In vivo Multiphoton FLIM Quantification and Characterization of Melanins in European Subjects with Different Variants in MC1R and SLC45A2 Key Pigmentation Genes

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1 INTRODUCTION

Since genetically determined human pigmentary characteristics are closely associated with photoaging and photo carcinogenesis, characterizing melanin in the skin is a challenging topic. For years, it was limited to *ex vivo* quantification on histological slides or HPLC quantification. More recently, using 2PEF/ Pseudo-FLIM multiphoton microscopy, the ability of *in vivo* noninvasive specific detection, quantification, and 3D distribution determination of melanin in the epidermis has been demonstrated[1]. To go a step further, discrimination between photoprotective eumelanin and photolabile and generating oxidative stress pheomelanin can also be assessed by multiphoton fluorescence lifetime imaging microscopy (FLIM), as demonstrated *in vitro*[2]. The objective of this study was to characterize the endogenous fluorescence lifetime properties of these 2 types of melanin and estimate their relative amount as well as their 3D density and z-epidermal distribution in normal human volunteers, carriers of different genotypes of 2 key pigmentation genes: MC1R whose "loss-of-function" variants are associated with Red Hair Color and increased risk of skin cancer and SLC45A2, one of whose variants, L374F, is conversely associated with dark hair and skin color and protection against skin cancer[3].

2 MATERIALS & METHODS

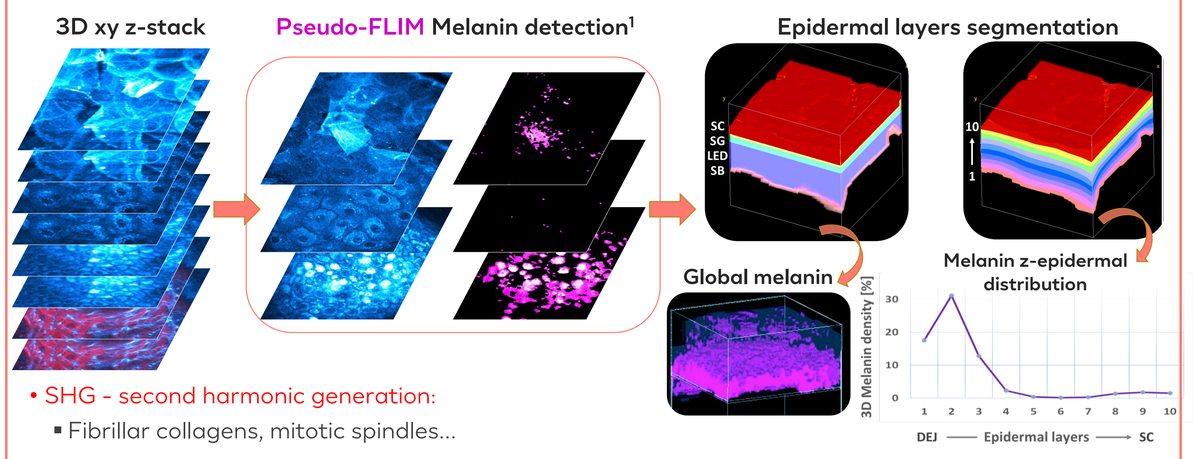
Subjects: 50 healthy adult volunteers (31 female, 19 male, 28-60 yo), carriers of:

1. Two MC1R deleterious variants and no L374F protective variant (n=10);
2. One MC1R deleterious variant, and no L374F protective variant (n=12);
3. No MC1R deleterious variant nor L374F protective variant (n=16);
4. At least one protective L374F variant and no deleterious variant of the MC1R gene (n=12).

Clinical study conducted in Paris, France (Nov. 2013 - Dec. 2014), in accordance with the Declaration of Helsinki principles. The protocol was approved by Saint-Louis Hospital Ethics Committee (EC ref. 2013/50) and all volunteers gave written, informed consent.

Methods: Subjects' pigmentary and sun sensitivity characteristics were collected. Colorimetry measurements (ITA values, Datacolor) were performed on both sides of the left forearms and on the buttocks as an area almost completely sunprotected. Multiphoton 2PEF/SHG 3D z-stacks and 2PEF-FLIM 2D images were acquired on dorsal and ventral forearms as previously described[1].

