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ORIGINAL ARTICLE

Chain dynamics of human dermis by Thermostimulated currents: A tool for new markers of aging

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Abstract

Background/purpose: The purpose of this clinical study was to identify dielectric markers to complete a previous thermal and vibrational study on the molecular and organizational changes in human dermis during intrinsic and extrinsic aging.

Methods: Sun-exposed and non-exposed skin biopsies were collected from 28 women devised in two groups (20-30 and ≥60 years old). The dielectric relaxation modes associated with localized and delocalized dynamics in the fresh and dehydrated state were determined by the Thermostimulated currents technique (TSC).

Results: Intrinsic and extrinsic aging induced significant evolution of some of the dielectric parameters of localized and delocalized dynamics of human skin. With photo-aging, freezable water forms a segregated phase in dermis and its dynamics is close to free water, what evidences the major role of extrinsic aging on water organization in human skin. Moreover, TSC indicators highlight the restriction of localized mobility with intrinsic aging due to glycation, and the cumulative effect of chronological aging and photo-exposition on the molecular mobility of the main structural proteins of the dermis at the mesoscopic scale.

Conclusion: TSC is a well-suited technique to scan the molecular mobility of human skin. It can be uses as a relevant complement of vibrational and thermal characterization to follow human skin modifications with intrinsic and extrinsic aging.

KEYWORDS
dielectric analyses, human skin aging, hydration, molecular mobility

1 | INTRODUCTION

Aging is a multifactorial process defined as the consequence of time dependent loss of function, resistance, and adaptability to stress in all organs.[1] Skin aging depends on both intrinsic and extrinsic factors (such as sun exposition), resulting from gradual molecular modification of the dermis, the major macroscopic sign being wrinkles.

The extracellular matrix (ECM) of dermis, containing as main components collagens, elastin and proteoglycans is actively implied in cutaneous aging,[2,3] as well as changes in hydration.[2,4] Collagen is the predominant component in the dermis (80% of the total dry mass), and collagens I and III constitute 90% and 10%, respectively, of the composition of dermal collagen fibrils. Among others functions, collagen fibrils confer tensile strength to the skin and are essential for the organization and stability of the dermal extracellular matrix.[5]

Human skin has been extensively characterized by histological, biochemical, and ultrastructural analyses studies to clarify the mechanisms involved in chronological (intrinsic) and photo (extrinsic) aging.[3,6,7] Nevertheless, due to multifactorial and slowness of the aging processes, that leads to subtle changes of biological function (including biochemical, morphological, physical aspects) complementary studies on aging are still of interest.[8-11]

In a previous work,[12] we showed that Differential Scanning Calorimetry (DSC) was a powerful technique to evaluate the hydric

organization of human dermis and the collagen stability. Via this technique, for the first time, the direct quantification of freezable and unfreezable water in human skin biopsies from two age groups has been performed. We found that freezable water, covering bulk water in excess but also confined water in mesopores roughly represents 2/3 of the total water in human skin in accordance with previous NMR, DVS, and DSC results.[13-15] Photoaging was shown to induce a significant increase in freezable water, associated with a decrease amount of unfreezable water, what highlights the preponderant effect of extrinsic aging on the hydric organization of the skin. This reorganization of water causes a very significant decrease in the ratio of unfreezable water to freezable water in aged sun-exposed skin (0.374 vs 0.490 in young sun-exposed skin, $P = .0039$).[12]

Moreover, it was shown that photo-aging led to a significant decrease in the collagen denaturation temperature,[12] reflecting a slight fragmentation of the collagen fibers with the cumulative UV exposition during life. This fragmentation can be induced by the decrease in unfreezable water that destabilizes collagen as previously described.[16-19] Finally, it was evidenced by DSC that chronological aging led to a temperature increase in the end of denaturation, whereas it remained constant in the sun-exposed young and aged skins. These heat-stable cross-links could be induced by the post-translational modifications such as the age-induced glycation products and/or carbonyl changes.[20-22]

