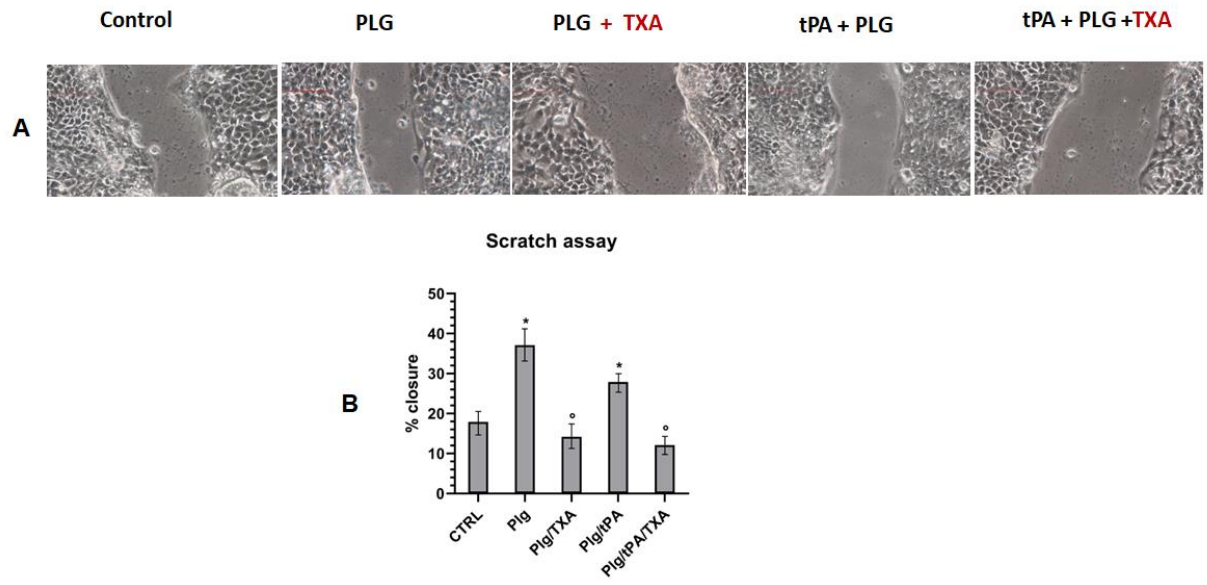

Figure 11. *Inhibitory effect of TXA treatment on Plg induced wound healing. Wound areas were formed in HaCaT cell confluent layers. Cells were treated with Plg (500nM) or Plg (500nM) + tPA (12nM) in the absence or presence of TXA (10mg/ml) for 18 h.*

A: *Phase-contrast images of the wounds at 18 h after treatments. Magnification 20X.* ***B:*** *The percentage of wound healing has been calculated as indicated in Materials and Methods. Values are presented as the mean ± S.D.; * significance vs. control, $p < 0.05$. ° significance vs. Plg or Plg/tPA, $p < 0.05$.*

4.1.4.2 Inflammatory factor production

In a first series of experiments, we analysed the effects of Plg in the absence and in the presence of tPA, as well as of TXA and α-Arb on PGE2, NO, and TNF-α production in both HaCaT and RAW 264.7 cells. As shown in Fig. 12, at 24 h in our experimental conditions no treatment induced changes in all the parameters investigated. As expected, all inflammatory molecules were significantly upregulated by LPS treatment, except for NO in HaCat cells (Facchin BM et al., 2022). When we investigated the effects of TXA, alone or in combination with α-Arb, in LPS treated RAW 264.7 cells, TXA induced a significant decrease in all the parameters investigated. Differently, α-Arb had no effect both when used alone or combined with TXA (Fig.13).

