EPIGENETIC MODIFICATIONS AND AUTOPHAGY ASSOCIATED TO UV RADIATIONS ON SKIN MODEL

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INTRODUCTION

Epigenetic refers to the study of heritable changes in gene expression that occur without a change in DNA sequence. Three distinct mechanisms appear intricately related in initiating and sustaining epigenetic modifications: RNA-associated silencing, DNA methylation and histone modification.
Human skin aging is characterized by a low-grade systemic inflammation, a condition that has been designated as "inflamm-aging". Mediators of the inflammatory response can be further associated to environmental stress and can induce genetic and epigenetic changes which contribute to the photo-aging process. Moreover, UV radiations induce autophagy, a catabolic process that regulates cellular response to UV and directly contributes to the process of photo-aging.
Different experimental protocols on a human 3D reconstructed skin model have been developed in order to recapitulate, at molecular and morphological level, the damages associated to different exposure times, doses and UV radiation type, which are responsible to induce inflamm-aging and epigenetic modifications in skin. In particular, the inflamm-aging model has been applied to assess the efficacy of a new generation of dermo-cosmetic products designed to provide an early and long lasting protection against aging and photo-aging processes.

In the present study, the following read-out parameters have been evaluated:

DNA methylation: it has been observed that chronic inflammation, also induced by UV irradiation, markedly accelerates acquisition of DNA methylation changes. Moreover, the DNA hypermethylation was consistent with the finding of enhanced levels of DNMTs in the UV-exposed mouse skin and UV-induced skin tumors (Katiyar 2012)

Specific miRNA pathway and NF-kb activation: it is associated to many processes linked to UV damage response such as inflammation and also autophagy. In particular, it has been reported that UV radiation can induce autophagosome formation and upregulation of autophagy markers through specific regulation pathways (Sample 2016).

INFLAMM-AGING MODEL

Inflamm-aging is believed to be a consequence of a cumulative lifetime exposure to subclinical inflammatory responses, tissue damage and production of reactive oxygen species that cause oxidative damage and also elicit the chronic release of inflammatory cytokines. This results in a vicious cycle, driving immune system remodelling and favouring chronic pro-inflammatory state and morphological I modification of the tissues (Baylis 2013).

In order to induce an **inflamm-aging stimulus**, a light mechanical stress (abrasion) has been used to induce the keratinocytes inflammatory response in association with exposure to 12 J/cm^2 UVA.
Inflamm-aging has been evaluated by MicroRNA (miRNA) analysis (mir-22 and mir-126) to identify an early regulatory pathway (6H after stress induction) and the expression level of inflammation (NF-kb) and ageing-associated target genes (CCN1, CCN2 and SIRT1) in the further 24H read out, associated to a morphological analysis.

Fig.1

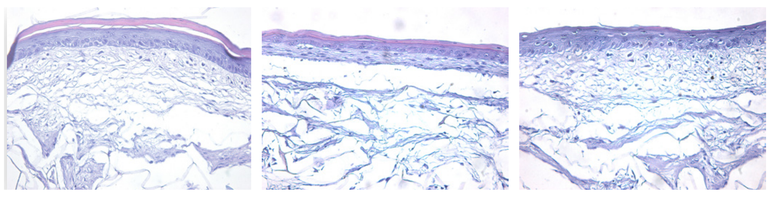

Fig.1. Haematoxilin and Eosin staining of FT-skin model in negative control (not irradiated) compared to Positive control (stressed by UV and abrasion) and Ceramage treated sample 6h after stress.

The morphology and architecture of the UVA treated sample appear severely modified in the dermal compartment (papillary upper dermis) which results thinner and less compact.
In the sample treated with Ceramage the extracellular matrix of papillar dermis appears fully restored.

EARLY REGULATORY PATHWAY BASED ON MiRNA

miRNA (miRs) are considered as modulators of distinctive pathways which are potentially related to inflammation, cellular senescence, and age-related diseases. miR-21 and -126, targeting messenger RNAs belonging to NF-kb pathway, are classifiable as senescence associated (SA-miRs), inflammation-associated (inflamma-miRs). They target the NF-kb pathway primarily through feedback loop aimed at induce pro-inflammatory response following signalling activation (Olivieri 2013).

miR-21 overexpression enhances the inflammatory response. Furthermore miR-21 is able to reduce the expression of potent human anti-inflammatory molecules such as interleukin (IL)-10 and TGF-beta.

miR-126 plays an important role in the modulation of inflammatory activity by down-regulating the expression of IKBA, an important inhibitor of the NF-kb signalling.

