Combination of in silico, *in vitro* and *in vivo* studies to *demonstrate* a new epigenetic mechanism linked to pigmented spots

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ABSTRACT

Pigmentation is a natural tanning process resulting in the production of melanin within melanosomes by melanocytes, and their distribution throughout the epidermis. Tyrosinase is a key enzyme of the melanin synthesis. With ageing, some pigmentary disorders appeared because of an over-production of tyrosinase in photo-exposed skin areas, triggering the appearance of pigmented spots. However, some new environmental assaults such as traffic-related air pollution were revealed as new contributors of pigmented spot formation, which open new perspectives.

Because melanin synthesis could be controlled through epigenetic mechanisms, we investigated if specific miRNA modulation could be responsible for melanin regulation.

We discovered by functional analysis coupled with bioinformatics that the miR-490 was able to regulate the melanogenesis process, with low functional redundancy, meaning that its defect couldn't be counterbalanced by other miRNAs.

Using gain and loss-of-function approaches *in vitro*, we found that miR-490 overexpression leads to reduce the production of melanin by human melanocytes through the repression by 50% of the tyrosinase protein synthesis.

Moreover, we demonstrated the ability of a *Lansium domesticum* leaf extract (LDLE) to stimulate the miR-490-5p expression and to decrease the tyrosinase protein synthesis in Asian melanocytes. It also inhibited the melanin production in Asian melanocytes - keratinocytes co-culture.

In vivo, LDLE at 0.3% decreased the surface of pigmented spots and their melanin content in 25 Chinese adult volunteers living in a daily air-traffic polluted environment and in a sun-exposed area in China.

Therefore, this extract, by a new discovered epigenetic mechanism, contributes to reduce the melanin content and size of pigmented spots, caused by daily environmental assaults.

INTRODUCTION

Melanin is a skin chromophore produced by melanocytes within melanosomes. It is transferred to adjacent keratinocytes to protect nuclear DNA and is responsible for the skin color [1]. Melanin synthesis is initiated by the rate-limiting enzyme called Tyrosinase (TYR) which converts tyrosine into dopaquinone. Then, dopaquinone undergoes several transformations until a final step of polymerization to obtain the melanin pigment. Age spots also known as lentigines, lentigo senilis, liver spots or solar lentigo, are age small, flat, darkened patches of skin (light brown to black in color) that occur on sun-exposed areas. Age spots are one of the most visible signs of photo-aging which concern almost 90% of elderly people and can appear before 40 years old. Pigment and melanosomes accumulate at the top of epidermal rete ridges [2]. However, their sizes are not modified but accumulation of massive pigment caps on the nuclei of basal keratinocytes are formed by a melanosomal complex. Moreover, the number of melanocytes increases by 2-fold [3] and their size and dendricity increase too [4]. Transcriptome analysis of solar lentigo skin compared to both adjacent non-lesional and sun-protected buttock skins evidenced strong up-regulation of melanogenesis-encoded genes such as TYR, TYRP1, MLANA (Melan A or MART1) and PMEL (Premelanosome protein) and genes related to inflammation [5].

Epigenetic mechanisms are generally considered to represent a regulatory interface between environmental cues and the genome. As ageing, photo-ageing is associated with environmental modifications which can affect epigenetically the skin. Several epigenetic mechanisms such as DNA methylation, histone modification or non-coding RNA contribute to gene and therefore protein expression changes during photo-ageing.

Among non-coding RNAs, microRNAs (miRNA) are members of a class of small functional non-coding RNAs. They play important roles in targeted-gene regulation and are responsible for transient modulation of specific protein expression. Thus, miRNAs are key regulators of all biological processes and more than 2000 miRNAs have been found in human. They negatively regulate gene expression at the post-transcriptional level through a direct binding to the 3'-untranslated region (UTR) of their target mRNA [6 ,7]. Bioinformatic algorithms showed that members of miRNA families are frequently targeting the same multigene set in a functionally redundant manner. On the other side, low redundant miRNAs are mostly highly specific, due to their impossibility of being counterbalanced by other miRNAs [8, 9]. However, these bioinformatic algorithms can only predict the sequences analogies of miRNA with the 3'UTR region of the mRNA of the targeted protein. Moreover, they produce a huge amount of data without experimental validation which may cause a misinterpretation of real relationship between the targeted proteins and microRNAs evidenced.

