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Stefan Puschmann
Christian-Dennis Rahn
Horst Wenck
Stefan Gallinat
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Stefan Puschmann, Christian-Dennis Rahn, Horst Wenck, Stefan Gallinat, and Frank Fischer
Beiersdorf AG, Research & Development, Unnastrasse 48, 20245 Hamburg, Germany

Abstract. Extracellular skin structures in human skin are impaired during intrinsic and extrinsic aging. Assessment of these dermal changes is conducted by subjective clinical evaluation and histological and molecular analysis. We aimed to develop a new parameter for the noninvasive quantitative determination of dermal skin alterations utilizing the high-resolution three-dimensional multiphoton laser scanning microscopy (MPLSM) technique. To quantify structural differences between chronically sun-exposed and sun-protected human skin, the respective collagen-specific second harmonic generation and the elastin-specific autofluorescence signals were recorded in young and elderly volunteers using the MPLSM technique. After image processing, the elastin-to-collagen ratio (ELCOR) was calculated. Results show that the ELCOR parameter of volar forearm skin significantly increases with age. For elderly volunteers, the ELCOR value calculated for the chronically sun-exposed temple area is significantly augmented compared to the sun-protected upper arm area. Based on the MPLSM technology, we introduce the ELCOR parameter as a new means to quantify accurately age-associated alterations in the extracellular matrix. [DOI: 10.1117/1.JBO.17.3.036005]

Keywords: multiphoton laser scanning microscopy; second harmonic generation; autofluorescence; skin aging; collagen; elastin; elastin-to-collagen ratio.

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1 Introduction

Human skin aging is a multifaceted phenomenon caused by intrinsic and extrinsic factors. While all human organs are subject to intrinsic aging, the process of extrinsic aging—mainly photoaging—is particularly prominent in human skin. Photoaging is superimposed on the inevitable intrinsic processes and is, to a large extent, due to environmental factors such as ultraviolet (UV) irradiation. Both types of aging induce profound changes in cutaneous morphology and structure. The physiology of the epidermis and dermis is altered, subsequently leading to increased fragility of the skin, laxity, and dry skin.[1]

Apart from collagen—the major component of the extracellular matrix (ECM)—the connective tissue contains an array of other components such as elastin, proteoglycans, fibronectin, and ECM proteins. In combination, these proteins are responsible for the dermal biomechanical properties. Whereas young skin is characterized by an intact, well-organized, and densely woven collagen network, this collagen is fragmented in the course of the aging process. At the same time, the synthesis of new collagen is down-regulated. Together these processes result in disorganized collagen fibers that accumulate within the ECM, impairing the structural integrity of the dermis.[2–5] While elastin is a minor ECM component with regard to its weight percentage, it provides the crucial basis for cutaneous elasticity. In photoaged skin, however, normal elastic fibers are degraded.[6] Elastotic material is deposited within the dermis forming amorphous structures and replacing the normal ECM structure and thus weakening skin resilience.

In the past, dermal structures were mainly studied *ex vivo* using histological or ultrastructural methods. A drawback of these invasive approaches is the fact that skin biopsies must be taken to allow for *ex vivo* analyses. In the course of sample isolation (i.e., excision of skin biopsies), the mechanical forces built up by the collagen and elastin network are subject to change, which by itself impacts the overall dermal tissue structure, thus inevitably obscuring the actual ECM status and its structural organization. Noninvasive microscopic *in vivo* procedures (e.g., optical coherence tomography, Raman microspectrometry, *in-vivo*-confocal laser scanning microscopy) may be used to circumvent these issues for visualizing the human dermis in situ. However, these methods lack high resolution and/or functional imaging, which impairs the interpretation of results.

To overcome these issues, we applied a multiphoton laser scanning microscope (MPLSM) for high-resolution three-dimensional *in vivo* imaging. This technique provides fast information under physiological conditions and is ideal to investigate age-associated dermal skin alterations quantitatively *in vivo*. It allows for the noninvasive evaluation of (extra)cellular structures by taking "optical" instead of a real skin punch biopsies without depending on contrasting agents.