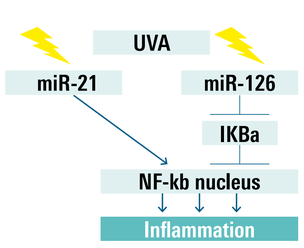

Fig.2

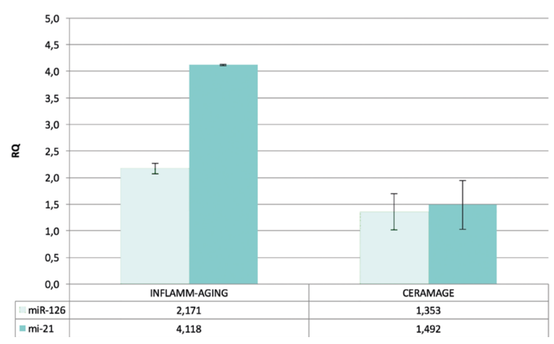

Fig.2. miRNA relative expression in Positive control and Ceramage treated in comparison with negative (control set as 1) after 6H. Values are expressed as average Relative expression (RQ) and error (RQ max-RQ min).

In the inflamm-aging model, at 6H the UVA radiation (12 J/cm2) has determined the up-rising of inflammatory response, via NF-kb pathway and based on direct over-expression of stimulatory (by mir-21 up-regulation) and indirect inhibition of negative regulator IKBa (by mir-126 up-regulation).
The treatment with **Ceramage** has exerted an early anti-inflammatory mechanism on regulatory miRNA expression, restoring an expression profile close to the negative control (not stressed control) thus confirming a significant efficacy in maintaining the homeostatic skin balance.

Fig.3

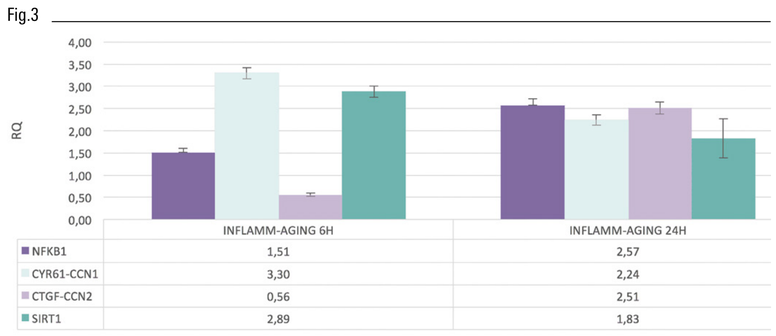

Fig. 3. Relative Gene expression in Inflamm-aged model at 6 and 24H after irradiation in comparison with negative control (set as 1). Values are expressed as average Relative expression (RQ) and error (RQ max-RQ min).

The tissue response to the induced stress, in terms of miRNA, has been further confirmed by the gene expression of NF-kb, in order to better describe the mechanism of action connected to inflamm-aging process in early and delayed read out.

NF-kb, the master regulator of inflammatory response which can also regulate several homeostatic responses such as apoptosis, autophagy and tissue atrophy (Salminen 2009) has shown an over-expression at 24H confirming its activation as a consequence of miRNA regulation.

Regarding the genes connected to senescence, CCN1 and CCN2, UVA irradiation has exerted a general stimulatory effect on both genes after 24H and a strong activation on CCN1 starting from 6H. This data confirm the involvement of these genes in UV-induced aging phenomenon as inflammation, collagen matrix degradation and metalloprotease activation (Quan, 2011; Jun and Lao 2016).

At 6H after irradiation, in inflamm-aging model an early defence response has been detected due to SIRT1 activation. This gene regulates various cellular processes including apoptosis, stress response, metabolism, and tumorigenesis. Increasing evidences have suggested that SIRT1 plays an important role in DNA repair (Fan 2010).

UV-INDUCED EPIGENETIC MODIFICATIONS

Ft-skin model has been irradiated with different doses and type of UV radiation as reported in Fig. 4 and the following parameters have been quantified after exposure.

- DNA Methylation
- Gene expression profile of the target genes NF-kb, CCN1, CCN2 and SIRT1

Fig.4

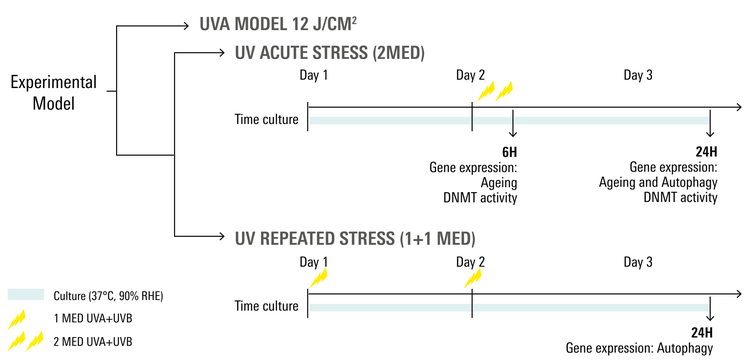

Fig. 4. Representative picture of experimental design for UV-induced epigenetic modifications.