In this study, we investigated if specific miRNA modulation could be responsible for skin whitening and improving age spot appearance. MicroRNA functional analysis coupled with bioinformatics first enabled us to identify the hsa-miR-490-3p (called miR-490) suspected to regulate the melanogenesis process. However, proving the direct link between the low redundant miRNA and tyrosinase protein level was essential.

The goal of our study was to identify a specific microRNA targeting *TYR* to reduce the pigmented spots. However, to reach clinical efficacy, we aim to:

- select a unique miR whose defect would not be counterbalanced by other miRNAs and further add an important low functional redundancy criterion for its selection
- prove the direct link between the low redundant miRNA and the tyrosinase protein
- demonstrate the efficacy of our strategy under UV stress *in vitro* and in a sunny polluted area *in vivo*.

METHODOLOGIE

<u>miR-490-3p in silico identification</u>

Our first step consisted to apply bioinformatic to virtually select:
- miRs targeting the 3'-UTR regulation sequence of *TYR* mRNA with a higher inhibitory potential compared to 5'-UTR area of the coding sequence;
- miRs with at least a 7-Mer hybridization potential within the 8 first miR nucleotides (seed sequence) to have a strong miRNA/*TYR* mRNA interaction.

The miRBase is a dedicated miRNAs database (http://microrna.sanger. ac.uk/) focused on providing integrated interfaces to comprehensive microRNA sequence data, annotation and publications of all published mature miRNAs. It helped us to predict associations between microRNAs and the targeted tyrosinase mRNA sequence, considering only Homo sapiens gene-microRNA information. Moreover, miRNA- mRNA interactions were figured out using a second tool called Ingenuity Pathway Analysis (IPA) (QIAGEN Redwood City, CA, www.qiagen.com/ingenuity) to select the relevant miRNA able to control the tyrosinase protein. This bioinformatic tool, is based on experimental results from the scientific community.

<u>Selection of miRNA with low redundancy</u>

As miRNA deficiency seems to be compensated by another miRNA, to evaluate the ability of miR-490 be compensated by another miRNA ie miR-490 redundancy, Targetscan and Diana microT develop specific algorithms to discover putative targets (http://diana.cslab.ece.ntua.gr/microT/ and http://www.targetscan.org/vert_61/). Thanks to these databases we used a new network-based method to analyse miRNA function [10] to create functional relationship between miRNAs. In this network, we define that miRNAs are partners if they share at least 50 percent of their predicted targets. If so, a link between the two miRNAs is drawn. In the network, the miRNA color is specific of their functional group. Three main groups exist:

1. The red and pink miRNAs are statistically involved in GTPase dependent signalization
2. The blue and cyan miRNAs are statistically involved in transcription process
3. The purple miRNAs have no statistically dominant functions

More precisely, this network may be used to identify miRNA with numerous partners that has high redundancy potential (ie the absence of this miRNA may be compensated by another miRNA) and

miRNA with few partners that has low redundancy potential (ie the absence of this miRNA may not be compensated by another miRNA). In this study, we particularly analyzed the position of miR-490-3p in this network and the ability of miR-490-3p to be compensated by another miRNAs (redundancy), was explored.

Concept proof: the miRNA miR-490-3p regulates the tyrosinase expression and melanin synthesis

Mimic miR-490-3p detection and functional validation by transfection: We cultured primary cell line of melanocytes to observe the endogenous modulation of the expression of miR-490-3p. Cells were transfected with 20nM of miR-490-3p-mimic. After transfection, cells were incubated at 37°C and respectively harvested after 48 and 72 hours for miRNA and protein analysis. Three biological replicates were performed.

miR-490-3p modulation by environmental factors: Experiments were carried out using Asian normal human foreskin melanocytes derived from surgery. At subconfluence, cells were exposed or not to UVB at 15 and 25mJ/cm2, and then total RNAs or total cellular proteins were extracted at different time points for Q-RT-PCR assessments (24h, 48h) or Western blot analysis (72h).