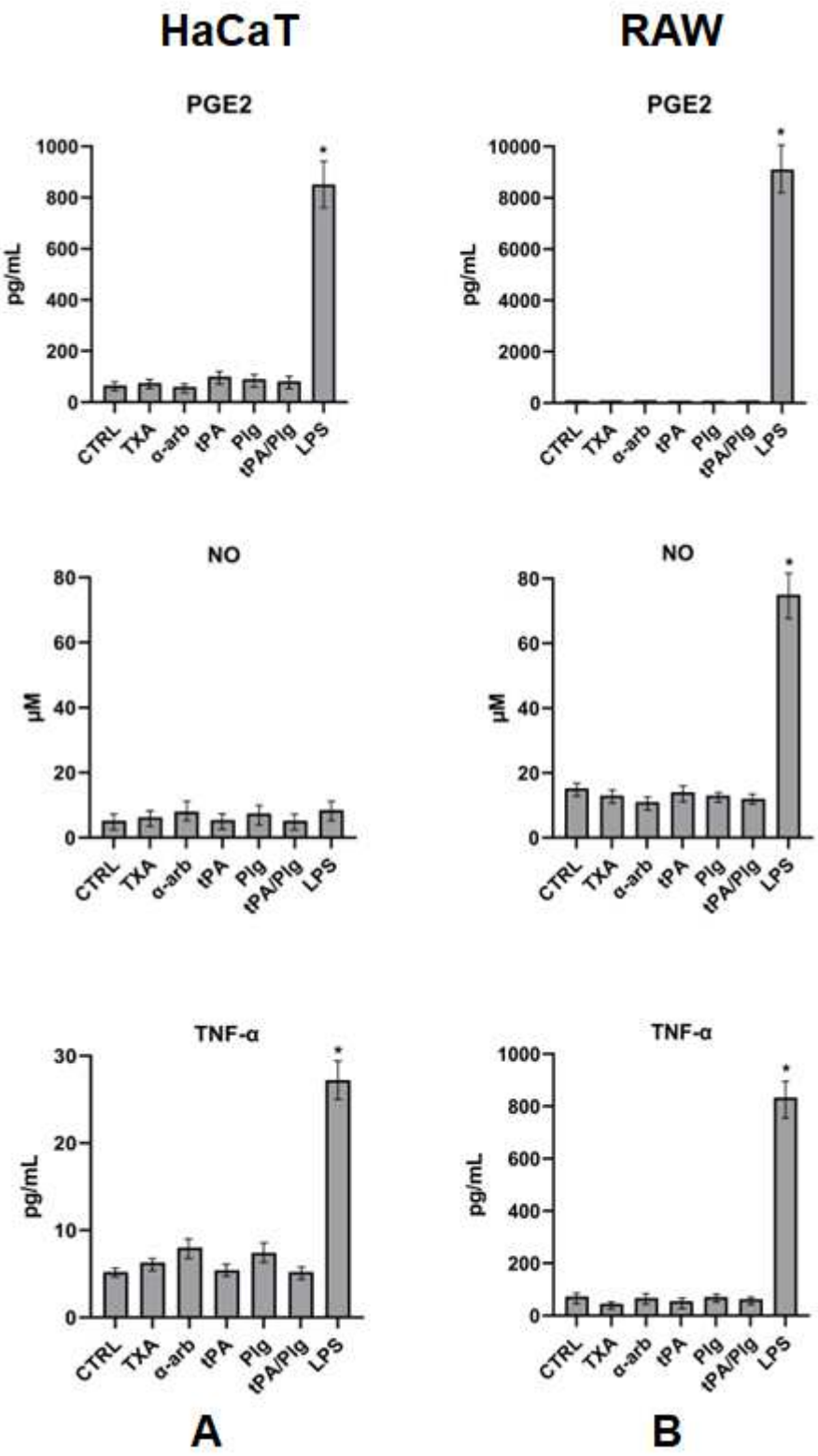

Figure 12. *Effects of TXA (10mg/ml), α-Arb (500 µM), Plg (500nM), PLg (500nM) + tPA (12nM) on PGE2, NO, and TNF-α production at 24 h in HaCaT (A) and RAW 264.7 cells (B). LPS (1µg/ml) was used as a proinflammatory control stimulus. Values are presented as the mean ± S.D.; * significance vs. control, $p < 0.05$.*

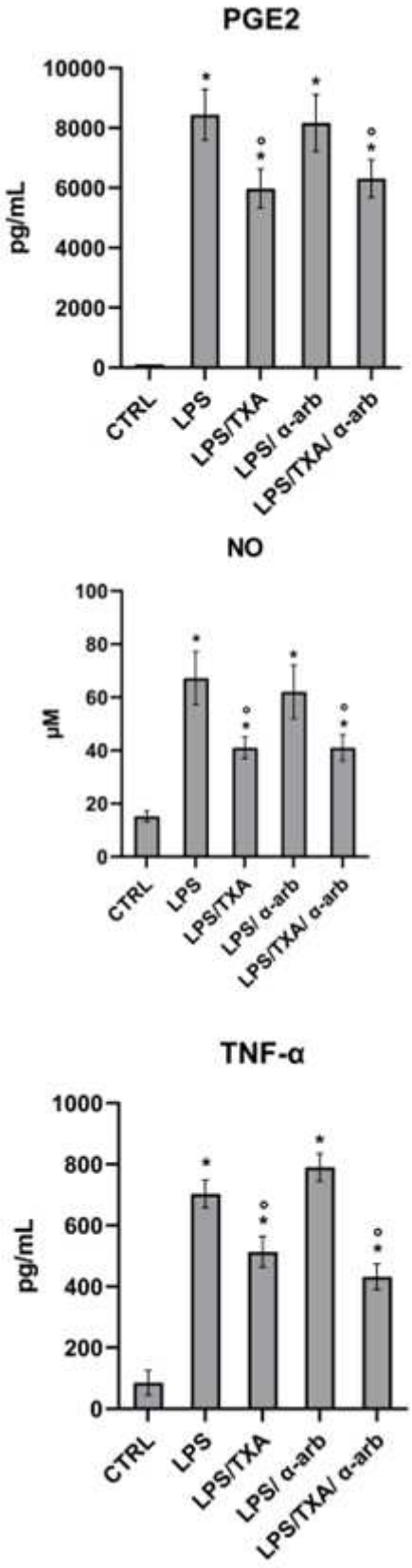

Figure 13. *Effects of TXA (10mg/ml), alone or in combination with α-Arb on PGE2, NO, and TNF-α production at 24 h in LPS (1μg/ml) stimulated RAW 264.7 cells. Values are presented as the mean ± S.D.; * significance vs control, $p < 0.05$; °significance TXA vs o TXA $p < 0.05$*

4.2 Microemulsion

4.2.1 Formation of microemulsion

To choose the optimal composition and determine changes of the microstructure of microemulsion it was necessary to build and analyse a ternary diagram. Samples with different composition of water, oil and surfactant-cosurfactant were prepared. The phase diagram of the system Surfactant/Cosurfactant (S+CoS), oil (O) and water (W) has been analysed for its external aspect. Samples which presented phase separation, crystallisation or non-isotropy indicated the area of non-existence of microemulsion (Fig. 14), while a completely transparent and homogeneous system indicated the area of its existence (Fig. 15).