To complete this previous study, we chose in the present work to use a dielectric technique, to scan the dynamics of the human skin. Due to its very low equivalent frequency, the Thermostimulated currents technique (TSC) allows to decouple relaxations modes in proteins and tissues from the conductivity that often hides the relaxation processes, and it is appropriate to scan the molecular mobility of collagen and elastin at the nanometric and mesocopic scales. In peculiar, it has been successfully used to identify localized and delocalized motions in keratin,[23] elastin,[24] aortic tissues[25] and irradiated rat skin[26] and to help the knowledge of the different organization levels in the pre-fibrillar state of elastin-like peptides.[27]

The aim of this work was to evaluate, for the first time, chronological and photo-aging with the TSC technique in a clinical study on biopsies from two age groups (20-30 and >60 years old).

2 | MATERIALS AND METHODS

2.1 | Study design and patients selection

This monocentric, comparative, open, study was conducted at the Pierre Fabre Skin Research Centre—CRP, Toulouse (France), in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines. The protocol was approved by the Sud-Ouest et Outre Mer III Committee for the Protection of Persons (*Ethics Committee, N° ID RCB 2014-A00698-39*) and the French Health Products Safety Agency (ANSM). Each volunteer signed a written informed consent.

Twenty-eight healthy female volunteers divided into two groups: aged 20-30 years and ≥60 years old with a SCINEXA score respectively < and ≥ at 2. SCINEXA (SCore for INtrinsic and EXtrinsic skin Aging) is a parameter which evaluate and differentiate intrinsic (<2) and extrinsic (≥2) aging.

Subjects meeting the following criteria were excluded: pregnant or breast-feeding women; skin diseases (lupus, psoriasis, atopic dermatitis …), wound healing disorder; signs of sun exposition on the selected areas; history of allergy to latex, lidocaine-medicated plaster, and chlorhexidine. Sun or UV exposure on sampled areas during the study period, corticosteroids, anticoagulants and phototherapy within the month preceding inclusion, cardiovascular agents and physical treatments (ie, radiotherapy) 6 months before inclusion were other reasons for non-inclusion, as were the use of retinoid or alpha-hydroxy acids at the test sites in the 2 weeks before inclusion.

2.2 | Samples treatment

Two punch biopsies (four mm in diameter) were taken from each volunteer: on sun-protected left buttock and left dorsal forearm resulting in four series: Young sun-Protected (YP), Aged sun-Protected (AP), Young sun-Exposed (YE) and Aged sun-Exposed (AE). The skin biopsy procedure was performed by a suitably qualified medical specialist. A local anesthetic has been administered to the site prior to excision of the biopsy. Following biopsy excision, the wound has been dosed appropriately, for example, with stitches and the site has been covered with a protective dressing.

Samples were immediately rinsed in Phosphate Buffered Saline solution to eliminate blood and were frozen in liquid nitrogen. Samples were then stored at −20°C. We performed stepwise thawing of the explants, (first stage at 5°C for 24 hours, and second stage at 20°C for 10 min) until TSC analysis.

2.3 | Thermally stimulated currents (TSC)

2.3.1 | Complex TSC curves

Complex TSC curves were carried out using a TSC/RMA Analyzer (SETARAM Instrumentation, Caluire, France).

Fresh biopsies were deposited between two specific shaped stainless steel electrodes (diameter 4 mm) surrounded by a Teflon guard allowing a precise matching of the thickness and a control of hydration. Before experiments the cryostat was flushed and filled with dry helium to insure good thermal exchange. The biopsy was polarized by a static electric field $E = 400\ V\ mm^{-1}$ from the poling temperature ($T_p = -20°C$) down to the freezing temperature $T_0 = -150°C$ with a fast cooling. Then, the field was turned off and the depolarization current was recorded with a controlled heating rate ($q = +7°C\ min^{-1}$).

Biopsies were then dehydrated in the TSC cell under primary vacuum at −50°C for 14 h, what can be considered as a freeze-drying. TSC curves corresponding to poling temperature of Tp = 25°C and Tp = 150°C, for a static field of $400\ V\ mm^{-1}$ were recorded with the same procedure as previously described.