Here, dermal collagen fibers are visualized based on their ordered, noncentrosymmetric molecular structure giving rise to the collagen-specific second harmonic generation (SHG) signal. Elastin, on the other hand, emits autofluorescence (AF) signals that can be used for evaluation of elastic fibers or elastotic material.

The MPLSM technology has already been used for a number of dermatological applications,[7,8] for example, the investigation of collagen dissociation induced by chemicals,[9] thermal

Address all correspondence to: Frank Fischer, Beiersdorf AG, Research & Development, Structure Research, Bf. 518, Unnastrasse 48, 20245 Hamburg, Germany. Tel: 49-40-4909-6638; Fax: 49-40-4909-3855; E-mail: frank.fischer@beiersdorf.com

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denaturation of collagen,[10] and effects of sunbed-induced skin aging.[11]

The aim of our study was to develop a parameter for the quantitative determination of dermal skin aging. We introduce the elastin-to-collagen ratio (ELCOR) as a new means to quantify accurately age-associated alterations in the ECM.

2 Materials and Methods

2.1 *Multiphoton Laser-Scanning Microscopy*

For noninvasive *in vivo* measurements avoiding the use of contrast agents, we developed, in collaboration with Jenlab GmbH (Jena, Germany), a CE marked multiphoton laser scanning microscope, called DermaInspect. This device contains a femtosecond laser, which provides a variable wavelength range (710 to 920 nm), a pulse repetition rate of 80 MHz, and a pulse width at the sample layer of about 150 fs. The DermInspect is equipped with a flexible mirror arm that allows optimal positioning over desired skin areas.

To image the dermal fiber network *in vivo*, collagen-specific SHG signals were measured utilizing an irradiation wavelength of 820 nm and SHG was recorded using a specific band-pass filter (410 ± 10 nm; AQ 410/20m-2P, Chroma Technology Corp., Bellows Falls, VT). Autofluorescence was determined using an excitation wavelength of 750 nm and emission was detected utilizing a 548 ± 150 nm band-pass filter (HQ 548/305m-2P, Schott AG, Mainz, Germany).

For imaging parallel to the skin surface, the focus of the laser beam was scanned *in vivo* within the papillary dermis at depths of 30 and 45 μm beneath the basal membrane. Each scan was 220×220 μm wide, and the image resolution was set at 512×512 pixels.

2.2 *In Vivo Studies*

After approval by an ethics committee, volunteers (ages 18 to 90 years) with skin types II and III were included in different *in vivo* studies. All donors provided written, informed consent. During the last 30 days prior to measurements, volunteers were required to desist from intensive sun exposure as well as visits to solariums. Also, the use of any medication two months prior to measurements was prohibited. Measurements were performed by trained and experienced personnel after acclimatization of volunteers for at least 30 min under standard atmospheric conditions ($21.5\,°C \pm 5.0\,°C$ and $45\% \pm 10\%$ relative humidity).

2.3 *Imaging of Intrinsic Aging In Vivo*

To study intrinsic aging *in vivo* using the MPLSM, SHG and AF signals of test areas located on the sun-protected volar side of one forearm of 45 healthy female subjects (15 young volunteers of ages 18 to 25; 15 middle-age volunteers of ages 35 to 45; and 15 older volunteers of ages >65 years) were acquired.

For measurements, the volunteers' right forearms were placed on a special holder utilizing a specifically designed *in vivo* adapter that also allowed for correction of volunteers' movements relative to the device. Areas of interest were imaged using two imaging modi at the same area. Each image was taken with 25 s acquisition time. We obtained AF images at 750 nm laser wavelength and SHG images at 820 nm laser wavelength. Images were taken at depths of 30 and 45 μm below the epidermal-dermal junction. All images were digitally stored for further quantitative analysis.

2.4 *Imaging of Photoaging In Vivo*

For the characterization of photoaging effects, 12 elderly females (>60 years) were included in this study. Test areas were the sun-exposed temple area and sun-protected volar upper arm. Measurements were performed as previously described. To investigate the effect of different measuring depths on the elastin to collagen ratio (ELCOR), measurements were performed at depths of 30 and 45 μm and results were calculated independently for each depth.