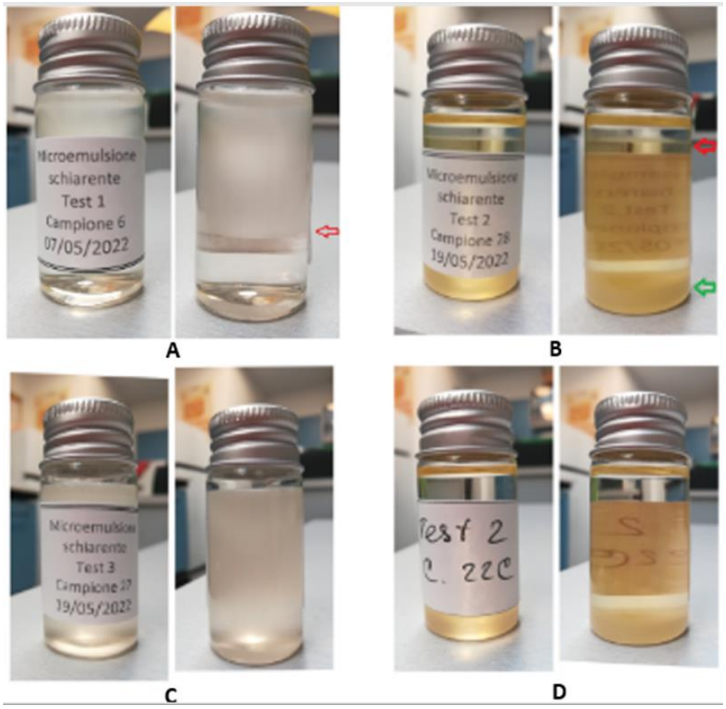

Figure 14. *Instability phenomena of microemulsion: phase separation (A), crystallization of active component and phase separation (B), non-isotropic emulsion (C), flocculation (D)*

Figure 15. *Microemulsion*

Those samples that presented a transparent system without separation interface were evaluated as points of microemulsion existence and are represented in the Fig 16 as a colored area. Every point of the ternary diagram represents a combination of three phases values (water (W), oil (O) and surfactant+co-surfactant (S+CoS)), expressed in mass percent (w/w). Every diagram represents the microemulsion without active components (A), with the only TXA 4% (B), the only α-Arb 2% (C) and with both TXA 4% and α-Arb 2% (D).

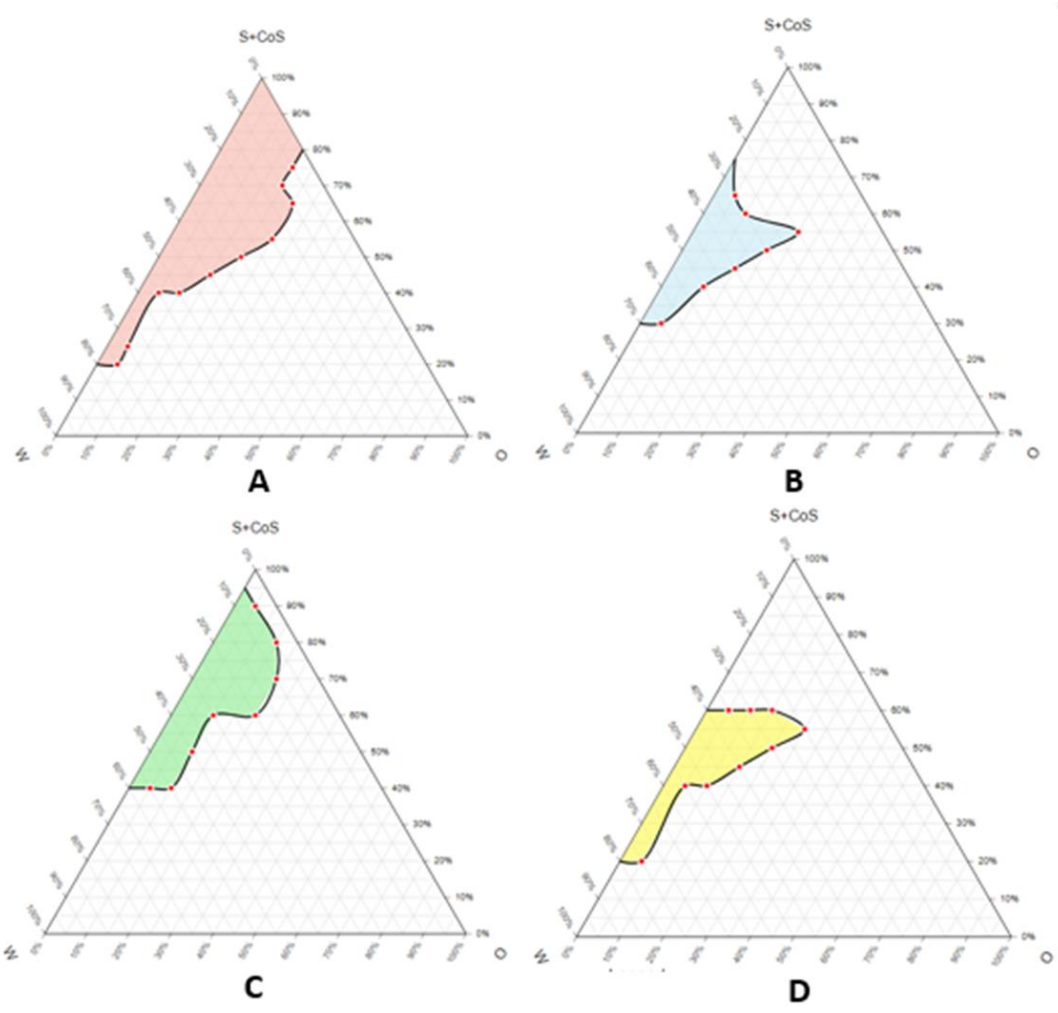

Figure 16. *Ternary diagram representing the area of existence of microemulsion without active components (A), microemulsion with TXA (B), microemulsion with α-Arb (C) and microemulsion with TXA and α-Arb (D). Every apex of the diagram represents a single component of the microemulsion: water (W), oil (O) and surfactant+cosurfactant mix (S+CoS); every side of the diagram represents a percentage of components in the dilution pathway. Colored areas represent areas of microemulsion's existence and red points represent a border composition between the area of existence and non-existence.*

The yellow area in diagram D indicates the concentration of each phase which determines the existence of microemulsion in the presence of both actives in the selected working conditions. Higher TXA concentrations were tested but showed instability phenomena of the microemulsion.