2.3.2 | Elementary TSC curves

Elementary TSC curves were obtained using fractional polarizations (TSC/FP) with a polarization window of 5°C. The field was removed and the sample cooled to a temperature T_0 = Tp−40°C. The depolarization current was recorded with a constant heating rate q = +7°C min^{-1}. The series of elementary curves was generated by shifting the polarization window by 5°C between −140 and −80°C.

2.4 | Statistical analysis

Quantitative values are shown as means ± SD. Statistical analysis have been done with SA software, using non parametric tests as required for samples size N < 10. Wilcoxon-Mann-Whitney test has been used to compare data between groups and Wilcoxon signed-rank test to compare data between areas (sun-protected and sun-exposed) from the same group. It was considered statistically significant threshold of *P* value less than .05.

3 | RESULTS AND DISCUSSION

3.1 | Dielectric characterization in the hydrated state (fresh biopsies)

In Figure 1 is reported the TSC curve of a skin biopsy in the sun-protected zone of a young subject corresponding to an initial poling of 400 Vmm^{-1} at −20°C.

This TSC curve is characterized by three relaxation modes labeled β_1, β_2, and β_3 located at −132.1, −95.9 et −84.2°C, respectively. To specify the origin of these modes, the experimental decomposition was performed using the fractional depolarization technique (TSC/FP) (Figure 2).

In this procedure, each isolated curve is well approximated by a single relaxation time, allowing the Bucci-Fieschi's analysis[28] and giving the distribution of elementary relaxation times τ_i involved in the different TSC modes in the scanned temperature range. By plotting the variation of $\tau_i(T)$ versus temperature (Figure 3), we noted that all the extracted relaxation times were well fitted by an Arrhenius' law:

$$\tau_i(T) = \tau_{0i} \exp\left({}^{E_{ai}}/_{RT}\right) \quad (1)$$

where R is the universal gas constant, Ea_i is the activation energy and τ_{0i} the pre exponential factor of the ith processus.

The variation in Ea and τ_0 vs temperature for all the isolated relaxation times are plotted in Figure 4 as well as the Starkweather's function [29] corresponding to non-cooperative processes (ΔS = 0).

Three regimes of increase in the activation energy are noted (I, II, and III) corresponding to the three relaxation modes. In the temperature range of the β_1 mode (regime I), the activation energy is comprised between 43 $kJmol^{-1}$ et 50 $kJmol^{-1}$, what approximately corresponds to twice the hydrogen bonds breaking (~23 kJ/mol). Moreover, the exponential factor is around 10^{-15} s. In the Eyring's theory,[30] this factor is related to the activation entropy by:

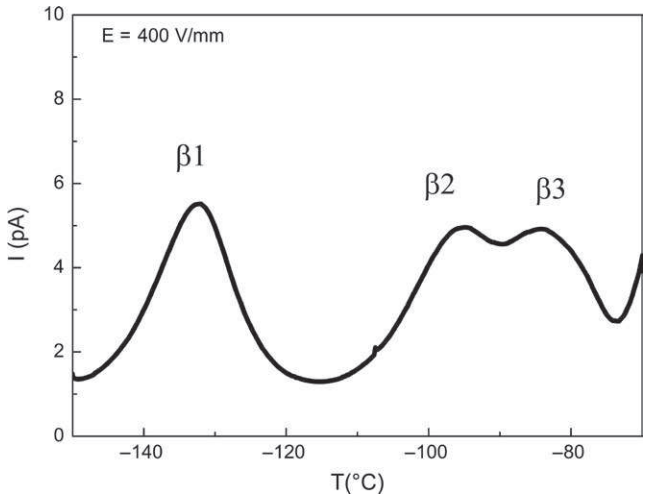

FIGURE 1 TSC curve of a fresh skin biopsy from the 20-30 years old group, sun-protected zone recorded at 7°C min^{-1} after an initial poling of 400 V/mm at −20°C. Enlargement in the [−150; −70°C] window