Q-RT-PCR after UV exposition: The expression level of miR-490-3p in Asian melanocytes after UVB exposition were determined by Q-RT-PCR using Taqman microRNA assay method One-step RT-PCR (C-Kit for Retro-transcription: TaqMan MicroRNA Reverse Transcription Kit and D-Master mix for Q-PCR: TaqMan Fast Advanced Master Mix) with miR-490-3p Exiqon primer after 24h-treatment with untreated control. Values of each experiment were measured using bio-computationally methods and the untreated control (UC) was used as target expression reference for each treatment normalization. The results were normalized using RNU6B expression. All the experiments were performed in triplicate for each biological replicate.

Tyrosinase protein inhibition after either mimic miRNA transfection or UV exposition: Total cellular proteins were extracted before quantitation with a BCA (bicinchoninic acid) protein assay and sample separation with Western-blot method. The membrane was incubated overnight at 4°C with primary anti-TYR mouse monoclonal antibody (Santa Cruz Biotechnology, USA) followed by 1 hour at room temperature with anti-mouse HRP-conjugated secondary antibody. Detection was performed with an ECL prime kit (Amersham Biosciences Europe, Germany).

Statistics: Results were normalized to the controls (UC or Ct-mimic) and were expressed as mean (%) ± standard error of mean (SEM) from triplicate assay. The statistical analysis was carried out using Student t test. The significance threshold was fixed at 0.05 for the p-value.

Selection of ingredient

The screening was performed in Asian melanocytes to select a miR stimulator/TYR inhibitor ingredient which activity was confirmed by melanin content evaluation in co-cultures of Asian melanocytes and keratinocytes.

Cell culture and treatment for Lansium domesticum leaf extract (LDLE) effect evaluation: LDLE is an aqueous plant extract stabilized with maltodextrin. *Lansium domesticum* is a species typical of Southeast Asian countries from Peninsular Thailand in the west to Borneo in the east (Indonesia), producing sweet and aromatic fruits, which are a popular dessert.
The different parts of the Lansium domesticum plant (fruit, peel, bark, seeds and leaves) display many biological activities such as antioxidant (Kee et al., 2015). The leaves are one part that can be valorized for use in cosmetic applications.
Experiments were carried out using Asian normal human foreskin melanocytes derived from surgery. Cells were or were not treated with LDLE for 1 day after confluence, and then total RNAs were extracted for Q-RT-PCR assessments.

miR-490 expression stimulation after LDLE treatment: The expression levels of miR-490 were determined by real-time RT-PCR using Taqman microRNA assay method One-step RT-PCR analysis (C-Kit for Retro-transcription: TaqMan MicroRNA Reverse Transcription Kit and D-Master mix for Q-PCR: TaqMan Fast Advanced Master Mix) with miR-490-3p Exiqon primers. RTq-PCR were performed on total RNAs extracted after 24h-treatment with LDLE at 0.034 and 0.07% or untreated

condition (UC, untreated control). Values of each experiment were measured using bio-computationally methods and the UC was used as target expression reference for each treatment normalization. The results were normalized using RNU6B expression. All the experiments were performed in triplicate for each biological replicate. Results were normalized to the untreated control (UC) and were expressed as the mean (fold change) ± standard error of mean (SEM) from triplicate assay. The statistical analysis was carried out using Student t test. The significance threshold was fixed at 0.05 for the p-value.

Tyrosinase protein inhibition after LDLE treatment: Experiments were carried out using Asian normal human foreskin melanocytes treated or not with LDLE at 0.034% for 3 days after confluence. Next, the cells were harvested and lysed with specific lysis buffer for protein extraction (CelLytic M cell lysis reagent from Sigma). Protein concentration was determined by BCA assay and the samples were adjusted at 0.5 mg/ml. Target proteins were identified by a capillary electrophoresis-based protein analysis system (Sally Sue; ProteinSimple, San Jose, California, USA) using primary antibodies against TYR and a horseradish peroxidase-conjugated secondary antibody detected by chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified by the Compass Software version 2.7.1 (ProteinSimple, San Jose, California, USA). Results were normalized to the untreated control (UC) and were expressed as the mean (%) ± standard error of mean (SEM) from 2 experiments in triplicate assay. The statistical analysis was carried out using the Mann-Whitney Test. The significance threshold was fixed at 0.05 for the p-value.