4.2.2 Dimensional analysis of microemulsion particles

At least 500 particles of microemulsion have been analysed in order to obtain a good statistical representation. The particle dimensional distribution has shown the values between 37 and 590nm for the length and between 23 and 560 for the strength. Median values respectively for the length and strength were between 215 and 146 nm. In addition, values of D10, D50 and D90, which represent respectively 10%, 50% and 90% of particles were found of 113, 215 and 352nm for length and 75, 146 and 234nm for strength.

Fig.17 shows a microemulsion's particle appearance at SEM microscopy at 15000x, 80000x, 125000x and 240000x.

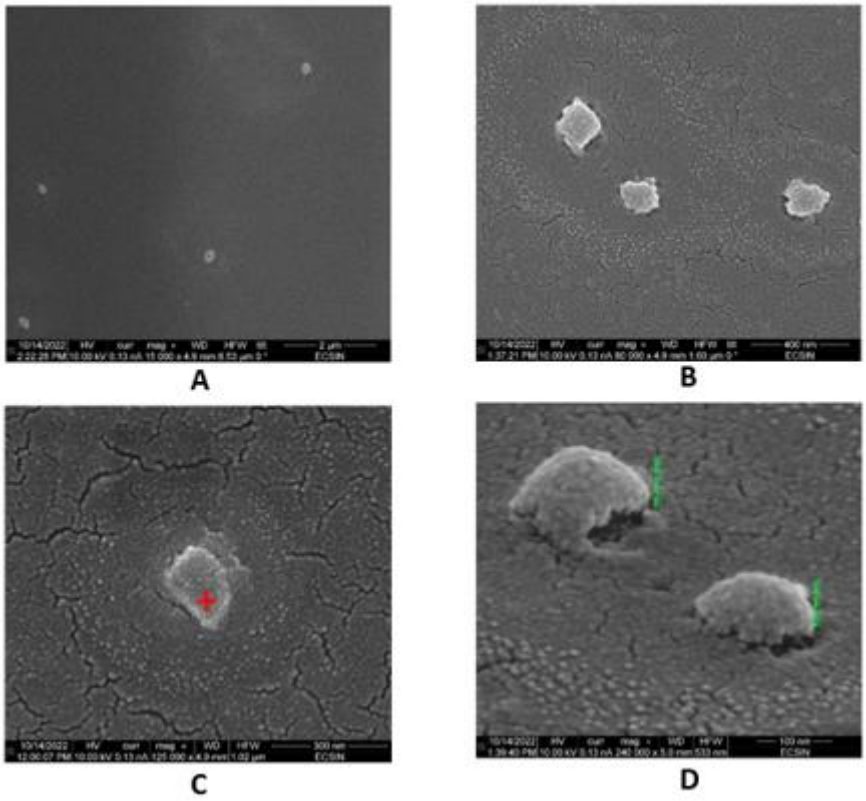

Figure 17. *Microemulsion particle size imagine with 15000x (A), 80000x(B), 125000x (C) and 240000x (D) magnification obtained with SEM microscopy*

4.2.3 Stability evaluation

To evaluate the stability of the microemulsion, several parameters of the product were measured within time: weight loss, pH, density and humidity at T0, T30, T60, T90 and T180 in different temperature and humidity conditions. The parameters analysis was performed in neutral container (glass) and in the final packaging (PE). All the measurements were performed in triplicate.

4.2.3.1 Comparison of variations at T0 and T180 between the product tested in neutral container and in PE packaging.

Since the packaging can influence the quality of the product and therefore its essential characteristics of efficacy, safety and stability, we have performed a comparative test between the product inserted into the neutral packaging (glass) and in the PE packaging. As shown in the Table 4, no significant variation was observed for all the parameters neither in neutral packaging nor in PE container, therefore, no significant interaction between the product and packaging was in course and the product can be considered stable. The only increase of density has been already observed between T0 and the first month of test (data not shown) and it was maintained till T180. It was probably due to the Ostwald ripening phenomenon, which consists in the incrementation of the particle dimension aimed to reach a thermodynamic stability (Wennerström H and Olsson U, 2009).

Table 4. *Comparison of variations at T0 and T180 between the product tested in neutral container and in PE packaging*

Parameter	**Product in neutral packaging (glass)**		**Product in PE packaging**		**p-value**
	T0	**T180**	**T0**	**T180**	
Weight*	11,82±0,42	11,76±0,33	85,16±0,85	83,6±0,94	p>0,05
pH	4,05±0,01	4,25±0,05	4,10±0,001	4,32±0,09	p>0,05
Density	0,717±0,01	1,039±0,001**	0,717±0,002	1,038±0,0007**	p>0,05
Humidity	56,52±0,001	56,806±2,14	56,52±0,001	55,95±4,69	p>0,05

*The evaluation was performed in neutral packaging with the capacity of 15mL, and in PE packaging with the capacity of 100mL

** Significance T180 versus T0

4.2.3.2 Comparison between testing condition and standard (+8°C)

As shown in Tables 5 and 6, in condition of accelerated test (+40°C) the humidity parameter presented the variation of more than 5% in both neutral and PE packaging. Moreover, in condition of freeze test (-20°C) more than 5% of humidity loss was observed in PE packaging. For the other parameters there were no significant variations.