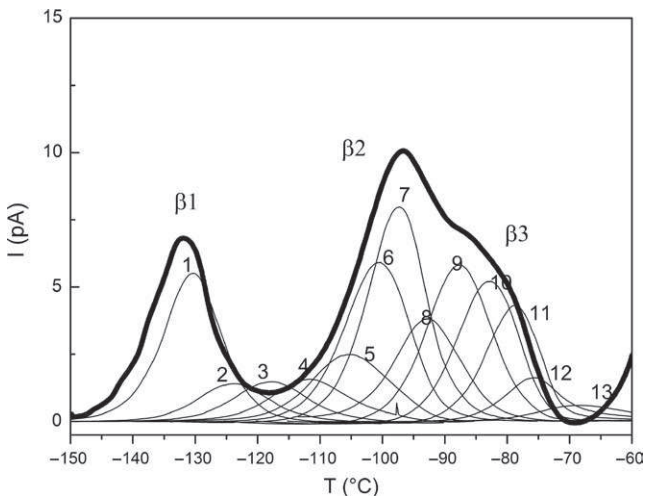

FIGURE 2 TSC/FP elementary curves in the [−150; −60°C] window of a fresh skin biopsy from the 20-30 years old group, sun-protected zone

$$\tau_0 = \frac{h}{kT} \exp - \frac{\Delta S}{k} \quad (2)$$

where h is the Planck constant and k the Boltzmann constant.

The activation entropy of the β_1 mode is around 30 $Jmol^{-1}K^{-1}$, indicative of a slight cooperative mode. On the contrary, the β_2 and β_3 modes are associated with activation energies of 50-100 $KJmol^{-1}$ and activation entropies of 70-180 $Jmol^{-1}K^{-1}$, indicative of more cooperative motions. By analogy with previous studies,[23,24,31,32] the β_1 mode, universally evidenced in highly hydrated biological systems (elastin, keratin, collagen, etc....) is associated with the reorientation of ice defects needing the breaking of two hydrogen bonds. It corresponds to the relaxational signature of freezable water. The β_2 mode is universally found in proteins and hydrated biological tissues.[24,32] It corresponds to the localized reorientation of the complex bound water/hydrophilic sequences. Several hypotheses have

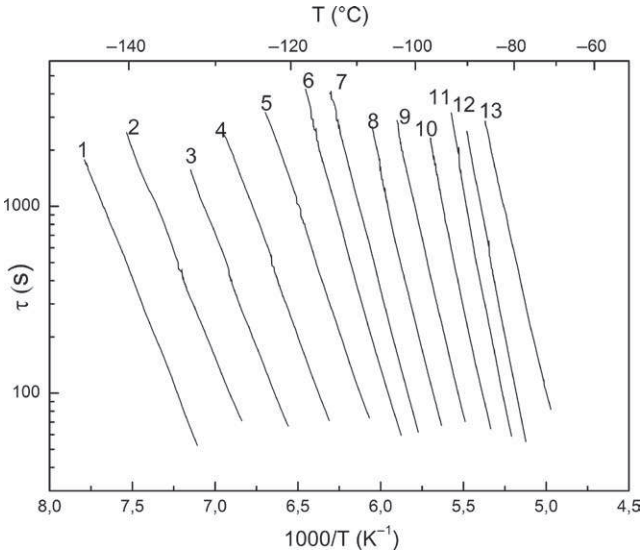

FIGURE 3 Variation in the elementary relaxation times vs reciprocal in a semi-logarithmic scale from the TSC/FP experiment of Figure 2