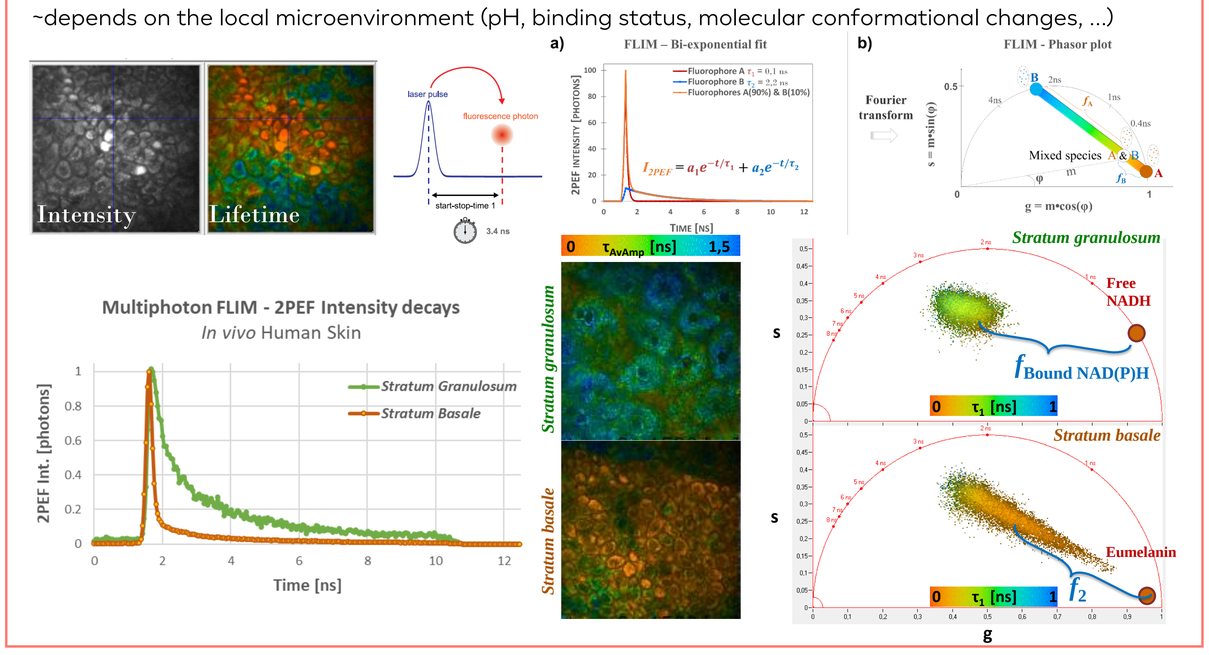
3D Multiphoton Pseudo-FLIM melanin quantification in human skin *in vivo*



- SHG - second harmonic generation:
  - Fibrillar collagens, mitotic spindles...
- 2PEF - two-photon excited fluorescence:
  - NAD(P)H, FAD metabolic coenzymes, keratin, melanins, elastin, porphyrins, tryptophan...

In vivo 2D Multiphoton Fluorescence Lifetime Imaging (FLIM)