Table 5. *Variation of parameters at different temperature conditions versus standard of the microemulsion in neutral container.*

Product in neutral packaging (glass)				
	Weight	**pH**	**Density**	**Humidity**
Photostability	0,17%±0,03	0,41%±0,09	0,00%±0,00	-0,74%±0,08
+40°C	2,26%±0,01	1,11%±0,15	0,42%±0,05	8,23%*±0,58
-20°C	-0,09%±0,02	-0,54%±0,03	-0,14%±0,02	0,00%±0,01
Stress test (+40°C/+8°C)	-0,18%±0,01	2,10%±0,24	-0,14%±0,04	-0,11%±0,02

*Significant variation versus established acceptance criterion of 5%.

Table 6. *Variation of parameters at different temperature conditions versus standard of the microemulsion in PE container.*

Product in PE packaging				
Test condition/parameter	**Weight**	**pH**	**Density**	**Humidity**
Photostability	1,07%±0,08	0,27%±0,09	0,00%±0,00	2,32%±0,24
+40°C	4,94%±0,46	0,97%±0,12	0,00%±0,02	17,11%*±2,73
-20°C	-0,43%±0,16	-2,16%±0,26	0,14%±0,04	-5,06%*±1,06
Stress test (+40°C/+8°C)	1,61%±0,22	3,42%±0,33	-0,14%±0,05	1,29%±0,13

*Significant variation versus established acceptance criterion of 5%.

4.2.3.3 Gravity acceleration

The test has been performed in condition of 6000 rpm for 30 minutes at T0, T30, T60, T90, T180 both for the microemulsion in neutral and in PE packaging. No sign of phase separation has been observed. Therefore, the microemulsion does not lose its form during a long time.

4.2.3.4 Microbiological test (Challenge test)

As established by the Regulation 1223/2009, the preservative system of microemulsion resulted adequate to break down the proliferation of microorganisms, satisfying criterion A.

4.2.4 Patch test on sensitive skin

Patch test was performed to determine the compatibility of the product with the sensitive skin tolerability. It was performed on 20 healthy volunteers with sensitive skin.

The tested product, resulted in a mean index of irritation is represented in the Table 7.

Table 7. *Mean index of irritation*

Mean index of irritation	**Timing and conditions**
0.00	30 minutes after patch removal (24 hours after patch application)
0.00	24 hours after patch removal (48 hours after patch application)

According to the evaluation scale used (Table 3) the product resulted not irritating with the average irritation index of <0.5.

4.3 Clinical data

20 patients with diagnosticated melasma and/or PIH were evaluated by two dermatologists during the first visit and after 2 months of application of the microemulsion. In all the cases no side effects like irritation, inflammation, redness, ochronosis, sensitisation, typical for the most of whitening treatments, were observed.

4.3.1 MASI evaluation

The reduction of melasma was evaluated by two independent dermatologists using a MASI score in 20 subjects. MASI is a globally accepted system for the evaluation of spots quality, considering their width, frequency and intensity. A statistically significant MASI

reduction of 21%±0,23 has been observed. Results of the evaluation are shown in the Table 8.

Table 8. *The improvement of MASI score at T0 and T2*

Mean±SD		**Improvement, %**	**N**
T0	**T2**	21%±0,23	20
11±7,71	8.90*±6,78		

*Significance T2 versus T0, p<0.05

4.3.2 Clinical evaluation using VISIA® System

The VISIA® Complexion Analysis System was used in 10 subjects to evaluate complexly the area, intensity and dimension of spots, translating it into the score for three main parameters involved in melasma: UV Spots, Brown Spots and Red Areas. Data analysis at T0 and T2 showed a statistically significant reduction of UV spots of 25,4%±0,15. A decrease was also observed in the other parameters (10,7%±0,14 of brown spots; 5,7%±0,16 of red areas), although differences did not result statistically significant (Table 9).

Table 9. *Improvement of UV spots, brown spots and red areas*

	Mean±SD		**Improvement, %**	**N**
	T0	**T2**		
UV spots	27,28±7,65	20,59*±9,14	25,4%±0,15	10
Brown spots	25,80±5,19	23,53±6,41	10,7%±0,14	
Red areas	14,04±4,66	13,45±5,77	5,7%±0,16	

*Significance T2 versus T0, p<0.05

In Fig.18, representative photos obtained by VISIA® System which showed the improvement of three parameters.