DNA METILATION

DNA methylation is an epigenetic mechanism involving the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine. DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA.
DNMT1 is the chief enzyme responsible for maintenance of mammalian DNA methylation during DNA replication.

Fig.5

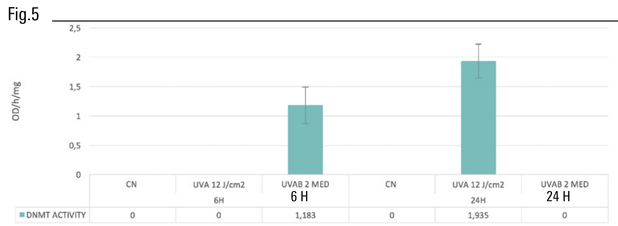

Fig. 5. DNA (cytosine-5)-methyltransferase 1 activity in different UV-irradiated model in comparison with negative control after 6 and 24H. Enzyme activity is expressed as OD per h per mg of total protein.

DNA methylation, evaluated by DNMT activity, has shown a different time-dependent pattern for UVA and UVA/B irradiation. In particular, 2 MED dose has induced methylation at earlier time-point (6H) probably due to stronger oxidative stress and DNA damage.
UVA irradiation alone has induced enzyme activation at longer timing (24H) that correlates with the damages occurring in the photo-ageing process.

Fig.6 **Acute UV stress Model (2 MED)**

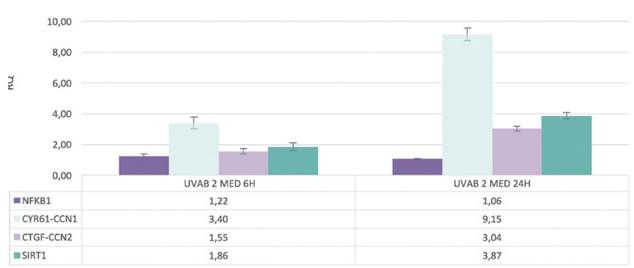

Fig. 6. Relative Gene expression in model at 6 and 24H after acute irradiation (2MED) compared with negative control (set as 1). Values are expressed as average Relative expression (RQ) and error (RQ max-RQ min).

In Ft-skin model irradiated with 2 MED the expression level of genes involved in the aging and inflammatory pathways has been differently modified in the 2 time points. At 6H the modification of senescence - involved transcriptional profile of gene CCN1 is visible, while at 24H a more complex response has been noticed. In particular, both the considered senescence genes CCN1 and CCN2 resulted overexpressed. Also the protective gene SIRT1 has shown an up-regulation indicating a mechanism of stimulus-response (i.e. damage-restoration) probably induced by acute oxidative stress.

AUTOPHAGY

Autophagy is an essential, homeostatic cellular process of "self-eating." Through this process, cells clear unwanted or damaged proteins, lipids and other cellular components, and in doing so regulate the availability of a number of cell signalling factors. Furthermore, autophagy-mediated recycling of cytoplasmic contents facilitates cell survival and adaptation during starvation, genotoxic and oxidative stress in normal cells. UVA, UVB and UVC have all been reported to induce autophagosome formation and upregulation of autophagy markers through specific regulation pathways (Sample 2016).

Autophagy has been evaluated by the gene expression profile of the following target genes ATG5, SESN1, SQSTM1, UVRAG, TSC1 (Mlitz 2016; Qlang 2013; Vessoni 2013; Di Nardo 2014).

Fig.7

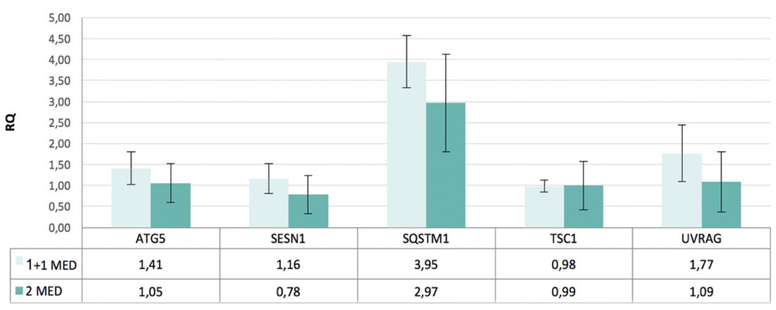

Fig. 7. Relative Gene expression in Acute UV stress Model (2 MED UVA/B) and repeated (1+1 MED) model at 24H after irradiation in comparison with negative control (set as 1). Values are expressed as average Relative expression (RQ) and error (RQ max-RQ min).