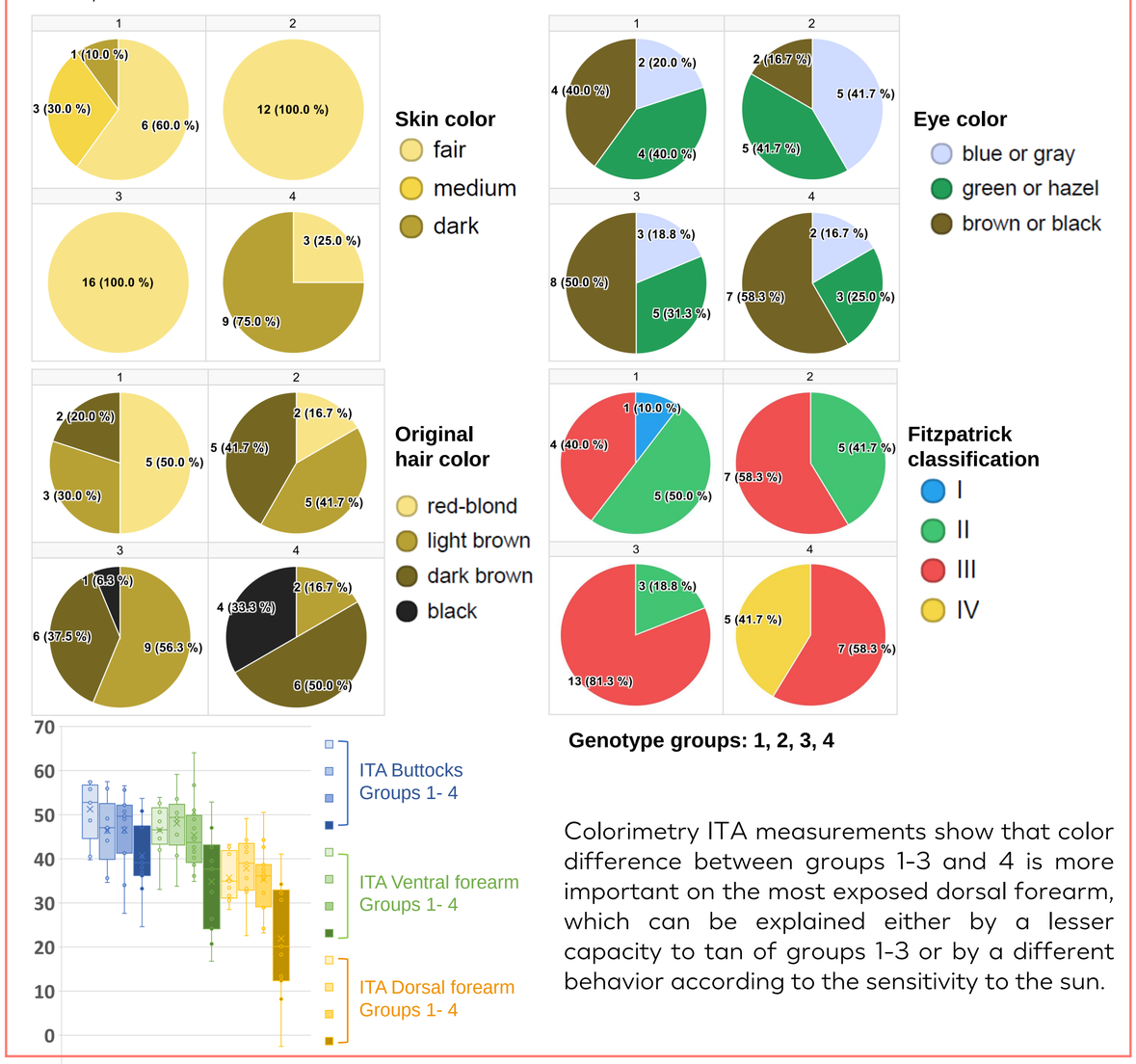
Fluorescence Lifetime (τ) - average time elapsed between excitation and fluorescence emission ~depends on the local microenvironment (pH, binding status, molecular conformational changes, ...)



3 RESULTS & DISCUSSION

Pigmentary characteristics and skin color according to genotype

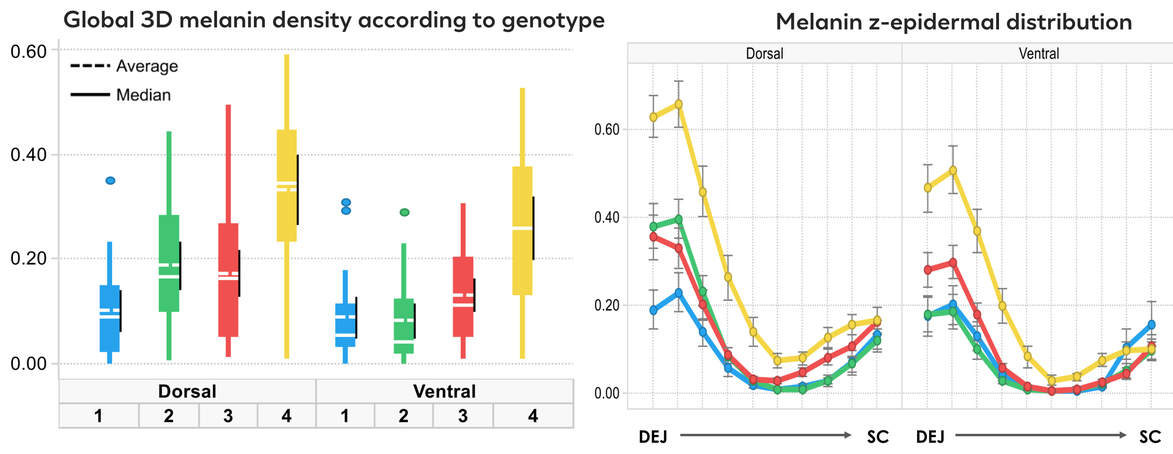
As expected, skin color (classified as fair, medium, or dark), eye color (classified as light [blue, green or hazel, or gray]) or dark [brown or black], and original (before graying) hair color (classified using four categories: red-blond, light or dark brown, and black) correlate with genotypic groups (1, 2, 3, 4) as well as skin types according to the Fitzpatrick classification (I: always burns never, II: always burns then tans, III : always tans, sometimes, and IV: always tans, never burns).