Melanin synthesis inhibition in co-culture after LDLE treatment: Experiments were carried out using a co-culture of Asian normal human foreskin melanocytes derived from surgery with keratinocytes (cell line HaCat) (Boukamp et al., 1988). Cells were mixed and cultured (50:50) for 6-7 days at 37°C and CO2 5% before treatments with DN-Aura® at 0.034% and 0.07%. for 4 days. The level of intracellular melanin was measured by recording the optical density at 475 nm of cells' homogenate. The results were expressed as the mean values (%) ± SEM from triplicate assay. The statistical analysis was carried out using the OneWay ANOVA test followed by Dunnet test. The significance threshold was fixed at 0.05 for the p-value.

Clinical trial

Study design: The study was carried-out as a double-blind, randomized, split-face study under dermatological control. The evaluation of the efficacy was compared to baseline (day 0) and a Benchmark. The test was performed in a sunny and polluted region in China, at Guangzhou (Canton, Est North of Hong Kong) between October and December. Two independent groups of 25 and 28 Chinese volunteers were involved in this study, where the product at 0.3%, Ascorbyl Glucoside (AA2G) at 2% and placebo cream were applied. The study was conducted during a period of 56 days, with check points at D0 and D56.

The study was done on Chinese female volunteers from 18 to 60-year-old, presenting pigmented spots on the face, belonging to phototype III and IV (according to Fitzpatrick grading scale). The specific inclusion criterion was the presence of well-defined pigmented spots on each half-face.

The product, the AA2G or the placebo creams were applied twice daily for 56 days to either the left or right side of a clean face. Each volunteer of the two groups tested 2 different products. The products were applied by volunteers under normal condition of use.

Evaluation method: At D0, selection of one significant pigmented spot (according to color intensity and contours) of 3 to 7 mm in diameter. The selection was done on each half-face.

The area of pigmented spot and the total quantity of melanin are measured by image analysis (Spectroscan™ - Orion Concept, France). The anti-pigmented spot effect is characterized by a decrease of both parameters. High resolution digital photographs (in cross polarized light) of the face were taken at D56 and at baseline (Nikon D300S).

Statistics: Results are expressed as the mean percentage of change compared to the baseline measurement (D0). The statistical analysis of the evolution of the parameters in function of products was done after the verification of the normality of distribution using Shapiro-Wilk test.

Afterwards, the statistical analysis of the evolution of the studied parameters for each product was performed with the Student t test in case the normality of distribution was confirmed.

RESULTS

miR-490-3p in silico identification

The comparison of 2 lists of miRNAs from bioinformatic analysis and published data, allowed us to select 8 miRNAs potentially able to control Tyrosinase synthesis in human melanocytes. Then, we experimentally verified that these miRNAs were expressed in human melanocytes (data not shown).

miR-490 is a low redundant miRNA

miRNA plays a major role in cellular functions. Deficiency of one miRNA may be compensated by another. Thus, numerous miRNAs share a large majority of theirs targets (family or hub). A network published in 2015 by balhun et al [10] identified miRNA partners sharing 50% of their targets. In this study, we analyzed the position of miR-490 in this network. miR-490 (Figure 1) takes part of purple group that have few partners and have few connections with other miRNAs. More precisely, Diana microT algorithms identified only 4 miRNAs that share at least 50% of their predicted targets with mir-490-3p.

Through in silico analysis, we identified 3 potentially interesting miRs targeting at least 2 proteins involved in melanogenesis including TYR enzyme. Then, because mir-490 seems to have a low redundancy potential, we hypothesize that its modulation may have a high effect on dependent cellular functions such as tyrosinase regulation.