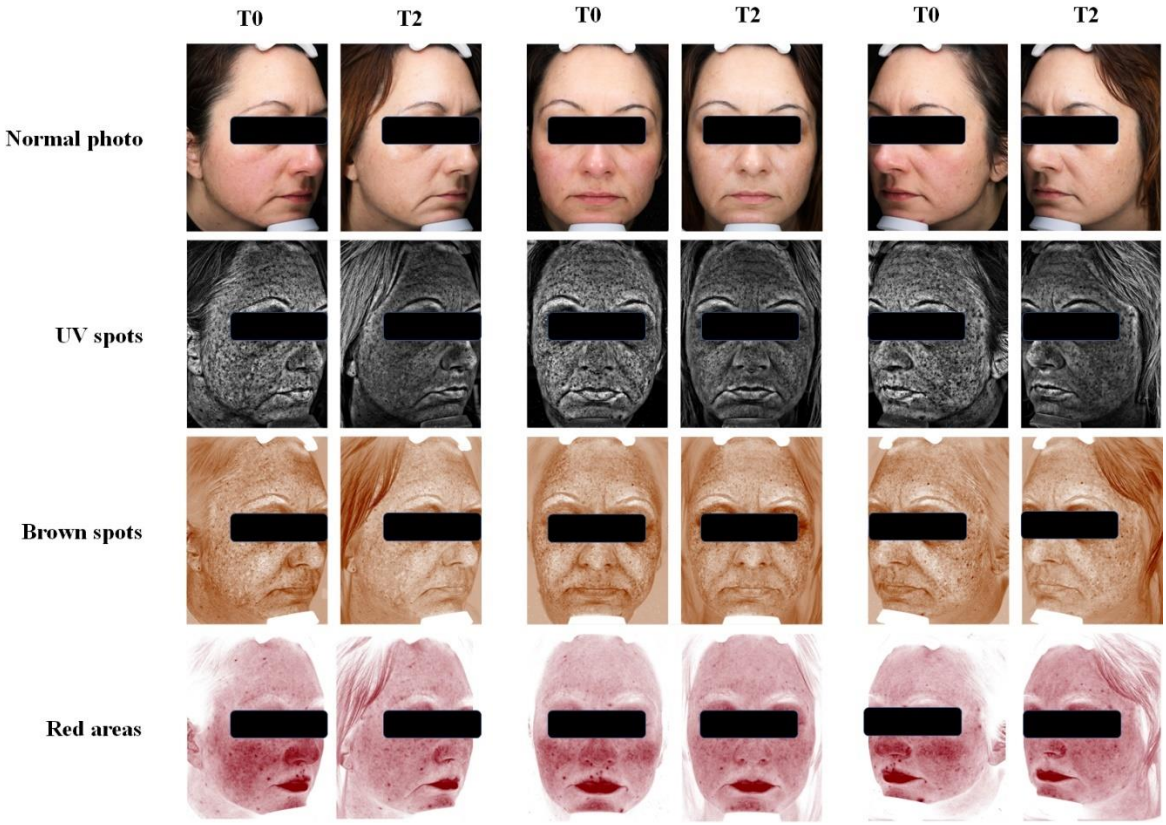

Figure 18. *Improvement of superficial UV spots, deep brown spots and red areas*

5. DISCUSSION

Skin hyperpigmentation disorders, first melasma, remain a challenge in dermatology and cosmetics. To date no specific treatment has solved and reached adequate amelioration, also because of the incomplete understanding of melasma pathogenesis and the molecular mechanisms involved. Complexity of melanogenesis originates from the multiple signalling pathways regulating the production of melanin in melanocytes and its transfer to KCs, as well as from the strict interplay and communication among melanocytes, other skin cells, and inflammatory cells which are regulated by several exogenous, mainly UVR, and endogenous stimuli (Koike S et al., 2020).

To date, both *in vitro* and *in vivo*, Tyr inhibitors have been the most known and investigated melanogenic inhibitors for melasma treatment, as Tyr is the key melanocyte enzyme involved in melanin biosynthesis. However, because of the growing knowledge in melanogenesis regulation, the recent trend in cosmetics is to target not only melanin synthesis but other melanogenic inducing events, also combining molecules with the potential to influence different activities and/or cells involved, to obtain better outcomes (Cassiano DP et al., 2022; Taraz M et al., 2017). On this basis, the main aim of this study was to generate a new cosmetic product for the topic treatment of melasma. To this purpose, we evaluated the combination of two molecules with different properties, specifically α-Arb and TXA, in a single formulation for topical use. α -Arbutin is an "old" whitening agent, known for its ability to inhibit melanogenesis by means of TYR inhibition by binding its copper active site (Boo YC et al., 2021). TXA is mainly known as an inhibitor of the Plg/Plm system and was selected for several reasons. First, TXA, as a potent antifibrinolytic agent, is considered safe and is commonly used as a drug in humans for several surgical conditions with excessive bleeding (Prudovsky I et al., 2022); second, several studies report that the Plg/Plm system is activated by UV, the main skin stress involved in increased skin pigmentation. Third, the Plg/Plm system has been associated to the regulation of inflammation and recent papers have emphasized the role of inflammation and cytokines in melanogenesis (Fu C et al., 2020; Hossain MR et al., 2021). Finally, some previous papers have shown the ability of TXA treatment, mainly by oral assumption, in reducing skin pigmentation in melasma (Chowdhary B et al., 2021; Feng X et al., 2021; Agamia N et al., 2021). The new cosmetic product generated in this project has been evaluated for different aspects: 1) the safety and potential efficacy of the combined

functional ingredients by using *in vitro* cell models; 2) the choice of the delivery system; 3) the stability of the finished product; 4) the efficacy in inducing skin whitening effect in a planned clinical study in subjects with melasma. All evaluations were performed in agreement with the Regulation 1223/2009 concerning cosmetic products and more specifically with the Regulation 655/2013 establishing rules about the correct communication based on scientific evidence. Despite data showing clinical efficacy (Chowdhary B et al., 2021; Feng X et al., 2021; Agamia N et al., 2021), few studies have investigated the TXA effects on skin cells involved in melanogenesis, reporting fragmentary and sometimes conflicting results (Desai S et al., 2019; Cho YH et al., 2017). Therefore, in the first phase of the project, we evaluated the effects of TXA and α-Arb in *in vitro* experiments by using cell lines commonly used as models in studies on melanogenesis and whitening agents (Chung S et al., 2019; Niwano T et al., 2015, Facchin BM et al., 2022). Both the safety as well as the potential efficacy in modulating events related to melanogenesis were investigated. Cell viability data showed a well TXA tolerability in all tested cell types. Only concentrations over 10 mg/ml and used for a prolonged 72-hour treatment, induced a significant decrease in cell viability in all investigated cell lines. This result agrees with previous studies performed both in melanoma cell lines as well as in other cell types including connective cells (Wang F et al., 2021, Eikebrokk TA et al., 2019, Law ME et al., 2022) and suggested concentrations to be used in further experiments. We also verified that 500 μM α-Arb, the dose selected in our experiments, did not modify cell viability, both when used alone or combined with TXA. As increased melanin production is one of the main melasma characteristics (Chung BY et al., 2014), then we evaluated the effects of TXA and α-Arb, alone or combined, on melanin production in B16/F10 cell line. To our knowledge, few reports have investigated the effects of TXA on melanin production in cultured melanocytes with conflicting results (Cho YH, et al., 2017, Maeda K and Tomita Yasushi, 2007). Our results confirm a melanocyte direct TXA inhibitory effect on melanin production in B16 cells, both in the absence and in the presence of α-MSH, used to stimulate melanin production (Cho YH, et al., 2017). Notably TXA effect was additive to that induced by α-Arb, suggesting that the combination of the two molecules may induce better outcomes when used *in vivo*. As previously proposed mechanisms for TXA effects include the modulation of PGE2 release (Endo K et al., 2021; Desai S et al., 2019) known as positive regulators of melanogenesis (Fu C et al., 2020), we also investigated potential changes in PGE2 levels during melanin production in B16/F10 cells. The absence of any change in PGE2 levels indicated that TXA and α-Arb effects were not related to the autocrine modulation of this