Acquisition time for each image was 13 s. Again, a mirror arm was used to guarantee optimal access to the test areas.

2.5 *Quantification of Skin Aging by ELCOR*

Using the MPLSM data, the determination of the occupied area fractions of the AF signal of elastin and of the SHG signal of collagen were carried out as follows: After image acquisition, image preprocessing and image analysis were performed. First, nondermal structures were excluded. Second, deshading and denoising of images and also automatic threshold determination based on the gray-level histogram were carried out.

Although the full image field that is occupied by either elastin or collagen fibers is used for analysis, in most cases, only a limited fraction of the entire image shows relevant fiber structures. The remaining parts may contain blood vessels or skin appendages. Because these structures interfere with the calculation of ELCOR, they are excluded by image-masking. The used masks are manually generated binary images.

Next, the ELCOR parameter is determined as follows: Provided that the image F_E most exclusively shows elastin fibers and the image F_C primarily displays collagen fibers, the ratio ρ of fibrous elastin to collagen tissue can be estimated from the corresponding occupied area fractions (OAFs) as follows:

$$\rho = \frac{\mathrm{OAF}[E]}{\mathrm{OAF}[C]},$$

with $\mathrm{OAF}|E| \in [0.1]$ being the area fraction of the (masked) image field that is occupied by elastin fibers in F_E and $OAF|C| \in [0.1]$ analogously for $\mathrm{OAF}|C|$ being the area fraction of the (masked) image field that is occupied by collagen fibers in F_C. Both OAF values can be calculated after binarization of the images F_E and F_C. Image binarization is conducted by automatic gray-level thresholding. Finally, ρ is termed the elastin-to-collagen ratio (ELCOR).

2.6 *Quantification of Skin Aging by SAAID*

For both studies, the SHG to AF aging index of the dermis (SAAID) was calculated as described previously.[12] In brief, $(\mathrm{SHG} - \mathrm{AF})/(\mathrm{SHG} + \mathrm{AF}) = \mathrm{SAAID}$ index, calculated inside a masked area of 55×55 pixel. For validation of the ELCOR parameter, SAAID values were set in relation to previous studies that already demonstrated the age dependence of SAAID *in vivo*.

2.7 *Statistics*

A significance level of 0.05 (alpha) was chosen for statistical analysis, based on two-sided hypotheses. For other parameters, the following analyses were conducted:

Imaging of intrinsic aging *in vivo*: Comparison among age groups via Mann-Whitney test.

Imaging of photoaging *in vivo*: Comparison among test areas via Wilcoxon test.

For analysis, Statistica and SAS software package for Windows V9.1.3 were used.

3 Results

3.1 *Determination of ELCOR in Intrinsic Aging*

To investigate age-dependent effects *in vivo*, we compared the ELCOR parameter calculated from data collected at sun-protected young, middle-aged, and aged skin areas. Data were obtained at two different measurement depths and the average signal was used for calculation of the respective parameter. Figure 1 shows example images taken from sun-protected skin and their corresponding masks for young and aged volunteers. Figure 2(a) depicts the ELCOR parameter obtained from sun-protected volar skin (young: 0.50 ± 0.01 au, $n = 15$; middle age: 0.54 ± 0.01 au, $n = 15$; and older age: 0.57 ± 0.01 au, $n = 15$). With respect to volar skin, the ELCOR parameter significantly increased with age (young versus middle aged, $\rho = 9.7 \times 10^{-11}$; middle aged versus old, $\rho = 1.2 \times 10^{-7}$; young versus old, $\rho = 8.1 \times 10^{-21}$).