The transcriptional profile has been evaluated after 24H post acute (2MED) and repeated (1+1 MED) stimulus, showing a similar pattern although the cumulative dose (1+1 MED) seems to be more effective in promoting autophagic process in comparison with single acute dose (2 MED),
In connection with the dynamic process, it can be noticed that at 24H only the effector gene SQSTM1 gene, a protein that induce autophagosome formation and protein degradation, is overexpressed. Precocious regulators and transcription factors such as ATG5, SESN1, TSC1 and UVRAG probably have already exerted their action at earlier time points.

CONCLUSIONS

- Inflamm-aging stimulus based on keratinocytes activation and UVA induced oxidative stress have determined damages at papillar dermis and an early inflammatory pathway based on early regulation via miRNA (mir-22 and Mir-126) of NF-kb pathway in FT-model. Moreover, UVA induced activation of senescence genes can be noticed after 24H in inflamm-aging model as well as SITR1 activation.
- Ceramage endocannabinoids-based product has shown to be effective in counter-acting up-rising of inflammatory response via a prompt blocking of pro-inflammatory miRNA, confirming its role as a new generation skin care product designed to excert a synerigic antiaging activity at epidermal and dermal level.
- The multi-parametric dynamic approach used in this study has highlighted the different temporary expression profile influenced by UV application indicating a more precocious response connected to miRNA up-regulation and CCN1 activation and a consequent later response involving senescence via CCN2 inflammation, via NF-kb and defence response via SIRT1.
- UV radiation induces DNA methylation as a consequence of oxidative stress in a time-dependent manner. In particular, after 6H UVA/B (2 MED) radiation was responsible for an early activation of DNMT while UVA (12 J/cm^2) has determined a late DNA damage (24H).
- In UVA/B model, at 24H autophagy gene expression can be detected in the phase of phagosome formation by the over-expression of SQSM1 gene indicating that at this particular time point the process of protein degradation is already started. Further analyses are planned in order to clarify regulative mechanism at earlier time-points and to verify the presence of autophagosomes by IHC staining of LC3 protein.

MATERIALS AND METHODS
Skin Model: Phenion® Full Thickness Skin Model (Henkel) composed by epidermal keratinocytes and dermal fibroblasts (derived from biopsy material from healthy donors) has been maintained in culture and used in different irradiation protocols. The full thickness model has been shown to be relevant to identify UV induced damages at dermal and epidermal level (Meloni 2010).
The transcriptional activity of target genes has been quantified by:
qRT-PCR on target genes: total RNA has been extracted from full thickness (RNAqueous kit, Life-technologies) and retro transcribed (High Capacity kit, Life-technologies). The produced cDNA has been used for relative RTqPCR analysis by ΔΔCt methods using GAPDH (as reference gene) TaqMan Assay and TaqMan Master mix in ABI7500 system Life-technologies for NF-kb, CCN1, CCN2 SIRT1, ATG5, SESN1, SQSTM1, UVRAG and TSC1.
RTqPCR on miRNA: miRNA has been extracted from full thickness (MirVANA kit, Life-technologies) and retro-transcribed (High Capacity kit, Life-technologies). The produced cDNA has been used for relative RTqPCR analysis by ΔΔCt methods using TaqMan Assay and TaqMan Master mix in ABI7500 system Life-technologies.
DNA metilation analysis: Total protein have been extracted from tissues and used to measure DNA methyltransferase activity by DNMT Activity Quantification Kit Colorimetric (Abcam). The ratio or amount of methylated DNA, which is proportional to enzyme activity and can be measured through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm.
UV Source: ORIEL 1 kW Sun Simulator (Mod. 81190) equipped with a Spectra-Physics Lamp (Mod. 6271 Xenon 1000 W), Filter WG 335-3 mm and 320-1,45 mm, flux uniformity 10 x 10 cm. The delivered UV doses have been monitored with a UV detector (PMA 2110-Solar Light Co. Inc.).
Test Item: Ceramage SERUM CONCENTRATE (Unifarco), containing Isopalmide (a synthetic endocannabinoid), has been tested for its capability to counteract inflamm-aging process and to keep skin homeoastatic balance. Endocannabinoids are a class of lipid mediators found in several tissues showing anti-inflammatory, immunomodulatory properties (Sancho 2003).

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