promelanogenic effector, at least in these cells. In vivo melanogenesis regulation relies on the close communication of melanocytes with different cell types, primarily KCs, and both KCs and inflammatory cells directly or indirectly impact on melanogenesis releasing inflammatory factors (Fu C et al., 2020; Hossain MR et al., 2021; Yuan XH et al., 2018). Several authors suggest that TXA, inhibiting Plm formation may be involved in the regulation of the proinflammatory events induced by the activation of the Plg system and that this activity may be involved in TXA skin whitening effects (Perper M et al., 2017; Wang N et al., 2004; Baker SK et al., 2020; Prudovsky I et al., 2022). Although a direct link of the Plg/Plm system with melanogenesis has not been reported, KCs bind Plg and produce Plg activators in response to both proinflammatory cytokines and exogenous stimuli, especially UV (Rox JM et al., 1996; Takashima A et al., 1992; Miralles F et al., 1998; Marschall C et al., 1999). Further, activation of this system regulates KCs and macrophage migration, as well as the release of inflammatory molecule in human monocytes, and mouse bone marrow-derived macrophages and astrocytes (Reinartz J et al., 1994, Syrovets T et al., 2012; Zalfa C et al., 2019; Pontecorvi P et al., 2019; Carmo AA et al., 2014). With the aim to investigate potential activity of TXA, as a Plg activation inhibitor, we treated cells with exogenous added Plg or Plg + tPA, in the absence of serum according to recent reports (Zalfa C et al., 2019; Pontecorvi P et al., 2019) and evaluated cell migration and inflammatory mediator release. Scratch assay data obtained in HaCaT indicated that 10 mg/ml TXA significantly inhibited Plg or Plg+tPA induced cell migration, in agreement with previous results reported in Plm treated RAW 264.7 cells (Carmo AA et al., 2014). No α-Arb interference on cell migration was identified (data not shown). This result shows that HaCaT could be regulated by TXA and confirms the TXA ability, at the concentration used in this study, to inhibit the Plg/Plm system and modulate KCs activities (Reinartz J et al., 1994; Bharadwaj AG et al., 2021). Although we did not measure Plm levels, we are confident that TXA effects were related to a TXA-induced decrease in Plm generation. In fact, it has been reported that TXA inhibits Plm formation in vitro (Longstaff C et al., 2019; Wu TB et al., 2020) and Plm and Plg by means of Plm formation promotes HaCaT migration by directly cleaving ECM components or indirectly through MMP activation (Szabo, I. et al., 2004; Reinartz J et al., 1994; Ny L et al., 2020; Bharadwaj AG et al., 2021). Melasma histopathological characteristics include BM degradation (Esposito ACC et al., 2022) and Plg/Plm system is activated by UV exposure in KCs (Takashima A et al., 1992; Miralles F et al., 1998; Marschall C et al., 1999). Therefore, our data suggest that TXA might inhibit UV induced matrix degradation preventing Plm formation and the consequent BM damage, in line with a previous study

performed in skin equivalents (Ogura Y et al., 2008*)*. Anyway, we cannot exclude other TXA molecular mechanisms (Law ME et al., 2022). Differently from scratch assay data, both in HaCaT and RAW 264.7 cells treated with tPA, Plg or Plg + tPA, no change in the levels of the inflammatory molecules investigated, including PGE2, NO and TNF-α was observed. These results are not in agreement with those reported in other cell types, including mouse bone marrow-derived macrophages and astrocytes, (Zalfa C et al., 2019; Pontecorvi P et al., 2019) by using the same experimental treatments and the Plm induced cytokine production in human monocytes (Carmo AA et al., 2014, Syrovets 2001, 2002). As our cells were active in the production of inflammatory molecules under LPS stimulation and cell migration was induced by Plg in HaCaT, we might speculate that in our experimental conditions, the amount of Plm was not sufficient to activate the expression of inflammatory factors. Also, our cells might differ from those used in previous studies for the expression or functionality of multiple components of the Plg/Plm system or protease activated receptor (PAR) which have been involved in cytokine modulation induced by Plm (Pontecorvi P et al., 2019). Further investigation and different experimental conditions are under investigation to clarify the reason for these apparent conflicting results (Bharadwaj AG et al., 2021). Finally, we investigated the potential anti-inflammatory activity of TXA in LPS treated RAW 264.7 cells showing that TXA could significantly inhibit the release of inflammatory molecules tested, in agreement with a similar effect, recently reported in rosacea-like mice and HaCaT treated with cathelicidin antimicrobial peptide (Camp) (Li Y et al., 2019). Interestingly, our data provide evidence that TXA may inhibit TLR-4 signalling, limiting release of inflammatory factors involved in melanogenesis regulation. Of note, these data are in line with previous studies showing reinforcement of TLR4 and TLR9 activation by Plm in monocytes (Shimazu H et al., 2017). Although further studies are required to elucidate the link between TXA and TLRs and the molecular mechanisms involved, these data appear of interest also taking into account recent findings which have identified a connection between TLRs and melanogenesis, and the TLR role in UV-mediated inflammation and other skin disease (Tam I et al., 2019; Koike S, Yamasaki K, 2020; Dickinson SE, Wondrak GT. 2019; Li Y et al., 2019).