Colorimetry ITA measurements show that color difference between groups 1-3 and 4 is more important on the most exposed dorsal forearm, which can be explained either by a lesser capacity to tan of groups 1-3 or by a different behavior according to the sensitivity to the sun.

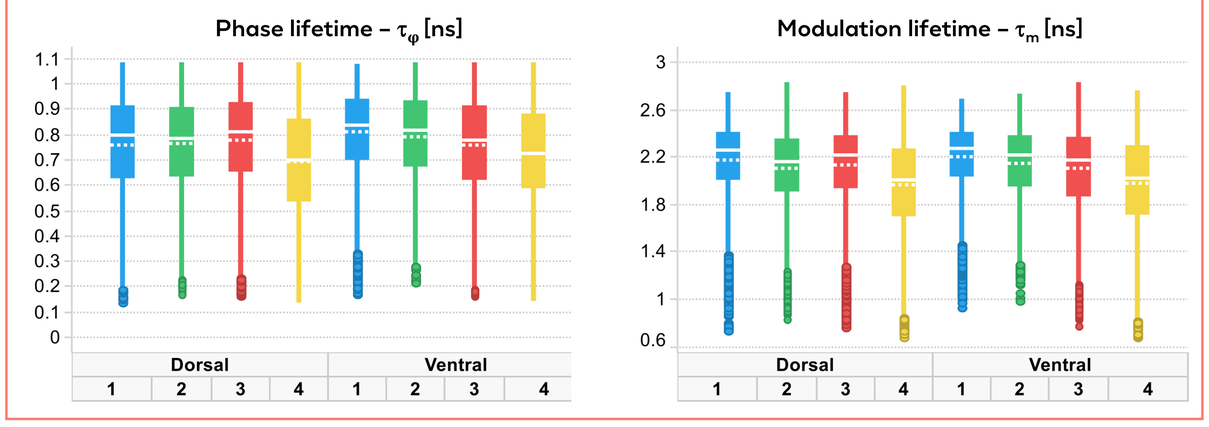
3D melanin density and epidermal distribution according to genotype

3D melanin density strongly differs between groups 1-3 vs 4 in both ventral and dorsal forearms with higher melanin levels on most photo-exposed side. Moreover, melanin density z-distribution profiles allow evidencing significant differences: limited to the basal epidermal layer in group 1 to occupying more than 2/3 of epidermis in group 4.



Melanin's fluorescence lifetime (eu-/pheo-melanin levels) according to genotype

Melanin in *stratum basale* has smaller phase and modulation fluorescence lifetimes in group 4 (mean±sd: τ_φ=0.70±0.16 ns; τ_m=1.69±0.25 ns) compared to group 1 (τ_φ =0.77±0.14 ns; τ_m=1.85±0.21 ns), suggesting higher contribution of eumelanin species in group 4, thus probably associated to different eu-/pheo-melanin levels (NB: native eumelanin has smaller τ_φ and τ_m lifetimes compared to native pheomelanin[2]). The data represent all the melanin pixels.



4 CONCLUSIONS

Multiphoton FLIM imaging, besides measuring melanin's amount and epidermal distribution, opens the possibility of quantifying eu-/pheo-melanin levels *in vivo*, thus allowing a better characterization of the populations at risk of accelerated skin aging and skin cancer for better evaluation and development of photoprotection products.

REFERENCES

[1] Pena et al. In vivo melanin 3D quantification and z-epidermal distribution by multiphoton FLIM, phasor and Pseudo-FLIM analyses. Scientific Reports 12, 1642 (2022). [2] Pena et al. Multiphoton FLIM Analyses of Native and UVA-Modified Synthetic Melanins. International Journal of Molecular Sciences 24, 4517 (2023). [3] Ibarrola-Villava et al. "MC1R, SLC45A2 and TYR genetic variants involved in melanoma susceptibility in Southern European populations: results from a Metaanalysis," European Journal of Cancer 48, 2183–2191 (2012).