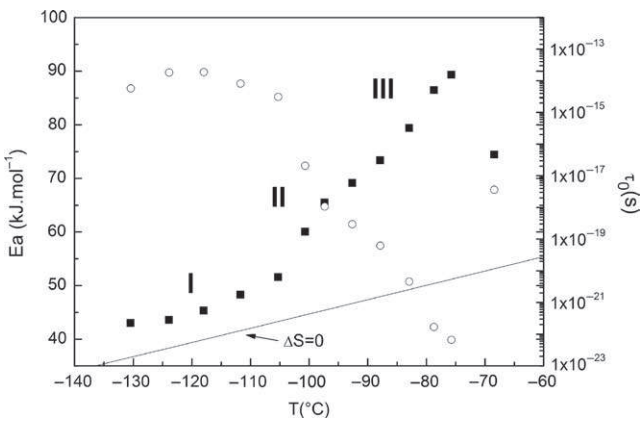

FIGURE 4 Activation energy and pre-exponential factor of elementary dielectric processes as a function of temperature from the TSC/FP experiment of Figure 2

been proposed for the β_3 mode: more cooperative, in pure hydrated proteins it has been ascribed to a long range rearrangement of the hydrogen bonds network.[32] It has also been associated with confined water interacting with hydrophobic groups.[24] In such a complex tissue as skin, it is difficult to propose a more accurate origin for this relaxation mode.

The TSC curves in the low temperature zone have been recorded for each fresh biopsy and the mean temperature maximum of the β_1, β_2 et β_3 modes have been reported in Figure 5.

The temperature maximum of the β_1 mode remains unchanged for the sun-protected skin of the two age groups: this mode is not affected by chronological aging only. In contrast, this mode is significantly shifted toward low temperature (P = .0239) for the aged sun-exposed skin when compared with young the sun-exposed skin. With photo-aging, the dynamics of the β_1 mode, ascribed to

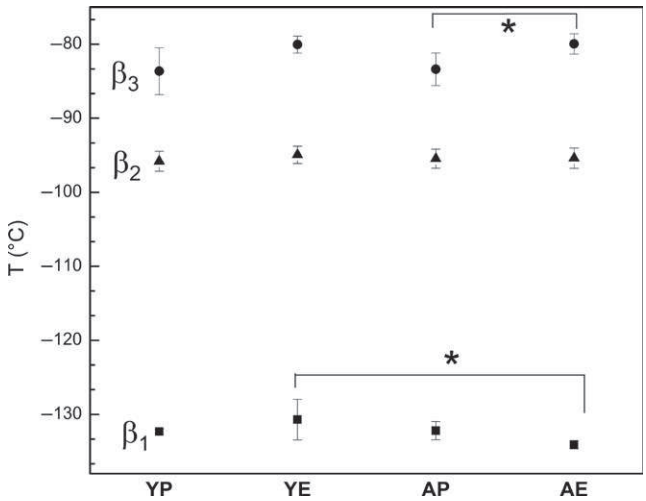

FIGURE 5 Mean temperature maxima of the β_1, β_2, and β_3 modes for the four series of fresh biopsies

freezable water, tends to be closer to that of pure ice.[33] This result evidencing phase segregation between bulk content water and proteins of the extracellular matrix supports and completes DSC[12] and Raman[17] analyses. This increasing of non-bounded water that forms a segregated phase in the photo-aged skin can be related to the decrease in echogenicity in the upper dermis of sun-exposed region in aged skin that could be due to a degradation of collagen, accumulation of GAGs and water in the upper dermis.[17,34]

Contrary to the β_1 mode, the temperature of the β_2 mode is constant for the four series of skin biopsies. The dynamics of the bound water/proteins complex is not impacted by chronological and extrinsic aging. Even if the amount of bound water significantly decreases with extrinsic aging as shown by DSC,[12] the first hydration shell of proteins remains saturated at the hydrated state, what certainly implies this temperature invariance of the β_2 mode.

Last, the β_3 mode is significantly shifted toward high temperatures for photo-exposed skin in the aged group when compared with photo-protected skin (P = .0156). The comparison is less straightforward in this case, dealing with two anatomically different zones: buttocks and forearms.

3.2 | Dielectric characterization in the dehydrated state (Freeze dried biopsies)

To avoid the intense dielectric answer of water, bringing an obvious complexity in TSC curves and compromising studies at temperatures above 0°C, it is often necessary to study the dielectric answer of proteins and biological tissues in the dehydrated state.