3.2 *Determination of ELCOR in Photoaging*

Because extrinsic factors predominantly determine human skin aging, we investigated the process of photoaging by MPLSM in elderly volunteers at different skin depths. The ELCOR parameter was determined and results from the sun-exposed temple area and the sun-protected upper arm were compared. Figure 3 shows example images taken from sun-exposed skin. As depicted in Fig. 4(a), at 30 μm below the basal membrane, the temple area yielded a value of 0.92 ± 0.03 au compared to 0.95 ± 0.03 au determined at 45 μm below the basal membrane. With respect to the sun-protected upper arm, the ELCOR parameter was calculated at 0.81 ± 0.02 au 30 μm below the basal membrane compared to 0.81 ± 0.02 au determined at 45 μm below the basal membrane. A significant difference between sun-exposed (temple skin) and sun-protected (upper arm skin) test areas in terms of ELCOR for elderly volunteers was detected at a depth of 30 μm ($n = 12$, $\rho = 9.9 \times 10^{-6}$) and 45 μm ($n = 12$, $\rho = 3.5 \times 10^{-6}$), respectively.

Compared to the sun-protected upper arm area, the ELCOR parameter calculated for the chronically sun-exposed temple area was significantly increased.

3.3 *Comparison of ELCOR and SAAID Parameters*

Using the data obtained in the *in vivo* study investigating intrinsic aging effects, the following SAAID parameters were calculated: $n = 45$; young $= 0.23 \pm 0.05$ au; middle age $= 0.22 \pm 0.04$ au; and older $= 0.12 \pm 0.06$ au [Fig. 2(b)]. Because data were obtained at three different measurement depths, the average signal was used for calculation of the respective parameter.