Analysis of the *in vitro* effects was associated with the development of the new cosmetic product. In previous studies showing TXA ability to control melasma, oral assumption was mainly used (Chowdhary B et al., 2021; Feng X et al., 2021; Agamia N et al., 2021). Here we aimed to realize a product for topical delivery, which represents an attractive alternative to combine drug utilization as well as avoiding potential systemic side effects (Nastiti

CMRR et al., 2017). Recently, microemulsions are of great interest among both pharmaceutical and cosmetic industry since they have a great ability of active component vehiculation and of gradual release deep in the skin, more efficiently than other preparations (Egito EST et al., 2021). As, this is true especially for hydrophilic molecules, like TXA and α-Arb we formulated a microemulsion. The existence of microemulsion was confirmed by a microscopic analysis which identified almost regular, non-spheric and well-dispersed particles of median dimension in the length 215 nm and in the strength 146 nm (Mcclements DJ, 2012; Nastiti CMRR et al., 2017). The resulted microemulsion contained an elevated amount of water and active ingredients, which allowed the use of high concentrations of the actives, specifically 4% TXA and 2% α-Arb (w/w). Interestingly, the resulted microemulsion might be used as a model for other cosmetic products containing hydrophile active components.

When we evaluated the stability parameters, data showed a high stability, even under different temperatures and packaging materials. Only an increase of density has been observed between T0 and the first month of test, probably due to the Ostwald ripening phenomenon (Wennerström H and Olsson U, 2009). However, this variation is not relevant for the stability since it was not changed for the rest of the testing period. Further, we observed that the product tends to change humidity in the condition of elevated temperature or reduced temperature, giving relevant indication for the industry to maintain the microemulsion in the humidity and temperature conditions between +8 and +25°C during both transport and storage. Anyway, the globally high stability indicated the capacity of the product to maintain its characteristics for a long time and in cosmetics, it is essential for its efficacy, safety evaluation and defining of a standard reference for the compliance of acceptability of the product. Also, microbiological analysis showed that the product is able to maintain its integrity independently of the external factors, and the result of Patch test has shown an excellent tolerability even in subjects with sensitive skin. Consequently, the new microemulsion can be considered already available for the following phases of the industrial development including manufacturing and placing on the market.

The cosmetic formulation was finally evaluated *in vivo* in subjects affected by melasma. Although to date, we were able to collect only few data, results have shown that the formulation was efficient and did not induce side effects as irritation, sensitisation, ochronosis or allergy reactions, typical for homologous treatments. Regarding efficacy in reducing melasma, the evaluation of MASI score has shown a significant improvement of 21%. We have attempted to compare our data with the efficacy of other treatments,

including oral or topical TXA, although direct comparison is not easy because of the high variety in the concentration, type of formulation, combination with other whitening treatments, treatment duration, as well as the variability of patient's response to the treatment (improvement of 5-50%). Nevertheless, our data are in line with literature data at two months treatment, both for oral (Sahu PJ et al., 2020; Feng X et al., 2021) and topical TXA treatments (Feng X et al., 2021; Austin E et al., 2019). Moreover, to our knowledge, only one previous clinical trial investigated treatment with Arb used alone (Paik JH, Lee MH et al., 2000) with a 10% reduction of MASI score. Therefore, at present clinical data do not permit to conclude about advantages in using combined actives in our formulation. In addition, in a group of patients the recent Visia® Complexion Analysis System was employed to evaluate efficacy showing an improvement in all the parameters analysed, in line with MASI results, although only reduction in UV spots was significant. This may be related to the limited number of subjects evaluated or may suggest a better response to treatment in the superficial epidermal hyperpigmentation.

Indeed, we are aware of the limitations of our clinical study because of the small sample size and the lack of comparison with subjects treated only with α-Arb or TXA, anyway we have shown that combination of TXA and α-Arb, introduced in a microemulsion, improves melasma *in vivo*, and it does not cause side effects typical for topic applications and oral TXA. The clinical trial in course foresees the increase of number of patients to 100 and the extension of treatment period from 2 to 4 months.

6. CONCLUSION

In conclusion, in this study we have developed a new cosmetic formulation combining molecules with potential different activities on the skin cells involved in melanogenesis. Our major findings are as follows: 1) *in vitro* results on melanocytes, KCs and macrophages, which play different roles in skin pigmentation, have shown that TXA, a known antifibrinolytic agent, has multiple biological functions that potentially may impact on *in vivo* melanogenesis and suggest that its combination with α-Arb, a common TYR enzyme inhibitor, may lead to a more powerful whitening activity than every single compound used alone. Interestingly, for the first time in mouse macrophage cell line we have shown that TXA inhibited the LPS induced release of proinflammatory molecules with promelanogenic activities, indicating interference among TXA and inflammatory events related to TLR activation. As TLRs are involved in melanogenesis, as well as in other inflammatory skin disease, we retain these data deserve further investigation; 2) the *in vitro* data were related to a good *in vivo* response to the new product. Certainly, with the obvious limitations of the number of enrolled subjects, data from the clinical study have shown that the new formulation is able to penetrate the skin, it is effective and it can induce significant amelioration of melasma, without installing of any side effect; 3) the evaluation of the formulation has shown that it meets cosmetic regulatory requirements as it is stable, well tolerable and essentially safe, so that it is currently under the phase of manufacturing and placing on market.

Although further clinical data are necessary for an adequate comparison of its efficacy to other treatments, these results encourage the use of microemulsion in cosmetic formulation, as well as the use of TXA in treating melasma and potentially other skin diseases, related to inflammatory events.

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