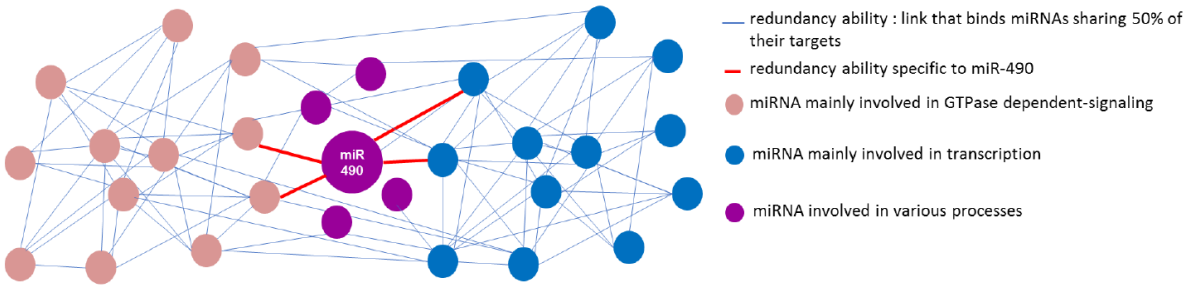

Figure 1: Representation of the miR-490 position in the network in which functional relationships between miRNAs are created if they share at least 50 percent of their predicted targets.

The miR-490-3p controls the Tyrosinase protein in melanocytes

However, whether miR-490-3p could virtually inhibit TYR and MLANA it was necessary to confirm its expression and effects in melanocytes. To confirm the previous bioinformatic prediction, we analyzed the protein synthesis in normal melanocytes from healthy donor after mimic miR-490 or control mimic transfections. Cells were incubated at 37°C and harvested 72 hours after transfection, for protein analysis. We showed that the transfection with mimic miR-490 significantly decreased the tyrosinase protein by 50% whereas the transfection with Ct-mimic did not modify the protein level (Figure 2).

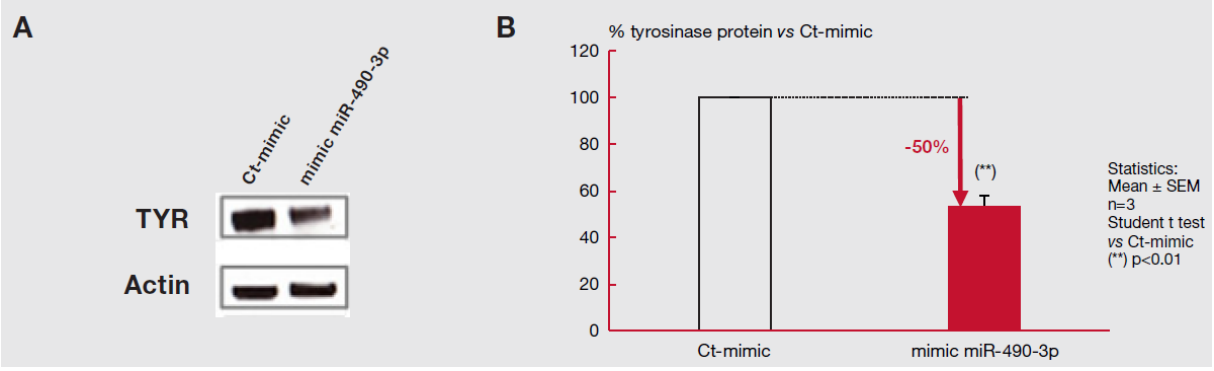

Figure 2: Tyrosinase (TYR) detection and functional validation of the role of miR-490-3p after transfection with a mimic. A - Western Blot analysis of TYR protein. B - Quantification of the TYR protein synthesis after mimic miR-490-3p transfection. Results are expressed in % against control mimic (Ct-mimic) transfected condition.