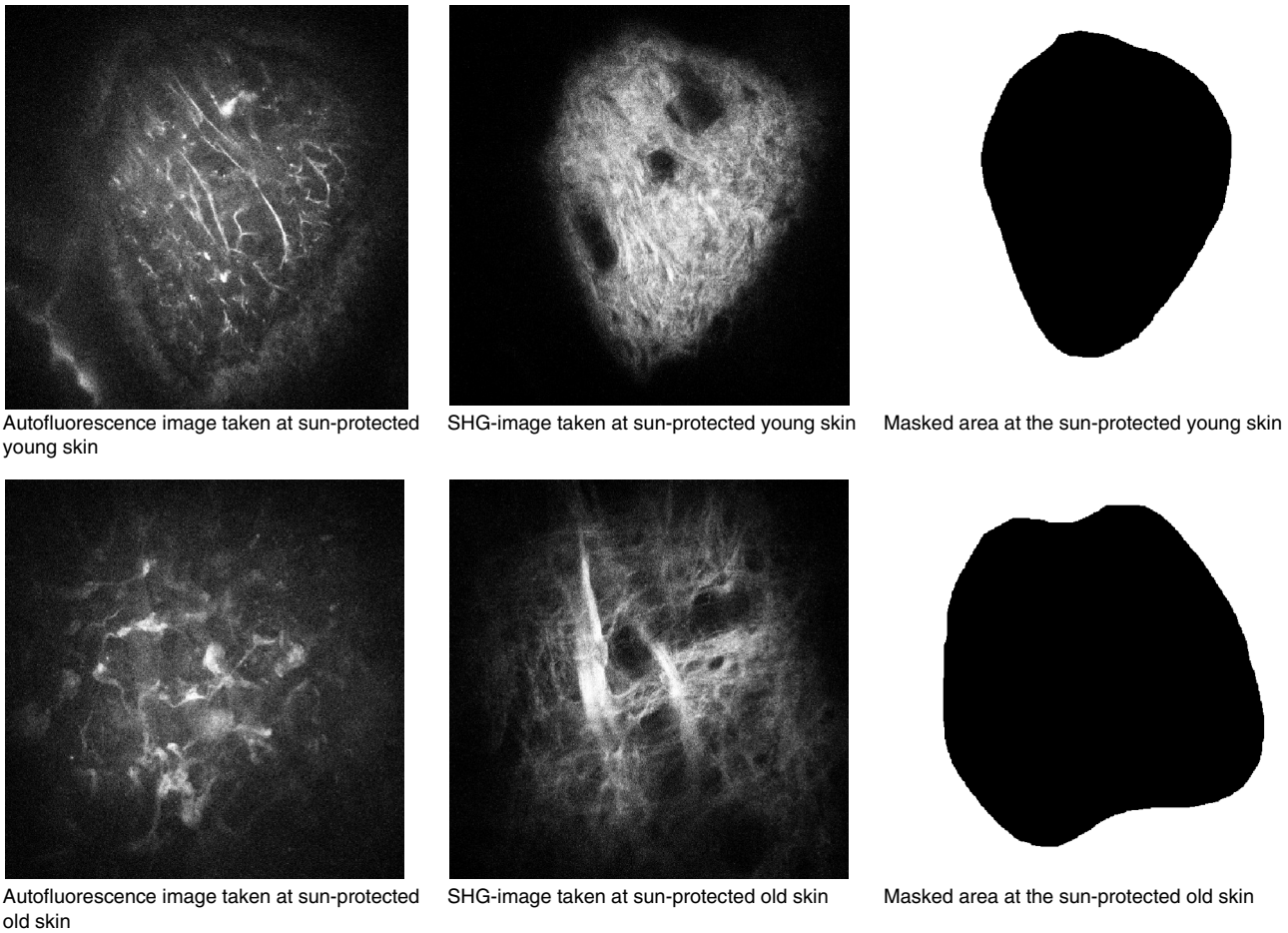

Fig. 1 Autofluorescence and SHG-images taken at a sun-protected area (upper arm).

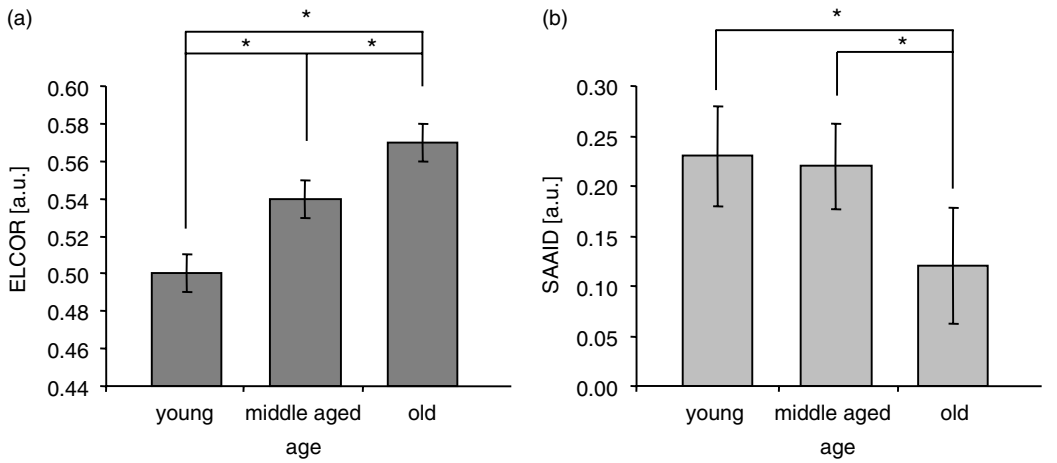

Fig. 2 ELCOR (a) and SAAID (b) values of test areas located on the sun-protected volar side of one forearm of young volunteers, volunteers of middle age, and older volunteers. [Results are shown as mean ± SEM ($n = 15$). Significant differences are marked with an asterisk (* for $\rho \leq 0.05$).]

The SAAID parameter decreased with increasing age. However, not all investigated groups yielded significant differences (young versus middle aged, $\rho = 0.619$; middle aged versus old, $\rho = 2.0 \times 10^{-4}$; young versus old, $\rho = 7.4 \times 10^{-5}$).

As shown in Fig. 4(b), the respective SAAID parameter for the photoaging study are as follows: at 30 μm below the basal membrane, the temple area yielded a value of -0.14 ± 0.08 au compared to -0.17 ± 0.09 au determined at 45 μm below the basal membrane. The sun-protected upper arm showed an SAAID value of -0.07 ± 0.04 au at 30 μm below the basal membrane compared to -0.09 ± 0.05 au determined at 45 μm below the basal membrane. No significant differences between sun-protected and sun-exposed areas could be detected using the SAAID.