In this case, skin biopsies are not characterized in their native state and most of the information associated with hydric organization is lost. Nevertheless, studies in the dehydrated state have previously shown their ability to provide markers of the intrinsic dynamics of the constitutive proteins of extracellular matrix such as cardiovascular tissues[25] and rat skin.[26] So it was essential to check this feasibility on freeze dried human skin.

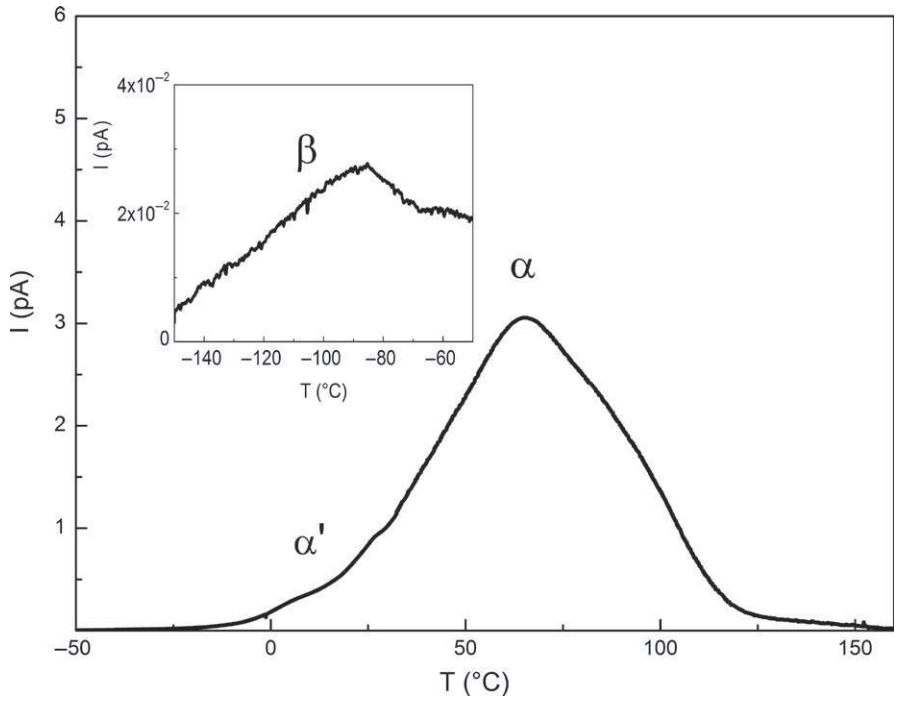

FIGURE 6 TSC curves of a freeze-dried skin biopsy in the sun-protected zone of a young subject. Insert graph: TSC curve recorded after an initial poling of 400 Vmm^{-1} at 25°C. Main graph: TSC curve recorded after an initial poling of 400 Vmm^{-1} at 150°C

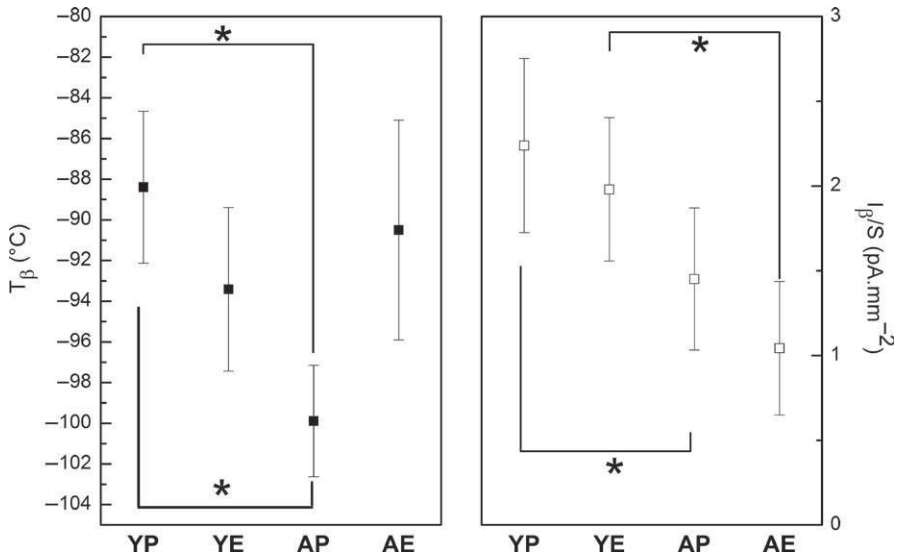

FIGURE 7 Mean temperature maxima and surface normalized intensities of the β mode for the four series of freeze-dried biopsies