Selection and *in vitro* efficacy of *Lansium domesticum* Leaf extract

As we have shown that the TYR protein could be inhibited by miR-490, we selected a plant extract able to increase the miR-490 and consequently to decrease the TYR protein and melanin synthesis in Asian melanocytes. We showed that LDLE at 0.07% was respectively able to significantly increase by 1.4-fold change the miR-490 (Figure 3A) and to decrease by 33% the TYR protein (Figure 3B). LDLE treatment at 0.07% inhibited by 34% the melanin production by melanocytes in Asian melanocytes/keratinocytes co-culture model. (Figure 4). So, LDLE regulates both miR-490 expression and TYR protein synthesis in Asian melanocytes

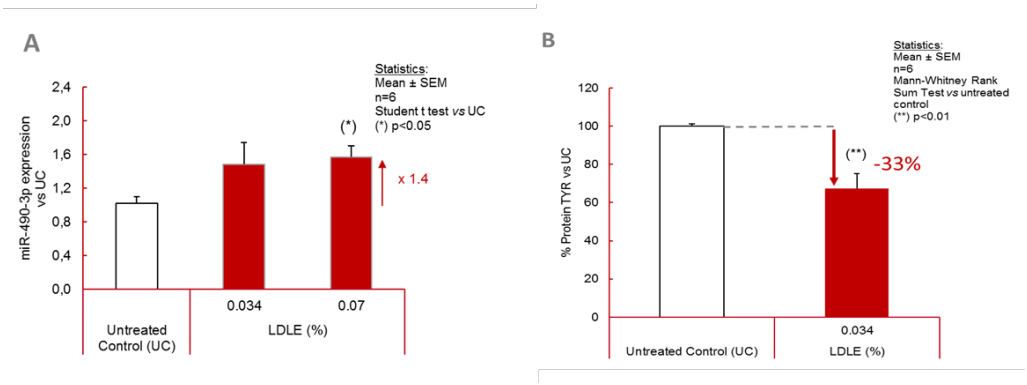

Figure 3: A-Quantification of the expression of miR-490-3p by Q-RT-PCR in human melanocytes after 24 hours with or without treatment. B-Quantification of TYR protein synthesis in human melanocytes, after 72 hours.

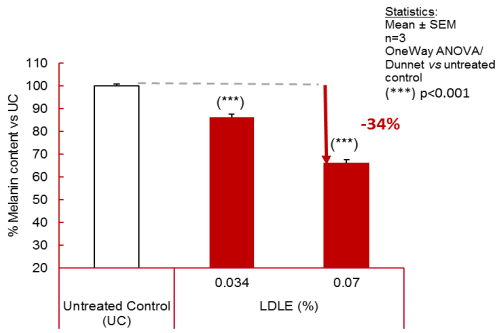

Figure 4: Quantification of melanin synthesis in melanocytes and keratinocyte co-culture, after 4 days; Values are expressed as a percentage of UC.

Validation of the modulation of miR-490-3p by UVB in Asian melanocytes

Chronic sun exposure, in particular UVB, is a major environmental factor triggering the increase of pigmented-spots in the skin.

We evaluated 2 doses of UVB on melanocytes corresponding to low and high level of UVB exposure. The lowest dose (15 mJ/cm²) didn't show any changes, demonstrated the necessity of the high irradiation level on miRNA modulation. High UVB irradiation (25 mJ/cm²) inhibited significantly miR-490-3p expression level in melanocyte cultures, showing a decrease by 25% ($p<0.01$) and by 29% ($p<0.01$) after respectively 24h and 48h (Figure 5A). As expected, tyrosinase protein was overexpressed by UVB irradiation, as shown by the Western Blot analysis (Figure 5B).