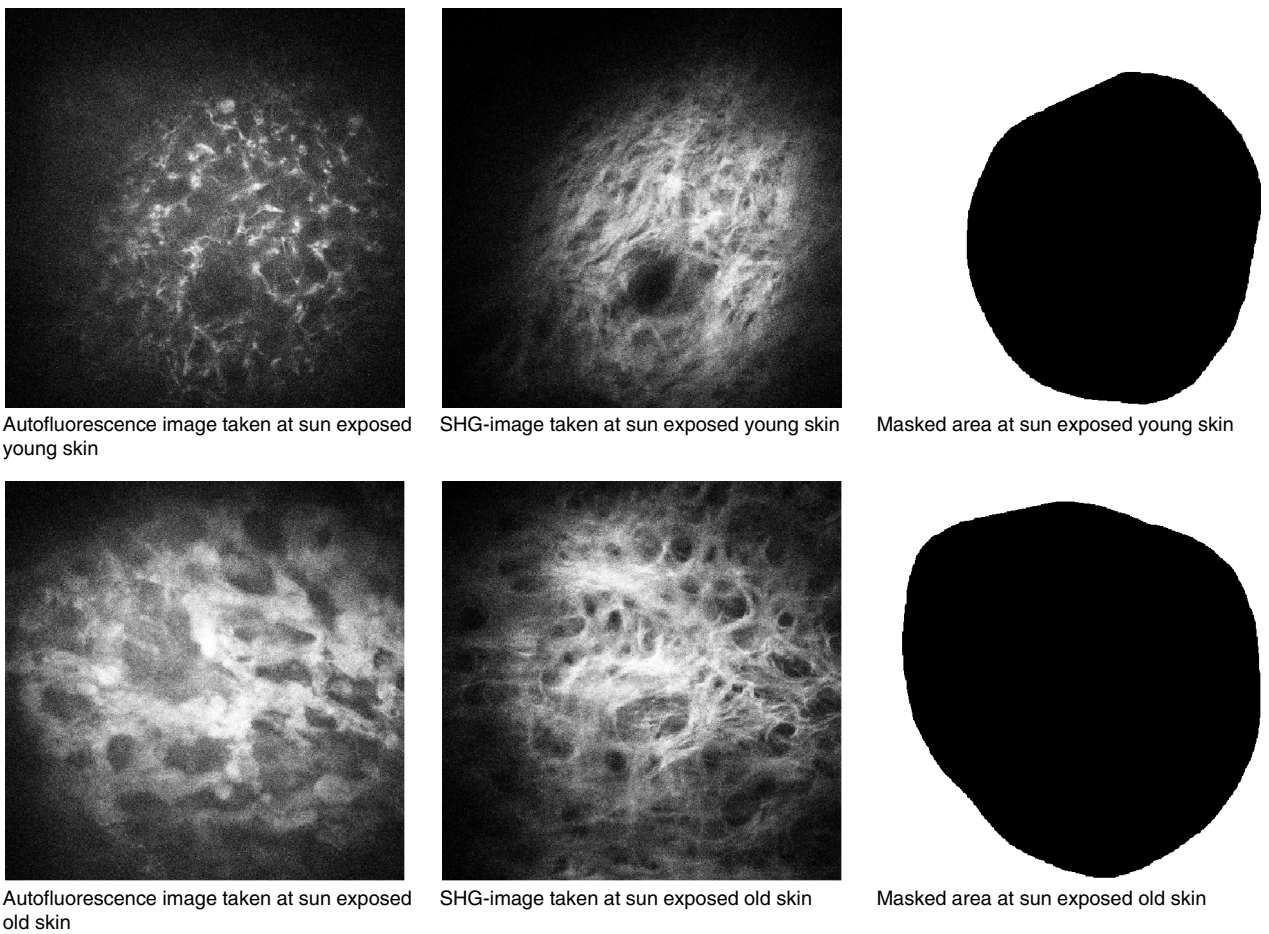

Fig. 3 Autofluorescence and SHG-images taken at a sun-exposed area (face).