In Figure 6 are reported the TSC curves of a freeze-dried skin biopsy in the sun-protected zone of a young subject corresponding to an initial poling of 400 Vmm^{-1} at 25°C and 150°C.

3.2.1 | Localized relaxations

In the low temperature zone a reproducible, large and weak relaxation mode centered on -95°C is evidenced. By analogy with previous works, this mode is universally found in freeze-dried proteins and tissues and labeled β mode.[25] It is ascribed to the reorientation of the polar groups/bound water (water of the first hydration shell). Associated with a localized motion, it corresponds to a marker of the intramolecular mobility. The TSC curves in the low temperature zone have been recorded for each fresh biopsy and the mean temperature maxima of the β modes have been reported in Figure 7, as well as the intensities at the maximum of the mode, normalized to the dry surface of each biopsy.

The β mode is significantly shifted toward low temperature with aging for sun-protected skin ($P = .0185$). Only the chronological aging leads to this evolution. The intensity of the β mode significantly decreases with aging, both in sun-protected (−35%, $P = .0469$) and sun-exposed (−47%, $P = .0244$) skins. It can argued that the amplitude of the localized mobility decreases with chronological aging, this effect being enhanced with cumulative effects of age and UVs. A similar behavior has been yet reported for collagen extracted from rat dermis and for skin rat submitted to ionizing

radiation.[26] The decrease in the localized amplitude can be connected to:

1. The increase in intramolecular and intermolecular links that restricts local mobility, in agreement with the increase in Tend in DCS experiments,[12] and that corresponds to the over cross-linking of fibrous proteins with aging,[3,35]
2. The decrease in the number of polar peptidic sequences, as already evidenced by FTIR with the decrease in collagenic fraction for extrinsic aging.[12]

It must be pointed out that the kinetics of this local dynamics, connected to the temperature of the β mode, is different according to the intrinsic and extrinsic aging.

3.2.2 | Delocalized relaxations

In the high temperature zone, a main complex relaxation mode is evidenced at 90°C. By analogy with previous works on pure collagen and rich collagen tissues,[25] it is ascribed to delocalized motions (some tens of nanometers) along polypeptidic chains of collagen and other structural proteins like elastin. In pure freeze-dried collagen, this relaxation mode has been associated with the dielectric manifestation of the glass transition, and it was found precursor of the denaturation phenomenon that occurs at 230°C in the dehydrated state.[25] Multiple and reproducible weak shoulders are detected in the [20; 50°C] zone (α'modes), and they are associated with the specific signature of collagen as already highlighted in pure collagen and collagen rich cardiovascular tissues.[36] Poorly distributed, isothermal and independent upon frequency, these specific modes are assigned to phase transition rather than relaxation modes.

The TSC curves in the high temperature zone have been recorded for each dehydrated biopsy and the mean temperature maxima of the α mode have been reported in Figure 8, as well as the intensities at the maximum of the mode, normalized to the dry surface of each biopsy.

A quasi-significant (P = .0549 YP vs AP) shift toward low temperature is noted for biopsies of the sun-protected zone of the aged group when compared with the young group, what reflects an effect of chronological aging. A similar behavior (P = .0508 YE vs AE) is found with aging for the biopsies of the sun-exposed zone. Moreover, even if the inter site comparison is less straightforward, there are not significant differences between temperature maxima of sun-protected and sun-exposed zones in the young group, as well as in the aged group. Chronological aging appears as the prominent factor for the shift of α mode toward low temperature.