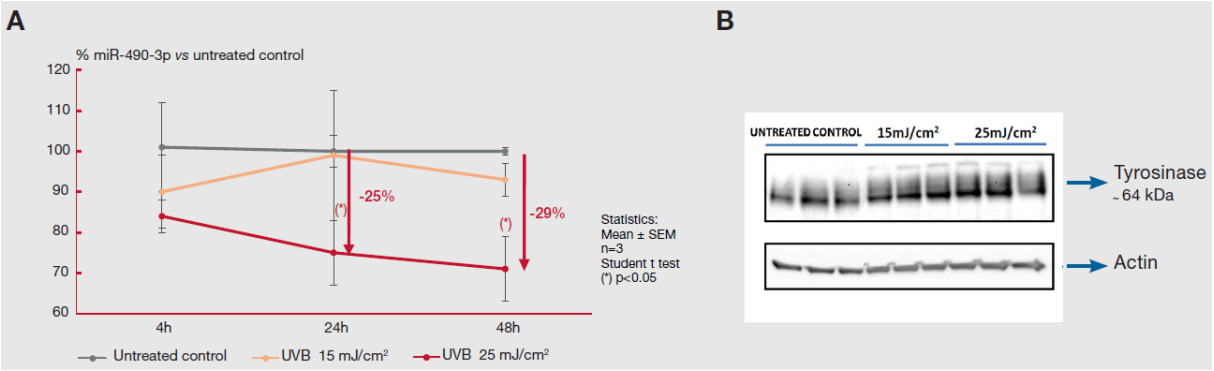

Figure 5: A - Kinetic study of miR-490-3p expression after UVB irradiations. Results are expressed in % against untreated control (UC). B - Western Blot analysis of TYR protein after UVB exposure (cells irradiated at 15 or 25 mJ/cm², analysis 72h after irradiation).

Clinical trial

In vivo, the LDLE at 0.3% was applied for 56 days by Chinese women living in a sunny and polluted region, compared to a placebo-controlled group.

In these conditions, the LDLE demonstrated a significant decrease by 23% ($p<0.01$) of melanin content of the pigmented spot on the face versus baseline (D0) (Figure 6A). This improvement was close to significance compared to placebo (-16%, $p<0.1$).

In the Benchmark group, the LDLE demonstrated a similar significant decrease of the pigmented spot melanin content by 20% ($p<0.01$) on the face vs baseline (D0) (Figure 6B). This improvement was significant compared to the Benchmark (-19%, $p<0.05$), which did not display any effect.

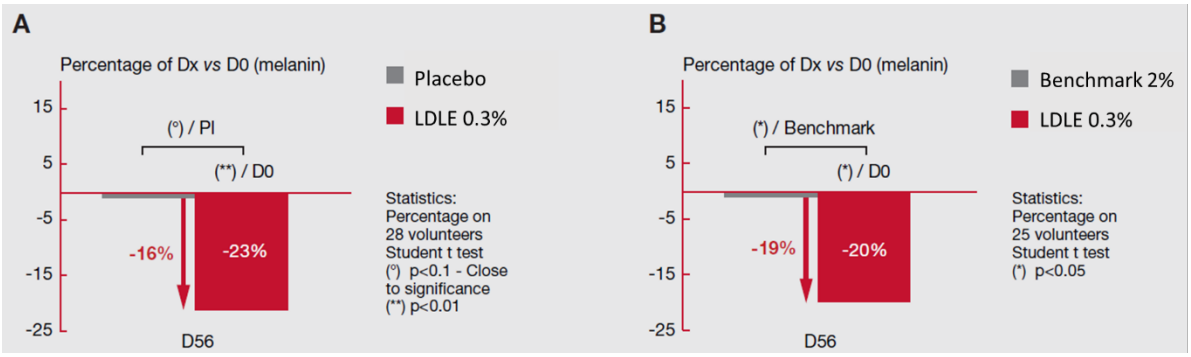

Figure 6: Percentage of pigmented spot's melanin content decrease after 56 days of application on the face vs baseline (D0) and compared to both placebo (A) and Benchmark (AA2G at 2%) (B).

CONCLUSION

Environmental factors affect the physiology of the skin, disturbing melanocyte functions in pigmented spots. Thanks to combination of in silico, *in vitro* and *in vivo* studies, we discovered that a specific miRNA, the miR-490-3p, directly inhibits the synthesis of tyrosinase protein, responsible for the appearance of pigmented spots. Thus, we have demonstrated that the less redundant and functionally proven miR-490-3p is a novel and specific epigenetic approach to efficiently reduce the pigmented spots on skin caused by daily environmental assaults such as the UV and pollution.

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