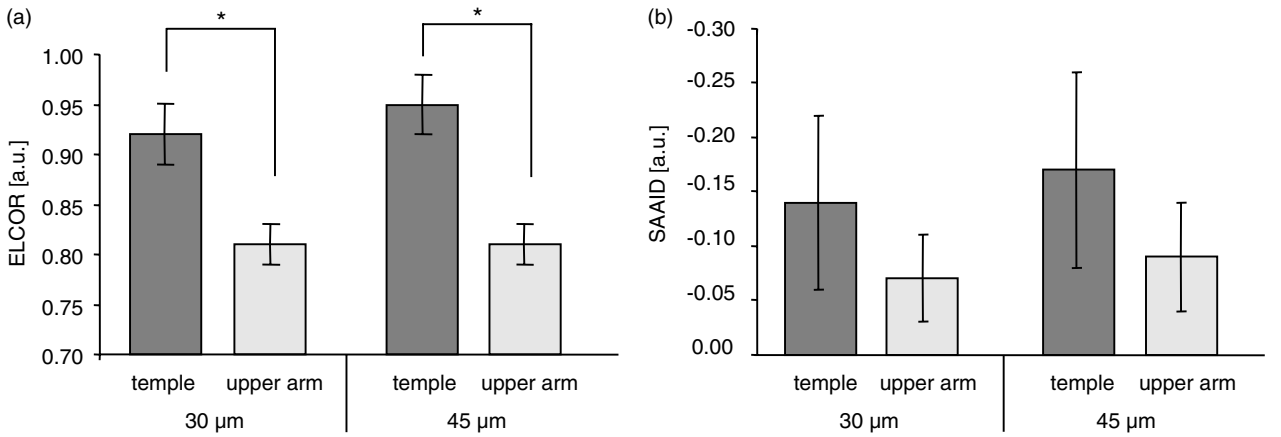

Fig. 4 ELCOR (a) and SAAID (b) values of test areas located on the chronically sun-exposed temple area and sun-protected upper arm. [Measurements were performed at depths of 30 and 45 μm below the basal membrane. Results are shown as mean ± SEM ($n = 12$). Significant differences are marked with an asterisk (* for $\rho \leq 0.05$).]

4 Discussion

Substantial changes in the collagen network and the formation of elastotic material are characteristic features of photoaged skin. The assessment of these dermal changes is usually performed by subjective clinical evaluation in combination with histological and molecular analysis. Because this approach is invasive and cutaneous tissue architecture as well as mechanical properties are damaged during the excision of skin biopsies, the development of a noninvasive technique is crucial for diagnostic and research purposes.

With the help of the high-resolution 3-D-MPLSM technology, it becomes possible to quantitatively investigate dermal skin alterations associated with photoaging and to determine extracellular characteristics of the ECM *in vivo*.[11–13] Accordingly, the objective of this study was to devise a new parameter based on MPLSM technology that allows for the quantification of structural differences in chronically sun-exposed and intrinsically aged human skin *in vivo*. With respect to changes in the dermal ECM, the optical discrimination between collagen fibers and elastic fibers is achieved by measuring collagen-specific SHG signals and elastin-specific fluorescence signals, respectively.

Several studies already successfully utilized MPLSM technology for dermatological research. In 2005, Lin et al.[12] introduced the aging index of the dermis (SAAID) and suggested that SAAID can be used for the qualitative and quantitative characterization of alterations of dermal structures associated with photoaging. SAAID is defined as the difference between SHG and AF signals normalized to the sum of both signals: $(\mathrm{SHG} - \mathrm{AF})/(\mathrm{SHG} + \mathrm{AF})$ and currently represents the standard parameter used for determination of skin aging utilizing the *in vivo* MPLSM technology. Performing *in vivo* multiphoton laser measurements on the forearms of volunteers, Koehler et al.[13,14] demonstrated that the SAAID parameter decreases with age and that the difference between sun-protected and sun-exposed skin areas increases during aging. A recent study also suggests that SHG and the SAAID index are useful indicators of facial skin aging *in vivo*.[15]

However, the use of SAAID is associated with certain restrictions. First, for determination of SAAID, only a small subregion of the entire dermis image is used for analysis. Furthermore, the subregion of interest is positioned subjectively in an ideally even illuminated image region. Second, it has to be noted that a comparison between different studies is delicate because SAAID can vary between positive or negative values.