In contrast, the intensity of the α mode remains constant for the biopsies of the sun-protected in the two groups of age, which implies that chronological aging has no effect on this parameter. A trend in decrease (P = .0625) is noted for the intensity of this mode for the sun-exposed zone of aged patients when compared with the sun-protected zone of the same patients, but in this case the site effect could be not negligible.

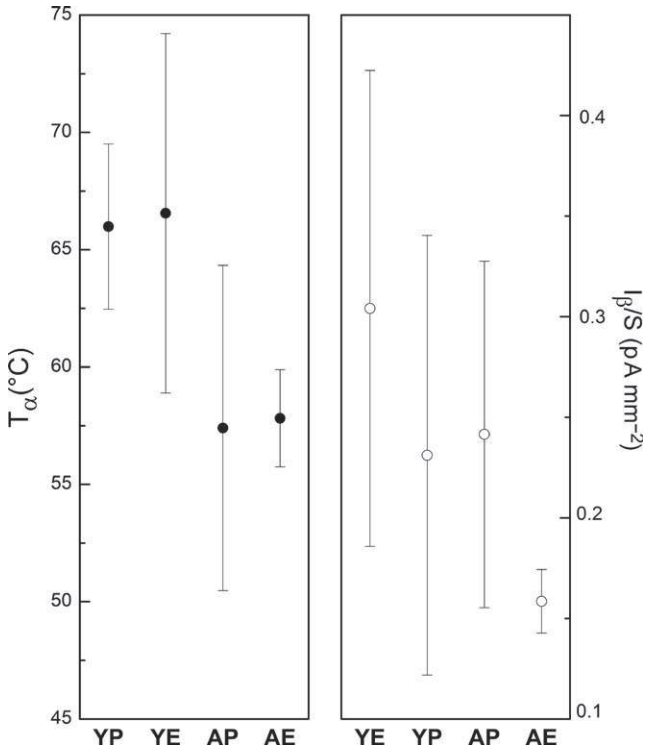

FIGURE 8 Mean temperature maxima and surface normalized intensities of the α mode for the four series of freeze-dried biopsies

In dermis, it can be assumed that α mode is mainly associated with the delocalized dynamics along collagen chains. The kinetics of this mode (associated with its temperature location) mainly evolves with chronological aging, even if the shift remains to be explained. In the case of elastin, another protein of the extracellular matrix, it was shown that α relaxation was shifted toward low temperature even at weak elastolysis level, although these variations were not detectable by DSC.[37] We can so suggest that this offset of the main mode with chronological aging is associated with defects in triple helical domains of collagen due to enhanced proteolysis such as observed in elastin with aging.[35]

The amplitude of the α relaxation mode tends to diminish with the cumulative effect of aging and UVs; such a behavior has been already observed for collagen extracted from rat skin and for rat skin exposed to ionizing radiations.[26] Both the increase in glycation products with chronological aging and the decrease in collagen fraction with photo-aging could explain the evolution of this indicator of the chain mobility of dermis structural proteins at the ten nanometers scale.

4 | CONCLUSION

TSC is a powerful technique to characterize human skin, giving accurate information on the hydric organization and chain dynamics of the ECM with a high reproducibility. It must be pointed out that for the first time, a dielectric study of human skin biopsies from two age groups has been achieved.

The combination of different techniques of thermal analysis (DSC[12] and TSC) is useful to extract new biomarkers of human skin modifications with intrinsic and extrinsic aging.

Chronological aging induces an increase in heat-stable cross-links, due to age-induced glycation and carbonyl modifications that have repercussion on the chain dynamics of the structural proteins of the dermis (restriction on the localized mobility). Photo-aging induces an increase in freezable water which forms a segregated phase in the photo-aged skin, with dynamics close to free water alone, highlighting the predominant role of extrinsic aging on water organization in human skin. Moreover, TSC indicators bring to the fore a cumulative effect of chronological aging and photo-exposition on the molecular mobility of the main structural proteins of the dermis at the mesoscopic scale, which will have consequences on the mechanical properties of the skin.

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