To overcome these disadvantages, we developed a more robust parameter, the elastin-to-collagen ratio, termed ELCOR. Based on the measurement of the elastin-specific AF signal and the collagen-specific SHG signal, the ELCOR parameter is defined as the ratio of the relative amount of elastin fibers to collagen fibers.

Both the SAAID and the ELCOR parameter are based on similar input data (i.e., SHG and AF signal) and share several characteristics: First, with both the SAAID and the ELCOR parameter, a relative quantification of intrinsic skin aging and photoaging can be performed. Second, both methods are equally fast to carry out and calculate. Third, because ELCOR and SAAID are both mainly dependent on a pure AF elastin signal (w/o SHG) and a pure collagen SHG-signal (w/o AF of other components), optical filters in the measuring device have to assure pure signals.

Apart from these common characteristics, the determination of the ELCOR parameter offers advantages. In contrast to SAAID, ELCOR is independent of total image intensity but based on image area comparisons instead. Also, most importantly, for the calculation of ELCOR, a large subregion of the entire 220×220 μm image scan is employed for analysis. This is crucial because subjective image selection, as used with SAAID, can be ruled out completely. With respect to minimizing operator bias, this more objective approach appears to be quite favorable. Additionally, because ELCOR is calculated utilizing the quotient rather than the difference between SHG and AF signals, ELCOR offers the benefit of a more robust parameter because it is mostly independent of intensity scaling. In contrast, intensity scaling is relevant for the SAAID.

In an earlier study using only the SHG signal, we already visualized the underlying structural changes in the collagen network of young and aged sun-exposed facial skin *in vivo* and determined collagen density changes in fibroblast-populated collagen gels.[16] Employing the ELCOR parameter for the comparison of young, middle-aged, and aged sun-protected skin areas, our data now showed that the ELCOR parameter significantly increased with rising age of volunteers. Also, the ELCOR values of chronically sun-exposed areas were significantly higher than the ELCOR values determined at sun-protected skin areas of older volunteers.

For the purpose of data validation and comparison, we determined for all ELCOR values calculated in this study, the corresponding SAAID values of young, middle-aged, and aged sun-protected skin areas and also of sun-protected and chronically sun-exposed areas of older skin. Taken together, these results demonstrate the validity of our experimental approach because decreasing SAAID values with age as well as lower SAAID values at sun-exposed compared to sun-protected skin areas are in line with the literature.[11] However, in contrast to SAAID, ELCOR yielded significant results for all investigated groups and skin areas. As previously discussed, these significant differences are not due to the MPLSM measurement, but rather a benefit of improved image-analysis tools demonstrating the robustness and insusceptibility of the new parameter. Our data show that ELCOR increases with age and can be used as a tool to quantify intrinsic skin aging and photoaging.

With respect to the investigation of photoaging, it has to be taken into account that ELCOR as well as SAAID were determined at different skin sides, the upper arm, and the temple area, which are characterized by a differing skin composition. Currently, we do not know which implications these different locations have on measurement parameters.

We determined the ELCOR parameter at different depths below the basal membrane to investigate the dependence of ELCOR on measurement depth. A determination of parameters at measurement depths of both 30 and 45 μm below the basal membrane seems reasonable because this approach yielded statistically sound data.

In conclusion, we introduced the ELCOR parameter based on the MPLSM technology as a means to study the cutaneous aging process *in vivo*. This novel parameter has proven useful to gain further insights into dermal skin aging and offers several advantages over the currently employed SAAID value.

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